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GENES AND DEVELOPMENT

Use of *c-myc* gene dosage in transgenic mice as a cell marker for muscle and myoblast transplantation studies

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Transplantation of muscle or myogenic cells in the mouse contributes to our understanding of hereditary neuromuscular diseases (Füchtbauer, E. M. *et al.* (1988) *PNAS* **85**, 1383–9; Partridge, T. A. *et al.* (1989) *Nature* **337**, 176–9) and may eventually advance their therapy in man.

The evaluation of transplantation experiments requires that donor cells can be recognized in the host even after prolonged periods of regeneration and after cofusion of donor and host myoblasts. We use a transgenic marker in the host, a high dose of *c-myc* (approximately 50 copies on chromosome 8; Beermann, F. *et al.* (1988) *Cytogenet. Cell Genet.* **49**, 311–2) that we have bred into the nude mouse to circumvent problems of immune rejection. Host and donor nuclei are recognized in frozen sections by *in situ* hybridization with the pCHTK2-myc probe. The method can be combined with standard histochemical methods applied to adjacent serial sections. This labelling system is being applied to transplanted mutant (X-linked muscular dystrophy, *mdx/mdx* or *mdx/y*; muscular dysgenesis, *mdg/mdg*; muscular dystrophy with myositis, *mdm/mdm*) muscles and myogenic cells to assess how pathological cell death is influenced in an environment of a nonmutant organism. In the case of MDX donor tissue the absence of dystrophin supplies an additional ('recessive') marker. We show that cofusion of donor and host muscle cells occurs even after transplantation of whole muscles, indicating breakdown of perimysial barriers during the process of regeneration.

Rejection of implanted myogenic cells and protective effects of cyclosporin A

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Expectations that myogenic cells would be less immunogenic than other cell types are contradicted by recent evidence showing complete rejection of human HLA-matched myoblasts (Gussoni, E. *et al.* (1992) *Nature* **356**, 435). Current experiments in our laboratory aim to elucidate factors that determine the survival of allografted myoblasts, mechanisms of rejection and ways to improve cell survival.

In a set of experiments we implanted C2n1sBAG mouse myoblasts (β-galactosidase transfected C2C12 subclone, H-2^k positive, H-2^d negative, provided by Drs S. M. Hughes and H. M. Blau, Stanford (1992) *Cell* **68**, 659; 10^6 cells per muscle) into cryodamaged (but not freeze-killed) soleus muscles of C57BL/6J mice (H-2^b) and CBA/J mice (H-2^k). β-Galactosidase (β-Gal) activity (marker for the grafted cells) was not detectable in cross-sections from regenerated muscles of incompatible C57BL mice already 25 days post-implantation, indicating rapid and complete rejection. Surprisingly, isometric muscle force 10 months after such implantation was significantly lower than in contralateral control muscles. In contrast, survival of the graft's progeny was found in 6 of 6 CBA/J mice studied 56–222 days post-implantation; however, only 1.3–17.2% of the muscle fibres on a single cross-section were β-Gal positive. Despite survival of injected cells and in contrast to the previously observed increase in tetanic muscle force after implantation of other compatible cell lines (Wernig *et al.* (1991) *J. Neurocytology* **20**, 982), isometric force measured *in vitro* again was largely reduced. At early post-implantation periods (2–28 days) a massive infiltration with T-killer (Ly2+) and T-helper (L3T4+) lymphocytes and acid phosphatase-/MAC1-positive macrophages was present. This was accompanied by the expression of MHC Class I and II molecules in β-Gal positive cells in the first 2 weeks which declined to zero at about 3 weeks, while myoblasts in culture were MHC negative. These results suggest that implanted allogenic myoblasts induce cytotoxic rejection even in presumably MHC-matched hosts. Most importantly, long-lasting deficits in force of regenerated muscle were found following such myoblast rejection.

In other experiments (F8B myoblasts implanted in incompatible CBA/J mice) short-term immunosuppression with cyclosporin A (CsA, 50 mg kg^{-1} per day) protected the implanted cells and their progeny for the period of treatment. Increase in muscle force, mass and fibre number much above control were found in two muscles studied 1–3 days after the last drug application, i.e. 29–31 days after implantation. At 31–136 days post-CsA treatment, however, gradual decline towards (but not below) control values was noticed (five muscles). During this time no MHC expression in muscle fibres was detectable while T-helper and T-killer lymphocytes were present. Thus, initial transient CsA treatment was to some degree effective in protecting the graft beyond the period of treatment in that later graft rejection occurred at a much reduced speed.

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Gene conversion within the skeletal myosin heavy chain multigene family of the rabbit

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Comparison of the nucleotide sequence of exon and intron regions of the head region of five developmentally regulated and tissue specifically expressed skeletal myosin heavy chain (MyHC) genes indicates that not only the coding sequences of rabbit MyHC isoforms are highly similar to the sequences of other MyHC isoforms, but also that the intron sequences show a high degree of homology between different mammalian species. Interestingly, there is only very weak homology between intron sequences of rabbit and chicken. The sequence data provide evidence that gene conversion events may have occurred among the isoforms. Similar gene conversion events are observed in the tandemly linked rat α- and β-cardiac MyHCs and in the light meromyosin region of developmentally-regulated chicken MyHCs isoforms (Moore *et al.* (1992) *J. Mol. Biol.* **223**, 383–7). The

data suggest that gene conversion events between duplicated myosin genes have played a significant role in the molecular evolution of the MyHC multigene families.

Multistep regulation of the murine β enolase gene

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We previously reported that β enolase belongs to a subset of muscle-specific genes which can be expressed at the myoblast stage (Lamandé *et al.* (1989) *PNAS* **86**, 4445). Terminal differentiation is accompanied by an increased expression in C2 as well as 10T1/2-derived myogenic cell lines. We now report that culture of myoblasts derived from azacytidine-treated 10T1/2 cells in a medium containing growth factors reversibly abolishes both MyoD1 and β enolase gene expressions. Transfection of 10T1/2 cells (which do not express β enolase gene) with cDNAs corresponding to each regulatory factor of the MyoD1 family demonstrates that β enolase gene expression always 'accompanies entering the myogenic lineage, whichever the factor responsible for the conversion.

Histological and biochemical studies (Keller *et al.* (1992) *Mech. Devel.* **38**, 41–52; Lucas *et al.* (1992) *Differentiation* (in press)) have provided indications on genetic and epigenetic factors which control β enolase gene expression during *in vivo* myogenesis. Transcripts are specifically detected at 7.5 dpc in the forming heart which will never express any of the known myfkins. In newly-formed myotomes, if β enolase gene is regulated by myogenic factors, according to their initial time of expression, myf5 and myf6 are the putative members of the family which could act on the gene. In hindlimb muscles, the increase in β enolase gene expression occurs at selected stages of development: a first limited accumulation of transcripts occurs in primary fibres, a large increase at the time of innervation and secondary fibre formation followed by a postnatal increase at the time of fibre specialization, with a lower expression of this gene in slow fibres than in fast fibres. Our data strongly suggest a role for thyroid hormone during the postnatal accumulation of β enolase transcripts. Denervation experiments show an important role of innervation in the maintenance of high levels of these transcripts in adult muscle.

In agreement with these *in vitro* and *in vivo* studies, sequencing of the gene and flanking regions has revealed numerous putative regulatory motifs, including TRE and MyoD1-binding consensus sequences. Functional analysis of these motifs is underway.

Regulation of mouse myosin alkali light chain genes

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In mammalian striated muscle there are four principal myosin alkali light chain isoforms associated with sarcomeric myosin: MLC1F and MLC3F in embryonic and adult fast skeletal muscle, MLC1A in embryonic skeletal and cardiac atrial muscle, and MLC1V in adult slow skeletal and cardiac ventricular muscle (Barton and Buckingham (1985) *J. Biochem.* **231**, 249–61). The mouse MLC1F and MLC3F isoforms are transcribed from distinct promoters at a single locus on chromosome 1, and activated at 10 and 15.5 days of development, respectively (Lyons *et al.* (1990) *J. Cell Biol.* **111**, 1465–76). A muscle-specific enhancer element 3′ to the rat and human *MLC1F/3F* genes (Donoghue *et al.* (1988) *Genes Devel.* **2**, 1779–90) is conserved in the mouse gene, and a 257 bp fragment confers strong myotube-specific expression on a -1.2 kb 1F promoter-reporter gene construct in C2/7 cells, and also increases expression approximately 30-fold from a -2 kb 3F promoter-β-galactosidase construct. Mouse MLC1V transcripts accumulate in slow fibres from embryonic day 16 and in the heart from day 8, being restricted to the ventricle by day 16 (Lyons *et al.* (1990) *J. Cell Biol.* **111**, 2427–36). Functional analysis in C2/7 cells has led to the identification of positive and negative regulatory elements in the proximal promoter of the mouse *MLC1V* gene. A minimal construct of -116 bp is strongly expressed in both C2/7 muscle cells and 10T1/2 fibroblast cells, while a -220 bp construct is muscle cell specific. Cotransfection experiments with a MyoD expression vector suggest that this specificity may be mediated in part by an E-box consensus site at -185 bp. We have initiated experiments using transfected mouse embryonic stem cells to investigate expression from this promoter during ES cell differentiation *in vitro* into embryoid bodies (often containing cardiac muscle; Doetschmann *et al.* (1985) *J. Embryol. Exp. Morph.* **87**, 27–45), and during *in vivo* development of ES cell-colonized chimaeric mouse embryos.

Myogenic lineage differentiation in transgenic mice: evidence for a regulatory element specific for desmin in skeletal muscle

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The 1 kb genomic fragment characterized to direct the cell-type specific and differentiation-dependent expression of desmin in myogenic C2 cells, was shown to act as a promoter and an enhancer in the skeletal muscles of transgenic mice. Important functional elements with this fragment have been mapped. First, sequences in a 0.2 kb stretch 5′ to the transcriptional initiation site contain potential binding sites for SP1, AP1, and MyoD1 transactivating proteins. In fact we know that this 0.2 kb fragment is sufficient to promote expression at a low level in cultured muscle cells. However, it is not sufficient for high level expression (Li and Paulin (1991) *J. Biol. Chem.* **266**, 6562–70). Second, high level expression of the desmin gene depends on a 280 bp muscle-specific enhancer located between -693 and -973 bp upstream of the transcription initiation site. This enhancer contains two different classes of binding sites. One is active in replicating myoblast and the other in myotube. The former possesses several GC-rich sequences and latter contains the binding site for myogenic factors belonging to the helix-loop-helix MyoD1 family and MEF2 (Li and Paulin, unpublished data). The 1 kb derived sequences proved to be active in transgenic mice when linked to nlacZ and the histochemical stain for LacZ provides a sensitive means for studying patterns of expression at the cellular level. Expression of the Des-nlacZ was skeletal muscle specific. Des-nlacZ transgene expression begins within the loose somitic mesoderm and continues throughout its conversion into myoblast, formation of myotubes and organization of muscle fibres of the trunk and limbs. The desmin-nlacZ transgene was also active in the derivative of the prechordal and para-axial mesoderm which gave rise to the cephalic muscles. A striking demonstration of the additional information that can be obtained by use of a histochemically detectable reporter is the finding that the Des-nlacZ transgene is expressed in migrating cells in the limb buds of 11-day-old embryos demonstrating that these cells are already determined for myogenesis. Cardiac and smooth muscles which are formed from splanchnic mesoderm were uniformly nlacZ negative.

Studies on the role of hydrophilic amino acids and phosphorylation in the dimerization of the MyoD family of myogenic regulatory factors

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The myogenic regulatory factors are phosphorylated nuclear proteins that dimerize and bind to an E-box motif, CANNTG, found in the regulatory regions of many of the muscle-specific protein genes. A structural motif common to all members of this family of regulators

consists of a basic stretch of amino acids, the DNA binding element, joined to a putative helix-loop-helix (HLH) region which serves as the dimerization domain. The function of these regulators appears to be controlled mainly through the nature of the dimer partner and the affinity of the dimer, either homo or hetero dimer, for the target E-box. We present evidence from studies of the vertebrate and *Drosophila* myogenic factors that suggests the specificity of dimerization is determined, in part, by the nature of the nonhydrophobic amino acids in the HLH domain and this dimerization can be modified via phosphorylation.

Conditional immortalization of normal and dysgenic mouse muscle cells by the SV40 large T antigen under the vimentin promoter control

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We have created new mouse muscle cell lines of an immortalized type, expressing normal differentiation at the myotube stage: sarcomeric organization, functional EC coupling, and triadic differentiation. The immortalizing recombinant DNA utilizes a deletion mutant of the regulatory region of the human vimentin promoter controlling the expression of a SV40 thermosensitive large T antigen, in which the small t sequence has been deleted. Skeletal mouse replicative myoblasts synthesized predominantly vimentin. After myoblast fusion the vimentin gene is strongly repressed in multinucleated syncytia. Furthermore, the normal activity of the vimentin promoter in myoblasts is increased in the large T antigen-expressing cells. We observed that continuous and rapid division of myoblasts occurs at permissive temperature suggesting that immortalization is achieved even though the small t antigen is absent. When fusion is induced by changing media conditions, large T antigen expression is totally repressed by the vimentin promoter. When the temperature is elevated to 39°C, the pre-existing large T antigen is inactivated. The resulting myotubes from normal mouse differentiate totally normally as indicated by their morphology, ultrastructure and electrophysiological properties. Mutant (muscular dysgenesis) immortalized cells express the same properties as mutant primary counterparts with no contraction, no slow Ca^{2+} current, no triadic differentiation. These immortalized cell lines are potentially very useful for further pharmacology, transplantation and cell biology studies. The vimentin promoter control of immortalizing recombinant DNA can be used for any mammalian normal and mutant muscle cell lines.

Integration of a myogenic cell line into murine skeletal muscle following *in utero* myoblast transfer

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The implantation of normal muscle precursor cells is currently being developed as a means of ameliorating inherited muscle diseases, such as Duchenne dystrophy. To date, effective dispersal and incorporation of donor myoblasts into mature skeletal muscle has only been achieved by experimentally traumatizing the muscle prior to myoblast transfer. While this approach facilitates the integration of donor myoblasts, the efficacy of the transfer procedure cannot be evaluated because it is superimposed on a regenerating system. We have demonstrated that myoblasts can successfully be transplanted into fetal murine muscle during a period of extensive secondary myotube formation. ^{3}H-thymidine-labelled MM14 myoblasts were injected into the posterior crus of Swiss Webster fetuses at gestational day 16. Tissue was collected at birth or at 2 days postpartum, and labelled donor myonuclei were identified on autoradiograms of longitudinal and transverse sections. In all samples, extensive incorporation of donor cells occurred at the injection site. In addition, hybrid myotubes containing labelled and unlabelled nuclei were identified in adjacent areas, suggesting that MM14 cells can undergo considerable migration before fusing with host myofibres. The extent of donor cell integration into fetal muscles greatly exceeded that of cells injected into untraumatized or regenerating adult extensor digitorum muscles. These results indicate that the transfer of myoblasts into immuno-incompetent fetuses whose muscles contain loosely organized connective tissue and in which there is extensive *de novo* myotube formation, has the potential to overcome the problems encountered when applying the technique to older hosts, namely obtaining an efficient dispersal and integration of donor cells. We have demonstrated, by immunoblotting, that myotubes derived from MM14 cells or from primary cultures of C57BL/10J muscle can produce dystrophin *in vitro*. Utilizing the technique of *in utero* myoblast transfer, we are currently investigating the dynamic effects of dystrophin replacement in mitigating the degeneration-regeneration cycles that occur in mdx skeletal muscle.

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Actin mutants and the study of myofibrillogenesis in *Drosophila melanogaster*

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The regular structure and repeating pattern of sarcomeres in a myofibril implies that myofibrillogenesis is a well regulated set of processes. The mechanisms of myofibril assembly are being investigated in *Drosophila melanogaster* by molecular genetic studies of the indirect flight muscles (IFMs) of *Drosophila*. These particularly regular, striated muscles provide a powerful experimental system for this, especially because the flies are so genetically amenable. Flight is a dispensable function in flies in the laboratory and mutants with nonfunctional IFMs are viable. Flightless mutants are easily recovered and have been found in most of the major muscle protein genes. These genes are easily manipulated permitting a wide range of genetic approaches.

We will describe our studies using both the IFM-specific actin gene, *Act88F*, and the single muscle myosin heavy chain gene, *Mhc*, to dissect the molecular processes of myofibrillogenesis in the fly. These studies include: (1) examination of the effects of homozygous null mutants on filament assembly and fibre shape; thick and thin filaments can assemble independently to a large extent, but while actin null mutations affect fibre shape, myosin nulls have no such effects. In flies homozygous for null mutations of both actin and myosin heavy chain, fibre shape is normal, implying that the aberrant fibre shapes may result from the presence of thick filaments in the absence of thin filaments; (2) using null mutations to examine the relationship between gene dosage and myosin and actin accumulation to probe the regulation of protein stoichiometry. Our evidence shows regulation of actin accumulation, that is compensation for reduced gene dosage, but the same results were not obtained with the myosin heavy chain. These observations have consequences for the regulation of muscle protein accumulation; (3) using missense mutations in the actin gene to explore the relationships between alterations to actin binding sites and affects on thin filament assembly *in vivo*.

Expression and organization of muscle-specific proteins during the development of the rabbit heart

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The expression and intracellular distribution patterns of muscle-specific proteins were studied during rabbit heart development using muscle-specific monoclonal antibodies against titin, myosin, tropomyosin and actin, as well as the intermediate filament proteins desmin, keratin and vimentin. From our panel, titin appeared to be the first muscle-specific protein to be exclusively expressed in the embryonic rabbit heart. On cellular differentiation, titin reorganizes from dot-like aggregates into a cross-striated pattern (in 9 to 30-somite embryos) via a transiently filamentous distribution. The expression and organization of the other muscle proteins was studied in relation to titin. Tropomyosin followed on titin with respect to its exclusive expression in the heart anlagen and its organization into a striated pattern. Myosin and desmin were organized into cross-striated patterns after titin and tropomyosin. Actin, keratin and vimentin were distributed in cytoplasmic filaments in the early embryologic stages we investigated. As the first contractions are already detected in 3-somite embryos, we conclude that the organization of titin, tropomyosin, myosin and desmin into a striated pattern does not seem to be essential for the initiation of muscle cell function in the heart anlagen. The sequence of expression and organization of the proteins we investigated is species dependent and varies in time in different regions of the developing heart.

Continuous basement membrane with specific synaptic elements is associated with aneurally cultured human skeletal muscle cells

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Human skeletal muscle cells are completely encompassed by a basement membrane (BM), which is specialized at the site of neuromuscular junctions (the synaptic BM). Immunofluorescence shows that muscle cells *in vivo* are surrounded by a continuous layer of heparan sulphate proteoglycans (HSPGs), laminin, type IV collagen and fibronectin. HSPGs are distinctively concentrated at the synaptic BM and so are molecules reacting with isolectin B4 from *Vicia villosa*.

Myotubes cultured on the serum substitute Ultroser G and brain extract show a continuous layer of HSPGs, laminin and type IV collagen. At sites of acetylcholine receptor clusters, HSPGs and lectin-positive molecules are distinctively concentrated resembling the *in vivo* situation. In contrast, myotubes cultured on serum-containing media do not display these characteristics. Electron microscopy reveals that myotubes cultured on Ultroser G/brain extract are surrounded by a continuous BM. Proteoglycans are present on the external site of the lamina densa, and associated in a regular fashion with collagen fibrils.

In conclusion, BM associated with myotubes cultured on Ultroser G/brain extract resemble in many ways the *in vivo* situation, including synaptic specializations. The culture system may be instrumental in studies on the structure and function of human muscular (synaptic) BM.

Inhibition of the ectoprotein-kinase activity of L_6 myoblasts involves an inhibition of their fusion

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We have recently reported for the first time in the literature that a Ca^{2+} dependent ecto-protein-kinase is responsible for myoblast fusion (J. L. Lognonné and J. P. Wahrmann (1986) *Exp. Cell Res.* **166**, 340–56). To investigate whether the myoblasts can fuse in the absence of ATP, we used a nucleotide affinity label, fluoro-sulphonyl benzoyl adenosine (FSBA).

We demonstrate that the incorporation of FSBA inhibits fusion by inhibition of the ectoprotein-kinase(s) of the cells. ^{14}(C)-SBA is incorporated into seven protein bands and among them into a 48 kDa band the phosphorylation of which is indispensable for myoblast fusion and which could thus be the kinase or one of the kinases itself (J. L. Lognonné and J. P. Wahrmann (1988) *Cell Differ.* **22**, 245–58). Similar results have been obtained with primary rat myoblast cultures which is in favour of the hypothesis that this is a physiological mechanism which could play a role in muscle regeneration

In vitro differentiation of embryonic stem cell (ESC)-derived cardiomyocytes and skeletal muscle cells is specifically modulated by retinoic acid

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Embryonic stem cells (ESC) differentiate *in vitro* into derivatives of all three primary germ layers: endoderm, ectoderm and mesoderm. A defined cultivation system ('embryoid bodies' differentiating in hanging drops) was developed for the differentiation of ESC into spontaneously beating cardiomyocytes, allowing investigations of chronotropic effects and electrophysiological responses of different cardioactive drugs *in vitro*. Cardiomyocytes differentiating from pluripotent ES cell lines (D3, B117) develop heart-specific adrenoceptors and cholinoceptors and signal transduction pathways as well as L-type Ca^{2+} channels. The contractions of spontaneously beating cardiomyocytes were concomitant with rhythmic action potentials very similar to those described for embryonic cardiomyocytes and sinusnode cells (Wobus *et al.* (1991) *Differentiation* **48**, 173–82). The differentiation system allows the investigation of functional expression of receptors dependent on the differentiation status of the cardiomyocytes.

The differentiation-inducing effects of retinoic acid (RA) on cardiomyocyte development was tested after treatment during the following days of *in vitro* differentiation of embryoid bodies: 0 to day 2 (endodermal differentiation), days 2–5 (endodermal and ectodermal differentiation) and days 5–7 (ectodermal and mesodermal differentiation). RA treatment resulted in a concentration-dependent specific modulation of mesodermal differentiation (into cardiomyocytes or skeletal muscle cells) when applied during days 2–5 of differentiation. The expression of muscle-determination genes (e.g. myogenin) was investigated with PCR-analysis. In addition, the chronotropic and electrophysiological responses of cardiomyocytes, differentiated under the influence of RA, were studied. The cellular system described may be useful as an *in vitro* model for studying commitment and cellular differentiation, and therefore, may be useful to reduce the use of living animals in pharmacology and reproductive toxicology.

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Patterns of myosin isoform expression by cultured myoblasts indicate that satellite cells appear during late chicken embryogenesis

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The emergence of avian satellite cells during development has been studied using markers that distinguish adult from fetal cells. Previous studies by us have shown that myogenic cultures from fetal (embryonic day 10) and adult (12–16-week-old) chicken pectoralis muscle (PM) each regulate expression of the embryonic isoform of fast myosin heavy chain (MHC) differently. In fetal cultures, embryonic MHC is

co-expressed with a ventricular MHC in both myocytes (differentiated myoblasts) and myotubes. In contrast, myocytes and newly-formed myotubes in adult cultures express ventricular but not embryonic MHC. In the current study, the appearance of myocytes and myotubes which express ventricular but not embryonic MHC was used to determine when adult myoblasts first emerge during avian development. By examining patterns of MHC expression in mass and clonal cultures prepared from embryonic and posthatch chicken skeletal muscle using double-label immunofluorescence with isoform specific monoclonal antibodies, we show that a significant number of myocytes and myotubes which stain for ventricular but not embryonic MHC are first seen in cultures derived from PM during fetal development (embryonic day 18) and comprise the majority, if not all, of the myoblasts present at hatching and beyond. These results suggest that adult type myoblasts become dominant in late embryogenesis. We also show that satellite cell cultures derived from adult slow muscle give similar results to those derived from adult fast muscle. Cultures derived from embryonic day 10 hindlimb (a mixture of presumptive fast and slow fibres) form myocytes and myotubes that co-express ventricular and embryonic MHCs similar to cells of the embryonic day 10 PM. Thus, adult and fetal expression patterns of ventricular and embryonic MHCs are correlated with developmental age but not with muscle fibre type. Employing Northern blotting and using probes for myogenic determination genes and MHCs, we started analysing whether there are differences in the expression of these genes between fetal and adult myoblasts; temporal differences in the expression of mRNAs for the myogenic determination genes could perhaps contribute to the difference between the two cell populations.

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Behaviour of conditionally immortal myogenic cells *in vitro* and *in vivo*

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Permanent cell lines are popular tools for the study of myogenic differentiation. The usefulness of such cells is largely restricted to *in vitro* studies, however, as they form tumours when implanted into muscle. We have obtained conditionally immortal cells from the limb muscles of the *H-2K*^{b}tsA58 mouse which is transgenic for an immortalizing gene producing a thermolabile form of the SV40 large T antigen (SV40tsA58 large T antigen). Expression of this gene is linked to an inducible promoter (*H-2K*^{b}) which can be driven by the presence of interferon-gamma (IFN-γ).

H-2K^{b}tsA58 myogenic cells can be maintained in a proliferative state under permissive conditions (33°C + IFN-γ). When the cells are transferred to nonpermissive conditions (39°C-IFN-γ), DNA synthesis stops within 48 h, and contracting myotubes form over the subsequent 7 days. The resultant myotubes express characteristic cytoskeletal proteins and their development is accompanied by a normal pattern of expression of myogenic regulatory factors such as myogenin.

When injected into the leg muscles of mdx nude mice, *H-2K*^{b}tsA58-derived clones form new muscle which is histologically indistinguishable from that formed by the injection of primary cells. Analysis of glucose-6-phosphate isomerase (GPI) isoforms in the injected muscles revealed mosaic host/donor fibres, demonstrated by the presence of GPI heterodimers consisting of one host and one donor subunit. Other fibres were derived entirely from injected cells: these contained dystrophin, but no hybrid GPI. There was no evidence of tumour formation up to at least 120 days after injection. Furthermore, several weeks after implantation of *H-2K*^{b}tsA58-derived cells, temperature/IFN-γ-sensitive clones were re-isolated from the injected muscle. These cells formed new muscle when injected into a second generation of hosts. This suggests that some of the injected cells did not participate in muscle formation but remained within the tissue while retaining their myogenic potential, a behaviour pattern which resembles that of satellite cells.

Myogenic cells derived from transgenic *H-2K*^{b}tsA58 mice can be used to generate large numbers of clonally-derived cells which do not exhibit the problems of senescence or neoplasia. The ease with which these cells can be cloned also provides the potential to isolate clones of different developmental or functional types.

Behaviour of myogenic cells *in vivo*

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Myoblast transfer is of interest both as a possible therapeutic procedure for the treatment of inherited myopathies and as a research tool for the study of muscle gene expression *in vivo*. Immortalized cell lines are commonly used in muscle research as they are easy to maintain and provide large numbers of well characterized cells. We have compared the *in vivo* behaviour of the myogenic lines C2C12 and AM1/2, conditionally immortal clones of *H-2K*^{b}tsA58 cells and primary cells prepared from neonatal mice.

In culture, *H-2K*^{b}tsA58 cells, C2C12, and AM1/2 cells are capable of apparently indefinite proliferation. Under appropriate conditions, these cells differentiate in a similar way to that observed with primary myogenic cell preparations. We have followed the expression of myogenic regulatory proteins such as myoD and myogenin, and muscle specific proteins such as dystrophin, myosin and actin, during myogenesis.

Whereas all the cell lines were capable of differentiating in culture, their behaviour *in vivo* differed considerably. Preparations of primary cells and *H-2K*^{b}tsA58 cells formed normal, dystrophin-positive muscle when injected into dystrophin-negative mdx mouse muscle. In contrast, C2C12 and AM1/2 cells formed little new muscle, and continued to proliferate as single interstitial cells which disrupted the normal pattern of the muscle.

These results suggest that immortalized cell lines have limited uses *in vivo* because of a tendency to form proliferating masses. However, the behaviour of the *H-2K*^{b}tsA58 cells was similar to that of primary cells and, therefore, these conditionally immortalized cells are likely to be of considerable value in experimental systems *in vivo*.

Development and maturation of the human diaphragm

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A study on the development of the diaphragm muscle has been carried out on 15 fetuses aged between 13 and 37 weeks of gestation and on three children who were 1 week, 5 months and 3 years old at the time when the biopsies were taken. Subjects with multiple congenital abnormalities or neuromuscular diseases were not included in this study. The formation and development of the different populations of muscle fibres was analysed by enzyme histochemistry (myosin ATPase and oxidative enzymes) and immunohistochemistry (using antibodies directed against vimentin, desmin and the different isoforms of the myosin heavy chain). Possible modifications which could occur in the various contractile proteins and especially myosin were revealed by electrophoretic (2D gel electrophoresis, native pyrophosphate gel electrophoresis, 6% PAGE-glycerol gel electrophoresis) analysis of muscle extracts.

Morphological studies have shown that the diaphragm develops from two successive generations of muscle fibres in a way very similar to that which we have described previously for the quadriceps. However, immunocytochemical and biochemical analysis of these

samples has revealed that although on the one hand there is a precocious expression of several of the different myosin light chains, there is an abnormally long persistence of both the embryonic myosin light chain MLC1emb and the fetal MHC. We have also been able to show the existence of the MHC IIX or IID in the human diaphragm.

Isolation and characterization of a developmentally controlled myosin heavy chain

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Vertebrate myosin heavy chains (MyHC) are represented in the genome by a multigene family. The MyHC genes are expressed in a spatially and temporally distinct pattern during development. To obtain molecular probes for developmentally-regulated MyHC isoforms in the rabbit, we screened a cDNA library, constructed from a 5-day-old rabbit, with an oligonucleotide covering the highly conserved ATP-binding site. We obtained two overlapping, but not identical, cDNA clones, called OCMYHCP1 and OCMYHCP2. OCMYHCP1 covers the head region up to the first actin binding site (23 and 50 kDa domains), whereas clone OCMYHCP2 covers half of the 50 kDa domain and the complete 24 kDa domain (up to the last proline). The nucleotide and deduced amino acid sequences of these cDNA clones show the usual strong homology with other sarcomeric MyHCs. The strongest homology can be found with a previously described cDNA, encoding a human perinatal isoform (Feghali and Leinwand (1989) *J. Cell Biol.* **108**, 1791–97).

In situ hybridization experiments show that these cDNAs are not expressed in adult muscle tissues but are detectable in early postnatal limb muscle. In contrast, a probe specific for the adult type IIB isoform is not detectable in early postnatal stages but stains heavily the muscle tissues of 19-day-old animals.

Analysis of expression and function of cell adhesion molecule M-cadherin in skeletal muscle cells

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Previously, we have identified cell adhesion molecule M-cadherin, a member of the cadherin multigene family, in myogenic mouse C2 cells (Donalies *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8024–8). M-cadherin is expressed at low level in C2 myoblasts but is upregulated in C2 myotubes. In adult tissues, M-cadherin mRNA is present in skeletal muscle and in low amounts in brain but is absent or almost undetectable in other tissues (e.g. heart or liver; Northern blot and PCR analysis). Thus at least in the adult organism, M-cadherin appears to be expressed predominantly in skeletal muscle cells.

As other members of the cadherin family have been shown to be involved in morphogenetic processes, we hypothesized that M-cadherin might play a role in morphogenesis of skeletal muscle cells such as in the control of myotube formation. To test this idea, two types of approaches were chosen: (1) synthetic peptides comprising the putative binding site of M-cadherin were added to rat L6 cell cultures induced to myotube formation, and (2) chimeric gene constructs from which anti-sense M-cadherin mRNA could be expressed, were stably introduced into L6 myoblasts and tested for their effect on myotube formation. In both types of experiments, the block of M-cadherin function inhibited the ability of L6 cells to form myotubes. These results clearly indicate that the control of myotube formation could be indeed one function of M-cadherin during myogenesis. This does not exclude, however, that M-cadherin might also play a role later in myogenesis, i.e. the development from the myotube to the adult muscle fibre.

Expression of α-cardiac and α-skeletal actin mRNA in the developing human heart

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In the human heart two different sarcomeric isoforms of actin, α-cardiac and α-skeletal, are expressed. These isoforms are encoded by two different genes that have a highly conserved nucleic acid sequence. The mRNAs coding for these two isoactins, however, differ markedly in the 3'- and 5'-untranslated regions. Using probes derived from the 3'UTR (Gunning *et al.* (1984) *J. Mol. Evol.* **20**, 202; Boheler *et al.* (1991) *J. Clin. Invest.* **88**, 323) we have investigated the spatial distribution of the respective mRNAs in the developing human heart between weeks 6 and 12 of development by *in situ* hybridocytochemistry. A high expression of α-cardiac actin mRNA was found in all myocardial structures in every heart examined, no spatial heterogeneity being observed. In contrast, at 6 weeks of development no expression of α-skeletal actin mRNA could be detected in the heart. In the subsequent stages of development, the relative amount of this mRNA increases. Interestingly, in these hearts the specific hybridization with the α-skeletal actin probe is confined to the developing papillary muscles, whereas no expression of α-skeletal actin mRNA is observed in the myocardium of the free walls of the ventricles or in the atrial myocardium. As (1) no functional differences between the α-skeletal and α-cardiac isoforms of actin are known, and (2) α-skeletal actin is the main isoform in adult human heart (Boheler *et al.* (1991) *J. Clin. Invest.* **88**, 323), the localized expression of α-skeletal actin mRNA in the myocytes of the developing papillary muscles after week 6 of development most probably reflects an advanced state of maturation of these structures.

Study on the conditions of gene transfer in mouse skeletal muscle

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The possibility of human gene therapy for the muscular dystrophies has been encouraged by the recent successes of gene transfer by direct injection or using viral vectors into rodent or primate muscle. However, those results have shown insufficient levels of expression and too great a variability to serve as a basis for human trials at present. We have undertaken to evaluate some of the conditions affecting the uptake and expression of purified plasmid DNA introduced by direct intramuscular injection. Mouse tibialis anterior (TA) muscles were assayed for luciferase activity at 1–60 days following a single injection of an SV40-luciferase promoter-reporter gene construct under various conditions. When the plasmid was administered by intramuscular injection, luciferase expression was highly variable, as has been found in previously reported studies. This was likely due, at least in part, to problems of physical distribution of the injected DNA and/or its accessibility to the fibres. For example, it was shown that when ink was injected into TA muscles, it remained almost entirely interfascicular in some muscles, whereas in others it would diffuse widely and surround individual fibres. The distribution of injected ink was more extensive and less variable if the muscles were pretreated by injection of a large volume of 25% w/v sucrose in PBS. Presumably the pretreatment forced the fibres apart and allowed a better distribution of the subsequently injected substance. Indeed, muscles injected with the SV40-luciferase plasmid had less variable expression if they were pretreated with a PBS or a PBS-25% sucrose solution. Gene expression after plasmid injection was dose-dependent

if muscles were pretreated, and was less variable if given in a large rather than a small volume. The effect of sucrose pretreatment on the distribution of fibres expressing the reporter gene was visualized in TA muscles by injection with an RSV-Lac Z promoter-reporter gene construct followed by sectioning and histochemical detection of β-galactosidase. Fibres expressing β-galactosidase were generally scattered around the injection site, but these fibres were not, for the large part, those which were damaged by the injection procedure (identified by the presence of neonatal myosin heavy chain). Luciferase reporter gene expression was transient if driven by SV40 promoter, but was high and sustained if driven by an RSV promoter.

Gene transfer in regenerating normal and *mdx* mouse muscle

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Gene transfer by either direct injection or using viral vectors has been carried out on muscle tissue or myogenic cell cultures in attempts to find viable strategies for gene therapy of the inherited muscular dystrophies. Because dystrophic muscle frequently contains many regenerating fibres we examined gene transfer in regenerating muscle. Mouse tibialis anterior (TA) muscles were induced to undergo a single cycle of degeneration-regeneration by the injection of 10 μM cardiotoxin (CaTx) and at various times following this treatment were subjected to direct or indirect gene transfer. At 1–15 days after CaTx treatment, an SV40-luciferase promoter-reporter gene construct was injected intramuscularly, and luciferase levels were measured 5 days later. Expression increased up to 11 days after CaTx injection, then reached a plateau. The levels of expression were at least equivalent to those obtained in normal muscle; however, there was significantly less variability in the results. The distribution of expression was also examined in regenerating TA muscles following injection of a recombinant adenovirus containing the Lac-Z reporter gene under the control of the same muscle-specific regulatory sequences (courtesy of B. Quantin and J-L. Mandel, Strasbourg) and found to cause widespread transfection of fibres throughout the entire muscle.

Transcriptional regulation of exogenous genes transferred into *Xenopus* tadpole muscle *in vivo*

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Somatic gene transfer into skeletal muscle *in vivo* could be a potentially powerful tool to analyse gene regulation; however, a drawback to this at present is the high variability of expression of the foreign gene. To circumvent this problem we have developed a procedure for double DNA injection into *Xenopus* muscle which allows simultaneous *in vivo* expression of the gene under physiological study and of a luciferase gene driven by a constitutive promoter. Chimeric DNA constructs were injected into the dorsal muscle mass of *Xenopus* tadpoles which were arrested in prometamorphosis by immersion in sodium perchlorate ($NaClO_4$). Expression of constructs containing the coding sequence for the chloramphenicol acetyl transferase (CAT) gene driven by either: (1) a minimal promoter downstream from the thyroid hormone (T_3) responsive element (T_3RE), or (2) the 1500 base pairs upstream to the transcriptional start site of the murine myosin heavy chain IIB (MHCIIb) promoter, was normalized against the activity of the cotransfected SV40-luciferase gene construct. It was shown that gene expression: (1) was strong and reproducible, (2) was highly correlated to the amount of injected DNA, and (3) increased continually over time (up to 4 months). On removal of $NaClO_4$, which allowed T_3-dependent metamorphic climax to ensue, apoptosis of the tail muscle occurred in a manner identical to that in control tadpoles and no further gene expression was measurable. Thus, this study demonstrates for the first time that somatic injection of chimeric DNA constructs into skeletal muscle may be used to analyse the physiological regulation of muscle gene transcription *in vivo*. The model also provides an alternative to *in vitro* systems for identifying *cis*- and *trans*-acting factors regulating transfected genes. We are currently investigating the thyroid hormone-dependent transcriptional regulation of expression from the T_3RE and MHCIIb promoters in our system.

Muscle and nerve–muscle cultures on microcarriers

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Microcarriers (MCs) were originally developed to enable large scale propagation of cells for the production of viral vaccines, interferon, recombinant products, etc. A wide range of MCs are commercially available, they differ in their form (spherical or cylindrical), composition (dextran, cellulose, gelatin, glass, etc), porosity (microporous or macroporous) and surface characteristics (positive or negative charge or coating with collagen or gelatin).

Fetal or neonatal primary cultures of striated and cardiac muscle cells and combined nerve–muscle cocultures are usually propagated in bidimensional monolayers. To culture these cells in a tridimensional organization, we have tested a variety of MCs as potential support for cell attachment and growth. From a large number of tested MCs, only two types were found suitable: DE-53 (Whatman, UK) and CultiSpher-GL (Hyclone, Sweden).

The DE-53 cellulose MCs are cylindrical in shape (40–50 μm diameter and 80–400 μm length) having a positive charge of 2 mEq/g dry materials. CultiSpher-GL are macroporous gelatin made MCs having a spherical shape (140–320 μm diameter).

Within 1–2 h after seeding, 70–90% of the cells attach to both types of MCs to form cells–MCs aggregates which remain floating in the growth medium. Myoblasts aggregates develop into myotubes after day 5 of cultivation and can be maintained in culture for more than 1 month. The aggregates obtained with CultiSpher-GL MCs differ from those obtained with the DE-53 MCs, in their size (0.5–1 versus 1–3 cm), fibre orientation (random versus parallel) and contraction (most of the CultiSpher-Gl aggregates contract actively, while the DE-53 aggregates contract only occasionally).

Cell growth and differentiation is obtained also in nerve–muscle MC cultures. Muscle cell differentiation occurs in these cultures in a similar pattern as described above; however, fusion of myoblast into myotubes occurs only towards week 2 in culture. The nerve cells sprout on the surface of the myotubes to form nerve muscle interconnections. In general, creatine kinase and acetylcholine receptors are formed in similar quantities and have a similar kinetics in monolayer and MC-cultures. Finally, cardiac muscle cells grow and contract spontaneously only on CultiSpher-GL MCs.

We foresee future use of these organized structures composed of MCs and highly differentiated nerve and muscle cells, in acute and chronic exposure to toxicological agents, ageing studies and implantation into injured or distrophic tissues.

MEMBRANES AND ION CHANNELS

ATP-driven ion pumps and voltage-sensitive ion channels in cultured muscle cells and/or skeletal muscle of myotonic dystrophy patients

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Myotonic dystrophy (MyD) patients show significantly higher increases in plasma K^+ concentration on ischaemic forearm exercise than controls (Wevers *et al.* (1990) *Muscle Nerve* **13**, 27–32). Defective regulation of ion transport could initiate or contribute to the abnormal cellular function in MyD. Na^+/K^+-ATPase, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and voltage-dependent Na^+ and Ca^{2+} channels were examined in cultured skeletal muscle cells and/or skeletal muscle of controls and MyD patients.

Na^+/K^+-ATPase is investigated by measuring ouabain binding and the activities of Na^+/K^+-ATPase and K^+-dependent 3-O-methylfluorescein phosphatase (3-O-MFPase). SR Ca^{2+}-ATPase is analysed by ELISA, Ca^{2+}-dependent phosphorylation and its activities on ATP and 3-O-methylfluorescein phosphate. The concentration of voltage-sensitive Na^+ and Ca^{2+} channels are quantified by saxitoxin and PN200-110 binding, respectively.

In cultured muscle cells, all ATPase parameters investigated increase with the maturation grade. The number of ouabain-binding sites and the activities of Na^+/K^+-ATPase and K^+-dependent 3-O-MFPase are higher in cultured cells than in adult muscle. The SR Ca^{2+}-ATPase concentration and the activities of SR Ca^{2+}-ATPase and Ca^{2+}-dependent 3-O-MFPase remain lower in cultured muscle cells compared with adult muscle.

In cultured muscle cells of MyD patients the concentration as well as the activities of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase are reduced. The lower K^+-dependent 3-O-MFPase activity and the decreased concentration and activities of SR Ca^{2+}-ATPase in homogenates of MyD muscle also indicate a reduced ATP-dependent ion transport. The ouabain-binding constant and the molecular activities of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase are similar in cultured cells and adult muscle of controls and MyD patients. Thus, the decreased activity of both ATPases is caused by a reduction of the number of their molecules. In MyD muscle, the content of voltage-sensitive Na^+ channels is doubled, whereas the concentration of voltage-sensitive Ca^{2+} channels is unchanged. The decrease of ATP-driven pumps and the increase of voltage-dependent Na^+ channels may contribute to the pathology of MyD muscle.

Immunocytochemistry of N-CAM in noninnervated skeletal muscle fibres

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The cell surface glycoprotein N-CAM (neural cell adhesion molecule) is supposed to be involved in cell contacts of neural cells and between neural cells and muscle fibres. In mature skeletal muscle fibres, N-CAM has been found at the junctional membrane of the motor endplate, in T-tubules immediately adjacent to the endplate, and in satellite cells. Light micrographs of denervated muscle fibres, however, show immunoreactivity on the entire surface of the fibre and, to a variable extent, also in its interior. The aim of this study was to clarify the intracellular localization of N-CAM in denervated and also in aneurally regenerating muscle fibres.

The soleus muscle of adult rats was denervated or denervated and injured to induce regeneration. The rats were perfused with paraformaldehyde after 2 days to 4 weeks, and individual muscle fibres were isolated and immunostained. Two polyclonal antibodies against N-CAM were used. The binding sites were labelled with a secondary antibody and the horseradish peroxidase-diaminobenzidine method. The fibres were osmicated, embedded in epoxy resin and studied by electron microscope.

Reactive sites were found at the plasma membrane, at triadic junctions, and in the membranes of clustered subsarcolemmal vesicles. Staining of triadic junctions produced a pattern of distinct cross striations when longitudinal sections for light microscopy were cut from EM blocks. Denervated and aneurally regenerating fibres did not differ. Innervated muscle fibres were nonreactive outside the endplate region. N-CAM is an integral membrane protein. Its expression in muscle fibres increases in the absence of innervation; hence, its presence at triadic junctions may simply reflect the fact that the T-system is part of the muscle fibre surface. The clusters of subsarcolemmal vesicles may be part of the Golgi system and thus participate in the exocytosis of N-CAM.

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Chloride channel regulation of mammalian skeletal muscles in different pathophysiological conditions

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In mammalian skeletal muscle, over two-thirds of the resting membrane conductance is the result of Cl^- permeability. Defects in the regulation of chloride conductance (G_{Cl}) are important in the pathophysiology of skeletal muscle. Indeed, a reduction of G_{Cl} results in a repetitive firing abnormality known as myotonia (Bryant and Morales-Aguilera (1971) *J. Physiol.* **219**, 367). Moreover, a decreased G_{Cl} was found in skeletal muscle of aged rats (De Luca *et al.* (1990) *Pflügers Arch.* **415**, 642). Long-term modulation of ion channels by phosphorylating pathways has a recognized role in the physiological tuning of cell functions (Levitan (1985) *J. Membr. Biol.* **87**, 177). Therefore, we have investigated the biochemical pathways involved in the modulation of muscle Cl^- channels to test the hypothesis that changes in some modulatory steps could account for the lowered G_{Cl} recorded under the above reported conditions. Toward this aim, using a computerized two microelectrode technique, we tested the effects of various agents that modulate protein kinase C (PKC), protein kinase A (PKA), Ca^{2+} and the guanidine nucleotide binding protein system, on G_{Cl} of rat extensor digitorum longus (EDL) muscle from adult and aged (29 month) rats and on G_{Cl} of external intercostal muscle from normal and myotonic goats. The PKC activator 4-β-phorbol-12,13 dibutyrate blocked G_{Cl} in adult rats (IC_{50} = 37.5 nM) (Tricarico *et al.* (1991) *Pflügers Arch.* **418**, 500) but was more potent in decreasing G_{Cl} in aged rats (IC_{50} = 2.2 nM). Staurosporine failed to restore the low G_{Cl} of aged rats but prevented the effect of phorbol ester in both age groups. Cholera toxin decreased G_{Cl} by 40% in adult (effect antagonized by staurosporine) but not in aged EDL. Elevation of cytosolic calcium by 1 μM A23187 produced a 25 and 40% decrease of G_{Cl} in adult and aged muscles, respectively. Similar effects were produced by the above agents on normal goat muscles (Bryant and Camerino (1991) *Pflügers Arch.* **417**, 605), showing that PKC, together with cytosolic Ca^{2+} and the involvement of a cholera toxin sensitive G protein, specifically modulates muscle Cl^- channels. An overactivity of PKC could be involved in the low G_{Cl} of aged rats. This is not the case in mytonic goat muscle; indeed, in general, none of the biochemical modulators that produce effects on normal fibres produced any restoration of G_{Cl} in the myotonic goat fibres.

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Analysis of membrane protein components of EC coupling in rat fast-twitch muscle during early postnatal development and ageing

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The characteristics of contraction of fast-twitch muscles are similar to those of slow-twitch muscle at birth. In the rat, the twitch becomes progressively quicker and reaches the adult response around 4–5 weeks after birth and it does not further change until advanced age (24 months), when it becomes relatively prolonged. Our results deal with ontogenic changes in rat tibialis anterior of specific protein markers of the junctional membrane domain of sarcoplasmic reticulum (SR) and of transverse tubules (TT), taking advantage of a recently described preparative procedure (Damiani *et al.* (1991) *BBRC* **175**, 858–65). Consistent with physiological data, a low density (Bmax about 8 pmoles/g m^{-1}) of high-affinity [^{3}H]ryanodine receptor (RyR) sites, and comparable with that of adult slow-twitch soleus, was observed in the tibialis of 1-day-old rats. The number of RyR sites increased steadily during development, and once adult values were attained at about 4 weeks after birth, it did not seem to change appreciably through ages 6–24 months, as did their affinity for ryanodine. The relative abundance of Ca^{2+} and LDL-binding protein, as well as calsequestrin, appeared to correlate closely with the density of RyR sites through the period investigated. The RyR of SR has been proposed to interact directly at the triad junction with the dihydropyridine receptor (DHP-R) on junctional TT, which is in turn believed to act mainly as a voltage sensor. According to the same theory, the number of voltage sensors should be twice that of ryanodine receptors. We provide evidence for the presence of a relatively large excess of DHP-R in muscle of adult rats, suggesting an age-related change in their functional role in relation to EC coupling.

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Expression of the major Cl^--channel CIC-1: regulation by muscle activity

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Genes for ion channels are major targets for hereditary muscular diseases. The excitability of skeletal muscle depends on the concerted action of different ion channels (Na^+, K^+ and Cl^- channels) and is developmentally regulated. In mature skeletal muscle, Cl^- conductance prevents self-excitation by stabilizing the resting membrane potential. Blockade or reduction of Cl^- conductance by drugs or mutations leads to a hyperexcitability of the muscle fibre and, as a chronic condition, to the muscle disease myotonia.

We have recently shown that the recessive autosomal myotonia of the mouse, ADR (genotype *adr/adr* (review: Jockusch, H. In D. Pette (ed.) *The Dynamic State of Muscle Fibres*, Walter de Gruyter, New York, 1990, pp. 429–43), is caused by a retroposon insertion into the muscular chloride channel gene, *adr/Clc-1* on Chr 6 (Steinmeyer *et al.* (1991) *Nature* **354**, 304–8). The levels of CIC-1 mRNA and the development of myotonic symptoms follow similar time courses. In neonatal rat (Steinmeyer *et al.* (1991) *Nature* **354**, 301–4) and neonatal mouse muscle as well as in cultured mouse myotubes, CIC-1 is expressed at very low levels. During the first few postnatal weeks, the CIC-1 message is steeply upregulated as is Cl^- conductance (Carmerino *et al.* (1989) *Pflüger's Arch.* **413**, 568–70). Here we investigate how the expression of the *Clc-1* gene responds to different patterns of muscle activity using the mRNA for α-actin as an invariant standard and that for parvalbumin (PV) to indicate the response of gene expression to the physiological state. In the rat, the level of CIC-1 mRNA was higher in fast glycolytic (EDL) than in slow (soleus) muscle; PV mRNA is high in the former and extremely low in the latter. Myotonia in humans and in the ADR mouse can be cured symptomatically by tocainide. This drug reduces the number of activatable sodium channels and as a consequence stabilizes the resting membrane potential. Myotonia downregulates and tocainide upregulates the expression of MHC IIB and PV. We show that CIC-1 mRNA is upregulated in muscles of tocainide treated wildtype and ADR mice as compared with untreated controls. Thus, this upregulation is independent of whether the CIC-1 mRNA is functional or not. In general, high levels of CIC-1 mRNA are positively correlated with the expression of MHC IIB and of PV and thus with phasic rather than tonic muscle activity.

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Chromosomal mapping of muscle expressed sodium channel genes in the mouse

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The generation and propagation of action potentials in muscle cells and neurons of vertebrates depend on Na^+ channels, the principal subunit of which is the α-subunit. At least five different α-subunits have been cloned and molecularly characterized in the rat alone. Of the relevant genes (using the nomenclature of the mouse), *Scn 1a*, *2a* and *3a* are expressed in the brain, whereas *Scn 4a* and an additional gene herewith termed *Scn 5a* are expressed in muscle. *Scn 4a* is the gene for the TTX sensitive Na^+ channel SkM1 of adult skeletal muscle, whereas *Scn 5a* codes for the TTX insensitive Na^+ channel SkM2 characteristic of neonatal and denervated skeletal as well as of cardiac muscle (for reviews: Mandel (1992) *J. Membrane Biol.* **125**, 193–205). To chromosomally map the muscle expressed genes *Scn 4a* and *Scn 5a* in the mouse, an interspecific backcross of *Mus musculus* and *Mus spretus*, strain SEG (C57BL/6J *wr/+* × *Mus spretus* +/+) F_1 *wr/+* × C57BL/6J *wr/+* (Kaupmann *et al.* (1992) *Genomics* **13**, 39–43) and the following probes were used. Rat-SkM1: cDNA probe 6-2-1 (nt. 2820-3580; interdomain II-III region) and the genomic probe 14.1/1.7 (5′ untranslated sequences). Rat-SkM2: isoform specific cDNA probe 15-2-1 (nt. 6203-7075; 3′ untranslated region). Restriction fragment length variants (RFLVs) specific for *Mus spretus* (SEG) were screened for by Southern hybridization using *Bam* HI, *Bgl* II, *Eco* RI, *Msp* I, *Pvu* II, *Rsa* I, *Sal* I, *Sca* I, *Sma* I, *Sst* I, *Taq* I and *Xba* I. The SkM1 probe 6-2-1 detected an SEG specific *Sst* I RFLV that cosegregated with markers on Chr 11 and, at low stringency, an additional *Pvu* II fragment that localized to Chr 2. The position of sequences on Chr 11 detected with probes 6-2-1 and 14.1/1.7, defines the locus for the adult Na^+ channel SkM1, confirming Ambrose *et al.* (1992, *Mammalian Genome* **3**, 151–5) with a different set of markers. The most likely gene order on Chr 11 with the map distances expressed in centimorgans is: cen-*Il*-3-6.1 ± 2.9-*Myhs*-8.1 ± 3.2-*Pfn*-22.2 ± 5.2-*Scn 4a*-2.6 ± 2.6-*Myla*. With the SkM2 probe defining gene *Snc 5a*, only one SEG specific fragment was detected, and this showed close linkage with *Mylc* on Chr 9. The most likely gene order is: cen-*Cyp*1a2-10.3 ± 4.9-*Tma*-9.1 ± 6.1-*Mod*1-18.2 ± 8.2-*Mycl*-9.1 ± 3.3-*Scn 5a*. *Scn 5a* does not fall into an established region of conserved human/mouse synteny, but is located close to a region, extending from *Acy*-1 (proximal) to *Mycl* with homology to human Chr 3p. Whereas the human homologue of *Scn 4a* is involved in certain

hereditary myotonias, no mutations in human or mouse are so far known for *Scn5a*.

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Chromosomal mapping in the mouse of K^+ channel genes expressed in brain, heart and skeletal muscle

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The *Shaker* gene of *Drosophila* codes for K^+ channels and gives rise to a variety of alternative transcripts (Pongs *et al.* (1988) *EMBO J.* **7**, 1087–96). In contrast, the diversity of *Shaker*-related K^+ channels in mammals is due to the existence of multiple intronless genes (review: Jan and Jan (1990) *TINS* **13**, 415–9) as concluded from sequence data.

To show the existence of multiple *Shaker*-related gene loci in mammals more directly we have started to chromosomally map K^+ channel genes in the mouse using cDNA probes of the rat channels Rk 1, 2, 3 and 4 (Roberds and Tamkun (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1798–802). Rk 2 identified a gene (provisional symbol *Rk 2)* with linkage to the Chr 3 markers *Amy-1* and *Hao-2* (most likely gene order: cen-*Hao-2-Rk 2-Amy-1),* whereas Rk 3 located a gene (provisional symbol *Rk 3*) on Chr 2 (most likely gene order: cen-*Neb-Tit-Scn 1a, 2a, 3a-Rk 3-Pax-6*; cf. Müller *et al.*, Klocke *et al.*: this meeting). The map positions of the *Shaker*-related genes identified with probes Rk 1 and Rk 4 and of a member of the *Shal*-related genes identified with probe Rk 5 (Roberds and Tamkun (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1798–802) are presently being determined.

The knowledge of the map positions in the mouse of K^+ channel genes might facilitate the mapping of homologous genes in humans and could reveal informations regarding the involvement of K^+ channels in genetic diseases.

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Molecular mechanism and modulation of AChR desensitization in vertebrate muscle

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Interosseus muscles of adult mice were dissociated enzymatically and prepared for patch-clamping within 3 h or cultivated in standard tissue culture for 14 days (denervated muscle), respectively. Outside-out patches of the adult and denervated mouse muscle fibres were superfused repetitively by pulses of acetylcholine (ACh) in concentrations between 0.1 and 1000 μM with the liquid filament switch. Concentration changes were effected within 0.2 ms. In adult muscle single-channel conductance was 64 pS, apparent mean open time was about 1 ms (*Pflügers Arch.* **417**, 509–16, 1991). Denervated muscle had a single channel conductance of 36 pS and a mean open time of 2.5 ms. Channel currents elicited by pulses of ACh were averaged. Between 1 μM and 10 μM ACh for adult muscle the peak responses increased proportional to the second to third power of ACh concentration and less steeply between 100 μM and 1000 μM ACh. The half-maximal response was at 100 μM ACh for adult and at 60 μM ACh for denervated muscle. The dose-response curves for the peak-current had double logarithmic slopes of 1.1–1.3, consistent with two binding sites at the embryonic nicotinic acetylcholine receptor (*J. Physiol.* **451**, 145–58, 1992). In comparison with the data of the adult receptors the dose-response curve of the embryonic receptors was shifted to the left, i.e. these receptors are more sensitive. Also the desensitization of the embryonic receptors is less complete compared with that of the adult ones. After 100 ms desensitization with 100 μM ACh the channel opening in adult muscle had decreased to less than 1/300 peak value, whereas in denervated muscle it decreased to just 1/100 peak value. The time constants of desensitization increased more steeply in denervated (20–50 ms with 1000 μM ACh to >1 s with 1 μM ACh) than in adult muscle (20–50 ms with 1000 μM ACh to 400–500 ms with 1 μM ACh). The rise time of peak current was measured from 0.1 to 0.9 of the peak current. It increased from 0.3 ms at 1000 μM ACh to a plateau value of 5 ms for adult and of 10 ms for embryonic receptors at concentrations <10 μM ACh. With these data an analysis of the possible routes of desensitization in nicotinic ACh receptors is done and a circular reaction scheme proposed.

Structural studies of glycosphingolipids from mouse and rabbit skeletal muscle

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Glycosphingolipids (GSLs) are ubiquitous components of the outer surface of animal cell membranes. Structures and functions of GSLs have been widely reviewed (Stults *et al.* (1989) *Methods Enzymol.* **179**, 167–214). Their localization suggests that they are involved in several membrane-mediated phenomena. Two classes of GSL function can be distinguished: one as receptors for bacterial toxins as well as for cell-cell interactions, the other as regulators through modulation of functional membrane proteins such as transducers and transporters (Igarashi *et al.* (1989) *Methods Enzymol.* **179**, 521–41). In this study we describe the isolation and structural characterization of neutral GSLs and gangliosides, sialic acid (Sia) containing GSLs, from mouse and rabbit skeletal muscle.

Neutral GSLs and gangliosides were isolated and purified by standard procedures (Müthing *et al.* (1991) *Glycoconjugate J.* **8**, 414–23) and structurally characterized by TLC-immunostaining (overlay technique) with specific monoclonal and polyclonal antibodies (Müthing *et al.* (1992) *Glycoconjugate J.* **9** (in press)).

Neutral GSLs of rabbit muscle showed a simple TLC pattern expressing only monohexosylceramide (MHC). In addition to MHC, lactosylceramide (Galβ1-4Glcβ1-1Cer), lacto-N-*neo*tetraosyl ceramide (nLcOse$_4$Cer, paragloboside; Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) and Forssman-GSL (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) were detected in the neutral GSL fraction of mouse muscle.

The major ganglioside in both species was G_{M3} (sialyl lactosylceramide), however, differing in their substitution with N-acetyl (NeuAc) and N-glycolyl neuraminic acid (NeuGc). Muscle gangliosides of rabbit are characterized by the absence of NeuGc, expressing exclusively G_{M3} (NeuAc), whereas G_{M3} (NeuAc) and G_{M3} (NeuGc) were found in a 1.85:1 ratio in mouse muscle. The presence of lactosamine-containing gangliosides was characteristic of both species. Sialyl lacto-N-*neo*tetraosyl ceramide (IV Sia-nLcOse$_4$Cer), sialyl lacto-N-*nor*hexaosyl ceramide (VI Sia-nLcOse$_6$Cer; VI Sia-[Galβ1-4GlcNAcβ1-]$_2$3Galβ1-4Glcβ1-1Cer) and sialyl lacto-N-*nor*octaosyl ceramide (VIII Sia-nLcOse$_8$Cer; VIII Sia-[Galβ1-4GlcNAcβ1-]$_3$Galβ1-4Glcβ1-1Cer) were found in both animals, with the exception of the latter ganglioside, in a relatively high concentration. Furthermore, minor amounts of the ganglio-series gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} were detected in muscle ganglioside fractions of both species.

In summary, G_{M3} represents the major GSL in mouse and rabbit muscle. Remarkable species variations were detected in the neutral GSL fraction. The presence of neolacto- and ganglio-series gangliosides was characteristic of both animals.

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Restoration of voltage-dependent Ca^{2+} release and intramembrane charge movement in cut muscle fibres internally perfused with D890

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Phenylalkylamine drugs are thought to act at an intracellular site of the dihydropyridine receptor, the putative voltage-sensor for internal Ca^{2+} release in muscle. Permanently charged derivatives like D890 remained without an effect on L-type Ca^{2+} currents and EC coupling when applied externally. We studied the restoration from inactivation (caused by a long-lasting depolarization) of calcium release and intramembrane charge movements in cut twitch muscle fibres of the frog at about 10°C. Restoration was determined by applying strong depolarizing and hyperpolarizing test pulses at various times after polarizing the membrane from 0 to −80 or −70 mV. Both events are suppressed by external D600 (Feldmeyer *et al.* (1990) *J. Physiol.* **421**, 343–62), a phenylalkylamine which is in equilibrium with an uncharged component. We added D600 (30 μM) and D890 (up to 50 μM) to only one or both end pools of the vaseline gap system used to voltage clamp the cut fibres. The entry of the artificial internal solution was followed by the increase of the absorbance at 550 nm caused by the metallochromic indicator dye Antipyrylazo III, which was used to detect Ca^{2+} transients. D600 blocked restoration whereas D890 had no significant effect. This observation is consistent with a smaller efficacy of D890 compared with D600 at the intracellular target site of the voltage-sensor for Ca^{2+} release from the sarcoplasmic reticulum.

Phosphoinositides in rabbit skeletal muscle membranes

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The composition of major phospholipids in mammalian skeletal muscle membranes seems to be well established (Borchman *et al.* (1982) *J. Biol. Chem.* **257**, 14136–9). However, detailed data on the composition and distribution of a group of minor phospholipids, the phosphoinositides, and thus on the capacity of the corresponding phosphoinositide-based signalling system are still lacking. The enzymes involved in this metabolism have been demonstrated and partially characterized (Varsanyi *et al.* (1986) *Biochem. Biophys. Res. Commun.* **138**, 1395–404, *Eur. J. Biochem.* **179**, 473–9, 1989; Hidalgo and Jaimovich (1989) *J. Bioenerg. Biomem.* **21**, 267–81). Ins(1,4,5)P_3 which is generated by this membrane associated enzyme system has been described to be involved in skeletal muscle Ca^{2+} release (Suarez-Isla *et al.* (1991) *J. Physiol.* **441**, 575–91) although its concrete function in this process is a matter of debate (Ashley *et al.* (1991) *Q. Rev. Biophys.* **24**, 1–73). On the other hand, Ins(1,4,5)P_3 acts as a competitive inhibitor of aldolase A (Koppitz *et al.* (1986) *Eur. J. Biochem.* **161**, 421–33) and its free sarcoplasmic concentration is substantially influenced by changes of the myoplasmic fructose(1,6)P_2 level (Mayr and Thieleczek (1991) *Biochem. J.* **280**, 631–40). The role of PtdIns(4,5)P_2 in skeletal muscle might not be restricted to Ins(1,4,5)P_3 release. PtdIns(4,5)P_2 itself could be involved in proteinkinase C activation (Chauhan *et al.* (1989) *Biochemistry* **28**, 4952–6). Amounts of phosphoinositides determined in skeletal muscle by means of radioactive methods (Lagos and Vergara (1990) *Biochim. Biophys. Acta* **1013**, 235–44) do not reflect the true concentrations of these inositol lipids. In this study we analysed the amounts and locations of PtdIns(4)P and PtdIns(4,5)P_2 in membranes of the rabbit skeletal muscle triad by nonradioactive mass determination. The method involved lipid extraction, deacylation and consecutive HPLC analysis of the resulting glycerophosphoinositol derivatives (Mayr (1988) *Biochem. J.* **254**, 585–91). The obtained total concentrations of PtdIns/PtdIns(4)P/PtdIns(4,5)P_2 in mol mol^{-1} phospholipid-phosphate were (n.i. = not investigated): Triads: n.i./9.4×10^{-6}/3.7×10^{-6}; T-tubules: 32×10^{-3}/1.4×10^{-3}/77×10^{-6}; Terminal cisternae: n.i./2.9×10^{-3}/not detectable.

Voltage dependent ionic currents and activation of contraction in cultured frog skeletal myocytes

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The voltage dependent ionic currents in cultured embryonic skeletal myocytes at stages of development 1–6 days were studied using the whole-cell patch clamp technique. Sodium (I_{Na}) and calcium (I_{Ca}) inward and potassium (I_K) outward currents were observed at all stages, as have been described previously (DeCino and Kidocoro (1985) *J. Neurosci.* **5**, 1471–82; Moody-Corbett and Virgo (1991) *NeuroReport* **2**, 437–40). I_{Na} did not differ from that which has been described in adult frog striated muscle fibres. Slow I_{Ca} was mediated by current through dihydropyridine sensitive Ca channels and did not differ in its kinetics from corresponding slow I_{Ca} in frog adult twitch muscle fibres (time to peak 402 ± 76 ms). In 10% of cells examined for I_{Ca}, this current was significantly slower (time to peak 1431 ± 79 ms) and similar to I_{Ca} which have been described in frog tonic muscle fibres (Huerta and Stefani (1986) *J. Physiol.* **372**, 293–301). Six types of I_K were registered, with time to peak (−10 mV) 5, 12, 20, 30, 50 ms (fast I_K) and more than 7 s (slow I_K). I_K with 5, 20 and 30 ms are predominant in the 3-day cultures and disappear in 6-day-old. Contractions of myocytes appear in response to depolarization after 1 day of culturing (Moody-Corbett *et al.* (1989) *Can. J. Phys. Pharm.* **67**, 1259–64). In our experiments contractions were recorded only qualitatively, as a break of the current recording line because the microelectrode loses its contact with cell membrane during the contraction. The experiments have shown that contractions to depolarizing pulses were observed only in the presence of inward I_{Ca} and always after the I_{Ca} has reached its maximum (in 770 ± 457 ms). Another condition for the activation of contraction to occur was the absence of outward I_K, which was abolished by addition of 4-aminopyridine and by Cs ions.

Effects of low extracellular calcium and calcium channel blockers on tetanic responses and fatigue in various types of frog twitch muscle fibres

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Two types of single twitch skeletal muscle fibres, easily fatigued (EF) and fatigue resistant (FR), were dissected from iliofibularis muscles of *Rana temporaria*. We found that in addition to functional differences, these two types of muscle fibres had ultrastructural ones in their different quantities of sarcoplasm, myofibrils, mitochondria and lipids. Low Ca^{2+} solution, verapamil and Ni^{2+}, blockers of slow Ca^{2+} channels, enhanced both the onset of the tetanic relaxation phase and fatigue, especially in EF fibres. FR fibres were much more fatigued and were similar to EF fibres after the combined action of low Ca^{2+} solution and verapamil. This effect was reversible. Although verapamil greatly prolonged refractory phase after tetanic responses and this effect was further enhanced in both muscle fibre types after fatiguing stimulation, Na^+ currents recorded were not changed. Fatigue also enhanced the onset of the tetanic relaxation phase. Caffeine contractures were not changed through all experiments. The results suggest that the coupling between T-tubule membrane depolarization and Ca^{2+} release from SR is similar in both muscle fibre types, but different amounts of extracellular Ca^{2+} ions are required for recovery from inactivation. The relation with slow Ca^{2+} channels is unclear.

Lipid composition and the pattern of triglyceride- and phospholipid-bound fatty acids of adult and cultured human skeletal muscle

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Specimens of human skeletal muscle tissue ($n = 8$), obtained as surgical waste, were used in part for direct lipid analysis and in part to prepare muscle cell cultures (Pröbstle *et al.* (1988) *Pflügers Arch.* **412**, 264–9). The lipids from the muscle tissue and the cultured cells were extracted with chloroform/methanol and the extracts were directly injected into an HPLC system. The stationary phase, silica gel, and the mobile phase consisting of acetonitrile, methanol and H_3PO_4, formed a pH gradient and a polarity gradient (Seewald and Eichinger (1989) *J. Chrom.* **469**, 271–80). Triglyceride (TG) and phospholipid (PL) fractions were collected, and the lipid-bound fatty acids (FAs) were analysed using gas chromatography following transesterification. To investigate the FA metabolism in the cultures, the myotubes were incubated with ^{14}C-labelled linoleic acid for 20 or 60 h. After 20 h of incubation >50% of the linoleic acid was in the PLs and only a smaller fraction was incorporated in the TGs. After 60 h of incubation this ratio was reversed and also ^{14}C-labelled arachidonic acid was detected. This indicates an intact fatty acid metabolism.

A comparison of the TGs in the total lipid extracts from adult and cultured muscle showed statistically (Student's *t*-test; $^*p < 0.05$, $^{**}p < 0.01$) significant differences (adult: $95.3 \pm 5.6\%$ of total lipid, cultured: $73.5 \pm 14.5\%$; **). A comparison of the PL pattern yielded statistically significant differences for the cardiolipin fractions (adult: $8.4 \pm 5.1\%$ of total PL, cultured: $1.6 \pm 1.5\%$; *) and for phosphatidylethanolamine ($7.0 \pm 4.9\%$ versus 16.6 ± 10.3; *). On the other hand, the fractional values for the other phospholipids in adult and cultured muscle were not different: phosphatidylinositol $2.6 \pm 2.2\%$ versus $1.6 \pm 1.2\%$; phosphatidylserine $3.3 \pm 3.3\%$ versus $5.6 \pm 4.2\%$; lyso-phosphatidylethanolamine $3.9 \pm 2.0\%$ versus 1.2 ± 1.0; phosphatidylcholine $64.7 \pm 21.2\%$ versus $67.9 \pm 12.8\%$; sphingomyelin $4.6 \pm 4.4\%$ versus $2.3 \pm 2.0\%$; and lyso-phosphatidylcholine $5.3 \pm 5.1\%$ versus $3.4 \pm 3.1\%$. The most common PL-bound fatty acids in both adult and cultured muscle were $C_{16:0}$, $C_{18:0}$, $C_{18:1(cis9)}$, $C_{18:2}$ and $C_{20:4}$. The FA patterns of the PLs isolated from adult muscle and the cultures were similar, although there was a clear trend to higher amounts of $C_{18:2}$ and $C_{20:4}$ in PLs isolated from cultures. PLs originating from adult muscle showed higher amounts and more diversified minor FAs. This might indicate the more individual nutritional and physiological situations that exist *in vivo*.

In summary, this study showed that phospholipids and TG- and PL-bound fatty acid patterns from both adult and cultured muscle are similar which implies that the major pathways of lipid metabolism in embryonic and adult human skeletal muscle cells are more or less the same.

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Tissue distribution of the chloride channel ClC-O in *Torpedo marmorata*

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The distribution of the voltage-gated chloride channel ClC-O (Jentsch *et al.* (1990) *Nature* **348**, 510–4) in *Torpedo marmorata* was investigated by indirect immunofluorescence. Antibodies raised in rabbits against fusion proteins containing the putative cytoplasmic loop of ClC-O detected the antigen both in the electric organ and in skeletal muscle of *Torpedo marmorata*.

The electrocytes were simultaneously incubated with the labelled anti-rabbit-IgG-antibody and differently labelled alpha-bungarotoxin. Alpha-bungarotoxin is a high affinity ligand for the nicotinic acetylcholine receptor (nAChR). The microscopic pictures of longitudinal sections clearly indicate that the anti ClC-O antibody recognizes an antigen which is localized at a different site in the membrane to that stained by alpha-bungarotoxin. This confirms previous data by Miller based on separations obtained by sucrose gradient centrifugation and demonstrates a clear polar distribution of ClC-O and the nAChR. ClC-O was also detected in skeletal muscle. In longitudinal sections indirect staining mainly appeared on the plasma membrane. Not only the surface of the muscle cells reacts with anti ClC-O antibodies but staining is also visible inside the cells. The Z-lines are distinctly stained. The staining can be removed by gentle treatment with Triton X-100. Cross-sections show thin stained membrane threads that have their origin in the plasma membrane and project to the centre of the cell. These results indicate that in *Torpedo marmorata* skeletal muscle cells the plasma membrane and the T-tubules are stained by anti ClC-O antibodies.

Major skeletal muscle chloride channel ClC-1 and its involvement in myotonia

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Mammalian skeletal muscle has a high chloride conductance at the resting potential, necessary for normal muscle excitability. The importance of skeletal muscle chloride channels is shown in the hereditary disease myotonia, where chloride conductance is drastically reduced and a hyperexcitability is manifested as muscle stiffness.

Starting from the *Torpedo* channel ClC-O (Jentsch *et al.* (1990) *Nature* **348**, 510–4), we have cloned a rat skeletal muscle chloride channel (ClC-1) (Steinmeyer *et al.* (1991) *Nature* **354**, 301–4). This chloride channel fulfils several features, characteristic for the major skeletal muscle chloride channel: (1) It is predominantly expressed in skeletal muscle. (2) ClC-1 is developmentally regulated; there is a dramatic increase of its mRNA level in rat muscle shortly after birth, which parallels the known increase in macroscopic muscle chloride conductance during postnatal development of skeletal muscle. (3) Expression of ClC-1 mRNA in *Xenopus* oocytes leads to chloride currents with the same voltage and time dependence as observed with intact skeletal muscle. (4) In addition, the channel is efficiently blocked by 9-anthracene-carboxylic acid, that elicits myotonia, when applicated to skeletal muscle. To test the hypothesis, whether the muscle chloride channel ClC-1 is mutated in myotonia, we investigated a myotonic mouse mutant, ADR (Steinmeyer *et al.* (1991) *Nature* **354**, 304–8). We have localized the *Clc-1* gene on mouse chromosome 6 and demonstrated a tight linkage of the Clc-1 locus to the disease. A close examination of the *Clc-1* gene revealed that in ADR mice a transposon of the ETn family has been inserted into an intron of the gene. This leads to the expression of aberrant forms of ClC-1 transcripts, none of which encodes a functional chloride channel. Thus, the destruction of the muscle chloride channel is the primary cause of mouse myotonia, implicating that this might also be true for human myotonia. We are currently extending our investigations to families with autosomal recessive generalized myotonia (Becker) and with autosomal dominant myotonia congenita (Thomsen).

Indanyloxyacetic acid sensitive chloride channels from outer membranes of skeletal muscle

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In mature mammalian muscle, the chloride conductance of the membrane is an important factor in the regulation of excitability. Up to now, no ligand was available for the biochemical characterization of muscle chloride channels. To localize and characterize these channels, we have employed indanyloxyacetic acid (IAA-94), a ligand previously used for epithelial Cl^- channels (Landry *et al.* (1987) *J. Gen. Physiol.* **90**, 779–98; (1989) *Science* **244**, 1469–72). IAA induced myotonic responses when microinjected into mature mouse muscle fibres, indicating a blockade of Cl^- channels from the cytoplasmic side. Membrane vesicles were prepared from rabbit skeletal muscle and separated by sucrose gradient centrifugation. Fractions obtained (in order of increasing density) were sarcolemma (SL), T-tubules (TT), sarcoplasmic reticulum (SR), triads and mitochondria (TR/M). The fraction enriched for SL was characterized by high specific binding capacity for ^{3}H-saxitoxin (Na^+ channel), whereas TT-rich fractions bound ^{3}H-PN 200-110 (DHP-receptor) with high specific activity. IAA sensitive Cl^- channels were found in the SL fraction but not in the SR. Highest specific activities in electrical diffusion potential sensitive ^{36}Cl-transport and ^{3}H-IAA-94 binding were found in the SL. SL vesicles were solubilized with CHAPS and subjected to IAA sepharose affinity chromatography. Specifically bound protein was eluted with 100 μM IAA-94 and either analysed by SDS-gel electrophoresis or reconstituted into phospholipid vesicles. The eluate contained two specifically bound polypeptides of M_{app} 60 kDa and 110 kDa and two additional bands of M_{app} 67 and 50 kDa which were also present in the wash. A 110 kDa polypeptide would be predicted for the product of the *Clc-1* gene (Steinmeyer *et al.* (1991) *Nature* **354**, 304–8) which is responsible for the stabilization of the resting potential (Steinmeyer *et al.* (1991) *Nature* **354**, 304–4). Functional IAA sensitive chloride channels, but very few IAA-insensitive Cl^- channels were found in the IAA eluate on reconstitution into lipid vesicles (Weber-Schürholz *et al. Biol. Chem.* (in press)).

We conclude that the IAA sensitive bursting chloride channels of the sarcolemma are likely to be responsible for its major chloride conductance, and thereby for the stabilization of resting potential. This hypothesis can be tested by analysing membranes from mutant myotonic muscle with impaired chloride conductance (cf. Wischmeyer *et al.*, this meeting).

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Using the myotonic ADR mouse to identify the major chloride channel of muscle: a patch-clamp study on lipid supplemented vesicles

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In the myotonic ADR mouse (genotype *adr/adr* (review: Jockusch (1990) In Pette (ed.) *The Dynamic State of Muscle Fibers*, W. de Gruyter, Berlin, pp. 429–43), the gene for the muscular chloride channel CLC-1 is functionally disrupted by the insertion of a retroposon (Steinmeyer *et al.* (1991) *Nature* **354**, 301–4). Therefore, by comparing membrane preparations from wildtype (wild-type, +/+ or +/*adr*) mice with those from *adr/adr* mice, one should observe a species of Cl^- channels missing in the latter. Patch-clamp analysis was done using lipid supplemented vesicles prepared from the sarcolemmal (SL) fraction of skeletal muscle (Weber-Schürholz *et al.*, this meeting). The spectrum of conductances (in symmetric 140 mM NaCl, pipette potential − 50 mV) for Cl^- ranged from $\leqslant$100 pS to $\approx$800 pS in wildtype SL, but only to 150 pS in ADR. Surprisingly, ADR mice symptomatically cured by chronic tocainide (Toca) treatment also showed conductances from $\leqslant$100 pS to 800 pS (Wischmeyer *et al.* (1992) *Eur. J. Physiol.* **420** (Suppl. 1) R96). However, there was a qualitative distinction between the Cl^- channels of wildtype and Toca-treated ADR mice: Only in wildtype, a bursting 100 pS/280 pS indanyloxyacetic acid (IAA) sensitive Cl^- channel was found, and this was also observed in native SL (e.g. blebs). We conclude that this is the major sarcolemmal Cl^- channel CIC-1 which is coded for by the myotonic gene *Clc-1* and is responsible for the stabilization of the resting potential. This finding corresponds to the fact that the membrane potential of muscle fibres can be destabilized by microinjection of IAA (Weber-Schürholz *et al.*, this meeting). The physiological significance of the IAA insensitive Cl^- channels is not known, as they were not found in native SL.

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Regulation of transcriptional activities of rat muscle nicotinic acetylcholine receptor subunit gene promoters in cultured muscle cells

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The five subunits α, β, γ, δ, and ε of the nicotinic acetylcholine receptor (AChR) are regulated differently during development and synaptogenesis. The synaptic specialization of the AChR complex is most likely controlled by neurogenic factors. In addition, factors linked to the acetylcholine-induced electrical activity and muscle-specific factors affect the gene expression of the AChR subunits. We have isolated untranslated 5′ flanking sequences of the β, γ, and ε subunit genes that confer muscle-specific expression on transient transfection of primary cultures of rat muscle. Analysis of the *cis*-acting regulatory elements of the different AChR subunit genes are expected to reveal significant similarities or differences which will help to understand the mechanisms regulating the expression of the corresponding subunit genes. Comparing the known AChR gene promoters little sequence identity appears to exist. In all cases, however, E-box elements are contained within these sequence regions. Their functional role as putative binding sites for myogenic factors such as MyoD, myogenin, MRF4, and the negatively acting Id was tested *in vitro* by cotransfection experiments. The results suggest that these factors can interact with the β, γ, and ε gene promoters but may affect transcriptional activities in a differential manner. Northern blot analysis experiments using total RNA from postnatal, adult, denervated and pharmacologically modified muscle tissue indicate that the myogenic factors may be linked to the electrical activity of the muscle but are probably not involved in synapse-specific events. Their regulatory role *in vivo* has not been proven unequivocally to date. To develop experimental models which allow the dissection of different regulatory pathways we used primary cultures from rat muscle. These experiments suggested that ultimately the *in vivo* situation cannot be replaced by cell culture systems and that the most promising approach to identify regulatory sequences on the AChR subunit genes may depend on the generation of transgenic animals using the different AChR gene promoter fragments.

Characterization of protein isoform-specific antibodies to study the expression of voltage-gated potassium channels in muscle tissue

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Voltage-gated potassium channels are important elements of all mammalian excitable cells, including muscle cells. Most cells express several subtypes of K^+ channels differing specifically in their functional and pharmacological properties. Owing to the lack of high affinity ligands (e.g. neurotoxins) for most K^+ channel proteins, only little is known of their cellular and subcellular distribution. As a first step to overcome these limitations, we have prepared monoclonal antibodies directed against limited sequence portions of K^+ channel proteins of the RCK family and have selected the antibodies as to their isoform specificity by means of a combined molecular genetic/immunohistochemical assay. Our assay is based on the application of *Xenopus* oocytes as suitable ectopic expression systems for voltage-clamp and patch-clamp studies of ion channels.

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Single channel conductivity events and oligo-channels of the monomer and dimer of the *Torpedo californica* nicotinic acetylcholine receptor

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The dimer ($M_r \approx 580\,000$) and the monomer ($M_r \approx 290\,000$) of the detergent-solubilized, affinity chromatographically-purified nicotinic acetylcholine receptor (nAChR) of *Torpedo californica* electrocytes exhibit different channel conductances and larger oligo-channels. Patch clamp data of the dimer, reconstituted in large lipid vesicles, show that the dimer double-channel behaves as a 'single-channel' of conductance $G(D) = 84 \pm 6$ pS at 0.11 M K^+ and 0.1 mM Ca^{2+}, 20°C. The vesicle-reconstituted monomer, if prevented from aggregation, exhibits a channel conductance, $G(M) = 42 \pm 3$ pS at 0.11 M K^+, 0.1 mM Ca^{2+}, 20°C, which is only half of that of the dimer. The conductance of the reconstituted dimer is the same, and shows the same inhibitory Ca^{2+}-dependence, as that of the *Torpedo* receptor expressed in *Xenopus* oocytes (Imoto *et al.* (1986) *Nature* **324**, 670–4). The equilibrium constant of the Ca^{2+}-inhibition is $K_{0.5}(Ca) = 0.48$ mM at 0.11 M K^+, 20°C; $G_0([Ca] \rightarrow 0) = 98 \pm 6$ pS and $G_\infty([Ca] \rightarrow \infty) = 27 \pm 6$ pS. The comparison of our data with the oocyte data implies that the dimer conductance reflects the simultaneous, concerted switching of the two constituent monomeric parts and that it is the dimer which is the physiological switching unit of the nAChR in the oocytes. The larger macrochannel events have conductance values which are multiples of the dimer or of the monomer conductances. Such oligo-channels caused by receptor protein oligomers would guarantee the steep depolarization required for the initiation of the postsynaptic action potentials causing muscle contraction and discharge of electric organs.

Cloning and characterization of two human voltage-gated potassium channels expressed in heart or in skeletal muscle

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Voltage gated K^+ channels may be very important in the regulation of the fast repolarizing phase of action potentials in the heart and thus influence cardiac action potential duration. Therefore, we have started to analyse the genomic organization and chromosomal localization of human K^+ channels. For this purpose, we have isolated two Yeast Artificial Chromosomes (YAC), involving the human homologous of the rat DRK1 gene (h-DRK1) and RCK4 gene (HBK4).

The RCK4 K^+ channel, a member of the rat RCK K^+ channel family, which is homologous to the *Drosophila Shaker* channels (Stühmer *et al.*, *EMBO J.* **8**, 3235–44) and the DRK1 K^+ channel are expressed in heart or skeletal muscle tissue. The RCK4 expresses an A-type K^+ channel. The channel is insensitive to TEA and DTX, but highly sensitive to variations in extracellular potassium concentrations, which affect the number of available active RCK4 channels (Pardo *et al.*, *PNAS* **8**, 2466–70). Variations in extracellular potassium concentrations have been implicated in the electrical instability of the heart following acute ischemia. The RCK4 gene is represented only once in the rat genome and the mRNA is not alternatively spliced like *Shaker*, but translated into one distinct A-type channel forming protein.

For mapping the human RCK4 analogue (HBK4) we isolated a YAC, which contains a 550 kb insert of human DNA. The HBK4 gene was mapped to chromosome 11p14.1 (Gessler *et al.*, submitted 1992). The gene maps close to the Wilms Tumor locus as well as to a region on chromosome 11 implicated in the autosomal dominant inherited LQT syndrome. Currently we investigate a possible linkage between this syndrome and polymorphismen associated with the HBK4 gene.

The DRK1 K^+ channel expresses a delayed rectifier K^+ channel. The channel is sensitive to 4-AP and TEA and can be found in heart and skeletal muscle tissue. We isolated a YAC, which contains a 590 kb insert of human genomic DNA, including the h-DRK1 gene. The chromosomal localization of h-DRK1 is currently under investigation. In contrast to the genes encoding RCK/*Shaker* related K^+ channel forming subunits, the human DRK1 gene (h-DRK1) possesses intron sequences in its translated part.

Because cardiovascular diseases are a major cause of morbidity and mortality in the industrialized world and inherited traits are involved in the pathogenesis of most cardiovascular disorders, the linkage of voltage gated K^+ channel genes to heart diseases is important for their understanding and the developing of possible therapies.

Regulation of the ryanodine receptor/Ca^{2+} release channel complex by luminal calcium

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$^{45}Ca^{2+}$ flux and single channel measurements have shown that the ryanodine receptor/Ca^{2+} release channel of sarcoplasmic reticulum is regulated in a concentration-dependent manner by cytoplasmic (*cis*) Ca^{2+}. While low concentrations in the micromolar range activate the Ca^{2+} release channel, higher concentrations ($\geqslant 100$ μM) slow down the Ca^{2+} release rate. It is, however, not known if luminal (*trans*) Ca^{2+} also affects the gating behaviour of the channel.

In the experiments presented here, the Ca^{2+} release channel of skeletal sarcoplasmic reticulum was isolated from rabbit back muscle and reconstituted into planar lipid bilayers of the Mueller–Rudin type as described by Lai *et al.* (*Nature* **331**, 315–9, 1988). Using K^+ as the channel conducting ion, it was possible to study the effects of changes in trans Ca^{2+} in the micro- to millimolar range. In the presence of micromolar *cis* Ca^{2+}, an elevation of *trans* Ca^{2+} up to 700 μM increased the open probability of the Ca^{2+} release channel while higher concentrations decreased channel activity. This inhibiting effect was antagonized by millimolar concentrations of *cis* ATP or caffeine. An increase of the free cytoplasmic Ca^{2+} buffer concentration (EGTA) shifted the concentrations of *trans* Ca^{2+} necessary for observing an activating or inactivating effect to higher values. Analysing of open and closed time distributions obtained from single channel recordings for different free *trans* $[Ca^{2+}]$ showed that mainly the life times of closed events were affected by changes in *trans* Ca^{2+}.

Influence of phosphate on the distribution of flux control in oxidative phosphorylation in rat skeletal muscle mitochondria

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The flux control coefficients of the adenine nucleotide translocase, the phosphate transporter and the H^+-ATPase were determined in rat skeletal muscle mitochondria using glutamate plus malate as substrates and soluble F_1-ATPase as load enzyme. For their determination from inhibitor titrations with carboxyatractyloside, mersalyl and oligomycin, the complete titration curve was analysed by means of nonlinear regression to a mathematical model describing the binding equilibrium of the applied inhibitor (Gellerich *et al.* (1990) *FEBS Lett.* **274**, 167–70). It was observed that the flux control coefficients of adenine nucleotide translocase, H^+-ATPase and the load enzyme F_1-ATPase at comparable rates of respiration strongly depend on the phosphate concentration in the incubation medium.

So, the flux control exerted by the adenine nucleotide translocase has in the intermediate states of mitochondrial respiration (approximately 120 nmol O_2 min^{-1} mg^{-1}) at 10 mM phosphate a value of about 0.37. At a phosphate concentration of 1 mM and comparable rates of respiration the flux control coefficient of the translocase decreased to about 0.20. Under these circumstances a sharp increase in the controlling influence of H^+-ATPase from 0.10 to 0.74 was detected.

It could be shown further that at this flux rate, the sum of flux control coefficients of adenine nucleotide translocase, H^+-ATPase, phosphate transporter and the load enzyme F_1-ATPase is very close to unity. This is an indication that under the conditions of intermediate state respiration all other reactions have a negligible controlling influence on oxidative phosphorylation in skeletal muscle mitochondria.

SKELETAL MUSCLE

Chemical kinetics of myofibrils prevented from shortening

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Myofibrils in suspension are unattached and when they are activated ($+Ca^{2+}$) and ATP added they contract at maximum velocity near zero load. We have already studied the chemical kinetics of unattached myofibrils (Houadjeto *et al.* (1992) *Biochemistry* **31**, 1564–9; Herrmann *et al.* (1992) *Biochemistry* (in press)). We have extended these studies to 'held' myofibrils, i.e. myofibrils that contract isometrically, by crosslinking them chemically using EDC. The crosslinked products were analysed after chymotryptic digestion by SDS-PAGE and Western blots (with actin or myosin antibodies). This revealed that the rods of myosin had been extensively crosslinked. The myosin heads were also crosslinked to actin but to a much less extent. When activated ($+Ca^{2+}$), the crosslinked myofibrils did not contract on the addition of ATP. The ATPase activities of native and crosslinked myofibrils were studied at 4°C. With both there were transient Pi burst phases of similar amplitudes ($\pm Ca^{2+}$). This suggests that the crosslinking does not affect the ATPase sites of the myofibrils. With Ca^{2+} activated native myofibrils the transient was followed by a rapid kcat (1.7 s^{-1}) which lasted for 1–2 s when there was a sharp break to 0.23 s^{-1}. The rapid kcat is that of shortening myofibrils and the slow kcat that of overcontracted myofibrils. With crosslinked myofibrils there was only one kcat (0.7 s^{-1}). This kcat is lower than that with native, unheld myofibrils. This in line with the conclusion of A. F. Huxley, namely that mechanical factors such as stress or strain affect ATP hydrolysis. With regulated ($-Ca^{2+}$) native myofibrils, the transient was followed by a single kcat (0.02 s^{-1}). With crosslinked myofibrils, the progress curve was identical to that with Ca^{2+}. Thus, crosslinked myofibrils are no longer regulated which could result from rigor activation (induced by the crosslinking of a few myosin heads to actin) or to a perturbation of the regulatory apparatus.

Angle-resolved fluorescence depolarization of dye-labelled muscle fibres

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Fluorescence depolarization techniques are commonly used in muscle research to investigate the orientational order and dynamics of crossbridges in muscle fibres. Steady state depolarization experiments on muscle fibres commonly utilize a fixed 90° scattering geometry. This allows the determination of only three independent polarization ratios (Wilson and Mendelson (1983) *J. Muscle Res. Cell Motil.* **4**, 671–93). Consequently, not all the information about order and dynamics can be accessed (van Gurp *et al.* (1988) In: B. Samori and E. W. Thulstrup (eds) *Polarized Spectroscopy of Ordered Systems*, Kluwer Academic Publishers, pp. 455–89) and the interpretation of the depolarization data remains ambiguous. To overcome this limitation we have developed an angle-resolved fluorescence depolarization technique which affords the determination of a larger number of independent polarization ratios. In these experiments the labelled muscle fibre is mounted vertically and excited with horizontally or vertically polarized light incident along the normal to the fibre axis. The fluorescence intensity, polarized vertically or horizontally, is also detected in the horizontal plane at different angles relative to the exciting beam. This approach enables us to extract the order parameters and correlation functions unequivocally.

Angle-resolved fluorescence depolarization experiments on muscle fibres labelled with Eosin-5-Maleimide are presented. It is important to note that the order parameters and correlation functions obtained from the experiments are determined by three independent factors: (1) the orientation and rotation of the crossbridges in the muscle fibre, (2) the orientation of the dye molecule relative to the crossbridge, and (3) the orientation of the excitation and emission transition moments in the molecular frame of the dye. We have determined the latter two factors for several dyes in separate experiments (van der Heide *et al.* (1992) SPIE Meeting on Time resolved Laser Spectroscopy III. *SPIE* **1640**, 681–9; van der Heide *et al.* (1992) *Eur. Biophys. J.* (in press)). This information is used to extract the intrinsic orientational distribution of the crossbridges in the muscle fibre.

Changes in disposition of various regions of actin monomer and myosin head during ATP hydrolysis cycle

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One approach to investigation of the mechanism of force generation of muscle is to study the movement of myosin heads and actin monomers as well as that of their domains or segments at various stages of the ATP hydrolysis cycle. This kind of information can be obtained by polarized microfluorimetry technique. In this method a fluorescent probe attaches specifically to protein. The changes in orientation are thought to reflect the movement in the corresponding site of the protein (Nihei *et al.* (1974) *Biophys. J.* **28**, 236; Borovikov and Kakol (1991) *Gen. Physiol. Biophys.* **10**, 245). In this work muscle fibres free of myosin, tropomyosin and troponin (ghost fibres) have been used. Various regions of actin monomer and myosin head were modified by fluorescent probes (phalloidin-rhodamine, ϵ-ADP, FITC, 1.5-IAEDANS, IAF, DASM, NBD-Cl, rhodamine-maleimide and fluorescein-maleimide). The stages of ATP hydrolysis cycle were

studied during interaction of proteolytic myosin subfragment-1 (S1) with F-actin of ghost fibre and Mg-ADP. It was shown that in the complex actin-S1 the orientation of fluorescent dyes located in actin and in myosin heads differed considerably at different stages of hydrolysis cycle of ATP. It is suggested that intramolecular movements in actin and myosin head participate in the mechanisms of muscle contraction (Huxley (1974) *J. Physiol.* **243**, 1–43).

Nucleotide-induced domain motion of subfragment-1 in glycerinated muscle fibres by spin labelling

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Rotational dynamics of myosin heads and their ordering in glycerinated skeletal muscle fibres was studied using maleimide, iodoacetamide and isothiocyanate spin labels attached to the fast-reacting thiol of myosin. The ordering of probe molecules was static on millisecond time scale in rigor at sarcomere length of 2.3 ± 0.1 μm, except isothiocyanate probe molecules; the segment that held the label rotated in the microsecond time range as revealed by saturation transfer EPR. This probe was able to distinguish between two S-1 states, the relative population of the states depended on the bound nucleotide.

The spectra of isothiocyanate spin-labelled fibres could be analysed in terms of two narrow distributions with mean angles of 74.5° and 54°. In rigor, the fractions were about 75 and 25% of the total EPR absorption. In the presence of MgADP at sarcomere length of 2.2–2.3 μm the conventional EPR spectra showed large changes in the ordering of isothiocyanate probe molecules towards new distribution; the $\theta = 54°$ population increased from 25 up to 76% at the expense of the other with no change in the mean angles of the distributions. In contrast, small effect of MgADP was observed on maleimide and iodoacetamide spin-labelled muscle fibres. ADP and Vi produced a large decrease of the ordering of the isothiocyanate probe molecules and a significant increase of the mobility of the labels evidencing large internal motion in the head region of myosin and/or the detachment of crossbridges from the thin filaments.

For direct calculation of the orientation distribution function of nitroxide spin label from the experimental spectrum, a new method is suggested which can be regarded as a reverse procedure of the spectrum simulation. Comparison of the calculation with the conventional procedure showed that the θ-dependence of the distribution function can be derived by the new method with good approximation (θ is the angle between the nitroxide z-axis and the static magnetic field).

CARDIAC MUSCLE

Which is better as a cardiac assistant?

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There are many studies of transplantation of skeletal muscle for cardiac assistance at the present time. We have tried to estimate a possible mechanism of interaction between skeletal and cardiac muscles with different contractile characteristics. The experimental model of myocardial mechanical nonhomogeneity (Pat. SU1560094A1), including two isolated rat muscles connected as parallel duplexes, has been used. The physiological regime of loading was applied to the complete duplex, but we could register a force of each muscle during shortening of the duplex. The first duplex consisted of good right ventricular papillary muscle and a fragment of soleus musculus (C-S duplex): their length and force were quite similar. Skeletal muscle was stimulated by single impulses (70 ms) and with a delay (150 ms) after stimulation of cardiac muscle. The second duplex consisted of the same good cardiac muscle and other papillary muscles with poor contractile characteristics (C-C duplex). In this case they were stimulated simultaneously. We have demonstrated that under relative load 0.3 P_0 the C-S duplex shortened to a greater extent (about 20%) than C-C duplex. The good cardiac muscle had found itself in a better loading condition rather than when it was a member of C-C duplex because in the period of the duplex's shortening, the skeletal muscle was not a significant additional load for cardiac muscle. Moreover, as soon as the excitation of skeletal muscle was started, the unloading of cardiac muscle took place. In C-C duplex, the weak cardiac muscle became an additional load for the good muscle from the moment of the duplex's shortening. As a result of this the range of shortening of both the good muscle and the whole duplex decreased. In our opinion this analysis of the interaction between muscles in duplexes may be useful for optimizing the regime of loading of transplanted muscle in the intact heart.

Survival of embryonic cardiomyocytes transplanted into host rat soleus muscle

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Embryonic cardiomyocytes are known to survive for 75 days when transplanted into the skeletal muscle bed of Nude mice (Jockusch *et al.* (1983) *Exp. Neurol.* **81**, 749–55). Here we transplanted embryonic rat heart cells alongside host rat soleus muscle to determine whether they would: (1) grow and beat in the presence of the host skeletal muscle, (2) migrate from the site of transplantation, and (3) form gap junctions and possibly fuse with the skeletal muscle. Small explants of heart tissue from E14 Fischer rat embryos were transplanted onto the surface of soleus muscle in adult host Fischer rats. Two weeks after transplantation, embryonic heart cells, prelabelled with BrDU (a nuclear marker) or DAPI (a fluorescent dye) were found to be present and beating on the surface of the host skeletal muscle. This was the case no matter whether the soleus muscle had been left undamaged or had received a small cut at the time of transplantation. At 4 weeks they continued to beat, and responded to acetylcholine (ACh, 10^{-4} M) by a slowing of the beating which was arrested 30 s after ACh application; the beating returned upon washing out the ACh. At 10–20 weeks after transplantation, the cells had migrated from their original site, along the entire length of the soleus muscle. Cardiac gap junction antibodies (gift from Gourdie and Green (1991), see *J. Cell Sci.* **99**, 41–55) were used to study the developmental state of these growing cells. Cardiac gap junctions were present at all times studied between 4 weeks and 6 months after transplantation of the heart cells. They were scattered initially, followed by the apparent alignment of junctions in some areas. At later intervals (more than 6 months after implantation) the transplanted cells were no longer spontaneously contracting. These cells may have no long-term survival in this environment, or may become incorporated into the host skeletal muscle tissue. Further studies will investigate this.

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Imaging of drug-induced calcium transients in rat cardiomyocytes

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Transients and waves of intracellular calcium were made visible by using the fluorescent calcium indicator fluo 3-AM. They were moni-

tored either by measuring the integrated fluorescence of single cells with a photomultiplier or by video microscopy. Serial images, which were acquired with a time resolution of 50 Hz, were analysed by means of image-processing with regard to the spatiotemporal kinetics of calcium redistribution. A small volume perfusion chamber was used which allowed for rapid application of drugs and their subsequent washout.

In control cells, spontaneous transients were observed rarely. They appeared as single narrow, longitudinal waves. Perifusion with 10 mM caffeine for 10 s usually elicited a train of 2–7 spikes. The images revealed bright waves of unifocal or multifocal origin, flashes and transitions from waves to flashes. Depolarization of the cells by short pulses of 30 mM KCl yielded single transients which were found to represent flashes, multifocal waves or mixed forms. When cardiomyocytes were preincubated with barbiturates (1–3 mM) for 20 min and then washed, they started spiking at frequencies varying from 0.02 to 2 Hz. Corresponding images revealed broad waves, so called 'tides'. Similar tides were observed during washout of 100 μM ouabagenin. 'Erratic waves' were also observed after barbiturate or ouabagenin incubation, which were characterized by irregular movements of calcium, for example along the short axis of the cell, or in spirals.

Calcium waves are assumed to spread by calcium-induced calcium release from the sarcoplasmic reticulum and subsequent reuptake. The modulation of calcium release and calcium uptake by different drugs could provide a means to further investigate calcium homeostasis.

Mitochondrial creatine kinase system in guinea pig hearts during ischaemia and reperfusion

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The subcellular distribution of ATP, ADP, creatine phosphate and creatine was studied in normoxic control, isoprenaline-stimulated and potassium-arrested guinea pig hearts as well as during ischaemia and after reperfusion. The mitochondrial creatine phosphate/creatine ratio was found to be closely correlated to the oxidative activity of the hearts. This was interpreted as an indication of a close coupling of mitochondrial creatine kinase to oxidative phosphorylation. To further investigate the functional coupling of mitochondrial creatine kinase to oxidative phosphorylation, rat or guinea pig heart mitochondria were isolated and the mass action ratio of creatine kinase determined at active or inhibited oxidative phosphorylation or in the presence of high phosphate, conditions which are known to change the functional state of the mitochondrial enzyme. At active oxidative phosphorylation the mass action ratio was one-third of the equilibrium value whereas at inhibited oxidative phosphorylation (N_2, oligomycin, carboxyatractyloside) or in the presence of high phosphate, the mass action ratio reached equilibrium values. These findings show that oxidative phosphorylation is essential for the regulation of the functional state of mitochondrial creatine kinase. The functional coupling of the mitochondrial creatine kinase to oxidative phosphorylation is indicated by the correlation of mitochondrial creatine phosphate/creatine ratios with the oxidative activity of the heart *in situ* as well as by the deviation of the mass action ratio of the mitochondrial enzyme from creatine kinase equilibrium at active oxidative phosphorylation in isolated mitochondria. These findings are in accordance with the proposed operation of a creatine shuttle in heart tissue.

Effect of Mg-ATPase inhibitors and calcium antagonists on the EDP-EDV relation in the isolated rat heart

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The diastolic pressure-volume relation in normoxic perfused rat hearts represents the elastic behaviour of the connective tissue in the ventricular wall. In our experimental setup isolated, Langendorff perfused, artificially stimulated, rat hearts were used. Left ventricular volume was measured by a microconductance catheter, inserted into the left ventricular cavity. Left ventricular pressure was measured by means of a catheter tip manometer located at the mitral orifice. The left ventricle was connected to a pressure controlled tube to set the end-diastolic pressure prior to stimulation. Contractions were kept isovolumically. The ventricle was first subjected to a series of increasing diastolic pressures and intraventricular pressure and volume were recorded. Then the perfusion was changed to one with a Mg-ATPase inhibitor, vanadate (1×10^{-3} M) or BDM (3×10^{-3} M). After incubation of about 15 min a same series of increasing diastolic pressures was recorded. Perfusion was returned to normal and the Mg-ATPase inhibitor was washed out. Then the perfusion was changed to one with a calcium antagonist, diltiazem (2×10^{-7} M). After 15 min of incubation, again a series of increasing pressures was recorded. Diltiazem was not as easily washed out as the Mg-ATPase inhibitors. All drugs reduced the systolic pressure to values close to zero, indicating a reduction of actively generated force as a result of perfusion with these drugs. The end-diastolic pressure was plotted against the end-diastolic volume resulting in EDP-EDV relations. The EDP-EDV relations show that at a particular pressure, especially at low values, the EDV is increased by diltiazem. Vanadate and BDM, however, exert hardly any effect on EDP-EDV relation. From these experiments we conclude that no phosphorylated myosin heads are involved in the EDP-EDV relation, but calcium-activated force is an important determinant of the EDP-EDV relation.

DNA-synthesizing cells in ascidian heart

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In vertebrate cardiomyocytes, DNA synthesis and mitosis occur in the presence of myofibrils. Neither developing nor mature vertebrate cardiac muscles display an undifferentiated myoblast pool. Cardiogenesis in invertebrates remains so far almost utterly neglected. However, some specific features of vertebrate cardiomyocytes might be better understood in terms of their phylogenetic origin. The aim of our study was to examine the replicative abilities of ascidian heart cells. The ultrastructural and autoradiographic investigation on heart cells of *Ascidia obliqua* has been carried out. The ascidian myocardium consists of myoepithelial cells, whose polarity is recognizable in basement lamina underlying the basal surface, centrioles, cilia, and well-developed Golgi complex located at the apical side, and a cross-striated myofibre per cell running along the basal membrane. After a short tritiated thymidine pulse, the label is found over some pericardial and myocardial cells. Electron microscope autoradiographs show that ^{3}HTdr-labelled nuclei in myocardium belong to myofibre-containing cells. Myonuclei in mitosis have not been observed. However, the occurrence of centrioles and high number of binucleated myocytes testify to their ability for karyokinesis. The presence of highly organized myofibres might possibly impede cytokinesis, whereas a complex mechanism of myofibre disassembling with Z-band resorption, similar to that in some vertebrate cardiomyocytes, is still lacking in ascidians. Thus, features such as polarity, well-developed secretory apparatus, presence of centrioles and cilia, and the ability of myocytes to synthesize DNA make the ascidian myocardium similar to the vertebrate embryonic one. Large numbers of ^{3}HTdr-labelled and prophasic nuclei are observed in the raphe region of the pericardium, the site of its transition to myocardium. This implies that there exists an active cell proliferation zone. The morphological features, in which the cells of raphe region are distinguished from ordinary pericardial cells, such as loss of labyrinth junctions and acquisition of nexuses,

give evidence of their premyocyte nature. This suggests that the ascidian myocardium, unlike the vertebrate one, cannot be related to a pure noncambial growing population.

Cyclical mechanical activity and hyperosmotic stress induce an immediate-early gene programme in myocardium

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It has recently been shown that in isolated neonatal cardiomyocytes continuous stretch induces the immediate-early gene (IEG) *c-fos* (Komuro *et al.* (1990) *J. Biol. Chem.* **265**, 3595–8) and protein synthesis (Mann *et al.* (1989) *Circ. Res.* **64**, 1079–90). However, these experiments could not distinguish whether mechanical activity by itself or concomitant depolarization were responsible for inducing the IEG programme. We therefore investigated whether: (1) potentially more physiological, cyclical mechanical activity could induce an IEG programme, (2) similar effects could be seen in adult cardiomyocytes, and (3) other forms of stress, such as hyperosmotic media, also induced an IEG programme. The 'early growth response gene-1' (*Egr-1;* Sukhatme *et al.* (1988) *Cell* **53**, 37–43) was investigated because we have recently demonstrated that reducing its expression by an antisense oligonucleotide could suppress the stimulation of protein synthesis induced by growth factors in adult cardiomyocytes indicating that the *Egr-1* gene product may be an essential component in the signal transduction pathway leading to growth (Neyses *et al.* (1991) *Biochem. Biophys. Res. Comm.* **181**, 22–7).

Single cardiomyocytes were isolated from adult (200–250 g) Wistar Kyoto rats. Then 2×10^5 cells were stimulated by biphasic electrical stimuli at 5% above threshold level (40 V cm^{-1}). Contraction was recorded using a computerized videomicroscopic system; mRNA was determined by Northern blotting.

After 30 min stimulation at 120 beats per min (b.p.m.), *c-fos* and *Egr-1* were induced 3–4 and 4–5-fold, respectively. At 60 and 300 b.p.m., induction was reduced by about 50%. Total actin controls showed no change.

BDM (2,3-butane-dione-monoxime), which desensitizes the contractile elements against calcium without altering membrane potential reversibly inhibited contraction. Full inhibition was seen at 9 mM BDM. At this concentration induction of *c-fos* and *Egr-1* was inhibited by >80%. Continuous depolarization by KCl (25 and 50 mM at isotonicity) for 30 min also induced *c-fos* and *Egr-1* (4- and 6-fold and 5- and 7-fold, respectively). Raising osmolarity from 290 to 315 and 370 mOsm by NaCl induced both genes (3- and 5-fold, respectively). This osmotic stress did not lead to shortening of the cells.

It is concluded that cyclical contraction of adult cardiac myocytes is sufficient to induce growth-related immediate early genes; no cofactors from other cells appear to be required. The mechanical component of contraction rather than the concomitant depolarization is responsible for this effect. Continuous, but not short repetitive, depolarization induces this gene programme. Adult cardiomyocytes may possess a sensor for osmotic stress which is coupled to the immediate-early gene response.

Microfilament–membrane attachment sites in chicken embryonic cardiocytes

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In striated muscle, the interaction of actin-based microfilaments with the plasma membrane is crucial for a variety of motility-related processes, such as isotonic and isometric contraction of myofibrils, the attachment of muscle cells to the basal lamina, and the formation of cardiocyte-specific cell–cell junctions (intercalated disks). A detailed knowledge of molecular composition, geometry and dynamics of these specialized regions is therefore of prime importance for the understanding of muscle function.

In this context, we have analysed the protein complement of three different types of microfilament–membrane attachment sites that are present in cultivated cardiocytes: (1) cell–substratum junctions (CSJ), (2) cell–cell junctions (intercalated disks, ICD), and (3) junctions between myofibrillar Z-disks and the plasmalemma (ZDJ, costameres; Pardo *et al.* (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1008–12). Chicken embryonic cardiac cultures were obtained from 7-day old embryos. After 4–7 days in culture, they were decorated with specific probes for myofibrillar and peripheral membrane proteins and analysed by fluorescence microscopy. These probes included FITC-phalloidin, and monoclonal or polyclonal antibodies against sarcomeric actin (HUC 1-1; Sawtell *et al.* (1988) *Cell Motil. Cytoskel.* **11**, 318–25), vinculin (Westmeyer *et al.* (1990) *EMBO J.* **9**, 2071–8), α-actinin (Schulze *et al.* (1989) *EMBO J.* **8**, 3587–93), insertin and zyxin (Wiegand and Jockusch, unpublished data), cingulin (Citi *et al.* (1988) *Nature* **333**, 272–6) and the vasodilator stimulated phosphoprotein, VASP (Reinhard *et al.* (1992) *EMBO J.* **11**, 2063–70). In addition, the distribution of these proteins was also monitored in the CSJ of cardiac fibroblasts present in the same cultures. Our data show that: (1) CSJ of cardiocytes and fibroblasts differ markedly in their protein composition, in accordance with Lu *et al.* (1992) *J. Cell Biol.* **117**, 1007–22). In particular, the regulatory protein zyxin (Crawford and Beckerle (1991) *J. Biol. Chem.* **266**, 5847–53) is only present in fibroblastic CSJ and not expressed in cardiac muscle, (2) VASP, a putative mediator between F-actin and a signal transduction pathway (cf. reference cited above) is a component of ICD, ZDJ and of fibroblastic and cardiocyte CSJ, and (3) the tight junction protein cingulin (cf. reference cited above) was detected in small dots associated with the ICD region, but not in the other junctional complexes analysed in this study.

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Cardiotin and vasculin, two recently characterized components in the cardiovascular system

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To understand the abnormal development of the mammalian heart, it is important to analyse the cell biological processes that underly the morphological changes during normal cardiogenesis and the formation of sarcomeric structures. In this respect specific cardiac muscle markers are indispensable. Using the monoclonal antibody technique, we have identified two new structural components of the cardiovascular system, which we designated vasculin and cardiotin. Vasculin is a 59 kDa protein and can only be detected in smooth muscle cells. Cardiotin is a high molecular weight protein and is exclusively found in cardiac and skeletal muscle tissues. No reaction with these antibodies was found in other human tissues. Cardiotin is localized at the periphery of the myofibrils. The cardiotin antibody is crossreactive in heart tissue of different species, i.e. rabbit, dog, goat, monkey and human. *In vitro* studies have shown that cardiotin is also expressed in differentiating human skeletal muscle cell cultures. Cardiotin expression seems to be diminished in myofibrils during cardiomyopathy. This indicates that this component may be a relevant constituent in normal heart function and that the antibody to cardiotin may become a useful tool in the diagnosis of congenital myopathies.

Effects of irregularities in the pacing rhythm and changed milieu factors on the shape of bi- and triphasic heart muscle contractions

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Under *in vitro* conditions the administration of certain drugs (e.g. milrinone, adrenaline, isoproterenol, dibutyrylcyclic AMP) to the electrolyte solution superfusing right ventricular guinea pig papillary muscles causes a curious change in shape of the time course normally observed for muscle contraction. Within the BOWDITCH staircase, following the postrest contraction as a transient phenomenon, biphasic (possibly multiphasic) twitches appear which can be stabilized for a prolonged period of time (2–5 h) by lowering the temperature, prolongation of the interstimulus interval and addition of calcium agonists. Staircase and potentiation phenomena of all kinds, well-known from monophasic twitches, occur in typical modifications, e.g. the postrest contraction is reduced to the (ryanodine insensitive) late component, and in premature multiphasic beats, only the first component is potentiated. Addition of the myotonia-inducing drug anthracene-9-carboxylic acid (AC-9; 10^{-4}–10^{-3} mol l^{-1}) to the superfusion fluid causes a conspicuous deformation of the curve reflecting the mechanical restitution in monophasic twitches. AC-9 proves to be a drug affecting, in particular, the second component of a biphasic contraction. In isoproterenol-induced, BAY K 8644-stabilized triphasic contractions, AC-9 causes an augmentation of the twitch up to 20-fold the developed tension of the control, particularly of the second and of the third component.

Effects of ADP, inorganic phosphate and low pH on rigor tension development in cardiac skinned fibres

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The effects of ATPase reaction products on rigor tension development were studied in rat ventricular Triton X-100 treated fibres. All solutions were calculated to contain (in mM): imidazole 30, Mg^{2+} 1, EGTA 10 (*p*Ca 7), Na^{+} 30.6, DTT 0.3, ionic strength 160 adjusted with K-acetate, in the presence or in the absence of phosphocreatine (PCr) 12, and variable MgATP concentrations. Under control conditions (pH 7.1, and no added inorganic phosphate (Pi) or MgADP), the pMgATP for half-maximal rigor tension ($pMgATP_{50}$) was 3.47 ± 0.04 in the absence and 5.09 ± 0.02 in the presence of PCr. This shift by 1.62 ± 0.05 induced by activity of myofibrillar creatine kinase can be taken as an index of the enzyme functional efficacy. The addition of 20 mM Pi did not change the $pMgATP_{50}$ in the absence of PCr but slightly decreased $pMgATP_{50}$ (5.01 ± 0.02) in the presence of PCr. Acidic pH (6.6) strongly increased $pMgATP_{50}$ both in the absence (3.90 ± 0.03) and in the presence (5.45 ± 0.02) of PCr. Conversely, MgADP (250 μM) decreased $pMgATP_{50}$ to 3.26 ± 0.06 in the absence of PCr; no rigor tension was observed in the presence of PCr. $pMgATP_{50}$ in the presence of ADP and low pH was close to the control value (3.44 ± 0.07). Neither acidosis nor increased Pi concentration influenced significantly the index of creatine kinase efficacy but combined action of these factors decreased it considerably to 1.18 ± 0.05. At acidic pH, maximal rigor tension was lower by 29% compared with control conditions while in the presence of MgADP, maximal rigor tension developed to 143% of control value; Pi had no effect. Thus, in addition to their known effects on active tension, MgADP and protons greatly affect rigor tension development in cardiac muscle.

Atrial natriuretic peptide (ANP) is synthesized, stored and secreted by adult rat ventricular cardiomyocytes in culture

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Adult rat ventricular cardiomyocytes (ARVC) in long-term cultures show extensive changes in morphology and in the expression of contractile proteins which are similar to that found in fetal rat ventricular cardiomyocytes (FRVC) (Eppenberger-Eberhardt *et al.* (1990) *Dev. Biol.* **239**, 269–78). In contrast to the contractile apparatus, mitochondria do not seem to be involved in the process of restructuring and of reprogramming gene expression (Eppenberger-Eberhardt *et al.* (1991) *J. Cell Biol.* **113**, 289–302). Additional to the capability of the cultured cells to contract synchronously, they display hormonal activity. Like FRVC, cultured ARVC synthesize, store and secrete atrial natriuretic peptide as we have demonstrated by immunofluorescence staining using an antibody against α-ANP (99-126) as well as by radioimmunoassay (RIA). In newly isolated rodshaped cells, as in tissue from adult ventricular wall, ANP is not synthesized and accumulated, and once in culture the cells do not begin to synthesize ANP before the myofibrillar apparatus is reorganized; then all cells with an intact myofibrillar apparatus contain ANP. The reactivation of a programme of embryonic gene expression, which includes expression of ANP in ventricle cells, suggests that ARVC in long-term cultures resembles a situation which has been shown to occur *in vivo* under the condition of hypertrophy.

Gene expression in cardiac hypertrophy

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Cardiac hypertrophy from chronic haemodynamic overload is the common end result of most cardiac disorders and a consistent feature of cardiac failure. It produces in the cardiac myocyte, through a mecanogenic transduction whose pathways are largely unknown, both qualitative changes (i.e. phenoconversions characterized by protein isoform switches) and quantitative changes (characterized by modulation of the expression of individual genes) (reviewed in Boheler and Schwartz, *Trends Cardiovasc. Med.* (in press)). In most instances it leads to the re-expression of a phenotype analogous to that seen in the fetal heart. The most thoroughly studied examples of isoform switches involve the myosin heavy chain (MHC) family (α- and β-MHC) and the actin family (skeletal α-act and cardiac α-act). The ratios of β- to α-MHC mRNAs, and of skeletal α-act to cardiac α-act mRNAs increase, and the precise mechanisms of these changes are unknown. Using RNA detection techniques, we recently found after the imposition of a haemodynamic overload that these MHC isogenes are antithetically regulated (β-MHC transcripts increase and α-MHC transcripts decrease in abundance) whereas the sarcomeric isoactins are not (skeletal α-actin transcripts accumulate while cardiac α-actin mRNA levels remain constant). To determine at which level these isogenes are regulated, we set up a nuclear run on assay with cardiac nuclei from intact rat hearts (Boheler *et al.* (1992) *J. Biol. Chem.* **267**, 12979–85). Radiolabelled nascent RNA transcripts were isolated and hybridized against single stranded probes corresponding to the + and − strands for isoactin and isomyosin isogenes. The relative transcriptional activities were compared with the relative accumulations of isomRNAs from each of these genes. Our results from 23–24-day-old rats support the conclusion that the primary mechanisms controlling the accumulations of the MHC and actin gene products during normal growth are transcriptional. Using this system we also detected the presence of an antisense RNA for the β-MHC that was transcribed at levels more than sevenfold above background and was absent when the experiments were performed in the presence of α-amanitin (2 mg

ml^{-1}). These results indicate that in heart, the major regulatory level for these contractile protein gene products is transcriptional but the presence of an anti-β-MHC transcript suggest that other mechanisms may be involved.

Adhesion of lymphocytes to mouse cardiomyocytes in tissue culture

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T cells are thought to be at least partly responsible for damage to heart cells in myocarditis. However, very little is known about the mechanisms by which lymphocytes bind to cardiomyocytes and the adhesion molecules and receptors involved in this process. We have developed a system for investigating interactions between these cells *in vitro*, using murine cells because of the availability of monoclonal antibodies. Ventricular cardiomyocytes of fetal mice (at 16–20 days gestation) were prepared by collagenase digestion, and after 20 h of incubation in full medium containing 10% fetal calf serum they were transferred to poly-L-lysine/laminin coated dishes and kept in serum-free medium containing insulin, transferrin, selenium, fetuin, EGF, ethanolamine, hydrocortisone and L-thyroxine (Nag *et al.* (1985) *In Vitro* **21**, 553–62, with modifications). During 3 weeks of culture the cardiomyocytes still retained contractile activity, with little evidence of overgrowth by contaminating fibroblasts.

Lymphocyte-binding assays were carried out between 6 and 14 days of culture. T lymphocytes were prepared from mouse spleens by removing monocytes and B cells on a nylon wool column. Activated T cells were obtained by culture of the resting cells with the T cell mitogen concanavalin A for 3 days. For the adhesion assay, the T cells were labelled with biotin and allowed to bind to the cultures of cardiomyocytes grown as monolayers in microtitre wells. Nonadherent lymphocytes were removed by washing and the monolayer incubated with streptavidin/peroxidase followed by *o*-phenylenediamine. The colour intensity was found to be directly proportional to the number of lymphocytes that had attached, as previously described (Pearce-Pratt *et al.* (1991) *J. Immunol. Meth.* **140**, 159–65). Using this procedure the average number of resting and activated T cells which adhered to the myocytes could readily be calculated from a standard curve of the binding of a known number of T cells to wells coated with antibody to the T cell receptor complex (anti-CD3). We are currently using monoclonal antibodies to specific T cell adhesion molecules (e.g. CD2, LFA-1, ICAM-1) to assess the significance and contribution of various cell–cell recognition pathways in the binding of T lymphocytes to heart cells.

Two possible ways of lymph-heart striated muscle regeneration in adult frog

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A study of the mechanically damaged caudal lymph-heart muscles of adult frog *Rana temporaria* carried out with the use of ^{3}H-thymidine electron microscopic autoradiography has revealed two possible ways of regeneration. With the first one, during the first postoperative week in most of the trauma-activated muscle fibres adjacent to the wound partial cellular dedifferentiation and subsequently, growth of the ends of these fibres towards the damaged area, is observed. With the second, during the second to third postoperative week, an activation of satellite cells of the lymph-heart muscles is registered as well as the formation *de novo* of young myotubes in the damaged area. Partial dedifferentiation of muscle fibres is accompanied by the ultrastructural reorganization of nuclei (chromatin decondensation, nucleolar hypertrophy) and of sarcoplasm. Changes in the sarcoplasm are expressed, on the one hand, in partial destruction of the contractile apparatus (disappearance of I-Z-I complexes, fragmentation of myofibrils, appearance of free myofilament bundles, etc.), and on the other hand, in the activation of organelles responsible for the RNA and protein synthesis, as well as active myofibrillogenesis. The DNA synthesis starting with the end of the first postoperative week observed in single myonuclei, is independent of the extent of partial dedifferentiation of muscle fibres in the area adjacent to the wound. The activation of the DNA synthesis in satellite cells of lymph-heart muscles also falls on the end of the first postoperative week.

Measurement of the sarcomere dynamics in isolated cardiac cells by image analysis

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The mean sarcomere length of both skeletal and cardiac muscle cells is often measured by the laser diffraction method. Laser diffractometry has been used to control the sarcomere length in clamp experiments (Granzier and Pollack (1990) *J. Physiol.* **421**, 595–615) as well as to measure dynamical changes of the sarcomere structure (Wussling *et al.* (1987) *J. Mol. Cell. Cardiol.* **19**, 897–907). The number of sarcomeres that generate the corresponding diffraction pattern is dependent on the diameter d of a commonly focused laser beam in the illuminated region. A theoretical limitation of d is given by $\lambda^* f/D$, where λ = wave length, f = focus length, and D = diameter of the unfocused laser beam. In practice, the spot size of a He-Ne laser beam in the focal plane is $\geqslant 100$ µm. This is in the order of an intact rodlike cardiac myocyte. Thus, it makes no sense to investigate *local* changes of the sarcomere length by laser diffractometry in that preparation.

Image analysis helps to overcome difficulties related to the measurement of local inhomogeneities in a small region of the sarcomere structure. Spontaneous contractile waves of isolated rat heart cells were analysed using the software IPLab of Signal Analytics Corporation for Fast Fourier Transform (FFT). The data of a CCD camera mounted to an inverting microscope was captured in a Macintosh IIci computer directly or via an Argus-10 image processor of Hamamatsu to freeze the contractile wave in a distinct portion of the cardiac cell. A video cassette recorder was used to store data for dynamical measurements. The 'diffraction pattern' obtained by the FFT of the cell's active region clearly differs from that of the relaxed portion even if the number of sarcomeres is less than ten (i.e. the region of interest is <20 µm). Our results demonstrate that image analysis is an appropriate tool to show local inhomogeneities of the sarcomere structure in cardiac myocytes.

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Influence of thyroid state on the contraction properties of ageing myocardium

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Our previous studies (Rom (1990) *Gerontol. Geriatr.* **11**, 4) have shown that water–protein interaction can be modified by hormone excess or deficiency. In the present work we tried to find such modifications in myocardium of long-term treated rats with different doses of T_3 according to the modified method (*Pflüger Arch. Eur. J. Physiol.* **411**, (1988) 620–7), using ^{1}H-NMR technique for measuring ^{1}H-proton transverse relaxing time in glycerinated heart muscle in rigor, contraction and relaxing media. A decrease in T_{2s} of hyperthyroid rats has been recorded when compared with age-matched controls; this fact can account for some modifications in structure and number of contractile

proteins and also for a stronger binding of H^+ to COOH groups. Concerning the T_{2l} parameter, an increase in its value has been recorded for hyperthyroid rats of different ages which can be related with an increase in interfilamentary water content because of high metabolic activity in animals having hormone excess. The hyperthyroid status in rats is characterized by an inverse correlation between T_{2s} and T_{2l} values and doses of hormone used. Phase contrast microscopy studies of sarcomere length in Ri, Co, Re media have shown an increase in active shortening capacity of sarcomere in hyperthyroid rats of different ages; this increase is related to the activation of myofibrillar ATPase. Radioisotopic techniques using ^{3}H-uridine and ^{3}H-tryptophan, have confirmed the increasing metabolic activity at the level of myocardium from hyperthyroid rats in comparison with their age-matched controls. Thyroid hormone excess acts as a modulator of metabolic activity of cardiac cell despite the animal's age.

SMOOTH MUSCLE

Smooth muscle cell differentiation in normal and hypertrophic rabbit detrusor muscle

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A panel of monoclonal antibodies specific for some cytocontractile and cytoskeletal proteins of smooth muscle cells (SMC) as well as procedures of immunocytochemistry and Western blotting have been used to study the differentiation pattern of detrusor muscle (DM) during development and after partial obstruction of rabbit bladder outflow. DM from 90-day-old animals contain desmin, α-actin of SM-type, SM myosin heavy chain isoforms 1 and 2 (SM-MHC1 and 2), but not vimentin or the nonmuscle (NM) myosin isoform recognized by NM-G2 antibody which are downregulated around birth. A second molecular transition occurs with SM-MHC2: this isoform is upregulated around the seventh day after birth, whereas SM-MHC1, desmin and α-actin are equally expressed throughout development. Analysis of cross-sectional areas of SMC of DM from fetal, newborn and adult rabbits indicate that there is a progressive increase in SMC size. This physiological hypertrophy is accompanied by a marked decrease in the number of DNA-synthesizing SMC, which become very scarce in the bladder of the adult animal.

In the rabbit bladders in which partial outflow obstruction was induced by a ligature at the level of the bladder neck, two distinct events take place in the wall of this organ: (1) a marked thickening of serosa, and (2) a pathological hypertrophy of SMC. Serosal thickening was characterized by the appearance of an heterogeneous population of myofibroblasts which undergo, during a 2-month time period, a marked differentiation toward a fetal-like SMC phenotype. By contrast, the NM myosin isoform recognized by NM-A9 antibody and vimentin become evident in the hypertrophic DM. In addition, SM-MHC2 becomes downregulated in obstructed bladders. Quantitative measurements of bromodeoxyuridine incorporation in these cells indicated that the labelling index increased progressively with the duration of obstruction.

These results indicate that in pathological DM hypertrophy SMC contain vimentin and a specific NM myosin isoform. This immunophenotype can be distinguished from that of the highly proliferative cells of thickened serosa which is characterized, in the chronic stages of obstruction, by the presence of a distinct NM myosin isoform peculiar to the fetal SMC phenotype.

Purification and properties of caldesmon-like protein from molluscan smooth muscle

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Caldesmon is a potential regulatory protein present in smooth muscle and nonmuscle cells. Two isoforms of this heat-stable molecule have been described. The low molecular mass caldesmon (lCaD) is detected in many cells while the high molecular mass caldesmon (hCaD) is specifically expressed in smooth muscle cells. The primary structure of these CaDs has been determined from mammalian and avian smooth muscle and nonmuscle cells (Yamashiro *et al.* (1991) *Nature* **349,** 169–72). Sequence comparisons indicate that these isoforms are generated by exon skipping of a single CaD gene (Humprey *et al.* (1992) *Gene* **112,** 197–204).

Bennett and Marston (*J. Muscle Res. Cell Motil.* **11** (1990) 302–12) have shown the presence of a hCaD-like protein in *Mytilus* anterior byssus retractor (smooth) muscle and oyster adductor translucent (fast) and opaque (smooth) muscle. Although smooth muscle was known to be the only source of hCaD, Bartegi *et al.* (*Eur. J. Biochem.* **155** (1989) 589–95) have reported on the presence of this protein in the striated muscle of *Pecten maximus.*

In the present work we have re-examined the heat-stable protein content in smooth and striated muscles of three different scallop species. We show that the heat stable hCaD-like protein is present only in the catch (smooth) muscle but it is completely absent in the striated muscle, while a lCaD-like protein can be isolated from both types of scallop muscle.

The hCaD-like protein was isolated from *Pecten maximus* adductor muscle and after purification, the properties of this molecule were analysed. This protein, with an apparent molecular weight of 120 kDa, was cosedimented with actin. Purified hCad attached to a solid support specifically retained myosin. This molecule acts as a potent inhibitor of rabbit skeletal actomyosin Mg^{2+} ATP-ase activity. CD measurements, susceptibility to proteases and solubility were studied. Sequence of a cDNA clone from ABRM shows close similarity with known vertebrate hCaD sequences further indicating that this invertebrate protein belongs to the caldesmon family.

Study on the secondary structure of caldesmon and its C-terminal fragments

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Smooth muscle caldesmon is a highly asymmetric protein with dimensions of about 76 × 2 nm. The studies on its secondary structure performed so far, have yielded inconsistent results. For example, the estimates of α-helix content from CD spectra were 10 or 40%, while the value predicted from amino acid sequence was >80%.

To determine all components of the secondary structure of caldesmon and its C-terminal fragments we have undertaken both empirical and theoretical studies. Among applied methods of the secondary structure evaluation from CD spectra, the most suitable appeared to be the modified method of Provencher and Glöckner (*Biochemistry* **22** (1981) 33–7). Using this method it was established that caldesmon is made up of 51% of helix, 9% of β-strand and 40% of other structures. The estimates of the secondary structure components in C-terminal 34 and 19 kDa fragments were 11 and 12% of helix, 22 and 20% of β-strand, 13 and 17% of β-turns, and 54 and 51% of other structures, respectively. Predictions of the secondary structure of caldesmon and its C-terminal fragments on the basis of the primary structure performed with ALB algorithm (Ptitsyn and Finkelstein (1983) *Biopolymers* **22,** 15–25) gave the values which agree well with those obtained from experimental data. Because in the case of caldesmon the predicted values were obtained for the unfolded chain state, it indicates that long-distance interactions are not involved in the formation of the tertiary structure of this protein. On the other hand, the best fit with experimental data of the predicted values

obtained for caldesmon fragments assuming their globular state, suggests that the conformation of the globular C-terminal part of the caldesmon molecule in intact protein and isolated fragments is somewhat different.

Interaction of caldesmon with phospholipids

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Caldesmon originally found in smooth muscle is also distributed in a wide variety of nonmuscle cells. As in some of these cells caldesmon is located in the vicinity of the membrane one can suppose that, like other actin-binding proteins, it can interact with membraneous lipids. The report of Vorotnikov and Gusev (*FEBS Lett.* **289** (1991) 213–6) showing interaction of caldesmon with soybean azolectin is consistent with this concept.

We have examined the interaction of caldesmon with liposomes composed of various phospholipids by tryptophan fluorescence spectroscopy. The results indicate that the effectiveness of phospholipids to change the tryptophan spectrum of caldesmon increases in the following order: cardiolipin < phosphatidic acid < phosphatidylinositol < phosphatidylserine. Phosphatidylcholine does not affect the spectrum. The strongest complex formed by caldesmon with phosphatidylserine ($K_{ass} = 1.45 \times 10^5\ M^{-1}$) is saturated at 50 moles of this lipid per mole of caldesmon. Both electrostatic and hydrophobic interactions contribute to its stability. The binding site is located in the 34 kDa C-terminal fragment of caldesmon. Binding of phosphatidylserine to this site results in displacement of calmodulin from its complex with caldesmon.

Expression of smooth muscle α-actin marks phenotypic differences of rat renal mesangial cells *in situ* and *in vitro*

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Mesangial cells (MC) of renal glomeruli possess some of the morphological characteristics of smooth muscle cells, and it has been proposed that they may contribute to the regulation of glomerular blood flow and filtration by dynamic contractility. This view has been supported by observations that MC in tissue culture are able to contract in response to vasoactive substances (Mene *et al.* (1989) *Physiol. Rev.* **69**, 1347–424). To further characterize the contractile apparatus of MC, a monoclonal antibody against smooth muscle alpha-actin (SM α-actin) (Skalli *et al.* (1986) *J. Cell. Biol.* **103**, 2787–96) was used to study the expression of this protein in kidney sections and mesangial cell (MC) cultures.

In the tissue sections, indirect immunofluorescence revealed intense labelling of vascular smooth muscle cells (SMC) and precapillary pericytes for SM α-actin. Glomerular cells including MC were negative, with the exception of scattered SMC in the wall of the intraglomerular segment of the efferent arteriole. In contrast, in MC cultures 50–95% of the cells displayed bright fluorescence. Immunoreactivity for SM α-actin first appeared 3 days after explanation of glomeruli, and increased until the primary culture reached subconfluence. In each subculture (one to ten) expression of SM α-actin was weak on day 1 and pronounced at subconfluence. Growth arrest of subconfluent cultures for 1–7 days in serum-free medium did not alter the percentage of cells positive for SM α-actin. However, exposure of MC to serum-free medium beginning on the first day of subculture curtailed expression of SM α-actin. Double-labelling with antibodies against proliferating cell nuclear antigen (PCNA) and SM α-actin revealed SM α-actin positive filaments in both replicating and resting cells.

In summary, our results demonstrate that some process or processes associated with cell proliferation and cell growth of MC are accompanied by *de novo* expression of SM α-actin. The relevance to the contractile behaviour of the difference in SM α-actin expression under *in vitro* and *in situ* conditions is unknown.

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New concept of muscle contraction mechanism: 2. Importance of two heads in contraction

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At present, much detailed knowledge concerning different aspects of muscle contraction has been accumulated. In spite of an intense effort, the correlation mechanism between the structural, mechanical, biophysical and biochemical properties of the working structures in the sarcomere is vaguely understood.

A new concept is proposed. All crossbridges act cyclically by shifting from the vicinity of the thick filament to the vicinity of the thin filament (Skubiszak (1990) Abst. First World Congr. Biomech., USA, p. 185). Sets of three HMM arranged at each 430 Å along the thick filament axis (Skubiszak (1990) *Cell Biol.* **14**, 207) act simultaneously. Each set arranged at each 143 Å works with the phase shift of $1/3T$ (T is the duration of a cycle). Each head of the same myosin molecule is roughly identical but the phase of ATPase taking place on each of them is shifted by T. In this way, if at the phase $t = 0$ one head (called A) has an ATP and the other (called B) has an ADP molecule bound to it, then, one cycle later, there will be ADP on the head A and ATP on the head B. Six simultaneously working heads belonging to the same thick filament act in keeping with four principles. Because of that, if at the beginning of a cycle three heads are involved in the attachment to the thick filament, then, $1/2T$ later, the remaining three heads will be involved in attachment to the thin filament. Two heads (with ADP or without nucleotide) will give strong attachments (owing to the spatial matching between the actin and myosin binding sites (Skubiszak (1992) *J. Muscle Res. Cell Motil.* **13**, 254)) and one head with ADP-P_i will give weak attachment (the matching does not exist). In the next cycle, the roles of the heads will exchange. In this way 12 characteristic states exist for each head during muscle contraction. On the basis of the above concept, an integral model of muscle contraction is described.

Molecular detection of caldesmon isoforms in mammalian tissue

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The polymerase chain reaction (PCR), Northern and Western blotting are currently being employed to detect mRNA and protein expression of caldesmon isoforms in normal and diseased tissue. The presently known isoforms differ in the central helical region of the protein (domain two) whose function is poorly understood.

mRNA expression is detected directly by Northern blotting, the isoforms being detected according to their size differential on electrophoresis through formaldehyde-containing agarose gels. PCR is used as an indirect detection method, the mRNA serving as a template for the synthesis of cDNA which can be amplified. The primers used in this technique are complementary to sequences either side of the cDNA coding for domain two of the CDh protein sequence, as this sequence is known to differ between caldesmon isoforms because of deletions and insertions, and are designed to amplify with equal efficiency cDNA of both CDl and CDh. The amplified cDNA products are then size fractionated by agarose gel electrophoresis, CDl giving a 320 bp product and CDh giving a 1091 bp product, according to the sequences published for human CDh (one sequence) and CDl (three sequences) (Bryan *et al. Gene* **112**, 197–204; Novy *et al. J. Biol. Chem.*

266, 16917–24). These two isoforms can thus be detected simultaneously from the tissue, cells and libraries.

The PCR technique is also being used to detect the presence of additional isoforms in mammalian tissue, which from nonmammalian sequence data are expected to differ in the region of domain two. Putative new isoforms have been amplified by PCR from a lambda gt10 human aorta cDNA library and are being analysed.

To detect protein expression we have used the C-terminal 288 amino acids of human caldesmon and amino acids 230-417, within domain two, of chicken gizzard caldesmon expressed in *E. coli* as antigens specific to all caldesmons and CDh, respectively.

A peptide corresponding to the unique junctional region of domains one and three of CDl was synthesized and used as an antigen-conjugated with KLH. CDh specific, CDl specific and nonspecific antibodies have been demonstrated on Western blots. Preliminary work indicates these antibodies can be used to investigate caldesmon isoform expression in human tissue by immunofluorescence microscopy.

High molecular weight, membrane-associated protein from smooth muscle

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In skeletal muscle, high molecular weight proteins, including titin (Mw = 3000 kDa), nebulin (Mw = 700 kDa) and dystrophin (Mw = 400 kDa) make up part of the structural network of the differentiated muscle cell. We have been probing smooth muscle for the presence of similar proteins with the aim of gaining insight into the organization of the cytoskeleton of these cells. Neither titin nor nebulin are present in smooth muscle tissue but other polypeptides in the molecular weight range 400–1000 kDa have been identified. This work describes the identification, partial purification and localization of a 1000 kDa protein (HMWP) from smooth muscle.

HMWP has been extracted from turkey gizzard smooth muscle using the procedure described by Feramisco and Burridge (*J. Biol. Chem.* **255**, (1980) 1194–9) for the purification of filamin and α-actinin. Owing to difficulties in separating HMWP from contaminating proteins, it could be purified to homogeneity only by electroelution from polyacrylamide gels. The electroeluted protein runs as a doublet of around 1000 kDa in 2–12% polyacrylamide gradient gels. Limited proteolysis by V8 protease and microsequencing of the products revealed no homology with other proteins: isolated peptides showed an unusual number of alternating valine-hydrophilic amino acid doublets, suggestive of a cross β-structure. Immunoblotting with an affinity-purified polyclonal antibody identified the HMWP in muscle as well as in nonmuscle tissues. In chicken, the most prominent staining was observed in smooth muscle in which HMWP was localized at the cell membrane in an alternating distribution with vinculin. The same localization has been established for smooth muscle dystrophin (North *et al.* (1992) abstract, this meeting).

Elasticity of the crossbridges in the skinned fibres of taenia coli in 'high' and 'low' rigor states

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To elucidate the mechanism of the force generation in the smooth muscle we carried out a detailed study of the mechanical characteristics of the skinned taenia coli preparations in rigor state. The 'low' rigor state (LR) was achieved after the removal of ATP from the relaxed preparation. When ATP and Ca ions were removed at the plateau of Ca activating tension the 'high' rigor state (HR) developed (Arner (1985) *Acta Physiol. Scand.* **124**, 206). At a steady level of rigor tension, quick releases and stretches were imposed (0.1–2% L in 1.5 ms) on the fibres and length–tension relations were constructed. It was found that: (1) the tension in the HR was 5.2 ± 1.5 times the tension in the LR state, (2) the immediate preparations stiffness evaluated from the slope of L–F plot was markedly lower in HR state than in LR: 100 ± 10 L^{-1} and 143 ± 10 L^{-1} for HR and LR, respectively, (3) the amount of shortening required to reduce rigor tension to zero was found to be 0.010 ± 0.002 L in HR and 0.007 ± 0.001 L ($n = 15$) in LR state. Experimentally obtained L–F relations were well approximated by the model in which crossbridges having linear stiffness were in series with passive elastic component and exponential elasticity. Small (0.1% L) sinusoidal (f = 200 Hz) length perturbations were superimposed on the fibre during the release step and fraction of the crossbridges stiffness as part of a total fibre stiffness was estimated by the method of Warshaw (*J. Gen. Physiol.* **91** (1988) 761). It was found that crossbridges in LR and HR states are stiffer than rigor fibres by 1.5 and 1.35 times, respectively. It may be suggested that different fibres compliance in HR and LR states are due to different patterns of distribution of the crossbridges between high- and low-force generating states during development of rigor by different ways.

Two domain organization of the smooth muscle membrane skeleton as revealed by double immunolabelling of dystrophin and vinculin

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The submembranous dense plaques or 'adherens junctions' of smooth muscle cells serve as anchorage sites for the actin filaments of the contractile apparatus. In most smooth muscles, the adherens junctions, typically marked by antibodies to vinculin, form continuous and longitudinal rib-like arrays at the cell surface: in thin cross-sections under the light microscope, this rib-like pattern gives rise to discontinuous, vinculin-positive streaks around the cell periphery. Electron microscopic studies have shown that the membrane domains between the adherens junctions are devoid of an electron-dense undercoat, instead being rich in membrane invaginations, or caveolae.

Using antibodies to dystrophin and vinculin, in double-labelling immunofluorescence microscopy and immunoelectron microscopy, we now show that dystrophin is specifically localized in the caveolae-rich domains of the sarcolemma of guinea pig taenia coli. A similar exclusion of dystrophin from vinculin-containing adherens domains is found in chicken gizzard. Additional labelling experiments revealed that β_1-integrin and fibronectin are confined to the vicinity of the adherens junctions, together with vinculin and tensin, a further peripheral membrane component of these structures. Laminin, on the other hand, was found to be distributed around the entire cell perimeter.

The membrane of the smooth muscle cell is thus divided into two distinct domains, featuring different and mutually-exclusive components of the membrane skeleton. This clear segregation suggests that smooth muscle may serve as a useful system for the identification of potential interacting partners of dystrophin.

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Identification of the functional domains of smooth muscle caldesmon

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Caldesmon is a major actin-binding protein in smooth muscle and

nonmuscle cells and is specifically located on the thin filaments. *In vitro* it has been shown to bind actin, tropomyosin, Ca^{2+}-calmodulin and myosin and to inhibit actin-activated myosin ATPase. To locate the various functional domains of the molecule, we are expressing mutant caldesmon DNAs in *E. coli* and performing *in vitro* assays on the purified proteins. We have previously reported the expression and functional properties of a full-length chicken gizzard caldesmon cDNA coding for 756 amino acids (Bryan *et al.* (1989) *J. Biol. Chem.* **264,** 13873–9) and a mutant ('N578') composed of amino acids 1–578 (Redwood *et al. FEBS Letts.* **270,** 53–6). We now report the characterization of four further mutant fragments: amino acids 1–128 ('N128'), 230–419, 606–756 ('606C') and 658–756 ('658C').

In common with other reports, actin binding was found to be localized in the C-terminus; only the two C-terminal fragments were found to bind to actin-tropomyosin in sedimentation assays. The 658C bound with an approximate kDa of 4 μM at 25°C in low salt buffer whereas the 606C fragment bound at least tenfold stronger under the same conditions. The measurement of binding of the fragments to pyrene-tropomyosin in solution found that N578, 230–419 and 606C all had affinities within one order of magnitude of the full length protein (approximate kDa = 0.5 μM); in addition, N128 was found to bind weakly. The occurrence of multiple interactions with tropomyosin may explain how caldesmon lies along the length of the thin filament despite only binding to actin at its C-terminus. Both N128 and N578 bound to smooth muscle myosin in sedimentation assays about tenfold less strongly than the full length protein; no other fragment showed any significant binding. Both the C-terminal fragments inhibit actin-tropomyosin-activated myosin ATPase; correlation of the inhibition with the amount of fragment bound to the thin filament showed that both 606C and 658C, in common with the full length protein, can inhibit approximately 14 actins. Inhibition by both fragments was found to be reversed by Ca^{2+}-calmodulin in a similar manner to that of the full length protein. The C-terminal 99 amino acids (658C) thus contain the coupled inhibitory and calmodulin binding sites of caldesmon.

Tension transient responses and frequency-dependent stiffness variations of taenia coli during its spontaneous contractions

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The kinetics of crossbridge cycle in skeletal and cardiac muscles have been studied either by obtaining tension (velocity) transient responses to sudden length (force) perturbations or by measuring the variation of the active stiffness with the frequency of the length perturbation. Although these two methods have a wide application in skeletal and cardiac muscles, only the former have been used in smooth muscles. The present study was carried out to measure the tension transient responses of taenia coli smooth muscles and to find the variation of stiffness with the frequency of the length perturbations.

In the experiments, seven taenia coli strips isolated from female guinea pigs were used. After they were mounted in an organ bath, they were allowed to develop spontaneous contractions. While rhythmic spontaneous contractions were being recorded, successive sudden stretches and releases were imposed continuously on the spontaneously contracting taenia coli and the average of the tension transient responses to each successive stretch was evaluated on-line. The amplitude of the length perturbations was maintained below 1% of the muscle length, and the duration of the stretch/release was 65 s.

The tension transient responses of spontaneously contracting taenia coli had the characteristic four phases of skeletal muscles. Initially a sudden tension increase coincident with the applied stretch was recorded. Then tension decreased and reached a minimum level in about 1–2 s. This early recovery phase was followed by a delayed tension rise which reached maximum around 5–12 s. Thereafter tension responses had the steady-state level. To obtain the variation of the stiffness with the frequency of the applied length perturbation the average tension transient responses and the imposed length perturbations were subjected to Fourier transform, and their ratio was calculated. In addition, phase difference between the tension and length signals and work done per cycle of the length perturbation were evaluated. It was found that the stiffness had a dip around 0.06–0.1 Hz. Within this frequency range the phase angle changed from negative to positive values, and work done per cycle of the length perturbation had positive values. In skeletal muscles the positive work region was considered to reflect the crossbridge cycle (Farrow *et al.* (1988) *J. Muscle Res. Cell Motil.* **9,** 261–74). Thus, our results indicated that the crossbridge cycle in taenia coli was around 0.06–0.1 Hz.

Sarcoplasmatic calcium increase retarded smooth muscle contraction kinetics

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Contraction kinetics were studied in the isolated portal vein (PV) and tracheal smooth muscle (TSM) of the rat by using the vibration technique (Klemt *et al.* (1981) *J. Physiol.* **312,** 281–96). The time constant of postvibration force recovery reflects the rate of crossbridge cycling, and steady-state force reflects the number of force-generating crossbridges. A 50 Hz sinus electrical field stimulation activated the contraction process in PV by membrane depolarization, and a 30 Hz 0.15 ms stimulation induced neurogenic release of acetylcholine in TSM. The time constants of force recovery after cessation of force-inhibiting vibration as calculated for the above control conditions averaged 0.64 ± 0.02 s (fast contracting PV) and 4.5 ± 0.1 s (slow contracting TSM). After incubation in calcium-free tyrode solution for 20 min and subsequent depolarization in calcium-free, potassium-rich tyrode solution for 3 min, a stepwise increase in extracellular calcium concentration between 0.25 and 97.5 mmol l^{-1} $CaCl_2$ increased force-generation stepwise. The ED_{50} was 10.8 mmol l^{-1} (PV) and 1.27 mmol l^{-1} (TSM). The time constant of postvibration force recovery as determined after 14 min incubation with 2.5 mmol l^{-1} $CaCl_2$ was 1.25 ± 0.02 s in PV and 16.76 ± 1.24 s in TSM. Owing to the downregulation of the crossbridge contraction kinetics, these time constants are distinctly higher in the tonically activated smooth muscle preparation than those calculated at control conditions. After 14 min incubation with 51.4 mmol l^{-1} $CaCl_2$ the time constants increased distinctly from 1.25 to 17.12 ± 1.11 s in PV and slightly from 16.76 to 21.49 ± 2.92 s in TSM. The force increase indicates a sarcoplasmatic calcium concentration increase with pronounced cross-bridge cycling retardation in PV only. Force development together with retarded actin–myosin interaction kinetics occurring at high sarcoplasmatic calcium improves the efficiency of contraction distinctly. This state of the contractile system is discussed with respect to smooth muscle spasm.

Characterization of smooth muscle of the urogenital tract by the detection of cyclic phosphodiesterase activity

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Increase of cyclic nucleotide monophosphate level was suggested to play a prominent role in mediating the relaxation of smooth muscle cells (cAMP by papaverine and prostaglandin E1, cGMP by EDRF). Cyclic nucleotide phosphodiesterase (PDE) modulates this relaxing effect by increasing the rate of breakdown of cyclic nucleotide. Smooth muscle tissue of the urogenital tract has not been characterized

thoroughly with respect to the composition of different PDE although this could be of great clinical interest.

Our present study was carried out to show the existence of PDE I to V in isolated human smooth muscle of urogenital tract. Tissue of the ureter and corpora cavernosa were tested in presence of selective inhibitors for PDE I to V. The tissue was obtained from patients undergoing operations for various syndromes like tumour excision, etc. The specimen was placed immediately after operation in PDE isolation buffer. The tissue was homogenized and the homogenate was centrifuged at 30 000g and the supernatant fraction was applied to a DEAE-sephacel column and eluted by using a 70–1000 mM Na-acetate gradient. Then 50 fractions were collected and the assay of PDE activity was performed by modification of the method of Thompson and Appleman (*Biochemistry* **10** (1991) 311–6). Using an organ bath for isometric tension measurement, the muscle tone of small strips of tissue ($n = 6$–8) of the smooth muscle tissue was recorded. Quazinone (cGMP inhibited PDE inhibitor), Rolipram (cAMP specific PDE inhibitor) and Zaprinast (cGMP specific inhibitor) were tested for their ability to relax 1 μM noradrenaline/80 mM KCl precontracted muscle strips. The EC 50 of these drugs was compared with papaverine as a nonspecific PDE inhibitor.

The supernatant fraction contained different PDE compositions in the preparations investigated. At least three peaks of activity were observed in each specimen; these may represent different molecular forms of PDE isoenzyme. Spontaneous contractions were found in 30% strips and could be abolished by all three PDE inhibitors. These PDE inhibitors relax noradrenaline/80 mM KCl precontracted strips dose-dependently. Quazinone and roliprame have shown to be the most potent PDE inhibitors and possess an EC 50 equal to papaverine.

Our data supports that cyclic nucleotide metabolism in urogenital tract smooth muscle is important for the relaxation of these tissues. The study indicates that PDE inhibitors may be useful drugs in treating syndromes in which muscle relaxation is required.

Expression and distribution of actin and gelsolin in the ovary of the mouse

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The existence of highly ordered microfilaments in elongated, spindle-shaped cells of the theca externa of the ovary (Burden (1973) *J. Morphol.* **140**, 467–76), and the immunohistochemical detection of smooth muscle cell α-actin with specific antibodies in the theca externa has led to the suggestion that these cells might contribute to the extrusion of oocytes during ovulation in mammals (Czernobilsky *et al.* (1989) *Virchows Arch. B* **57**, 55–61). We investigated the distribution and expression of actin, and an actin-binding protein, gelsolin (Yin (1988) *BioEssays* **7**, 176–9) in mouse oocytes and ovary to possibly further substantiate this notion. Using gelsolin-specific pairs of primers and the reverse transcription-polymerase chain reaction (RT-PCR) it was possible to amplify a partial, gelsolin-specific sequence of ovarian cDNA indicating that there may be substantial levels of gelsolin mRNA present in the mouse ovary. In Western blots using mono- and polyclonal anti-gelsolin antibodies against smooth muscle from pig stomach which crossreact with mouse gelsolin, the concentration of gelsolin over total protein appears to be at least one-sixth of that detected in uterus, a tissue extremely rich in smooth muscle cells. In accordance with this, gelsolin was also detected by immunohistochemistry in cryosections of mouse ovary. It appears to be especially prominent in epithelial, endothelial and theca externa cells which are also rich in actin. Although the role of gelsolin in epithelial cells where it seems to be enriched in the circumferential belt near the lateral cell surface remains elusive, its presence in cells of the theca externa supports the smooth muscle-like character of these cells (Chaponnier *et al.* (1990) *Eur. J. Biochem.* **190**, 559–65). As gelsolin also leads to an increase in the activity of the actin-dependent myosin-ATPase (Strzelecka-Golaszewska *et al.* (1985) *FEBS Lett.* **177**, 209–16), it might play a role in the spatiotemporally controlled contraction of these cells during the process of ovulation.

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Chicken arterial smooth muscle consists of two phenotypically different cell types

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Many studies of vascular smooth muscle cells assume that these cells are homogeneous. However, two morphologically distinct cell types have been identified in avian arterial smooth muscle: classical smooth muscle cells situated in obliquely oriented lamellae and interlamellar cells lacking smooth muscle characteristics. We are studying the two cell types in chicken aortic smooth muscle by characterizing, *in vivo* and in culture, the expression of smooth muscle proteins, especially those of the contractile apparatus and the basement membrane. We have demonstrated, using immunocytochemistry, that in the adult the lamellar cells express smooth muscle specific alpha-actin, myosin and calponin as well as desmin (positive cells), and the interlamellar cells do not (negative cells). Additionally, lamellar, but not interlamellar cells, colocalize with the basement membrane components, collagen type IV and laminin. Both cell types have shown to express vimentin. In aortic arches from 10-day embryos (E10), we could detect collagen type IV and laminin in a multilamellae organization; however, cells expressing smooth muscle cytoskeletal proteins were rare and appeared primarily at the periphery of the vessels. By E13, both the extracellular and cytoskeletal proteins were co-expressed in the lamellae. From this stage through adulthood, patterns of expression of the extracellular and intracellular proteins were similar. We further developed methods for the isolation and culture of the aortic smooth muscle cells and determined, employing tissue from E16, that on the first and second day of primary cultures approximately 10% of the cells express the characteristic smooth muscle cytoskeletal proteins (excluding desmin whose expression is rather rare). The rest of the cells are negative in respect to the specific cytoskeletal proteins. Preliminary results indicate that the percentage of the positive cells may increase with time in culture. In addition, we have begun defining the mitotic state of the two populations by immunocytochemical visualization of bromodeoxyuridine incorporation. We have shown that both positive and negative cells are proliferating. Thus the two cell phenotypes are not a result of a so-called 'phenotypic modulation' from a contractile to a proliferative phase. In summary, the positive and negative cells may represent two distinct cell lineages or they may be lineally related. These two populations may contribute differently to the function and pathology of the vessel.

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Structure of the contractile apparatus of ultrarapidly frozen smooth muscle

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Little is known of the structure of the contractile apparatus in smooth muscle because there are several major problems inherent in investigating smooth muscle ultrastructure. For example, filaments do not necessarily lie parallel to the cell surface, and are often not straight. They are also, by necessity, labile structures. Furthermore, most electron microscopic techniques require a chemical fixation step which, by its very nature, induces artefacts in the structure being studied. Using ultrarapid freezing followed by freeze fracture, deep etching and

platinum carbon replication, we are developing a strategy eliminating the use of chemical fixatives while allowing the visualization of the ultrastructure in three dimensions. In our preliminary studies we see great potential for the use of this technique in the study of the contractile apparatus of smooth muscle. The actin filaments within intact, nonchemically fixed, smooth muscle cells are very well preserved, such that the 5.4 nm repeat of the actin subunits along the filaments (which appear to run roughly parallel to each other) is clearly visible. The complex nature of the contractile network is apparant. Filaments appear to run for only short lengths in the plane of fracture. At points close to the cross fracture through the plasma membrane, filaments are observed which form a net-like structure completely different from that of the actin filaments. We have not yet observed thick filaments, possibly because of the high ratio of actin to myosin and the difficulty in identifying small portions of fractured myosin filaments. Initial freeze substitution results for ultrarapidly frozen material with and without tannic acid provide complementary information to that obtained from freeze fracture. Our initial results here, do reveal both thick and thin filaments; with the contrast of the filaments clearly enhanced in the tannic acid treated tissue. Currently we are carrying out studies of the structure in Triton-extracted cells (using a method which preserves the contractility of the muscle). Immunogold labelling will be carried out on tissue prior to freezing and freeze fracture to pinpoint the different proteins of the contractile apparatus.

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Synergistic and antagonistic modulation of actomyosin ATPase by caldesmon, tropomyosin and gelsolin

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The actin activation of the myosin ATPase activity is modulated by a number of actin-associated proteins. Among them both gelsolin and smooth muscle tropomyosin activate the ATPase, while caldesmon has an inhibitory effect. Because all these proteins coexist in smooth muscle as well as in nonmuscle cells the interaction of each single one of these with actin may affect the interaction of the others. Therefore we have added various combinations of the three proteins to F-actin and have studied the modulatory effects of these filaments on the myosin ATPase activity.

The results show that titration of the gelsolin-activated actomyosin ATPase (gelsolin/actin 1:100 mol/mol) with smooth muscle caldesmon diminishes the activation effect. Conversely, titration of the actomyosin ATPase inhibited by caldesmon with gelsolin decreases this inhibition. Both effects are potentiated significantly by smooth muscle tropomyosin at stoichiometric ratio to actin (1:6 mol/mol). As both caldesmon and tropomyosin are distributed along the whole length of the actin filament the results can be interpreted as a protective effect exerted by both proteins against the filament severing activity. However, a relative activation of myosin ATPase by gelsolin in the presence of tropomyosin plus caldesmon was observed even at high ratios of caldesmon to actin. The persistence of this activating effect even in the presence of both caldesmon and tropomyosin indicated that the filament-severing process was not totally inhibited under these conditions.

These findings were corroborated by fluorometric actin-severing assays which permitted a quantification of gelsolin activity in the presence of the two other actin binding proteins. It was found that smooth muscle tropomyosin alone reduced the severing efficiency by only 28% while caldesmon caused a reduction by 33%. The combination of the two proteins led to a reduction of gelsolin activity by maximum 70%. Even at physiological ratios of both caldesmon and tropomyosin to actin a complete protection of actin filaments from severing by gelsolin was not obtained.

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Myosin light chain kinase and regulation of actin–myosin interaction in smooth muscle

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The key regulatory enzymes of smooth muscle myosin, namely myosin light chain kinase (MLCKase) and myosin light chain phosphatase (MLCPase), are copurified with actomyosin and filamentous myosin in amounts sufficient for the activation and inhibition of actomyosin ATPase. These enzymes are tenaciously bound to myosin filament (Sobieszek (1990) *J. Muscle Res. Cell Motil.* **11**, 114–24) but not to myosin monomers, indicating that the filament architecture is important for their tight association with myosin. Their apparent affinities for filamentous myosin are subnanomolar at approximate molar stoichiometries relative to myosin of 1:30 for the MLCKase (and CM) and 1:10 000 for the MLCPase. My primary interest has been to clarify the kinetic features of the phosphorylation–dephosphorylation reaction as well as to investigate the mechanism of Ca^{2+} and CM activation of the MLCKase.

The kinetic studies, carried out with the isolated regulatory light chain (LC_{20}) as substrate (Sobieszek (1991) *Eur. J. Biochem.* **199**, 735–43), indicated that the phosphorylation reaction has an ordered sequential mechanism in which MgATP bounds first to the kinase, followed by the LC_{20} light chain; subsequently, ADP departure precedes that of the phosphorylated LC_{20}. For the MLCPase, the order of product release during the dephosphorylation reaction was different; the dephosphorylated LC_{20} was released after inorganic phosphate (see Nowak *et al.*, this Meeting). This reaction order is consistent with independent binding studies and indicates a significant role of the ATP induced conformational change of myosin in the phosphorylation reaction.

I have recently shown that, at relatively high concentrations, MLCKase exhibits some allosteric properties (Sobieszek (1991) *J. Mol. Biol.* **220**, 947–57). During gel filtration chromatography the kinase was eluted as a large complex of about 600 kDa M_r. The oligomerization was also suggested from a cooperative activation of the kinase by its substrates (the LC_{20} light chain and intact myosin) and from a concentration dependence of its specific activity. From the 1:1 CM to MLCKase binding stoichiometry a hyperbolic form of activation of the kinase by CM was expected; however, also highly cooperative activation was observed. This property was shown to result from a Ca^{2+}/CM dependent modification of the kinase leading to its apparent inhibition (Sobieszek *et al.*, this Meeting).

Calcium-calmodulin dependent modification of smooth muscle myosin light chain kinase leading to its cooperative activation by calmodulin

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It has been shown recently that at relatively high molar ratios of myosin light chain kinase (MLCKase) to calmodulin (CM) almost complete inhibition of the kinase activity was taking place (Sobieszek (1991) *J. Mol. Biol.* **22**, 947–57). This inhibition resulted in a highly cooperative activation of the MLCKase by CM while the opposite activation (of CM by the kinase) was as expected hyperbolic (uncooperative). This difference in the activation was observed only for the kinase preparations preincubated with the substoichiometrical amounts of CM and only if micromolar concentrations of Ca^{2+} were present. We have shown that under the latter conditions affinity of the kinase for CM was reduced about 20–50-fold so that the CM present could not activate the kinase resulting in the apparent inhibition. This inhibitory effect was variable and depended not only on a concentration ratio of the kinase to CM but also on the MLCKase preparation. For most of the preparations full inhibition required

between 5 and 15 min of preincubation at 25°C and a 3–6-fold molar excess of the kinase above that of CM. The inhibition was reversible in a sense that a full activity could be obtained after saturation of the kinase by additional CM. As a result, an initiation of the phosphorylation reaction by the kinase (no preincubation) produced essentially noncooperative activation by CM while for the assays initiated normally by ATP (preincubation prior to assay) the activation was cooperative. The inhibitory effect did not require ATP (excluding phosphorylation type modifications of the kinase) and was not observed for the kinase preincubated with CM (at substoichiometrical ratios) at higher concentration. Thus, Ca^{2+}/CM-dependent dephosphorylation possibly promoting oligomerization of the kinase could produce the observed reduction of the kinase affinity for CM, but the nature of modification involved remains to be established.

Purification and characterization of myofibrillar myosin light chain phosphatase from turkey gizzard smooth muscle

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Although several phosphatases have been isolated from smooth muscle (Tulloch and Pato (1991) *J. Biol. Chem.* **266**, 20168–74), the identity of the phosphatase responsible for the dephosphorylation of myosin *in vivo* has not yet been established. All of these phosphatases dephosphorylating the myosin regulatory light chain (LC_{20}) would appear to be of the cytosolic type, as they were purified from whole smooth muscle extracts. In contrast, a myofibrillar phosphatase tightly associated with the contractile apparatus and copurified with the myosin light chain kinase (MLCKase) (Sobieszek and Barylko (1984) Stephens, N. L. (ed) *Smooth Muscle Contraction*, Marcel Dekker, New York) is a more likely candidate for the *in vivo* form of smooth muscle myosin light chain phosphatase (MLCPase). We have further purified this MLCPase by an improved affinity chromatography procedure which included a purification step on a thiophosphorylated LC_{20} light chain column. The purified phosphatase appeared to form a complex with the MLCKase which on SDS-gels showed a few bands of similar intensity, of which one corresponded to the MLCKase, and another to a subunit responsible for the MLCPase activity (35 kDa). By inclusion of Ca^{2+}/CM during the affinity chromatography the monomeric 35 kDa subunit was separated from the complex and purified to an apparent homogeneity. This subunit had a relatively high affinity for the phosphorylated LC_{20} light chain ($K_m \sim 3$ μM) and was strongly inhibited not only by the thiophosphorylated LC_{20} ($K_i \sim 4$ μM) but also by the product, the unphosphorylated LC_{20} ($K_i \sim 150$ μM), both of the LC_{20} species inhibiting the enzyme in a competitive manner. The other product, inorganic phosphate, was not inhibitory, indicating that was released from the phosphatase before the departure of the LC_{20} light chain. The phosphatase was activated twofold by 2 mM $MnCl_2$ and was inhibited by okadaic acid, a common inhibitor of the type PP1 and PP2 protein phosphatases, with an IC_{50} value of about 250 nM. On the basis of the currently established properties, the MLCPase cannot be readily classified by conventional criteria. We are currently attempting to show whether the above described complex and an analogous endogenous complex of smooth muscle actomyosin and filamentous myosin are identical.

Functional properties of C-terminal fragments of human caldesmon

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We have used a partial clone of human caldesmon isolated from a human fetal liver library by N. D. Avent, University of Bristol, UK, for production of various mutant fragments. The clone codes for the C-terminal 288 amino acids (equivalent to amino acid 506–793 of human CDh sequenced by Humphrey *et al.* (*Gene* **112** (1992) 197–204) or amino acid 476–737 of gizzard CDh as sequenced by Bryan *et al.* (*J. Biol. Chem.* **264** (1989) 13873–9). Caldesmon can be structurally divided into four domains. Our clone corresponds to domains 3 and 4, which is known to contain binding sites for tropomyosin, actin and Ca^{2+}-calmodulin in gizzard caldesmon. Mutant fragments have been produced by using the polymerase chain reaction (PCR) with mutant oligomers and subcloning the produced cDNA fragments into a T7 RNA polymerase based expression vector. The whole clone (33 kDa) and one mutant (9.1 kDa), which codes for amino acid 683–767 (human) and 626–710 (gizzard), respectively have been expressed and purified so far. The peptides were identified by SDS-PAGE and by immunoblotting using a polyclonal antibody which was raised against sheep aorta caldesmon. We used BL21 (DE3) cells for expression; the cells were lysed, treated with DNAse, boiled in high salt and centrifuged to give a crude extract of the heat stable peptides in the supernatant. Final purification was achieved by ion exchange on Sepharose S. The yield was approximately 1.2 and 5 mg per litre of culture for the 33 kDa (H1) and the 9.1 kDa (H2) peptide. H1 inhibits the tropomyosin-enhanced actin activated MgATPase as potently as gizzard or sheep aorta caldesmon and binds skeletal muscle actin with a kDa = 1 μM. H1 also bound to smooth muscle myosin with a dissociation constant in the micromolar range, slightly less than whole sheep caldesmon. This suggests that there is a hitherto unknown myosin binding site in addition to the one near the N-terminus. This was confirmed by thrombic digestion of sheep and gizzard CDh; the '35 K' fragment (domains 3 and 4) bound to myosin and actin. As fragment 606C (see abstract of Redwood) does not bind to myosin it seems likely that the newly identified myosin binding site is in domain 3. H2 has no inhibitory activity and does not bind tropomyosin or myosin whereas a weak binding affinity was found for actin–tropomyosin. H2 binds Ca^{2+}-calmodulin which agrees with binding affinities found using oligopeptides contained in this fragment (Zhan *et al.* (1991) *J. Biol. Chem.* **266**, 21810–4; Marston, unpublished data).

GENE EXPRESSION AND ISOFORM TRANSITIONS

Effects of neuromuscular activity on gene expression in skeletal muscle

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Owing to the expression of sarcoplasmic and myofibrillar proteins as multiple isoforms, skeletal muscle fibres are capable of changing their phenotype in response to altered functional demands. Chronic low-frequency stimulation (CLFS) is a suitable model for studying effects of increased neuromuscular activity on gene expression in skeletal muscle. It affects all functional elements of the muscle fibre studied to date and induces sequential fast-to-slow fibre type transformations (Pette and Vrbová (1992) *Rev. Physiol. Biochem. Pharmacol.* **120**, 116–202). The fast-to-slow fibre type transitions include both qualitative and quantitative changes in gene expression, some of which occur in parallel, whereas others occur sequentially. The time courses of isoform exchanges vary between different proteins and even between subunits of a given protein, e.g. myosin light and heavy chains, troponin subunits TnT, TnI, and TnC. In several cases, changes at the protein level have been found to be preceded by similar alterations in the amounts of specific mRNAs, most likely resulting from altered transcriptional activities. However, several observations point to the significance of additional post-transcriptional and post-translational regulatory mechanisms, such as protein degradation (e.g. with regard to the sarcomeric exchange of myosin isoforms) or differential changes in intracellular distribution (e.g. glucose transporter translocation and redistribution of hexokinase). The question as to the primary signals

related to the initiation of the transformation process has been addressed in a recent study (Green *et al.* (1992) *Pflügers Arch.* **420**, 359–66). Measurements of metabolite levels in short-term and long-term stimulated muscles of the rabbit showed dramatic effects of CLFS immediately after the onset of stimulation, e.g. decreases in phosphocreatine or glycogen, increases in lactate, etc. Most of these changes were transient and, therefore, could be excluded as playing a role in triggering muscle fibre transformation. However, the observed immediate and persistent depression of the ATP phosphorylation potential, i.e. $ATP/(ADP_f \times Pi_f)$, might represent an important signal for inducing fast-to-slow fibre transformation, especially in view of the central role of the phosphorylation potential in metabolic control.

Asynchronous fast-to-slow fibre transitions in chronically stimulated rabbit muscle

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Chronic low-frequency stimulation of rabbit fast-twitch muscle leads to a progressive fast-to-slow transformation of the fibre population (Pette and Vrbová (1992) *Rev. Physiol. Biochem. Pharmacol.* **120**, 116–202). As shown by single fibre studies and analyses of the myosin heavy chain (HC) isoform pattern at both the protein and mRNA level, the final step of this transformation consists of a switch from the fast HCIIa to the slow HCI. In the present study, we have investigated this final step at the single fibre level by *in situ* hybridization of HCI mRNA. *In situ* hybridization with a digoxigenin-labelled 350 bp cRNA probe (Aigner and Pette (1990) *Histochemistry* **95**, 11–8) was performed on paraformaldehyde-fixed and paraffin-embedded cross and longitudinal sections. Studies on tibialis anterior (TA) and extensor digitorum longus (EDL) muscles showed that the amount of HCI mRNA increased in a time-dependent manner during chronic low-frequency stimulation. However, increases in HCI mRNA did not appear uniformly throughout the fibre population but was restricted to specific fibres. The number of these fibres increased with time. After 30 days of continuous stimulation, 36% of the fibres in TA muscle and 56% of the fibres in EDL muscle reacted positively for HCI mRNA. After 60 days, HCI mRNA-positive fibres amounted to 59% in TA and 87% in EDL. Electrophoretic analyses of the same muscles showed that the percentage of fibres expressing HCI mRNA was linearly correlated with the relative amount of HCI at the protein level. Furthermore, our studies revealed that the fast-to-slow fibre type transition occurred in an asynchronous manner in the two muscles. Most likely, this results from the heterogeneity of the fast fibre population which initially contains type IID and type IIA fibres. Assuming that the fast-to-slow transition consists of a switch from HCIIa to HCI, the transformation of the type IIA fibres which are present before the onset of stimulation will precede that of the type IIA fibres which are eventually formed from type IID fibres under the influence of chronic low-frequency stimulation. Because the percentage of type IIA fibres is low in unstimulated EDL and TA muscles (approximately 30%), the bulk of HCI mRNA expressing fibres can be expected to appear only after longer stimulation periods.

Chronic stimulation-induced elevations in hexokinase II activity are correlated with increased mRNA content and enhanced enzyme synthesis

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Severalfold increases in hexokinase (HK) activity have previously been observed in low-frequency (10 Hz) stimulated rat tibialis anterior (TA) muscle. It was also shown that this increase was uniquely from an increase in the protein content of HK II isozyme (Weber and Pette (1990) *Eur. J. Biochem.* **191**, 85–90). In the present study, we investigated to what extent this increase relates to changes at the level of transcription and translation. For this purpose, the left tibialis anterior muscle of male Wistar rats was continuously stimulated at 10 Hz for different time periods (3, 6, 12, 24 h and 2, 3, 5, 7, 10, 14 days). In one series of experiments, total RNA was extracted from contralateral and stimulated TA muscles and analysed for HK II mRNA by hybridization with a digoxigenin-labelled cRNA derived from a specific cDNA. We thank A. Thelen and J. Wilson, East Lansing, for generously supplying the cDNA. In a second series of experiments, relative synthesis rates of HK II were measured using the *in vivo* labelling technique with ^{35}S-methionine described in the above cited publication. Measurements of HK activity were performed on the same samples. Increases in HK activity were recorded as soon as after 3 h of stimulation. Further increases in HK activity were recorded until 3 days of stimulation when a maximum (fivefold increase) was reached. Enzyme activity remained elevated at this level until 14 days of stimulation. Rapid increases in mRNA content were followed by steep increases in enzyme synthesis rate. The mRNA content reached a maximum (30-fold increase) after 12 h of stimulation, whereas the synthesis rate of HK II reached its maximal value (20-fold) after 24 h of stimulation. After 2 days of stimulation, the mRNA content had declined, but was still elevated 13-fold. Its decay became slower with longer stimulation periods, but HK II mRNA was still fourfold elevated after 14 days. The elevated synthesis rate started to decrease in the 2 day stimulated muscle, following a time course similar to the decay in mRNA. These results suggest that the increase in HK II isozyme protein is primarily from enhanced transcriptional and translational activities.

Analysis of human muscle biopsies with PCR and *in situ* hybridizations

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Human muscle adapts to changes in its pattern of use with alterations in the concentrations of key enzymes and structural proteins. Data from animal models indicate that such modulations are accompanied by changes in the steady-state concentrations of their respective RNAs. Our focus is the adaptation of human muscle to exercise training. Muscle tissue is obtained from needle biopsies (Bergström technique) weighing 50–150 mg, which yield only 10–40 µg total RNA. RT-PCR allows quantitative assessment of specific RNAs in as little as one cryostat section. A requirement of quantitative PCR is the control of the efficiency of the process during the logarithmic phase which in our hands is followed by the incorporation of radioactive label in its product. Thus we are able to distinguish less than twofold differences in RNA concentration. A further approach involves *in situ* hybridization to localize particular RNAs in the different fiber types. We use ^{33}P-labelled RNA probes in these experiments. This isotope has an energy spectrum comparable to ^{35}S, but probes labelled with ^{33}P nucleotides are less prone to oxidative modifications than ^{35}S probes, which eliminates some of the background problems. *In situ* hybridization with ^{33}P-labelled myosin light chain 1f mRNA on chicken muscle gave signals that were at least as distinct as the ones with ^{35}S-labelled probes. In human muscles hybridizations with a probe specific for carbonic anhydrase III showed preferential localization in type I fibres, which corresponds to studies with antibodies specific for carbonic anhydrase III protein.

Myosin heavy and light chain composition in skinned fibres of rat soleus muscle after hindlimb suspension

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Skeletal muscles, especially slow-twitch weight bearing muscles, are

known to be altered by exposure to microgravity. In this study, rat soleus (SOL) muscle atrophy was induced by 2 weeks of hindlimb suspension (2 week HS), a model commonly used to mimic weightlessness. It was established that 2 week HS induced slow-to-fast changes of the contractile activity, and a parallel fast myosin heavy chain (MHC) isoform expression in soleus fibres of HS rats (Reiser *et al.* (1987) *J. Appl. Physiol.* **63**, 2293–300). The aim of our study was to determine, by using electrophoretic analysis of SOL single skinned fibres, whether the myosin light chain (MLC) distribution was modified after 2 week HS, and to what extent this transformation might be correlated to the modification of the MHC isoform expression. Control and 2 week HS SOL fibres were examined for MHC and MLC composition by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis. Our results indicated that HS induced a parallel modification of the MHC and MLC isoform expressions in the rat SOL muscle: after 2 week HS, 40% of the analysed SOL fibres ($n = 30$) exhibited both fast (type IIA-IIX) and slow (type I) MHCs and co-expressed fast and slow MLC sets: they were considered as intermediate fibres. This type of fibre represented only 10% of the studied SOL control fibres ($n = 30$). Moreover, a variability in the expression level of fast MHC and MLC isoforms was observed in the intermediate fibres of HS SOL: some fibres exhibited more fast than slow MLC isoforms, in relation to a greater amount of type IIA-IIX than type I MHCs. Densitometric analysis was performed and revealed a greater similarity between the MHC and MLC_2 fast isoform expressions. Nevertheless, whatever the fast MHC expression level, fast MLC_1 isoform was always expressed in the same proportion. All these results indicated that after HS, the MLC composition was modified in the slow SOL fibres, additionally to the changes in the MHC composition previously described. Furthermore, this study suggested that 2 week HS induced alterations in the contractile protein gene expressions, with a close transformation of the MHC and MLC_2 expressions.

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Opposite hormone regulations by androgenic and thyroid hormones of *V*1 myosin isoform expression in skeletal and cardiac muscles

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The VI myosin isoform is normally found in cardiac ventricles and is known to be composed of α-type heavy chains and of slow-type light chains. Its synthesis is developmentally regulated: it constitutes about 50% of the myosin in newborn rabbits, but only a few per cent in rabbits > 1 month. We have recently shown (d'Albis *et al.* (1991) *Basic Appl. Myol.* **1**, 23–34) that rabbit masticatory muscles contain a developmentally-regulated VI myosin isoform, composed of α-type heavy chains, also detected by immunocytochemical methods (Bredman *et al.* (1991) *Histochem. J.* **23**, 160–70), and of slow-type light chains. In masticatory muscles, VI synthesis starts during the third week after birth, increases similarly in males and females during the first 2 months, and then stabilizes at a level of about 70% in females, while it decreases to a few per cent in males. No other skeletal muscle has been shown to contain the cardiac VI myosin isoform. The hormonal regulation of VI in cardiac and masticatory muscles was compared. Thyroid hormones had no significant effect on the postnatal expression of VI in the masticatory muscles, but increased its postnatal expression in cardiac ventricles. In contrast, androgenic hormones decreased the expression of VI in the masticatory muscles, but had no significant effect on its expression in cardiac ventricles. The hormonal regulation of the synthesis of VI appears therefore to be tissue dependent, with hormones having opposite effects in two different types of striated muscle.

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Histochemical profiles of three fast fibre types in muscles of mouse, rat and rabbit

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Three fast-twitch fibre types have previously been delineated in rat muscle by electrophoretic studies of myosin heavy chain (MHC) patterns in histochemically defined single fibres (Termin *et al.* (1989) *Histochemistry* **92**, 453–7). The present study was undertaken to establish the histochemical profiles of these fibre types also in mouse and rabbit. Histochemical fibre typing was based on myofibrillar actomyosin ATPase (mATPase) activity with the use of various methods. NADH-tetrazolium reductase histochemistry was also applied. In addition, cross-sectional fibre area was determined by computer-assisted microphotometry. Generally, the histochemical profile of the three fast fibres in mouse muscle was similar to that in the rat. However, some differences existed between the staining patterns of these two species, especially of type IIB and type IID fibres. Species specific differences were even more pronounced when comparing the rabbit with rat and mouse, i.e. reversed staining patterns could be seen. In addition, type IIB fibres were not present in all the same muscles as in rat and mouse. For example, psoas, tibialis anterior and extensor digitorum (EDL) muscles of the rabbit contain no pure type IIB fibres. In these muscles, type IID was the predominant fast fibre type. Type IIB fibres were found to be numerous in rabbit adductor magnus, vastus lateralis and gastrocnemius muscles. Therefore, histochemical identification of fibre types IIB and IID could be performed only in these muscles. The presence of type IIB fibres in these muscles was confirmed by electrophoretic analyses. Thus, MHCIIb could be detected only in these muscles but not in psoas, TA and EDL muscles. Our comparative study proves the existence of fibre type IID also in the rabbit. In addition, it suggests that the fibre type IID in the rabbit has erroneously been classified, i.e. as fibre type IIB. In respect of the oxidative capacity and the mean cross-sectional fibre area, IIB fibres are the least oxidative and the largest, IID fibres intermediate, and IIA fibres the most oxidative and the smallest in all three species. This order applied if the three fibre types were compared with each other in the same muscle; however, differences in the size of a given fibre type were observed if different muscles of the same animal were studied.

Time course of changes in GLUT-4 glucose transporter translocation and increases in mRNA content of low-frequency stimulated rat muscle

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This study was initiated by the observation that low frequency stimulation (CLFS) induces severalfold increases in both synthesis and total amount of hexokinase II isozyme in rat fast-twitch muscle. The present study investigates effects of chronic low-frequency (10 Hz) stimulation on both distribution and total amount of the muscle-specific GLUT-4 glucose transporter. The left tibialis anterior (TA) muscle of male Wistar rats was subjected to CLFS for different time periods (0.5, 1, 3, 6, 12, 24 h and 2, 3, 5, 7, 10, 14 days). In one experimental series, total RNA was extracted from contralateral and stimulated TA muscles and analysed for GLUT-4 mRNA by hybridization with a digoxigenin-labelled cRNA derived from a specific cDNA. Synthesis of GLUT-4 protein was assessed in a second series by *in vivo* labelling with ^{35}S-methionine and measurement of the radioactivity in an electrophoretically separated protein band identified by immunoblot analysis. In a third series, the intracellular distribution of GLUT-4 was assessed by immunoblot analysis per-

formed on membrane preparations isolated by differential and sucrose density centrifugations. The specific cDNA and the specific antibody used in this study were kindly supplied by Dr D. E. James, St Louis. According to our results, GLUT-4 was rapidly translocated into the sarcolemma after the onset of stimulation, whereas increases in its synthesis rate occurred later. Thus, GLUT-4 was 35% elevated in the sarcolemma 30 min after the onset of stimulation. Significant (two to threefold) increases in both synthesis and amount of GLUT-4 were first observed in 5 day-stimulated muscles. Longer stimulation periods did not lead to further increases. These moderate changes in synthesis rate corresponded to similar increases in GLUT-4 mRNA content. Our results suggest that the immediate response of the muscle to increased contractile activity consists of an enhanced translocation of GLUT-4 from an intracellular pool into the sarcolemma. The redistribution of the transporter is then followed by a delayed and moderate increase of its synthesis.

Regulatory proteins of calcium in ischaemic fast and slow skeletal muscle

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In muscle cells Ca^{2+} storage, release and uptake are regulated by the protein of the sarcoplasmic reticulum (SR) (Michalak (1985) In *The Enzymes of Biological Membranes*, edited by Martonosi, Vol. 3. Plenum Press, New York, London, pp. 115–55). Calreticulum is one of several Ca^{2+}-binding proteins which is localized within the lumen of the SR (Michalak *et al.* (1980) *J. Biol. Chem.* **255,** 1317–26) and it is known to bind Ca^{2+} with both high and low affinity and capacity (MacLennan *et al.* (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37,** 469–77). Calsequestrin, another SR Ca^{2+}-binding protein, has been localized specifically to the lumen of the skeletal and cardiac muscle SR (MacLennan *et al.* (1983) In *Calcium and Cell Function*, edited by Cheng, Vol. 4. Academic Press, New York, pp. 151–73). The study was carried out on the rat ischaemic fast (extensorum digitorum longus, EDL) and slow (soleus, SOL) skeletal muscle. We have isolated both the Ca^{2+}-binding proteins by SDS-PAGE methods (Laemmli (1970) *Nature (Lond.)* **227,** 680–5). After electrophoresis gels were either stained with Coomassie Brilliant blue, or the fractions were transferred to nitrocellulose membranes and tested for $^{45}Ca^{2+}$ binding with a $^{45}Ca^{2+}$overlay technique (Michalak *et al.* (1990) *J. Biol. Chem.* **265,** 5869–74). The results show that the differences of protein fractions were larger between ischaemic and normal skeletal muscle. It suggests that lack of normal circulation is the main reason for the changes in the conformational state of Ca^{2+}-binding proteins, and of decreased activity of these muscles.

Protein amounts and relative synthesis rates of alkali LC1F and LC3F in rabbit muscle

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Studying effects of chronic low-frequency stimulation on the myosin light chain (LC) pattern of rat fast-twitch muscle, we have previously observed a discrepancy between protein amounts and relative synthesis rates of alkali light chains LC1f and LC3f. Alkali LC3f protein was found to decrease although its synthesis rate remained at a high level (Bär *et al.* (1989) *Eur. J. Biochem.* **178,** 591–4). This observation led us to compare in the present study the relationship between protein amount and relative synthesis rate for LC1f and LC3f in rabbit tibialis anterior muscle. Relative protein amounts of the two alkali light chains were determined by photometric evaluation of Coomassie blue-stained two-dimensional electrophoresis gels. For the measurement of relative synthesis rates, we used *in vivo* labelling with ^{35}S-met as described in the above reference. The radioactivity incorporated into the light chains was determined by photometric evaluation of autoradiographs obtained from the two-dimensional gels. The measurements were performed on a total of ten rabbits. At the protein level, the LC1f/LC3f ratio amounted to 2.76 ± 0.48 (means $\pm$ SD). The corresponding ratio of the relative synthesis rates was 1.1 ± 0.29 (means $\pm$ SD). The difference between these two values was explained by the fact that, referred to protein amount, ^{35}S-met incorporation into LC3f exceeded by far that of LC1f. This finding suggested a higher turnover rate of LC3f as compared with LC1f and pointed to a specific regulation of the degradation of LC3f. Measurements of relative protein amounts and synthesis rates in chronically stimulated TA muscles provided similar results, i.e. the relative synthesis rate of LC3f as referred to protein amount exceeded by far that of LC1f. This was remarkable in view of the fact that the protein amount of LC3f was found to decrease under the influence of chronic low-frequency stimulation. Taken together, these findings on normal and transforming muscles indicate that the cellular level of LC3f is controlled not only by transcription and translation, but is also subject to post-translational regulation.

Coordinated changes of troponin T and myosin heavy chain isoforms in chronically stimulated rabbit muscle

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Chronic low-frequency stimulation of rabbit fast-twitch muscle induces fast-to-slow fibre type transformations with transitions in myosin heavy chain (HC) expression in the order of HCIId→HCIIa→HCI (Pette and Vrbová (1992) *Rev. Physiol. Biochem. Pharmacol.* **120,** 116–202). We were interested to study how this transformation applies to the major troponin T (TnT) isoforms, especially with regard to possible correlations to the changes in myosin isoforms. Changes in TnT isoforms were studied by determining relative amounts of the fast and slow isoforms at the protein level, as well as by assessing relative synthesis rates by *in vivo* labelling (Bär *et al.* (1989) *Eur. J. Biochem.* **178,** 591–4). Relative protein amounts were determined by photometric evaluation of Coomassie Blue-stained two-dimensional gels. The radioactivity incorporated into the different TnT isoforms was determined by photometric evaluation of autoradiographs obtained from the two-dimensional gels. Myosin HC isoforms were analysed by gradient gel electrophoresis. Measurements on tibialis anterior (TA) muscles stimulated for 8, 11, 16, 35 and 60 days confirmed the above given sequential transitions in myosin expression. HCIId which represented the major fast isoform (60%) in normal TA muscle, reached a level of $<5\%$ after 35 days of stimulation. This change was accompanied by parallel decreases in synthesis rate and protein amount of TnT_{1f} and TnT_{2f}, the major fast TnT isoforms in unstimulated TA. The decay in synthesis rate was steeper than in protein amount. Conversely, myosin HCIIa which amounted to 35% of all HC isoforms in normal TA muscle, increased under the influence of low-frequency stimulation, reaching a maximum of approximately 90% in 35 day stimulated TA muscles. Thereafter, the relative amount of HCIIa decayed, reaching a value of 35% after 60 days of stimulation. The changes in HCIIa were parallelled by similar increases and decreases in TnT_{3f} at both synthesis rate and protein amount. The exchange of myosin HCIIa with the slow isoform HCI took place in muscles stimulated longer than 35 days and was accompanied by similar increases in synthesis rate and protein amount of the two slow TnT isoforms, TnT_{1s} and TnT_{2s}. These results suggest the existence of patterns of coordinated expression for fast and slow isoforms of myosin heavy chains and troponin T.

Muscle fibre types of the rat levator palpebrae muscle

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The levator palpebrae muscle (LP) belongs to the extraocular muscles and is innervated by motoneurons located in the oculomotor nucleus. Because of the lack of systematic informations about the fibre type composition especially in comparison with oculorotatory muscles (OR), the LP of adult rats was studied by histochemical (ATPase after acid and alkaline preincubation) and immunocytochemical (using monoclonal antibodies (AB) against different myosin heavy chains (MHC)) methods. The results were compared with those obtained from OR and some skeletal (diaphragm, soleus, extensor digitorum longus) muscles. The LP of the rat is a small muscle containing 500 ± 150 (mean + SD) fibres. Of these fibres $15.4 \pm 6.3\%$ were type I (diameter 21.6 ± 4.7 μm) only reacting with AB against type I-MHC; $24.9 \pm 6.9\%$ of the fibres showed positive reactions with an AB against type IIA-MHC (diameter 17.8 ± 3.6 μm). About half of these fibres ($12.3 \pm 8.7\%$) also exhibited positive reactions with the type I-MHC-AB and belong therefore to the type IIC category. The rest of the fibres ($57.6 \pm 11.9\%$) showed only positive reactions with an AB against IIA + IIB-MHC and were therefore classified as IIB (diameter 28.7 ± 5.7 μm). In comparison with the OR the LP has no layer organization and there are no fibres expressing special MHCs (embryonic, neonatal, slow-tonic, cardiac α) as regularly observed in the OR. Apart from the smaller diameter of all its fibre types the LP has more similarities to a skeletal muscle than to an OR one.

Effect of temperature on muscle protein mRNA levels in embryonic salmon (*Salmo salar* L.)

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The rate of embryonic development is dependent on temperature in various species (Krogh (1914) *Allg. Physiol.* **16**, 163–77). This holds true also for muscle development in Atlantic salmon. Stickland *et al.* (*Anat. Embryol.* (1988) **178**, 253–7) demonstrated a marked difference in cellularity in salmon embryos reared at 5° and 10°C, respectively. At 10°C white muscle showed an increase in fibre hypertrophy and a reduced rate of hyperplasia compared with fish raised at 5°C. In this study the mRNA levels for muscle protein (actin and myosin) were determined in salmon embryos reared at 5°, 8° and 11°C. Samples were taken at Gorodilov stages 25, 27, 29, 31 and 33 (hatching) (Gorodilov (1983) *GosNIORKh.* **200**, 107–26) and total mRNA was isolated from the 'body' of the embryos, which consists almost entirely of muscle as head and yolk sac had been removed. This RNA was analysed by means of Northern blotting and slot blotting. Probes for actin and myosin derived from a genomic carp library (Gerlach *et al.* (1990) *Am. J. Physiol.* **28**, R237–44) were used. The specificity of the probes was checked on Northern blots and the levels of specific message semi-quantified using slot blots. To standardize the measurements total mRNA levels were determined by means of hybridization with oligo-dT. The actin and myosin mRNA levels were found to be dependent on the developmental stage and the incubation temperature. Both muscle proteins exhibited a similar pattern. At hatching the levels were found to be inversely related to the incubation temperature, i.e. lowest at 11°C, intermediate at 8°C and highest at 5°C. At 5°C and 8°C an increase of the muscle protein mRNA (mpmRNA) levels during development was found. However, at 11°C mpmRNA levels were higher at stage 27 than at hatching. These results were found for myosin and actin mRNAs whether based on total message or on total RNA. It therefore seems that at the higher temperature the transcription of muscle protein is switched on at an earlier age than at the lower temperature. The levels of muscle protein mRNA are a product of transcription and degradation. However, as higher temperatures increase the rate of protein production, it is evident that the mRNA levels are not a direct reflection of protein synthesis. Other regulatory mechanisms such as RNA stability or translation rate must play a role. The high amount of mpmRNA found at 11°C may support the shift to hypertrophy, and therefore protein accumulation, found by Stickland *et al.* (1988, as above) in salmon raised at an increased temperature.

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Modulations of embryonic and muscle-specific enolase gene products in rat heart during ontogenesis and after pressure overload

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In mammals, the glycolytic enzyme enolase exists as dimers formed from three subunits encoded by different genes. In adult tissues the α subunit is found in many cell types, but the γ and β subunits are specifically expressed in neurons and muscle cells, respectively. During striated muscle maturation, a progressive transition occurs from the embryonic αα to αβ and ββ isoforms. In mature skeletal muscle the ββ isoform is largely predominant while a substantial amount of α subunit containing isoforms remains in the heart (Fletcher *et al.* (1978) *Dev. Biol.* **65**, 462–75; Kato *et al.* (1985) *Biochim. Biophys. Acta* **841**, 50–8).

We have studied the developmental expression of enolase transcripts in the cardiac muscle. *In situ* hybridization experiments show that the muscle-specific enolase gene is already activated in the mouse cardiac tube: at 7.8 dpc, the β transcripts are clearly detectable in this tissue. As the heart further develops to form atrial and ventricular compartments, these transcripts are expressed over the whole structure (Keller *et al.* (1992) *MOD* **38**, 41–52).

Quantitative Northern blot analysis performed in the rat during fetal and postnatal development indicates that the switch of enolase isozymes mainly results from modulations of the levels of the corresponding mRNAs.

We have also quantitated α and β mRNA levels after cardiac pressure overload. It is well established that adaptative cardiac hypertrophy secondary to pressure overload is accompanied by changes in gene expression. Re-expression of fetal myosin after overpressure induces the thermoeconomical decrease in the velocity of contraction (reviewed in Schwartz *et al.* (1992) *Am. J. Physiol.* **262**, R374–9). The enolase being implicated in energy metabolism, it was therefore of particular interest to study its isozymic pattern during cardiac hypertrophy.

Haemodynamic overload was induced by thoracic stenosis in 3-week-old rats. At the earliest time examined, 3 days after surgery, there is already a large decrease of muscle-specific enolase mRNA level in the left ventricle, while the level of α mRNA is not greatly modified. These results suggest that muscle-specific enolase gene expression may have an important connection with the functional state of the cardiac muscle, in agreement with previous results obtained in skeletal muscle (Kato *et al.* (1985) *Biochim. Biophys. Acta* **841**, 50–8; Lucas *et al. Differentiation* (in press)).

Transient neonatal denervation alters MHC isoform composition of adult muscle: implication in preventing dystrophic deterioration

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Transient neonatal denervation (t-den) of the dystrophic (129ReJdy/dy) mouse EDL prevents the spontaneous degeneration-regeneration

and myofibre loss characteristic of dy/dy muscle. At 100 days after t-den, the dy/dy EDL contains 2X the number of myofibres of age-matched, unoperated dy/dy EDL, a number equal to that in normal (+/+) and in t-den +/+ muscle (Moschella and Ontell (1987) *J. Neurosci.* **7**, 2145–52). To understand the mechanism responsible for the sparing of fibres in t-den dy/dy EDL, myofibre type frequency and absolute numbers of myofibres of each type in these muscles have been determined with immunohistochemistry and compared with similar data from unoperated +/+ and dy/dy EDL and from t-den +/+ EDL. Unoperated 100-day dy/dy EDL contain only 32% of the number of MHC2B fibres found in unoperated +/+ EDL. Both types of unoperated muscles have similar numbers of myofibres containing MHC2A and MHC2X, suggesting that fibres with MHC2B are most affected by the dystrophic process. Approximately 20% of the extrafusal myofibres of the unoperated dy/dy muscle contain MHC-neo, while this isoform is not expressed in the extrafusal myofibres of unoperated +/+ muscles. Reinnervation following t-den of both types of muscles results in a similar decrease (approximately 62%) in the number of myofibres containing MHC2B and an increase in the number of myofibres containing MHC2A and MHC2X. Moreover, < 2% of the myofibres of t-den dy/dy muscles contain MHCneo. As t-den of both types of muscles has no effect on fibre number, the change in the frequency of myofibre types is a result of alterations in MHC gene expression. It is suggested that this alteration may be because neurons that supply myofibres containing MHC2B may be at a selective disadvantage in their ability to reinnervate neonatally denervated normal and dystrophic muscles as compared with neurons innervating fibres containing MHC2A and MHC2X. The selective effect of dystrophy (in unoperated dy/dy muscle) on fibres containing MHC2B, the alteration of MHC gene expression in the dy/dy muscle after t-den (i.e. a reduction in the number of myofibres containing MHC2B and an increase in the number of myofibres containing MHC2A and MHC2X) and the subsequent sparing of dystrophic myofibres in the t-den dy/dy muscle are consistent with an hypothesis that alterations in fibre type (toward a slower phenotype) may play a role in the rescue of the dy/dy muscle fibres after transient neonatal denervation.

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Altered myofibrillar protein expression in electrically-stimulated skeletal muscle

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The skeletal muscle phenotype most appropriate for use in long-term cardiac assist procedures remains to be defined. Electrical stimulation of the muscle is used to achieve muscle contraction in the cardiac cycle and the fast-to-slow fibre-type transformation required for fatigue resistance. Here we discuss altered expression of contractile proteins analysed in sheep ($n = 12$) latissimus dorsi muscle (LDM) which had been electrically stimulated for time periods up to 20 weeks (at burst frequency; 35 Hz, pulse amplitude; 3 V, burst duration; 240 ms, pulse width; 240 μs every 1:4 cardiac cycles). The myosin heavy chain and regulatory light chain subunits of the thick filament had near complete transformation of their slow isoform counterparts by 12 weeks stimulation (94 and 92%, respectively; unstimulated control LDM values were 22 and 42%, respectively). The proteins of the thin filament transformed towards their slow isoforms after the myosin heavy chain and regulatory light chain had begun to switch. After 12 weeks stimulation, the tropomyosin slow isoform accounted for 64% of the α-subunits (unstimulated control: 26%).

Troponin I transformation did not begin until after 4 weeks stimulation and had reached 78% slow isoform by 12 weeks (unstimulated control: 55%). Troponin T analysis revealed five fast and two slow isoforms in the sheep. Of the two slow isoforms, the isoform of lowest apparent molecular weight was not detected until 20 weeks stimulation while that of highest apparent molecular weight was detected at all time points. All of the fast troponin T isoforms were detected in the LDM at 20 weeks of stimulation, although the three of highest apparent molecular weight progressively decreased over this period.

The isoform transitions in the thin filament proteins began after that of myosin heavy chain and regulatory light chain subunits of the thick filament and without the same degree of coupling suggesting that a wide heterogeneity of fast and slow isoform interactions are possible in the thin filament.

Effect of porcine somatotropin on the carcass and fat tissue composition in different sexes of finishing pigs

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Exogeneous long-term application of recombinant porcine somatotropin (pST) induced a faster growth of lean meat and reduced the fat tissue growth rate. We treated 174 German Landrace pigs daily with 0, 2 mg or 4 mg pST over a period of about 75 days. The improvement of the carcass quality is reflected in a higher lean meat percentage and in the increase of the longissimus muscle area. The daily administration of pST clearly decreased intramuscular lipid concentration in boars, females and castrates. This reduction is dose dependent for females and castrates.

The increased meat and protein content accompanied by a decreased fat content did not include negative effects on meat quality.

The chemical fat content of the whole carcass has decreased by about 30–40% in all sexes. The fat concentration of backfat was lower in pST treated pigs with a concomitant increase of water content. The relative proportion of polyunsaturated fatty acids of backfat raised in boars, females and castrates in comparison to controls.

The pST-application caused an improvement of carcass composition and a deterioration of fat characteristics indicated by higher water content, lower backfat thickness and increased fatty acid proportion of backfat.

Effects of different stimulus patterns on the expression of myosin isoforms in myotube cultures from skeletal muscle of newborn rat

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Chronic nerve stimulation markedly affects the expression of phenotypic properties in adult mammalian muscles (Pette and Vrbová (1992) *Rev. Physiol. Biochem. Pharmacol.* **120**, 116–202). The present study was undertaken to investigate effects of chronic stimulation on aneural myotube cultures. Culture conditions were improved in order to maintain long-term myotube cultures. Myoblasts from hindlimb muscles of newborn rats were used. Laminin in combination with fetal calf serum provided optimal conditions for maintaining cultures up to 35 days. Two days after seeding, cultures were continuously stimulated for periods of 20 days. The following stimulus patterns were used: (1) 10 Hz continuously, (2) 250 ms lasting bursts at 10 Hz every 4 s, (3) 250 ms lasting bursts at 100 Hz every 4 s, and (4) 250 ms lasting bursts at 100 Hz every 100 s. Myosin heavy chain (HC) patterns of the cultures were investigated electrophoretically and by immunoblot analyses. The following results were obtained: 22-day-old control myotubes expressed, in addition to the embryonic HC_{emb} as major isoform, adult fast isoforms HCIIb and HCIId and small amounts of the neonatal isoform, HC_{neo}. This pattern was significantly altered by chronic stimulation. Continuous 10 Hz stimulation (pattern 1) markedly decreased the relative amounts of HCIIb and HCIId and induced small amounts of the slow isoform HCI. HC_{emb} was still the

predominant isoform. Phasic low (10 Hz, pattern 2) and high (100 Hz, pattern 3) frequency stimulation further repressed the adult fast isoforms and markedly enhanced the expression of HCI. Also under these conditions, HC_{emb} was the major isoform. No difference existed between the effects of patterns 2 and 3. Phasic low-amount, high-frequency stimulation (pattern 4) shifted myosin expression towards fast isoforms. Under these conditions, HCI was not expressed and HC_{emb} decreased. Conversely, HC_{neo} and HCIId were elevated with HCIId becoming the major fast isoform. Taken together, our results show that direct stimulation of cultured aneural myotubes has marked effects on the myosin HC isoform pattern. Specific alterations with regard to shifting myosin expression towards slow or fast isoforms were induced by different stimulus patterns. Finally, the frequency of the single stimuli appeared to be less important than the temporal pattern of burst delivery.

Myofibrillar protein composition of single identified muscle fibres of the locust, *Locusta migratoria*

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The anterior coxa rotator muscle of the locust (M92) is composed of three fibre types termed I, IIa and IIb. They differ in innervation pattern, activity and pH-stability of myofibrillar ATPase (mATPase) and in their mechanical responses, such as maximum shortening velocity and pCa-tension relationship (Müller *et al.* (1992) *J. Comp. Physiol. B* **162**, 5–15).

Myofibrillar protein composition of the three types has been analysed on the level of single identified muscle fibres. In the fibre types I and IIb, two different myosin heavy chain (mHC) isoforms (I; II) could be separated on SDS gradient gels (nomenclature different to mammalian muscles). Myosin heavy chain isoform I is present in fibres with slow maximum shortening velocity and low mATPase activity which tolerates alkaline preincubation (type I). Expression of mHC isoform II, however, can be correlated with fast contraction and high mATPase activity which is labile to alkaline preincubation and typical for type IIb fibres. In intermediate fibres (type IIa), both mHC isoforms can coexist within the same muscle fibre.

Also myofibrillar calcium regulatory proteins, such as troponin T and tropomyosin occur in different isoforms which are alternatively expressed in different muscle fibres. They can be correlated with *p*Ca-tension relationships of the three fibre types. The data allow a classification of myofibrillar proteins into specialized 'fast-' and 'slow-type' isoforms. Their functional significance for muscle performance is discussed.

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Correlation of contractile gene expression and morphogenic events in mouse crural muscles during development

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The time course of primary and secondary myotube formation and of innervation of the mouse extensor digitorum longus (EDL) and soleus muscles has been established. We have evaluated contractile protein gene expression in these and other crural muscles with ^{35}S-labelled cRNA probes specific for various transcripts for actin and myosin and correlated gene expression with morphogenic events in these muscles. Moreover, we have compared contractile protein gene expression in the limbs with the expression of these genes in the somites (Sassoon *et al.* (1988) *Development* **104**, 155–64; Lyons *et al.* (1990) *J. Cell Biol.* **111**, 1465–76). While the general pattern of early activation of the cardiac genes is found in both myotomal and limb muscles, there are differences in the pattern of early activation of a number of genes. Transcripts for contractile protein genes (α-cardiac actin, MLC_{1A}, MLC_{1F}, MHC_{emb}) can be detected in the limb at 11.5 days gestation, simultaneously with the appearance of mRNAs for myogenin and MyoD. The α-skeletal actin mRNA is detectable in the limb muscles after α-cardiac actin, MLC_{1A} and MLC_{1F} transcripts accumulate; whereas, α-skeletal actin mRNA is found in somites concurrent with the appearance of α-cardiac actin mRNA and prior to any myosin transcripts. MHC_{pn} mRNA accumulates in the limb, prior to mRNA for $MHC_{\beta/slow}$. In somites MHC_{emb} and $MHC_{\beta/slow}$ mRNAs appear concurrently, prior to MHC_{pn}. These differences in the pattern of expression of somitic versus limb muscle suggest that there is no strict, coordinated pattern of expression of contractile protein genes in mouse muscle. At 14 days gestation, a period when only primary myotubes are present in the crural muscles, transcripts for α-cardiac and α-skeletal actin, MLC_{1A}, MLC_{1F} and MLC_{3F}, and MHC_{emb} $MHC_{\beta/slow}$ and MHC_{pn} have accumulated in the primary myotubes. At this stage there is no differential accumulation of any transcript among crural muscles. At 16 days gestation, variation in mRNA accumulation for some genes among and within crural muscles becomes evident. Two factors account for this differential distribution: the stage of muscle maturation and future myofibre type. In anterior crural muscles, which mature approximately 2 days before posterior crural muscles, accumulation of transcripts for α-skeletal actin and MLC_{3F} is greater than in posterior crural muscles. In future slow muscles, $MHC_{\beta/slow}$ mRNA accumulates in greater amounts and MHC_{pn} transcripts are present in lesser amounts than in other crural muscles. At birth, the soleus muscle exhibits strong signal with probes for $MHC_{\beta/slow}$ and MLC_{1V}. While the level of transcript accumulation for the developmental isoforms, MHC_{emb}, MLC_{1A} and α-cardiac actin, is greatly reduced in most crural muscles by birth, these transcripts persist in the soleus muscle.

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Myosin isoform expression in rat heart muscles during long-term adaptation to cold and to heat

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The isoenzyme composition of contractile proteins determines the limits of mechanical muscle performance. Atrial and ventricular heart muscles of adult rats contain mainly myosin with α-heavy chains (α-MHC) and high ATPase activity. Work overload and/or low thyroid hormone levels tend to shift expression towards myosin with β-MHC and low ATPase activity. To test long-term adaptation adult female rats were kept for 26 weeks at 5°, 21° and 32°C. Food intake, body weight, heart weight, heart rate, serum levels of triiodothyronine (T3), of total and free thyroxine (T4), iodine uptake by the thyroid, myosin-HC and myosin light chain (MLC) composition in right and left ventricles and in atria, and histochemical ATPase reaction at pH 10.5 on tissue cryosections were followed. Rats at 5°C developed cardiac hypertrophy (ratio of heart weight/body weight 25–32% higher than at 21° and 32°C) within the first 3 weeks which persisted over the whole period. Heart rates remained similar at all temperatures. Food intake was twice as high at 5°C than at higher temperatures. Serum thyroid hormone levels remained within normal range at 5° and 21°C. At 32°C, total and free T4 dropped within the first few days and subsequently increased slowly two to fourfold. T3 decreased at 32°C slightly and remained after 5 weeks 22–32% below that at 5° and 21°C. Iodine uptake was faster in cold-adapted animals at 5°C. The content of α-MHC showed no change at 5°C, a moderate decrease corresponding to age at 21°C and a large drop at 32°C within 5 weeks on the expense of β-MHC to a stable level around 40% in both right and left ventricles. This coincided with the reduction of T3 and was accompanied by drastic decrease in histochemical ATPase reaction on tissue cryosections. No change in α-MHC was visible in atria. The results indicate that a slight reduction in serum T3 levels at 32°C in

the absence of work overload, shifts the isoform expression towards β-MHC in ventricles but not in atria.

Quantitative determination of myosin heavy chain mRNA isoforms by means of a nonradioactive reverse transcriptase/polymerase chain reaction assay

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A method was elaborated for measuring absolute amounts of three different myosin heavy chain mRNA isoforms in total RNA preparations from different rabbit skeletal muscles. The method is a combination of primer-directed reverse transcriptase and polymerase chain rections (RT/PCR) with 5'-digoxigenin-labelled oligonucleotides. After polyacrylamide gel electrophoresis, the reaction products were electrotransferred to a nylon membrane, visualized by chemiluminescence and evaluated by photographic documentation. Quantitative evaluation was based on reactions performed in the presence of external standards with identical sequences at defined concentrations. Studies on the coamplification of the three sequences had given unsatisfactory results, most likely from interactions of the three oligonucleotide pairs. In addition, it appeared necessary to elaborate sequence-specific reaction conditions. Therefore, defined amounts of the three standard cRNAs, prepared by *in vitro* transcription, were used for determining the exponential phase of the RT/PCR. In addition, these standards served for establishing in separate reactions for each sequence the necessary calibration curves. This approach made it possible to measure, in the range of constant efficiency between 10^3 and 10^6, the number of specific mRNA copies per total RNA yield, as well as per muscle weight. The detection limit of the method was found to be lower by one or two orders of magnitude. However, technical problems limit the reliability of the method at these extremely low concentrations. Nevertheless, the method seems to be applicable to future quantitative determinations of specific myosin heavy chain mRNA isoforms at the single fibre level.

Expression patterns of troponin T and myosin heavy chain isoforms in single fibres of rat muscle

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Similar to the family of sarcomeric myosin heavy chains (HC), troponin T (TnT) exists as multiple isoforms in skeletal muscle. The multiplicity of myosin HC and TnT isoforms is more pronounced in fast-twitch than in slow-twitch skeletal muscles. Thus, three fast myosin HC isoforms (HCIIb, HCIId and HCIIa) and four major fast TnT isoforms (TnT_{1f}, TnT_{2f}, TnT_{3f} and TnT_{4f}) have been identified in fast-twitch mammalian skeletal muscles as opposed to one slow myosin HC (HCI) and two slow TnT isoforms, TnT_{1s} and Tn-T_{2s} (Pette and Staron (1990) *Rev. Physiol. Biochem. Pharmacol.* **116**, 1–76). We investigated whether and to what extent relationships exist between the expression of fast and slow myosin HC and fast and slow TnT isoforms in rat muscle. For that purpose, we isolated single muscle fibres from freeze-dried fibre bundles of several rat skeletal muscles and used fragments of the same fibres for analysing their myosin HC and TnT isoform patterns. Myosin HC isoforms were separated by gradient PAA gel electrophoresis and visualized by silver-staining. TnT isoforms were identified after one-dimensional electrophoresis by immunoblot analyses with a commercially available monoclonal anti-fast TnT and a specific polyclonal antibody prepared against the slow TnT isoforms. The following results were obtained from analyses on a total of 106 fibres from tibialis anterior, gracilis, adductor magnus, vastus lateralis, and soleus muscles: (1) type IIB fibres contain HCIIb together with $TnT_{4f} > TnT_{2f}$, (2) type IID fibres contain HCIId together with $TnT_{1f} > TnT_{2f}$, (3) type IIA fibres contain HCIIa together with $TnT_{3f} > TnT_{2f}$, and (4) slow-twitch type I fibres contain myosin HCI together with $TnT_{1s} > TnT_{2s}$. These results agree with findings on rabbit muscle (Schmitt and Pette (1990) In *Dynamic State of Muscle Fibers*, Berlin, New York, de Gruyter, pp. 293–302), except that in our previous study some type IID fibres had been erroneously classified as type IIB.

Neoformation of intrafusal fibres in rat muscle spindles after neonatal de-efferentation

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We have studied the myogenesis induced by neonatal de-efferentation in rat muscle spindles 1–4 months after operation using light and electron microscopic methods. The average number of intrafusal fibres in de-efferented muscle spindles ($n = 100$) from 5–6-week-old rats had doubled in comparison with the usual four fibres in control spindles; up to 20 intrafusal fibres per spindle cross-section were found 4 months after de-efferentation. The additional intrafusal fibres originated from activated intrafusal myosatellites and by different forms of fibre division. The original fibres extended throughout the whole encapsulated portion of the spindle, whereas supernumerary fibres were found only in the A region. The diameters of the supernumerary intrafusal fibres varied from relatively large to very small. On the basis of their mATPase activity and pattern of expression of MHC isoforms and of M-protein, the vast majority of the supernumerary fibres could be classified as nuclear bag_1, bag_2 or chain fibres; some supernumerary fibres with small diameters did not belong to any of these fibre types. The supernumerary fibres were derived from all three intrafusal fibre types, but more of them originated from the nuclear chain than from nuclear bag_1 or bag_2 fibres. Immunocytochemical data suggested the existence of at least two types of intrafusal satellite cells. One type related to the nuclear bag fibres which give rise to myotubes that express slow tonic MHC and differentiate immunocytochemically into nuclear bag fibres phenotypes, if they share the sensory innervation. The other type of satellite cells attains, after fusion into myotubes, the nuclear chain fast phenotype irrespective of its innervation. It is noteworthy that de-efferentation, which causes wasting of extrafusal fibres, initiates myogenesis and maintains additional intrafusal fibres within the spindle capsule. Our results are consistent with the idea that sensory innervation is essential for muscle spindle formation and for the differentiation of the nuclear bag phenotype.

Myosin heavy-chain composition in striated muscle after immobilization

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As previously observed, the particular isoforms of myosin heavy chains (MHC) react selectively and variously to muscle denervation or tenotomy. The MHC-1 and MHC-2B are susceptible to lack of innervation (Jakubiec-Puka *et al.* (1990) *Eur. J. Biochem.* **193**, 623–8), whereas the isoforms characteristic for the fatigue-resistant fibres such as the MHC-1, MHC-2A and MHC-2S (MHC-2X) are susceptible to tenotomy (Jakubiec-Puka *et al.* (1992) *Biochem. J.* **282**, 237–42). In the present work investigation of immobilized muscle was undertaken to complement information concerning regulation of the MHC content and its dependence on the type of muscle. The total content of MHC and their isoform patterns were studied by biochemical methods in the slow-twitch (soleus) and fast-twitch (gastrocnemius) rat muscles immobilized for 4 days in a neutral or in a shortened position. It was found that: (1) the atrophy was larger in the immobilized slow muscle than in the fast muscle on the same leg, (2) the total MHC content decreased more in the slow than in the fast muscle, and (3) shortened position of the immobilized muscles increased their atrophy. The ratio MHC-1/MHC-2S remained almost unchanged in the immobilized

slow muscle, showing similar diminution of both isoforms. In the immobilized shortened fast muscle the MHC-2A/MHC-2B ratio decreased, showing loss of the MHC-2A mainly, whereas the MHC-2B content remained almost unchanged. The results, especially those of the immobilized-shortened muscles, are similar to the previous ones of the tenotomized muscles.

The results allow for the following conclusions: (1) MHC-1, MHC-2A and MHC-2S, isoforms characteristic for the fatigue-resistant fibres, are very susceptible to immobilization, (2) the MHC-2B is insensitive to immobilization, and (3) the fast MHC isoforms differentiate in sensitivity to immobilization. The results from the immobilized muscles were analysed and compared with those from the denervated and tenotomized muscles. Such comparison indicates that both the muscle activity and 'the trophic nerve influence' are the factors controlling the muscle content of the MHC isoforms.

Identification of the pMHC20-40 cDNA clone as specific to myosin HCIIb in identified single muscle fibres from rabbit adductor magnus by PCR after reverse transcription

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The existence of various subtypes within the fast fibre population of mammalian skeletal muscle necessitates the assignment of fast myosin cDNA clones to histochemically-defined fibre types. Of the different methods which can be applied to this problem, we have chosen to analyse specific mRNAs from microdissected histochemically and biochemically-defined single muscle fibres. For this purpose, specific oligonucleotides were used for reverse transcription and polymerase chain reaction (PCR). The oligonucleotides chosen were from the 3′ end of the translated and from a part of the untranslated hypervariable region of the pMHC20-40 cDNA (Maeda *et al.* (1987) *Eur. J. Biochem.* **167**, 97–102). Total RNA was extracted from freeze-dried fibre fragments microdissected from 100 µm thick muscle cross-sections using a modified oil-well technique. Reverse transcription was performed by adding reverse transcriptase, nucleosidetriphosphates and oligonucleotide primers to the extract under paraffin oil. The total assay mixture (1.12 µl) was then transferred into the PCR assay. After 25 cycles, the reaction was stopped and the 236 nt band specific to the pMHC20-40 clone was visualized after polyacrylamide gel electrophoresis by Ethidium Bromide. The identification of the fibres displaying the specific signal was based on the following procedure: thin (9 µm) serial sections were histochemically stained for myofibrillar actomyosin ATPase after preincubations at various pH values (4.3, 4.55, 9.6) and for NADH tetrazolium reductase. In addition, fragments of the same fibres were analysed for their myosin heavy chain (HC) complement by gradient gel electrophoresis under denaturing conditions. All histochemically and biochemically-defined type IIB fibres displayed the specific 236 nt band in the reverse transcriptase/PCR assay. Conversely, no positive reaction was detected in type IID and type IIA fibres. Analyses on single fibre fragments from psoas, tibialis anterior and extensor digitorum longus muscles gave negative results. This is in accordance with other findings from our laboratory that pure type IIB fibres were not detected in these muscles and that type IID is their predominant fast fibre type. Taken together, these results identify clone pMHC20-40 as specific to HCIIb.

Muscle growth and myosin isoform transitions during postnatal development of *Poecilia reticulata*

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Developmental changes in the myosin composition of lateral muscle in *Poecilia reticulata*, a small, ovoviviparous teleost fish, were studied by mATPase histochemistry and immunostaining with antibodies specific for a variety of myosin isoforms. At birth, fibres of the deep layer and superficial monolayer are already distinct. The histo- and immunohistochemical profile of deep layer fibres is of the 'fast' type, but it is not identical to adult fast white muscle. This juvenile myosin composition (JW) is replaced by the adult fast-white myosin isoform during the next few days. In the epaxial and hypaxial regions of the monolayer, the myosin composition present at birth (J1) is soon replaced (within 3 days) by another (J2). In some of these fibres, this J2 composition persists throughout life but in others it is slowly replaced by the adult slow-red muscle isoform. Close to the lateral line all monolayer fibres are already in transition between the J2 myosin and the adult slow-red form at birth, and rapidly complete the transition to the slow-red form. These fibres, with others generated *ex novo* in an underlying hyperplastic zone, form the red muscle layer of the adult.

The pink muscle develops during the first month after birth, and by 31 days it consists of an outer, middle and inner layer, each with a distinct histo- and immunohistochemical profile. A few middle layer fibres are already present at birth and the outer layer fibres first appear 3 days later. The thin inner layer, which is probably a transitional form between the middle pink and adult white isoforms, appears at about 31 days. A morphometric analysis of the white muscle showed that this layer grows mainly by hypertrophy. Even at EM level, no satellite cells or myoblasts were found in the white muscle except in the far epaxial and hypaxial regions in the first 10 days only. However, a zone of hyperplastic growth just under the superficial monolayer close to the lateral line presumably contributes fibres to the red and pink muscle layers.

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Muscle-specific adaptations in patients with dysfunction of the cervical spine

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Biopsies of neck or cervical erector spinae muscles obtained at operation on 22 patients aged from 18 to 76 years with cervical dysfunction of different aetiologies were examined histochemically. Muscle fibres were classified according to the pH lability of their myofibrillar ATPase. Additionally, reactions for succinate dehydrogenase, cytochrome *c* oxidase, and phosphorylase were performed. For the immunolocalization of parvalbumin (PV) monoclonal antibodies were employed. The intra- and interindividual fibre distribution in the investigated muscles varied considerably. As expected, the most postural muscles (longus colli and splenius capitis, respectively) exhibited the highest percentage of type I fibres. In the neck muscles (sternocleidomastoid, omohyoid, and longus colli) signs of muscle fibre transformation were uniformly observed within the first 2 years after the onset of symptoms. This was the case for all aetiologies (accompanied with hypo- or hypermobility). However, the direction of the muscle fibre transformation could not be determined unequivocally as only singular biopsies have been harvested. Patients with a case history of >2 years no longer showed signs of muscle fibre transformations. The vertebral muscles (splenius capitis, rectus capitis posterior major and obliquus capitis inferior) constantly lacked signs of muscle fibre transformation. The main findings in these patients (all rheumatoid arthritic) were denervation and reinnervation of both type I and type II fibres probably caused by the primary disease itself. The degree of fibre atrophy and type grouping was independent of the duration of symptoms. In the muscles investigated, extrafusal fibres were constantly devoid of PV. However, the intrafusal fibres showed a strong immunoreactivity corresponding to the one found in rodents.

The PV content of the muscle spindles could be responsible for the minimal amount of PV present in human muscles.

HCIId but not HCIIb is the predominant fast myosin isoform in rabbit skeletal muscle

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Using S1-nuclease mapping, we performed studies on the distribution of myosin heavy chain (HC) isoforms at the mRNA level in various skeletal muscles of the rat and rabbit. A cDNA probe specific to HCIIb mRNA of the rat did not hybridize with RNA preparations from fast-twitch muscles of the rabbit, namely tibialis anterior and extensor digitorum longus. On the other hand, a cDNA specific to rat HCIIa mRNA gave a strong signal with the same RNA preparations. In view of the presence of two electrophoretically separated fast myosin HC isoforms in these muscles, we concluded that the slower migrating isoform corresponded to HCIIa, whereas the faster migrating band represented HCIId, but not HCIIb. HCIId is a fast myosin HC isoform which was previously detected by electrophoretic analyses of single fibres in rat muscle (Termin *et al.* (1989) *Histochemistry* **92**, 453–7). These observations prompted us to investigate the pattern of fast myosin HC isoforms in the rabbit in more detail, especially with regard to the existence and assignment of HCIIb and HCIId. Our studies included S1-nuclease mapping assays for mRNA isoforms, as well as electrophoretic studies of the fast myosin HC isoforms at the protein level. The following rabbit muscles were investigated: adductor magnus (AM), diaphragm (DIA), extensor digitorum longus (EDL), extraocular muscles (EOM), gastrocnemius (GAS), masseter (MAS), plantaris (PL), psoas (PS), tibialis anterior (TA), tongue muscle (TO) and vastus lateralis (VL). The following results were obtained: (1) electrophoretic analyses showed the presence of HCIIa and HCIId in all muscles under study, (2) HCIIb was demonstrated electrophoretically only in AM, EOM, GAS and VL, (3) S1-nuclease mapping for HCIIb mRNA was negative in the case of DIA, EDL, MAS, PL, PS, TA and TO, and (4) however, strong signals for HCIIb mRNA were detected in RNA preparations from AM and EOM. GAS and VL were not examined at the mRNA level. Taken together, these findings suggest that the major fast myosin HC isoform in rabbit skeletal muscle fibres is HCIId, but not HCIIb. Contrary to fast-twitch muscle of mouse and rat, where HCIIb is a prominent fast isoform, HCIIb is expressed in the rabbit in only a few muscles. This conclusion is fully confirmed by our histochemical findings.

Muscle fibre types in lower vertebrates of different types of locomotion

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The aim of the present study was to find how fibre composition of the hindlimb muscles (sartorius and gluteus magnus) in two Anuran species – a frog (*Rana esculenta*) and a toad (*Bufo bufo*) reflects differences in their type of locomotion: saltatorial and pacing, respectively. Part of muscles was frozen and analysed histochemically for myofibrillar ATPase (pH 10.4, 10.5, 4.0, 4.2, 4.35, 4.50), cytochrome c oxidase, and SDH activity. Part of muscles was fixed in Bouin, embedded in paraffin and analysed immunohistochemically for parvalbumin PV (antibody 235; Celio *et al.* (1988) *Cell Calcium* **9**, 81–6). Some of muscles were fixed, frozen and examined histo- and immunohistochemically following method by B. Zawadowska and M. Muntener (*Muscle and Motility*, Vol. 2. Intercept Ltd., Andover, Hampshire, UK). In controls immunoincubation with primary antibody was omitted. Sartorius muscles of the frog and toad contained 10.6 and 55.3%, respectively, of type I fibres. Gluteus magnus muscles contained about 2–3% of this fibre type. In sartorius muscles of the frog type IIA composed 28.5% and type IIB composed 60.9% of all fibres. In sartorious muscles of the toad these amounts were 19.8 and 24.9%, respectively. IIA and IIB fibres in toad were more oxidative (high SDH and cytochrome *c* oxidase activity) than relative fibre types in the frog. Also PV immunostaining was more intensive in both II types of fibres in the toad than in the frog. In conclusion, investigated muscles in the toad are composed of fibres more resistant to fatigue (high oxidative metabolism) than the same muscles in the frog. It can be considered as adaptation to a walking gait of the toad. The results of the study suggest also that PV content correlates with ATPase activity in the hindlimb muscles of the Amphibian species compared.

Molecular physiology and modulation of myosin gene expression in mammals

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The isoforms of the myosin heavy chain (MHC) multigene family are molecular markers for muscle physiology. By determining which MHC types are present at different times of histogenesis in various muscles, it has been possible to describe the various pathways of isoform transitions. The physiological influences involved in some of these transitions have been identified through analysis of muscle development and induced regeneration in adult muscles. Study of postnatal development has allowed us to investigate the control of the neonatal-to-fast MHC (IIA, IIB, IIX) transitions, while the study of regeneration has permitted us to focus on the mechanism of slow MHC induction in adult muscle fibres. It can be inferred that the physiological controls of MHC transitions involves contractile activity in a general way, and that they act possibly through myotrophic factors and certainly via hormonal effects. Although an explanation of the underlying mechanisms of these controls can only be formulated in general terms at present, one main goal of future studies is to combine molecular manipulations of MHC genes with novel ways of reintroducing them into cells and animals. We are presently concentrating on the adult fast IIB MHC gene because it is a stage- and fibre-specific marker, and its expression is modulated by neuronal and hormonal influences. The study of this gene is being carried out to analyse two distinct levels of control. The first level concerns a definition of the promoter-based molecular mechanisms involved in gene transcription. We have uncovered a striking evolutionary conservation, acting over some 300 million years, among distinct sequence motifs of avian and mammalian fast-type MHC promoters. These sequences, and in particular a set of 'myosin AT-rich' or mAT motifs, are important elements in establishing transcription initiation. Studies of the promoters are being carried out using cell culture models and especially transgenic mice and direct injection of cloned promoter fragments into muscle tissue. The second level of MHC gene control involves the position of the IIB MHC gene within the linked multigene MHC locus. Long range activator effects may be operative in determining the fine regulation of IIB MHC expression, and manipulation of this gene within its locus will be carried out through the use of homologous recombination.

Duchenne muscular dystrophy gene: structure and products in muscle and nonmuscle tissues

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The huge gene, which is defective in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy, spans approximately 2500 kb

and is one of the largest genes known to date. The transcription product of the gene in the muscle is a 14 kb mRNA, encoding a 427 kDa rod-shaped protein called dystrophin, which is associated with the sarcolemma. A 14 kb mRNA produced by the same gene, which encodes a protein very similar to the muscle type dystrophin but is regulated by a different promoter, is present in the brain. Recently we identified a 6.5 kb mRNA transcribed from the DMD gene. This transcript, first identified in liver and hepatoma cells, is the major DMD gene product in many nonmuscle tissues including brain. It is downregulated during *in vitro* myogenesis and is undetectable in differentiated muscle fibres. This mRNA is transcribed from a relatively small region (approximately 150 kb) at the 3′ end of the DMD gene. Cloning and *in vitro* transcription and translation of the entire coding region shows that the 6.5 kb mRNA encodes a 70.8 kDa protein (Dp71), which is a major product of the DMD gene. It contains the C-terminal and the cysteine rich domains of dystrophin, seven additional amino acids at the N-terminus and some modifications formed by alternative splicing in the C-terminal domain. It lacks the entire large domain of spectrin-like repeats and the actin binding N-terminal domain of dystrophin. Cell fractionation experiments have shown that Dp71 is highly enriched in the membrane fraction. The expression of the three DMD gene products during embryonic development and the possible implication of the findings with regard to the ontogenetic activation and the evolution of the giant DMD gene are under consideration.

Expression of myosin isoforms in human satellite cells in culture

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Satellite cells were isolated from quadriceps and masseter muscles of a 5-month-old child. The cultures were shifted to differentiation conditions, and the pattern of myosin expression was determined by 2D-electrophoresis, 6% SDS-glycerol electrophoresis and immunocytochemistry using specific antibodies. Satellite cells, whether they come from masseter or quadriceps, express MLC1emb, MLC1f, MLC2f and MLC3, and no slow MLC. The myosin heavy chain pattern includes embryonic, fetal, and slow MHC. These cells were passed several times, and after ten passages, we observed a modulation in this pattern: the expression of MLC1f is then stopped. This is the only modulation detected. The same result was found in a culture of satellite cells isolated from an older patient (60 years); however, this modulation appeared as soon as the first passages. The same modulation was also observed in cells derived from a rhabdomyosarcoma after chemotherapy.

Our results demonstrate that satellite cells, derived from different muscles, express the same programme of differentiation, thus suggesting that the differences observed *in vivo* would be the result of epigenetic influences. This programme is very similar to the one found in primary fibres during development. In addition, our results show that a modulation of expression of MLC1f, resulting in the absence of this marker, occurs when these cells are passaged. It seems that this modulation can also be the result of ageing *in vivo*. The models which can explain such a modulation will be discussed.

Genomic structure and chromosomal localization in mouse of the α-tropomyosin gene

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Tropomyosins (TM) are ubiquitous proteins of 33–45 kDa, associated with thin filaments and stress fibres of animal muscle and nonmuscle cells. The TM genes of various species have been cloned and characterized. In human and rat they comprise up to 13 exons and code for diverse tissue specific expressed isoforms, regulated via an alternative splicing mechanism. At present four different structural genes are known in human and three in rat (Lees-Miller *et al.* (1990) *Mol. Cell. Biol.* **10**, 1729–42). In the mouse, no information on the structure and chromosomal localization is available for TM genes.

We have studied a TM sequence obtained from a mouse muscle cDNA library. On the cDNA and genomic level, this sequence is very similar to the rat αTM gene (Goodwin *et al.* (1988) *J. Biol. Chem.* **266**, 8408–15). The cDNA sequence for the mouse αTM specific isoform is identical to the mouse TM fibroblast isoform 2 (TM2), except for its 3′ end. We conclude that the cDNA sequence codes for the αTM and the TM2 transcripts, and designated the corresponding structural gene *Tma* (provisional gene symbol). This identification was confirmed by restriction pattern analysis of mouse DNA and of the genomic TM clone, and by partial DNA sequencing of an αTM specific exon.

By use of interspecific backcrosses (Kaupmann *et al.* (1992) *Genomics* **13**, 39–43) the mouse *Tma* gene was assigned to chromosome 9, the most likely gene order being *Cen-Cyp1a2-Tma-Mod1-Myllc-Scn5a*.

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CONTRACTILE PROPERTIES AND MOLECULAR THERAPY

Structural basis for force decline in EDL-muscle from *mdx* mice after eccentric contraction

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EDL-muscles from mdx mice display an irreversible decline in maximal isometric force after eccentric contractions (Moens *et al.*, this Meeting). As only about 50% of the force decline could be explained by membrane-damaged fibres, we propose and test two hypotheses to explain the remaining 50% force decline.

The first hypothesis is that the observed force decline can be fully explained by membrane-damaged fibres, but the extent of the latter is underestimated. It is possible that the dye that we used to detect membrane-damaged fibres only stained a limited area around a membrane lesion. As we studied sections taken from the centre of the muscle, we may have not counted fibres that were membrane-damaged more towards the tendon. To test this hypothesis, transverse sections were cut at 150 μm intervals along the length of an EDL muscle (mdx) with a large force decline. Then, the muscle was reconstructed and the longitudinal distribution of staining (membrane damage) was studied. It appeared that still the observed force decline could not be fully explained by the percentage of membrane-damaged fibres.

A second hypothesis is that the unexplained part of the force decline is caused by damage at the myotendinous junction (MTJ). It was reported earlier that eccentric contractions can cause separation of basement- and plasma membrane at MTJs of normal muscle (Miller (1977) *Am. J. Sports Med.* **5**, 191–3), and that dystrophin is present in abundance at the MTJ (Tidball and Law (1991) *Am. J. Pathol.* **138**, 17–21; Byers *et al.* (1991 *J. Cell Biol.* **115**, 411–21). Therefore, absence of dystrophin might render this location relatively more susceptible to mechanical strain in mdx mice. To study MTJs by electron microscopy, ultrathin longitudinal sections were cut at 100 μm intervals from blocks that contained both tendon and muscle. Preliminary results show no fibres that are both healthy and display separation of basement- and plasma membrane at the MTJ. So far, our results indicate yet another cause for the unexplained part of the force decline observed after eccentric contractions in EDL muscles from mdx mice. One possibility is that in mdx mice eccentric contractions give rise to a disruption of sarcomeres, without destruction of the fibres.

Series elasticity in normal, dystrophic and transplanted mouse muscles

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A common way of investigating crossbridge properties is to record tension during imposed changes in muscle length. However, the series compliance of the muscle may interfere with the results, so that measured tension does not accurately reflect changes taking place in the contractile machinery. We have measured the stress–strain relationship of the series compliance in soleus and extensor digitorum longus (EDL) from both C57BL/10 and mdx mouse strains, and in transplanted muscles. Methods were adapted from Jewell and Wilkie (*J. Physiol.* **143** (1958) 515–40): muscles were submitted to isotonic releases from L_0 against various loads P ($<P_0$). To reduce the inertia of the system, loads were applied by springs instead of weights. The length transient comprised two phases: (1) elastic recoil of the series compliance, and (2) active shortening of the crossbridges. The length change of the series compliance (expressed as $\Delta L/L_0$) was plotted against stress (P/P_0). Results showed that: (1) No significant difference exists between the stress–strain relationships of normal and dystrophic soleus and EDL. Transplanted muscles may be slightly more compliant than controls, but the difference is not significant. The series compliance may therefore be neglected in experiments designed to compare these muscles. (2) The stress–strain relationship of mouse muscles is not linear as in amphibian muscles, but is well described by a single exponential equation. (3) A step shortening of 2% L_0 (25 nm per half sarcomere) can bring tension from its isometric value to zero. This is close to the value found in whole frog sartorius (Jewell and Wilkie (1958) *J. Physiol.* **143,** 515–40), but much higher than that measured in isolated fibres at clamped sarcomere length (Ford *et al.* (1981) *J. Physiol.* **311,** 219–49). Clearly, most of the series compliance of whole mouse (and frog) muscles resides in structures other than the sarcomeres, probably the tendons. (4) The series compliance of mouse muscles is much less than that of frog muscles, except for very large load steps (i.e. for $P < 0.05\ P_0$), and the strain is not significantly different from zero if $P > 0.4\ P_0$. In this condition, the contribution of series compliance is therefore negligible in whole mouse muscles.

Three-dimensional presentation of contractile properties of stretch-activated arterial smooth muscle

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Bayliss proposed first that stretch may activate constriction of blood vessels (Bayliss (1902) *J. Physiol.* **28,** 220–31). As this proposal changes in both electrical and mechanical properties of vascular smooth muscles during/following stretch have been reported in numerous cases (for review see Johnson (1980) In *Handbook of Physiology*, vol. II. Bethesda, American Physiological Society, pp. 409–42. The biochemical events behind these myogenic reactions were clarified by Bárány *et al.* (Ledvora *et al.* (1984) *Clin. Chem.* **30,** 2063–8; Bárány *et al.* (1985) *J. Biol. Chem.* **260,** 7126–30; Bárány *et al.* (1990) *Biochem. Biophys. Res. Com.* **173,** 164–71). They demonstrated the following: stretch induces phosphorylation of the 20 kDa light chain of myosin; the extent of phosphorylation is a function of the applied stretch reaching a maximum at 1.7 times the resting length; the stretch-induced phosphorylation is catalysed by myosin light chain kinase, not by protein kinase C and decays as a function of time. The fully stretched arterial strips cannot generate active tension even in the presence of any exogenous stimulating agents indicating the lack of overlapping filaments. When stretched arterial strips are released active tension develops without any exogenous stimulating agents.

During our experiments reported in this paper helical strips from pig carotid arteries were activated by stretching them to 1.7 times their resting length then the stretched muscle strips were released. Active tension developed which was recorded. The extent of release as well as the time-elapse between the stretch and release were varied systematically. In this way we were able to investigate the active tension development at different muscle lengths and at different myosin light chain phosphorylation levels. Our results demonstrate that the amplitude of stretch–release-induced active tension is a monotonous function of light chain phosphorylation and shows a maximum as a function of muscle length. These data are presented in a three-dimensional active tension–light chain phosphorylation–muscle length space.

Microstructural alterations in longissimus muscle of pigs with PSE meat condition

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To study the muscle structure in relation to alterations in meat quality, especially in PSE (pale, soft, exudative) condition, muscle fibre properties were determined in shot biopsy samples of longissimus muscle from 90 Landrace pigs. Biopsy samples were taken sequentially 95 and 195 days of age. After slaughter the carcasses were classified into three groups according to their meat quality: I (normal), II (slightly PSE meat) and III (extremely PSE-meat).

As a result significant differences were found in muscle fibre diameter as well as in fibre type composition between pigs with normal and PSE meat, progressively from 95 to 195 days of age. In pigs with PSE meat condition the number of white fibres, as classified by negative histochemical NADH-tetrazoliumreductase reaction, was increased and the percentage of intermediate fibres was decreased; whereas the number of red fibres remained unchanged. In pigs with PSE the diameters of all three fibre types were enlarged. These changes of fibre population indicate high glycolytic potential of muscle and also predisposition of PSE meat.

The results suggest that microstructural alterations in skeletal muscle tissue in the live animal are one of the reasons for biochemical disorders leading to pale, soft, exudative meat post mortem.

Effects of electrostimulation on the contractile properties of unloaded soleus: a study on *in situ* muscle and isolated skinned fibres

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The purpose of this study was to investigate the effects of electrical stimulation counteracting the changes in the unloaded rat soleus muscle. This aim was achieved by imposing different patterns of activity on the soleus nerve during 10 days of hindlimb suspension (HS). Two patterns were tested during HS: a slow-like pattern of activity (HSS1 rats) and a fast-like one (HSS2 rats). Both HSS1 and HSS2 groups were compared with the control (CON) and the HS rats. The contractile properties of the soleus muscle in the different situations described above were studied in two experimental conditions: *in situ* and using isolated EGTA skinned fibres. The kinetic properties estimated for the *in situ* muscle and for the isolated fibres indicated that HS induced slow to fast changes in the slow soleus muscle. Our results showed that the application of the slow pattern avoided these transformations. Indeed, the decrease of about 22% in the time to peak and in the half relaxation time observed in HS was prevented in HSS1. What is more, the tension response to calcium and strontium ions (Tension/pCa and pSr) and the P/t_{max} kinetic parameter indicated that the soleus fibres from the HSS1 group exhibited characteristics identical to those of the CON group. On the contrary, the application of the fast-like pattern seemed to favour the slow to

fast fibre type transformation. Our results also showed that the loss of muscle mass and the decrease in fibre diameter observed after HS were not prevented by any of the two patterns. Moreover, the decline in maximal tension occurring in HS conditions as well for the isolated fibres and the whole muscle was not counteracted in the two stimulated groups. In conclusion, our results demonstrated a compensation of the HS-induced kinetic changes in the soleus muscle by means of a pattern of stimulation resembling slow motor unit activity. The present study emphasized the nervous influence on the plasticity of skeletal muscle and outlined the contrasting effects of two specific patterns of stimulation on HS animals. Furthermore, we suggested that the kinetic changes were predominantly modulated by the nervous command while the atrophy might be under another influence, such as mechanical strains.

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Analysis of the 'catch-like' effect of muscle contraction in Crustacea

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A number of hysteresis phenomena during muscle contraction in arthropods has been described. The most prominent among these phenomena is the so-called Blaschko–Catell–Kahn effect. Blaschko *et al.* (*J. Physiol.* **73** (1931) 25–35) found that, in crayfish, repetitive low-frequency stimulation of a motoneuron causes the corresponding muscle to develop a force plateau. Switching to a high stimulation frequency for a few milliseconds causes the muscle to generate a higher force peak. After returning to the original low stimulation frequency, the force drops to a value much higher than the first plateau. Thus it appears that the muscle can maintain an increased amount of force without additional expenditure of energy. Because of this superficial similarity to the 'catch' contraction of some molluscan smooth muscles (e.g. the anterior byssus retractor of *Mytilus*), the term 'catch-like' effect has been used since the late 1960s to describe such hysteresis phenomena (Wilson and Larimer (1968) *Proc. Natl. Acad. Sci. USA* **61**, 909–16).

For a systematic investigation of the catch-like effect, walking leg closer muscles of the crab *Eriphia spinifrons* and the crayfish *Pacifastacus leniusculus* were used. The results show that the effect depends not only on the stimulation frequency of the motoneurons or the amplitude of the force peak, but also on the length of the muscle fibres, the degree of stretch applied to the muscle or even the stiffness of the force transducer. This suggests that the catch-like effect is rather analogous to the 'residual force enhancement' described for vertebrate muscle (Edman *et al.* (1982) *J. Gen. Physiol.* **80**, 769–84). Similar to the residual force enhancement, the catch-like effect can be evoked by quickly stretching the muscle, provided the muscle length lies within the descending limb of the force-length relationship.

One hypothesis to explain residual force enhancement proposes that the increase in force is caused by nonuniformity of sarcomere lengths along the muscle fibre (Morgan (1990) *Biophys. J.* **57**, 209–21). Based on this hypothesis and the actual force–length relationship of the crab closer muscle, a computer model was developed to test whether such sarcomere length non-uniformities suffice to explain the catch-like effect. It was found that all the results could be explained by such a model. The postulated differences in sarcomere lengths along a muscle fibre could be shown in the electron microscope.

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Reactivity of skinned skeletal muscle fibre after denervation

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Chronically denervated muscles of vertebrates are capable of producing a lot of physiological and histological modifications, which could be similar to the modifications which appear in different types of human muscular dystrophies. Thus, it is well known that the resting membrane potential of mammalian skeletal muscle, decreased within a few hours following denervation (Hörr and Lorkovic (1985) *Neurosci. Lett.* **56**, 175–81). To explain this depolarization, on increase in the sodium permeability, P_{Na}, of the membrane and on inactivation of the Na-K pump have been discussed (McArdle (1983) *Progr. Neurobiol.* **21**, 135–98). The chronically denervated muscle are capable of producing contractile force if exposed to elevated K concentrations or to ACh (Thesleff (1960) *Physiol. Rev.* **40**, 734–52). Skinned skeletal muscle fibres were prepared by chemical means from soleus (SOL) and extensor digitorum longus (EDL) of Wistar rats. The denervated EDL fibres have contractile characteristics which were distinct from those of the normal fibres. However, some characteristics of the denervated EDL were quite similar to those of the normal EDL fibres. Denervated EDL fibres were the least sensitive to pCa. Denervated SOL muscle fibres have contractile characteristics which were similar with those present in normal soleus muscle. However, the denervated SOL fibres were the most sensitive to pCa, because these fibres formed probably a distinct population. The maximum tensions induced by Ca^{2+} were smaller in denervated fibres than in normal fibres obtained from equivalent muscles. In conclusion, it is possible to detect significant physiological differences in the mechanism of force activation brought about by different types of muscular dystrophies.

Experimentally-induced myopathic changes in the contractile apparatus of striated muscle

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Several anomalies appear in the contractile structure of myopathic muscle; they are usually believed to be degenerative. However, some of them were also found in the intensively active muscle during reorganization of the contractile apparatus for adaptation to the altered muscle length (Jakubiec-Puka (1985) *J. Muscle Res. Cell Motil.* **6**, 385–401; Jakubiec-Puka and Carraro (1991) *J. Anat.* **178**, 83–100). In the present work the ultrastructure of the contractile apparatus was studied in the rat leg muscles subjected to several experiments such as immobilization, work overloading, or maintainance in a shortened or a lengthened position. Several changes such as Z-line streaming, extension of the Z-line, its spreading into the I- and A-band and rod-like structures were observed in the muscle maintained in extension, and also occasionally in the muscle maintained in shortening. The foci of disorganization of the contractile structure, resembling 'minicores' from myopathic muscle, were found in the muscle maintained in shortening, both immobilized and active. On the other hand, in the immobilized-shortened muscle some of the so-called 'post-mortem' changes were found. When such a muscle was electrically stimulated, a segmental necrosis appeared transiently in fragments of some fibres. All these anomalies were found in the muscles where anabolic processes were very pronounced while serious necrotic changes were practically absent and the contractile structural appearance soon recovers to its normal state. Thus, those anomalies should not be considered as degenerative changes, but rather as the transient structures in the course of remodelling and reorganization of the contractile structure, including its partial elimination, during adaptation to working under altered conditions. When the muscle maintained in extension was work overloaded, quite different changes were observed: contraction bands, focal damages of the contractile structure, fibre necrosis with phagocytosis, and numerous 'empty tubes of sarcolemma'. Thus, certain degenerative and necrotic processes can take place in such a muscle. It must be stressed, that in other fibres

of the same muscle the above-mentioned 'adaptive' myopathic changes have occurred. It seems that simultaneous work overloading and extension of the muscle bring about a particular risk of damage and degeneration.

Twitch, tetanus and potassium contracture of phasic and tonic frog muscles with adrenaline and CGP

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Phasic and tonic bundles (10–20 fibres) were isolated from m. iliofibularis of frogs (*Rana temporaria*) and mounted horizontally in a small Perspex chamber supplied with Ringer solution. Mechanical responses were triggered by direct stimulation or 160 mM K^+ solution and recorded on an oscilloscope. Adrenaline and CGP (2-methyl-3-methoxycarbonyl-4-(2'difluormethoxyphenyl)-5 oxo 1,4,5,7-tetrahydrofuro-(3,4-b)pyridine) were added to the Ringer solution at least 10 min before the test contraction. The temperature was 20–22°C.

Tonic bundles did not respond to single pulses except small twitches appearing in a few preparations containing some phasic fibres within the tonic bundle. Tetanic contractions were induced by trains of 2 s in duration. On phasic bundles the twitch/tetanus ratio at 40 Hz was near 0.5 and the tetanic tension at 20, 40 and 70 Hz was less different than in tonic preparations. Both in phasic and tonic bundles the maximum of potassium contracture attained similar tension as maximum tetanus. Exposure to adrenaline (10 μM, 10 min) enhanced the tension development of all contractile responses but more the submaximum tension (twitch, 20 Hz-tetanus) than maximum tetanus or potassium contracture. No differences were obtained between responses to adrenaline of phasic and tonic fibres. CGP exposure (10 μM in 0.1% DMSO, 10 min) decreased the contractile responses of both fibre type preparations in Ringer solution and changed the relaxation slope of tetanic tension as well as of potassium contracture. There occurred a protraction of the relaxation time lasting more than 3 min. During this period single pulses were able to trigger twitch tension on tonic bundles in the order of 30% of the 70 Hz tetanus although the preparations showed no twitches before CGP. This response to single pulses may be due to the high intracellular level of free Ca^{2+} caused by a modification of the Ca^{2+} re-uptake after tetanus.

Increased susceptibility to eccentric contractions in *mdx* EDL limb muscle

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We have tested whether mdx muscles are more vulnerable to mechanical stress than muscles of the control strain (C57BL/10). To this end, we submitted the muscles to eccentric contractions, because of the high levels of stress that are obtained in that way. Extensor digitorum longus (EDL, $n = 20$) and mdx soleus (SOL, $n = 12$) muscles were stimulated tetanically to isometric contraction six times every 3 min *in vitro* at 20°C. When the force reached a plateau, the muscles were stretched 1 mm at a velocity of 1 fibre length per second.

In a control series of isometric contractions force remained constant throughout the series in all muscles and strains tested.

After eccentric contraction, maximal isometric force did not change substantially from the initial levels developed by SOL mdx muscles or control muscles (EDL: $n = 14$; SOL: $n = 12$). However, maximal isometric force declined by 5–10% compared with the preceding eccentric contraction (in the series of six) in mdx EDL. This resulted in a force decline of 30–60% of the initial isometric force at the sixth tetanus. Moreover, the muscles did not recover even after 60 min of rest.

We measured the percentage of membrane damaged fibres by bathing the muscles after the mechanical experiments for 1 hour in a Krebs solution containing 1% Procion Red. This dye is known to be excluded from intact cells, but to penetrate cells with damaged membranes. We found that the percentage of membrane-damaged fibres is positively correlated with the muscle force decline.

The increased susceptibility to eccentric contractions of mdx EDL compared with mdx SOL muscles may be explained by the higher levels of force developed by EDL muscles. This suggests that muscle damage occurs when high levels of stress are imposed onto the muscles, supporting the hypothesis that muscles lacking dystrophin are more susceptible to mechanical strain, such as hypo-osmotic shock (Menke and Jockusch (1991) *Nature* **349**, 69–71).

Force–velocity relation and isomyosin content of soleus muscle from six strains of mice

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The force–velocity relation of isolated soleus muscle was evaluated through a series of isovelocity releases from maximal isometric tetanus at 20°C ($n = 77$). The force constant a/Fo and the velocity constant b were computed using a nonlinear least squares procedure. a/Fo was equal to 0.11 in all strains, but b increased from 0.34 to 0.64 muscle fibre length per s in the order: ky (male) < ky (female) < NMRI (female) = SJL (male) < NMRI (male) = mdx (female) < DBA (male) < mdx (male) = C57 (female) < C57 (male). All soleus muscles contained two isomyosins (SM2 and IM) and their corresponding myosin heavy chains (MHC1, also called MHC-β, and MHC2As, subscript s to recall that this chain is isolated from soleus muscle). The fraction of MHC2As relative to the total myosin content increased in the various strains from 5 to 65%, in the same order than in b. Therefore, b was a linear function of MHC2As ($r^2 = 0.96$). The velocity constant for MHC2As (or isomyosin IM) or MHC1 (SM2) can be evaluated from extrapolation of this function to 100% or 0% MHC2As: b is equal to 0.8 Lf/s for MHC2As and 0.3 for MHC1. Soleus from dystrophic mdx mice are faster, equal or slower than control muscles, depending on which strain or sex is selected for comparison. Soleus from male mice are faster than soleus from female ones (except for strain ky, a mutant with severe kyphoscoliosis). These effects are simply explained by the differences in isomyosin proportions observed between strains. Since b for EDL muscle is in the range 1.5 to 2.5, it appears that MHC2As (isomyosin IM) resemble more type I fibres from soleus muscle than type II fibres from EDL muscle.

Duchenne myotubes are more susceptible to hypo-osmotic shock than dystrophin expressing human controls

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In Duchenne muscular dystrophy (DMD) and in X-linked muscular dystrophy of the mouse (MDX), a defect in the gene for the subsarcolemmal cytoskeletal protein dystrophin causes muscle fibre necrosis (review: Slater (1989) *Curr. Opin. Cell Biol.* **1**, 110–4). Dystrophin is associated with both transmembrane and cytoskeletal proteins, yet its physiological function is not known.

We have previously demonstrated that dystrophic MDX (mdx/mdx or mdx/y) mouse myotubes are more susceptible to hypo-osmotic shock than wildtype (+/+ or +/y) controls (Menke and Jockusch (1991) *Nature* **349**, 69–71). As this difference was not influenced by blocking K^+, Cl^- or Ca^{2+} channels nor by a Ca^{2+} ionophore (Menke and Jockusch (1991) *Eur. J. Cell Biol.* **32**, 33) it is likely to be due

to the absence versus presence of functional dystrophin. Immunofluorescence of cross-sections of cultured mouse myotubes stained with the 'Dys2' anti-dystrophin monoclonal antibody in fact demonstrated a subsarcolemmal localization of dystrophin. In human myotubes the presence of dystrophin in control cultures was shown by immunoblotting.

To compare the osmotic stability of dystrophic and normal human myotubes, myoblasts were obtained from biopsies of five DMD patients and from seven control patients who had undergone surgery for orthopedic reasons. Cell cultures displaying visible cross-striation in $\geqslant 5\%$ of the myotubes were analysed. For hypo-osmotic shock experiments cultures were exposed to test solutions of 10–150 mOsm (standard value 280 mOsm) and the release of the muscle specific enzyme creatine kinase was measured as an indication of membrane damage. On the basis of the amount of enzyme released as a function of osmolarity of the shock solution, we found that DMD myotubes were significantly less stable than dystrophin containing controls; in case of the 95 and 50 mOsm containing test solutions the statistical difference was $\alpha = 0.0001$ and 0.002, respectively (Student's t-test). We conclude that already in early stages of muscle fibre development dystrophin may mechanically stabilize the cell surface.

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Muscle pathology and density of satellite cells in the mouse mutant 'muscular dystrophy with myositis' (mdm/mdm)

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The *mdm* mutation ('muscular dystrophy with myositis') was first described by Lane in 1985 (Lane (1985) *Mouse News Letter* **73**, 18) as a lethal mutation on a C57Bl/6J background. Homozygous MDM (genotype *mdm/mdm*) mice can be recognized from day 12 onward by a stiffness and a humpback posture. The *mdm* locus has been mapped to chromosome 2 (Lane (1985) *Mouse News Letter* **73**, 18) which also contains the locus for the myofibrillar protein titin (Labeit *et al.* (1990) *Nature* **345**, 273–6).

By use of light and electron microscopy we studied degenerative and regenerative processes in skeletal muscles of MDM mice as well as the fine structure of heart muscle cells. Degeneration of skeletal muscle starts within the first two postnatal weeks in the slow soleus muscle and approximately 1–2 weeks later in fast muscles, so that the time course of degeneration seems to be dependent on the type of muscle. However, from day 40 onward only regenerated fibres are present and still ongoing degeneration is insignificant. This fast and effective regeneration most probably is due to a strongly increased density of satellite cells (SC) in all ages examined so far. Thus, the number of SC is very high at day 20 but, instead of decreasing as in the wildtype (WT), it has increased up to day 80. Especially in the slow soleus muscle of 80-day-old MDM mice the level of SC is up to six times higher than in WT. The observed high numbers of SC cannot be explained alone by an activation by a degenerative process as the number of SC increases (at least in the tibialis anterior) between day 40 (where the phase of degeneration has terminated) and day 80 by a factor of two. In the slow soleus the number of SC is very high in all stages examined and does not show the common negative correlation with age.

At the EM level the most prominent features of adult MDM skeletal muscles are Z-band streaming of individual myofibrils, a very high content of glycogen as well as centrally situated fibre nuclei showing that practically all fibres have undergone degeneration/regeneration. Z-band spacing as well as A- and I-band lengths are not different from controls. First results indicate that in the heart muscle degeneration takes place to a considerable extent as well. Degeneration was found in ventricular cells in all stages (20–80 days) examined so far.

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Alterations of protein degradation and two-dimensional protein pattern and the influence of calcium-channel blockers in cultured MDX and DMD muscle cells and isolated MDX muscle fibres

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The *mdx* mouse is an established animal model for human Duchenne muscular dystrophy (DMD). It is not known how the defective dystrophin gene may cause pathological muscle degeneration. In addition to a diminished membrane stability (Menke, 1991), impaired calcium homeostasis occurs in MDX and DMD muscle (Turner, 1988; Mongini, 1988), and may be either a secondary, or even a crucial step in muscle destruction.

Measurements of protein degradation were done by [^{35}S]-methionine pulse-chase-experiments. In comparison to control myotubes degradation of short-lived proteins, which are more involved in regulatory processes, is increased in 5 day MDX myotubes by a factor of 2.5. Likewise, the degradation of long-lived proteins in 5 day MDX myotubes is elevated by 33%. Our results on protein degradation (chase-time 24 h) in isolated muscle fibres with a maximum at the age of 3 weeks correlates with published histological data. The degradation rate alters from 76% at 2 weeks, to 85% at 3 weeks, and returns to 52% at 5 weeks, whereas the degradation in wildtype muscles remain at basically the same level of 10–17%. Total intracellular [^{35}S]-methionine labelled proteins were separated by high resolution 2-D-PAGE. The net content of several proteins in cultured myotubes of MDX and DMD origin and isolated MDX muscle fibres is decreased. The net content of protein p51/5.1 ($M_r = 51{,}000$, IEP = 5.1) is reduced in human DMD myotubes by 66% compared with wildtype. In MDX muscle of several differentiation states protein p51/5.1 is decreased from 47% in cultured myoblasts to 33% in 9 day cultured myotubes, and finally to 6% in 9 weeks old interosseus muscle fibres. The calcium-channel blockers Dantrolene and Verapamil markedly reduced degradation of many proteins in 9 day MDX myotubes.

The degradation of protein p51/5.1 is especially reduced by Dantrolene by about 91%, and by Verapamil by about 95%, whereas wildtype myotubes showed only little effect (Verapamil) or none at all (Dantrolene). The function of protein P51/5.1 is as yet unknown.

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Novel immobilization model in the rat: functional, histomorphological and biochemical changes in atrophying skeletal muscle

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Muscle atrophy is induced by immobilizing one hindlimb of the rat (Wistar, male, about 300 g BW) with the help of a 'boot' made by assembling two moulded shells around the leg. The procedure has several advantages, e.g. the shells weigh only a few grams, can be opened any time when needed, can be used several times and the immobilization angle is the same for all animals. This procedure limits but does not prevent joint mobility, allows weight bearing and the animals maintain normal posture during the immobilization period, although leg extension is not possible. Disuse atrophy is reflected by a muscle mass loss of about 35% after 3 weeks of immobilization, the soleus and plantaris being most affected. Functional parameters were measured during direct electrical muscle stimulation (gastrocnemius-

plantaris-soleus group) *in situ* under isometric and isotonic conditions for 30 min. Contraction force, assessed isometrically, of muscles suffering from disuse declined significantly from 9.4 ± 0.54 N (45 s stimulation) to 4.5 ± 0.06 N (30 min stimulation). Normal muscle showed decreasing contraction force from 10.6 ± 0.63 N (45 s) to 5.9 ± 0.13 N (30 min). The area under the tension curve over 30 min was determined to be 11 657 ± 292 for normal muscle and 9281 ± 268 (−20.4%, $p < 0.001$) for atrophic muscle. Compared with normal muscle, contraction rate decreased by 35.4% and relaxation rate by 29.9% in muscles which suffered disuse. In isotonic measurements (load: 100% BW), work performed during last 500 contractions was 9.3 J for normal and 6.7 J for atrophic muscle. In histological sections of soleus muscle it was observed that the number of type IIa fibres was increased in atrophic muscle (9.1 ± 1.85% versus 0.8 ± 0.3% in normal muscle); the type I fibres atrophying most (−30% area/fibre versus normal muscle). Electrophoresis of immobilized soleus revealed an increase in myosin HC IIa corresponding to histochemistry. No change in the distribution pattern of creatine kinase isoforms BB and MB was detectable. Thus, there seems to be no apparent necrosis and subsequent regeneration. Torbafylline, a xanthine derivative possessing antiischaemic activity in skeletal muscle (Okyayuz-Baklouti (1989) *Eur. J. Pharmacol.* **166**, 75–86; Dawson *et al.* (1990) *Int. J. Microcirc. Clin. Exp.* **9**, 385–400) was tested in this model.

Muscle structure development based on sequential biopsy in response to pST in pigs

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Finishing Landrace pigs were used to study the effects of long-term application of porcine somatotropin (pST) on the development of muscle structure characteristics. The animals were treated daily by im injection with 2 mg, 4 mg pST (Pitman-Moore, Inc.) or a placebo from about 120–200 days of age. Transverse sections were reacted for NADH-tetrazolium-reductase and acid-stable ATPase or chromalum-carmin-eosin.

pST treatment caused a considerable repartitioning of feed energy from fat to meat. It stimulated the hypertrophy of skeletal muscle fibres resulting in 6–11% larger fibre diameters at the end of treatment without dose-related differences. The response was greatest in barrows and least in boars. Dose-dependent differences occurred in the course of fibre growth. In barrows and gilts muscle fibres responded somewhat later to 2 mg pST compared with 4 mg pST. The total muscle fibre number was not influenced. Also the nucleus/plasma ratio declining with age remained unchanged suggesting that pST stimulated satellite cell proliferation and protein accretion of the multinucleated fibres to the same extent.

In general, fibre type frequencies as slow twitch oxidative (STO), fast twitch oxidative (FTO) and fast twitch glycolytic fibres (FTG) were not affected by pST suggesting a lack of shifting in energy producing metabolism or contractile properties. Accordingly no significant deterioration in meat quality traits of carcass longissimus was evident. Percentages of abnormal fibres as small dark angulated ones were not enhanced by pST, whereas light violet ones were slightly increased in barrows only. Giant fibres counted in barrows carcass muscle samples remained unchanged by pST.

Effect of temperature change on Donnan potentials in rabbit psoas muscle

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In the past 10 years it has been demonstrated that the Donnan potentials in the A- and I-bands of glycerinated rabbit psoas muscle can be measured using microelectrodes, and hence the net fixed electrical charge on the contractile proteins can be calculated (Naylor *et al.* (1985) *Biophys. J.* **48**, 47–59). It has also been shown (Bartels and Elliott (1985) *Biophys. J.* **48**, 61–76) that there is a dramatic difference in the A-band potentials between the relaxed and rigor states. In rigor the A-band charge is 40% higher than that in the I-band; in the relaxed state, however, the charges are about equal. The large increase of the A-band charge in the rigor state cannot be from the binding of one ATP molecule per active site on the myosin head alone, and is therefore clear that there is a charge amplification effect taking place. It has been postulated (Elliott (1980) *Biophys. J.* **32**, 95–7) that the increase in the net fixed charge may be due to the immobilization of anions, which bind to the electrically-effective surface of the myosin filament. A possible mechanism which may facilitate charge amplification is ion binding (or ion association) which may occur at 'Saroff sites', by weak hydrogen bonds onto networks of charged side chains (Loeb and Saroff (1964) *Biochem.* **3**, 1819–26).

It has been reported that temperature has a profound effect on the visibility of the myosin layer lines in relaxed rabbit muscle (Wray (1987) *J. Muscle Res. Cell Motility* **8**, 62). To gain insight into this phenomena the A- and I-band potentials were measured as a function of temperature. Our hypothesis is that anions bound to the electrically-effective surface of the myosin filaments are governed by Boltzmann's theorem, and will be released at higher temperatures, and that this charge release increases the order of the filament lattice.

Experimentally we found that both the A- and I-band potentials fall off sharply as the temperature is increased, in rigor and in relaxed muscle. As the temperature reduction of the A-band potential in relaxed muscle appears to coincide with the appearance of myosin layer lines, it seems likely that the two observations are connected, and that the layer lines appear when the charge falls, because the excess charge acts as a disordering agent in rabbit muscle.

Histochemical and contractile properties of the oesophageal muscle fibres in a myotonic mouse mutant

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The investigation of the physiology and histochemistry of skeletal muscles of a hereditary neuromuscular syndrome of the mouse ('arrested development of the righting response', ADR) has shown that they are characterized by after-contractions and a retarded relaxation after tetanic stimulation, and by a predominance of oxidative and a lack of glycolytic fibres (Reininghaus *et al.* (1988) *Muscle Nerve* **11**, 433–9). The oesophagus of the mouse contains two layers of singly innervated cross-striated muscle fibres, physiologically characterized by a slow contraction time, a low fusion frequency, and a high twitch-tetanus ratio; but they exhibit also some properties of a fast muscle (e.g. post-tetanic and cold potentiations), and they are also fast by histochemical fibre type characterization. We have studied the contractile and histochemical properties of oesophageal muscle of the ADR phenotype and compared them with those of the wildtype (WT). The ADR oesophageal muscle fibres do not exhibit any indications of myotonia. The twitch and tetanus parameters are virtually the same as those of the WT. Especially there are no signs of after-contractions or a prolonged relaxation after repetitive stimulation, as observed in skeletal muscles of the same ADR animal. There is also no shift in the oxidative capacity of the ADR oesophageal muscle. The reasons for the protection of the oesophageal muscle against myotonia remains unexplained.

Regulation of neuromuscular interaction by thiazolium derivatives

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Thiamine (vitamin B_1) is a factor which is necessary for the mechanism of neuromuscular transmission. Thiamine receptors are localized on presynaptic membranes. Thiamine interaction with presynaptic receptors provokes a facilitation of neurotransmitter release from nerve endings. There are two heterocyclic rings in the structure of thiamine: thiazolium and pyrimidinium. The actions of synthetic thiamine derivatives with a modified pyrimidine ring were investigated in isolated frog n. ischiadicus–m. sartorius preparations. Thiamine derivatives were used with such structures as: 3-R-4-methylthiazolium chloride (R-C_6H_5: substance BMT, R-$C_{11}O_2H_{21}$: substance DMT) and 3-R-4-methyl-5-β-hydroxyethylthiazolium chloride (R-C_6H_5: substance BMHT, R-$C_{11}O_2H_{21}$: substance DMHT, R-$C_{13}O_2H_{23}$: substance DDMHT). DMT, DMHT, DDMHT in the concentration 1×10^{-6}–1×10^{-4} mol l^{-1} and BMT, BMHT in the concentration 1×10^{-3} mol l^{-1} decreased the amplitude and quantal content of endplate potentials, the frequency of miniature endplate potentials, the blocked spontaneous and nerve stimulation evoked quantal neurotransmitter release from nerve endings, but did not influence the muscle fibre's resting potential. DMHT also blocked α-latrotoxin evoked quantal neurotransmitter release from nerve endings. DMHT (7 mg kg^{-1}) or DDMHT (5 mg kg^{-1}) intraperitoneal injection led to rats horizontal and vertical activities, 'burrow' reflex and 'gruming' depression in 'open field' experiments and to depression of locomotor activity in mice in actographic experiments. Probably the derivatives used interact with thiamine-sensitive presynaptic receptors owing to the thiazolium ring and thus block endogeneous thiamine binding to these receptors and thiamine action in the neuromuscular junction.

Irradiation prevents compensatory hypertrophy of, but not fibre type changes in, overloaded rat extensor digitorum longus muscle

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Appropriate experimental manipulation of active and/or passive tension of a fast-twitch skeletal muscle stimulates proliferation of resident satellite cells. Their progeny fuse with contiguous muscle fibres, which subsequently hypertrophy and alter their myosin heavy chain gene expression. In the work reported herein we tested whether compensatory adaptation of chronically overloaded fast-twitch skeletal muscle can occur in the absence of satellite cell activity.

Procedures were carried out on anaesthetized, young adult, male Wistar rats. Chronic overload of the right extensor digitorum longus (EDL) muscle was produced by removal of the synergist tibialis anterior muscle. Reproductive sterilization of proliferative (satellite) cells was achieved by prior exposure of the right hindlimb to 2500 rad irradiation. Control animals received one of the two treatments (ablation or irradiation) only or no treatment. Four weeks after ablation the mass of the experimental and contralateral control EDL muscles, and the numerical frequency and cross-sectional area of their type I, IIa, IIx and IIb fibres in immunohistochemically-stained serial transverse sections was measured.

Ablation of the tibialis anterior gave rise to EDL muscle hypertrophy, characterized by a 20% increase in both muscle mass and fibre cross-sectional area. Prior irradiation of overloaded EDI muscle not only prevented compensatory hypertrophy, but also impeded normal EDL muscle growth. Overloaded EDL muscle, with or without prior irradiation, contained 30% fewer type IIb fibres and a corresponding larger number of type IIa and IIx fibres than the control; a significant percentage of these fibres coexpressed either type IIa and IIx myosin heavy chain or type IIx and IIb myosin heavy chain. Irradiation alone of EDL muscle did not affect the normal fibre type numerical frequency.

The results of the present work indicate that satellite cell mitotic activity is required for only certain muscle adaptations to chronic overload: adaptations in muscle size are dependent on satellite cell activity; adaptations in myosin heavy chain gene expression are independent of satellite cell activity.

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Defects of the human mitochondrial genome

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An increasing number of human neuromuscular diseases are associated with biochemical defects of the mitochondrial oxidative phosphorylation system. Some of these diseases are related to deletions or point mutations of the mitochondrial genome. Muscle biopsies from patients with Kearns–Sayre syndrome or chronic progressive external ophthalmoplegia show more than 50% deletions of the mitochondrial genome (e.g. Moraes *et al.* (1989) *N. Engl. J. Med.* **320**, 1293–9). We present data showing that in some of the other patients point mutations lead to the mitochondriopathy.

Second, we analysed by PCR amplification and sequenced muscle tissue from a patient with a MERRF syndrome (myoclonic epilepsy with ragged red fibres) and her mother and sister where the classical point mutation ($tRNA^{lys}$) was not present. In addition, we demonstrated a new family with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) where we could confirm the previously reported point mutation of the $tRNA^{leu}$ at position 3243 (Goto *et al.* (1990) *Nature* **348**, 651–3). Mitochondrial protein synthesis in cultured fibroblasts from patients with MERRF syndrome revealed diminished incorporation of ^{35}S-methionine into lysine containing peptides, suggesting that the $tRNA^{lys}$ becomes the rate limiting component of the mitochondrial translational apparatus.

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Effect of hyperosmolarity on the mechanical properties of rat slow- and fast-twitch muscles

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The effect of hyperosmolarity on the resting tension and on the directly evoked twitch and tetanic tension development has been investigated in isolated slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscles of the rat. Physiological saline was made hypertonic by the addition of the nonpermeant solutes sucrose, mannitol or urea (200–800 mosmol). Hyperosmolarity induced, in a dose-dependent manner, transient monophasic contractures in both muscle types, but the SOL was somewhat more sensitive. The resting tension rose to a peak within 1–3 min and declined slowly afterwards and could be maintained up to 60 min. Using urea, occasionally, a second phase of tension rise was observed starting after about 20 min with a maximum after 35 min. Sucrose or mannitol depressed the tension development of twitches and fused tetanic contractions of both types of muscles also dose-dependently, but the decrease was more pronounced in the EDL. Urea is less effective in depressing the contractile responses. The time parameters of the twitch (contraction and half-relaxation times) and the tetanus rise and fall were prolonged. The reversibility of the changes is only complete after an exposure of the muscles with lower concentrations of the nonpermeant solutes (up to 400 mosmol). As for reasons for the changes of the mechanical properties, an osmotically-induced release

of calcium ions from the sarcoplasmatic reticulum and a shrinkage of the muscles by water loss are discussed.

Contraction modes of the gizzard muscle and body wall muscles in the earthworm *Lumbricus*

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Different myosin isoforms and actomyosin ATPases in the earthworm muscles have been described in the past (D'Haese and Carlhoff (1987) *J. Comp. Physiol. B* **157**, 171–9, 589–97). Here we describe some experiments on the mechanical properties of these muscles. In addition the ultrastructure was investigated at contracted and at very stretched state.

About 2 × 10 mm long strips of the cordless body wall cut either along the longitudinal muscle axis or along the circular muscle and in addition 2 × 10 mm long stripes of the gizzard muscle were electrically stimulated. The isometric active forces and the passive tensions were determined at various lengths of the muscle stripes and the calculated length–tension relationships of the three different muscles were compared. In all of them a clear optimal length exists for force development. Nevertheless, the longitudinal muscle was able to exhibit active force even at a very stretched state.

The forces of the isometric twitch produced by the circular muscle were about 50% larger than that developed by the longitudinal muscle; the force exhibited by the gizzard muscle was considerably smaller. In comparison, when the gizzard muscle was stimulated tetanically, it developed the largest force. After a single stimulus (5 ms) the circular muscle reached 90% of its maximal contraction strength within 700 ms, the longitudinal muscle within 1800 ms and the gizzard within 1600 ms. After reaching the maximum of its contraction the muscles showed a distinctly different way of relaxation. The circular muscle relaxed fastest, the active force declined to 50% of its maximum value within 3.2 s, in the gizzard muscle within 4.4 s and in the longitudinal muscle within 8.5 s. The contraction of the longitudinal muscle is composed of at least a fast and a slow component for force development: a phasic increase of tension reaching its climax after 120 ms was followed by a temporary rapid relaxation which again was superimposed by the slower contraction component mentioned before. The contractions of circular and gizzard muscles may also consist of more than one component. The highest contraction velocity is found in the longitudinal muscle. High amount of mitochondria and a greater diameter of the thick filaments are probably in line with a comparably lower ATPase activity and a slower time course of contraction.

Hymenochirus: a new donor of suitable muscle preparations?

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Although most of our knowledge on the subject of muscle has been derived from the study of suitable whole muscles, fibre bundles and single fibres of the frog, the strict interpretation and observance of the laws concerning wildlife conservation now existing in the European countries prohibits the catching of and dealing with indigenous amphibians. The experimental procedures in isolated skeletal muscle preparations of the dwarf claw-frog (*Hymenochirus* species; body weight of the adult approximately 0.5 g) to be described were undertaken with the thought that a reasonable compromise can be made between the essentials for a fruitful work in the range of muscle physiology and the legitimate demands of the conservationists. The breeding, keeping and feeding (*Tubifex, Daphnia, Enchytraeus*) of these mini-frogs in a rich-planted aquarium seems to be without difficulties. On the strength of four basic representative muscle experiments using the miniaturized suspension method as well as the laser-light diffractometric method, evidence is presented in support of the suitability of some isolated skeletal muscle preparations of *Hymenochirus* in research and practical training courses ((1) single contraction, incomplete and complete tetanus; (2) block of the neuromuscular transmission; (3) fatigue; and (4) potentiation of the contraction during caffeine contracture).

Effect of anions on the contractile properties of the fast- and slow-twitch muscles of the rat

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The study is to clarify the effect of anions such as nitrate (NO_3^-), bromide (Br^-), and iodide (I^-) on the mechanical properties of rat fast-twitch (extensor digitorum longus, EDL) and slow-twitch (soleus, SOL) muscles. The contractile parameters of isolated single muscles of 3-week-old rats were investigated by replacing chloride ions (Cl^-) of the Tyrode solution by the corresponding anions (either with NO_3^-, Br^- or I^-). The muscles were investigated at 20°C and 35°C bath temperature. Twitch peak tension, contraction and half-relaxation times of control and test twitches were analysed.

The anions increased the twitch tension output of the isometric contractions of both fast- and slow-twitch muscles. The extent of twitch amplitude potentiation corresponds to the lyotropic order of the anions; $Cl^- < Br^- < NO_3^- < I^-$, in which I^- displayed the maximum level of twitch tension augmentation. Varying the temperature of the bath showed no variation in order of potentiation of the anions or percentage of twitch amplitude enhancement between the two muscles except the SOL muscle in I^- bath at 20°C which appeared to have exceptionally higher twitch amplitude. Besides, NO_3^- and I^- prolonged the contraction and half-relaxation time of both muscles while Br^- did not.

Changes in the quadriceps muscle of paraplegic patients after electrical stimulation: histological and histochemical examination of biopsy specimens before and after therapy

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Recent advances in Functional Electrical Stimulation have enabled paraplegic patients to stand and make simple movements, an achievement which greatly increases their quality of life. A limitation to this technique is that the muscles of these subjects fatigue rapidly. Experiments with implanted electrodes in animals have shown that certain patterns of stimulation can alter muscle fibre types producing a more fatigue-resistant muscle. Rutherford and Jones (*J. Neurol. Sci.* **85** (1988) 319–31) have shown that stimulation of the adductor pollicis in humans changes the fatiguability of the muscle. We have used a 3 month programme of percutaneous stimulation of the quadriceps in seven patients who had been paraplegic for varying lengths of time ranging from 11 months to 9 years. Muscle biopsies taken before and after treatment were examined to assess changes in fatigue resistance, as evidenced by changes in myosin expression. Prestimulation specimens were examined and changes in muscle fibre structure and biochemistry which had occurred as a result of the spinal cord injury were documented. Using these findings as a base line we examined poststimulation biopsies for changes in fibre areas and fibre type percentages using ATPase staining. Staining with monoclonal antibodies to fast and slow myosin and neonatal myosin were also examined. In the initial biopsy, the proportion of slow fibres decreased with the length of time the patient had been paralysed. Subjects still showed up to 40% type 1 fibres at 15 months after injury and proportions were not changed after stimulation. One subject, para-

plegic for 9 years, showed a complete absence of type 1 fibres and no restoration after stimulation. Of the 4 subjects paralysed for between 3 and 6 years three showed an increase in fibres expressing slow myosin as a result of stimulation. All but one subject showed an increase in mean fibre areas after stimulation.

EXCITATION–CONTRACTION COUPLING AND CALCIUM ACTIVATION

Dual action of diltiazem in the development of paralysis and restoration

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The Ca^{2+}-antagonist diltiazem interacts with the voltage-sensor/Ca^{2+}-channel molecule ('DHP-receptor') in the T-tubule membrane of skeletal muscle fibres. Measuring Ca^{2+}-current and force development in short toe muscle fibres of the frog it was shown that transition to a secondary slow inactivated state (I_0, the paralysed state) of the DHP-receptor molecule is accelerated by diltiazem (Böhle (1992) *J. Physiol.* **445**, 303–18). As the effect of diltiazem occurs without a shift of the steady-state restoration curve, it is suggested that the same I_0 state is also achieved under control conditions, albeit with a slower time course. It is concluded that the activation energy barrier of the transition from the fast inactivated state (I) to I_0 is lowered by diltiazem. In further experiments the backward reaction from I_0 to I was investigated. Surprisingly this reaction was slowed down by diltiazem. As an explanation it is assumed that there exists a different pathway for the backward reaction and that the activation energy barrier of the transition from I_0 to I is higher in the presence of diltiazem.

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Precise determination of cytosolic calcium concentration in skeletal muscle cells from normal and dystrophic (MDX) mice

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Fura-2-AM probe has been widely used to determine cytosolic calcium concentration ($[Ca^{2+}]_i$) because it combines high sensitivity and easy diffusion inside cells. Fura-2 responds to calcium by shifting the excitation peak of fluorescence; the ratio (R) of fluorescence signals excited at two different wavelengths is independent of dye content, cell thickness and setup sensitivity. A precise calibration is required to calculate $[Ca^{2+}]_i$ according to Grynkiewicz *et al.*'s (*J. Biol. Chem.* (1985) **260**, 3440–50) equation: $[Ca^{2+}]_i = Kd \cdot \beta \cdot (R - Rmin)/(Rmax - R)$.

We investigate each calibration parameter *in situ* to accurately compare the absolute resting $[Ca^{2+}]_i$ of isolated skeletal fibres from normal (C57) and dystrophic (mdx) mice. Each cell was scanned with excitation wavelengths ranging from 300 to 425 nm. From this measurement, 340/360 and 340/380 ratios were calculated. Rmin and Rmax were measured in α-toxin permeabilized preparations, in absence and in excess of calcium, respectively. Apparent Kd, i.e. $Kd \cdot \beta$, was determined by incubating the cell in different calcium buffered solutions. The Kd value was found to be the same in the two strains. However, we unexpectedly observed that the excitation spectrum of the probe was different in normal and in dystrophin-lacking fibres, resulting in significantly different β, Rmin and Rmax values. This finding strongly challenges previous studies relying on cuvette calibration or assuming an identical behaviour of the dye within normal and dystrophic fibres (Mongini *et al.* (1988) *Neurology* **38**, 476–80; Turner *et al.* (1988) *Nature* **335**, 735–8). Indeed, using these *in situ* calibration parameters, we show that, in spite of a higher fluorescence ratio detected in mdx, as previously reported, $[Ca^{2+}]_i$, at rest, is not different in control and dystrophic muscles.

Mechanical and ATPase studies of calcium effects on turnover kinetics of crossbridges in chemically skinned soleus fibres from the rabbit

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We studied turnover kinetics of crossbridges in detergent-treated rabbit soleus fibres (slow type I fibres) using the protocol given by Brenner (*PNAS* **85**, 3265–9) for fast psoas fibres: fibres were subjected to isotonic contractions and restretched to initial length. Then the fibre was held at constant length and redevelopment of force back to the isometeric steady state was observed. The complex time course of force redevelopment showed a predominant phase which could be fitted by a single exponential from which the rate constant, k_{redev}, was calculated, which is assumed to represent the sum of all turnover rate constants. The rate constant of force redevelopment (k_{redev}) of the slow soleus fibres is similar calcium-dependent, but by about an order of magnitude smaller than that of fast psoas fibres (Hutzler *et al.*, *Pflügers Arch.* **420**, R92). We suggested that crossbridge turnover kinetics are Ca^{2+}-sensitive. This is inconsistent with the concept of regulation by crossbridge recruitment.

Increasing calcium in the solution increased k_{redev}. At high activation, calcium influences mostly the apparent rate constant f_{app} that describes the transition from weak- to strong-binding crossbridge states. At low activation levels ($< 50\%$ F_{max}) other parameters together with f_{app} must change with Ca^{2+} in the slow fibres, for instance the rate constant for the transition from strong- to weak-binding states g_{app} or the mean force per crossbridge F_0.

To test these possibilities isometric ATPase in fibres were measured at different $[Ca^{2+}]$ as ATPase/force is proportional to g_{app}/F_0. Throughout activation, isometeric ATPase was continuously measured by an enzyme-NADH coupled system at 340 nm in an UV spectrophotometer. The fibres were incubated in solutions containing (in mM): EGTA 1, MgATP 5, PEP 1, NADH 0.1, LDH 10 U/ml, PK 10 U/ml; pH 7.0; 10°C; $\mu = 0.17$ M; *p*Ca ranged from 8 to 4.5, measured by a calcium sensitive electrode (ETH129). The apparent rate constant f_{app} increased from 0.03 s^{-1} (*p*Ca 6.2) to about 0.6 s^{-1} (*p*Ca 4.5). The other rate constants g_{app} and f_{app}^- were not detectably changed. At low activation levels ($< 50\%$ F_{max}) the mean force per crossbridge F_0 assumed to be decreased by about 50%. The number of active cycling crossbridges did not significantly vary during activation.

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Intracellular effects of calcium antagonists on the contractile system in skinned heart tissue

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Small preparations of heart muscle were obtained from rat hearts. The apex was cut off and the right ventricle was cut open from apex along the septum to the atrium. The wall of the ventricle was folded open and fixed on a piece of cork. This preparation was skinned by means of freeze drying. Small trabeculae and small strips of skinned heart muscle could be obtained, and activated by means of Ca^{2+} containing solutions. The free $[Ca^{2+}]$ was calculated according to Fabiato and Fabiato (*J. Physiol. Paris* **75** (1979) 463). Sarcomere length was determined and set by means of laser-diffraction. Verapamil or diltiazem were added to all the solutions, yielding a concentration of 10^{-6} M. Isometric developed tension was measured with a force transducer (Scientific instruments KG3-BAM3). After measuring a

control calcium sensitivity curve (the relationship between the pCa and the developed tension), one of the two calcium antagonists was measured after which the preparation was stretched towards longer sarcomere length and the protocol was repeated. Three parameters of these calcium sensitivity curves were used to analyse the obtained data; the maximal developed force (T_{max}), the pCa value at which half-maximal tension has been reached (pCa$_{50\%}$) and the slope of the curve (n_{Hill}) at this pCa$_{50\%}$ point. It proved that in the presence of therapeutic plasma concentrations of calcium antagonists, T_{max} increased by 10–70% (diltiazem) and by 5–25% (verapamil), possibly via a change in Mg-ATPase activity. From the Hill-plot, which plots the logarithm of T_{rel}/T_{max}-T_{rel} as a function of pCa, it can be derived that the sensitivity of troponin-C (Tn-C) to calcium in presence of diltiazem has increased by about 0.2 pCa units, meaning the same amount of force is produced at only half the $[Ca^{2+}]$ of the control solution. However, the Hill-plots do not reveal information whether this is due to an interaction of the calcium antagonists with the calcium-specific site or with the high affinity sites of Tn-C. The n_{Hill} has increased slightly implying an increase in cooperativity of calcium binding sites on Tn-C and troponin–tropomyosin complexes. From this we conclude that intracellularly calcium antagonists merely work as agonists.

Evidence by fura-2 imaging for a skeletal-type excitation–calcium transient coupling in limited areas of myotubes from mutant mice with 'muscular dysgenesis'

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The muscular dysgenesis (mdg/mdg) in mouse is a recessive autosomal mutation whose most striking feature is the failure of skeletal muscle excitation–contraction (EC) coupling. The absence of the α_1-subunit of the dihydropyridine (DHP)-receptor which is correlated with the alteration of the gene encoding the DHP-receptor and the lack of I_{slow}, is considered as the cause of EC coupling dysfunction in mutant cells. This statement is further supported by restoration strategies which allow expression of the α_1-polypeptide and recovery of both EC coupling and I_{slow}. The lack of functional EC couplings is considered to postulate implicitly the nonexistence of spontaneous or evoked transients of intracellular free calcium concentration ($[Ca^{2+}]_i$) (see for ref. Adams and Beam (1990) *FASEB J.* **4**, 2809–16; Powell (1990) *FASEB J.* **4**, 2798–808). Recently, it was shown by fura-2 imaging technique, that at an early stage of skeletal muscle cells ontogenesis, 3–5-day-old rat myotubes developed in culture may display spontaneous or electrically-induced transients of $[Ca^{2+}]_i$ which are completely disconnected from any detectable contraction. The amplitude, time course and spatial distribution of these $[Ca^{2+}]_i$ transients are independent of external $[Ca^{2+}]_i$, satisfying the criteria for a skeletal-type EC coupling (Grouselle *et al.* (1991) *Pflügers Arch.* **418**, 40–50). The $[Ca^{2+}]_i$ of myotubes in culture obtained from normal and mdg/mdg new born mice was investigated by fura-2 imaging technique. At rest, dysgenic myotubes maintained a mean $[Ca^{2+}]_i$ of 97 ± 8 nM which was moderately more elevated in comparison with normal muscle cells (79 ± 5 nM). But the spatial distribution of $[Ca^{2+}]_i$ within normal myotubes appeared uniform whereas mdg/mdg myotubes display obvious heterogeneity in $[Ca^{2+}]_i$ distribution with distinct localized areas of higher than mean ($[Ca^{2+}]_i$). Surprisingly, electrical stimulation induced, in more than 80% of observed dysgenic myotubes, skeletal-type $[Ca^{2+}]_i$ transients without contractions within small domains located almost exclusively at the extremities of the mutant cells. The time course of the $[Ca^{2+}]_i$ increase and reuptake was three to six times slower compared with normal muscle cells. These results provide strong evidence that: (1) Ca^{2+} homeostasis is markedly impaired in dysgenic myotubes, and (2) a membrane-depolarization Ca^{2+}-release coupling is present in localized areas of mdg/mdg myotubes indicating that a voltage-sensor is functional but whose molecular support is unknown.

Calcium activation and cooperativity in force development in skinned smooth muscle of rat aorta

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Rat aorta tissue was skinned by means of freeze-drying. From this tissue transversal segments of 3–4 mm were prepared and glued between a strain gauge force transducer (AME 801 Sensonor) and a fixed steel rod. Adventitia and intima were removed from the preparation. The preparation was activated with calcium containing solutions at constant lengths. The free calcium concentration was calculated according to Fabiato and Fabiato (*J. Physiol. Paris* **75** (1979) 463). The calcium sensitivity curve, the relation between the negative logarithm of the free calcium concentration (pCa) and the developed tension, was measured as a function of preparation length. The length–tension relation shows an optimum. From the experiments, the pCa50% (the pCa value at which 50% of the maximal developed tension has been reached) and the Hill coefficient, n (the slope of the sensitivity curve at the pCa50%) seem to be independent of the length of the preparation. From the measured data the Hill plots, the relation between the logarithm of $(F_{rel}/1\text{-}F_{rel})$ in which F_{rel} is the normalized tension and pCa, were constructed. A straight line in this plot indicates that the cooperativity of the calcium binding sites to calmodulin is independent to the free calcium concentration. However, the slope of the plot decreases from a mean value of 3.7 at low calcium concentrations (high pCa-values) to a mean of 2.6 at higher calcium concentrations (lower pCa values), indicating that the cooperativity of the binding process of Ca^{2+} to the contractile machinery at low pCa values is much less than at high pCa values. This change in cooperativity can be explained by the observation of two kinds of calcium binding sites on calmodulin (Ovaska and Taskinen (1991) *Proteins: Structure, Function and Genetics* **11**, 79–94). Comparison of normal rats with preliminary results of hypertensive rats shows that the pCa50% in SHR increases, meaning a higher sensitivity to calcium.

Model of Na^+ and Ca^{2+} coordinate involvement in skeletal muscle activation

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The study of excitation–contraction (EC) coupling in skeletal muscle in the past few years was focused on the molecular machinery and mechanisms involved in T-SR transmission. Nevertheless, the evidence of the most favoured hypotheses are not yet conclusive (Rios *et al.* (1991) *J. Muscle Res. Cell Motil.* **12**, 127–35), especially for the fast-twitch skeletal muscle fibres of vertebrates. Based on the results of recent studies we have suggested the model of coordinate involvement of Na^+ and Ca^{2+} ions in EC coupling. During a single spike excitation some amount of Na^+ ions enter through the open Na^+ channels of T-tubulus membrane (TM) into T-SR junctional space. The cations activate a Na^+-Ca^{2+} exchange (reversed mode) and as positive charges cause the voltage gated Ca^{2+} release channels in SR junctional feet to open (I open state), primarily triggering a release of Ca^{2+} from SR. In turn these Ca^{2+} ions and entering via Na^+-Ca^{2+} exchanger from outside as a secondary trigger interact with Ca^+ receptors of SR Ca^{2+} channels and induce the main single Ca^{2+} release (II open state of Ca^{2+} channels) from the SR into myofilament space. During rapid repetitive stimulation (tetanus) twitches are fused and the arising depolarization of TM opens its L-type Ca^{2+} channels for extracellular Ca^{2+} ions. The latter ones induce a prolonged main Ca^{2+}

release from the SR. During high K^+ contracture, voltage sensors of L-type Ca^{2+} channels are affected by a constant membrane depolarization. In this case the first rapid contraction is followed by a main long-lasting Ca^{2+}-induced Ca^{2+} release (with positive feedback, as during smooth tetanus) from the SR. Recent studies have revealed arguments in favour of Na^+ and Ca^{2+} coordinate involvement in EC coupling in skeletal muscles of vertebrates.

Role of intracellular calcium in normal and Duchenne myotubes: rise of intracellular calcium levels by osmotic shock, high extracellular calcium ions and acetylcholine

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Increase of the intracellular free Ca^{2+} plays a key role in the abnormal condition of dystrophic muscle fibres and probably leads to cell death and dystrophy in later stages (Martonosi *et al.* (1989) *Biochim. Biophys. Acta* **991**, 155–242). In Duchenne muscular dystrophy (DMD) and in the X-linked muscular dystrophy (MDX) of the mouse, the lack of the submembranous cytoskeletal protein dystrophin leads to fibre necrosis and dystrophy. Myotubes from dystrophic muscle are less stable in hypo-osmotic solutions than control myotubes (Menke *et al.* (1992) *Eur. J. Cell Biol. (Suppl.)* **36**, 52), indicating a mechanical function of dystrophin. We have studied the resting levels and the increases of intracellular free Ca^{2+} in single myoblasts and myotubes from DMD and control persons using the fluorescent Ca^{2+} indicator fura-2. The resting levels of Ca^{2+} in DMD and control myotubes were not significantly different and ranged between 108 and 124 nM ($n = 60$ cells each from two DMD and two control persons). After the application of a hypo-osmotic solution of 140 mosm, the intracellular free Ca^{2+} level increased within 2–3 min by a factor of 1.5–2 in both DMD and control myotubes. In most of the cells, the response was transient and the Ca^{2+} concentration decreased within 10 min towards the initially measured resting level. Faster and smaller transient responses were seen after application of external solutions containing 15 mM Ca^{2+} or 300 µM ACh ($n = 60$, two patients in each case). Our experiments do not confirm former results of increased intracellular Ca^{2+} levels in DMD myotubes. The homogeneous reaction of DMD and control myotubes to the modified external conditions applied here may be explained by insufficient maturation of the cultures. The cells responded to ACh and 15 mM Ca^{2+} in the expected way. The slow rise in intracellular Ca^{2+} induced by the hypo-osmotic solution probably reflects an influx of Ca^{2+} ions through the surface membrane caused by mechanical stress or a cellular reaction in connection with their volume regulation. Obviously, the cells react with a Ca^{2+} sequestering mechanism within a few minutes. The readjustment of the intracellular free Ca^{2+} concentration cannot be explained by Ca^{2+}/Na^+ exchange, as the extracellular Na^+ concentration was 10 mM for these osmotic shock experiments and the osmolarity was adjusted by sucrose.

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RAS Proteins modulate Ca^{2+}-sensitivity of smooth muscle contraction

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G-proteins may be involved in receptor-mediated Ca^{2+} sensitization of smooth muscle contraction (Kitazawa *et al.* (1991) *J. Biol. Chem.* **266**, 1708–15), but the G-proteins responsible are not known. We investigated the effects of two small molecular weight G-proteins, wildtype (w) Ha-*ras* (p21[Gly 12]) and its oncogenic mutant (m) (p21[Val 12]), on submaximal contraction (0.5 µM Ca^{2+} and 1 µM calmodulin) of β-escin skinned mesenteric microarteries from guinea pigs. Addition of GTP (0.01–10 µM) increased submaximal tension development dose-dependently. In the presence of either w-$p21^{ras}$ or m-$p21^{ras}$ (3 µM), this dose-response relationship was shifted upward ($p < 0.05$). Tyrphostin (50 µM), a synthetic inhibitor of tyrosine kinases, partially inhibited force induced by 3 µM w-$p21^{ras}$ with 10 µM GTP ($p21^{ras}$ + GTP: 215 ± 11% of Ca^{2+}-induced force, with tyrphostin: 151 ± 7%). Tyrphostin had no effect on Ca^{2+}-induced force. In microarteries which had been presensitized to Ca^{2+} by activation of endogenous G-proteins with the nonhydrolyzable GTP analogue, Gpp(NH)p (0.3 µM), addition of w-$p21^{ras}$ (3 µM preactivated with 0.3 µM Gpp(NH)p) induced a transient partial relaxation (tension remaining 20 min after addition of w-$p21^{ras}$: 75 ± 4%) which was followed by a slow tension recovery (110 ± 9% at 3 h), whereas the presensitized force by Gpp(NH)p alone declined slowly (72 ± 17% at 3 h). In a second series of experiments, submaximally activated microarteries were first incubated with Gpp(NH)p (0.3 µM) and then challenged with increasing concentrations of GTP (0.01–10 µM) with and without $p21^{ras}$ (3 µM). GTP alone had little effect under this condition. In the presence of $p21^{ras}$ (both wildtype and mutant) and low concentrations of GTP (0.01–1 µM), force was partially inhibited. The inhibition of force was significantly larger in the presence of m-$p21^{ras}$ than w-$p21^{ras}$ (w-$p21^{ras}$ + 0.1 µM GTP: 8 ± 4%; m-$p21^{ras}$ + 0.1 µM GTP: 27 ± 6%). At 10 µM GTP, there was no difference in tension with or without the $p21^{ras}$ proteins. Nucleotide-free $p21^{ras}$ affected neither Ca^{2+}-induced force nor presensitized force by Gpp(NH)p. Thus, the activated $p21^{ras}$ seems to modulate Ca^{2+}-sensitivity of smooth muscle contraction in both directions, sensitization and desensitization, depending on cell conditions (possibly the state of intrinsic G-proteins), and the sensitization effect may be partially related to tyrosine kinase pathways.

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Intracellular effects of calcium antagonists and Mg-ATPase blockers on the contractile system in skinned skeletal muscle fibres

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Single fibre preparations of thermally-skinned gracilis muscle from the rabbit were activated by means of Ca^{2+}-containing solutions. The free [Ca^{2+}] was calculated according to Fabiato and Fabiato (*J. Physiol. Paris* **75** (1979) 463). Sarcomere length was set and controlled by means of laser-diffraction during the whole protocol. Isometric tension was measured with a force transducer (AME Sensonor 801). After measuring a control series, a drug was added to all test solutions. We used a calcium antagonist (1 µM diltiazem; 1 µM verapamil) or a Mg-ATPase blocker (25 µM metavanadate (V_i); 3 mM BDM). The fibre was then stretched to a new sarcomere length and the protocol was repeated. Calcium sensitivity curves (the relation between *p*Ca and the developed tension) were constructed for each length. Three parameters of these curves were used to analyse the data obtained: the maximal developed tension (T_{max}), the *p*Ca-value at which 50% of T_{max} is reached ($pCa_{50\%}$) and the slope of the sensitivity curve at $pCa_{50\%}$ (n_{hill}). Looking at the first parameter, it is found that BDM and V_i both reduce T_{max} by 20–30% at the stated concentrations. This phenomenon is independent of sarcomere length. Diltiazem and verapamil both increase T_{max} by 8–20 and 25–35%, respectively. The enhancement using diltiazem is dependent on sarcomere length. This is not found with verapamil. The other two parameters can be better observed in the Hill-plot. This is the relation between the logarithm of ($T_{rel}/(1-T_{rel})$)

and pCa; T_{rel} being the relative developed tension. $p Ca_{50\%}$ is decreased by BDM (0.12 units) and increased by diltiazem (0.06 units), both independent of sarcomere length. Verapamil increases $p Ca_{50\%}$ only at sarcomere lengths below 2.8 μm. V_i does not change $p Ca_{50\%}$ at all. The last parameter, n_{hill}, is not affected by diltiazem, verapamil and BDM, implying a parallel shift of the Hill-plot. V_i, however, enhances n_{hill} by at least 25%. In conclusion, all drugs change T_{max}. Remarkably, calcium antagonists act as agonists inside the cell, whereas ATPase blockers lower T_{max}, probably by stabilizing a product of the ATPase cycle. In addition, diltiazem, verapamil (SL < 2.8 μm) and BDM all change the responsiveness of the myofilaments to Ca^{2+}. Finally, changes in the shape of the Hill-plot suggest that both verapamil and V_i mainly interact with the high affinity sites of troponin C. Verapamil reduces the cooperativity between the high affinity calcium binding sites. V_i increases the cooperativity, mainly at high [Ca^{2+}], suggesting that the drug also has an affinity for Mg^{2+}.

Measurement of free calcium using an ion selective electrode after flash photolysis of caged calcium with a low cost flash unit

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Photolysis of caged calcium (Nitr5) can be used to study excitation–contraction coupling and muscular mechanics. Expensive high energy light sources are routinely used for excitation. As an alternative, the performance of an ultra low cost Xenon flash unit was investigated. A 300 J short arc Xenon flash lamp (Heimann®) was mounted in an ellipsoid reflector and driven by a modified Metz®60 CT 4 photo flash unit up to 240 J input energy and 4 ms flash duration. A 20 μl cuvette containing the test solution was placed in a complementary ellipsoid reflector. The glass for this reflector was blown in a milled mould and coated with aluminium. An ion selective calcium electrode was used to measure the free calcium concentration before and after flash. Test solutions were composed of 10 mM KCl for ionic strength adjustment and 11 different background concentrations of calcium chloride: 10^{-7}, 10^{-6}, 10^{-5}, $5*10^{-5}$, 10^{-4}, $1.5*10^{-4}$, $2*10^{-4}$, $2.3*10^{-4}$, $3*10^{-4}$, $5*10^{-4}$, 10^{-3} M with or without 1 mM Nitr5. With a background Ca concentration below $2.3*10^{-4}$ M, Nitr5 behaved as a calcium chelator, as indicated by the decreased ion selective electrode (ISE) potential after adding Nitr5. At higher background concentrations the response to a flash became less clear as a result of decreased sensitivity of the electrode in this range. By projecting the datapoints before and after flash to the x axis, we can estimate that five flashes released $5*10^{-5}$ M Ca and that 1 mM Nitr5 was saturated with Ca at a background concentration of $2.3*10^{-4}$ M Ca, i.e. five flashes released 5% of 1 mM Nitr5 and 23% of Nitr5 was not Ca loaded. In conclusion this method appears to be a useful tool for analysing both the behaviour of Nitr5, and flash efficacy for photolysis. This low cost instrument has the ability to evoke a physiological calcium jump by photolysis of Nitr5, provided that this substance is in a saturated condition.

Contractile responses to flash photolysis of intracellularly loaded caged calcium in intact bladder muscle strips

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Previous research on pig urinary bladder smooth muscle (*Am. J. Physiol.* **261** (1991) R138–44) has indicated that for acetylcholine and presumably also for electrically stimulated contractions, the influx of extracellular calcium is the rate limiting process in the excitation–contraction coupling. To bypass this rate limiting process, caged calcium (NITR 5/AM, Calbiochem) was loaded into intact muscle strips. Strips, 2 mm in length and 150 μm in diameter, were suspended in a specially developed 20 μl drop-organbath in modified Krebs solution kept at 37°C with an infrared halogen lamp. The strip was mounted between two tweezers, 400 μm diameter (K. Güth, Heidelberg), one of those attached to an AE801 force transducer (Sensonor). Electrical stimulation was applied via two platinum ring electrodes on either end of the strip (alternating ± 3 V, 100 Hz, 5 ms). After three stimuli, the strip was loaded for 15 min with a 2 μM NITR5/AM solution. For this loading solution one part of a solution of 1 mM Nitr5/am in DMSO, containing 25% Pluronic F127 (BasF Wyandotte) was dissolved in 500 parts of modified Krebs. After loading one control electrical stimulation was applied and 5 minutes later the strip was flashed using a 240 J Xenon flash lamp (described in the abstract above). For this purpose the drop organbath and suspensory device were placed in a custom-made aluminium coated glass ellipsoid reflector and the transducer was sufficiently protected from light. Electrical stimulation before loading with Nitr5 resulted in a maximum developed force of 70 μN. Electrical stimulation after loading generated 60 μN, while the responses upon flash photolysis were variable in different strips with a maximum of 40 μN. This preliminary study indicated that it is possible to load and photolyse a sufficient amount of Nitr5/AM in a small unskinned smooth muscle preparation at 37°C and thus evoking a relatively large contraction using low-cost flash equipment.

Role of the carbonic anhydrase of sarcoplasmic reticulum in excitation-contraction coupling

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We have previously shown that sarcoplasmic resticulum (SR) vesicles from rabbit muscles possess a membrane-bound carbonic anhydrase (CA) (Bruns *et al.* (1986) *J. Physiol.* **371**, 351–64). To elucidate the functional role of SR-CA we have performed a series of experiments designed to test the following hypothesis: protons may act as a major counter-ion exchanging across the SR membrane when Ca^{2+} is released into the sarcoplasm or taken up by the SR. SR-CA could then accelerate the CO_2 hydration reaction sufficiently so that it can provide or buffer protons at rates comparable to the rapid Ca^{2+} fluxes. The following experimental findings are consistent with this hypothesis. (1) Isolated rat skeletal muscles incubated in the CA inhibitors chlorzolamide (CLZ) or cyanate show a slow-down of time-to-peak and relaxation of single twitches. (2) In small fibre bundles prepared from fast and slow rat muscles, we demonstrate that CLZ and other membrane-permeable CA inhibitors (methazolamide, ethoxzolamide, dansylsulfonamide, L-645,151) produce the same effects, which are in all cases reversible. In contrast, a sulphonamide that is poorly membrane-permeable, acetazolamide, does not affect the contractile behaviour of fibre bundles. (3) These effects are mirrored in Ca^{2+} transients measured in fibre bundles with fura-2. CA inhibitors cause a slow-down of rise time and decay time of the Ca^{2+} signal elicited by muscle activation.

The conclusion from observations 1–3 is that inhibition of the (intracellular) SR-CA causes a retardation of Ca^{2+} release from and Ca^{2+} reuptake into the SR by slowing down the speed of the reaction $H^+ \leftrightarrows HCO_3^- \leftrightarrows CO_2$ and that this leads to corresponding changes in contractile behaviour. (4) CA activity measurements using a ^{18}O-technique show that most of the CA activity of the SR is located in the interior of the SR, i.e. where it is most necessary in view of the limited reaction volume of the intra-SR space. (5) The same technique allows us to determine the HCO_3^- permeability of SR vesicles to be 2×10^{-7} cm s^{-1}, which contrasts with the very high figure of 10 cm s^{-1} reported in the literature for H^+ permeability of SR. This indicates that it is indeed H^+ rather than HCO_3^- that compensates the charges of Ca^{2+} moving across the SR membrane.

P_i effects on crossbridge turnover kinetics in skinned skeletal muscle fibres activated by NEM-S1 or calcium

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Previous studies have shown that not only calcium but also binding of myosin heads to actin with high affinity (strong binding crossbridges) can activate the contractile system. To examine the effects of strong binding crossbridges on regulation of muscle contraction in more detail, we used NEM-modified subfragment 1 of myosin (NEM-S1) as an analogue for strong binding crossbridge states. NEM-S1 was characterized in solution studies regarding ATPase-activity and the nucleotide and calcium dependence of NEM-S1 binding to actin (see Schnekenbühl *et al.*, this Meeting). For analysis of the effect of strong binding crossbridges on the structurally intact contractile system, we diffused NEM-S1 into chemically skinned rabbit psoas fibres. Following the time course of diffusion and binding to actin by X-ray diffraction and mechanical measurements (e.g. active force generation) showed that diffusion is a rather slow process. Therefore we incubated the fibres for at least 3 days with NEM-S1 (in the presence of ATPγS) before studying its effects on the contractile properties of the muscle fibres.

After preincubation with 2 μM NEM-S1 the fibres develop smaller active force at *p*Ca 8.0 than at full calcium activation while the rate constant for force redevelopment (k_{redev}) is somewhat faster. There are two possibilities which could account for small isometric force while k_{redev} is very fast. (1) Crossbridges in the presence of NEM-S1 can complete the crossbridge cycle very fast, i.e. g_{app} could be large and therefore the ratio of isometric fibre ATPase over isometric force were expected to be high. Preliminary ATPase measurements did not support this idea. (2) An increase of the rate constant for the return of crossbridges from the strong binding states back into the weak binding states without completing the ATP hydrolysis cycle (f^-_{app}) could also account for the fast k_{redev} in the presence of NEM-S1. As this transition is associated with reversal of the P_i-release step, its kinetics are expected to be dependent on inorganic P_i-concentration $[P_i]$. Testing this idea by changing $[P_i]$ in the presence of NEM-S1 at low and high calcium concentration (*p*Ca 8 and *p*Ca 4.5) showed that k_{redev} increases much more with $[P_i]$ at *p*Ca 8 than at *p*Ca 4.5. At *p*Ca 4.5 the P_i effect appears to be independent of NEM-S1. This high sensitivity of turnover kinetics for $[P_i]$ in the presence of NEM-S1 at low $[Ca^{2+}]$ suggests that activation by strong binding crossbridges is associated with a fast f^-_{app} which becomes inhibited at high $[Ca^{2+}]$. In turn, these findings imply that reducing $[Ca^{2+}]$ in the presence of strongly bound crossbridges results in disinhibition, i.e. an increase in f^-_{app} that might become important for muscle relaxation.

Pharmacological and physiological changes of excitation–contraction coupling in rat skeletal muscle

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In skeletal muscle, excitation–contraction coupling (EC coupling) links depolarization of transverse tubules (T-tubules) to contraction. The main role in this phenomenon is played by dihydropyridine (DHP) receptors in the T-tubules and ryanodine sensitive Ca^{2+} release channels in the sarcoplasmic reticulum (SR). However, EC coupling might be modulated by physiological or pharmacological changes of either membrane electrical properties or subcellular structures involved in Ca^{2+} handling. To gain insight into these mechanisms we evaluated EC coupling of rat skeletal muscle under different experimental conditions by measuring the voltage dependence of mechanical threshold (MT) in extensor digitorum longus (EDL) muscle fibres using the two microelectrode 'point' voltage clamp (holding potential = −90 mV). Using this approach we have already found that MT changes significantly during aging, e.g. the EDL muscle from 29-month-old rats contract at more negative potentials than those from 3–4-month-old rats (De Luca and Conte Camerino (1992) *Pflügers Arch* **420**, 407). The change in Ca^{2+} homeostasis occurring in skeletal muscle during aging (Larsson and Salviati (1989) *J. Physiol.* **419**, 253) may account for the observed shift of MT; however, the low chloride conductance (G_{Cl}) which characterizes EDL muscle from aged rats (De Luca *et al.* (1990) *Pflügers Arch* **415**, 642) may also play a role. To evaluate the relationship between G_{Cl} and EC coupling, we have tested the effects of R-(+) and S-(−) isomers of 2-(p-chlorophenoxy) propionic acid (CPP) on MT. The R-(+) and S-(−) enantiomers, at concentrations as low as 3 μM, increase and decrease G_{Cl}, respectively (De Luca *et al.* (1992) *J. Pharmacol. Exp. Ther.* **62**, 364). These compounds modified MT only at high concentrations (1 mM) and with effects that seemed to rule out any involvement of G_{Cl}, since R-(+) lowered MT whereas S-(−) shifted MT toward more positive potentials. Finally, we tested the hypothesis that the sulphonic amino acid taurine, which is normally found in mammalian skeletal muscle, might have a role in EC coupling by increasing Ca^{2+} uptake by SR (Lake *et al.* (1991) *Biophysis. J.* **59**, 64a). Toward this aim, we measured MT in EDL muscles that had been depleted of taurine by chronic 4 week treatment of the rats with guanidinoethane sulphonate (GES, 1% in drinking water). The MT was shifted toward more negative potentials in the GES treated EDL, with mean values of rheobase voltage (test pulse duration = 200–500 ms) of -71.1 ± 2.3 mV ($n = 11$) versus -61.4 ± 2.2 mV ($n = 22$) in the controls. The *'in vitro'* application of 60 mM taurine on the GES-treated muscles almost completely restored MT (-64.8 ± 2.9 mV; $n = 10$). The above finding suggests that a deficiency of taurine affects the EC coupling of skeletal muscle, corroborating the role of this amino acid in modulating the cytosolic Ca^{2+} concentration.

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Nicotinic and purinergic receptors present on outer haircells of the inner ear are similar to those of embryonic skeletal muscle

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Of the two types of cochlear hair cells involved in the processing of the sound signal the inner haircells (IHC) transduce afferent acoustic information, while the function of the outer haircells (OHC) is not clear. OHC answer stimuli with motile responses, suggested to modulate sound. Electromotility results in fast, high-frequency movements; other stimuli (K^+, ACh, ATP, Ca^{2+}, IP_3) cause a slow movement. OHC and skeletal muscle cells resemble each other in several ways. Actin, myosin and associated proteins are known to be present, as are cholinergic proteins such as choline acetyltransferase and choline esterase. Activation seems to result in slow movement of OHC in the presence of intracellular ATP and Ca^{2+}. Cytoplasmic Ca^{2+} activates C-type K^+ channels at the basolateral cell membrane (Gitter *et al.* (1992) *Hear. Res.* **60**, 13–9) and is involved in the repolarization of the electromechanical transduction. An active PI cycle in OHC was shown (Sehacht *et al.* (1988) *Hear. Res.* **31**, 155–60). Subsurface cisternae may store intracellular Ca^{2+}. No source for IP_3 receptor have been shown. By pharmacological means, we (Plinkert *et al.* (1991) *Hear. Res.* **53**, 123–30) recently identified a nicotinic acetylcholine receptor of the skeletal muscle type at the basolateral pole of OHC. Furthermore, a P2-receptor subtype as in skeletal muscle (Häggblad and Heilbronn (1987) *Neurosci. Lett.* **24**, 199–204) was identified. Its activation results in increased cytoplasmic Ca^{2+} via a G-protein-PL C cascade. Using novel ATP-derivatives (Zimmet *et al.* (1992) *Nucleotides and Nucleosides* (in press)) the pharmacology of the two P2R was found to be similar. A small part of Ca^{2+} increase came via influx from

DHP-sensitive channels. Cytoplasmic Ca^{2+} increase was slower than in muscle and more persistent. The reason for this may be found in the artificial storage conditions of OHC or the persistent high Ca^{2+} may have activated destructive proteases. The cell membrane of the ATP-activated OHC must have been hyperpolarized through stimulation of Ca^{2+}-stimulated K^+ channels.

Influence of Ca^{2+} on the structural transitions in N-domain of troponin C: regulation of skeletal muscle contraction

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Troponin C (TnC) and calcium ions play an important role in regulation of skeletal muscle contraction. It has been shown (Miroshnichenko and Shuba (1989) *Doklady Academii Nauk, Ukraine* **9**, 72–6; **3** (1992) 128–32) that the model of regulation by sterically blocking the interaction of myosin heads with actin filaments by tropomyosin is physically untenable. A new concept of regulating muscle contraction is proposed. It consists of the fact that TnC alone is able to determine the muscle contractile activity. The absence of Ca^{2+} in calcium-specific sites I and II did not allow the determination of what structural changes take place in this molecule during contraction switch. TnC structural analysis (molecular modelling, computer graphics) show that in the sites I and II the structures favourable for Ca^{2+} coordination may be formed. It takes place during conformational changes of TnC disordered regions (aminoacid residues 31–41, site I and 64–76, site II) as a result of polypeptide chain rotation around single bonds, changing the ϕ and ψ angles within permitted values. Such an approach makes it possible to clarify which amino acid residues participate in the formation of the structure of TnC N-domain containing Ca^{2+}. Hence, Ca^{2+} and TnC interaction causes only insignificant change in the topography of the latter. In adjacent sarcomere structures TnC acts as a specific 'catch'. Ca^{2+} and TnC interaction slightly alters the shape of this catch, thus eliminating sterical restrictions for filament sliding. Such principle of muscle contraction is based on real three-dimensional sarcomere macrostructure and fits well the skeletal muscle contraction model (Miroshnichenko and Shuba (1990) *Uspehy Fisiol. Nauk* **21**, 3, 3–18) of myosin filaments twisting into the gaps between actin threads.

CONTRACTILE AND REGULATORY PROTEINS

Molecular movements in the actomyosin complex: F-actin-promoted internal crosslinking of the 25 kDa and 20 kDa heavy chain fragments of skeletal myosin subfragment 1

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Reciprocal internal displacements of segments or domains of the S-1 heavy chain on binding of nucleotides and actin are thought to form the molecular mechanism driving energy transduction by the actomyosin complex. Nucleotides-mediated distortions of the S-1 heavy chain were previously characterized through nucleotide-dependent intramolecular crosslinking within or between particular regions of the tryptic 25, 50 and 20 kDa heavy chain fragments. But the nature and location of the heavy chain movements specifically induced by F-actin have not been precisely identified. Herein, we describe for the first time the F-actin-promoted changes in the spatial relationship of strands in the NH_2-terminal 25 kDa and COOH-terminal 20 kDa fragments detected by their exclusive chemical crosslinking in the rigor F-actin–S-1 complex with *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). The quantitative electrophoretic analysis of this reaction showed the extensive conversion of the 95 kDa heavy chain of the actin-bound S-1 into a new 135 kDa species (yield: 50–60%), whereas the heavy chain mobility remained unaffected when actin was omitted. The 135 kDa entity retained the fluorescence of AEDANS–S-1 but not of AEDANS–actin indicating that it was not a crosslinked acto-heavy chain adduct. Its extent of production was dependent on the S-1/actin molar ratio and was maximum near a ratio of 1:4. The MBS treatment of acto–S-1 led also to some covalent actin–actin oligomers which could be suppressed, by using trypsin-truncated F-actin lacking Cys-374, without altering the generation of the 135 kDa heavy chain derivative. The MBS reaction on the complex of F-actin and tryptic (25-50-20 kDa)-S-1 resulted in a new 40 kDa band, comigrating with actin, which was composed of the 25 kDa N-terminal and 20 kDa C-terminal fragments as it incorporated the fluorescence of the anthroyl group specifically attached to the former peptide and the fluorescence of AEDANS selectively bound to the SH-1 thiol in the latter segment. Blocking SH-1 and SH-2 thiols did not abolish this interfragment crosslinking. However, it was totally suppressed by millimolar concentrations of MgADP without dissociation of the acto–S-1 complex as assessed by cosedimentation. Thus, in the strong binding state, relative movements of the 20 and 25 kDa regions were induced by F-actin and could be modulated by nucleotides. The crosslinked site of the 25 kDa domain is a lysine within residues 145–204 which include the phosphate-binding glycine-rich loop of the ATPase site. This flexible region and the crosslinked mobile segment of the 20 kDa peptide, whose identification is underway, may contribute to the mechanism of interactivity between the actin and ATPase sites of myosin during force generation.

A 100 kDa actin-modulator protein with fully reversible actin-binding properties from the tail muscle of the crayfish *Astacus leptodactylus*

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Astacus tail muscle contains a Ca^{2+}-dependent 100 kDa *Astacus* actin-modulator (ACAM). We have purified this protein to near homogeneity by a procedure involving extraction in the presence of EGTA, ammonium sulphate fractionation and anion exchange chromatography followed by gel filtration. ACAM severed actin filaments at high rates and strongly promoted nucleation of actin polymerization. Both reactions were completely dependent on the presence of Ca^{2+}. Activation occurred in a narrow range of Ca^{2+}-concentrations around 10^{-7} mol^{-1} l. Native electrophoresis of ACAM–actin mixtures (using IAEDANS-labelled actin) in the presence of Ca^{2+} reveals three distinct types of complexes which were identified as binary, ternary and quaternary complexes of ACAM with actin. The type of complex formed and the ratio of the different complexes depended on the actin/modulator ratio in the mixture. The cooperativity in the formation of the ternary and quaternary complexes appeared to be very low. The three different complexes were isolated by gel filtration. On addition of EGTA all actins were released from all three complex types. The fully reversible interaction of ACAM with actin was employed for further purification of ACAM by high resolution gel filtration.

By proteolytic cleavage with chymotrypsin two fragments of nearly identical molecular weight were obtained (50 and 48 kDa). This property, in addition to the Ca^{2+}-dependent actin severing and nucleation activity, identifies ACAM as a gelsolin-like protein, although with significantly higher molecular weight than the known vertebrate gelsolins. However, unlike gelsolin which only forms binary and ternary complexes with actin, the binary complex being EGTA-resistant, ACAM seems to bind three actin molecules and all ACAM–actin complexes are EGTA-sensitive. The latter property is also found for the other actin modulating proteins villin and adseverin. However, neither gelsolin nor villin antibodies recognized ACAM on immunoblots, nor was a Ca-dependent bundling observed as is characteristic for villin. It is therefore concluded that ACAM

represents a novel type of actin modulating protein related to, but not identical with, both gelsolin or villin.

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Effects of tropomyosin and tropomyosin-troponin on the actin-myosin crosslinking sites

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Chemical crosslinking experiments with crosslinking reagents of various specificity and different spans were used to define the effects of tropomyosin–troponin on the actin-S1 binding sites.

The presence of tropomyosin alone or associated with troponin was found to have no effect on the gel electrophoretic pattern of the actin-S1 crosslinked products formed on crosslinking reactions induced by EDC (I = 0Å), ethoxycarbonyl 2-2 ethoxy 1-2 dihydroquinoline (I = 0Å), dimethyl suberimidate (I ⩽ 11 Å) or para-azidophenylglyoxal (I = 7 Å) (performed at a tropomyosin–troponin/actin/S1 molar ratio of 1.5:7:3). Identical results were obtained in the absence as in the presence of Ca^{2+} and/or the nucleotide, Mg^{2+}-ADP. In contrast, when glutaraldehyde (I ⩾ 9 Å) was used as crosslinking reagent, tropomyosin alone as well as in the presence of troponin (with or without Ca^{2+} and/or ADP) abolished the formation of the actin-S1 crosslinked product of apparent MW 200 kDa with no significant effect on the 165 kDa actin-S1 product. This result evidences that tropomyosin either blocks an actin-S1 binding site or masks one of the glutaraldehyde anchoring points on the actin molecule. Similar experiments performed with proteolytic S1 derivatives demonstrated that tropomyosin inhibited the glutaraldehyde-induced crosslinking between actin and the central 50 kDa fragment of the S1 heavy chain. The location of this tropomyosin dependent actin-S1 crosslinking site on the actin molecule is now under investigation.

Except for the glutaraldehyde-induced crosslinking experiment, tropomyosin alone or with troponin does not affect any of the actin-S1 crosslinking sites studied. This result is in good agreement with recent X-ray diffraction and electron microscopic reconstructions (Milligan *et al.* (1990) *Nature* **348**, 217–22; Holmes and Kabsch (1991) *Current Op. Struct. Biol.* **1**, 270–80) which placed these crosslinking sites (segments 1–7, 20–28 and 92–102 of actin) on the outer surface of the subdomain I of actin, distant from tropomyosin located in a grove between subdomains 3 and 4 near the filament axis. Moreover, the absence of Ca^{2+} dependence of the crosslinking reaction between S1 or S1–ADP complex and the reconstituted thin filament could have two explanations: either during the on/off regulatory switch, tropomyosin–troponin inhibits the actin–S1 interaction at different sites than those revealed by the crosslinking approach or under our experimental conditions (in particular with an actin/S1 ratio close to 2:1), most of the actin binding sites are in the 'on' state even in the absence of Ca^{2+} as proposed by Trybus and Taylor (*Proc. Natl. Acad. Sci. USA* **77** (1980) 7209–13).

Interaction of smooth muscle talin and talin–vinculin complexes with actin

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Talin and vinculin are developmentally regulated marker proteins for adherens-type junctions in differentiating muscle cells. *In vitro*, talin serves a dual function: nucleating actin polymerization and anchoring into lipid membranes. When complexed with vinculin, the binding and polymerization rate constant with actin at 20°C of approximately 0.3 μM and approximately $4 \times 10^{-3}\ s^{-1}$, respectively, remain unchanged. The thermodynamic data exhibit all signs of 'van der Waal's' binding forces for actin–talin and actin–(talin-vinculin). Furthermore, the insertion of talin into hydrophobic domains of lipid layers occurs irrespective of preincubation with vinculin (Goldmann *et al.* (1992) *Biochemistry* **31**, 7665–71).

Fluorescence microscopy of rhodamine-phalloidin labelled actin filaments shows that the interaction between actin and talin results in an increase of filament numbers over length. This finding is also supported by microrheologic studies. Frequency-dependent measurements give evidence that the presence of talin and talin–vinculin suppresses the internal oscillating mode of actin chain dynamics, which is the result of reduction of average filament length by a factor of 4–5 (Ruddies *et al.* (1992) *Eur. Biophysics J.* (unpublished data)). The observed increase in filament stiffness and viscosity decrease of talin and talin–vinculin nucleated actin gels are consistent with previous data (Kaufmann *et al.* (1991) *FEBS Lett.* **284**, 187–91).

Effect of skeletal muscle myosin regulatory light chains on the aggregation of myosin active fragments

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Comparison of aggregative features of active rabbit skeletal muscle myosin fragments was carried out at 42°C by using the light scattering method. The following myosin fragments were studied: (1) chymotryptic subfragment 1 (Ch-S1) containing a shortened heavy chain (96 kDa) and a single essential light chain (ELC), (2) Mg^{2+}-papain subfragment 1 (Mg-S1) having a 102 kDa heavy chain (elongated at the expense of inclusion of a hinge region between the head and the rod) and both ELC and regulatory light chains (RLC), and (3) heavy meromyosin (HMM) containing phosphorylated or dephosphorylated RLC.

A pronounced stabilizing effect of Ca^{2+} ions on myosin head structure in the case of both phosphorylated and dephosphorylated HMM and Mg-S1 is shown. Aggregation of Mg-S1 containing all light chains was slower in comparison with preparations containing only ELC. A similar slowing of aggregation was observed also for HMM. Denaturation changes in the head structure was promoted when Ca^{2+} was substituted for Mg^{2+}; the evidence for this was a more rapid aggregation. Aggregation processes became even more intensive at addition of EDTA.

The results obtained suggest that in the presence of divalent cations regulatory light chains have a stabilizing effect on most labile structures of the myosin head, namely on essential light chain and through it on the 50 kDa heavy chain fragment. A coordinatory bond between the RLC and ELC via Ca^{2+} ion probably plays the key role in this stabilization.

It was also established that on RLC phosphorylation the stabilizing effect of metals on myosin active fragments was decreased: aggregation of phosphorylated HMM was initiated earlier and reached higher levels in comparison with dephosphorylated preparations.

Our results provide evidence for the weakening of phosphorylated RLC binding to the myosin head and may be an indirect proof of the participation of phosphorylated RLC in regulation of skeletal muscle contraction, possibly as auxiliary mechanism.

Myosin subfragment 1 inhibits dissociation of nucleotide and calcium from G-actin

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In recent years, there has been a growing number of experiments indicating that actin plays a more active role in the actomyosin-based motility. To understand the molecular mechanism underlying this phenomenon it is necessary to elucidate the effects of myosin on the structure of actin in both G- and F-forms. We report here that

structural changes in the vicinity of the nucleotide and calcium binding sites of G-actin occur on binding of myosin subfragment 1 (S1). The interaction between the two proteins was studied under the conditions in which the actomyosin complex is stable and does not undergo polymerization (Chaussepied and Kasprzak (1989) *Nature* **342,** 950). The dissociation of the ATP analogue, εATP, from G-actin has been followed by recording a large decrease of the fluorescence intensity of εATP on transferring the etheno-base from the protein environment to the solution. The rebinding of the released nucleotide and its eventual accumulation has been prevented by the use of alkaline phosphatase (Purich and MacNeal (1978) *FEBS Lett.* **96,** 83) or apyrase. In all cases the initial reaction rates have been measured. The rate of εATP dissociation from G-actin in the absence of S1 was found to be approximately 5×10^{-4} s^{-1}, which corresponds to the release of εATP from G-actin without prior Ca^{2+} dissociation. The rate of εATP has been measured as a function of S1A2 concentration; when saturating concentration of S1A2 was used, the dissociation rate was reduced to approximately $0.5–0.7 \times 10^{-4}$ s^{-1}. When EDTA was used to chelate the free and bound calcium the rate of εATP release was in the order of approximately 5×10^{-2} s^{-1} (this value represents the rate of Ca^{2+} dissociation from G-actin). The presence of saturating concentration of S1A2 lowered this rate constant by approximately an order of magnitude. Similar results were obtained with S1A1. The accessibility of the base of the actin-bound εATP in the absence and in the presence of ATP has been assessed by employing acrylamide quenching. It was found that S1 did not change the solvent accessibility of the base which, when bound to actin, remained well shielded from water and quencher(s). It has been reported that subtilisin-cut actin retains most of its functional properties of the native protein, with the notable exception of failing to support movement in the *in vitro* motility assay. When the cleaved actin was used instead of native protein, S1 induced conformational changes that led to a partial inhibition of the nucleotide and calcium dissociation. This behaviour closely resembled the effects of S1 exerted on native actin. The results are discussed in terms of the conformation of actin in the complex with S1 as a pre-'F' actin state (Chen *et al.* (1992) *Biochemistry* **31,** 2941).

^{31}P-NMR-spectroscopical characterization of the phosphorylation domain of cardiac troponin I

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Troponin as isolated from bovine heart is phosphorylated at ser23 and/or ser24 in the heart-specific region of troponin I (Swiderek *et al.* (1988) *Eur. J. Biochem.* **179,** 335–42; Mittmann *et al.* (1990) *FEBS Lett.* **273,** 41–5). It contains a mixture of non-, bis- and two monophosphorylated troponin I species. At neutral pH each form gives rise to a distinct ^{31}P-NMR signal between 4.0 and 5.0 p.p.m. The signal at the lowest field represents troponin I phosphorylated at ser24, upfield the signals of the form phosphorylated at ser23 and the bisphosphorylated form are observed. The chemical shift of each form is dependent on pH. In the bisphosphorylated form both phosphates exhibit very similar *p*Ka-values of about 6.0 owing to an interaction of these phosphate groups with acidic groups of either troponin T or C. Isolated troponin I only exhibits two ^{31}P-NMR signals with chemical shifts identical to those observed with the monophospho forms or with a bisphosphorylated synthetic peptide representing the phosphorylation domain. Reconstitution of the holotroponin complex re-establishes the three signal spectrum. An interaction of the phosphate groups with the acidic Ca^{2+} binding subunit, troponin C, seems to be most probable maybe by changing the protonation degree of each phosphate group in the bisphosphorylated troponin I. It is also possible that binding of the C to the I subunit induces a structural change of the bisphosphorylated N-terminal region bringing the two phosphate groups closer together.

Localization of the regulatory light chain on the heavy chain of fast skeletal muscle myosin

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Biochemical and electron microscopic studies suggest that the regulatory light chain (RLC) binds near the junction of the subfragment-1 (S1) with the rod. We have prepared the S1 moiety from rabbit fast skeletal muscle myosin by limited proteolysis with alpha-chymotrypsin (chymo-S1), thrombin (thromb-S1) and papain (pap-S1). All three S1 varieties contain LC1 and LC3 in similar proportion as in the parent myosin. Chymo-S1 and thromb-S1 are devoid of the RLC while pap-S1 still retains it. Papain is known to cleave the myosin HC past L-842. When the chymo-S1 is further digested with trypsin its HC ends with R-809 (Tong and Elzinga (1990) *J. Biol. Chem.* **265,** 4893–901). The next possible split point for alpha–chymotrypsin would be between F-814 and C-815. If this were the case the HC of chymo-S1 would be shorter than that of pap-S1 by 27 amino acid residues (AA). Molecular mass determination in SDS-gel electrophoresis yielded a difference of 3260 kDa between the two HC corresponding to around 29 AA with an average weight of 112. The HC of thromb-S1 appears in electrophoresis to be 1160 kDa larger than the heavy chain (HC) of chymo-S1, and 2100 kDa smaller than pap-S1. This indicates that the HC of thromb-S1 has around ten AA more than chymo-S1. The only possible split point for thrombin in the difference peptide between chymo-S1 and pap-S1 is between R-821 and A-822. This corresponds well to our molecular mass determinations. The sequence of the 20 AA of the HC peptide between the putative cleavage point of thrombin and papain is almost identical among five myosin HC species from different skeletal muscles. They all have in common the stretch (826–834)–VKHWPWMKL–containing two tryptophans (W). We showed that the HC of pap-S1 contains two more tryptophans than thromb-S1 and chymo-S1. Binding studies revealed that only pap-S1 can bind exogenous RLC. The results indicate that the RLC binds to a stretch of maximal 20 AA in the myosin HC (822–841) containing the two additional tryptophans.

Modification of the ATPase activity of cofilaments of insect myosin and paramyosin by projectin

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Two most interesting myosin associated proteins of invertebrate muscles are paramyosin and projectin. Paramyosin seems to determine the diameters of synthetic thick filaments (Dufhues *et al.* (1991) *Comp. Biochem. Physiol.* **99B,** 871), projectin their length (Kölsch *et al.* (1992) *J. Muscle Res. Cell Motil.* **13,** 240).

To study a possible influence of these proteins on the actin-activated ATPase activity of insect myosin, aggregates of locust (*Locusta migratoria*) myosin alone or in combination with paramyosin and/or projectin were formed by overnight dialysis against low ionic strength buffers (see Dufhues *et al.* (1991)). Locust myosin, paramyosin and projectin were purified by FPLC Mono Q chromatography (Ziegler *et al.* (1990) In *Muscle and Motility,* edited by Maréchal and Carraro, Vol. 2. New Hampshire, Intercept, pp. 9–14), paramyosin from *Mytilus edulis* (ABRM) according to Bailey, modified by Bullard *et al.* (*J. Mol. Biol.* **75** (1973) 359) and rabbit actin from acetone dried powder (Nonomura *et al.* (1975) *J. Biochem. Tokyo* **78,** 1101). The molar ratios of the proteins used in the assays were 1 M myosin per 0.5 M paramyosin and 0–0.04 M projectin.

The actin-activated ATPase activity (optical test) of cofilaments of

locust myosin and projectin formed in the absence of paramyosin, was only slightly increased by projectin. The actin-activated ATPase activity of cofilaments formed in the presence of locust myosin, *Mytilus* paramyosin and locust projectin was considerably increased, the amount of increase depending on the amount of added projectin. A fourfold increase could be obtained by adding 0.04 M projectin per molar myosin and 0.5 M paramyosin (ABRM) to the dialysing assay. Experiments with locust paramyosin showed similar results. No ATPase activity could be observed in pure projectin or paramyosin preparations.

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Caldesmon inhibits actin-tropomyosin activation of myosin MgATPase by a cooperative reduction in the rate limiting step (product release)

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Caldesmon inhibition of actin–tropomyosin activation of myosin MgATPase activity was investigated. More than 90% inhibition of ATPase activation correlated with 0.035–0.1 caldesmon bound per actin monomer over a wide range of conditions. Caldesmon inhibited sheep aorta actin–tropomyosin activation of skeletal muscle heavy meromyosin (HMM) by 85% but had no effect on the binding affinity of HMM.ADP.Pi to actin. At ratios of 2 and 0.12 subfragment-1(S1):1 actin, addition of caldesmon inhibited the ATPase activation by up to 95%, but did not alter the fraction of S1.ADP.Pi associated with actin–tropomyosin. We concluded that caldesmon inhibited A.tm.myosin ATPase by slowing the rate limiting step of the activation pathway.

An expressed mutant of caldesmon comprising just the C-terminal 99 amino acids bound actin ten times weaker than whole caldesmon but otherwise inhibited actin–tropomyosin activation with the same potency and same mechanism as intact caldesmon. Thus the entire inhibitory function of caldesmon resides in its extreme C-terminus.

At concentrations comparable to the ATPase measurements S1 displaced caldesmon from native thin filaments both in the absence (rigor) and the presence of MgATP. We therefore concluded that caldesmon could displace S1.ADP.Pi from actin–tropomyosin only under exceptional circumstances.

In the absence of tropomyosin much larger quantities of caldesmon were required to inhibit acto-S1 ATPase, such that 90% inhibition correlated with one caldesmon bound per 0.7–1.0 actin (compared with 0.07 CD/A.tm). S1.ADP.Pi and caldesmon binding to actin were measured simultaneously at both 0.5 μM S1 per 50 μM A and 8 μM S1 per 4 μM A. Inhibition was directly proportional to S1.ADP.Pi displacement from actin. Thus caldesmon inhibits actin by competing with S1.ADP.Pi for a binding site on actin.

We conclude that tropomyosin in smooth muscle thin filaments is essential to propagate the inhibitory signal (reduction in V_{max}) from a single actin–caldesmon contact to up to 14 other actins and that inhibition in the absence of tropomyosin involves a competition between a different site on caldesmon and S1.ADP.Pi for actin, which is not physiologically relevant.

Modificatory effect of exogenous C-protein on contractile properties of isolated myofibrils

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C-protein is the most abundant nonmyosin component of both skeletal and cardiac muscle thick filaments. Its function may be connected with regulation of contractile activity (Offer *et al.* (1973) *J. Mol. Biol.* **173,** 653–76; Moos and Feng (1980) *Biochim. Biophys. Acta* **632,** 141–9; Hartzell (1985) *J. Mol. Biol.* **186,** 185–95; Udaltsov *et al.* (1991) *Biochem. Intern.* **25,** 337–44). One of the ways to test this supposition is selective extraction of C-protein from striated muscle (Hartzell and Glass (1984) *J. Biol. Chem.* **259,** 15587–96; Hofmann *et al.* (1991) *J. Gen. Physiol.* **97,** 1141–63). Another approach is to incorporate exogenous C-protein in muscle, where it is absent. We found the conditions for the binding of rabbit skeletal muscle C-protein to scallop cross-striated adductor myofibrils (20 mM NaCl, 20 mM Tris-maleate buffer, pH 7.0). The binding resulted in the decrease (by 30–50%) of the superprecipitation (SPP) rate of isolated scallop myofibrils. The addition of C-protein to rabbit psoas myofibrils (75 mM KCl, 1 mM $MgCl_2$, 20 mM Tris-maleate buffer, pH 7.0) also led to the decrease in SPP rate. It is known that C-protein is located only in the middle of each polar half of skeletal muscle thick filament. The effect observed by us may be the ability of exogenous C-protein to bind to the thick filament sites, free from endogenous C-protein (Moos (1981) *J. Cell Biol.* **124,** 571–86). The inhibitory effect of added C-protein on the SPP rate of myofibrils isolated from skeletal muscle is in a good agreement with the data on effects of C-protein extraction from skeletal muscle (Hofmann *et al.* (1991) *J. Gen. Physiol.* **97,** 1141–63). Partial extraction of C-protein led to a reversible increase in active tension of striated muscle fibres. Taken together these data support the view that C-protein can modify muscle contractile function.

Characterization of NEM-S-1-binding to regulated actin in solution and its effects on actomyosin ATPases

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The thin filament of striated muscle can be activated by calcium-binding to TnC and/or by attachment of strong-binding crossbridges to actin (Bremel and Weber (1972) *Nature* **238,** 97–101). To study whether activation of regulated actin by binding of Ca^{2+} to TnC has the same effects on actomyosin ATPases as activation by attached strong-binding crossbridges, we intended to use N-ethylmaleimide-modified myosin-subfragment 1 (NEM-S-1) as an analogue for strong-binding crossbridges. First we examined whether NEM-S-1 under all of our conditions (i.e. different nucleotides) represents a strong-binding type crossbridge analogue. We measured the binding of NEM-S-1 to regulated actin by either radioactive labelling of NEM-S-1 to determine the fraction of bound NEM-S-1 after mixing with regulated actin and centrifugation or by fluorescence-labelling of actin with pyrenyl-iodoacetamide and following the quench of the fluorescence signal on addition of NEM-S-1. Our experiments showed that without any nucleotide NEM-S-1 binds to actin very tightly. Unexpectedly, actin-affinity of NEM-S-1 is nucleotide-dependent. In the presence of ATP, NEM-S-1 binds to actin with an affinity intermediate between the myosin-ATP state and the rigor state. However, the binding of NEM-S-1 to regulated actin is sensitive to Ca^{2+}, independent of the present nucleotides. This suggests that despite the effects of nucleotides on actin-affinity, NEM-S-1 under all conditions represents a strong-binding crossbridge state. As shown by others (Nagashima and Asakura (1982) *J. Mol. Biol.* **155**) NEM-S-1 causes activation of ATPase-activity by binding to actin. To compare actomyosin ATPases when actin is activated either by presence of NEM-S-1 or by Ca^{2+}, we followed actin-activated ATPase-activity of native S-1 that was chemically crosslinked to actin.

In parallel experiments when NEM-S-1 is diffused into single muscle fibres we find that in the structurally intact contractile system NEM-S-1 has effects that differ from those of Ca^{2+} (see Kraft *et al.*, this Meeting).

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Analysis of the weak myosin-binding site on actin

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We recently reported that an 18-residue peptide, comprising residues 77–95 of the actin structure, forms part of the myosin binding site on actin (Alessi *et al.* (1992) *J. Muscle Res. Cell Motil.* **13**, 220). In actin, this region is largely α-helical, with an unwinding of the helix at the C-terminus (α-helix→5-turn helix), and is located at the top and 'side' of subdomain 1. This peptide binds equally well to S1 alone and to an S1-ATP analogue state, suggesting it forms a contact point in the weak binding acto-S1–ATP complex which is consistent with recent image reconstruction studies (Milligan and Holmes, unpublished data). Extensive analysis of this peptide in solution and when bound to S1 has been carried out by NMR and its structure determined from NOESY spectra using the simulated annealing protocol of XPLOR. In 50% aqueous trifluoroethanol (TFE), the peptide was essentially α-helical over most of its length with both the backbone and side chains aligned. The helix was slightly curved and further analysis showed that there was a discontinuity between H_{87} and H_{88} that was most apparent in the Ramachandran plots and seemed to originate at a break in the regular organization of the side chains; up to H_{87} the side chains were aligned C→N and after H_{88} they were aligned N→C. In most α-helices the side chains align either N→C or C→N without the break. In 90% H_2O, the helical character of the backbone is still apparent although the side chains are more flexible and the C-terminal end unwinds out of the α-helix, i.e. in some respect it is more like the structure found in actin. Using the transferred NOESY method, the structure of the peptide bound to S1 in 90% H_2O was also determined. This was found to be much more like that in 50% TFE as evidenced by the larger number of transferred NOEs than NOEs found in water in the absence of S1. The backbone was mostly helical and the side chains more ordered. In particular, residues W_{86}-H_{87} were held relatively rigid indicating that these two amino acids were the major contact points with S1. In actin, H_{87} (and H_{88}) are readily available for interaction with S1 but W_{86} is buried. However, limited molecular dynamics calculations based on minor changes in winding of the C-terminal region of this helix show that W_{86} can be exposed (and H_{88} buried) at this discontinuity point in the helix. This could be a means whereby S1 could deform the actin structure and transmit information through the molecule.

As controls it has shown that peptides containing the α-helical region 113–125 also retain their structure in solution but do not bind to S1. This is the major surface feature of the 'back' of the subdomain 1. We are presently investigating peptides containing the 338–348 helix, which is a dominant feature of the 'front' of subdomain 1.

Comparison of chicken and pigeon calsequestrins

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Chicken skeletal muscle calsequestrin (CS) exhibits many properties different from those of other classes, like mammals and amphibia (Damiani *et al.* (1986) *J. Muscle Res. Cell Motil.* **7**, 435–45). The primary structure deduced from cDNA sequence showed that the homology was higher with dog cardiac CS than with rabbit skeletal CS (Yazaki *et al.* (1990) *Biochem. Biophys. Res. Commun.* **170**, 1089–95). Recently, it has been suggested (Volpe *et al.* (1990) *Neuron* **5**, 713–21) that, at least in chicken, CS is also expressed in cerebellum microsomal fraction and that this isoform is probably identical to that of skeletal muscle. To assess if this feature is unique to chicken or shared by other avians, we isolated pigeon skeletal muscle CS with methods usually applied to other calsequestrins. Results show that pigeon CS and chicken CS have the same apparent molecular weight as estimated by electrophoresis both at neutral and alkaline pH. The electrophoretic patterns obtained after controlled proteolysis of both CSs by different proteolytic enzymes are very similar. ^{45}Ca-binding data show that both CSs bind calcium in a lower amount than rabbit CS, even though pigeon CS values seem to be closer to that of cardiac CS. The primary structure of pigeon CS was investigated by microsequence analysis of native purified protein and of fragments obtained by controlled proteolysis. All stretches so far analysed were identical to the corresponding regions of chicken CS. Finally, immunoblots of pigeon microsomal fractions obtained from different tissues show that pigeon CS is also expressed in cerebellum. As pigeon and chicken CS appear to be identical proteins and to be expressed in cerebellum, we conclude that avians probably possess a unique gene for calsequestrin.

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Nonenzymatic glycosylation of myofibrillar proteins: effect of age and diabetes

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Nonenzymic glycosylation is a reaction between simple sugars and proteins, namely lysine residues. It occurs *in vivo* especially in ageing and diabetes mellitus and can be also studied *in vitro*. Nonenzymic glycosylation of myosin and myofibrillar proteins from normal muscle, diabetic muscle and muscle of old animals was studied. It was found that in cardiac muscle and to a lesser extent also in skeletal muscles of the rat, nonenzymic glycosylation of myosin increases with age. Skeletal muscle myosin from diabetic humans and also that from diabetic rat cardiac muscle are more glycated when compared with control myosin preparations. Glycosylation of myofibrils with ribose *in vitro* results in a decrease of ATPase activity, which is proportional to the amount of glycosylated proteins. Electrophoretic studies revealed that myosin is the protein which is predominantly glycosylated. Incubation of myofibrillar proteins with ribose in the presence of 2-mercaptoethanol, aminoguanidine or diethylene-triaminepentaacetic acid resulted in a less pronounced glycosylation and also the inhibition of myofibrillar ATPase activity was diminished. Furthermore, the content of SH groups of myofibrillar proteins incubated with ribose is lower than those containing in addition 2-mercaptoethanol.

Inhibition of actin-activated Mg^{2+}-myosin ATPase by IMP and AMP: alternative explanation for muscle fatigue during strenuous exercise

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Muscle fatigue during strenuous exercise is not well understood, just like contractile failure in heart muscle during hypoxia. Often the reduction in force generation capacity has been related to metabolic changes in muscles. Increasing concentrations of metabolites such as ADP, H^+ and P_i have been reported to inhibit force generation by inhibition of myosin ATPase. However, inhibition of myosin ATPase activity by these metabolites can only partly explain fatigue. We suggested that fatigue is a regulatory mechanism to maintain the balance between ATP synthesis and ATP consumption and thus prevent an 'energy crisis' in the muscle (Berden *et al.* (1986) *J. Physiol.* **381**, 85P; Westra *et al.* (1992) *Pflügers Arch.* **420** (Suppl. 1), R 93). We hypothesized that inosine-5'-monophosphate (IMP), which is formed in great amounts in muscle during exhaustive exercise, might be involved in the downregulation of actin-activated myosin ATPase. To test this hypothesis we used a preparation of contractile proteins (actin without troponin and tropomyosin, and myosin) isolated from fast twitch rabbit muscle. Mg^{2+}-myosin ATPase activity and actin-

activated Mg^{2+}-myosin ATPase activity were measured in an ATP regenerating medium under control conditions and in the presence of different amounts of actin, IMP and AMP.

The results showed that: (1) Mg^{2+}-myosin ATPase activity was not inhibited by IMP or AMP, (2) actin-activated Mg^{2+}-myosin ATPase activity was inhibited by IMP and AMP to about 70% at 15 mM, and (3) the effect of IMP and AMP were dependent on the molar ratio actin/myosin.

From the results we suggest that IMP does not inhibit Mg^{2+}-myosin ATPase activity but prevents complex formation between actin and myosin. This would explain the decrease in force during exhaustive contractions in skeletal muscle. IMP is expected to bind to one of the actin-binding sites on myosin and not on the ATP binding site.

AMP has a similar effect to IMP. Although the formation of AMP in skeletal muscle during exhaustive exercise is too low to be of physiological significance, it might be of interest for the explanation of contractile failure in heart muscle, as large amounts of AMP are formed during hypoxia in heart muscle.

Dissociation of acto-HMM by inorganic pyrophosphate: estimation of actin–myosin binding constants

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Inorganic pyrophosphate (PPi) is a valuable ATP analogue: it binds to the ATP-binding site of myosin and it dissociates the actomyosin complex into actin and myosin. The dissociation can be easily followed by monitoring light scattering (although in this case 'super-opalescence' can pose a severe problem) or actin fluorescence (if actin is appropriately labelled). In this study we measured the dissociation of smooth and skeletal acto-HMM by increasing PPi concentrations as influenced by tropomyosin–troponin, EGTA, phosphorylation. The main results were that smooth and skeletal acto-HMM were dissociated by the same range of PPi concentrations, that tropomyosin profoundly shifted the dissociation curve to higher PPi concentrations, that EGTA increases the dissociating effect of PPi only slightly in 'regulated' acto-HMM. The main goal of our study was to extract from the dissociation curves quantitative information about the binding constants of actin–myosin binding. Under the assumption that there is equilibrium between free actin and myosin, acto–myosin, PPi–myosin and the ternary complex acto–myosin–PPi an equation was derived that predicts the degree of acto–myosin dissociation as a function of PPi concentration. By a nonlinear least-square fit procedure we found that this equation describes our data reasonably well and is thus able to give information about the relevant binding constants. It emerged that the dissociation curve relating the degree of dissociation to PPi concentration is not so much dependent on the absolute size of the binding constants of actin–myosin or PPi–myosin binding but rather on their ratio. But despite these limitations valuable information can be extracted by this method, e.g. that the shift of the dissociation curve to lower PPi concentrations which can be observed after phosphorylation of smooth HMM is mainly due to a reduction in concentration of the ternary complex acto–HMM–PPi.

Studies on myosin isoform composition in synchronous and asynchronous insect flight muscle: evidence for heavy chain homodimers

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Myosins from the asynchronous flight muscles of fleshfly (*Phormia terrae novae*) and the synchronous flight muscles of locust (*Locusta migratoria*) were purified by FPLC ion exchange chromatography on Mono-Q. They were analysed by SDS-PAGE on 2–15% gradient gels. Locust myosin shows two light chains with slightly lower molecular weight than the two corresponding light chains of fleshfly. A third light chain band (31 kDa) of fleshfly myosin could not be demonstrated for locust myosin. Using different methods, two types of heavy chains could be separated. In glycerol-SDS-PAGE, the molecular weight of one isoform was identical in both flight muscles (205 kDa), the second type had a slightly higher mobility in the case of *Phormia* (202 kDa) and an essentially lower mobility in the case of myosin from locust flight muscles (215 kDa). Isoelectric focusing in 8 M urea-containing gels showed identical isoelectric points at about pH 6.8 for one of the heavy chain isoforms in either flight muscle myosin. The second heavy chains had lower isoelectric points which were near pH 6.5 for the myosin preparation from freshfly and at about pH 6.6 for that of locust. Native myosin isoenzymes from both types of flight muscles could also be separated into two bands when pyrophosphate gel electrophoresis was used (d'Albis *et al.* (1979) *Eur. J. Biochem.* **99**, 261). One of the isoenzymes from both muscles had identical mobility in these gels. The second isoform migrated faster in the case of myosin from fleshfly flight muscles, but slower in the case of locust myosin. Densitometric scans of pyrophosphate and focusing gels showed good agreement in the molecular ratio of the native isoenzymes (1.3:1 for locust and 1.5:1 for fleshfly) and of the dissociated myosin heavy chains (1.2:1 and 1.6:1, respectively). Additionally, rod length determinations of rotary shadowed myosin molecules revealed two populations of rods with lengths of 152 ± 3 nm ($n = 39$) and 165 ± 4 nm ($n = 37$) for myosin from fleshfly flight muscles or 152 ± 3 nm ($n = 79$) and 165 ± 4 nm ($n = 70$) for locust myosin. The results led to the conclusion that the two heavy chain isoforms of myosin from insect flight muscles associate as homodimers. As the rod lengths of the two myosin populations were found to be identical in both flight muscles, the peptide responsible for the relatively large molecular weight of one myosin heavy chain from locust flight muscles (215 kDa) is assumed to be located in the head portion of the molecule. In cooperation with the different light chain composition such differences might contribute to the distinct contractile properties of either type of flight muscle.

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Characterization of a mutated troponin I with two cysteine residues substituted

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Troponin I (TnI) purified from rabbit skeletal muscle tends to produce aggregates because of oxidation of its three cysteine residues (Cys48, Cys64 and Cys133). Oxidation of TnI occurs also in the Tn complex, causing problems in various experiments, especially crystallization trials. To seek for a more stable TnI, we have performed the present work. First, we have cloned TnI from a rabbit skeletal muscle cDNA bank, expressed it in *E. coli* and established a purification procedure. Then, by substituting two cysteine residues (Cys64 and Cys133) we created a mutated TnI, TnI252. TnI252 formed complex together with TnC and TnT as the authentic TnI does. The reconstructed Tn complex with TnI252 showed the same Ca^{2+}-dependent inhibitory effect on the actin-activated myosin S1 ATPase activity as the authentic Tn complex. These results suggest that the function of TnI was not affected by the substitution of the two cysteine residues. TnI252 still tends to form dimers. However, when it is in the Tn complex, the oxidation is blocked even in the absence of DTT, indicating that the third cysteine residue, Cys48, is buried inside the complex. In conclusion, as an alternative of the authentic TnI, TnI252 is suitable for various studies, especially for crystallization of Tn complex.

Actin interfaces in dystrophin and myosin molecules

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The structure–function relationship of most actin-binding proteins and their respective interactions with G- or F-actin are poorly documented in comparison with the recent progress on the 3-D structure of G-actin (Kabsch *et al.* and Holmes *et al.* (1990) *Nature* **347**, 37–44 and 44–9). Protein engineering could help to reduce this information gap. Indeed, this technique has provided native protein fragments corresponding to limited domains interacting with actin. The recombinant actin-binding fragments are of controlled size and purity, and obviously could be mutagenized and crystallized. Such chimaeric constructs are relevant to most of the available structural analysis methods including NMR, crystallography, etc. The putative actin-binding domains of Xp21-linked dystrophin and of human cardiac α and β myosin heavy chains have been expressed as soluble recombinant fragments in *E. coli* They were fused to the bacterial maltose-binding protein, which permitted their easy purification.

Pelleting, NMR and electron microscopy experiments indicated that some recombinant fragments near the dystrophin N-terminal end recognized F-actin at only a few positions along the filament. Native dystrophin preparations have confirmed this actin interaction.

Pelleting, light scattering and electron microscopy experiments indicated that recombinant myosin heavy chain fragments around the connecting region (residues 518–722) interact with F-actin in an ATP-dependent manner. Such myosin fragments decorated F-actin filaments in the same way as the S1-fragments. Different affinities for actin were detected between the α and β constructs, indicating the role of some myosin heavy chain subdomains in the functional specificity of each cardiac isomyosin.

Crystals of different dystrophin or isomyosin actin-binding fragments have now been produced. It is expected that information on the fine structure of these typical actin-binding domains will thus be obtained.

Structure and assembly of myosin and tropomyosin coiled-coils

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The molecular structure and interaction geometry of coiled-coils from both myosin rod and tropomyosin has been investigated using X-ray crystallography and electron microscopy in conjunction with molecular biology methods (Atkinson and Stewart (1991) *J. Cell Sci.* **99**, 823–36; (1992) *J. Mol. Biol.* **226**, 7–13). Both coiled-coils are constructed from two in-register and parallel alpha-helices that twist round one another with an average pitch near 140 Å. A 9 Å map obtained from spermine-induced crystals of tropomyosin shows that the precise value of both the radius and the pitch of the coiled-coil varies along the molecule, probably in response to different sizes of residue in the hydrophobic interface between the two helices (Whitby *et al.* (1992) *J. Mol. Biol.* **227**, 441–52). The tropomyosin crystals are constructed from sheets of antiparallel molecules and there are two different interaction geometries with a sheet, as well as a more complex geometry between sheets. The tropomyosin molecules follow a sinuous path probably as a result of their being distorted by the interaction forces between them in the crystal. Two-dimensionally ordered crystalline sheets of skeletal myosin long S-2 also show curved molecules that have three different interaction geometries, underlying the complexity of the interactions between coiled-coils likely to be important for the construction of thick filaments. Thick filaments from frog, fish and rabbit all have essentially similar structures in which the myosin heads are perturbed from an ideally helical arrangement and this may reflect a rather complex series of different interaction geometries within thick filaments. The role of charge periodicities in myosin rod interactions has been investigated using a series of mutant LMM constructs expressed in *E. coli*. From these experiments it is clear that the complementation of zones of different charge is not the driving force behind assembly, although complementation of these charges is probably important in determining the precise interaction geometries available. Deletion mutants suggest that an important determinant of solubility (and thus interaction energy) is located near the C-terminus of the molecule.

Self-assembly mechanism of nonsarcomeric myosin

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Myosin self-assembly is like crystallization, in as much as the number and size of the filaments produced depends on the rate at which monomers are supplied to the assembly reaction, and on the number of nuclei available. This can be demonstrated using either smooth muscle or scallop skeletal muscle myosin. Both these myosins adopt an inert folded monomeric conformation in the presence of MgATP. If excess MgATP is removed from the medium, the folded molecules gradually lose the products of MgATP hydrolysis (approximately 0.002 s^{-1}), and pop open, becoming available to the assembly reaction. Supplying molecules for assembly by this very slow mechanism yields relatively few, very long (several μm) filaments. By contrast, supplying molecules rapidly, via a downwards salt-jump for smooth muscle myosin, or (more elegantly) via a Ca-jump for scallop myosin, yields very large numbers of filaments containing only a few molecules each. Supplying smooth muscle myosin molecules by light chain phosphorylation yields filaments of intermediate (1–2 μM) size.

Disassembly of the long filaments can be induced by adding back MgATP, and is seen in the electron microscope to take place by loss of material from the ends of the filaments. The mechanism appears to be that molecules dissociating from the filaments are captured into the folded conformation. The dissociation rate, across the whole filament population, is about 0.02 s^{-1} (25°C, physiological saline).

Calculation of the microscopic rates for molecular exchange demands that the number of molecules per unit length of filament be known. For smooth muscle myosin, electron microscopy reveals that the filaments consist of stacked layers of one to several side-polar molecular sheets, each sheet having two anti-parallel molecules per 14.3 nm along its length. Using this value, the microscopic dissociation rate per sheet is 2.1 s^{-1} per end, and the association rate, at 10 μM steady-state free monomer concentration, is 2.1×10^5 M^{-1} s^{-1}, again per filament end.

The importance of nucleation to the assembly process can also be demonstrated in the smooth muscle system. In the absence of added nuclei, long filaments are formed, whereas in the presence of added nuclei (in the form of preassembled short filaments), the formation of long filaments is prevented.

We have most recently been interested in the process by which free molecules become incorporated into filaments. There are reports in the literature that several different myosins require nonhelical sequences at the tip (C-terminus) of the tail in order to self-assemble. Using directed mutagenesis, we have investigated the effects of deletions and alterations in this region on the self-assembly of brush border myosin rods (headless myosin tails). We found that deletion of the tailpiece inhibited, but did not block, self assembly. The tailpiece had the effect of lowering the steady state free monomer concentration about 50-fold. Mutagenesis indicates that the detailed sequence of the tailpiece is not important for function. What is important is that it be nonhelical. We are considering the possibility that the tailpiece promotes assembly by forcing a stagger between molecules which prefer to otherwise overlap one another fully.

CYTOSKELETON AND MYOFIBRILLAR ASSEMBLY

Immunocytochemical localization of gelsolin in muscle and nonmuscle cells

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Gelsolin has three different actin binding sites which enable both an end capping of actin filaments and a lateral binding to two adjacent subunits of F-actin (Pope *et al.* (1991) *FEBS Lett.* **280,** 70–4). As *in vitro* gelsolin severs actin filaments and caps the (+)-end of F-actin in the presence of Ca^{2+} the lateral association always seems to be connected with a severing process. Previous immunochemical localizations *in vivo* have shown an association of gelsolin with the microfilament system, e.g. the cortical network (Yin *et al.* (1981) *J. Cell Biol.* **91,** 901–6; Hartwig *et al.* (1990) *J. Histochem. Cytochem.* **38,** 1145–53). Localization of gelsolin on stress fibres has also been demonstrated but these results are still contradictory (Carron *et al.* (1986) *J. Cell Biol.* **102,** 237–45; Cooper *et al.* (1988) *J. Cell Biol.* **106,** 1229–40).

Using monoclonal and polyclonal antibodies for immunofluorescence we have localized gelsolin in a variety of different cell types. In contrast to the results *in vitro* we showed that gelsolin may be associated with actin filaments without disrupting the actin structures. (1) Cultured fibroblasts showed continuous staining of stress fibres together with a diffuse staining of the cytoplasm. Under specific culture conditions an increased number of fibroblasts also showed polygonal actin networks which consisted of actin- and α-actinin-containing foci connected by actin filaments associated with tropomyosin (Lazarides (1976) *J. Cell Biol.* **68,** 202–19). The polygonal actin structures were found to be continuously stained by anti-gelsolin, including the foci. (2) Differentiated myotubes of myogenic cell cultures revealed a periodical pattern of cross-striation within the sarcomere. Staining of isolated myofibrils demonstrated that, dependent on the degree of contraction, the cross-striations varied. In expanded sarcomeres the staining pattern consisted of relatively wide bands in the I–Z–I range whereas contracted sarcomeres had in addition to a narrow I–Z–I staining a fluorescence signal in the centre of the A-band. This indicates that gelsolin is located along the whole actin filament length and the discontinuous pattern observed is the result of sterical hindrance effects of the myosin–actin interactions. (3) In cultured smooth muscle cells the myofibrils appeared as large stress fibre-like structures which revealed a gelsolin fluorescence pattern congruent to the rhodamine-phalloidin staining of actin. Owing to the close coincidence of the gelsolin staining with the pattern of actin in the investigated cytoskeletal structures gelsolin is shown to be bound to the filamentous actin, even in the presence of EGTA, and possibly without severing the filaments. The continuous staining of filaments, especially in skeletal muscle, indicates a lateral association with F-actin rather than a capping of filaments.

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Myosin-associated proteins C-, H- and 86 kDa proteins, are all members of the immunoglobulin C-2 superfamily

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Thick filaments of all vertebrate striated muscles contain, in addition to myosin, a group of myosin binding proteins (MyBPs) which include: C-protein, H-protein (mammals), 86 kDa protein (birds), titin (connectin), skelemin, M-protein, myomesin, M-creatine kinase, AMP-deaminase. Invertebrates contain paramyosin, twitchin, projectin, mini-titins and core protein(s). Twitchin (Benian *et al.*, 1989), C-protein (Einheber and Fischman, 1990), titin (Labeit *et al.*, 1990), smooth muscle light chain kinase (smMLCK; Olson *et al.*, 1990), mini-titin (Nave and Weber, 1990; Nave *et al.*, 1991, skelemin (Price *et al.*, 1991), projectin (Ayme-Southgate *et al.*, 1991) and M-protein (Masaki *et al.*, 1991) all contain two sets of internal domains: one is homologous to the Ig C-2 repeats (Williams and Barclay, 1988), the other related to the fibronectin (Fn) type III repeats. As this same duality of domain structure is found in N-CAM (Cunningham *et al.*, 1987) and a large group of cell surface molecules which function in cell–cell or cell–ligand interactions, it is reasonable to suspect that the type III and especially the C-2 domains might function in protein–protein recognition and adhesive interactions within the myofibril. To further our understanding of this protein family and map functional domains, we have cloned and completed the primary structures of fast-type chicken C-protein, chicken 86 kDa protein, human fast- and slow-type C-proteins, and human H-protein. It is clear that all these proteins are members of a common protein family. Over their C-terminal 46 kDa sections, all have approximately 70% sequence identity, the same domain structure: type III, C2-type III, C2. A myosin-binding assay has been established and we can demonstrate that the last C-2 repeat contains virtually all of the myosin-binding activity of chicken C-protein. As 86 kDa protein and H-protein have very similar C-terminal sequences, it is likely that these molcules bind to myosin in the same C-2 domain.

Myofibrillar assembly may be regulated by the C-terminus of titin

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Titin is so far the largest known protein (M, approximately 3000 kDa) and spans sarcomeres of striated muscle from M- to Z-lines (> 1 μM). Its early expression and intimate association with thick filaments suggests a role in myofibrillar assembly. Consistent with that idea, titin A-band sequences have been shown to provide ordered patterns of myosin and C-protein binding sites, and the pattern repeat appears to match the 430 Å thick filament repeat (Labeit *et al.* (1992) *EMBO J.* **11,** 1711–6). Titin is expressed early during myogenesis and is present before ordered myofibrillar assembly starts. It is unknown how its interaction with other muscle proteins and its early embryonic striated organization is developmentally controlled.

We have now cloned the titin C-terminus and are structurally and functionally characterizing this M-line section of the molecule. While A-band titin has structural molecular ruler-like functions, the M-line region of the molecule is likely to participate in the regulation of myofibrillar assembly. Expression of isolated titin motifs in *E. coli* and binding studies demonstrate that a transition of myosin-binding to nonmyosin binding motifs occurs towards the M-line. Sequence analysis shows that the titin C-terminus is composed of class 8 × class II motifs being separated by a heterogeneous family of interdomain insertions. One such insertion consists of four tandemly repeated KSP motifs previously found in neurofilament subunits H and M. In neurofilaments, phosphorylation of the KSP motif is developmentally regulated and supposed to control neurofilament assembly. In muscle, differentiating myotubes express titin-KSP-kinases and their expression is regulated during myogenesis. We suggest that titin-KSP-phosphorylation is involved in the control of sarcomere assembly in striated muscle. We are presently characterizing titin-KSP-kinase activities to test their possible involvement in regulation of myofibrillar assembly.

Dynamic light scattering study on the two proteolytic fragments of gelsolin

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The gelsolin molecule consists of two functionally distinct subdomains of approximately equal molecular weight (approximately 40 kDa) which can be separated by proteolytic cleavage. Both domains have binding sites for actin and for Ca^{2+}, but only the C-terminal half shows a calcium dependence of actin-binding. The N-terminal fragment retains the actin filament severing properties of intact gelsolin, but this function is no longer Ca^{2+}-sensitive.

We have purified the two subdomains of gelsolin by ion exchange chromatography after proteolytic cleavage with thermolysine. Dynamic light scattering measurements in the absence of calcium revealed an identical translational diffusion coefficient for both fragments. The frictional ratio f_0/f_{exp} (1.33–1.39) was nearly the same as obtained for intact gelsolin (1.37) in aqueous solution (Patkowski *et al.* (1990) *Biopolymers* **30,** 427–35), indicating a comparable molecular shape for the whole molecule as well as for the two subdomains. On addition of calcium ions the translational diffusion coefficient of the C-terminus decreased by almost 10% while there was no change observed for the N-terminus. This result indicates that the large ligand-induced conformational change seen for intact gelsolin is located on the C-terminal subdomain of the molecule.

The calculated increase in the ratio f_0/f_{exp} to 1.46 for the C-terminal domain was also observed for the intact gelsolin protein. Additional information on the rotational motion of native gelsolin obtained by depolarized dynamic light scattering revealed that the increase in the frictional ratio is related to an increase in the molecular volume while the molecule assumes a more spherical shape on Ca^{2+} binding. Therefore, we can conclude that the increase in the frictional ratio for the C-terminus in the presence of calcium ions is also probably caused by a transition to a more open structure. From an increase of f_0/f_{exp} from 1.33 to 1.46 for the C-terminal fragment we calculate a decrease in the partial specific volume by about 30%, neglecting changes in the molecular shape. This value is unusually large and we assume that the transition to a more open structure is accompanied by a conformational change into a more spherical shape of the C-terminal subdomain of gelsolin.

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Chromosomal localization in the mouse of genes for structural components of the myofibril: titin, nebulin and C-protein slow

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Associated with the thick and thin filaments of the myofibril are proteins with presumed structural roles, titin, C-protein and nebulin. Titin (Labeit *et al.* (1992) *EMBO J.*; Wang (1991) *PNAS* **88,** 7101–5; Labeit *et al.* (1990) *Nature* **345**; Fürst *et al.* (1988) *J. Cell Biol.* **106,** 1563–72), one of the largest known proteins (3 MDa), is considered to control thick filament assembly. C-protein (Einheber and Fischman (1990) *PNAS* **87,** 2157–61), another thick filament associated protein, is distributed in the crossbridge region, its function is not known. C-proteins occur in developmental and in adult fast and slow isoforms. Nebulin (Labeit *et al.* (1991) *FEBS Lett.* **282,** 313–6; Jin and Wang (1991) *FEBS Lett.* **281,** 93–6) a large protein (600–900 kDa) confined to skeletal muscle, is directly attached to the thin filament and may act as a 'protein-ruler' regulating thin filament length. We have mapped the genes for these myofibril-associated proteins using RFLPs in interspecific backcrosses between the laboratory mouse *Mus musculus* (C57BL/6J) and *Mus spretus* (cf. Kaupmann *et al.* (1992) *Genomics* **13,** 39–43). The genes for titin, *Tit*, and for nebulin, *Neb*, were localized on mouse Chr 2, with the gene order Cen-*Vim*-16.9 cM-*Gsn*-20 cM-*Neb*-7.6 cM-*Tit*-25 cM-*Pax*-6-18.8 cM-*a*. Our linkage analysis has placed the C-protein slow locus, *Cpt-s* (provisional gene symbol), on Chr 10: Cen-*Myb*-21 cM-*Pah*-8.1 cM-*Cpt-s*-. . . . Using hamster/human cell hybrids, the human homologue *CPT-S* has been assigned to Chr 12, within an established region of conserved synteny between the man and mouse.

Genes for myofibril-associated proteins may be involved in muscle diseases. *Neb* and *Tit* map close to the gene for murine 'muscular dystrophy with myotosis' (*mdm*) (Lane (1985) *Mouse News Lett.* **73,** 18). No disease genes are known to map to the region of Chr 10 in which we have localised *Cpt-s*.

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Invertebrate myosin filament: parallel subfilament arrangement in wall of solid filaments from honeybee, *Apis mellifica*

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Transverse serial sections (100–140 nm thick) of solid myosin filaments of the honeybee, *Apis mellifica*, were photographed in a JEM-200 electron microscope at 200 kV. Regions close to the Z-band or the M-band were not used for study. Micrographs were obtained at a magnification of 100 000. The images were digitized and areas including one myosin filament and six surrounding actin filaments were windowed and computer processed by rotational filtering. In all, 87% of the myosin filaments showed sixfold symmetry at radii of 6–10 nm in their power spectra, confirming the results of earlier works (Beinbrech *et al.* (1988) *J. Mol. Biol.* **201,** 557–65). To determine if the subfilaments were arranged parallel to the filament backbone, two methods were used. First, the three images of each myosin filament in the three serial sections were superimposed on the sixfold centre without rotation. In 85% of the resulting images a strong peak for sixfold symmetry at radii of 6–10 nm was found. After superimposing the images the averaged images showed sixfold symmetry and six pairs of subfilaments, which gives evidence for parallel arrangement of the subfilaments relative to the filament axis. This result was confirmed by the second method in which a three-dimensional reconstruction was made. An average image was made from the images of the same 17 myosin filaments from each of the three sections by superimposing on the sixfold centre without rotation. The data for the three-dimensional reconstruction were collected by tracing the outlines of the myosin- and actin-filament structures in the three successive sections. Four layers of outlines were used for each serial section, resulting in 12 layers of the reconstruction. Two stereo-pairs of the reconstruction, one simulating a cross-section view, the other simulating a longitudinal view, show a parallel arrangement of the six pairs of subfilaments.

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Stress fibre-like structures at the muscle tendon junctions of growing carp

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The muscle tendon junction (MTJ) of growing muscle is a site where force transmission (from myofibrils to the tendon) as well as the addition of new sarcomeres occurs (Schattenberg (1973) *Z. Zellforsch.* **143,** 587–96). We studied the structure of MTJs in red and white muscle fibres of growing carp (*Cyprinus carpio* L.; 18–25 cm standard length; kept at 23°C, increase in weight 15–20% per week).

MTJs of red axial muscle fibres have long finger-like protrusions that contain no sarcomeres. The last row of sarcomeres and the terminal membrane are connected by long (up to 20 μm) stress

fibre-like structures (SFLSs). In white fibres myofibrils extend into much shorter protrusions and are connected to the terminal membrane by less conspicuous bundles of thin filaments (length generally 2–5 μm). This difference between the fibre types may be related to the difference in metabolism: the aerobic red fibres having a greater need for extramyofibrillar space (for e.g. mitochondria).

Immunohistochemistry on the MTJs of red fibres, in combination with a phalloidin reaction, shows in addition to a reaction with the sarcomeres, a diffuse reactivity with anti-titin (clone T12; Fürst *et al.* (1988) *J. Cell Biol.* **106**, 1563–72) extending into the distal ends of the projections. A relatively high reactivity with anti-desmin appears to be located between the SFLSs.

In both fibre types new sarcomeres are formed adjacent to the last dark Z-line. Especially in red fibres new sarcomeres already terminated by a Z-line are partly surrounded by SFLSs that extend from more proximal Z-lines to the terminal membrane. This suggests that force transmission is a major function of these conspicuous SFLSs. The presence of a dark Z-line seems to be necessary for the addition of new sarcomeres.

Biphasic pattern of gelsolin expression and gelsolin–actin interactions during myogenesis

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During myogenesis actin is constantly being reorganized in respect to a variety of factors controlling the number and length of filaments and their interaction with other cellular structures. Gelsolin is one candidate for the regulation of actin reorganization.

Immunoblotting analysis and ELISA show that in chicken, quail, and mouse myocultures the content of gelsolin increases by a factor of three to five from myoblasts to the state of cross-striated myotubes. On the other hand, in neonatal mouse muscle the gelsolin content decreases rapidly between days 4 and 8 after birth by a factor of four. These results combined indicate a biphasic pattern of expression during muscle development. The stability of gelsolin in terminally differentiated myotubes was investigated by pulse chase experiments with ^{35}S-methionine. Analysis of immunoprecipitates from cultures at different time points after labelling revealed that gelsolin was stable for at least 24 h indicating a relatively high half-time of 48 h.

Fractionation of early and terminally differentiated myotubes into Triton X-100 soluble and insoluble components in the presence of EGTA revealed 80–90% of the gelsolin as soluble. When fractionated in the presence of Ca^{2+}, about 50% of the gelsolin was associated with the cytoskeletal components both in early and mature myotubes. Native electrophoresis of soluble fractions from Triton/EGTA extracts showed that approximately 50% of the gelsolin was present as a complex with actin. To determine whether the gelsolin was bound to the barbed end of short actin filaments we subjected the Triton extracts of myotubes from different states of development to high speed centrifugation. In all cases only 5–10% of the gelsolin was found to be sedimentable. These experiments show that the cellular pool of soluble gelsolin in myogenic cells consists mainly of free gelsolin or EGTA-resistant gelsolin–actin complexes, but not of capped actin filaments. This is in contrast to observations with polymorphonuclear leucocytes where the major proportion of Triton-soluble gelsolin is associated with a labile soluble pool of F-actin (Watts and Howard (1992) *Cell Motil. Cytoskel.* **21**, 25–37).

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Interactions made by titin

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Single molecules of the giant protein titin (chain weight approximately 3000 kDa) are thought to span from M- to Z-line. The fixed distance from the M-line of titin epitopes in A-band as sarcomere length is varied suggests this part of the molecule is an integral thick filament component. The I-band region of titin probably acts as an elastic connection between the end of the thick filament and the Z-line. These connections centre the A-band in the sarcomere and are the main route of mechanical continuity through relaxed muscle fibres. Titin can therefore be expected to make a number of interactions with other myofibrillar proteins at different places in the sarcomere. Here we describe the use of a simple solid-phase binding assay to screen for such interactions.

Rabbit skeletal muscle titin was purified by a rapid method that allows isolation of approximately 20 mg of undegraded protein in <24 h. SDS gels of the purified preparations show, in addition to the main band at approximately 3000 kDa, two bands at approximately 170 kDa and one band at approximately 100 kDa (which is probably not α-actinin). Electron microscopy of the purified titin straightened by solvent flow shows molecules approximately 900 nm long with a head at one end.

Serial dilutions of candidate interacting proteins were spotted onto nitrocellulose paper which was then reacted with the purified titin. After washing, bound titin was detected by monoclonal antibodies (visualized by the horseradish peroxidase assay). Interactions were detected with myosin, C-protein, X-protein (a slow muscle C-protein isoform) and AMP-deaminase. Tests with myosin proteolytic fragments indicated that binding was to the LMM part of the molecule. No binding was seen to α-actinin and H-protein (another crossbridge region protein), or to various control proteins with which interactions were not expected (BSA, keyhole limpet haemocynin and cytochrome C). A weak reaction was seen with actin, the significance of which is not clear, since *in situ* antibody labelling suggests titin acts independently of thin filaments. The binding to AMP-deaminase was not unexpected as localization studies have previously suggested that this enzyme may be attached to the elastic part of titin (Cooper and Trinick (1984) *J. Mol. Biol.* **177**, 137–52). The binding to C-protein, X-protein and LMM are consistent with the suggestion that A-band titin is closely associated with thick filaments (Whiting *et al.* (1989) *J. Mol. Biol.* **205**, 163–9). The present data are also consistent with binding results obtained using titin constructs expressed from cDNAs (Labeit *et al.* (1992) *EMBO J.* **11**, 1711–6).

Associations of titin spots with other sarcomeric or cytoskeletal proteins during human myofibrillogenesis: immunofluorescence and immunoelectron microscopic study

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Differentiating human skeletal muscle cells were used to study the nature of titin spots appearing during early stages of myofibrillogenesis (van der Ven *et al.* (1992) *Cell Tissue Res.* (in press)), and their associations with other sarcomeric or cytoskeletal proteins. Postmitotic mononuclear myoblasts and young myotubes were stained with antibodies against titin in conjunction with antibodies specific for desmin, vimentin, α-actinin, α-actin, sarcomeric myosin and β-tubulin. Randomly distributed titin spots were found in mononuclear cells expressing desmin, vimentin, α-actinin, α-actin and β-tubulin. At the light microscopic level, no obvious associations of titin with any of these proteins were observed at this stage of development. However, in a minority of cells showing a punctate titin localization, the titin spots were found colocalized with myosin. Subsequently stress fibre-like structures (SFLS) containing α-actinin and α-actin were found, decorated with titin spots. In more differentiated cells, titin was localized in a filamentous, stress fibre-like pattern, together with

α-actinin, α-actin and myosin. To gain resolution, preembedding immunoelectron microscopic studies were performed using the antibodies specific for titin. In differentiating mononuclear cells, titin aggregates decorated with up to 15 gold particles were identified. These aggregates were initially localized close to, dispersed or aligned with 10 nm filaments, but not to microtubuli or microfilaments. Maturation proceeded with an association of titin aggregates with SFLS, resulting in the formation of nascent myofibrils. From these results we conclude that titin spots as seen in immunofluorescence microscopy, consist of aggregated titin molecules. These aggregates are neither associated with microtubuli, as was suggested earlier (Fulton and Isaacs (1991) *BioEssays* **13**, 157–61) nor with microfilaments. However, an association of the aggregates with 10 nm filaments, that precedes their association with SFLS, seems more likely.

Biogenesis and turnover of contractile structures in cultured muscle cells

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Isolated, rod-shaped adult rat cardiomyocytes (ARC) were kept in long-term cell cultures and the changes of cardiomyocyte morphology were investigated by fluorescence staining with RHO-Phalloidin or antibodies specific for contractile proteins. Three-dimensional pictures were reconstructed by artificial shadowing of confocal data sets using the software package IMARIS developed in our laboratory. After isolation, rod shaped cells full of myofibrils and spherical cells devoid of clearly defined sarcomeric structures were observed. During prolonged culture, all cells regenerate myofibrils, and eventually cells contain nonsarcomeric as well as sarcomerically organized cytoskeletal structures. Treatment of cells with drugs like cytochalasin D lead to alteration of cytoarchitecture.

The molecular properties of the different members of the myosin light chain isoprotein family were investigated by transfection experiments using epitope-tagged myosin light chain (MLC) cDNA (Soldati and Perriard (1991) *Cell* **66**, 277–89). The sorting of the different MLC was shown to be isoprotein specific, and with chimeric constructs it was demonstrated that isoprotein-specific incorporation into myofibrils was dependent on the presence of the middle domain of MLC-1f/3f. The members of the MLC isoprotein family can be arranged into a sequence of increasing affinity to myofibrils. A hierarchical order of myofibrillar assembly is postulated.

Similar experiments with constructs containing epitope-tagged α-cardiac, α-smooth muscle, γ-enteric and γ-cytoplasmic actins were carried out. Expression of these epitope-tagged actins in ARC shows that these isoforms are localized at different sites within the cells. While the γ-enteric, α-cardiac and α-skeletal actin showed a marked preference for sarcomeres, the α-smooth muscle isoprotein had an intermediate specificity and could either preferentially be incorporated into stress fibre-like filaments (SFLF) while in other cells it was observed in both SFLF and myofibrils. Most striking results were obtained with γ-cytoplasmic actin: large, even more flattened cells, containing newly induced filopodia filled with the transfected actin, while F-actin was depleted from the perinuclear myofibrillar region were observed.

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Structure of titin-associated proteins

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Immunoelectron microscopic experiments have led to the hypothesis that titin may interact with the thick filament via a set of myosin-associated proteins. One prime candidate was C-protein (Fürst *et al.*, 1989). We have established a fast method for the isolation of C-protein from bovine skeletal muscle. Electron micrographs reveal short rods with a relatively uniform length of about 50 nm. *In vitro* binding assays using radiolabelled C-protein demonstrated strong and specific decoration of myosin rods as well as purified titin II. Antibodies specific for C-protein were used to isolate cDNA clones from a fetal human skeletal muscle λgt11 library. Clone HC38 is 3833 bp long and contains a single large open reading frame encoding a protein of 1138 amino acids. The start of this sequence fits the sequence obtained directly from the N-terminus of bovine C-protein. An alignment of a partial chicken cDNA clone (Einheber and Fischman, 1990) and of all the bovine peptide sequences along the human sequence revealed 50% identity and 70% similarity between avian and mammalian proteins. The specific repeat pattern of domains of the immunoglobulin (Ig) CII or fibronectin type III puts C-protein into the recently defined group of intracellular members of the immunoglobulin superfamily.

The two titin-associated M-line proteins, called 165 K and 190 K protein (Nave *et al.*, 1989) have also been purified and monoclonal antibodies were raised. These antibodies were used to isolate cDNA clones from a λgt11 cDNA library of human fetal skeletal muscle. Further screens of this and a λgt10 library have yielded a series of overlapping clones covering the entire sequence of the human 165 K protein and about 60% of the 190 K protein. A sequence alignment identified both proteins as highly related members in the immunoglobulin superfamily.

There's more to adherens junctions

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One general feature of cells, both in tissues as well as in cultures, is the ability to form contacts with neighbouring cells and with components of the extracellular matrix. A subclass of the latter are the so-called adherens junctions (focal contacts in cultured cells), at which the actin cytoskeleton is connected to the matrix via a protein complex that includes a transmembrane receptor (integrin). These contacts are a central point of interest as sites of transduction for signals that lead to remodelling of the cytoskeleton. We have established a method to isolate focal contacts of cultured cells in sufficient amount and purity to characterize their protein composition directly, using high resolution two-dimensional gel electrophoresis. The protein composition of focal contacts isolated from different cell types proved to be highly conservative. Apart from already known and characterized components (integrins, vinculin, talin and tensin), there was a surprisingly high number of unknown proteins which we have called adherens junction associated molecules (AJAM). Selective extraction and radiolabelling allowed us to classify them as matrix components, cell surface proteins, actin-associated- and membrane-associated proteins.

To characterize these components in more detail we have been using smooth muscle as a source of adherens junction proteins. Smooth muscle extracts, obtained using different conditions, namely water alone, detergent or high pH (at low ionic strength), were analysed on two-dimensional gels and polypeptides matched with those found in fibroblast focal contacts. In addition to known proteins, several new polypeptides were identified that co-electrophoresed with fibroblast AJAMs. These new proteins are now under investigation. On the basis of the extraction experiments and parallel electron microscopic studies of co-extracted muscle strips we could distinguish at least three domains of adherens junctions: the membrane domain, containing integrins and integral membrane proteins; a core domain, associated with the membrane, where tensin is located; and a peripheral domain, probably associated with actin filaments, containing paxillin, vinculin and talin. Putative, new AJAMs were identified in all three domains.

Sequence information about an actin-severing protein from the earthworm *Lumbricus terrestris*

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A Ca^{2+}-dependent 40 kDa type actin severing protein (termed Earthworm Actin Modulating protein, EWAM) from the bodywall of *Lumbricus terrestris* has been isolated and characterized earlier (D'Haese and Hinssen (1987) *J. Comp. Physiol. B* **157**, 615–23). We tried to investigate that protein's domain structure by constructing a muscle-specific cDNA library in a lambda expression vector and by peptide sequencing after LysC-digestion. Sequencing led to five peptides, each of them about 10–15 amino acids in length, which could be aligned to the amino acid sequences of gelsolin, severin and fragmin. Two peptides, separated by about 150 amino acids, lie in highly conserved regions of the modulator, namely the peptides 'KIWRI' and 'FILD'. From these peptides oligonucleotides were designed so they could serve as primers to the polymerase chain reaction (PCR). To facilitate cloning of the possible PCR products recognition sequences of two restriction enzymes were included. The PCR delivered a product of 450 bp, which is the expected size according to the alignment. After ligation into a pUC plasmid vector and cloning, the PCR product was sequenced. Up to now 360 bp of DNA could be determined. The derived sequence of 120 amino acids is discussed in its alignment to the actin modulating proteins gelsolin, severin and fragmin. Like severin and fragmin EWAM can be aligned to the N-terminal half of gelsolin. The deduced amino acid sequence lies in the C-terminal half of EWAM about 30 amino acids away from the actual C-terminus and includes the segment two with its motifs B, A and C according to the terminology of Way and Weeds (*J. Mol. Biol.* **203** (1988) 1127–33).

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Combination of dynamic light scattering and hydrodynamic calculations to elucidate the molecular shape of vinculin in aqueous solution

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The structural protein vinculin has been localized in a wide variety of microfilament–plasma membrane attachment sites (cf. Geiger and Gillsberg (1991) *Cell Motil. Cytoskel.* **20**, 1–6). Vinculin integrity is essential for muscle development (Barsteadt and Waterston (1991) *J. Cell Biol.* **114**, 715–24), but its precise role in this process is not known.

We have studied the dynamics of intact chicken gizzard vinculin and its 94 kDa N-terminal head by quasielastic light scattering. A comparison of the experimentally-determined translational diffusion coefficient with detailed hydrodynamic calculations provided information on the molecular size and shape of vinculin in solution.

The dynamic light scattering results on the 94 kDa head fragment, as obtained by V8 proteolytic cleavage, revealed an almost spherical shape for the N-terminus. The slight anisotropy could be modelled by an oblate ellipsoid with semiaxes $a = 2.82$ nm and $b = 3.10$ nm. This result indicated that the C-terminal domain is primarily responsible for the anisotropic structure of intact vinculin as manifested in its unusually low translational diffusion coefficient. Assuming a rod-like shape for the tail fragment, we performed hydrodynamic calculations on various molecular structures built from spherical subunits. Our dynamic light scattering results are in agreement with the concept of a 'balloon on a string' (Milam (1985) *J. Mol. Biol.* **184**, 543–5) for the molecular shape of vinculin in solution. The existence of dimer and oligomer forms of vinculin can be excluded from our results. A comparison of the dynamic light scattering data with the known sequence data (Coutu and Craig (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8535–9) of vinculin suggests that a proline rich region, found at the beginning of the tail sequence allows for some intramolecular flexibility of vinculin molecules. These molecular properties probably enable vinculin to effectively organize and bundle microfilaments at the cytoplasmic face of the plasma membrane.

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Use of 2.5 Hz and 10 Hz stimulation to examine coordination in the phenotypic response of fast skeletal muscle to increased use

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Transformation of fast muscle into slow muscle by chronic electrical stimulation is brought about by a sequence of profound changes in the expression of proteins involved in the major molecular systems of the muscle. The isoform complements of proteins involved in calcium transport, the generation of ATP, myofilament regulation and force generation are all modified. Recently cDNA probes have been used to study changes in the levels of the corresponding mRNAs. The results indicate that many of the protein changes are the results of regulatory processes taking place at a pretranslational level. It is well known that a fast muscle subjected to continuous 10 Hz stimulation acquires properties which make it indistinguishable from a naturally occurring slow muscle and time courses are available for many of the underlying processes. Changes in the capillary bed, the calcium-handling systems, the oxidative capacity and the myosin isoform profile progress over different time courses. Indeed, the levels of several of the oxidative enzymes show an overshoot to 10 times the control value, declining later to twice the control. During the secondary decline, the myosin isoforms show their greatest rate of change. It is possible that the different systems are interdependent and that the observed phase differences reflect causal relationships. We have found that the rate and extent of transformation are less with stimulation at 2.5 Hz than at 10 Hz. We will present evidence for this, based on maximum force, shortening speed and power output, enzyme levels in key metabolic pathways, metabolite levels measured by HPLC of samples freeze-clamped in situ, and levels of mRNA coding for fast and slow myosin heavy chains from muscles stimulated for 2, 4, 6, and 12 weeks. After 12 weeks of continuous stimulation at 2.5 Hz, the maximum shortening velocity is 68% of control: for 10 Hz the figure is 30%. Nevertheless, the fatigue-resistance of the 2.5 Hz group is as high as that of the 10 Hz group.

We do not yet know enough about the cellular mechanisms underlying stimulation-induced transformation to propose either that the time taken to reach a new stable state would be *longer* with 2.5 Hz than with 10 Hz stimulation (because the signal for change is lower), or *shorter* (because a smaller change would be sufficient to adapt to the new demand). We believe that a careful study of the rate and extent of the modifications of the major molecular systems in muscle with different patterns of stimulation will provide important clues to their possible interaction and degree of coordination.