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Cellular and subcellular expression of myosin mRNA in normal and transforming muscle fibres

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mRNA coding for slow myosin heavy chain I (MHCI) was assessed by *in situ* hybridization in cross and longitudinal sections of paraffin-embedded rabbit skeletal muscles with the use of a non-radioactive, digoxigenin-labelled cRNA probe. The specificity of the hybridization signal was verified by Northern blot analyses and confirmed by the cellular distribution which was strictly confined to type I fibres. In cross-sections of normal fibres, the MHCI mRNA was mainly found in a patchy manner around and between the subsarcolemmal myonuclei. Longitudinal sections displayed a cross-striational pattern with staining of the I-bands. The intensity of the signal decreased towards the fibre core. A similar intracellular distribution was observed in transforming fibres of fast-twitch muscles subjected to chronic low-frequency stimualtion. As compared to normal type I fibres, transforming fibres stained more intensely with dense accumulations of MHCI mRNA in and around the subsarcolemmal myonuclei. In addition, the I-bands were more intensely stained. As compared to normal type I fibres, e.g. in soleus muscle, the staining of the myonuclei and the amount of the message in he perinuclear areas appeared non-homogeneous in the transforming fibres. The number of transforming fibres increased with the duration of chronic low-frequency stimulation, indicating that differences existed between the responsiveness of different fibres. This observation was confirmed by protein analyses performed on samples of the same muscle used for *in situ* hybridization. Thus, the number of fibres expressing MHCI mRNA was correlated with the relative amount of MHCI protein. This correlation supports the notion that the altered myosin isoform expression in transforming fibres is primarily the result of changes at the transcriptional level.

Location of an S1 binding site on an α-helical region of actin

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An 18-residue peptide from actin comprising of residues 77–95 (T_{77}.N.W.D.D.M.E.K.I.W.H.H.T.F.Y.N.E.L_{94}) has been synthesized and purified. According to the recent published actin structure (Kabsch, W. *et al.* (1990) *Nature* **347**, 37–44), residues 79–92 form a surface α-helix at the top of subdomain 1. In aqueous solution at pH 7.8, and over a temperature range 10–26° C, extensive two-dimensional NMR studies have shown that this peptide adopts an α-helical structure from W_{79}-H_{88}. At this point the helix is disrupted but appears to reform towards the C-terminus. Even in the helix-inducing solvent, trifluoroethanol, where the helical regions are strengthened, this helix break is still found. NMR studies have also shown that this peptide binds to S1, predominantly around the helix disruption region and at selected other amino acids. The specificity of this binding is shown by the fact that the peptide is readily displaced from S1 by actin and that the interaction still occurs when the ionic strength is raised to 0.5 M. After iodination with ^{125}I, the peptide was crosslinked to S1 with the zero length crosslinker EDC. Radioactivity was only associated with the tryptic 50 kDa domain and has so far been located in the N-terminal 40 kDa fragment of this domain. The exact interaction site is currently under investigation. Rather interestingly, this peptide does not appear to inhibit or activate the actin-stimulated Mg.ATPase of S1. We have been using this peptide to probe the architecture of S1 by using the distance dependent broadening effect of suitably located nitroxide spin labels on the peptide ^1H-NMR signals. A spin-labelled ADP analogue (Alessi, D. *et al.* (1991) *J. Chem. Soc.* (in press)) in the active site of S1 was found to broaden resonances from the peptide bound to S1 indicating that the two sites are <1.5 nm apart. (The spin-labelled ATP has been shown to be hydrolyzed by S1 and to support muscle contraction.) Furthermore, a nitroxide spin label attached to Cys 707, situated in another actin-binding site on S1, also broadens resonances arising from this actin peptide, emphasizing the spatial proximity of the two actin-binding sites on different domains of S1. Reduction of both spin-labelled moieties *in situ* with ascorbate removes their paramagnetic effects. Comparison of the binding of this peptide to S1 and to an S1.ATP analogue state (pPDM-S1), the latter representing a weak-binding S1.ATP conformation, reveals that the actin peptide binds differently and more weakly to the pPDM-S1. That is, this actin-binding site can distinguish between the strong- and weak-binding S1 states.

Intracellular Mg^{2+} in fatigue of isolated mouse muscle fibres

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The tension decline of single mouse muscle fibres fatigued by repeated tetanic stimulation occurs in three phases: initially tension falls rapidly to about 85% of the control (phase 1), then follows a period of almost constant tension (phase 2) and finally there is a rapid tension decline (phase 3). We have previously shown the tension decline during phase 3 to be caused by reduced Ca^{2+} concentration ($[Ca^{2+}]$) during tetanus, but the cause of this reduction was not clear (Westerblad & Allen (1992) *J. Gen. Physiol.* (in press)). It has recently been shown that a moderate increase of the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) may inhibit the voltage-dependent Ca^{2+}-release from the sarcoplasmic reticulum (SR) (Lamb & Stephenson (1991) *J. Physiol.* **434**, 507–28). As $[Mg^{2+}]_i$ may be expected to rise in fatigue due to breakdown of ATP, we have used Mag-fura-2 to monitor $[Mg^{2+}]_i$.

Mag-fura-2 was pressure injected into single fibres isolated from the flexor brevis muscle of the mouse. Fatigue was produced by repeated 350 ms tetani which were given until tension was reduced to about

30% of the original. Before fatiguing stimulation the mean $[Mg^{2+}]_i$ was 0.66 mM ($n = 5$). During fatiguing stimulation $[Mg^{2+}]_i$ remained almost constant during phase 1 and 2. During phase 3 $[Mg^{2+}]_i$ rose rapidly and reached a mean of 1.12 mM at the end of fatiguing stimulation. A similar tension decline accompanied by increasing $[Mg^{2+}]_i$ was obtained in fibres where the glycolysis and oxidative metabolism were blocked.

In conclusion, the rapid tension reduction at the end of fatiguing stimulation, which is caused by declining tetanic $[Ca^{2+}]_i$, is related to an increase of $[Mg^{2+}]_i$. The impaired SR CA^{2+}-release may be caused by the increased $[Mg^{2+}]_i$ inhibiting the SR Ca^{2+} channels. Alternatively, reduced ATP concentration may impair the function of the SR Ca^{2+} pumps and consequently reduce the SR Ca^{2+}-content.

Channelling of ADP between myosin and creatine kinase co-immobilized onto the surface of a film

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Myosin and creatine kinase from rabbit muscle have been covalently immobilized on Immunodyne film. The couple activity of creatine kinase and myosin ATPase was compared in solution and between films bearing an activity ratio 1, 4 and 22: the efficiency of coupling was very high for all the films and it was very bad in solution. The facilitated diffusion of ADP in the unstirred layer near the film surface explains this efficiency coupling without hypothesis of an intimate coupling between the enzymes. In addition, immobilization mimics the channeling of ATP or ADP observed with myofilaments.

The myofilament lattice during isotonic shortening of intact single muscle fibres of the frog

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Single intact muscle fibres from tibialis anterior muscles of *Rana temporaria* were isolated intact and exposed to an X-ray beam (beam dimensions 0.3 × 4 mm, wavelength 0.15 nm) at the synchrotron facility DESY. The high beam intensity permitted 5 ms resolution of equatorial intensity changes (10 and 11) using averages of 20 tetani. Sarcomere length was recorded using a laser diffractometer system. The time courses of intensity and fibre stiffness changes under isometric conditions have been reported (Cecchi *et al.*, (1991) *Biophys. J.* **59**, 1273–83).

Isotonic shortening of a tetanized fibre caused a lattice expansion which occurred in two phases: a rapid phase associated with the fall of force, and a slower phase while force remained at its isotonic level. Unlike the lattice expansion accompanying the tetanus rise in fixed-end contractions which can be accounted for by changes in sarcomere length, expansion during isotonic shortening could not be wholely explained by changes in fibre length. Upon termination of shortening, the lattice was gradually compressed during tension recovery, which occurred under isometric conditions. In unstimulated fibres, this compression was absent. We therefore propose that these lattice spacing changes result from a radial component of crossbridge force.

The magnitude of this radial component is of importance in choosing models of crossbridge mechanism. The radial force required to compress the relaxed lattice from 43.1 nm to 41.8 nm (the mean spacing change on recovery of axial force from zero to P_0) can be estimated from Matsubara and colleagues (1985; *J. Mol. Biol.* **173**, 15–33) to be 10^{-10} N per thick filament. The corresponding axial force per thick filament in the intact fibre should be 5×10^{-10} N. If both axial and radial forces are orthogonal components of a force vector between the actin and myosin filaments, an angle of 11° between the myosin filament and this force vector at the plateau of the length tension relationship would be required.

Force-velocity-relation of the rabbit inferior oblique muscle: influence of temperature

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The extraocular muscles (EOM) are among the fastest cross-striated mammalian muscles. Isolated inferior oblique muscles (IO) of rabbits were stimulated directly *in vitro* at 20, 25, 30 and 35° C to produce fused tetanic contractions, and they were released at various controlled velocities. The Hill hyperbolic relation, the velocity constant b (in muscle fibre length per s) and the force constant a/P_0 were computed by a non-linear least square procedure. At body temperature the maximum shortening velocity (V_0) of IO was 19.1 ± 2.0 muscle length per s (mean ± SE) corresponding to a maximum shortening velocity of the sarcomer of 57.3 ± 6.0 μm s^{-1}. This figure is comparable with that measured in EOM of rats. In consideration of the diameter of the eyeball and the length of the IO V_0 resembles an angular rotation velocity of the eyeball of 2400 ± 200° per s. Assuming that the resting tension of an EOM is about 10% of P_0 the shortening velocity at that load is about seven muscle lengths per s corresponding to an angular velocity of 850° per s and this figure is of the same order of magnitude as the fastest saccadic eye movement (600° per s). A drop in the temperature of the bathing solution decreases P_0 to 71% and V_0 to 9.3 ± 2.0 muscle length per s at 20° C. The V_0 of the IO decreased with a Q_{10} (temperature coefficient) of 1.5 in cooling from 35 to 25° C and of 1.75 in cooling from 30 to 20° C. These data are comparable to those of fast-twitch rat skeletal muscles. The force constant a/P_0 was not significantly changed in the temperature range investigated.

A-band width in contracted and relaxed intact single fibres, studied by a new quick-freeze apparatus

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It is generally accepted that thick filaments do not change length during muscle contraction. Nevertheless, structural studies have yielded conflicting results. Therefore, we have readdressed this issue in single intact fibres. As chemical fixation may interfere with activation phenomena or otherwise affect thick filament length, we have used quick-freezing followed by freeze-fracture or freeze-substitution to determine A-band width in the electron microscope.

Quick-freezing was accomplished with a newly built quick-freeze apparatus that uses liquid nitrogen cooled cryopliers, which are moved upward pneumatically to freeze the fibre. The fibre is stimulated electrically through two hooks which are also used to keep the fibre suspended in physiological solution. Tension is recorded up to the moment of freezing, and sarcomere length is determined by laser diffraction. Isotonic releases were performed by removing the stop that holds the left hook in fixed position. In this way, the fibre is allowed to pull the hook in the axial direction, thereby lifting a weight that is suspended from a beam, rigidly connected to the hook. Instants of shortening and freezing are precisely timed.

Freeze-fracture appears less suitable for determining A-band width in intact fibres, because of uncertainty in distinguishing the filament ends (Baatsen *et al.* (1987) *Biophys. J.* **51**, 474a). Freeze-substitution in a mixture of methanol, osmium tetroxide, glutaraldehyde and uranyl acetate yielded excellent ultrastructural preservation of intact fibres.

Analysis of sarcomere length changes indicated that several fibres, which were believed to have shortened isotonically, must have been stretched artefactually. Therefore, we found it only justified to pool the results of all fibres. To this end, A-bands were measured with a ruler on calibrated micrographs. Mean A-band width amounted to 1.46 μm in activated and 1.40 μm in relaxed fibres. A significant difference at the 5% level. The difference of these values with the generally accepted A-band width of 1.6 μm may be attributed to shrinkage occurring during the freeze-substitution process. Using thin filament length as a calibration, no more significant difference in mean A-band width could be detected.

Passive force response of isolated frog muscle fibre to fast stretches

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The experiments reported here were made to verify if weakly bound bridges, which have been demonstrated in relaxed skinned muscle fibres (Brenner *et al.* (1982) *PNAS* **79**, 7288–91) are also present in intact frog fibres. Single fibres from tibialis anterior and lumbricalis muscles of the frog were stretched at constant speeds and the passive force response was analysed. A laser diffractometer was used to monitor the actual sarcomere length-changes. The passive force response to ramp stretches includes four different phases: (1) a rapid increase at the beginning of the stretch, (2) a much slower exponential rise, (3) a rapid drop at the stretch end, and (4) a much slower exponential return of the initial level. The analysis of the data shows that: (1) the amplitude of phase 1 (which correspond to the force response of skinned fibres attributed to weakly bound bridges) is proportional to the stretching speed even at speed higher than 10^5 nm per half-sarcomere per second, (2) the delay between the speed of sarcomere-length changes and force response during the acceleration period is < 10 μs, and (3) the amplitude of phase 1 increases with increasing the sarcomere length. These results show that phase 1 is very likely a viscous response whose amplitude is inversely proportional to the extension of myofilament overlap. This suggests that weakly bound bridges are not present in significant amount in passive intact fibres.

Tetanus inactivation by Verapamil related to $[Ca^{2+}]_0$ in frog single skeletal muscle fibres

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Dihydropyridine-receptor, a protein localized mainly in the T-tubule-membrane (Borstotto *et al.* (1984) *Eur. J. Biochem.* **142**, 449), has been proposed as the voltage-sensing molecule for E-C coupling in skeletal muscle fibres. this protein may enter an activated state that triggers Ca^{2+} release from the SR, and an inactivated state which is stabilized by low $[Ca^{2+}]_0$ and by binding with Verapamil and other calcium blockers (Brum *et al.* (1988) *J. Physiol.* **398**, 475).

We report that inactivation of isometric tetanic contraction by Verapamil 30 μM persists longer in the presence of low $[Ca^{2+}]_0$ solutions. Experiments were performed at 18° C on single muscle fibres isolated from the anterior tibialis muscle of frog (for technical aspects see Cecchi *et al.* (1984) *Pflüg–Arch.* **401**, 396). Fibres were tetanically stimulated with train pulses of 240 ms duration and 100 Hz frequency. The duration of inactivation was estimated by evoking a twitch at various times after the train pulses.

When fibres were treated with Verapamil 30 μM in presence of low $[Ca^{2+}]_0$ (300 μM) solution, inactivation lasted 2.6–37.5 times, as compared with that induced by Verapamil in normal Ringer.

These results show that in frog muscle fibre the activity of the DHP-reactor can be modulated by $[Ca^{2+}]_0$.

Troponin gene expression in the developing and adult human heart

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The troponins I, T and C (TnI, TnT and TnC) form a protein complex on the thin filament of striated muscles which acts as a calcium-sensitive molecular switch involved in regulating muscle contraction. Three isoforms of troponin I have been identified and are the major isoforms present in cardiac muscle (TnIc), fast skeletal muscle (TnIf) and slow skeletal muscle (TnIs). These isoforms are encoded by three separate genes and there is no evidence for alternative splicing. We have cloned a full length cDNA encoding the human cardiac isoform (Vallins *et al.*, (1990) *FEBS Lett.* **270**, 57–61) and have used this, together with a PCR-derived TnIs probe and antibodies specific for troponin I isoforms, to examine expression in the developing and adult human heart.

Monoclonal antibodies which react with all three troponin I isoforms, detect a predominant isoform in early fetal heart ($\leqslant 20$ weeks gestation) which is distinct from the adult cardiac isoform TnIc, but which has the same electrophoretic mobility as TnIs. Later samples ($\geqslant 24$ weeks) contain the same protein with increasing amounts of TnIc. By 9 months postnatal development TnIc is the only detectable isoform. All fetal samples tested contain mRNA for TnIs supporting identification of the development isoform as TnIs.

Although there is a clear transition in the level of accumulation of these proteins, the mRNAs encoding TnIc and TnIs appear equally abundant throughout fetal development. The increase in TnIc protein accumulation which occurs in late fetal development is not, therefore, regulated through increased mRNA accumulation. After birth, TnIs mRNA decreases and is absent by 9 months postnatal development, following the level of TnIs protein. Normal adult cardiac muscle does not contain TnIs but we have detected TnIs protein in several pathological samples including those from right ventricular pressure-overlaod, dilated cardimyopathy and ischaemic heart disease.

In conclusion, we have demonstrated the expression of a fetal isoform of troponin I during human cardiac development. This fetal isoform is probably the same protein as that found in adult slow skeletal muscle (TnIs). Furthermore, we have shown that the developmental regulation of troponin I expression probably involves both transcriptional and post-transcriptional mechanisms, and that the fetal isoform is re-expressed in several cases of cardiac pathology.

Implication of short actin sequences in actin binding protein (ABP) interfaces

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Extensive studies have established that actin fragments support a large part of the binding properties of actin towards various ABPs, which are able to interact with G-actin (i.e. myosin head, DNase I, Vitamin D binding protein (DBP), gelsolin, α-actinin, filamin . . .).

Our investigations, using immunochemical probes, fluorescence analysis and affinity chromatography, demonstrated that a limited number of small actin peptides would carry the binding activity.

Taking into account the crystallographic model of actin-DNase I complex (Kabsch *et al.* (1990 *Nature* **347**, 37–44), we located these peptides near the N- and C-terminal extremities or in exposed loops of the subdomain I (myosin head, DBP, α-actinin, filamin) and the subdomain III (gelsolin).

Thus, the 1–7, 18–28, 95–113 and 360–372 sequences are implicated in myosin head interface, the 123–132 and 360–372 sequences

in the α-actinin interface, the 105–120 and 360–372 sequences in the filamin interface, the 305–326 sequence in the gelsolin interface and the 360–372 sequences in the DBP interface.

The binding properties of the related natural or synthetic peptides has been shown to be affected by regulation factors as Mg^{2+} pyrophosphate (myosin head), Ca^{2+}/EGTA (gelsolin) and Mg^{2+}(α-actinin).

Myosin crossbridge: two heads or a pair of legs?

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Force-generating step of the crossbridges in single skinned fibres from rabbit psoas muscle with myosin heads covalently crosslinked to actin with EDC was synchronized using Joule temperature jump (T-jump) from 4–6 to 29–37° C (Bershitsky and Tsaturyan (1985) (1989). The crosslinked fibre was washed with a high-salt (I.S. = 0.6 M) relaxing solution (HSR) where unlinked heads were detached from actin. After 30 min treatment with EDC at 11–13° C and pH 7.1 (partial linking), the fibre stiffness in the HSR solution was 0.35–0.45 of that in the rigor one. As EDC crosslinks two myosin heads independently (Tawada and Kimura (1986), a significant fraction of the cross bridges was linked with only one head in the partially crosslinked fibre. After the partially linking, the T-jump induced a tension rise with the rate constant of 250–400 s^{-1} in the HSR solution. The tension increment was identical to that induced by the fibre stretching by up to 8 nm per a half-sarcomere in the same solution. After 120–150 min linking, the fibre stiffness in the HSR solution was about the same as that in the rigor one showing the most myosin heads were crosslinked. Under the complete linking, the T-jump induced only an immediate tension drop due to the fibre thermal expansion in both HSR and rigor solutions. Crosslinking with EDC does not prevent the head mobility (Svensson and Thomas (1986) and only slightly decreases the ATPase activity in the fibre (Glyn and Sleep (1985)). Thus, the data presented here show that crossbridge cannot make the force-generating step when both its heads are attached to actin. Therefore, this two heads have to move along the thin filament(s) similar to a pair of alternately stepping legs.

Specific crosslinking of the SH1 thiol of skeletal myosin-S-1 to F-actin and G-actin

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Previously, we reported that maleimidobenzoyl G-actin (MBS-G-actin), which was resistant to the salt-and-myosin S-1-induced polymerizations, reacts reversibly and covalently in solution with S-1 at or near the F-actin binding region of the heavy chain (Bettache *et al.* (1989) *PNAS* **86**, 6028). Here, we have readily converted the MBS-G-actin into MBS-F-actin in the presence of phalloidin and salts. The binding of S-1 to the two actin derivatives, which carry a free reactive maleimidobenzoyl group, was investigated comparatively in crosslinking experiments performed under various conditions to probe further the molecular structure of the actin-heavy chain complex before and after the polymerization process. Like MBS-G-actin, the isolated MBS-F-actin, which does not undergo any intersubunit crosslinking, bound stoichiometrically to S-1 generating two kinds of actin-heavy chain covalent complexes migrating on electrophoretic gels at 180 h and 140 kDa. The relative extent of their production was dependent on pH for both G- and F-actins. At pH 8.0, the 180 kDa species were predominant and at pH 7.0, the amount of the 140 kDa adduct increased at the expense of the 180 kDa entity. The 140 kDa complex was suppressed by blocking Cys-707 (SH1) in S-1 but not all by the specific modification of Cys-697 (SH2). The addition of MgADP (1 mM) increased selectively the yield of the 140 kDa product with both native S-1 and SH2-blocked S-1. The crosslinking of the MBS-actins to split S-1 showed the conjugation of actin to the 50 kDa fragment in the 180 kDa species and the 20 kDa fragment in the 140 kDa derivative. Finally, the crosslinking of MBS-F-actin to S-1 led to the superactivation of the Mg ATPase subtantiating its ability to stimulate the S-1 ATPase as the native protein. Collectively, the data suggest that in the G-and-F-MBS-actin-S-1 complexes, the lysine on actin carrying the maleimide arm is within 0.9–1.0 nm from two different S-1 heavy chain segments, one of which includes the SH1 thiol, and which may be spatilly related forming together a unique actin recognition site in S-1.

Characterization of viscous and elastic properties of crossbridges

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From step changes in length of a skeletal muscle fibre and measurement of the resulting force transient the viscoelastic impedance of the half-sarcomere can be determined as a function of frequency. The procedure is applied to tension transients measured in frog fibres skinned by freeze-drying. For fibres in rigor the amplitude of the impedance increases with a power of approximately 0.15 of the frequency and phase shift is constant over a range in frequency from about 100 Hz to at least 50 kHz. It was investigated if this phase shift was dependent on viscosity of the incubation fluid. Therefore, a rigor solution was used with 20% w/v glucose added, which doubles viscosity of the solution. The procedure of determination of the viscoelastic impedance contains correction for viscosity and density of the incubation medium outside the fibre. As a result, effects from the viscosity of the fluid within the half-sarcomere can be explored. No difference could be detected in the impedance determined from measurements with the fibre in rigor (sarcomere length: 2.15 μm, 4° C) and with the fibre, transferred subsequently to the rigor/glucose solution. Apparently the viscous effect observed in rigor is not from interaction of cross bridges and/or filaments with the surrounding fluid. Available data indicate (see also poster of M.E.M. de Winkel e.a) that this viscoelastic effect of the fibre in rigor is the result of properties of the crossbridges. Consequences of the viscoelastic compliance of rigor cross bridges in connection with the crossbridge cycle during activation as to crossbridge range and time course of relaxation of the compliance will be discussed.

Experimental model of mechanical non-homogeneity of myocardium and its dependence on the stimulation frequency, Ca-ions concentration and catecholamines

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Two papillary cat muscles connected in a parallel duplet were used as an experimental model of mechanically non-homogeneous myocardium (Blyakham *et al.*, Pat. SU1560094A1). The physiological regimes of loading was applied either to each muscle of duplet or to the complete duplet. In the first series of experiments, inotropic factors were applied to the duplets consisted of the muscles with about equal lengths, cross-sections, similar slope of force-length and force-velocity relationships (homogeneous duplet). In the second series, the same inotropic factors were applied to duplets with original mechanical non-homogeneity. It was resulted that in homogeneous duplets the mechanical characteristics each of the duplet muscle became different under the inotropic factors (i.e. the duplet became non-homogeneous).

For example, the shortening increase ΔL under 0.5 P_0 for one of muscle of duplet was 35% and 80% for another when 10^{-7} M of epinephrine was added; 25% and 48% when Ca^{2+} was increased from 2.3 to 4.6 mM; 16% and 32% when the stimulation frequency was increased from 0.3 to 1.0 Hz. However, under the same factors the duplet ΔL values at 0.5 of duplet P_0 were significantly lower (10%, 7% and 11% respectively). In the second series, it was founded that at the same inotropic factors the paradoxical effects were observed. For example, when epinephrine was added or the frequency increased under the same relative load the duplet ΔL can be even lower than before positive inotropic factors. Thus, in non-heterogeneous myocardium the effect of positive inotropic factors can be either decreased or even inverted. This depends on the loads redistribution between muscles in duplet, differences of time course on contraction-relaxation cycle, and so, on asynchronism of developing force and shortening of muscles in duplet.

Unimpaired Ca^{2+} homoestasis is found in dystrophin-lacking smooth muscle from mdx mice

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The genomic lesion underlying Duchenne-type muscular dystrophy (DMD) in humans and mouse mutants (mdx) results in the absence of a cytoskeletal-like protein, dystrophin. How muscle dystrophy evolves from this defect is still unknown. In mdx striated muscles an increased cytosolic Ca^{2+} concentration has been described, associated with an increased protease activity. If the absence of dystrophin was responsible for anomalous leaks to Ca, $[Ca^{2+}]^i$ would have to increase in a much more dramatic way in smooth muscle cells than in striated muscles because of the very large surface-to-volume ratio of the former. We present here the first study of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$ in dystrophin-lacking smooth muscle from mdx mice. We compred the cytoplasmic calcium concentration, monitored by the fluorescent Ca-indicator Fura-2, in the muscular wall of the vas deferens from mdx mice and their genetically-related control animals (C^{57}). The experimental protocol aimed at measuring values and kinetics of $[Ca^{2+}]_i$ (1) in the resting stage, (2) in experimental conditions with either an increased (10 mM $[Ca^{2+}]_0$) or an inverted (2 mM $[EGTA]_0$ electrochemical gradient for Ca^{2+}, (3) in activating conditions, obtained by prolonged depolarization in isotonic KCl solution, by application of P_{2x}-purinergic and α-adrenergic agonists and by transmural electrical stimulation. In normal krebs, $[Ca^{2+}]_i$ was 107 ± 4 nM in mdx ($n = 50$) and 103 ± 3 in C_{57} ($n = 53$); the increase of $[Ca^{2+}]_0$ to 10 mM changed those values to 133 ± 13 and 129 ± 15 nM respectively. Peak values were similar for mdx and C_{57} during contractions by 140 mM K^+ (288 ± 49 and 293 ± 68 nM) or by pulse trans for 5 s (179 ± 8 and 180 ± 9 nM). Moreover, in this last condition the kinetics of Ca^{2+} decay during relaxation were identical for both strains ($t_{1/2}$ of 1.16 ± 0.01 s and 1.21 ± 0.02 s). Our results thus indicate that dystrophin-lacking smooth muscle can maintain normal values of $[Ca^{2+}]_i$ at rest and react normally to changes of the electrochemical gradient of Ca^{2+}. Also the amplitudes and the kinetics of fast or slow intracellular Ca^{2+}-transients are similar in both strains independently of the mode of activation. No progressive Ca overload could be observed after prolonged or repeated contractions. The intracellular Ca homeostasis appears therefore to be unimpaired in this mdx smooth muscle.

Photochemical crosslinking of the skeletal actin-myosin head complex

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To better characterize the actin-myosin interface, we have developed a new photochemical crosslinking of the F-actin-myosin head (S1) complex using a photoactivable heterobifunctional crosslinking reagent, the p.azidophenyl glyoxal (APG, span = 9.3 A). This crosslinking reagent was chosen for its specificity towards Arg residues via the glyoxal moiety (new crystallographic and electron microscopic data have suggested the involvement of Arg residues of actin in the F-actin-S1 interface), and for its non specific reactivity associated with the nitrene part.

The crosslinking reaction was carried out in a two-steps process to specifically modify the Arg residues of the actin molecule and to protect the ATPase function of S1. During the first step, APG was let to react with F-actin overnight in the dark. After removal of the excess of reagent by ultracentrifugation and addition of S1, the crosslinking reaction was initiated, during the second step, by photoactivation of the nitrene moiety of APG under ultra-violet flash at 365 nm. Analysis of the actin-S1 crosslinked products by SDS PAGE showed four major types of actin-S1 heavy chain complexes with apparent MWs of 165 K, 175 K, 200 K and 240 K and one actin-light chain 1 complex migrating with an apparent MW of 70 K. Actin modification by APG did not affect its binding properties or the F-actin induced activation of the Mg^{2+}-ATPase of S1. On the other hand, the formation of the covalent actin-S1 complexes was correlated with an increase of the S1 ATPase activity. Based on crosslinking yields of 13–25% (depending on experimental conditions), we could determine an averaged turnover rate for these covalent complexes of $33 \pm 8\ s^{-1}$ ($n = 9$) which is similar to the values obtained at infinite F-actin concentration as well as to those obtained with other covalent complexes (Bertrand *et al.* (1988) *Biochemistry* **27**, 5728–36). Using various proteolytic S1 derivatives associated with fluorescent and immunochemical detection of the cross-linked products, we found that the crosslinking occurs between F-actin and the central 50 K and C-terminal 20 K fragments of S1. Moreover, the heterogeneity of the crosslinking products obtained with the 50 K containing fragments reveals that F-actin was covalently linked to various segments of the 50 K fragment. Finally, the modification of F-actin by a fluorescent analogue of APG, the 7-diethylaminocoumarin-3-glyoxal, showed that only 1–1.2 Arg residues of F-actin were reactive towards the glyoxal-carrying reagent and therefore were candidate for crosslinking to S1.

The precise identification of the residues involved in the crosslinking as well as the effect of the nucleotides and the regulatory proteins on the crosslinking pattern is now under investigation.

Structural kinetics of frog muscle during isometric contraction by time resolved two-dimensional X-ray diffraction

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Structural events associated with tension generation have been studied by time resolved X-ray diffraction under conditions where initially three-dimensional order in the contractile apparatus is either present or absent. In both cases layer lines appear owing to the attachment of myosin heads to actin at specific sites and with a configuration that differs from that seen in rigor. The data clearly reveals the presence of two distinct states for the attached myosin heads, respectively in a non-tension and a tension generating configuration. Throughout tension generation asynchronous axial motions of the myosin heads are not significantly different from those at rest. Analysis of the two-dimensional X-ray diffraction patterns (10 ms time resolution); (Bordas *et al.* (1991) *Adv. Biophys.* **27** (in press)) suggests the following sequence of events. (1) A very early activation phase involving motions and/or conformational changes of troponin and tropomyosin. (2) An order/disorder transition arising from the filaments losing their axial alignment relative to each other and the myosin heads rotating azimuthally and moving radially towards the actin filaments.

(3) Attachment of the myosin heads at specific sites of the actin filament in a non-tension generating state. (4) A tension of generating phase, characterized by a change in orientation of the myosin heads towards a more perpendicular orientation relative to the muscle axis. (5) A relaxation phase during which the myosin heads return to an axial spacing and axial orientation similar to that at rest with a time course paralleling tension, but in which the full recovery of the three dimensional order characteristic of the rest state returns much more slowly. Each of these phases have different kinetic rates and are present in differing degrees throughout the diffraction pattern. The two 'attachment' conformations may be ascribed to the weak and strong binding states observed in solution but the kinetic rates have to be modified to include the lag time owing to activation and the structural constraints imposed by the structure of the filaments.

Ca^{2+}-dependent effects of myosin subfragment-1 on the structure of 1,5-IAEDANS-labelled tropomyosin in regulated thin filaments of ghost fibres

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Earlier studies using polarized microphotometry have shown that tropomyosin modulates the alterations in structure of actin in ghost fibre that take place on the binding of myosin heads. These alterations were Ca^{2+} -dependent in the presence of troponin or calmodulin-caldesmon complexes (Dobrowolski *et al.* (1988) *Biochem. Biophys. Acta* **956**, 140–150). In an attempt to understand the mechanisms of governing the alteration in actin structure we have studied the Ca^{2+}-dependent effects of myosin subfragment-1 (S-1) on the structure of 1,5-IAEDANS-labelled tropomyosin bound to actin in ghost fibres. Ca^{2+} regulated thin filament was reconstructed in ghost fibres by incorporating either caldesmon-gizzard tropomyosin-calmodulin or skeletal muscle troponin-tropomyosin complex. The results show that in the presence of caldesmon and calmodulin, S-1 causes Ca^{2+}-dependent alterations of smooth tropomyosin conformation and flexibility similar to those induced by S-1 in skeletal tropomyosin in the presence of troponin. In both cases, S-1 binding changes the angular distribution of tropomyosin relative to the fibre axis and decreases random movement. Ca^{2+} increases this effect. The decreased mobility of tropomyosin in ghost fibres is best explained by assuming that S-1 strengthens the bonding between F-actin and 1,5-IAEDANS-labelled tropomyosin in ghost fibres.

It is suggested that the basic physiology role of tropomyosin is to modulate the alteration of actin structure. The mechanism of the modulation seems to be related to the changes of the tropomyosin-actin interaction.

Hormonal excess and deficiency conditioning the water-protein inter-relationship in skeletal muscle and myocardium

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It has been observed that the water-protein inter-relationship can be modified as a function of many pathological states of the body. In the present work we tried to find such possible modifications in skeletal muscle and myocardium obtained from Wistar rats acute and chronically treated with high levels of hormones known to affect the water content of the tissues, and producing alteration of function as well.

We have measured ^{1}H-transverse, relaxation time in skeletal muscle and myocardium of animals treated with thyroid, certioesteroid and androgen hormones. We also have tested RNA and protein synthesis and the state of the receptors for the above mentioned hormones. We have also taken into account the age influence on those phenomena using in our tests in 6-month and 22-month-old animals.

The results showed an age dependency of all studied parameters. We also observed the T_2 (long) modification in testosterone and hydrocortisone treated animals. Dose-dependent proton transverse relaxation time variations were proved.

Dependence of force and shortening velocity on MgATP concentration in skinned rat ventricular trabeculae

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The relationships between MgATP concentration and isometric force and maximum shortening velocity (V_{max}) were investigated in thin trabeculae from the right ventricle of young rats. The preparations were chemically skinned and mounted by means of aluminium clips between a force transducer and an electromagnetic puller. Sarcomere length was set at 2.1 μm. The trabecula was activated by transferring it from a calcium-free relaxing solution first into a preactivating and then into an activating solution (pCa 4.45, 12° C). In each activation the trabecula was subjected to 10–15 load clamps to obtain the complete force-velocity curve. The latter was fitted to Hill's equation and the value of V_{max} was determined. After activation the preparation was returned to the relaxing solution and the Na_2ATP concentrations of the preactivating and the activating solution were changed. The Na_2ATP concentration was varied from 0.01 to 12 mM between different activations and the respective force-velocity relations were determined. V_{max} increased with the ATP concentration in a hyperbolic fashion. Michaelis-Menten equation was fitted to the relation between V_{max} and ATP concentration and a K_m of 53 μM was obtained. This value is considerably lower than what has previously been reported for skeletal muscle (0.25 mM; Cooke and Bialek (1979) 0.47 mM, Ferenczi *et al.* (1984)). Maximum isometric tension was related at the ATP concentration in a biphasic manner, with a peak between 100–500 μM. At higher ATP concentration tension declined steeply, whereas at lower concentrations the decline in tension was less pronounced. The results indicate that cardiac muscle is less sensitive to changes in ATP concentrations than mammalian or amphibian skeletal muscle.

Myosin heavy chain and myosin light chain composition and shortening velocity in skinned fibres of rat skeletal muscle

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Eighty-seven single chemically skinned muscle fibres were dissected from soleus, plantaris and tibialis anterior muscles of the rat. From each fibre two consecutive segments about 2 mm long were cut. One segment per fibre was maximally activated at 12° C, 2.5–2.7 μm sarcomere length, and subjected to load clamps to obtain force-velocity relations. Maximum velocity of shortening (V_{max}) was calculated using Hill's hyperbolic equation. V_{max} values ranged between 0.40 and 2.81 L s^{-1}. The fibre segments used for mechanical experiments were thereafter characterized by monoclonal antibodies (Mabs) against myosin in heavy chains (MHCs). The second segment of each fibre was studied as regards MHC and myosin light chain (MLC) composition by SDS-PAGE electrophoresis. Combining the results obtained by Mabs and SDS-PAGE it was possible to characterize fibres according to their MHC content very precisely. Among the 87 fibres studied, 12 fibres contained one MHC, eight fibres 2A MHC, six fibres 2B MHC, 20 fibres 2X MHC, three fibres 1 and 2A MHC,

27 fibres 2B and 2X MHCs, 11 fibres 2A, 2B and 2X MHCs. The mean V_{max} values ± SD (standard deviation) for the seven groups were: 0.596 ± 0.103 for type 1 fibres, 1.739 ± 0.466 for type 2A, 1.574 ± 0.395 for type 2B, 1.692 ± 0.409 for type 2X, 0.581 ± 0.110 for type 1-2A, 1.695 ± 0.558 for type 2B-X, and 1.540 ± 0.555 for type 2A-B-X. Differences were statistically significant between type 1 and type 1-2A and the other types; differences were not statistically significant among fibres containing MHCs fast: 2A, 2B and 2X. The ratio between MLC3f and MLC2f, was studied in 45 fast fibres of known MHC content and V_{max}. MLC3f to MLC2f ratio can be used as an index of the relative content of the two alkali light chains (MLC1f and MLC3f). No relation was found between V_{max} and MLC3f/MLC2f pooling all fast fibres together. Considering only fibres containing 2X MHC a positive relation was found between V_{max} and MLC3f content; however the slope of the linear regression did not reach statistical significance. Our results seem to suggest that V_{max} of fast fibres is more likely related to alkali MLC than to fast MHC content.

Dynamic actin interaction of force-generating crossbridges. Implications for crossbridge action in muscle

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We previously demonstratred that in skinned segments of rabbit psoas muscle fibres under relaxing conditions, in rigor, or in the presence of nucleotide analogues crossbridges dynamically interact with actin, i.e. dissociate and reassociate from and to actin rapidly on the time scale of active turnover (Brenner *et al.* (1981) *Proc. Natl. Acad. Sci. USA* **79,** 7288–91; (1986) *Biophys. J.* **50,** 685–91). From preliminary stiffness measurements on Ca^{2+}-activated fibres we proposed that similar dynamic actin interaction also occurs while crossbridges occupy force-generating states (Brenner (1986) *Basic Res. Cardiol.* **81,** 1–15 (1986); *Ann. Rev. Physiol.* **49,** 655–72). We established conditions where other reactions, e.g. transitions between the various attached force-generating states (Huxley and Simmons (1971) *Nature* **233,** 533–8) are insignificant and thus do not contribute to the force response to imposed length changes (stretches). From plots of force *versus* imposed length change, recorded under these conditions, we can demonstrate that crossbridges in the force-generating states also dynamically interact with actin and apparently resume contribution to force generation rapidly after reattachment (Brenner (1988) *Pflügers Arch.* **412,** R79). From stiffness-speed relations (Brenner *et al.* (1986) *Biophys. J.* **50,** 685–91) we can derive rate constants for dissociation between 50–1000 s^{-1}, while the rate constant for reassociation is at least an order of magnitude larger (high actin affinity). We will present experiments for testing whether such dynamic actin interaction is limited to one of the force-generating states or may rather be a general feature. Implications for crossbridge action in muscle will be discussed.

Reversible inactivation of ATPase properties of myosin subfragment 1 by reaction with mercuric chloride

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The reaction of mercuric chloride with myosin subfragment 1 (S1) has been examined. After modification with a 1 M excess of mercuric chloride, reverse phase HPLC analysis of tryptic S1 showed that the 50 kDa tryptic fragment was converted into two fractions eluting at lower acetonitrile concentrations, signifying that this domain of the modified protein behaved as though it was more hydrophilic than its unmodified counterpart. Premodification of SH1 and SH2 with N-ethylmaleimide has no effect on the subsequent changes induced by reaction with mercuric chloride demonstrating that these changes occurred without the participation of these thiols. Modification of the mercuric chloride treated protein with IAEDANS showed substantial labelling of the 20 kDa fragment but some label at the 50 kDa could also be observed. Furthermore, limited tryptic digestion of the mercuric chloride treated protein, indicated that the 50 kDa was now vulnerable to attack. These data suggest that the structure of the 50 kDa segment is perturbed and that this perturbation involves the region between cys-402 to cys-540. This finding substantiates previous work by others that changes in the 50 kDa segment of the protein can lead to inactivation of the ATPase properties of S1 and indicates the importance of this domain for expression of the ATPase properties of the protein. Treatment of the inactivated protein with 0.05 M DTT at 4° C for 90 min resulted in regain of activity and the reconversion of the 'modified' 50 kDa forms observed on HPLC to the unmodified form of this fragment. The finding that there are two modified forms suggests that these may arise from sulphydryl-Hg-sulfhydryl bridges involving the three thiols located in this segment of S1.

Sources of rapid elasticity in intact frog cardiac cells

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Examination of the quick change in tension following the imposition of fast length steps to skeletal muscle fibres led to the conclusion that a rapid component of elasticity is located in the crossbridges (Ford *et al.* (1977)). Similar experiments have been performed on cardiac preparations (Steiger (1977)) but no clear results could be obtained mainly because of the complex nature of multicellular heart preparations. An attempt is made in this study to examine the behaviour of the rapid elasticity of cardiac muscle using single intact cells enzymaticlly isolated from frog heart. Cells were held horizontally at slack length (s.l. around 2.10 μm) by two suction micropipettes in Ringer soution. Tension was measured by photoelectrically recording the elastic deflection of one of the micropipettes, which acted as a cantilever force probe (frequency response 600–900 Hz). Length changes complete in 2 ms were applied just before the peak of an isometric twitch by means of a loudspeaker-coil motor. The tension change during the length step was an apparently elastic response whose characteristics were analysed by plotting the extreme tension (*T1*) reached during the step against the step amplitude. The resulting force-extension relation was linear for small steps and reached the length axis between −1 and −2% cell length (*lo*). The extrapolation from the linear part of the curve intercepted the length axis between −0.7 and −1.2% *lo*. Both the observed and extrapolated values from the amount of shortening required to bring tension to zero are much smaller than those previously reported for multicellular heart preparations. The force-extension curve of a cell was independent of the amount of developed force suggesting that the largest part of the rapid elasticity of cardiac cells resides in the crossbridges.

Characterization of Caldesmon C-terminal actin binding polypeptides

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We have suggested earlier that caldesmon inhibits actin activated ATPase activity of myosin by competing with the weak myosin-ATP interaction with actin (Chalovich *et al.* (1987) *J. Biol. Chem.* **265,** 5711–16) Caldesmon also inhibits the potentiation of ATPase activity caused by tropomyosin. The competition of caldesmon with myosin for binding to actin has been confirmed by the effects of caldesmon and its proteolytic fragments on the mechanics of single psoas fibres (Brenner *et al.* (1991) *PNAS* (in press)). We have now characterized two caldesmon derived polypeptides which have many similarities to intact

caldesmon but which differ in the stoichiometry of binding. Fragments with M_r of 20 kDa begin near Lys 579 and the 8 kDa fragment begins at residue 597 in the caldesmon sequence. While we observed a stoichiometry between 1 : 7 and 1 : 10 for the binding of caldesmon to actin in solution (Velaz *et al.* (1989) *J. Biol. Chem.* **265**, 2929–34) we now find that the 20 kDa and 8 kDa fragments bind with stoichiometries of 1 : 2 and 1 : 1 (or 1 : 2), respectively. The binding of intact caldesmon, but not the fragments, to actin is enhanced by tropomyosin. Despite these differences, caldesmon and the 20 and 8 kDa fragments inhibit ATPase activity in a calcium-calmodulin dependent manner and are competitive with the binding of skeletal S-1-ATP to actin. These results suggest that the region of caldesmon from residue 597 through about residue 688 (estimated from molecular weight) are important in the function of caldesmon.

Calcium fluxes and cholesterol uptake in three types of muscle under the affect of D_3 vitamin and calcitonin

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Vitamin D and calcitonin influence the calcium metabolism in some key points relatively well known today. However, there are important reasons to presume that these molecular agents also act at the level of the membranes of various cells, not necessarily specialized in the body equilibration as concerns the calcium balance. Our investigations were carried out on skeletal, myocardial and aortic tissues obtained from control animals and from animals exposed to high levels of D_3 and calcitonin, for different periods. On the explanted biological material calcium incorporation and cholesterol uptake were tested.

Calcium fluxes decreased under D_3 in the skeletal muscle, increases in myocardium and remain unchanged in aorta. Calcitonin produces increases of calcium uptake in the skeletal and cardiac muscle tissues and practically does not influence calcium distribution in aorta. D_3 generates a clear cut increase of cholesterol accumulation in aorta.

Creatine kinase binding and possible role in the guinea pig carotid artery

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Activity and role of creatine kinase (CK) associated with contractile proteins of smooth muscle have been investigated using skinned guinea pig carotid artery. Total CK activity was 47.0 ± 9.3 U g^{-1} w/w and agarose electrophoresis showed BB, MB, and MM isoforms (BB-CK being the predominant isoenzyme). After membrane solubilization for 1 h with triton X-100, CK was specifically associated with the myofibrils, representing 22% of the preskinned CK activity. When relaxed fibres (approximately 300 μm) were exposed to pCa 9 in the presence of 250 μM ADP, 0 ATP, and 12 mM PCr, tension was not significantly different from rest tension, but changing to pCa 4.5 caused the fibres to generate 45.9 ± 4.5% of maximal tension, a similar response was observed in the absence of exogenous ADP. However, no significant increase in tension was seen with fibres exposed to fluoro-1-dinitro-2,4 benzene (an inhibitor of CK). When a high-tension rigor state was achieved (250 μM ADP, 0 ATP, 0 PCr, pCa 9 and then pCa 4.5), the addition of 12 mM PCr effected significant relaxation (67.1 ± 6.3% of control). Exposing the fibres to rigor conditions from resting tension did not induce a contraction.

These observations implicate that an endogenous form of CK is intimately associated with the myofilaments of the Triton X-100 skinned carotid fibres and that it is capable of producing ATP from ADP for submaximal tension generation as well as significant relaxation from rigor conditions. It was also shown that ADP is bound to the myofibrils and available for rephosphorylation by CK. These results suggest co-localization of ATPase, MLCK, and CK on the contractile proteins of the carotid artery with ADP. This enzymic association may play a role in the compartmentation of adenine nucleotides in smooth muscle.

Mechanical power output by the red muscle fibres of dogfish

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Most of the muscle mass in dogfish consists of white muscle fibres used during short bursts of swimming. In addition, there is a thin layer (only a few mm thick) of red muscle fibres just under the skin on the sides of the fish; these are active during slower, substained swimming.

The mechanical and energetic properties of fibre bundles were investigated by measuring force and heat production during sinusoidal movement. The amplitude of the movement was kept constant at about 10% of the fibre length. The stimulation was a brief tetanic burst lasting for 33% of each cycle of movement. The timing of the stimulation within the movement cycle and the frequency of sinusoidal movement were varied to find the conditions optimal for mechanical power output and efficiency (mechanical power/total energy output).

We have previously reported (Curtin and Woledge (1991) *J. Muscle Res. Cell. Motil.* **12**, 95) that for white fibres, peak mechanical power is produced when the frequency of movement (about 4 Hz) is higher than that giving peak efficiency (about 2 Hz).

We now report measurements for red muscle fibres. These fibres produced maximum power at a lower frequency of movement (about 1 Hz) than white fibres (about 4 Hz). The red fibres appear to produce heat at only a modest rate compared with their power output, indicating that these fibres are efficient at converting chemical energy into useful mechanical power.

Immunolocalization of dystrophin skeletal muscle

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Identification of the exact position of dystrophin in the skeletal muscle cell is an essential step towards understanding its function. We have been using antibodies to different areas of the dystrophin molecule to gold-immunolabel cryosections of human biopsy material to try to identify where each domain lies in relation to the plasma membrane. Initial studies using antibodies (Dy4/6D3) to the spectrin-like domain of the molecule showed that this rod section is situated close to (approximately 15 nm) the cytoplasmic face of the membrane. The repeat spacing of the molecules is approximately 120 nm) (Cullen *et al.* (1990) *Proc. R. Soc. Lond.* **B240**, 197–210). Gold labelling of an antibody (Dy8/6C5) to the last 17 amino acids of the C-terminus showed the probe most commonly located over the membrane itself, towards the outer face (Cullen *et al.* (1991) *Neuromusc. Disorders* **1**, 113–19). This suggests that the C-terminal end of dystrophin is inserted into the membrane itself, perhaps in association with the membrane glycoproteins which have been reported to be tightly associated with dystrophin (Campbell *et al.* (1981) *Nature* **338**, 259–62; Ervasti *et al.* (1990) *Nature* **345**, 315–19). Using a third monoclonal antibody (Dy10/12B2) we are currently engaged in localizing the N-terminal end of the molecule. In subsequent work we intend to improve the resolution of our localization by conjugating the gold probe directly to the primary monoclonal antibody, thereby reducing the 'noise' introduced by using a secondary labelling antibody.

Role of myotube-derived mitogens in myoblast proliferation during limb histogenesis

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During muscle histogenesis, different generations of muscle fibres are formed by fusion of different classes of skeletal myoblasts (embryonic, fetal and satellite cells). Embryonic myoblasts withdraw from mitotic activity to form primary myofibres during the early phases of limb development (about 12 d.p.c. in the mouse) while fetal myoblasts continue to proliferate and do not fuse into secondary fibres until several days later (16–17 d.p.c.). This different behaviour might be explained by: (1) differential sensitivity to different growth factors present in the limb; (2) expression of receptors with different affinity for the same growth factor(s), and (3) differential sensitivity to agents that block differentiation, without acting as specific mitogens. Experiments performed in several laboratories have shown that different growth factors and the corresponding message are present in developing muscle of vertebrates. We previously showed that ACTH and related peptides are selective mitogens for myoblasts (Cossu *et al.* (1989) *Dev. Biol.* **133**, 331). By immunofluorescence and radioimmunoassay, we have localized ACTH-like peptides in the neural tube and in the muscle anlagens of post-implantation mouse embryos. As this localization is compatible with these peptides acting as mitogens on myoblasts, we investigated which cell type might release ACTH-like peptides as well as other mitogens. By using a microassay for mitogen production, we have determined that myotubes, but not muscle fibroblasts or neuroblasts, release into the medium mitogens that support myoblast proliferation in a low serum medium. A blocking antibody against ACTH dramatically reduced the number of myogenic but not of fibrogenic cells. Based on these data and on others from different laboratories, we propose a model of paracrine growth control in limb development, based on a temporary regulated growth factor production by differentiated myotubes to support myoblast proliferation.

Actin severing proteins from invertebrate muscles are immunologically and structurally related to gelsolin and fragmin

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Obliquely striated annelid muscle contains a Ca^{2+}-dependent 40 kDa type actin severing protein (D'Haese and Hinssen (1987) *J. Comp. Physiol.* **B156**, 615–23). The protein was found to be functionally similar to slime mould fragmin and vertebrate gelsolin. However, unlike other 40 kDa severing proteins, this actin modulator binds two actins with high cooperativity in the presence of Ca^{2+}. Only one of these actins is released from the complex by EGTA as shown by gel filtration and native electrophoresis. Earthworm body wall muscle contains two functionally identical but immunologically distinct isoforms of the actin modulator with molar masses of 43 and 45 kDa. Amino acid sequence analysis of peptides purified from Lys-C digests of the 45 kDa isoform revealed homologies to fragmin/severin and to the N-terminal part of vertebrate gelsolin. Polyclonal antibodies highly specific for each isoform were raised and, in conjuction with antibodies against fragmin and gelsolin, distinct immunological relationships between the three proteins were established. Anti-fragmin strongly cross-reacted with both isoforms of the annelid protein but not with gelsolin. Anti-gelsolin does not recognize fragmin and differentially reacts with the two earthworm proteins. The two antibodies against the earthworm isoproteins interact significantly weaker with gelsolin than with fragmin. Investigations with muscle crude extracts obtained from a number of species of different invertebrate phyla (e.g. coelenterata, arthropoda, echinodermata) contained considerable actin severing and nucleating activities. Analysis of fractions from gel filtration experiments revealed that the invertebrate muscle actin modulators were all of the 49 kDa type, with the sole exception of a crustacean tail muscle (*Astacus*) which contains a protein of approximately 100 kDa, possibly similar to gelsolin.
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Complex patterns of myosin expression in masticatory muscles

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The majority of biochemical, physiological, histochemical and immunocytochemical studies of mammalian skeletal muscle have been carried out on the limb muscles. However, these results cannot be generalized to skeletal muscles with different types of innervation or which have distinct embryological origins such as the masseter. Histochemical studies have revealed striking differences in the properties of this muscle depending upon the species and in man it has been shown to be very different from the limb muscles. As the histochemical properties are known to be related to the type of myosin isoforms which are expressed by the muscle, we have carried out a comparative electrophoretic and immunological study of the adult masseter muscles from several different species of mammals. Mouse and rat masseter muscles contain typical type II myosins in addition to variable amounts of neonatal (or developmental) myosin. The rabbit masseter, on the other hand, in addition to type I and II myosins, contains a large amount of the ventricular myosin V1. In man, the masseter is even more complex as in addition to the type I and type II myosins it also expresses developmental myosins (fetal and embryonic MHC, embryonic MLC), and ventricular myosin. It is tempting to think that each animal has evolved a complex pattern of expression of different myosin isoforms to meet the specialized functional requirements of this muscle.

Do caldesmon and calponin coexist on the same actin filament?

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Caldesmon and calponin are actin-binding proteins located on thin filaments of smooth muscle. The capability of both these proteins to inhibit actin-activated ATPase activity of myosin implicates their role in the actin-linked system of smooth muscle regulation complementary to myosin phosphorylation. As functional properties of caldesmon and calponin are similar, the question arises if these proteins operate on the same or on separate actin filaments. To answer this question we have studied the effect of caldesmon on the binding of calponin to actin, and that of calponin on the binding of caldesmon to actin. The results revealed that both proteins displace each other from their complexes with actin. As a consequence, the inhibition of the ATPase by both caldesmon and calponin used at a substoichiometric ratio to action is additive, whereas the maximum inhibition of the ATPase is caused by this of the two proteins which is in an excess.

Mutual exclusion of caldesmon and calponin from F-actin indicates that these two proteins are distrubuted between two classes of thin filaments. The results are consistent with the recent data of Lehman (*J. Muscle Res. Cell Motil.* **12**, 221–4 (1991)) on separation caldesmon-rich and calponin-rich smooth muscle thin filaments by immunoprecipitation.

HCP, a putative component of the junctional protein complex between transverse tubules and terminal cisternae

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Concerning mechanisms involved in excitation-contraction coupling at the triad junction of skeletal muscle, present views are divided between a direct interaction between the DHP receptor and the ryanodine receptor (RyR), or an indirect interaction through bridging proteins (Kim *et al.* (1990) *Biochemistry* **29**, 9281), such as a 165–170 kDa Stains All blue stained component on SDS-PAGE of the isolated sarcoplasmic reticulum (SR) from rabbit fast-twitch muscle. This protein was characterized as a histidine-rich Ca^{2+}-binding and lipoprotein (LDL)-binding protein, and cloned by Hofmann and colleagues (1989, *J. Biol. Chem.* **264**, 8260). Using isopycnic sucrose density fractionation of SR membranes and ^{125}I-LDL and $^{45}Ca^{2+}$-ligand blot techniques, HCP has been found to be greatly enriched in junctional TC and in the purified junctional face membrane (JFM) of both fast-twitch and slow-twitch muscle. Junctional SR of chicken pectoralis muscle is shown to be similarly enriched in a LDL-binding protein component(s), which however migrate in the 130–116 kDa range of electrophoretic mobility, thus suggesting that HCP may be species-specific, rather then being specific to the type of muscle. As investigated in the chicken, the time of appearance of an accumulation of HCP in SR membranes during ontogenic development appears to be concomitant with that of the RyR. Rabbit HCP is shown to be fully retained in calsequestrin (CS)-depleted JFM after treatment with high ionic strength solutions, and to be selectively detached from membranes by subsequent treatment with 1 mM EGTA or EDTA at alkaline pH. The lack of binding of HCP to ^{125}I-CS, using protein overlay techniques and other kinds of experimental evidence, such as its accessibility to exogenous protein kinase A, in intact TC vesicles, like that of the RyR, but unlike CS, suggest that HCP is associated to the cytoplasmic side of JFM, probably through binding to an integral membrane protein involving Ca^{2+}-bridges. This membrane localization suggests a role of HCP in electromechanical coupling in adult skeletal muscle.

Functionally distinct states of myosin ATPase site hydrophobic pocket

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As we and other authors (Burke *et al.* (1986) *J. Biochem.* **261**, 12330) have shown previously, degradation of 50 kDa fragment during the trypsinolysis of myosin S1 is much more rapid than that of 27 and 20 kDa fragments and correlates with the decrease of ATPase activity of S1. Degradation of the 50 kDa fragment is much slower in the presence of actin, ATP and ADP. Probably, the change of S1 structure lability in the presence of specifically interacting agents is functionally important. This investigation treats the problem of denaturative effect of temperature on 50 kDa fragment of myosin S1 in the presence of various nucleosidetriphosphates (NTP) and nucleosidediphosphates (NDP). Denaturation changes were evaluated by trypsinolysis extent. It is shown that by protecting action NTPs and NDPs may be arranged as follows: ATP > CTP > UTP > GTP (1), ADP > GDP > CTP > UDP (2). It is known that by the fluorescence increase caused mainly by tryptophanyls of the 50 kDa fragment various NTPs may be arranged just as in (1); the same is the sequence of lifetime decrease of longliving intermediate complexes M**NDP · P_i (Werber *et al.* (1987) *Biochemistry* **26**, 2903). These results suggest that the decrease of the lifetime of M**NDP · P_i complex and fluorescence increase are a result of the strength of the NTP binding in the active centre, resulting in the increase of the S1 rigidity. Special importance has the interaction of the NTP nitrous base with the 'hydrophobic pocket' of the ATPase centre. The decisive factor of this interaction is probably the NH_2-group (proton donor) in position 6 of the purine ring of ATP and position 4 of the pyrimidine ring of the CTP, and ≡N-group (proton acceptor) in positions 1 and 3, respectively; this stipulates the leading position of ATP and CTP in the series (1). In the case of NDP interaction with ATPase site these groups lose their importance and hydrophobic forces become more valid. Therefore the series (2) is headed by ADP and GDP. Summing up these results we suggest the existence of at least two conformational states of the ATPase site 'hydrophobic pocket'. One of them is responsible for decelerating the ATPase reaction of the stage of M**ADP · P_i. Another state takes place at actin binding when the structure of 'hydrophobic pocket' undergoes reverse changes.

Length-dependent calcium sensitivity of skinned human and fetal single skeletal muscle fibres

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Small bundles of human (M. Deltoid and M. Quadriceps) and fetal (arm) skeletal muscle were skinned by means of freeze-drying. Single muscle fibres were prepared of these bundles. Sarcomere length were set and monitored during the whole experiment using laser diffraction. The fibres were activated by containing solutions with free calcium concentrations calculated according to Fabiato and Fabiato (*J. Physiol (Paris)* **75**, 463 (1975)). Muscle type was characterized by means of the shift in sensitivity in the strontium activated fibre and the fitted number of binding sites according to the Hill model (Fink *et al.* (1990) *J. Physiol (Lond.)* **420**, 337). The calcium sensitivity curve, the relation between pCa (the negative logarithm of the free calcium concentration) and the developed tension, was measured as a function of sarcomere length. From these measurements the Hill plots, the relation between the logarithm of $(F_{rel}/(1-F_{rel})$, in which F_{rel} is the normalized tension, and pCa, were constructed. The Hill coefficient n, the number of binding sites involved in the onset, fitted from the calcium sensitivity curves ranged from two to six depend on the muscle type (slow, fast or intermediate). While the tension developed in slow and fetal fibres continuously increased at higher $[Ca^{2+}]$, the tension in fast fibres obtained an optimum at $[Ca^{2+}]$ of 2 μM followed by a decline in tension when $[Ca^{2+}]$ was increased. The fitted parameters of the Hill equations proved to be dependent upon sarcomere length similar to the dependency in mammals: increasing the sarcomere length resulted in an increase of the $pCa_{50\%}$, the pCa value at which 50% of the maximal tension has been induced, while n only increases for slow fibres. The Hill plots proved to be curved indicating that the onset of the contraction process cannot be described by a Hill process. At higher pCa values (lower calcium concentrations) the degree of cooperativity, estimated from the slope fo the Hill plot, proved to be higher than at low pCa values. Experiments performed on fetal tissue give qualitatively the same results, although the calcium sensitivity curves were shifted to higher pCa values and their slopes proved to equal those of slow fibres.

Effects of UL-FS-49 on action potential and contraction in single cardiomyocytes

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In ventricular conductive tissue use-dependent interactions between the pacemaker current and the bradycardic agent UL-FS-49 have been observed (van Bogaert *et al.* (1990) *Eur. J. Pharmacol.* **187**, 2). We studied the effects of UL-FS-49 on action potential and contraction in single ventricular cardiomyocytes ($n = 20$). Single cardiomyocytes

were isolated from guinea pig hearts by collagenase perfusion through the aorta. Action potentials were measured by whole cell patch clamp with one electrode (stimulation rate 0.8 Hz). Each run ten action potentials were averaged. A stabilization period of 5 min was allowed between control conditions and steady drug perfusion. No significant changes in resting potential were observed after addition of UL-FS-49. However, UL-FS-49 (10^{-6} M and 10^{-5} M) has a time- and use-dependent effect on the duration of the action potential. After 10 min of perfusion with the drug (10^{-6} M), the duration of the action potential was reduced by 25% ($n = 5$). This reduction could be preceeded by a transient prolongation of the action potential when stimulation was temporarily interrupted ($n = 5$). To compare electrical and mechanical activity in the myocyte, twitch contractions were induced by electrical stimulation. During control conditions and after addition of the drug, three stable twitch contractions were analysed. Contractions were recorded on a video-tape; shortening was measured by still-frame analysis. In control conditions peak shortening averaged 15% of the initial cell length. When UL-FS-49 (10^{-6} M) was added, the amplitude of shortening was reduced by 23% after 5 min ($n = 6$) and by 29% after 10 min of drug perfusion.

This reduction in contractile activity could be explained by the decrease in action potential duration, which could be from the reported depressive effect of the drug on the calcium current (Tytgat *et al.* (1991) in press), thus leaving less free intracellular calcium to activate the contractile proteins.

Effects of an active pre-stretch on skeletal muscle forces during and after stimulation

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In previous experiments it was observed that muscle-tendon complexes were able to produce more work during shortening after a pre-stretch. Extra work was produced even in conditions when the whole shortening occurred at lengths smaller than muscle optimum length (L_0 = length with the highest tetanic force), whereas in single amphibian fibres pre-stretch effects were only observed at lengths greater than L_0 (Edman *et al.* (1978) *J. Physiol.*). In the present study the influence of muscle length was investigated on isometric force enhancement as a result of an active pre-stretch in mammalian skeletal muscle-tendon complexes. Contractions (1 s duration) were induced in rat medial gastrocnemius muscles (temperature $36 \pm 1°$ C). The complexes were stretched by 2.4 mm at a velocity of 20 mm s^{-1} from different starting lengths; the range of final lengths was Z_0 to $L_0 + 4$ mm. The stretch was either passive (control) or stimulation started during the stretch (pre-stretch). The length dependency of the isometric force enhancement was similar to that described by Edman and colleagues (1978). However, also a significant enhancement was observed at lengths smaller than L_0. After relaxation of the pre-stretch contraction force remained at a higher level than expected from the passive length-force relation. This non-stimulated force enhancement (NSFE) persisted for 1–3 min and was also length-dependent. However, at lengths smaller than L_0 no enhancement occurred. The relationship between NSFE (ΔN) and length above L_0 (Δmm) is given by $y = 0.07 + 0.20x$; $r = 0.78$; $n = 69$; $P < 0.001$. NSFE could be removed by a length decrease to L_0 and subsequent re-stretch to the previous length in the non-stimulated state. During re-stimulation after the pre-stretch contraction at the maintained length total force was not enhanced compared to control. However, NSFE was found again after the end of this stimulation. No progressive loss of function indicating muscle damage was observed. Both stimulated force enhancement and NSFE could result from a stretch-induced force bearing system parallel to the crossbridges (Sugi and Tsuchiya (1988) *J. Physiol.*). Additionally NSFE may result from a very slow detachment of some actively stretched crossbridges.

Frequency dependence of the elastic impedance of sarcomeres of skeletal muscle fibres

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Tension transients, induced by quick length changes of isometrically mounted muscle fibres, reveal information about the elastic properties of muscle fibres. In this study the frquency dependence of the elastic impedance of a sarcomere of skeletal muscle fibres of the frog (*Rana esculenta*) was examined over a wide range, from approximately 5 Hz up to 50 kHz.

Fibres of the iliofibularis muscle were skinned by freeze-drying and incubated in relaxing and rigor solutions (ionic strength 160 mM, 4° C). Rigor solution did not contain Ca^{2+} and the tension developed during isometric incubation in rigor solution was $0.3 \cdot T_0$ (T_0 = the tension developed at maximal activation of pCa = 4). The sarcomere length was measured by means of a laser diffraction pattern and set to 2.15 µm before rigor development; fibre length and its diameter were measured by means of a microscope (accuracy 5%).

The elastic impedance Z was determined from the tension transients under the assumption that a single fibre segment could be compared with a uniform rod composed of small identical units, connected in series with a density of 1060 kg cm^{-3}. The phase shift of elastic impedance appeared to be zero up to 30 Hz. Then a slight increase was observed up to 1 kHz, while above 1 kHz the phase shift remained constant. The magnitude of elastic impedance was constant up to 30 Hz. Subsequently it increased linearly with some power of the frequency ω, in good approximation to the formula: $Z(j\omega) = E_0 \cdot (1 + j\omega/\alpha)^{\nu}$. For the parameters E_0, α and ν, the results indicate that for rigor fibres compared with relaxed fibres the power ν is two times less, the elasticity constant E_0 is about ten times higher, and α is about the same. Differences found can not be explained by differences in interfilament distance; they must result from the presence of crossbridges themselves.

Different contraction kinetics of the guinea pig tracheal smooth muscle after activation by carbachol and leukotriene C4

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We compared the effects of LTC4 and carbachol (carb) on the isometric contraction parameters of the isolated guinea pig tracheal smooth muscle. The initial force increase after LTC4 application showed a longer latency and a lower rate of force increase than after carb application. The initial peak forces of 19.16 mN (carb) and 15.08 mN (LTC4) were reached after 21.4 and 39.8 min, respectively.

Within 2 s, length vibration (100 Hz, sinus) relaxes the preparation to 54.4% (carb) and 38.3% (LTC4) of maximum force. After cessation of vibration, there as a distinctly lower rate of force recovery in the LTC4-activated preparation (time constants 243 s (carb) and 413 s (LTC4)). After multiple vibrations carried out in the course of one single activation period we found a pronounced stepwise decrease in the steady-state force during carb-activation, and only slightly diminished force during LTC4-activation. Control experiments with carbachol and histamin activation, however, showed similar contraction kinetics.

Our results suggest the existence of a special signal transducing pathway for LTC4 and at a force-controlling mechanism differing from that for carb. The crossbridge down-regulation and the stepwise diminished force observed are interpreted as the result of a greater fraction of non-cycling latchbridges in the carb-activated preparation. In the LTC4-activated preparation, the crossbridges seem to remain in a cycling state despite the slower contraction kinetics calculated. Further evidence was obtained for a gradual change of crossbridge

cycling rates under sustained contraction possibly resulting in latch-bridges as the final state.

Time resolved X-ray diffraction studies of skeletal muscle during isotonic and isometric contraction

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To investigate the crossbridge cycle during isotonic contraction at low load the following protocol for time resolved X-ray diffraction studies was employed. An initial 600 ms tetanic stimulus was applied to the muscle held isometrically and at 30% peak tension during the recovery phase (Bordas *et al.* (1991) *Adv. Biophys.* **27** (in press)) a further 600 ms stimulus was applied with an almost simultaneous length shortening. The length shortening was at approximately 0.75 muscle lengths per s and was of 200 ms duration. Throughout this period the tension remained at 27.5% to within $\pm 2.5\%$. After this time the muscle was held isometric for the remainder of the stimulus, thus combining isotonic and isometric contractions within the same stimulus. The initial and final sarcomere lengths were approximately 2.6 and 2.3 μm respectively. Data were obtained using an area detector in 1D mode with a time resolution of 4 ms over the entire contraction sequence.

The results suggest that no axial asynchronous cycling of the heads occurs to any appreciable extent in either isotonic or isometric contraction. It appears that the attachment of myosin heads following reactivation proceeds continuously during the isotonic phase and increases with the degree of overlap. However, the reorientation of the myosin heads to a more perpendicular position relative to the muscle axis, typical of the tension generating state in isometric conditions, only occurs from the moment that tension is allowed to rise again at the end of shortening.

The time constants for the troponin motions associated with activation are essentially identical in isometric or isotonic conditions (approximately 15 ms half-time). No motions of the myosin heads occur until this activation phase is completed.

Changes in the muscle proteins of the mdx mouse

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The protein composition of extracts of the gastrocnemius, tibialis anterior and extensor digitorum longus muscles of mdx and control mice were compared by two dimensional electrophoresis. Increases were observed in serum albumin, proteins of apparent molecular weights 50 000, 38 000 and a group of six basic proteins with molecular weights ranging from 24 000 to 35 000 in the mdx mouse. In most cases the changes in the unidentified proteins represented increases in components either barely detectable in control muscles or not present in high enough amounts to stain with Coomassie Blue at the loadings used. On the other hand, no marked decrease in intensity of the approximately 250 protein spots seen in control muscle samples was observed. There were no marked differences in the isoform pattern of the myosin light chains or tropomyosin present in muscles at comparable ages in the mdx and control mice. Similar results were obtained in mdx mice at 21, 70 days and 1 year despite the fact that at 21 days the impairment of muscle function was marked but has recovered at 70 days. Control mice fast muscles contained five isoforms of lactic dehydrogenase with the H_4 form staining the faintest but increasing in intensity with age. A similar pattern was seen in mdx muscle but with the H_4 form less intense than in the controls.

When changes in the muscle proteins of the dy mouse mutant were compared with those in the mdx mouse only the change in the six basic protein pattern was unique to the latter condition. In the dy mouse changes in the tropomyosin and myosin light chain isoform patterns were observed implying that a phenotype change was occurring in response to the dystrophic process. This did not appear to be the case to any substantial degree in the mdx mouse where it might be expected, if according to the current view, the absence of dystrophin had been overcome by regeneration of the muscle.

Mobility of the contractile proteins in glycerol-extracted muscle tested by NMR and EPR

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Tests carried out on glycerol-extracted rabbit psoas showed both a change of the level of fixed electric charge in muscle during contraction and a variation in the mobility of the protein surfaces under the same condition. Our experiments consisted in the determination of the transverse nuclear magnetic relaxation time of the protons (^{1}H-NMR) in the presence or in the absence of Mn^{2+}, the determination of the sodium distribution in muscle (using ^{23}Na-NMR), the measurement of ATP splitting during the contraction (using ^{31}P-NMR) as well as in the tests concerning the Mn^{2+}, accumulation is the glycerinated fibres during activity (based on the EPR method).

Sodium is excessively accumulated in the glycerol-extracted, contracted muscle. In the same functional condition the correlation time of the protein movements is increased. These facts are examined in relation with the presumed molecular basis of contraction. The increase of the electric charge on filaments may enhance the stiffness of the contractile proteins. Huxley's theory of 1957 probably should be interpreted in a more realistic and more comprehensive model of contraction.

Exercise metabolism in the *mdx* model of Duchennes muscular dystrophy

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The *mdx* mouse is useful for studying Duchennes muscular dystrophy (DMD) metabolism *in vivo* because it has a defect in the homologous region to the DMD gene (*Science* **244**, 1587 (1989)), and both DMD patients and *mdx* mice lack dystrophin (*Cell* **51**, 919 (1987)). ^{31}P-NMR was done on a 400 MHz magnet and a Bruker Aspect 3000 spectrometer. The sciatic nerve was stimulated via implanted electrodes. The knee and ankle were fixed and tension in the achilles tendon was monitored. Three frequencies were used, 1 Hz, 10 Hz and 100 Hz. Maximum tension was maintained at 1 Hz but not at the higher frequencies. Metabolic indices decline steadily in the 1 Hz protocol but reach a minimum early and begin to recover during exercise at higher frequencies. This metabolic recovery, coupled with a decline in tension, indicates that the cause of fatigue is not metabolic. Tensions per g dry weight were similar in the *mdx* and control strain, C57B1/10 ScSn. Thus metabolic differences are not from a diminished force generation in dystrophic muscle. The ratio PCr/(PCr + Pi) is lower in *mdx versus* control muscles at rest and during exercise at all frequenceis. A reduced PCr/PCr + Pi is observed in mitochondrial myopathies (*Ann. Neurol.* **18**, 189 (1985)) and with a shift from Type II to Type I fibres (which occurs in DMD and in *mdx* muscle) (*Biochim. Biophys. Acta* **1096**, 115 (1991); *Cell* **52**, 503 (1988)). There was increased acidification during exercise in *mdx versus* control muscle, and a reduced rate of pH recovery. The increase in Type I fibres in *mdx* muscle makes it unlikely that this acidification is from increased lactate production. It is possible that the Na/H exchanger V_{max} has been reduced by a decrease in the sodium gradient if increases in

total sodium (Dunn and Radda (1991) *J. Neurol. Sci.* (in press)) are proportional to increases in intracellular free sodium.

Transitory increase in myoplasmic Ca^{2+} concentration during isometric relaxation of frog muscle fibres

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Intracellular free calcium concentration and contractile force were recorded in frog isolated muscle fibres that were loaded with fluo-3 AM. The fibres were mounted horizontally on the stage of a Zeiss Axiovert-microscope between a force transducer and an electromagnetic puller and were stimulated at regular intervals to produce a single twitch or a partially or completely fused tetanus at 3–5° C. The microscope was provided with a epifluorescence attachment and low-power (×10) objective and the fluorescence signals were recorded by means of a phototransistor (bandwidth 3 kHz).

The calcium signal started to decay soon after the last stimulus during tetanus and was approximately 90% of its maximum value when force began to decline. At a time when the calcium signal had reached about 40% of its peak value, which coincided with the shoulder of the isometric myogram, there was a secondary transient rise of the calcium signal. This secondary calcium transient followed the same time course as the non-uniform segmental length changes that occur during the relaxation phase after the tension shoulder (Cleworth and Edman (1972) *J. Physiol.* **227**, 1–17). Both stretch and shortening ramps applied to fibre immediately before the tension shoulder enhanced the calcium transient. The same movements imposed on the fibre at rest did not affect the calcium signal significantly. The results suggest that filament sliding in both shortening and elongating segments during relaxation leads to an increased rate of dissociation of calcium from its binding sites on the thin filaments.

Contractile process

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It is almost four decades since the sliding-filament model was published but the physical-chemical process of muscle contraction within that model remains obscure. Existing models have often used mechanical analogies (rotating levers, oars, springs and so on). It may now be timely to concentrate on the electrical regimen in the overlap zone, and in particular on the potential well that must exist between the thick and the thin filaments; the depth of this well will be in the range of 50–100 mV (Elliott and Bartels (1982) *Biophys. J.* **38**, 195–9). We have shown experimentally that the absence of ATP increases the myosin (thick filament) charge by about 40%, and this will clearly alter the depth of the myosin (thick filament) charge by about 40%, and this will clearly alter the depth of the potential well (Bartels and Elliott (1985) *Biophys. J.* **48**, 61–76; Bartels *et al.* (1983) *J. Physiol.* **358**, 80P).

The myosin head (or S1 moiety) will span the potential well, and different sites on that head will therefore experience different potential gradients. The ATP-binding site is very probably close to the bottom of the potential well (the least negative region) while the sites of interaction with the myosin back bone and the actin molecule will be near to the top of it. Given these facts, and given that the control function seems to be exerted by ions and proteins in the high potential region (Ca^{2+} together with troponin/tropomyosin, or the myosin light chains) there is a strong possibility that the myosin S1-ATPase may act as an ion-directing pump, using the energy of ATP-hydrolysis to shift ions up potential gradients, as indeed ATPases appear to do in so many other biological situations. This possibility is not contradicted by 'biological assay' type experiments because these use a substrate, usually glass, that is negatively charged at the surface and will thus mimic the *in vivo* potential well.

If ion-pumping is part of the contractile process, the clear changes in order of the filament lattice that can be seen in several published X-ray pictures, between the relaxed rigor and contracted states, could be manifestations of charge movements, as both the temperature-jump effect on the myosin layer line pattern and the change in spacing of the 14.3 nm meriodional reflection can be readily explained if higher electrical charge is equated to disorder. Models for this will be discussed. To understand order-disorder transitions in helical systems a new approach to helical diffraction is desirable, particularly in multi-start helices such as myosin, and this is currently under development (Worthington and Elliott (1989) *Acta Cryst.* **A45**, 645–54; Worthington (1990) *Acta Cryst.* **A46**, 777–783).

Adult rat ventricular and atrial cardiomyocytes in culture

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Adult rat ventricular cardiomyocytes (VCM) can be kept in long-term culture undergoing extensive morphological changes accompanied by degradation and eventual regeneration of structures of the contractile and cytoskeletal apparatus (Eppenberger *et al.* (1988) *Dev. Biol.* **130**, 1–15; Eppenberger–Eberhardt *et al.* (1990) *Dev. Biol.* **139**, 269–78), whereas the mitochondria are mostly conserved and reorganized during redifferentiation of the cultured VCM (Epperberger–Eberhardt *et al.* (1991) *J. Cell Biol.* **113**, 289–302).

Adult atrial cardiomyocytes (ACM), directly after isolation slender rod-shaped or already rounded up, attach after 1 day to the substratum, and after 3–4 days a very polymorphic well-differentiated cell population has developed: flat, thin or small bipolar ACM and cells with a small body extending several long processes, all these different cell types making contact. Immunofluorescent studies using antibodies against mitochondrial creatine kinase (Mi-CK), α-smooth muscle (sm) actin and myomesin were performed. It was found that, although practically all cells are cross-striated, in contrast to VCM only part of them express Mi-CK in their mitochondria. Their pattern of cross-striation is different from that of VCM insofar as that myofibrils are not concentrated in the perinuclear region, but are mostly distributed throughout the ACM. Alpha-sm actin appears in most cells in a sarcomeric pattern, contrary to the characteristic staining pattern in VCM where strong staining is observed within stress-fibres extending from the myofibrils. These preliminary results suggest an accelerated de- and redifferentiation process in the cultured ACM. To study whether the different types of ACM keep their characteristic properties also in culture is the aim of further investigations.

Characterization of the properties and role of the central domain of gizzard caldesmon

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The amino acid sequence of gizzard caldesmon deduced from its cDNA allows correlation of those regions of the molecular isolated

following proteolysis and showing anomalous gel migration properties with their corresponding locations in the primary structure of caldesmon. These data provide information on the distinctive structural feature possessed by different proteolytically-defined domains of the molecule. We describe here the isolation and characterization of the central part of the molecule that is made up of repeating amino acid sequences which, on the basis of their composition, are unlikely to possess the resistance to proteolysis that we observe. NMR and CD studies indicate that this domain of caldesmon, present in smooth muscle but absent in non-muscle caldesmon, contains a high helical content and displays marked structural stability to denaturation by temperature or area. We demonstrate, using a synthetic peptide based on the repeat sequence that occurs in this region, that the properties associated with this domain are based on the intrinsic structural propensity of the repeat sequence. We discuss the biochemical and biophysical properties of the central domain in the context of the role of native caldesmon in smooth muscle regulation. These data are further relevant to other muscle proteins that possess similar repetitive primary sequences, e.g. dystrophin. We compare the properties of this central portion of caldesmon with those of the actin and calmodulin-binding region at the C-terminal end of the molecule.

Properties of the covalent G-actin-myosin head complexes in the presence and in the absence of DNaseI

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We have demonstrated that a stable complex between G-actin and skeletal myosin head (S1) can only be obtained with S1 isoenzyme carrying the A2 light chain subunit (Chaussepied and Kasprazak (1989) *Nature* **432,** 950–3). This peculiarity is mainly the result of differences in the critical concentrations of the G-actin-S1(A1) (Cc = 0.35 μM) and the G-actin-S1(A2) (Cc = 9 μM) complexes (Cc were determined by measuring the polymerization-induced increase of the pyrenyl-actin fluorescence intensity after 20 h incubation at 20° C in 2 mM HEPES, 0.1 mM $CaCl_2$, 0.2 mM DTE, ±0.1 mM ATP).

In addition to their tendencies to polymerize at high concentration, the G-actin-S1 complexes are strongly sensitive to ionic strength (dissociating and polymerizing effects) and to nucleotides (dissociating effect) (Chaussepied (1991) *Biophys J.* **59,** 410a). To obtain G-actin-S1 complexes under destablizing conditions, we have produced and isolated various covalently-linked G-actin-S1 complexes in the absence and in the presence of DNaseI known to stablize the complexes in their monomeric forms. Crosslinking experiments performed under non-polymerizing conditions (actin-S1 = 9 μM), resulted in different gel electrophoretic patterns of the crosslinked products obtained with G-actin-S1 (without or with DNaseI) and with F-actin-S1 when the crosslinking reaction was catalyzed by EDC, glutaraldehyde, ethoxy-carbonyl 2-2-ethoxy 1-2-dehydroquinoline (EEDQ), or dimethyl suberimidate (DMS). These findings support previous results suggesting that changes occur in the actin-S1 interface during actin polymerization (Chaussepied and Kasprzak (1989) *J. Biol. Chem.* **264,** 20752–9). Purification of the EDC-crosslinked G-actin-S1 complexes by gel filtration on Sephadex G-150 (without DNaseI) or TSK4000 (with DNaseI) column yields to pure monomeric complexes. Though these complexes did not present a significant actin-activated S1 Mg^{2+}-ATPase, their polymerization by 2 mM $MgCl_2$ (only without DNaseI) was accompanied by an activation of the S1 Mg^{2+}-ATPase similar to that observed with the reversible F-actin-S1 complexes.

In summary, this work describes the isolation of a covalent ternary DNaseI-G-actin-S1 complex suitable for crystallographic studies, but also it provides for the first time the possibility to study the actin-myosin interface either in the monomeric form or in a polymeric form with filaments fully decorated by covalently linked S1.

Pressure-induced tension transients in skinned isolated rabbit psoas muscle fibres at submaximal calcium concentrations

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We have previously reported that the steady tension in a maximally Ca-activated glycerinated muscle fibre is depressed by increased hydrostatic pressure. We argued that this depression of active tension is the result of an effect of pressure on a transition between low and high force crossbridge states (Fortune *et al.* (1989) *J. Muscle Res. Cell Motil.* **10,** 113–23). We report here the pressure sensitivity of tension at different calcium concentrations. All experiments used single glycerinated rabbit psoas fibres (12° C, pH 7.9, μ = 0.2 M). An increase in hydrostatic pressure to 10 MPa caused no shift in the midpoint of the force-pCa relationship. However, at lower calcium concentrations, tension was potentiated by up to 15% whereas it was depressed by up to 15% at higher concentrations.

The tension transients following rapid release (1 ms) of elevated pressure shows three phases at maximally activating calcium concentrations (pCa 4.52); a rapid decrease in force (phase 1) in phase with the pressure fall followed by a two phase recovery of tension at 28 and 2.5 s^{-1} respectively (refered to as phases 2 and 3 respectively; Fortune *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88,** (in press). This three phase transient was evident at all calcium concentrations over the range pCa 4.52–5.88. However, whilst phase 2 contributed to a tension recovery at all concentrations of calcium following pressure release, phase 3 contributed to either a tension increase (above the midpoint) or a tension decrease (below the midpoint). The reciprocal relaxation time ($1/\tau$) and the normalized amplitude of phase 3 were reduced at lower calcium levels. Data from the above single fibre at pCa = 4.52 and 5.88 gave $1/\tau = 2.5$ and 0.7 s^{-1} respectively with amplitudes of +7% and 11% of T_0 in each case (where T_0=steady tension at atmospheric pressure in each case). In contrast, the amplitude and $1/\tau$ of phase 2 were independent of calcium concentration.

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Interaction of the regulatory light chain with the heavy chain of skeletal muscle myosin

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The regulatory light chain (RLC) of rabbit skeletal muscle myosin contains two unique Cys in the C-terminal portion in position 128 and 157 of a total of 169 amino acid residues. Treatment of myosin with dithiobis-nitrobenzoic acid (DTNB) in the absence of divalent metal ions (Ca^{2+} or Mg^{2+}) induces a disulphide bond between the two Cys of zero-distance length. Divalent metal ions (Me^{2+}) are known to bind to the first EF-domain (residues 27–57) near the N-terminus of the RLC. The presence of Me^{2+} prevents the formation of this intrachain disulphide bond. The two Cys of the RLC can also be crosslinked by dibromobimane (with a length of 0.3–0.6 nm) in Me^{2+}-dependent manner. Bifunctional thiol reagents with a length of more than 0.6 nm (up to 1.9 nm) are able to form an intrachain between the two Cys in the RLC in the presence and absence of Me^{2+}. This indicates that binding of Me^{2+} to the RLC in myosin affects its C-terminal portion so that the two Cys may become separated up to 0.6 nm from one another. The prevention of formation of the intrachain bridge by DTNB or the shorter bifunctional reagents in the presence of Me^{2+} depends on the interaction of the RLC with the HC. When the RLC is isolated all reagents induce an intrachain bridge in the presence and absence of Me^{2+}. After induction of the intrachain bridge in myosin or in actomyosin the RLC can be removed even in the presence of

Me^{2+} either by repeated washings at low ionic strength or by gel chromatography at high ionic strength. Isolated RLC with an intra-chain bridge between its two Cys was never able to rebind to myosin. Myosin whose RLC had been removed forms polymeric aggregates as shown by column chromatography. As a consequence the actin-activated ATPase activity is lowered. Reconstitution with isolated RLC, however, is able to revert these alterations. Myosin becomes monomeric again and its actin-activated ATPase is restored. The RLC is therefore essential for the proper functioning of the two myosin head portions in their interaction with actin.

Rabbit skeletal muscle α-tropomyosin: cloning, sequence determination and expression in *E. coli* and insect cells

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For biophysical and crystallographic studies of tropomyosin (Tm), it is of great importance to establish an expression system from which variants of Tm is obtained of great quantity and to high homogeneity. In this study the rabbit skeletal muscle was used as the source of cDNA, as Tm from this muscle has been most thoroughly investigated. The cDNA was screened from the cDNA bank derived from the leg muscle of an 11-day-old rabbit. The nucleotide sequence of the cDNA completely agrees with the peptide sequence of this protein (Stone and Smillie (1978) *J. Biol. Chem.* **253**, 1137–48) and nearly identical, with all silent replacements of 59 base pairs, to the rat skeletal muscle counterpart.

The protein was expressed in *E. coli* (using expressions vector pTrc99) as well as in insect cultured cells by use of the baculovirus expression system. Previous studies (Hitchcock-DeGregori and Heald (1987) *J. Biol. Chem.* **262**, 9730–5; Bartegi *et al.*,, (1990) *Euro, J. Biol.* **194**, 845–52) indicated that the protein expressed in *E. coli* was not acetylated at its N-terminus, being devoid of the head-to-tail polymerizability. The expression in the insect cells could be associated with some post-translational chemical modifications of the expressed protein of eucaryotic origin. Actin-binding of Tm expressed in the insect cells was almost as strong as the native Tm, indicating this Tm is acetylated at its N-terminus.

Functions of troponin T 25 K fragment of rabbit skeletal muscle neonatal variant (β-TNT) expressed in *E. coli*

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To crystallize the troponin (Tn) complex, the heterogeneity of troponin T (TnT) within the complex could be one of the major obstacles. To circumvent this problem, Tn could be reconstituted from a single species of TnT expressed in *E. coli* and TnI + TnC prepared from muscle.

In our previous work, four variants of TnT cDNAs were isolated and sequenced (Fujita *et al.* (1991) *J. Muscle Contr. Cell Motil.* (in press)). All the cDNAs, being derived from the rabbit skeletal muscle of an 11-day-old animal, encode the neonatal variants (β-TnT). In β-TnT exon 17 is expressed, while exon 16 in the adult variants (α-TnT), resulting in replacement of a segment of 14 residues near the C-terminus (sequence 229–242 according to the conventional numbering system; Pearlstone *et al.* (1977) *J. Biol. Chem.* **252**, 983–89).

In the present study, we have expressed in *E. Coli* a 25 K β-TnT fragment which is deficient about 50 residues from the N-terminus. The truncated cDNA was inserted in a T7 expression vector pET3d and BL21 (DE3) cells were transformed to express the protein. The protein predominantly went into inclusion bodies, from which the protein was recovered soluble either with 6 M urea, with various polyanions or with high concentrations of LiCl. After two steps of column chromatography of the extract, 25 Kβ-TnT was obtained to homogeneity.

This fragment, together with Tm, TnI and TnC which are all prepared from the rabbit muscle, confers calcium sensitivity of acto-S1 Mg-ATPase as the intact α-TnT does. As the 26-K α-TnT fragment also sensitizes the acto-S1 Mg-ATPase to the same extent as the intact α-TnT (Ohtsuki (1984) *J. Biochem. (Tokyo)* **95**, 1337) the present result suggests that the sequence replacement α−/β− has no major effect on the calcium sensitizing action of TnT.

Thin filament linked regulation: the Ca-switch in troponin C

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Binding of Ca^{2+} to troponin C (TnC), a component of the regulatory complex in striated muscle, initiates the activation of the actin-myosin system leading to force development and contraction. X-ray studies have shown that TnC consists of two globular domains linked by a single α-helix. (For a review see Strynadka and James (1989) *Ann. Rev. Biochem.* **58**, 951–98.) Comparison of the structure of the Ca^{2+}-sound C-terminal domain with that of the Ca^{2+}-free N-terminal domain led to the proposal (Herzberg *et al.* (1986) *J. Biol. Chem.* **261**, 2638–44) that Ca^{2+}-binding to the N-terminal triggering sites causes a movement of helices B and C with respect to helices A and D opening up a hydrophobic patch for TnI binding. Introduction by site directed mutagenesis of a pair of Cys residues (Grabarek *et al.* (1990) *Nature* **345**, 132–5) and the subsequent formation of a disulphide bridge between the B-C linker and helix D reduces the Ca^{2+}-affinity of, and blocks the interaction of TnI with, the N-terminal domain resulting in the loss of regulatory activity tested in the myofibrillar ATPase system. These experiments indicate that helix-helix separation in the N-terminal domain of TnC is required for initiating triggering by Ca^{2+}. Direct demonstration of Ca^{2+}-induced changes in helix-helix separation comes from studies on mutants of TnC in which pairs of Cys residues introduced by site-directed mutagenesis were labelled either with a pyrene derivative showing changes in the excimer spectrum or with an appropriate donor-acceptor pair permitting distance determination by resonance energy transfer. Introduction of a disulphide bridge in the C-terminal domain weakens TnI-binding but does not abolish the regulatory activity. Further aspects of the TnC-TnI-Ca^{2+} interaction and their role in regulation will be discussed in light of the demonstrated movement of TnI away from actin on Ca^{2+}-binding (Tao *et al.* (1990) *Science* **247**, 1339–41).

Effect of caldesmon phosphorylation on its interaction with tropomyosin and myosin

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Avian gizzard caldesmon was phosphorylated by casein kinase of the second type. Under conditions used the enzyme predominantly phosphorylated Ser-73 of caldesmon. Interaction of phosphorylated and unphosphorylated caldesmon with smooth muscle tropomyosin and myosin was investigated by means of affinity chromatography, ultracentrifugation and fluorescence spectroscopy. Under certain conditions N-terminal chymotryptic peptide (M_r 25–27 kDa) of caldesmon containing the site of phosphorylation interacts with immobilized smooth muscle tropomyosin. This indicates that except of the earlier described tropomyosin-binding site located in the

C-terminal part of caldesmon there is a second tropomyosin-binding site which is formed by the first 165–170 amino acid residues of caldesmon. Phosphorylation by casein kinase II diminishes the affinity of both native caldesmon and its N-terminal peptide to immobilized tropomyosin. Similar effect of phosphorylation was observed in experiments on caldesmon-induced increase of fluorescence of DNS-labelled tropomyosin. At low ionic strength native caldesmon and its N-terminal chymotryptic peptide cosediment with smooth muscle myosin. The portion of caldesmon peptide determined in the myosin pellet gradually decreases with the extent of exogenous caldesmon phosphorylation increases from 0 to 60%. The data presented indicate that casein kinase II-induced phosphorylation may affect the interaction of caldesmon with contractile and regulatory proteins.

Crosslinking of actin with N-(4-azidobenzoyl)-putrescine: a test of the F-actin model

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We have synthesized a new photoactivatable crosslinker for proteins, N-(4-azidobenzoyl)-putrescine, and have used it to measure the proximity of two side chains in adjacent actin monomers in F-actin. The reagent was incorporated into rabbit skeletal muscle G-actin, by transglutaminase, at residue Gln-41. The modified actin was polymerized, and crosslinking was induced by photogeneration of an active nitrene. Oligomers were separated from uncrosslinked monomers by S-200 gel filtration, and a crosslinked peptide complex was isolated from an Arg digest of the actin. The crosslinked peptides included residues 40–62 and residues 96–116. Sequence analysis showed that the actual crosslink was between Gln-41 and Lys-113. By examining a model of the reagent, N-(4-azidobenzoyl)-putrescine, we estimate the maximum distance between the distal nitrogen atom of the putrescine moeity (which is bound to Gln-41), and the active nitrene (which binds to the ε-amino group of Lys-113) to be 10.7 Å, and the maximum distance between the α-carbons of Gln-41 and Lys-113 to be 22.3 Å. Previously it was shown (Elzinga and Phelan, *PNAS* **81**, 6599) that phenylene-dimaleimide can crosslink Cys-374 and Lys-191. Measurement of a model of this crosslink shows that the sulphydryl group of Cys-374 and the ε-amino group of Lys-191 are no more than 9.8 Å apart, and that the maximum distance between the α-carbons of these amino acids is 21.4 Å.

These crosslinks provide crucial tests for the model of F-actin published by Holmes and colleagues (*Nature* **347**, 44). Although atomic coordinates of the side chains will be required for a definitive analysis, the model seems to accomodate the distances estimated by the crosslinks. The Cys-374:Lys-191 crosslink would involve monomers in opposite strands, while the Gln-41:Lyn-113 crosslink would involve sidechains from adjacent monomers in a given strand. The crosslinking results seem to corroborate the F-actin model.

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Rate of crossbridge dissociation following a rapid shortening step is greater in phasic than in tonic contraction of smooth muscle of guinea pig taenia coli

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Numerous studies have shown that in smooth muscle the shortening velocity and the energetic tension cost in smooth muscle are smaller during short-lasting 'phasic' than long-lasting 'tonic' contraction. One hypothesis to explain this is impeded dissociation of attached crossbridges with dephosphorylated 20 kDa myosin light chains ('latch' bridges; Hai and Murphy (1988) *Am. J. Physiol* **244**, C99). Direct evidence of this would be collateral effects on the stiffness of the muscle fibre. In guinea pig taenia coli strips stimulated by high-K^+ at 37° C, stiffness under isometric and unloaded shortening conditions was probed by rapid releases (0.3 ms) to slack length and then restretches to beyond the initial length (L_i). The restretches were performed after varying time to allow periods of unloaded shortening. Before the release, the strips had been stimulated for either 15 s (phasic contraction) or 5 min (tonic contraction). Force was identical in either stimulation mode. Stiffness was evaluated by plotting force (F) *versus* length (L) from data sampled at 100 kHz as described by Arheden and Hellstrand (*J. Physiol.* **442**, 601). Stiffness (slope of the L-F plot at L_i) was consistently smaller during restretch than during the original release from isometric contraction ('isometric stiffness'). Increasing the interval from release to restretch from 0.5 ms to several s progressively displaced the L-F plots towards smaller L, owing to intrinsic shortening of the fibre before the restretch. This displacement was slower in the tonic than in the phasic contractions, verifying a lower unloaded shortening velocity. Isometric stiffness was identical in either stimulation mode, but stiffness during restretch was smaller in phasic contractions. This was apparent already at 1 ms after the release and accentuated at later times. These results show that although the number of attached crossbridges at a given isometric tension is unaffected by the development of 'latch, the rate of net crossbridge dissociation on transition to unloaded shortening is descreased.

Transient kinetics of rigor and relaxed rabbit psoas myofibrils

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The chemical kinetics of myofibrillar ATPases were compared with those of myosin-S1 and crosslinked actoS1. The experiments were carried out by the rapid flow quench method in a solvent of near physiological composition but because of difficulties at higher temperatures, at 4° C. Rigor was ensured by 0.1 mM $CaCl_2$, and relaxed myofibrils by 2 mM EGTA. The data obtained was interpreted by the scheme:

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M.ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M.^*ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$$

$$M^{**}ADP.Pi \xrightarrow{k_4} M + ADP + Pi$$

where M represents myosin heads with or without actin interaction. The initial binding steps (K_1, k_2) were studied by the cold ATP chase and the cleavage (k_3, k_{-3}) and release of products (k_4) steps by the Pi burst method. The second order binding constant, k_2/K_1, was similar for the four materials ($1 \times 10^6 M^{-1} s^{-1}$) but there were significant differences in the kinetics of the cleavage step. Thus, with relaxed and rigor myofibrils the constants were identical ($k_3 = 17\ s^{-1}$, $k_{-3} = 1\ s^{-1}$) but they were different from those with S1 ($k_3 = 12\ s^{-1}$, $k_{-3} = 4\ s^{-1}$). The kinetics of the release of products (k_4) was rate limiting with all the four materials: with rigor myofibrils and crosslinked actoS1 $k_{cat} = 1.5\ s^{-1}$ but with relaxed myofibrils and S1 it was $0.015\ s^{-1}$. Thus, the main effect of calcium activation in rigor myofibrils is to increase 100 times the release of products. But with reference to S1, actin also has an effect on the cleavage step, even in relaxed myofibrils. This suggest that the myosin heads could be attached in both relaxed and rigor myofibrils.

Effect of small release during sarcomere-isometric tetanus on subsequent force development

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We investigated the effect of small shortening imposed on frog muscle fibres during sarcomere-isometric tetani. Sarcomere length was initially

kept constant, then slightly shortened (1–5% of initial length), and clamped again during the remainder of the tetanus. Force level after the imposed shortening was higher than before the shortening. It was higher than predicted by the descending limb of the force-length relation: by 50% at SL 3.2 μm. This unexpected effect is termed shortening-induced force enhancement. Two independent methods were used to check for inhomogeneity of sarcomere length within the sampled region: striation imaging and analysis of the intensity profile of the first diffraction order. Both methods agreed: sarcomere-length inhomogeneity in the sampled region was too small (±0.03 μm, SD) to account for the observed force enhancement. We studied the dependence of the force enhancement on size, velocity, and timing of the release. Only size has a significant effect. Releases of 20 nm per half-sarcomere were sufficient to produce an almost full increase; larger ones increased the force only slightly more. The enhancement was essentially absent at SL 2.2 μm, but increased progressively with increasing sarcomere length.

A particularly interesting feature of the result was that the phenomenon could be found only in sarcomeres that stretch during fixed-end tetani; those (near the ends) that shorten did not exhibit the enhancement. The results could be only partially reproduced in skinned fibres, where shortening induced only a very small force increased, not significantly different from the expectation based on the descending limb. However, the small increase was found only in regions that lengthen during fixed-end contractions (randomly located along the fibre), but not in those that shorten.

The entire phenomenon, including the distinction between sarcomeres that shorten ('generators') or lengthen ('sustainers') during fixed-end contraction, is predicted in a recently published model of contraction (Pollack (1990) *Muscles and Molecules: Uncovering the Principles of Biological Motion*, Ebner & Sons, Seattle). Enhancement occurs as 'sustaining' sarcomeres shorten, their myosin molecules melt, and they become 'generators'.

The enhancement phenomenon also accounts for the diversity of reported length-tension relations. Those relations obtained using length-clamped sarcomeres have invariably shown a classical, linear descending limb: length-clamped sarcomeres have undergone no shortening, and therefore show no enhancement. Shortening-induced enhancement does occur, however, in fixed-end contractions, where stronger sarcomeres do have the opportunity to shorten (and stretch the weaker ones). Such contractions are therefore expected to yield the higher, flatter length-tension relations that are consistently reported under such conditions (Pollak (1990)). Thus, the distinction between the higher, flatter-tension relations reported under fixed-end conditions and the linear descending limb observed under length-clamp conditions is explained.

Expression of a 33 kDa C-terminal fragment of human caldesmon in *E. coli*

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Caldesmon is an actin-binding protein present in smooth muscle and non-muscle cells. It inhibits the activation of myosin Mg^{2+}-ATPase by actin-tropomyosin. Seven different full-length caldesmon sequences have been determined so far; two are the high MW isoforms (87–93 kDa) found in smooth muscle tissue while the other four are low MW isoforms (55–65 kDa) found in non-muscle tissue. The difference between the two MW isoform groups is a central repetitive sequence believed to be in α-helical conformation, which is only found in the bigger smooth muscle caldesmon. However, the main functions of caldesmon have been found in the C-terminal third of the molecule. A 35 kDa C-terminal fragment produced by limited proteolysis with thrombin shows *in vitro* binding to actin, tropomyosin and Ca^{2+}-calmodulin. It shows simular inhibition of the myosin Mg^{2+}-ATPase activity activated by actin-tropomyosin as the whole protein.

We have expressed a C-terminal fragment of a human caldesmon spanning amino acids 476–736 according to the numbering of Bryan and colleagues (*J. Biol. Chem.* **264**, 13873–9 (1989)). The cDNA was cloned by N. D. Avent (University of Bristol, UK) from a human fetal liver library. It has an insert of 78 nucleotides coding for 26 amino acids compared with the chicken gizzard sequence of Bryan and codes for 288 amino acids. The clone was inserted into a T7 RNA polymerase based expression vector. The protein was expressed in BL21 (DE3) cells and identified by SDS-PAGE and Western blotting using a polyclonal antibody raised to sheep aorta caldesmon. The *E. coli* cells were lysed, DNAse treated, boiled in high salt and centrifuged to give a crude extract of the heat-stable peptide in the supernatant. Final purification was achieved by gel filtration on Superose 6. The yield was approximately 3 mg ml^{-1} of bacterial culture. The 33 kDa peptide showed anomalous migration (49 kDa) on SDS-PAGE, a property typical of caldesmon. It inhibited potently the tropomyosin-enhanced actomyosin ATPase activity. The inhibition could be reversed by Ca^{2+}-calmodulin. So far the expressed human caldesmon fragment has shown quantitatively and qualitatively very similar properties to full length chicken gizzard caldesmon. Unexpectedly the fragment has been found to bind myosin, a property which is localized in the N-terminal part of chicken gizzard caldesmon.

Mechanical properties of normal and mdx mouse sarcolemma: bearing on function of dystrophin

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Sarcolemmal vesicles (20–90 μm diameters) prepared from normal or mdx mice (Burton *et al.* (1989) *Muscle Nerve* **11**, 1029–38) were aspired under video observation into pipettes of uniform bore (6–24 μm diameters) so as to impose an increase in surface area. This method yields information on: (1) elastic modulus of area expansion (dyn cm^{-1}), (2) lysis tension (dyn cm^{-1}), and (3) critical area strain (% area increase) (Bovelle *et al.* (1990) *J. Physiol.* **429**, 4P). For 132 vesicles from normal muscle *versus* 179 mdx vesicles results (mean ± SE) were: (1) 6.2 ± 0.2 *versus* 5.2 ± 0.2; $P < 0.01$, (2) 2.0 ± 0.1 *versus* 2.0 ± 0.1, (3) 316 ± 13 *versus* 277 ± 9; $P < 0.05$.

Membrane blebs and vesicles can be raised also by exposing fibres to hypotonic solutions, and osmotic fragility of normal and mdx fibres has been compared by Menke and colleagues ((1991) *Nature* **349**, 69–71) who found mdx fibres to lyse more readily by about 20 mOs. From our above results it follows, from Laplace and van't Hoff, that sarcolemmal blebs or vesicles can sustain an osmotic gradient no greater than 1.0 mOs and that the small difference between the tensile strength of mdx and normal sarcolemma corresponds to about 0.1 mOs. The observed higher fragility of mdx fibres cannot therefore be attributed to a lower tensile strength of mdx sarcolemma.

An alternative hypothesis that comprehends all the available information is that dystrophin is essential for the normal folding of the surface membrane (Dulhunty *et al.* (1975) *J. Physiol.* **250**, 513–40). If dystrophic muscle possessed less spare membrane it would be more prone to damage when the fibres undergo extension and/or swelling. This hypothesis is open to morphometrical and electrophysiological test.

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Reactivity of fast and slow skeletal muscle, after denervation: comparison with muscular dystrophies

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Investigations have been undertaken on the reactivity of the striated muscle fibre, after chronic denervation using comparison fibres isolated

both from the extensorum digtorum longus (EDL) and rat soleus muscle. Some of the results have been compared with those obtained in similar experimental conditions in Duchenne muscular dystrophy (DMD).

At 60 days after denervation, the sensitivity to the pCa value is more reduced in the soleus muscle as compared with EDL. Yet, in both cases, the reactivity of the denervated muscle is slower than the normal muscle, i.e. the amplitude of the contraction-relaxation cycles, as well as their duration, is significantly prolonged. The maximum contraction tension, at pCa = 4.4, was observed as having close values in both muscles.

After chronic denervation, the sarcolemma is hyperpolarized, spontaneously; although atypical action potentials were obtained, the spike value ranges between 5 and 10 mV: the duration of the action potential has been calculated at −25 mV, being 0.7 ms compared with 0.4 ms in the normal muscle. The time required for attaining the maximum value of the K contracture is slower in the case of EDL and shorter with one soleus muscle, although in both experiments the contractions are weakly maintained.

The time necessary for muscle recovery to its initial condition, after removal of the hyperpotassium solution of the bath, increases: the soleus muscle maintains the potassium contracture for a longer period of time. Some sarcoplasma proteins are absent in the denervated muscle, as compared with the normal, yet within the 10–120 kDa range of molecular weights some proteins may be observed, appearing only after chronic denervation; also some types of progressive muscular dystrophys may be met. Major contractile proteins are nevertheless present in each type of gel.

Study of the sarcolemma proteins evidences that, after denervation, two groups of four fractions each to appear, more evident in the case of the soleus muscle, ranging between 10–50 kDa. Part of these fractions could be identified in the case of DMD also, and entitles us to think that, in some respects, the denervated muscle has characteristics of a dystrophic muscle.

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 result of regulatory processes taking place at a pretranslational level. It is well known that a fast muscle subjected to continuous 1.0 Hz stimulation acquires properties which make it indistinguishable from a naturally occurring slow muscle are time courses and 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 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 of 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.

Expression of GH receptor mRNA, intermediate filaments and smooth muscle actin in regenerating rat muscle: differences between normal and hypophysectiomized animals

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The early phase of muscle regeneration is considered to be regulated mainly by local factors, while hormones such as growth hormone (GH) and thyroid hormones are of importance for the final maturation of the regenerated cells. To further understand the regulation of muscle generation it is of interest to know at which stage of maturation regenerating muscle cells begin to express GH receptors *in vivo*.

In the present study expression of GH receptor mRNA was investigated by *in situ* hybridization in regenerating muscle from normal and from hypophysectomized rats. Muscle injury was induced in one EDL muscle by ischaemia preceded by glycogen depletion. The regenerating muscles were sampled 2, 3, 4, 5 and 7 days after the injury. Cryostat sections were prepared and processed for *in situ* hybridization. A digoxigenic-labelled (Boehringer Mannheim, Germany) RNA probe directed against the extracellular part of the rat GH receptor was used. The hybridization was visualized by a monoclonal antibody directed against digoxigenin, followed by a conventional immunohistochemical detection system. In serial sections expression of vimentin, desmin and smooth muscle actin were demonstrated by immunohistochemistry using monoclonal antibodies.

In both normal and hypophysectomized rate distinct expression of GH receptor mRNA could be demonstrated in the regenerating muscle cells at the late myoblast/myotube stage. The GH receptor expression appeared to decline with increasing maturation of the regenerated muscle fibres. The regeneration process appeared to be slower in the hypophysectomized rats as evidenced by persistent expression of vimentin and smooth muscle actin in the regenerated cells after 7 days. Furthermore, in these animals expression of GH receptor mRNA was delayed.

The finding that GH receptor mRNA is expressed already at the myoblast stage indicates that GH may influence also the early phase of muscle regeneration *in vivo*. To what extent lack of GH in the hypophysectomized rats contributes to the delayed regeneration process remains to be elucidated.

Stress models to test for the biological role of dystrophin in muscle cells

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The elucidation of the molecular basis of Duchenne and Becker dystrophies (DMD and BMD) has led to the discovery of a submembraneous cytoskeletal protein, dystrophin (Hoffman *et al.* (1987) *Cell*

51, 919–28), which plays a hitherto undefined role in the structural maintenance of muscle fibres. In the mouse, nonsense mutation in the homologous mdx gene (Sicinski *et al.* (1989) *Science* **244**, 1578–80) leads to truncation and loss of the dystrophin molecule and to necrosis of muscle fibres. Lost muscle fibres in the MDX (mdx/mdx or mdx/y) mouse are, however, efficiently replaced by regeneration.

Using osmotic shock, we have detected differences in the stabilities of muscle cells from normal and MDX mice as well as of cultured myotubes (Menke and Jockush (1991) *Nature* **349**, 69–71). Others have found elevated activities of sarcolemmal Ca^{2+} channels in DMD and MDX myotubes (e.g. Fong *et al.* (1990) *Science* **250**, 673–6). In principle, dystrophin could influence osmotic stability either directly, by 'mechanical strengthening' of the membrane/cytoskeletal complex, or indirectly, by influencing compensatory ion fluxes. Blockers of Ca^{2+}-, K^+- and Cl^--channels as well as a Ca^{2+} ionophore, were used to analyse the contribution of ion fluxes to osmotic stabilities of isolated mature muscle fibres and a cultured myotubes. The differences in stability between wildtype and dystrophin-less cells were not eliminated by these manipulations of ion fluxes or by supression of metabolism. Other indirect effects of the absence of dystrophin on cell stability must be considered, such as long-term protease activation, the quality of the extracellular matrix or abnormal morphogenesis.

Stress experiments on myotubes are applicable to heterokaryons and transfected cells as well as to biopsy material from DMD or DMD patients.

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Mathematical modelling of myocardial non-homogeneity contribution into the contractile function of the myocardium

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Earlier we developed a mathematical model for cardiac muscle contraction that allowed for inactivation realized through the effects of cooperativity of contractile proteins. The model was used to analyse the mechanical function of a non-homogeneous myocardium. To simulate the latter we chose, as the simplest system, a duplet in which muscles with different mechanical properties were connected in series and in parallel. The mechanical non-homogeneity was given by various sets of parameters reflecting the molecular mechanisms of muscle contraction and the elastic properties of muscle. Numerical experiments showed that the basic effect due to the non-homogeneity consists in the non-additivity of the mechanical characteristics of a muscle, for example, of the relationship end-systolic length–end-systolic force, the muscles being combined in a non-homogeneous duplet. As a rule, non-additivity consisted in a negative inotropic effect. Only in the case of the difference V_{max} the members of the duplet displayed additivity (and only over a certain range of end-systolic lengths). Moreover, for a parallel duplet whose members differed only in serial compliance a positive inotropic effect was observed. Analysis showed that the cause of non-additivity is the redistribution of loads between muscles (in a parallel duplet), redistribution of lengths (in a serial duplet), changes in the rate of contraction of each muscle compared to contraction in isolation, shifts in the time of attainment L_{ex}. Also, the model predicts that additional inactivation of contractile proteins in a duplet against isolation is a substantial factor to non-additivity. Among the factors of nonhomogeneity studied the basic determinants are the difference in amplitude between the isometric tensions developed by each muscle in isolation and the asynchronism in the development of these tensions. Good agreement with our experimental results was obtained.

Tightly bound Mg^{2+} is necessary to restore the inhibitory activity toward DNaseI and polymerizability of actin split at Val-43 by a novel protease from *E-coli*

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Earlier studies (Khaitlina *et al.* (1988) *FEBS Lett.* **228**, 172) indicated that actin split between Gly 42-Val 43 by a novel protease from *E. coli* A2 strain is unable to polymerize and to interact with DNaseI. Using an increase in the fluorescence of N-(1-pyrenyl) iodoacetamide label attached to Cys 374 to monitor actin polymerization, we confirmed here that the split actin, even at the concentration as high as 76 μM, does not assemble into filaments on addition of 0.1 M KCl. The five-fold increase in the fluorescence was however observed on addition of 2 mM $MgCl_2$, alone or subsequent to 0.1 M KCl. Similar results were obtained when 0.1 M KCl alone was added to the split actin pretreated with 20–100 μM $MgCl_2$/0.2–1.0 mM EGTA. Polymerization of the *E. coli* protease-split actin under these conditions was confirmed by measuring an increase in viscosity of the solutions and by electron microscopy. The results show that the occupation of the high-affinity metal ion-binding site Mg^{2+} is necessary for restoring the polymerizability of the split actin. The critical concentration for polymerization of the *E. coli* protease-split Mg-actin in 0.1 M KCl was 2.5 μM, about three-fold higher than the critical concentration for polymerization of actin split with subtilisin between residues 47–48, and about 30 times higher than that for intact actin. Polymerization of the *E. coli* protease-split actin was not accompanied by the appearance in excitation spectrum of the pyrenyl label of the peak at 365 nm characteristic of intact F-actin and observed also in the polymer of subtilisin-split actin. Treatment of the polymerized *E. coli* protease-split actin with N,N-1,2-phenylenebismaleimide did not reveal the intermolecular cross-link formation between Cys 374 and Lys 191. These data suggest that distortion of the polypeptide chain structure around Gly 42-Val 43 on splitting the bond between these residues is transmitted to the C-terminal segment, and that the resulting alteration in the conformation of this latter region also contributes to weakening the intermonomer interactions.

Replacement of the tightly bound Ca^{2+} by Mg^{2+} resulted also in partially restoring the ability of the split actin to inhibit the activity of DNaseI. Taken together, the results presented here indicate that the tightly bound divalent cation has small but significant effect on the polypeptide chain region around Val 43 which is known to be directly involved in the DNaseI binding and to be part of an actin-actin interface. Replacement of the tightly bound Ca^{2+} by Mg^{2+} seems to partially restore the native conformation of this region in the *E. coli* protease-split actin.

Ca^{2+}-free contraction of the skinned guinea pig taenia coli preparations with unphosphorylated myosin light chains

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To elucidate the possible role of the actin-linked regulator system in smooth muscle we studied the contractile characteristics of the skinned (Triton X-100) preparations from the guinea pig taenia coli muscle treated with low concentrations of glutaraldehyde ([GA] = 0.025%, crosslinking time 100 s). It was found, that the fibres crosslinked in the 'high' rigor state were capable of developing isometric tension or contracting at zero load after the addition of MgATP (5 mM) in the absence of Ca ions (pCa 9). Maximal value of the Ca^{2+}-free tension was found to be 60–65% as compared with the Ca-activated (pCa 4) one in a control specimens. The fibres crosslinked in the relaxed state ([MgATP] = 5 mM, pCa 9) were not contracted and developed any tension in the absence of Ca ions. The treated specimens in large part

retained the ability to develop Ca-activated tension. The phosphorylation level of the myosin 20 kDa light chain (LC) in the fibres developing maximal Ca-free tension was found to be the same as in control preparations in the relaxed state, as was evidenced by urea-glycerol PAGE (Sobieszek (1986) *Electroph.* **7**). The results presented are explained on the basis of the assumption that GA can 'freeze' the 'on' state in the thin filaments, which takes place after the cooperative attachment of the rigor crossbridges to the actin. It can be supposed that the permanently turned 'on' state of the thin filaments is a sufficient condition for the maintenance of the large value of tension in a skinned smooth muscle preparation at low Ca ions concentrations and unphosphorylated 20 kDa LC. The fibres modified in such a way may represent a model of the 'latch' state in the intact smooth muscle.

Effect of Ca^{2+}-CmD complex on the cooperative behaviour of the cross bridges in the skinned smooth muscle preparations

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To examine if the Ca-CmD complex can influence the interactions between myosin crossbridges and actin in the smooth muscle we studied the relaxation of the rigor tension in the skinned (Triton X-100) chicken gizzard preparations in the absence and presence of Ca-CmD (pCa 5, [CmD] = 1 μM). We used preparations which have been stored after skinning in the relaxing solution at 4° C over 24 h. It was found that such preparations have lost their ability to contract in Ca-activating solution (pCa 4), but are able to develop significant rigor tension (about 40% of max Ca-activated) in the absence of Ca ions. Skinned fibres were transferred into 'low' rigor state, and the dependence of tension *versus* [MgATP] was studied. The tension rise was observed after the low concentrations of MgATP (< 50 μM) has been added. The value of such tension increment was found to increase markedly (up to 10%) in the presence of Ca-CmD complex. Phosphorylation level of the myosin LC 20 kDa in the presence of the Ca-CmD was determined by urea-glycerol PAGE (Sobiezsek (1986) *Electroph.* **7**) and was found to be the same as in a control preparation. The results presented demonstrate the ability of Ca-CmD complex to facilitate the interaction of the unphosphorylated crossbridges with the actin in skinned smooth muscle preparations, that has been shown previously *in vitro* (Sobue (1982) *Biomed. Res.* **3**). As the force developed in smooth muscle at low [MgATP] in the absence of Ca ions is thought to be caused by cooperative turning 'on' of the thin filaments by rigor crossbridges (Somlyo (1988) *J. Gen. Phys.* **91**) it can be supposed that mechanisms of the observed disinhibition may involve the cooperativity within the thin filaments.

Polarized microfluorimetry investigation of the conformational changes of F-actin in myosin-free ghost single fibre induced by binding of glycolytic enzyme lactate dehydrogenase

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The changes in F-actin conformation of myosin-free single ghost fibre induced by binding of lactate dehydrogenase (LDH-EC 1.1.1.27) have been studied by polarized microfluorimetry (Borovikov and Chernogriadskaya (1979) *Microsc. Acta*, **81**, 383). In this method some residues of actin are modified by fluorescent labels and their orientation distribution is studied. It is suggested that the changes in orientation and mobility of dyes attached to F-actin reflect the changes in conformation of the corresponding regions of this protein (Yanagida and Oosawa (1978) *J. Mol. Biol.* **126**, 507; Nowak *et al.* (1989) *Biochem. Biophys. Acta* **999**, 289).

Glycerinated single ghost fibres free of myosin, tropomyosin and troponin (Borovikov and Gusev (1983) *Eur. J. Biochem.* **913**, 363) were used after the modification of F-actin by fluorescent probes (phalloidin-rhodamine and 1.5-IAEDANS). Myosin subfragment-1 (S-1) was obtained from rabbit psoas muscle. The molar ratio of LDH to F-actin was found by densitometric scans of the gels of SDS-PAGE. Experimental data were analysed in terms of a mathematical model described earlier (Kakol *et al.* (1989) *Biochem. Biophys. Acta* **913**, 1).

The formation of complex between LDH and F-actin was accompanied by the changes in parameters of the intrinsic and extrinsic polarized fluorescence of F-actin of ghost fibres. Computer analysis of polarized fluorescence has shown that binding LDH to F-actin decreases the angles of emission and adsorption dipoles and increases the angle between the F-actin axis and the fibre axis, thus suggesting that F-actin in ghost fibre becomes more flexible. It was found that LDH stimulated actin-activated Mg^{2+}-ATPase of myosin S-1 by 30%. F-actin ghost fibres depressed LDH activity to 20% of initial values. The study shows that LDH adsorption to F-actin of ghost fibres induced the structural changes in F-actin as well as in catalytic properties of the metabolic enzyme itself.

It was assumed that the coupling of energy-providing mechanism with that of muscle contraction is realized through the conformational changes in F-actin.

Binding region for the regulatory light chain on the heavy chain of skeletal muscle myosin

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Electron microscopic mapping with antibodies against the regulatory light chain (RLC) of skeletal muscle myosin places it near the basis of the myosin head portions where they join the rod (Winkelmann and Lowy (1986) *J. Mol. Biol.* **188**, 596–612). Myosin subfragment-1 (S1) prepared from rabbit skeletal muscle myosin by papain (P-S1) still contains the RLC. S1 prepared by chymotrypsin (C-S1) only retains the LC1 or LC3 but no RLC. Removal of the RLC from P-S1 leads to polymeric aggregation of the P-S1. C-S1 does not bind RLC and remains always monomeric. The heavy chain (HC) of P-S1 is about 3300 Da larger than that of C-S1. This corresponds to around 30 amino acid residues. This difference peptide seems then to be responsible for the binding of the RLC to P-S1. The sequence of subfragment-2 (S2) prepared by trypsin starts at Ser-844 (Capony and Elzinga (1981) *Biophys. J.* **33**, 148a). Papain splits the HC near that region around Leu-842 (Lu (1988) *Proc. Nat. Acad. Sci. USA* **77**, 2010–13) almost where the S2 sequence begins. The difference peptide between C-S1 and P-S1 extends therefore upstream from around Leu-842. C-S1 after treatment with trypsin (tryptic-C-S1) ends at Arg-809 (Gallagher and Elzinga (1980) *Fed. Proc.* **39**, 2961a). Between typtic-C-S1 and P-S1 is a stretch of 22 amino acid residues whose sequence is not known for rabbit skeletal muscle myosin HC. However, five different vertebrate myosin HC including cardiac, skeletal and embryonic varieties all contain the sequence stretch Trp-Pro-Trp-Met-Lys-Leu. We determined therefore the Trp content in the HC and found 5.21 for C-S1 (5 Trp are in the sequence of rabbit muscle myosin C-S1) and 7.20 for P-S1. The two more Trp found in the longer HC of P-S1 suggests that they are also present in that region of rabbit skeletal muscle myosin HC whose sequence is not yet known. The difference peptide which binds the RLC is therefore located between Arg-809 and Ser-844.

Peptide competition of actin activation of S1 ATPase by an amino terminal actin fragment

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The amino-terminal region of actin to participate in the binding of myosin subfragment 1 (S1) during the crossbridge cycling thereby activating the magnesium-dependent myosin ATPase. An actin fragment containing the amino terminal residues 1–44 mimicked this activating effect. In an *in vitro* assay using isolated S1 from rabbit skeletal muscle the peptide increased the magnesium-dependent S1 ATPase activity by a factor of 2.2 and 3.3 with peptide concentrations of 8.3 μM and 16.6 μM respectively. S1 concentration was 0.1 μM. Under identical conditions another actin peptide containing residues 1–18 had no influence on the S1 ATPase activity. The acto-S1 ATPase on the other hand was inhibited by nearly 70% after addition of the actin peptide 1–44 (16.6 μM), half-maximal inhibition occurring at a peptide concentration of 6.0 μM. S1 concentration was 0.1 μM, actin concentration 2.0 μM. At higher actin concentration larger peptide concentrations were required for inhibition. Again the actin peptide 1–18 had no effect, i.e. the acto-S1 ATPase activity was altered. These results suggest that the actin peptide 1–44 may compete with actin for its binding site on myosin. Obviously it contains regions important for actin-myosin interaction that are not contained by the peptide 1–18. This conclusion is in agreement with that of van Eyk and colleagues ((1991) *Peptides as Probes in Muscle Research*, Heidelberg Springer:, pp. 15–31) who found that another actin peptide (amino-terminal residues 1–28) slightly (by approximately 30%) activated the S1 ATPase.

Length determination of coaggregates of locust myosin and mollusc paramyosin by projectin

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Locust myosin and projectin were purified by FPLC Mono Q chromatography (Ziegler *et al.* (1990) *Muscle Motil* (edited by G. Maréchal and U. Carraro) Vol 2, p. 9 Intercept. New Hampshire) in the presence of PMSF, leupeptin and pepstatin A. Molecular weight determination was performed according to Laemmli ((1970) *Nature* **227**, 680, and Hu and colleagues ((1990) *J. Muscle Res. Cell Motil* **11**, 497) using cross-linked rabbit myosin HC as standards. Two projecting bands could be observed between the myosin HC tetramers and pentamers, suggesting a molecular weight of 900 kDa and 1000 kDa respectively. Rod-like structures and aggregates with herringbone patterns were obtained after overnight dialysis of projectin solutions against low ionic strength buffers (50 mM KCl, 20 mM Tris/maleate, pH 7.5). The length of the rod-like structures was 1–2 μm, their diameters 4–6 nm.

If mollusc paramyosin and locust myosin (0.5 mole PM per mole M_{HC}) were codialysed overnight against a low ionic strength solution (see above) filament-like coaggregates were formed with lengths of 1–5 μm. These lengths could be reduced to 1–2 μm if up to 0.04 mole projectin per mole M_{HC} and per 0.5 mole PM were added before dialysis (1.5 μm is about half of the length of native thick filaments). Binding studies with gold-labelled antibodies showed that, as in native filaments, gold particles bind to the ends of the aggregates only. This indicates that projectin is exposed only at the ends and unlike titin not along the entire length of the filaments.

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Some aspects of functional activity of chick embryo skeletal muscles

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The myofibrillar ATPase activity and the extent of superprecipitation (SPP) for natural actomyosin from the muscles of chicken embryos aged 8–19 days *in ovo* were determined. Breast and soleus muscles were studied. The SPP extent for the first and second muscle types increases in the course of development 2.2-fold and 1.5-fold respectively. During the embryonic life the ATPase level rises 4.5-fold. The changes observed are supposedly connected with a delay of the myosin light chain biosynthesis in the embryonic muscles. SPP of actomyosin from the embryonic muscles shows a very low sensitivity to Ca. ATPase activity of the natural AM also has a tendency to decrease only at high concentrations of EGTA. One of the reasons of low ATPase activity of early actomyosin could be a poor development of the calcium regulatory systems. Besides, a poorly developed sarcoplasmic system in the embryonic muscles seems to be incapable of serving as a regulator of intracellular calcium. Changes were found in the secondary structure of myosin and its rod part during differentiation. The method of O^{18} exchange reaction was used to compare the functioning of the active sites of ATPases of myosines from embryonic and adult muscles. A similarity was found in ATP hydrolysis intermediates between embryonic and adult muscles. This suggests the existence of common stages in the utilization mechanism of ATP energy in different forms of biological motility.

^{31}P-NMR-spectroscopy of cardiac troponin

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Cardiac troponin is a phosphoprotein which is involved in the regulation of muscle contraction. Troponin C binds Ca^{2+} and Mg^{2+} (Holroyde *et al.* (1980) *J. Biol. Chem.* **255**, 11688–93). In the intact organ both subunits, T and I, contain up to two phosphoserines, respectively. The phosphorylation domain of troponin I shows a common motif of three arginines followed by two phosphorylable serines (Leszyk and Collins (1987) *Biochemistry* **26**, 7042–7). Upon β-adrenergic stimulation 1 mol P/mol troponin may be incorporated into this subunit affecting the Ca^{2+}-affinity of troponin C (England (1976) *Biochem. J.* **160**, 295–304; Moir *et al.* (1980) *Biochem. J.* **185**, 505–13). Until now, the mechanism of this signal transduction is unknown. However, former ^{31}P-NMR and Ca^{2+}-measurements led to the hypothesis that Mg^{2+}-binding to Mg^{2+}-specific sites of troponin C is involved (Beier *et al.* (1988) *Eur. J. Biochem.* **176**, 327–34). At room temperature, Mg^{2+} shifts at least two signals of the three signal^{31} P-NMR-spectrum of troponin I (resulting from a mixture of the mono- and bisphosphorylated forms) to lower p.p.m. values. This shift occurs at Mg^{2+}-concentrations < 0.8 mM $MgCl_2$. No apparent line broadening of the resonance occurs on Mg^{2+}-saturation of phosphotroponin. Thus, there seems to be no direct linkage between these phosphate groups with Mg^{2+}. Measurements using phosphotroponin with different contents of phosphate in the I subunit and none in the T subunit, will show which forms (mono- or bisphosphorylated or all) will respond to Mg^{2+}. A Mg^{2+}-binding curve will be established.

Hyperplastic and hypertrophic growth of axial muscle of *Cyprinus carpio* L. (Teleostei)

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We examined hyperplasia and hypertrophy in growing white axial muscle by analysing the frequency distribution of the cross-sectional

area (diameter) of the muscle fibres. In fish smaller than 8 cm standard length (SL) growth by hyperplasia appeared to be predominant. Between 8 cm and 20 cm SL the relative importancy of hypertrophy increased. In fish larger than 20 cm SL hypertrophy was the more important process and in fish of about 40 cm SL only hypertrophy was observed. This transition is accompanied by a decrease in the slope of the growth curve of white axial muscle of carp (Oikawa and Itazawa (1984) *Copeia*, 800–3). This is in agreement with an earlier observed correlation of hyperplasia to a high rate of growth in fish (Weatherley *et al.* (1980) *Can. J. Zool.* **58**, 1535–41).

The DNA's protein ratio of the muscle decreased by 50% between 5 cm and 15–20 cm SL and increased again in fish larger than 20 cm SL. *In vitro* experiments showed an increasing proliferation potential of myosatellite cells over the same size range. Myosatellite cells isolated from carp of 5 cm SL have a low level of proliferation; 80% of these cells are postmitotic. Myosatellite cells isolated from carp larger than 15 cm SL have a higher proliferation level; circa 50% are postmitotic. The occurrence of a high percentage of postmitotic myosatellite cells together with a decreasing DNA to protein ratio during hyperplastic growth, suggests the existence of a subpopulation of highly differentiated myosatellite cells that is mainly used in hyperplasia.

Weak crossbridge attachment to actin at near physiological conditions ($\mu = 170$ mM, $T = 20°$ C) and its significance for force generation

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Previously we showed that caldesmon reduces relaxed fibre stiffness in skinned rabbit psoas fibres at low ionic strength and low temperature apparently by decreasing the number of crossbridges that can attach to actin in the weak binding states. In parallel to relaxed stiffness, active force generation was inhibited at least by the same amount, although caldesmon had no effect on crossbridge attachment in the strong binding states like in the presence of $MgPP_i$ or in rigor. This suggests that the selective inhibition of weak crossbridge attachment to actin is sufficient to inhibit active force generation.

Since these experiments were carried out at low ionic strength and low temperature, we extended our studies to more physiological conditions. We show that at high ionic strength (120–170 mM) and higher temperature (20° C) the remaining fibre stiffness under relaxing conditions is also the result of weak crossbridge attachment to actin as caldesmon specifically reduces this fibre stiffness. Under these more physiological conditions the inhibiting effect of caldesmon on the weak crossbridge attachment to actin again results in a parallel decrease of active force. Using $MgATP_\gamma S$ instead of MgATP, we find that caldesmon interferes more effectively with weak crossbridge attachment to actin at high Ca^{2+}-concentration such that now the observed inhibition of weak crossbridge attachment and of active force is almost identical. These results provide further evidence that weak attachment of crossbridges to actin is essential for their transition into the force generating states.

Vacuolization of skeletal muscle fibres during spreading degeneration

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The development of spreading degeneration (SD) in isolated frog skeletal muscle fibres is accompanied by strong vacuolization of the part of fibre adjacent to the necrotic boundary. If prior to damage the fibres were incubated, in Ringer solution with ferritin, most of the vacuoles at a distance of 1–2 mm or more from the boundary of SD contained some amount of ferritin. It is concluded that these vacuoles originate from swelling of the T tubules. Although SD is irreversible, the vacuolization disappears in 5 to 10 min of incubation of damaged fibres in Ringer solution with 200–400 mM of glycerol. The same effect is produced by some other low-molecular non-electrolytes penetrating the muscle membrane at a rate comparable to that of glycerol. Similar vacuolization of the T-system occurs during SD in fast muscle fibres (EDL muscles) of young rats, whereas in slow (SOL) muscles it is very poorly expressed. In close proximity to the necrotic boundary the vacuolization is mostly caused by swelling and fusion of the adjacent terminal cisternae of sacroplasmic reticulum (SR). Mitochondria also participate in this vacuolization. The vacuolization is expressed the better, the slower the SD is. After incubation in Ringer solution with glycerol this vacuolization remains unchanged.

It is assumed that vacuolization of the T-system in the areas distant from the necrotic boundary is from the leakage of K^+ from the damaged muscle fibres. In the proximity to the boundary of SD the vacuolization is associated with Ca^{2+} release from SR and activation of muscle proteases.

Biochemical evidence for the presence of an actin-like protein in prokaryotic cyanobacteria group

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Spirulina platensis cells were sonicated in the presence of 0.6 M KI-containing buffer. After chromatography, SDS/PAGE analysis showed a major protein migrating as a 46 kDa band and a minor pigmented component of 26 kDa. Reactivity of the 46 kDa component with anti-actin antibodies induced by oxidized skeletal muscle actin anti-(Ox actin) was revealed by immunoblotting.

It was observed by ELISA an apparent K_D of $1.6\ 10^{-7}$ M for anti-(Ox actin) antibodies and 1.4×10^{-7} M for anti-(285-375 peptide) antibodies. In comparison no reactivity was obtained for a purified fraction EF/TU of *E. coli*.

The 46 kDa protein could be purified by affinity chromatography over sepharose linked anti-(Ox actin) antibodies or sepharose linked skeletal myosin head. The protein was polymerized in the presence of skeletal myosin head or salts.

DNase I binding with the 46 kDa (apparent K_D of 1.5×10^{-5} M) was followed with anti-DNase I (ELISA). Further inhibition of DNase I activity was evidenced.

Using negative staining in electron microscopy we have attempted to characterize the 46 kDa actin-like protein. Short filaments of about 6 nm in diameter were observed in the presence of salts, myosin head and spermine. In addition myosin was detected by specific antibodies induced by modified rabbit skeletal myosin.

Evidence that nebulin acts as a protein-ruler to regulate the length of thin filaments

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Nebulin is a massive (approximately 800 kDa) protein abundant in vertebrate skeletal muscle. Its properties, role and exact location are not known, but antibody labelling has suggested an association with thin filaments where a role as a 'protein-ruler' controlling filament

length has been postulated. Electron microscopy shows that in many muscles thin filaments have constant length, indicating they are assembled from exact numbers of actin, tropomyosin and troponin subunits. As the filament is many times longer than these molecules, the observed length precision is difficult to explain without a template or ruler spanning the entire structure. Here we describe evidence supporting the protein-ruler idea.

A 560 residue partial amino acid sequence was obtained from nebulin, by PCR extension of a previously reported human nebulin cDNA clone. The derived sequence consists entirely of a repeated motif containing about 35 residues and super-repeats of $7 \times 35 = 245$ residues. These patterns are likely to persist throughout the molecule. About 25% of the residues are conserved between different repeat-motifs and about 70% between super-repeats. Comparative data show there is also a high degree of conservation between species; in a 200-residue section common to the rabbit and human molecules, there was 92% conservation at the nucleotide level and 98% at the protein level. Thus mutations have occurred, but only conservative amino acid substitutions have been allowed.

The sevenfold periodicity in the super-repeats suggests that interactions with actin and/or tropomyosin in thin filaments. The repeat-motifs are likely to be largely α-helical and an 800 kDa α-helical molecule would have a length of roughly 1 μm, comparable to the filament length. We propose that one super-repeat spans 38.5 nm along the filament, similar to tropomyosin. This would require an axial translation of 0.16 nm per residue, close to the value of 0.15 for a continuous α-helix. Between muscles where thin filament length is different but exactly specified, nebulin was found to vary in size roughly in proportion to filament length. In cardiac muscle, where filament length varies by up to 30%, nebulin is absent. These data are therefore consistent with the hypothesis that nebulin acts as a protein-ruler to control exact assembly of thin filaments.

Flash photolysis of a caged Ca^{2+} chelator (diazo-2) increases relaxation rate of single intact *Xenopus* muscle fibres

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There is no consensus about what factor is rate limiting for relaxation in skeletal muscle; the two main possibilities are crossbridge detachment rate and rate of lowering of cytoplasmic (Ca^{2+}). We have used the recently available caged Ca^{2+} chelator diazo-2 (Adams *et al.* (1989) *J. Am. Chem. Soc.* **111**, 7957–68) in an attempt to resolve this question.

Single fibres (type 1) from lumbrical muscles of *Xenopus* were incubated for 1–2 h in 5–10 μM diazo-2/AM in Ringer solution with 1–2% DMSO and mounted in a small trough with a quartz glass window. Short, 70 Hz tetanic contractions (21–23° C) were studied and the initial, slow phase of relaxation was analysed (the isometric phase before the 'shoulder'). Following incubation in diazo-2/AM twitch force diminished, tetanic force rose more slowly and relaxation rate decreased. A flash from a Xenon lamp was discharged either at 16–18 ms after the last tetanic stimulus (F1) or at 30–40 ms (F2). In both cases relaxation rate immediately after the flash was markedly increased compared with the rate at the corresponding time of a pre-flash contraction. Compared with control values, before diazo-2 incubation, the early flash (F1; $n = 5$) increased relaxation rate (measured as tension fall over 10 ms) from 3.3 ± 0.5 to $3.6 \pm 1.2\ s^{-1}$ which was still lower than the maximum rate of the slow phase ($4.9 \pm 0.7\ s^{-1}$). The late flash (F2; $n = 6$) had a more striking effect and increased the mean rate from 4.7 ± 0.9 to $8.1 \pm 3.0\ s^{-1}$. The results indicate that speed of relaxation of intact frog fibres is sensitive to lowering of $(Ca^{2+})i$ and suggest that Ca^{2+} removal is rate-limiting for relaxation under the present conditions.

Domain structure of the myosin head

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The structure of the myosin head in the active fragments of myosin from rabbit skeletal muscle was studied by differential scanning microcalorimetry. Three independently melting regions (domains) were revealed in different types of myosin subfragment-1 (S1). Selective denaturation of the middle 50 kDa segment of the S1 heavy chain resulted in the disappearance of the heat sorption peak corresponding to the melting of the first, the most thermolabile domain. The thermal denaturation of the isolated C-terminal 20 kDa fragment of the S1 heavy chain correlates with the melting of the third, the most thermostable domain in the S1 molecule. No additional domains were revealed in the heads of heavy meromyosin containing 'neck' region and regulatory light chains. On the basis of results obtained and of data in the literature we suggest the domain model of the myosin head. According to this model the first, the most thermolabile domain, corresponds to the N-terminal part of the 50 kDa segment in the S1 heavy chain located on the tip of the myosin head. The widest part of the head corresponds to the second domain, formed by different parts of all three segments of the S1 heavy chain (23, 50 and 20 kDa). The third, the most thermostable domain seems to correspond to the C-terminal half of the C-terminal 20 kDa segment of the S1 heavy chain together with associated alkali light chain. It forms the narrow part of the head joined to the 'neck' region connecting the head and the rod part of the myosin molecule. Apparently the 'neck' region itself and associated with them regulatory light chain do not form the separate calorimetric domain in the head.

Specific force in relation to growth in rat skeletal muscle

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In the present study changes in morphological and functional characteristics during growth have been investigated in rat EDL muscle. Experiments were performed at 37° C using anaesthetized male Wistar rats (pentobarbitone 60 mg kg^{-1}, of approximately 40, 60 and 120 days old and 2 years old. Mean body masses were approximately 150, 250, 400 and 700 g, respectively. Muscle optimum length (length with the highest tetanic force) was assessed with short tetani (150 ms; stimulation frequencies 100–130 Hz). At optimum length, muscle belly length and the most proximal fibre bundle length were measured using a pair of compasses. Thereafter the EDL muscle was dissected and weighed. The physiological cross-sectional area of the muscle (CSA) was obtained from the muscle mass, the density and the fibre length.

It was found that muscle mass increased by approximately 220% ($P < 0.05$) from 0.081 g in the youngest group to 0.260 g in the group with a body mass of 400 g; in the same growth period CSA increased by approximately 160% ($P < 0.05$) from 0.07 cm^2 to 0.18 cm^2. No further changes occurred up to old age. Changes with respect to the length measurements were much smaller. First, fibre and muscle length both increased by approximately 30% ($P < 0.05$) up to a body mass of 250 g. Thereafter fibre length remained rather constant, whereas muscle length further increased. Tetanic force increased enormously (approximately 266%) from the youngest group up to the group with a body mass of 400 g. Thereafter no further increase in tetanic force was observed. Because the increase in force was much greater than the increase in CSA, force in relation to CSA (specific force) increased by 48% ($P < 0.05$) from the youngest group to the group with a body mass of 250 g. Thereafter no significant change in specific force was observed. Thus, an increase in specific force of rat EDL muscles

occurred early in life time, up to 60 days of age. The time course of the increase in specific force coincides with the main increase in fibre length. According to Close (*J. Physiol.* (1964)) this would be up to 5 weeks after the end of differentiation of rat muscle fibres into fast and slow twitch.

Actin and creatine in growing soleus and EDL muscle of mouse

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Soleus and extensor digitorum longus muscles of white mice (NMRI) aged 15–100 days were extracted in KCl 80 mM, TES 1 mM, dithiothreitol 0.1 mM, pH 7. After centrifugation, total creatine (Ct = creatine + phosphocreatine) was measured in the supernatant. The precipitate was dissolved in SDS and actin was separated by electrophoresis on slab gels 12% PAA and stained with Coomassie Blue. Images of the gels were digitized and the light intensities of the actin zones were integrated, using a specialized software (ELPHOR, BIOCOM) and compared with standard zones of actin. At all ages EDL muscles have higher concentrations of actin and Ct then soleus muscles. At 100 days of age, soleus muscle has 16 µmole per g of Ct and 0.65 µmole per g of actin, while in EDL muscle these concentrations are respectively 23 and 1.05 µmole per g. When pooled over all ages and muscles, actin is a linear function of Ct: [actin] = 0.055 (±0.004)*[Ct-3.8 (±0.6)]. The data suggest existence of two pools of creatine. One pool located in sarcomeres is functionally dependent on actin concentration, keeping a constant ratio of 18 molecules of Ct for every molecule of actin during growth and differentiation into fast or slow muscle. The second pool, presumably located in sarcoplasm remains at a constant concentration of 3.8 µmole per g and may act as a shuttle of P between the first pool and glycolytic/oxidative metabolic pathways.

Caldesmon binds to myosin and myosin rods and crosslinks thick and thin filaments

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It is well established that caldesmon binds to actin ($K_b = 10^7$–10^8 M^{-1}) and to tropomyosin ($K_b = 10^6$ M^{-1}) and that it is a potent inhibitor of actomyosin ATPase. Caldesmon can also bind tightly to myosin. The myosin binding domain of caldesmon is probably in the first 128 amino acids as a chymotryptic fragment (1–165), an NTCB fragment (1–150) and the sequence 1–128 expressed in *E. coli* from chicken gizzard cDNA all bind to myosin. We investigated the binding of smooth muscle and non-muscle caldesmon isoforms (CD*h* and CD*l* respectively) to myosin using proteins from sheep aorta. Both caldesmon isoforms bind to myosin with indistinguishable affinity. The affinity is about 10^6 M^{-1} in low salt buffer, but is weakened by increasing [KCl] reaching 10^5 M^{-1} in 100 mM KCl. The stoichiometry of binding is about 3 caldesmon per myosin molecule. Stoichiometry and affinity are not dependent on whether myosin is phosphorylated on the presence of Mg^{2+} and ATP provided the ionic strength is maintained constant. The caldesmon binding site of smooth muscle myosin is located in the S-2 region, consequently both HMM and myosin rod bind to caldesmon. Over a range of conditions myosin and myosin rod binding to caldesmon were indistinguishable. Skeletal muscle myosin has no caldesmon binding site.

Smooth muscle myosin rods form side-polar filaments in low salt buffer in which the backbone packing of LMM into the filament shaft is clearly visible in negatively stained EM images. Sometimes the S-2 portions can be seen 'frayed' from the filament shaft. When caldesmon is bound the filament shaft appears to be about 20% thicker and the frayed effect is dramatically increased; long filamentous 'whiskers' are often seen curving out from the filament shaft. Similar structures are observed with smooth muscle and with non-muscle caldesmon.

Myosin also binds to caldesmon when it is incorporated into the thin filament, however, this interaction is qualitatively different. Measurement of smooth muscle HMM binding to native thin filaments in the presence of 3 mM MgATP shows there is a high affinity binding (Kb. 10^6 M^{-1}) which is independent of [Ca^{2+}] and of the level of myosin phosphorylation. The stoichiometry is one HMM molecule per actin monomer which is equivalent to up to 14 HMM bound at high affinity per caldesmon. Negatively stained EM images of the HMM.ADP.Pi-thin filament complex have failed to show any attachment of HMM to the thin filaments. Although the interaction of individual myosin molecules with thin filaments and A_{14}CD is not stable the interaction of an entire thick filament containing 20–100 myosins can be visualized. When rod filaments are added to actin + caldesmon or to native thin filaments the rod filaments are strongly associated with the actin filament bundles. This reflects the ability of the S-2 portion of myosin to bind to caldesmon while it is incorporated into the thin filament. The majority of rod filaments are lined up parallel and in close proximity to actin filaments. However, as we found with HMM, we could not detect any material crossing between the thick and thin filaments. Similar crosslinking is observed with non-muscle caldesmon.

The ability of caldesmon to bind with high affinity to thick and thin filaments at the same time is probably unique. In the smooth muscle cell caldesmon-containing thin filaments are found together with myosin filaments in the 'contractile domain' in parallel arrays not unlike those shown in our synthetic systems. Thus caldesmon ought to be able to crosslink thick and thin filaments *in vivo*.

Properties of calponin isolated from sheep aorta thin filaments

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Calponin is a 35 kDa heat-stable actin binding protein which is abundant in smooth muscles. It has been suggested that it might have a regulatory function in the smooth muscle thin filaments. We have isolated native thin filaments from a variety of smooth muscles; the activation of myosin MgATPase by thin filaments is Ca^{2+} regulated and there is strong evidence that regulation is mediated by caldesmon; however, we had not examined the possibility of a role for calponin.

SDS gel electrophoresis of native thin filaments reveals no calponin, however on urea-SDS gels, where the tropomyosin bands are moved to a mobility lower than actin, a 35 kDa band can be identified. Quantitative gel scans of 12 thin filament preparations yielded a mean band area ratio 35 kDa: caldesmon of 0.36 ± 0.11, indicating a molar ratio of about 0.9 calponin per caldesmon (0.056 calponin per actin). The 35 kDa protein was isolated under mild conditions and identified as calponin on the basis of (1) precipitation in 30% ammonium sulphate, (2) molecular weight, (3) amino-acid composition, and (4) actin binding.

Sheep aorta calponin inhibited actin activation of myosin Mg ATPase, a maximal inhibition of 80% being achieved with 0.4 calponin per actin. Inhibition was independent of tropomyosin. The potency of inhibition was one-tenth that of caldesmon although both are present in equal molar quantities in the native thin filaments. Calponin inhibition was slightly less in the presence of 0.1 mM Ca^{2+} but was not affected by added Ca^{2+}·calmodulin up to 30 µM under a variety of ionic conditions where calmodulin can reverse caldesmon inhibition. Thus there is no evidence in favour of calponin acting as a regulator of smooth muscle thin filaments.

The possibility of indirect calponin action on caldesmon function was investigated. When calponin was added to caldesmon-actin-tropomyosin its inhibitory effect was additive. When calponin was

added to Ca^{2+}-calmodulin-caldesmon-actin-tropomyosin under conditions where calmodulin reversed caldesmon inhibition, normal calponin inhibition was observed. Calponin did not modify inhibition under conditions where calmodulin was ineffective at reversing caldesmon inhibition. Thus there is no evidence that calponin interacts with caldesmon or with the caldesmon-calmodulin interaction.

Comparison between the normal and fatigued rigor states in frog sartorius muscle

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Two different rigor states have been identified in frog sartorius muscle using X-ray diffraction, which depend on whether the muscle is isometrically stimulated during rigorisation.

Sartorius muscles bathed in Ringers solution containing 1 mmol iodoacetic acid (pH 7.0) (Huxley (1968) *J. Mol. Biol.* **37**, 507–20) and stored at 4° C for 24 h in a nitrogen atmosphere, gave X-ray diffraction patterns typical of the rigor state (Huxley (1968) *J. Mol. Biol.* **37**, 507–20; Huxley and Brown (1967) *J. Mol. Biol.* **30**, 383–434; Haselgrove and Huxley (1973) *J. Mol. Biol.* **77**, 549–68). Muscles similarly treated for 1 h at 8° C and then subjected to 6-s isometric tetani every 5 min gave substantially different patterns. Typically, 10–15 tetani were required to put a muscle into rigor. The onset of rigor began with a sudden loss in the three-dimensional order typical of the resting pattern and a progressive increase in the intensity of the rigor layer lines.

The off-meridional rigor layer line at 14.4 nm is unstimulated rigor was observed at 14.57 nm in stimulated rigor (as in isometrically contracting muscle). The third order meridional is at 14.4 nm in both rigor states although its intensity is stronger in the case of stimulated rigor. The first layer-line intensity is weaker in the stimulated rigor patterns suggesting a different orientation and/or conformation of the crossbridge. Strong sampling, absent in unstimulated rigor, was observed in the first, second and third rigor layer-lines in the radial position of the [1, 1] and [1, 0] reflections. The [1, 1] equatorial intensity was substantially larger under stimulated rigor conditions although the [1, 0] had a similar intensity.

These results demonstrate a large degree of structural variability in the rigor states which are dependent on the precise conditions of rigorisation. Consequently, it is questionable whether rigor can be used as a standard structural state for quantitative comparisons of crossbridge attachment. This work shows the site of attachment of the myosin heads to actin, and the crossbridge orientation and conformation, differ depending on the manner in which rigor is induced and may bear no resemblance to the situation in isometric contraction.

Natural involution of the muscular component of the proximal sesamoid ligament in sheep

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In ungulates, the proximal sesamoid ligament (PSL, a homologue of the interosseus muscle) consists initially of alternating bands of muscle and tendon. However, the muscular component is progressively reduced, being replaced by fat and connective tissue within a few months after birth. We have studied this precocious involution of the muscle fibres of the PSL in an attempt to find indications of its cause. Histochemical and immunohistochemical analysis of the PSL revealed that there is a relatively rapid transformation of many fibres from type II (about 50% are type II by 105 days gestation, although they still contain neonatal myosin at this stage) to type I. Prenatally, the muscle appears in good condition morphologically, and is relatively mature. By 1 month postnatal the muscle fibres are almost all type I, with just a few type IIC. By contrast, the masseter muscle, which in sheep is also exclusively type I in the adult, goes through the same transition but much more slowly. Morphological indications of the process of involution are already just visible at birth (occasional necrotic fibres, central nuclei and an infiltration of fibroblast-like cells), but only occurs in those peripheral fibres adjacent to the tendinous sheets. Over the next few weeks this process intensifies and gradually spreads inwards to reach even the central fibres, resulting in an invasion of the muscular component by connective tissue, and the appearance of fibrosis. Strikingly, the areas of necrosis are not at any time accompanied by attempts at regeneration; no fibres with either the morphological characteristics of the myosin composition of new fibres were found during this process of natural involution. Various possible causes have been considered: the most likely candidate is that the primary cause is an active invasion by connective tissue elements which also produce a local factor inhibiting satellite cell activation and regeneration.

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Effect of muscle fibre type of anaerobic energy release during bicycling

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Human type II fibres may have a larger anaerobic capacity than type I fibres. To examine this further 19 healthy young men cycled for 1–3 min till exhaustion. The anaerobic energy release was quantified in two different ways. It was first determined for the whole body by the accumulated O_2 deficit. In addition the anaerobic energy release in the quadriceps muscle was determined from changes in muscle lactate and creatine phosphate concentrations. The fraction of type II fibres in this muscle was determined from at least four biopsies from each subject by staining for the myosin ATP-ase activity.

The fraction of type II fibres in the quadriceps muscle ranged between 27% and 76%. The accumulated O_2 deficit was 2.28 ± 0.05 mmol O_2 per kg. There was no relationship between the fraction of type II fibres in the quadriceps muscle and the accumulated O_2 deficit ($r = -0.31$, $p = 0.95$). The muscle lactate concentration rose 29 ± 1 mmol kg^{-1} wet weight muscle during exercise. The creatine phosphate concentration fell 12 ± 1 mmol kg^{-1}. Consequently the anaerobic ATP-production in working muscle was 56 ± 2 mmol ATP per kg wet weight muscle. There was no relationship between the anaerobic ATP-production and the fraction of type II fibres in this muscle ($r = 0.2$, $p = 0.2$).

The data from this study suggest that in man there is no difference in the anaerobic capacity of type I and type II muscle fibres.

F-actin-calponin interactions

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The contraction of smooth muscle requires the interactions of the phosphorylated myosin head with F-actin and ATP. These interactions seem to be modulated by the specific actin-binding proteins, caldesmon and calponin which are present within the natural thin filament and whose association with actin is reversed by Ca^{2+}-calmodulin and by phosphorylation. The two regulatory proteins inhibit the actomyosin ATPase cycle according to different mechanisms. While caldesmon blocks the interaction between actin and the S-1-ATP(-ADP-Pi) complex, calponin appears to inhibit the kinetic steps related to Pi and/or ADP release (Abe *et al.* (1990) *J. Biochem.* **108**, 835). To highlight the unknown mode of calponin action, we have analysed the molecular structure of the F-actin-calponin complex, in comparison with the F-actin-caldesmon complex previously described (Bartegi *et al.* (1990) *J. Biol. Chem.* **265**, 2231), using

carbodiimide crosslinking, limited proteolysis, cosedimentation assays, amino acid sequencing and spectrofluorometry. EDC-crosslinking between turkey gizzard calponin and skeletal F-actin yielded a single adduct (M_r = 76 kDa) which was stained with both anticalponin and antiactin antibodies. The crosslinking process was not affected by the presence of caldesmon which continues to be crosslinked to actin as in the absence of calponin, suggesting no competition between the two proteins. In contrast to caldesmon, calponin failed to crosslink to actin which was activated alone by EDC in an initial step, although both proteins cosedimented under the employed experimental conditions. Furthermore, while caldsmon bound to the acidic N-terminal actin segment, calponin bound and crosslinked to the 35 kDa C-terminal fragment of subtilisin split F-actin. A restricted chymotryptic digestion of calponin generated a N-terminal 22 kDa peptide and a C-terminal 13 kDa fragment as identified by microsequencing. A more extensive proteolysis converted calponin into a single, stable N-terminal 13 kDa peptide. This peptide bound to Ca^{2+}-calmodulin-sepharose but did not cosediment with F-actin. Only the 22 kDa entity was able to cosediment with and to crosslink to F-actin. As the latter peptide differs from the N-terminal 13 kDa fragment by a C-terminal extension of 38 amino acids (Vancompernolle *et al.* (1990) *FEBS Lett.* **274**, 146), this difference segment seems to contain the actin-binding domain of calponin. This site is completely protected by F-actin which prevents the production of the calponin peptides.

Lobster tail muscle tropomyosin: homogeneity of both intact and non-polymerizable species

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For some biophysical studies, especially crystallographic studies, it is crucial to obtain both intact and non-polymerizable tropomyosin (Tm) of great quantity and at high purity. Tm preparation from the rabbit skeletal muscle is problematic in twofold. First, the rabbit preparation consists of two isomers, α- and β-Tm, which can not be easily isolated from each other to a high purity. Second, digestion of rabbit α-Tm by carboxypeptidase A results in a heterogeneous population of molecules differing in the number of C-terminal residues removed by the enzyme. Even by employing the improved protocol of enzyme reaction (Mak and Smillie (1981) *BBRC* **101**, 208) and by taking precautions to dephosphorylate serine-283 (Mak *et al.* (1978) **75**, 3588), the truncated Tm was still substantially heterogeneous (Walsh *et al.* (1984) *J. Mol. Biol.* **182**, 265).

In the present study, as an invertebrate alternative Tm, the Tm from lobster tail muscle was studied. This Tm consists of a single species and homogeneous preparations of truncated and non-polymerizable Tm can be easily obtained in a great quantity (300 mg from 300 g muscle).

The peptide sequence analysis indicated that this Tm has the C-terminus sequence of 11 amino acids which is homologous to that of the thracic isoform generated from Tm-I gene of *Drosophila melanogaster* (Basi and Sorti (1986) *J. Biol. Chem.* **261**, 817), substantially differing from the rabbit α-Tm. The amino acid composition is also distinct from the rabbit counterpart. In spite of the chemical differences, physical properties are not distinguishable to the rabbit α-Tm. The truncated lobster Tm showed low viscosity at low salt concentrations, and a reduced affinity to F-actin. Recently crystals of the truncated lobster Tm have been obtained. The morphologies of the crystals, especially the 13.6 nm (41 nm × one-third) spacing striations seen under EM of crashed crystals, indicate that the molecules are packed side-by-side, unlike the conventional Bailey-type crystals where molecules form the network. In conclusion, the lobster tail muscle Tm is a useful alternative to rabbit skeletal muscle α-Tm, and the crystals may be suitable for X-ray crystallography at an atomic resolution.

Expression and isolation of fragments of the carboxyl-terminal region of dystrophin

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Dystrophin has several distinct structural domains: an amino-terminal domain with sequence homology to the actin binding domain of α-actinin (Hammonds (1987) *Cell* **51**, 1); a long, central, spectrin-like domain which is predicted to confer an elongated shape on the molecule; and a carboxyl-terminal domain (Koenig *et al.* (1988) *Cell* **53**, 219–28). This latter region contains a cysteine-rich domain (extending from amino acid 3080 to 3360), which shows some similarity with the carboxyl-terminal region of the slime mold α-actinin and which is suggested to contain two potential EF-hand sequences, followed by a region which is apparently unique to dystrophin and exhibits tissue-specific variations by alternative splicing (Feener *et al.* (1989) *Nature* **338**, 509–11).

At present, the function of dystrophin is unclear. As part of a study aimed towards understanding the function of this protein in muscle cells we have cloned different domains of dystrophin cDNA, using PCR technology, and have expressed them as glutathione-S-transferase (GST) fusion proteins. Three different fragments of the carboxyl-terminal region have been expressed. Fragment 1 encodes the carboxyl-terminal 200 amino acids, Fragment 2 encodes the cysteine-rich region (amino acid 3107–3400) and Fragment 3 encodes the carboxyl-terminal 491 amino acids. The fusion protein carrying Fragment 1 was found in the soluble fraction of an *E. coli* extract. The solubility of this fusion protein may be related to the relatively high predicted hydrophilicity and surface probability of Fragment 1 and suggests that the fragment was properly folded during expression in *E. coli* and may, therefore, have its functional properties preserved. This protein has been purified by glutathione affinity chromatography, followed by FPLC chromatography, and has been further characterized. In contrast, the fusion proteins carrying Fragments 2 and 3 appear to be only partially soluble as only small amounts are detectable in the soluble fractions of *E. coli* extracts. Despite this, we have been able to purify the fusion protein carrying Fragment 2 using glutathione affinity chromatography. These fusion proteins have been characterized by their biochemical, biophysical and immunological properties.

In conclusion, it appears that the carboxyl-terminal 200 amino acids of dystrophin form a protein fragment which is fully soluble, but that this solubility is markedly reduced when the fragment includes portions of the cysteine-rich region. These results suggest that the two domains may play different roles in the function of the carboxyl-terminal region of dystrophin.

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Mechanical power and isomyosins in transplanted soleus muscle of normal and dystrophic (mdx) mice

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Nine soleus muscles of mdx mice and 12 soleus muscles of the control strain C57BL/10 were orthotopically homotransplanted. At 1 month after transplantation, the grafted muscles and contralateral muscles were isolated, their force-velocity relationship at 20° C were analysed together with their composition in myosin isoforms. Transplantation induces the same modifications in mdx soleus muscles as in C57 soleus muscles: a decrease of 30–40% of the maximal isometric stress (*So*), an increase of the velocity constant *b*, computed according to Hill's equation, and no change of the force constant *a*/*So*. The myosins

composition vary in parallel with mechanical parameters, showing a decrease in slow type isoforms together with an increase in fast types isomyosins. The maximal mechanical power per unit volume [$P_{max} = So*b*f(a/So)$] of mdx soleus muscles is slightly lower than in normal muscles. However, the maximal power developed by the transplanted muscles reaches values equal to or higher than that of their contralateral muscles. Thus, mdx dystrophy does not seem to affect the power recovery after transplantation.

Regulation of myosin heavy chain expression in the hypertension-induced hypertrophied rat heart by cAMP

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It is known that both genetically-determined and artificially-induced hypertension lead to cardiac hypertrophy and shift the myosin heavy chain (MHC) expression to the β-MHC form. The cause of this change of gene expression was unknown. To contribute to the understanding of this phenomenon, we investigated MHC expression in the left ventricle (PP-PAGE), basal and isoprenaline-stimulated (10 μM) cAMP levels (membrane fractions), and the degree of cardiac hypertrophy in control rats (Wistar-Hagemann (WH), Wister-Kyoto (WKY)) and several rat models of hypertension: one clip-one kidney (1C-1K), desoxycorticosterone-treated rats (DOCA), rates with reduced renal mass (RRM), and spontaneously hypertensive rats (SHR) (eight animals per group were investigated). The degrees of hypertension developed correlated positively with the degree of cardiac hypertrophy ($p < 0.01$). Furthermore, we observed a statistically significant ($p < 0.05$) correlation between both basal and isoprenaline-stimulated cAMP levels in which the higher the degree of hypertrophy the lower both basal and stimulated cAMP levels. In addition we found that the lower the cAMP levels (or the higher the degree of hypertrophy) the lower the expression of αMHC isoenzymes ($p < 0.05$). These data suggest that the decreased α-MHC expression upon hypertension-induced cardiac hypertrophy is mediated via decreased cAMP levels probably from increased expression of Giα proteins. Indeed, it could be shown previously (Gupta *et al.* (1991) *BBRC* **174,** 1196–203) that stimulation of cAMP production in cardiac cells increases transcriptional rate of the α-MHC gene.

Diversity of the dystrophin isoforms identified by help of monoclonal antibodies

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The presence of dystrophins in human, mice and chicken muscles was studied by immunofluoresence assays, by immunoblot detection and by immunogold electron microscopy. To characterize these dystrophin isoforms we used monoclonal antibodies raised against sequences 1173–1728, 1840–2266 and 3357–3660 as immunogen.

A protein with homology to dystrophin was first identified as expressed at the neuromuscular junction of normal individuals, Duchenne Muscular Dystrophy (DMD) patients, as well as in muscle of normal and mdx mice. Two different dystrophins or dystrophin isoforms were detected in the human uterus, one was located at the periphery of the blood vessels and the other specific of the periphery of the myometrial cells.

Biopsies of DMD muscle patients, lacking the first 52 exons of the Xp21 dystrophin gene show presence of the NMJ dystrophin associated molecule as well as vessel dystrophin isoform. Therefore it must have been translated from a different gene transcript. Specific distribution of dystrophin in gizzard chicken smooth muscle while spectrin is not, or in cardiac muscle where pectrin is, suggests that dystrophin could play a specific role depending on its muscular origin. Only specific monoclonal dystrophin antibodies allow such analysis and as a practical consequence, their use for diagnostic purposes as well as the muscle biopsies should be carefully chosen before any conclusions can be drawn from the results.

Studies on the phosphate-binding of myosin subfragment-1 with beryllium and aluminum fluoride complexes

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It has been recently shown that aluminum (AlF_4^-) and beryllium fluoride (BeF_3^-) complexes are good structural analogues of phosphate (Chabre (1990) *TIBS* **15,** 6–18) and, like vanadate, they form stable S1-MgADP-AlF_4^- or S1-Mg-ADP-BeF_3^- complexes (Maruta *et al.* (1991) *Biophys. J.* **59,** 436a). Here the characterization of these S1 complexes are reported. It was found that S1 completely lost its K^+ (EDTA) activated ATPase activity following incubation with MgADP and stochiometric concentration of BeF_3^- at 25° C for 15 min. Incubation of S1 with MgADP and somewhat higher than stoichiometric concentration of AlF_4^- also led to the inhibition of S1 ATPase. Incubation of S1 with $BeCl_2$ (or $AlCl_3$) and MgADP without fluoride or incubation with fluoride and MgADP but without $BeCl_2$ (or $AlCl_3$) did not inhibit the ATPase activity. When excess reagents were removed by gel filtration following the formation of the S1-MgADP-BeF_3^- complex, the ATPase activity remained low and only partially recovered after keeping the complex of 0° C for 1 week. The tryptophan fluorescence emission of S1 significantly increased upon addition of $BeCl_2$, or $AlCl_3$, if MgADP and fluoride are also present. The increment in fluorescence was similar to the increment observed upon addition of MgATP to S1. The results indicate that BeF_3^- and AlF_4^- complexes, together with MgADP, are trapped at the ATP binding site of S1. We have shown recently that near UV irradiation in the presence of vanadate cleaves S1 heavy chain at three specific sites located at 23 kDa, 31 kDa and 74 kDa from the N-terminus (Muhlrad *et al.* (1991) *Biochemistry* **30,** 958–65). The 23 kDa cleavage site is at Ser-180 (Cremo *et al.* (1989) *J. Biol. Chem.* **264,** 6608–11), which is located at the consensus ATP binding site of S1. When the S1-MgADP-BeF_3^- complex was subjected to vanadate-induced photocleavage S1 was cleaved only at 74 kDa from the N-terminus, while the cuts at the 23 kDa and 31 kDa sites were completely inhibited. This shows that vanadate cannot bind to the phosphate binding subsite of the consensus ATP binding site if this is occupied by the BeF_3^- complex. The findings of the photocleavage support our earlier assumption that both the 23 kDa and 31 kDa sites are part of the consensus ATP binding site, as the cut at both sites is inhibited in the S1-MgADP-BeF_3^- complex.

Monte Carlo simulation of muscle activation by calcium

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Hill (*Biophys. J.* (1983) **44,** 383–96) proposed a model of calcium regulation of muscular contraction in which there were cooperative

interactions between adjacent regulatory units along the thin filaments. We have used the Monte Carlo technique to follow the behaviour of an ensemble of 3000 regulatory units, grouped into 100 linear chains of 30 units each, when the free calcium concentration is rapidly elevated. These conditions are analogous to our experiments on muscle activation following the rapid release of calcium by laser-flash photolysis of the photolabile calcium chelator nitr-5 (Ashley *et al. Biophys J.* **53**, 564a). In these experiments the half-time for force development was calcium sensitive when small amounts of calcium were released but the half-time for force development was relatively insensitive to free calciuim when calcium release was sufficiently large to produce more than 50% maximal force. This experimental result may be explained by a model in which the rate of transition from a relaxed state to a force-generating state is dependent on the state of the adjacent regulatory units on the thin filament. In addition, this model suggests that the affinity for calcium of the regulatory sites on troponin C may be independent of crossbridge state. This would be thermodynamically feasible provided that the system was in a non-equilibrium state (i.e. there is an energy flux through the system) (Ashley *et al.* (1991) *Quart. Rev. Biophys.* **24**, 1–73) and would imply that the kinetics of calcium exchange with the regulatory sites on the thin filament are much more rapid than crossbridge cycling.

Binding of LC1 (A1) light chains and troponin-I to actin

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All LC1 (A1) light chains from vertebrate striated muscle myosins are characterized by possessing a 40-residue N-terminal extension which is rich in $(X\text{-}Pro)_n$ tandem repeat sequences and possesses a fully methylated N-terminus and an actin-binding site. The $(X\text{-}Pro)_n$ section acts as a relatively rigid, extended structure displaying high segmental mobility. By chemically synthesizing regions of this extension and testing the various peptides abilities to bind to actin by ^{1}H-NMR, it has been found that actin-binding is confined to the N-terminal ten amino acids and that the N-terminal methylation is not essential for this interaction to take place. Thus, the actin-binding site of this light chain exists at the end of an extended 'spacer' segment permitting interaction to occur away from the body of the S1 molecule. The relationship between the N-terminal of the LC-1 light chain and Tn-I in the regulatory process of the thin filament is under investigation in skeletal muscle and also in heart muscle where a cardiac-specific phosphorylation of Tn-I shifts the tension-pCa curve to the left, enhancing relaxation. Using purified proteins, it was found that both cd-Tn-I and phosphorylated cd-Tn-I bound to F-actin with a pronounced positive cooperativity (Hill coefficient = 1.3–1.5) and with 1 mol cd-Tn-I binding per mol of actin. In the presence of TM, cd-Tn-I still bound with positive cooperativity but phosphorylation of cd-Tn-I removed this effect and weakened the interaction slightly. It is simplest to interpret these data in terms of a two-state allosteric binding model in which cd-Tn-I binds to one actin monomer in the filament and affects the conformation and thus the Tn-I binding properties of other monomer units in the filament. Some indication that long range conformational effects are occurring is shown by monitoring cd-Tn-I binding to actin polymerized with the fluorescent ϵ-ATP analogue. cd-Tn-I binding increases the fluorescence of the actin-bound nucleotide some 20–25% despite the wide separation of the nucleotide and Tn-I sites on actin. In the presence of cardiac troponin-C the cooperative binding of cd-Tn-I is eliminated in the presence of Ca^{2+} and the interaction is considerably weakened. These data imply a more central role for Tn-I in governing actin filament structure and suggest that this may be the central interaction in switching the thin filament from an active to an inactive state. The LC-1 light chain would then serve to act as a moderating influence on this interaction.

Studies on cellular myosin II heavy chain isoforms using isoform-specific antibodies

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Comparison of the amino acid sequences of the COOH-termini of myosin heavy chains from bovine brain (Murakami *et al.* (1990) *J. Biol. Chem.* **265**, 1041) and human macrophage (Saez *et al.* (1990) *PNAS* **87**, 1164) indicates that these myosins are genetically distinct isoforms of cellular myosins; they are related, but the sequences are very different at the end of the heavy chain. We prepared antisera against synthetic peptides having sequences that are isoform-specific; the immunogen for anti-IIA was a macrophage sequence and for anti-IIB a sequence from brain myosin. These antisera did not recognize muscle myosins, but did recognize cellular myosins from several mammalian species. On Western blots of tissue extracts, in which the cellular myosin heavy chain isoforms were electrophoretically separated, we found that anti-IIA and anti-IIB stained different myosin heavy chain bands, and did not cross-react. Most tissues were found to contain both isoforms, but the ratios between them varied. Brains from bovine, mouse, and rat showed a weak band stained by anti-IIA, and two strong bands stained by anti-IIB. These myosin heavy chain bands are designated MIIA, $MIIB_1$, and $MIIB_2$. Blood platelets contained only MIIA, while kidney showed $MIIB_2$ and MIIA bands of equal intensity. We observed the $MIIB_1$ isoform only in brain, and the $MIIB_1$ to $MIIB_2$ ratio varied in different regions of the brain. These results indicate that mammalian cells contain the MIIA and MIIB isoforms of cellular myosin in variable ratios, and that the MIIB isoform has subtypes, perhaps resulting from alternative splicing, one of which is brain-specific. A commercial anti-platelet myosin antibody stained MIIA but not $MIIB_1$ or $MIIB_2$. Immunohistochemistry of brain sections showed that anti-IIA and anti-platelet myosin antibodies stained blood vessels, while anti-IIB stained most neuronal cells selectively.

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Lactate production of single muscle fibres from *Xenopus* during fatiguing intermittent stimulation

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Lactate content and lactate efflux were measured to determine the contribution of glycolysis to ATP production during fatiguing stimulation of skeletal muscle fibres. Single type 1 fibres were dissected from m. iliofibularis of *Xenopus laevis* (Lännergren and Smith (1966) *Acta Physiol. Scand.* **68**, 263–74) and mounted in an experimental chamber at a sarcomere length of 2.3 μm. The fibre was rinsed for 1 h with oxygenated, phosphate-buffered Ringer's solution (pH = 7.2) at 20° C. Then, the volume of the chamber was reduced to about 0.5 ml, and the chamber was covered with a plastic coverslip. Oxygen saturated with water vapour was blown between the Ringer's surface and the coverslip to prevent anoxia of the fibres. The fibres were stimulated intermittently (40 Hz) to produce one 250 ms tetanus every 5 s. Fibres were stimulated for 1.5, 5, 10 or 20 min, after which they were quickly frozen with solid carbonic acid and freeze dried. The lactate content of the fibres and of the Ringer's solution was determined, and normalized by the dry weight of the fibre.

Lactate content of the fibre increased during the first 5 min of intermittent stimulation from 8 ± 4 μmol g^{-1} (mean ± SD, ($n = 5$)) to a steady level of 147 ± 49 μmol g^{-1} ($n = 6$). Force was 74 ± 9% of original after 5 min ($n = 6$), 34 ± 16 ($n = 5$) after 10 min, and 22 ± 8 ($n = 5$) after 20 min. Lactate efflux rate, 2.9 ± 0.6 μmol. g^{-1} min^{-1}, was fairly constant between 5 and 20 min of intermittent stimulation.

Assuming P/lactate = 1.5, it can be concluded that during the first 5 min of intermittent stimulation the rate of glycolytic ATP

production is 0.23 nmol mm^{-3} s^{-1}, whereas the maximum rate of oxidative ATP production is 0.15 nmol mm^{-3} s^{-1} (van der Laarse *et al.* (1989), *J. Muscl Res. Cell Motil.* **10**, 221–81). However, after 5 min of intermittent stimulation, when the lactate content of the fibre remains constant, the rate of glycolytic ATP production is only 0.02 nmol mm^{-3} s^{-1}.

Ionic currents in isolated human cardiomyocytes

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Calcium-tolerant single cardiac myocytes were obtained by enzymatic digestion from auricular appendages of five infants and from the heart of a cardiac transplant recipient. Electrophysiological properties of these myocytes were measured at room temperature using whole-cell voltage clamp techniques. In ventricular cells, we observed the inward calcium current (I_{Ca}), transient outward current (I_{to}), inward rectifier potassium current (I_{K1}), but not the delayed rectifier potassium current (I_K). The amplitude of I_{K1} was bigger in ventricular than in atrial myocytes, while I_{to} was more pronounced in atrial than ventricular cells. The steady-state current-voltage relationship (measured at 400 ms) was N-shaped in ventricular cells, but not in atrial ones. In atrial cells, I_{to} could be activated only from negative voltages, and inactivation of I_{to} was induced by application of depolarizing prepulses. The steady-state inactivation of I_{to} showed a sigmoid voltage-dependence. In ventricular cells, the inactivation of I_{Ca} had a biexponential time course, while the recovery from inactivation was monoexponential. The ionic currents that underlie human ventricular electrical activity appear to most resemble those reported earlier in canine and rabbit ventricular cells, in contrast to ones described in guinea pig or rat.

Basal lamina in the process of crayfish muscle formation

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Basal lamina of crayfish muscle fibre, characteristed by its thickness, significantly greater than in mammalians, represents a suitable experimental model for studying its role in the process of regeneration and growth of muscle fibre. In the work presented we concentrated on a morphological study of basal lamina in regenerating crayfish muscle tissue in the period of proliferation of myoblasts. Samples from m. extensor carpopoditi of *Astacus fluviatilis*, 23–50 days after mechanical injury were processed for electron microscopy and examined by Jeol JEM/EX microscope. Ultrastructural study showed that basal lamina of regenerating fibres became enormously thicker. Instead of a fibrous structure typical for normal muscle fibre, less compact and homogeneous membrane material was found. In this surrounding activation of satellite cells, proliferation of myoblasts and formation of myofibrills were taking place. Basal lamina accompanied myoblasts penetrating into the inner regenerating parts of muscle fibre. Whereas satellite cells in a crayfish muscle are by their whole surface in a direct contact with basal lamina of a myofibre, effect of basal lamina on the satellite cell proliferation can be supposed (Bishoff (1990) In *Myoblast Transfer Therapy* (edited by R. Griggs and G. Karpati) pp. 147–158, New York: Plenum Press).

Nerve occurrence in basal lamina and morphological similarity of nerve covering with basal lamina material suggest the idea about the continual sheet between nerve and muscle fibre surface. According to the basal lamina thickness it does not seem likely that satellite cells in regenerating muscle could easily cross such a basal lamina. From this point of view basal lamina could represent a material for possible migration of satellite cells within the framework of muscle fibres, eventually for migration from nerve to muscle fibre (Lebart-Padebas (1984) *Tissue Cell* **16**, 767–77).

Duchenne muscular dystrophy gene: products and regulation of expression

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Duchenne Muscular Dystrophy (DMD) is an X-linked disorder resulting in progressive degeneration of muscles, leading to death in the second or third decade of life. About one-third of the affected children suffer also from some degree of mental regardation. The major transcript of the DMD gene in the skeletal muscle is a 14 kb mRNA encoding a 427 kDa membranal protein. A very similar isoform, regulated by a different promoter, is expressed in the brain. Recently, we identified a new mRNA, transcribed from the same gene, which seems to code for a major product of the DMD gene, differing greatly in its structure from dystrophin. It is only 6.5 kb long. It contains the sequence of the 3′ untranslated region, and the sequence encoding the c-terminal domain and the cystein-rich domain, but seems to lack most or all the sequences encoding the spectrin-like repeats and the actin-binding domains, as well as the first exon of muscle- and brain-type dystrophin mRNAs.

The tissue distribution of the novel mRNA is very different from that of the 14 kb muscle and brain isoforms. It is found in liver, brain, testis and several other non-muscle tissues and is barely detectable in skeletal muscle. Moreover, it is the major DMD gene transcript in glia and neuronal cells. This raises the possibility that this gene product is involved in the aetiology of mental regardation which is associated with a significant fraction of DMD cases.

Primary structure of scallop striated and smooth adductor muscle myosin heavy chains

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The primary structure of the scallop, *Aequipecten irradians* striated adductor muscle myosin heavy chain (MHC) has been determined by cloning and sequencing its cDNA. In the subfragment-1 region it has a 59–62% and 52–53% sequence identity with sarcomeric and non-sarcomeric MHCs, respectively. The coiled-coil rod resembles other sarcomeric myosins and ends in a Ser-rich nonhelical tail-piece.

Segmental comparison with other MHCs in the head region reveals regional differences in functional constraint. Sequence differences in the 50 K domain and in the N-terminal two-thirds of the 20 K domain reflect clear division between sarcomeric and non-sarcomeric myosins. On the other hand, the 25 K domain and the light chain binding segment of the regulatory domain (occupying the neck region) has higher homology with MHCs of regulated myosins, indicating that certain heavy chain sites might be critical for regulation.

We have isolated a distinct MHC cDNA fragment from a catch (smooth) adductor muscle library. A 26 residue segment within the HMM/LMM-hinge differs from the striated sequence by 50%, while the remainder of the sequences are identical. Sequencing of amplified catch mRNA revealed no additional differences from the 50 K domain to the C-terminus. Partial sequencing of amplified genomic DNA proved the presence of two alternative exons in the hinge-coding part of the apparently single scallop muscle MHC gene. These results suggest that the hinge region of the rod may have some important role in the isoform-specific functions of MHCs.

Reduction of force production in single fibres of frog skeletal muscle by BDM and its effects of cellular components

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In the field of muscle physiology BDM (2,3-Butanedione-monoxime) initially became known mainly as a substance which reduces tension development in skeletal, cardiac and smooth muscles without affecting the excitability of the surface membrane system. As it was assumed to interfere rather specifically with the contractile apparatus proper, the hope came up that BDM could be used as an ideal uncoupler of EC-coupling. In experiments set up to test this hypothesis more closely, a dose dependent decrease of twitch amplitude, reaching a steady level within a few seconds, was found on continuous stimulation at 1 Hz. In addition, the relative potency of the substance in reducing twitch amplitude was negatively correlated with sarcomere length (SL) in the range of SLs tested (2.5–3.2 μm). At 2.8 μm SL the single twitch amplitude was reduced to 50% in presence of about 3.5 mM BDM. Furthermore, within a short time of continued stimulation dose and frequency dependent occurrences of all or nothing twitch failure were observed, when stimulation frequencies and [BDM] above 2 Hz and 5 mM respectively were used. This resulted in various ratios of blockage between stimuli and twitches, from maintained 2:1 to 4:1 blocks to sometimes total inexcitability at (BDM) above 17 mM. All these effects were reversible within seconds on washout of BDM. Based on these findings the question arose if BDM could possibly lower the myoplasmic ATP level, which in turn might influence the ATP-sensitive potassium channel, finally reducing the excitability of the plasma membrane and by that producing use dependent twitch failure. Measurements of the rate of oxidative phosphorylation in rat liver mitochondria revealed a linearly dose-dependent reversible reduction on the respiratory control rate. O_2-consumption on addition of ADP was reduced to 50% at 30 mM. Determinations of the Mg-ATPase activity of myofibrils revealed a similar dose-dependent sensitivity to the substance, while the Mg-ATPase activity of myosin alone or of acto-myosin were more sensitive to BDM at concentrations below 10 mM. The activity of all three preparations of contractile proteins was reduced to about 50% at 30 mM BDM. It is interesting to note that in contrast the K-ATPase of isolated myosin was slightly increased by BDM. On comparison of the sensitivity of the different systems it is evident that none of the actions observed *in vitro* alone is sufficient to explain the effects observed on intact single fibres and that in addition the depressive effect on Ca^{2+} release, described by other researchers, is needed to explain the effects observed on whole muscle fibres.

Effects of changes in pH on the rate of relaxation of single skinned fibres of *Rana temporaria* on photolysis of the caged-calcium chelator, DIAZO-2

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A slowing of the rate of relaxation of muscle fibres is a characteristic feature of fatigue. In a fatiguing fibre the concentrations of ADP, P, and H^+ are all increased. We have investigated the direct effect of H^+ on crossbridge transitions during relaxation, using single chemically skinned fibres and the caged calcium-chelator, diazo-2 (Mulligan and Ashley (1989) *FEBS Lett.* **255**, 196–200). Diazo-2 enabled us to produce a rapid (< 2 ms) decrease in the free Ca^+ within the skinned fibres at three different pHs. The time course of the tension transients during relaxation were closely fitted (P. Fit, Biosoft) to two exponentials, representing a fast phase (k_1) and a slow phase (k_2), the proportions of which were P and Q respectively, representing at least two crossbridge transitions. All values are mean ±SEM (Table 1).

	k_1 (s^{-1})	P	k_2 (s^{-1})	Q
pH 6.5 ($n = 9$)	24.4 ± 0.1	71.6 ± 1.7	9.2 ± 0.3	31.2 ± 1.4
pH 7.0 ($n = 15$)	42.3 ± 1.4	75.7 ± 2.4	12.0 ± 0.7	27.1 ± 2.6
pH 7.5 ($n = 9$)	83.6 ± 1.0	56.9 ± 0.3	9.5 ± 0.1	46.5 ± 0.3

It is suggested that the effect of pH 6.5 was to slow the rate of k_1 by a direct action on crossbridge kinetics. At pH 7.5, the rate of k_1 was greatly increased and the proportions of k_1 and k_2 were changed, suggesting that there is a complex effect on more than one crossbridge transition.

Relaxation of single frog fibres on photolysis of the caged calcium-chelator, DIAZO-2, is slowed by ADP

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Diazo-2 is a photolabile calcium chelator which on photolysis rapidly (rate > 2000 s^{-1}) changes from a chelator of low Ca^{2+} affinity (K_d 2.2 μM) to a chelator of higher affinity (K_d 0.073 μM) for Ca^{2+} (Adams (1989), *J. Am. Chem. Soc.* **111**, 7957–68). We have used Diazo-2 to rapidly uptake Ca^{2+} within single skinned fibres from the semitendinosus muscle of the frog, *Rana temporaria*, and investigated the effect of a high ADP concentration upon the rate of relaxation. A total concentration of 10 mM ADP led to an increase in the half-time of relaxation from 73.5 ± 5.9 ms ($n = 6$) (mean ± SEM), with no ADP, to 122.9 ± 8.3 ms ($n = 7$) ($p < 0.05$, Student's t-test) in the presence of ATP. The force transients were analysed with a non-linear least squares regression technique (N.A.G. routine EO4FDF). This indicated that the curves consisted of two exponential phases. The control relaxations had a fast phase with a rate of 11.0 ± 0.31 s^{-1} and a slower phase with a rate of 0.53 ± 0.07 s^{-1}. The presence of 10 mM ADP had no effect on the slow phase which was 0.54 ± 0.08 s^{-1}, however, the fast phase of relaxation had a decreased rate of 7.9 ± 0.45 s^{-1}. The remnant of force remaining after the fast phase was the same in the two groups.

These results suggest that ADP has a direct action on the contractile proteins to slow relaxation in muscle, distinct from any action on the intracellular free Ca^{2+}. This direct action leads to a slowing of the fast phase of the relaxation process.

Reversal of the Fenn effect in rat skinned cardiac trabeculae at low levels of activation

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The relationship between energy turnover and muscle performance was investigated at different Ca^{2+} concentrations and at different sarcomere lengths in skinned cardiac trabeculae at 20 °C. The preparations were skinned for at last 1.5 h in relaxing solution with 1% (vol/vol) Triton X-100. The ATPase activity was measured photometrically by enzymatic coupling of the regeneration of ATP to the oxidation of NADH. The NADH absorbance in the bath was measured at 340 nm (Steinen *et al.* (1990) *Am. J. Physiol.* **259**, c349–57). The experiments were carried out under isometric conditions and when the length of the preparation was varied repetitively at a frequency of 24 Hz, in a pulse-shaped fashion, by ±2.5% of its initial length. Muscle length was adjusted so that resting sarcomere length was 2.2 μm or 1.8 μm. Composition of the solutions was (in mM): BES 100 (pH = 7.1, adjusted with KOH); PEP 10; NADH 0.9; (Ca)EGTA 20; MgATP 5; free Mg^{2+} 1; ionic strength 200 (adjusted with K-propionate); Na-azide 5; Ap_5A 0.2,

4 mg ml^{-1} pyruvate kinase, 0.24 mg ml^{-1} lactate dehydrogenase and 10 μM oligomycin. Total ATPase activity was corrected for resting ATPase activity measured in relaxation solution (20 mM EGTA).

At maximal activation (pCa = 4.27), the ATPase activity (mean ± SEM) increased during the length changes to 1.60 ± 0.08 ($n = 6$) of the isometric value at 2.2 μm and at 1.8 μm, to 1.38 ± 0.6 ($n = 4$) of the isometric value. At pCa ≅ 5.1 no effect of the length changes was observed. At pCa = 5.5, where isometric force was 34% of the maximal value, a reduction in ATPase activity was found to 0.28 ± 0.05 (2.2 μm) and to 0.58 ± 0.03 (1.8 μm) of the respective isometric values at that pCa. Near the threshold of force development (pCa = 5.74), the effect of the length changes was small or absent.

These results indicate that the Fenn effect is present in cardiac trabeculae and that it is dependent of the degree of activation. The increase in ATPase activity upon length changes at high Ca^{2+} concentration and the decrease in ATPase activity at low Ca^{2+} concentration suggest, in a simple two-state crossbridge model, the presence of a dual effect of mechanical disturbance on crossbridge kinetics. We propose that g_{app}, the overall rate of transition from the force generating to the non-force producing state (rate limiting at high Ca^{2+}), is increased during the length changes and that the reverse rate, f_{app} (rate limiting at low Ca^{2+}), is decreased during the length changes.

Characterization of troponin-C calcium binding sites in cut frog skeletal muscle fibres

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Ca-binding properties of regularity sites on troponin-C controlling muscle contraction were investigated. Measurements were performed in a single Vaseline-gap voltage clamp system using cut skeletal muscle fibres of the frog.

Intracellular Ca^{2+} concentration changes were evoked by long (100 ms) and short (10 ms) depolarizing pulses. Antipyrylazo III Ca^{2+} transients recorded at membrane potentials belonging to the just visible movement were only analysed. Assuming that at this threshold movement the troponin saturation is the same independently of the duration of the depolarizing pulse, and that the mebrane potential does not influence the Ca-binding properties of troponin-C, possible rate constants of the Ca troponin reaction were calculated at different saturation maximums (from 5 to 95%).

It has been found that the curve describing the time course of the Ca^{2+} binding on the toponin reaches its maximum at the same time supposing different saturation levels. A linear relationship was established between the reciprocal values of the maximum saturation and the calculated dissociation constants (K_D).

Our results prove that characterization of intracellular Ca^{2+} binding proteins can be carried out in intact skeletal muscle fibres.

Interaction of actin with the N-terminal region of dystrophin

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Although it is not yet possible to isolate native dystrophin in amounts adequate to enable detailed study of its properties the fact that its primary structure is known presents other opportunities for investigating its function. One approach is to use proton NMR to identify regions of dystrophin that interact with other components of the cytoskeletal-membrane system. With this objective we have studied the perturbations of the proton NMR spectra of synthetic polypeptides corresponding to defined regions of the N-terminus of human dystrophin on interaction with F-actin and its peptide fragments. The results have enabled us to identify two sites on dystrophin. Actin-Binding Site 1 (ABS1) which is present in the region represented by residues 10–32, and Actin-Binding Site 2 (ABS2) located in the region of residues 128–156. These two sites have specific points of interaction with regions of the actin molecule that are exposed in the double helical chain structure of the F-actin filament. ABS1 interacts with amino acid side chains in the region represented by residues 83–117 and ABS2 with those in the region represented by residues 350–375 of the actin polypeptide chain.

Although these sites can be independently identified by studies on the individual peptides it is not yet possible to say how close they are on the dystrophin molecule and whether they form part of one large site. In any case the specificity of the two point attachment will ensure that the two molecules are linked in a fixed conformation. The presence of two actin-binding sites may indicate that one dystrophin molecule binds to different actin subunits, either in the same or the other polymer chain, of the F-actin filament.

The N-terminal regions of dystrophin, α-actinin and β-spectrin exhibit considerable homology which is particularly strong in the regions corresponding to the actin-binding sites of dystrophin. This implies that these proteins may bind to the same regions on actin as dystrophin.

Actin binding fragment of caldesmon inhibits tension development in skinned smooth muscle fibres from chicken gizzard

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The function of caldesmon in the regulation of smooth muscle contraction is still poorly understood. In this study, we tested the effect of caldesmon and its actin- or myosin-binding fragments on isometric force in Triton skinned chicken gizzard fibres. To avoid interference with activation due to binding of caldesmon and the fragments to calmodulin (CaM), tension was elicted by the CaM-insensitive, intrinsically active proteolytic fragment of myosin light chain kinase (I-MLCK, 0.18 mg ml^{-1}) which was allowed to diffuse into the fibres with or without (control) peptides in an EGTA rigor buffer without added CaM over a period of 30–40 min. The fibres were depleted of endogenous ATP by incubation in glucose (10 mM) with hexokinase (400 U per ml). Tension development was initiated by switching to a MgATP containing relaxing solution in the continued presence of I-MLCK (I-MLCK-RS) with or without peptides. In the presence of a 20 kDa actin binding fragment of caldesmon (0.4 mg ml^{-1}), tension development in the I-MLCK-RS was significantly less ($p < 0.05$) than in the control preparations (1.9 ± 0.6 N cm^{-2}, $n = 12$, as compared to 4.8 ± 0.4 N cm^{-2}, $n = 25$, values are means ± SEM). Lower concentrations of the 20 kDa fragment had only a small effect on force (e.g. about 20% inhibition to the presence of 0.27 mg ml^{-1}). Phosphorylation of myosin light chains was not reduced by the peptide. The addition of 15 μM Ca^{2+} and 1 μM CaM induced a further increase in force in both control fibres (7.1 ± 0.7 N cm^{-2}, $n = 18$) and in fibres treated with the 20 kDa fragment (5.8 ± 0.9 N cm^{-2}, $n = 9$) suggesting reversal of the inhibitory effect by Ca^{2+} and CaM. Reversal of the inhibition appeared to require both Ca^{2+} and CaM, as washout in I-MLCK-RS or addition of 1 μm CaM to the I-MLCK-RS was ineffective. By contrast, a myosin binding fragment (0.4 mg ml^{-1}) appeared to have no effect on force, while intact caldesmon (0.4 mg ml^{-1}) reduced force only by about 26% ($n = 6$).

Interaction of F-protein (phosphofructokinase) with actin- and myosin- containing filaments

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F-protein, identified as the key glycolytic enzyme phosphofructokinase (PFK) can be present in skeletal muscle myofibrils both in soluble and particulate forms and equilibrium between them depends on muscle state. It was assumed so far that in myofibrils PFK was associated with thin filaments (Clarke *et al.*, (1985). Using sedimentation, electron microscopy and antibody methods we have recently demonstrated the PFK binding to reconstructed myosin filaments and to thick ones in muscle (Freydina *et al.* (1986)).

Comparison of quantitative parameters showed that PFK bound to myosin and actin filaments with similar and rather high affinity ($K_a \sim 10^5$–10^6 M^{-1}). K_a does not depend on pH-value and Ca^{2+}-level. The estimated number of PFK binding sites per $M_r = 80\,000$ (PFK monomer) at pCa5 averages 2.4, 2.8, 0.7 for myosin and 0.4, 0.2, 0.2 for actin at pH 6.5, 7.0, 7.5, respectively. The increase on Ca^{2+}-level from pCa8 to pCa4 results in the increase of the number of binding sites on myosin and actin. The presence of C-protein in myosin preparations does not change K_a but does decrease the number of PFK binding sites. Studies on quantitative parameters of PFK binding to reconstructed thin filaments are now in progress. As shown by *in vitro* experiments the bound form of PFK differs from soluble one in enzymatic properties which may be involved in regulation of glycolysis in muscle cell (Clarke *et al.*, (1985)). Our data allows to suppose that the increase of bound PFK upon such muscle states as intensive exercises, ischameia, anoxia and so on can favour the elevation of the total activity of the enzyme and thus the intensification of ATP-production.

Probing caldesmon structure-function by expression and mutagenesis of caldesmon cDNA

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Caldesmon is a major actin-binding protein in smooth muscle and non-muscle 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. A full length chicken gizzard caldesmon cDNA coding for 756 amino acids (Bryan *et al.* (1989) *J. Biol. Chem.* **264**, 13873–9) has been expressed in a T7 RNA polymerase-based expression vector system and the caldesmon purified. The expressed protein behaves almost identically to purified native chicken gizzard caldesmon in *in vitro* assays for inhibition of actin-activated myosin ATPase and actin, tropomyosin, Ca^{2+}-calmodulin and myosin binding. A mutant caldesmon composed of amino acids 1.578 had no inhibitory effect and did not bind to actin or Ca^{2+}-calmodulin. It bound to tropomyosin with a five-fold reduced affinity and to myosin with a greater than 10-fold reduced affinity thus indicating a requirement for the C-terminal 178 amino acids in all of caldesmon's functions. However, in contradiction with this result a mutant composed of the first 128 amino acids was found to have a similar affinity for smooth muscle myosin as the full length protein, in agreement with Velaz and colleagues (*J. Biol. Chem.* **265**, 2929–2934 (1990)). Further mutants being constructed and expressed include fragments of the C-terminal portion of the protein (amino acids 606–756 and 658–756) and a fragment (amino acids 230–417) which is unique to the smooth muscle isoform and contains the central repeating region.

Structural and enzymatic comparison of human cardiac myosin from young and old patients with or without mitral valvae insufficiency

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The study has been done on 40 patients aged between 25 and 78 years: 20 controls and 20 patients with heart failure from mitral valvae insufficiency. Left ventricular tissue was used for MgATP-ase assay and for sarcomere measurements in contraction, relaxation at 0.5 mM, 1 mM and 2 mM ATP. We tried to see whether: (1) enzymatic properties of myofibrillar proteins are depressed in heart failure and during aging; (2) if the content myofibrillar proteins is decrease in heart failure, and (3) if contractility of saromere is modified. A significant reduction in MgATP-ase activity and in content of myofibrilar proteins has been recorded in comparison with ageing hearts from normal patients. The depressed activity suggests that the interaction between thick and thin filaments within myofibrillar lattice may be abnormal even though the enzymatic activity of myosin is normal. A significant correlation has been made between MgATP-ase activity and sarcomere shortening capacity in diseased heart. In aging hearts there is no modification in ATP-ase activity even a drop in SH group content has been recorded. It seems possible that the loss does not imply S_1 or S_2 regulatory types of SH residues. Myosin from ageing heart is not affected from a structural and functional point of view like that from diseased heart.

Sarcomere shortening peculiarities in glycerinated cardiac and skeletal muscle of ageing rats

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Optical microscopy and ^1H NMR techniques have been used to investigate the effect of age on the behavior of contractile apparatus from glycerinated rat sartorius and papillary muscle in rigor, contraction and relaxation at different ATP concentration.

During ageing, a significant decrease in active shortening capacity of sarcomere from skeletal muscle have been observed in comparison with heart muscle. It is possible that a certain proportion of cross-bridges to be no longer functionally efficient. ^1H NMR studies on young rat sartorius and papillary muscle in Ri, Co and Re media have revealed a decrease in T_{2s} and T_{21} in comparison with old rats. The smallest values for T_{2s} both for skeletal and cardiac muscle have been recorded in contraction media in comparison with Ri or Re media. This fact should be accounted for a strong binding of H^+ on carboxyl groups of contractile proteins. The increase in T_{2s} and T_{21} in old rats does not derive from an increase in water concentration within muscle, and this can be accounted for a modification in quantity of dissociable proteinic groups. Skeletal muscle is much more susceptible to ageing modifications than cardiac muscle.

Muscle development in *Sparus auratus* (sea-bream)

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We are studying the growth and development of skeletal muscle in a fast-growing fish, *Sparus auratus*, the sea-bream. A survey of larval, fry and adult stages has been carried out by mATPase histochemistry and myosin isoform immunohistochemistry. By 14 days post-hatching three fibre 'types' are clearly distinguishable: (1) a superficial monolayer of fibres which do not react with antibodies to slow myosin, but react strongly with an antibody selective for mammalian fast myosin and have an mATPase activity which is both acid- and alkali-stable, (2) the main bulk of the deep muscle which reacts weakly with most

antibodies but has a moderately alkali-stable ATPase, and (3) two fibres located superficially either side of the lateral line which react strongly with antibodies to slow myosins and have an alkali-labile ATPase. Over the next 2–3 weeks there is no change in the types present, but deep layer and slow-positive fibres increase in number. By 60 days, three distinct muscle layers lie superficial to the deep muscle at the level of the lateral line: an outermost layer similar to the previous superficial monolayer (above), then a slow-positive layer presumably derived from (3) above, and finally a third layer like the outermost one. Subsequently, the outermost layer gradually disappears, the slow-positive layer increases in size to form the classical slow muscle layer, and the third layer appears to form the pink (intermediate) muscle. The growth of the slow layer is probably caused at least in part by hyperplasia, as from about 60 days very small diameter fibres start to appear in it. By 60 days the deep muscle also shows signs of a new hyperplastic process, as it consists of a mosaic of fibres of different diameter. In older subjects (fry aged > 100 days) this mosaic becomes more striking and some differences in ATPase activity between the large and smaller diameter fibres become evident.

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Muscle tissue formation after crayfish muscle injury

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Ultrastructural changes associated with myoblast maturation following degeneration of crayfish skeletal muscle are of central interest in describing muscle regeneration in invertebrata. Muscle fibres from m. extensor carpopoditi of *Astacus fluviatilis* after mechanical or thermal damage were processed for electron microscopy at different time intervals and examined in a Jeol JEM/EX microscope. Conditions sufficient for initiating satellite cell proliferation and fibre survival were found (unpublished data). Near the places of disintegrated myofibrillar material activated satellite cells proliferated in groups, forming clusters with the same cytoplasmic material (Novotova and Rydlova (1990)). The changes of satellite cells in the process of crayfish muscle regeneration. (*Abstracts of International Symposium on Motoneuronal Plasticity*, p. 79, Bonn, 13–16 September). Different stages of myoblast development within basal lamina were evident. The early stage of regeneration was characterized by numerous outgrowths of mother myoblast containing rough endoplasmic reticulum, free ribosomes, vesicles different in size, electrondense bodies and microtubules in their cytoplasm. Formation of new undifferentiated myofilaments and membrane systems in developing myotubes were observed. Differentiation of myofilaments and creation of mitochondria were typical for the later stages of regeneration. Mentioned changes could be found from the third week after injury. After about 50 days new myofibril-like areas packed in basal lamina, comparable with the similar ones occurring in young growing crayfish muscle, were found.

Effect of sucrose feeding on the structure of guinea pig urinary bladder

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Urinary bladder hypertrophy occurs in several pathological states, for example, in bladder outflow obstruction and as a consequence of diabetes. It was of interest therefore to see if the histological response of the bladder in experimentally-induced outflow obstruction was similar to that seen when bladder load is increased by continuous sucrose feeding (50 g l^{-1} sucrose/H_2O w/v in place of drinking water) (Kudlacz *et al.* (1989), *Diabetes* **38**, 278–84).

In this study, it was found that although there was an increase in bladder weight in both treatments, the changes in the bladder structure of sucrose fed animals (after 4 and 8 weeks sucrose feeding) was very different from those seen in bladders from guinea pigs after outflow obstruction. Gross examination of bladders from sucrose fed animals showed that they were thin walled, atonic and had greatly increased blood supply. Light microscopy of the bladders of sucrose fed animals confirmed that there was a pronounced increase in blood vessel diameter compared with control bladders. Bladders from sucrose fed animals showed less organized packing of muscle bundles than control animals, with an increase in interstitial space, a patchy uptake of stain and lipid deposits between muscle bundles.

Examination at the electron microscope level confirmed the patchy uptake of stain and showed that sucrose feeding results in very different bladder structure from that found in bladders in outflow-obstructed animals. Stereological parameters such as the volume fractions of muscle cells, nuclei and collagen showed that sucrose fed and control animals were similar, while obstructed animals showed an increase in the collagen component and a reduced volume fraction of muscle cells. Muscle cells from sucrose fed animals showed a loss of cell-to-cell contract in comparison with both untreated and outflow obstructed animals. Regular apposition and spacing of intermediate junctions is shown in both untreated and outflow obstructed animals, but is disrupted in sucrose fed animals.

In conclusion we find that the bladders from sucrose fed guinea pigs closely resemble those from animals with streptozotocin-induced diabetes (Lincoln *et al.* (1984) *Br. J. Urol.* **56**, 24–30), but are very different from those from guinea pigs with experimental urinary outflow obstruction.

Oligomerization of calsequestrins

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Calsequestrin is a low-affinity high-capacity calcium binding protein localized intraluminally within the terminal cisternae of sarcoplasmic reticulum. Many isoforms of this protein has been described in different species and tissues. Recently we reported the primary sequence of adult chicken fast-twitch skeletal muscle calsequestrin as deduced from the cDNA sequence (Yazaki *et al.* (1990) *Biochem. Biophys. Res. Commun.* **166**, 898). Chicken calsequestrin seems to be an exception to the rule of higher homology among tissues rather than among species, being very similar to dog cardiac type. Interestingly, both of them seems to have lower level of capacity for calcium-binding (from one-half to two-thirds of that measured in rabbit skeletal calsequestrin). One possible explanation for this reduced capacity is the formation of dimers, as proposed firstly by Mitchell and colleagues (*J. Biol. Chem.* **263**, 1376 (1988)). We reinvestigated this aspect by using a cross-linking approach. Preliminary experiments carried out in solution in the presence of a carbodiimide derivative (EDC), or homobifunctional N-hydroxysuccinimide esters (DTSSP, sulpho-DST), or a homobifunctional imidoester (DTBP) seem to indicate that chicken calsequestrin undergoes dimer formation more easily than other calsequestrins from other sources.

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Latex beads as accelerator of myoblast fusion

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We have shown previously (Ritter et al. (1988) *DAN* **105**, 239) that an addition of polyvalent substances including latex beads (1800Å) to chick muscle cells in culture brought about an acceleration of the

myoblast fusion and differentiation. Myotubes appeared on the first day after plating. A mechanism of such acceleration remains obscure. We made an attempt to clarify a role of cytoskeletal actin microfilaments in this process. There are data (Wuestenhube *et al.* (1987) *J. Cell Biol.* **105,** 1741) relating to the connection between the plasma membrane and underlaying microfilament network. Basic cell activities such as adhesion, motility, endocytosis, etc. are thought to involve interactions between the plasma membrane and microfilament network. We have supposed that latex beads influenced on the cell membrane bringing to endocytosis on the one hand and to the increase of actin microfilaments on the other hand. By method of electron microscopy we have found latex beads inside the cells. Besides the chick muscle cells in culture were fixed and labelled with Rhodamine-Falloidine in different periods after plating. The comparison of normal cultured cells and the ones treated with latex beads has shown that the amount of actin microfilaments (stress-fibrils) increased greatly in the second case. The abundance of microfilaments appears to lead to the increase of the myoblast mobility. We suggest that both processes may play an important role in the acceleration of the myoblast fusion.

Are cyprinid fish trainable?

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Training studies on teleosts which have been done so far are restricted mainly to salmonids with that being the 'classical trained fish'. Cyprinid fish are thought to be not trainable. Recent studies on metabolism and aerobic capacity (Lackner *et al.* (1988) *J. Exp. Biol.* **140,** 393–404; Hinterleitner *et al.* unpublished data) have shown that *Leuciscus cephalus* and *Chondrostoma nasus* do adapt to endurance training. In the present investigation the same species as mentioned above were subjected to an endurance training programme for 17 weeks. By means of histochemistry and electronmicroscopy morphometrical analyses was done on red, intermediate and white myotomal muscle. Significant increases in red and intermediate muscle mass, red fibre diameter and capillarization, mitochondrial and lipid compartment can be observed in trained fish. Thus endurance training results in adaptational changes of the aerobic capacity on light and electronmicroscopical level.

The loss of the Ca^{2+} sensitizing effect of GTPγS in Triton-skinned smooth muscle is associated with the loss of small GTP-binding proteins

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Mesenteric microarteries (internal diameter *in situ*: 80–120 μm) and second branch coronary arteries from guinea pig and smooth muscle fibre bundles from lamb trachea (diameter approximately 300 μm) were mounted on an isometric myograph and permeabilized with 50 μg ml^{-1} β-escin in relaxing solution for 35 min at 25 °C. In the mesenteric resistance arteries, the non-hydrolysable GTP analogue GTPγS enhanced tension dose-dependently (0.01–10 μM) at a fixed submaximal but not at maximal [Ca^{2+}] buffered with 10 mM EGTA and in the presence of 1 μM ionomycin thereby shifting pCa_{50} from 6.24 to 6.37 in the presence of 1 μM GTPγS. The Hill coefficient decreased from 4.2 to 2.2. Submaximal force was also increased by norepinephrine (10 μM) with GTP (100 μM) and this effect was blocked by prazosin (1 μM). In the coronary arteries and in smooth muscle from lamb trachea, submaximal force was enhanced by GTPγS in a similar way but in the latter maximal force was also increased. By contrast, in preparations skinned with Triton-X-100, no response to GTPγS up to 100 μM was observed, whereas okadaic acid (5 μM) still enhanced contractions. Following homogenization, proteins of intact, β-escin- and Triton-skinned microarterial and lamb trachea smooth muscle tissues were separated on 12.5% SDS-gels, and electroblotted to nitrocellulose membranes which were incubated with [a-^{33}P] GTP (1–5 nM yielding 1 μCi ml^{-1}). Radioactivity associated with proteins was detected by autoradiography. In the intact and β-escin-skinned but not in the Triton-skinned preparations, GTP was bound to two low M_r weight proteins (approximately 27 and 25 kDa) the binding of which could be blocked by 10 μM GTPγS. In the Triton-skinned preparations very little GTP was bound. This suggests the presence of at least two small GTP-binding proteins in intact and β-escin-skinned but not in Triton-skinned smooth muscle preparations. Whether they are involved in the GTPγS- and agonist-induced increase in Ca^{2+} sensitivity is not known.

Increased energy cost of contraction during repetitive isometric contractions

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Repetitive submaximal isometric exercise leads to a gradual reduction in muscle strength accompanied by a gradual increase in oxygen uptake suggesting an increased energy cost of contraction (Vøllestad *et al.* (1990) *J. Appl. Physiol* **63,** 1150–6). Heat production was calculated from the rate of temperature increase (measured with intramuscular thermocouples) during test contractions at 30% and 50% of maximal voluntary force (MVC) lasting for 15 and 10 s respectively. These contractions were introduced into a protocol of intermittent (duty cycle = 0.6) isometric exercise at 30% of MVC carried out until exhaustion (49 ± 3 min). The experiments were carried out on four healthy subjects (27 ± 2 years of age).

Heat production at the 30% level increased from 17 ± 3 W kg^{-1} (w/w) in control to 34 ± 3 W kg^{-1} (w/w) at exhaustion. The respective values at the 50% level were from 38 ± 9 W kg^{-1} (w/w) to 64 ± 12 W kg^{-1} (w/w). Relative to the control values the rate of heat production was more than doubled at both force levels. The whole body O_2 uptake increased by 60 ± 5% over the entire exercise period and the muscle temperature increased from 34.1 ± 0.4°C to 37.5 ± 0.5°C.

The results indicate that the increase in O_2 consumption is the result of an increased metabolic rate during contraction. The similar time courses of the changes in heat production at both the 30 and 50% force levels suggests that increased activation of less energy efficient type II fibres does not contribute siginficantly to the increased metabolic rate. Metabolic changes at the level of each active fibre thus seems to be an important factor in the economy of force maintenance during repetetive isometric exercise.

Sarcomere-length induced uniform deformation of the hexagonal myofilament matrix in skinned skeletal muscle fibres of the rabbit

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Rabbits were anaesthetized with sodium pentobarbital (30 mg kg^{-1}). Strips of gracilis muscle were dissected and abruptly deep-frozen in liquid nitrogen for 30 min. Thereafter the strips were stored at −25 °C for 3 weeks to achieve thermally skinning. Skinned single muscle fibres were immersed in solutions containing two different levels of activator calcium (pCa: 4.4; 6.0). Sarcomere length was varied from 1.6 to

3.5 μm and recorded by laser diffraction. Slack length was 2.0 μm. Small-angle equatorial X-ray diffraction patterns of relaxed and activated fibres at different sarcomere lengths were recorded using synchrotron radiation (Daresbury Lab). The position and amplitude of the diffraction peaks were calculated from the spectra according to the method of Yu and colleagues (*J. Mol. Biol.* **132**, 53–67 (1979), based upon the hexagonal arrangement of the myofilament matrix, relating the (1.0)- and (1.1)-diffraction peaks in this model by $\sqrt{3}$. The diffraction peaks were fitted by five Gaussian functions (1.0, 1.1, 2.0, 2.1 and Z-line) and residual background was corrected by means of a hyperbole. The coupling of the position of (1.0)- and (1.1)-peak was expressed as factor related to $\sqrt{3}$: FAC = d(1.0)/d(1.1)/$\sqrt{3}$. In relaxed state this coupling factor decreased at increasing sarcomere length (0.988 ± 0.002 at 2.0 μm; 0.90 ± 0.01 at 3.5 μm). At shorter sarcomere lengths also a decrease of the coupling factor has been seen (0.960 ± 0.005 at 1.6 μm), giving rise to an increased uniform deformation of the hexagonal matrix, when sarcomere length is changed from slack length.

From these experiments we conclude that a change in sarcomere length (from slack length) increases the non-random deformation of the actin-myosin matrix.

Modified procedure for isolation of single smooth muscle cells from pig urinary bladder: relation between calcium concentration and isolated cell length

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Isolation of smooth muscle cells from bladder tissue has been proven extremely difficult, probably because of both the high concentration of collagen (Glerum *et al.* (1987) *J. Muscle Res. Cell Motil.* **8**, 125–134) and the many close contacts between the cells (Gosling *et al.* (1983). London: Gower Medical). Furthermore, the isolated cells are generally of moderate lengths. A method was developed for isolating smooth muscle cells from pig urinary bladder using low concentrations of collagenase and papain with the aid of a repeated resuspensation device. Previous findings suggested a relation between $[Ca^{2+}]$ in the isolation solution and isolated cell length. Different Ca-concentrations were used in a number of isolation procedures to measure this dependency. Concentrations as low as 3.5 mg per 10 ml of collagenase and of papain were sufficient to obtain a yield of 10^3–10^4 viable single smooth muscle cells, which was proven with a vital fluorescence test. Compared with the average length of cells in intact muscle bundles (191 μm), cells isolated in a Ca concentration < 1.0 mmol l^{-1} seemed to be relaxed with cell lengths of 280–320 μm. In an isolation medium containing 1.0 mmol l^{-1} Ca the cells were contracted as compared with cell length in intact muscle bundles. If the cells are considered cylindric then there must have been a considerable decrease in cell volume in Ca concentrations exceeding 1.0 mmol l^{-1}. However, at this moment there is no evidence that this phenomenon is found in cells in contracting muscle bundles.

Structural effects of Al-F and Be-F as analogues of Pi in skeletal muscle myosin

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Mechanical evidence (Schrumpf and Wray (1991) *J. Muscle Res.* **12**, 100) suggests that, in skeletal muscle myosin (M), complexes of fluoride with Al or Be can bind to the M.ADP complex in place of Pi, forming long-lived analogues of the states M.ATP/M.ADP.Pi. In this respect they resemble vanadate ion (Vi). We have sought conditions in which the effect of this replacement on crossbridge structure can be investigated. X-ray patterns of relaxed rabbit psoas fibres show a large temperature-dependence of crossbridge arrangement (Wray (1987) *J. Muscle Res.* **8**, 62), interpreted as reflecting a coupling between nucleotide state and global conformation (M.ATP, favoured at 0 °C, appears as 'disordered'; M.ADP.Pi, favoured at 20 °C, appears as 'ordered'). When relaxed fibres were incubated with Be and F ions, the relationship of structure to temperature changed gradually (over ca 6 h), such that higher temperatures were required for the appearance of the 'ordered' crossbridge arrangement. Incubation with Al and F appeared to produce a smaller shift in the opposite direction. ATPase measurements suggested that occupancy was only partial; it is likely that with complete replacement of Pi the effect on structure would be significantly larger. Removal of F led to slow reversal to the original temperature behaviour.

These results are consisted with the idea that the 'weakly-binding' species M.ATP/M.ADP. Pi can exist in two states differing in global structure, whose proportions depend on the state of the active site. Each the four substituents Pi, Vi, Be-F and Al-F may dictate a characteristic dependence of enzymatic state and structure on temperature, perhaps related to their preference for different coordination states. In particular, Be-F may promote the 'disordered' structure otherwise associated with the species M.ATP.

The ability of Al-F and Be-F to stabilize particular myosin states was also seen in their effects on trytophan fluorescence. When S1.ADP was mixed with Al-F, the fluorescence increased by approximately 17.9% ($t_{1/2}$ approximately 370 s, 100 μM Al, 20 °C). With Be-F, the fluoresence increase was smaller (7.6%) but apparently faster approximately 65 s, 100 μM, 20 °C). These fluorescence enhancements may reflect the slow formation of the species M.ADP.Be-F and M.ADP-Al-F, whose fluorescences might correspond to those of M.ATP and M-ADP.Pi respectively. The slowness of binding is in agreement with the time-course of effects on mechanics and structure.

New conception on muscle contraction mechanism: 1. structural matching between the thick and thin filaments

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It is generally accepted that the contraction of skeletal muscle is induced by the crossbridges, i.e. by myosin filaments. To the actin filaments a more passive role has usually been assigned. But there is good evidence that motion can be generated by the interaction of single myosin molecules with actin ones forming a cable. Moreover, it is well known that the thick and thin filaments are helical structures with different subunit periodicities (385 Å for the actin filament and 430 Å for the myosin filament) whereas the interaction between myosin and actin is rather aggregated. A spatial arrangement of the two kinds of filaments into hexagonal bundles relates a precisely defined orientation of crossbridges. Because of that, the crosslinks arranged at 60° around the myosin filament and at 120° around the actin filament. All those experimental facts mean that for an attachment a good structural matching between myosin and actin filaments is necessary.

Employing a new model of the thick filament (Skubiszak (1990) *Cell Biol.* **14**, 207) and the generally accepted structure of the thin filament it is shown that myosin HMM-segments unwinding away from the thick filament core can cause a deformation of the actin filament: the axial translation and the angular separation between neighbouring subunits in the genetic helix change from 27.5 to 28.6 Å and from 154.28 to 168°, respectively. In this way, an ideal match arises in the overlap zone: the specific sites on the actin monomers are oriented strictly opposite the specific sites on the corresponding myosin heads.

Expression of myosin heavy chain isoforms in human intrafusal fibres

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Muscle spindles are mechanoreceptors formed by encapsulated bundles of small diameter intrafusal fibres innervated by both sensory and motor axons. Rat intrafusal fibres coexpress several myosin heavy chain (MHC) isoforms, some of which are muscle spindle specific and have a distinct pattern of expression along the length of nuclear bag_1, nuclear bag_2 and nuclear chain fibres (Pedrosa *et al.* (1990) *Histochemistry* **85**, 105–13).

We studied the expression of MHC isoforms along the length of intrafusal fibres in human lumbrical muscles. Twelve muscle spindles were serially sectioned over a distance of 5 mm. The sections were stained with two sets of monoclonal antibodies (Abs) against slow tonic (STO), slow twitch (STW) and fast twitch (FTW) MHC and with an antiserum directed against neonatal (NN) MHC. Generally, nuclear bag_1 fibres were strongly stained with the STO Abs along their entire length and with the STW Abs in their polar zones, whereas the staining with each of the two FTW Abs differed significantly. Nuclear bag_2 fibres were strongly stained along their whole length with the STO and STW Abs, however, one of the two STW Abs did not stain their equatorial region. They were moderately to strongly stained with the NN Ab and displayed regional variations in the level of staining with both FTW Abs. Nuclear chain fibres were strongly and uniformly stained with the FTW and NN Abs. Fibres whose pattern of immunoreactivity did not fit those described above were also observed.

Thus, human intrafusal fibres coexpress several MHC isoforms, each with a characteristic distribution along the fibre length, as described in other species. However, the organization and pattern of MHC expression in human spindles are much more complex.

Pathologic intra-organelle crystallization of mitochondrial creatine kinase (Mi-CK) in mitochondrial myopathies: Mi-CK is the major constituent of crystalline type I and II mitochondrial inclusions

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The mitochondrial myopathies (MM) are a heterogeneous group of disorders in which a defect in mitochondrial metabolism is thought to be the primary cause of the disease. Specific enzyme deficiencies can be demonstrated by biochemical examination in many patients, but there still remain patients who have abnormalities with unidentified enzyme defects. These abnormalities include the Kearns-Sayre syndrome and the MELAS (MM, encephalopathy, lactic acidosis and stroke-like episodes) syndrome (Walton (1988) *Disorders of Voluntary Muscle, V* edn, Churchill Livingstone), both characterized by the presence of 'ragged red' (RR) fibres in the muscle biopsy specimen. Characteristic aspects of the pathology in the RR-fibres are mitochondrial over-accumulation and the occurrence of highly ordered crystal-like inclusions in the mitochondrial intermembrane spaces. Using optical diffraction techniques, it has been possible to demonstrate that these inclusions are true crystals (Farrants *et al.* (1988) *Muscle & Nerve* **11**, 45–55). On the basis of shape, size, pattern, unit cell dimension, specific location in the intermembrane space and their occurrence in different muscle fibre types, two distinct types of crystals, type I and type II crystals, can be distinguished. Understanding the role of these crystals in relation to the patients' pathology, either in terms of causative mitochondrial dysfunction or of mitochondrial response to the disease, requires a detailed knowledge of their chemical composition.

Therefore, the biochemical nature of these inclusion crystals has been studied using digestion and immunocytochemical techniques on frozen, freeze-substituted or paraform-/-glutaraldehyde fixed and LR-White resin embedded thin sections. The crystals showed complete RNase resistance and moderate resistance to pronase P digestion. No immunoreactivity was found using rabbit-anti-human cytochrome oxidase (COX) or rabbit-anti-baboon cytochrome c antibodies. On the other hand, the crystals demonstrated high immunoreactivity with rabbit-anti-chicken mitochondrial creatine kinase (Mi-CK) antibodies, whereas appropriate controls remained negative. The anti-Mi-CK immunolabelling of the crystals was uniform, irrespective of the orientation of the crystals to the plane of sectioning. However, the type II crystals were always more heavily labelled than the type I crystals. Mi-CK immunoreactivity was also found using periodate pretreated upon sections after conventional EM-fixation. This indicates that, like the Mi-CK immunoreactivity was also found in mitochondria or adult rat cardiomyocytes cultured in creatine-free medium (Eppenberger *et al.* (1991) *J. Cell. Biol.* **113**, 289–302), the pathological inclusions shown here are also made up of Mi-CK as the major component. Image processing of type II crystals revealed arrays of regularly ordered square-shaped particles with dimensions of component. Image processing of type II crystals revealed arrays of regularly ordered square-shaped particles with dimensions of component. Image processing of type II crystals revealed arrays of regularly ordered square-shaped particles with dimensions of 10×10 nm and with a central cavity. Thus, the building blocks of the crystals are very reminiscent of isolated Mi-CK octamers (Schnyder *et al.*, (1988) *J. Biol. Chem.* **263**, 16954–62). Therefore, it is concluded that the crystalline mitochondrial inclusions seen under pathological conditions consist mainly of Mi-CK octamers. The question of whether the appearance of mitochondrial crystals is causative to or a consequence of the disease cannot be answered yet, but the results point to a general alteration of high-energy phosphate metabolism in these patients.

Ca^{2+}-dependent cell adhesion: analysis of M-cadherin in developing muscle

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Cadherins, a multigene family of Ca^{2+}-dependent cell adhesion molecules are thought to play a role in morphological processes including myogenesis. Of the cadherins known, only N-cadherin has been found in developing skeletal muscle. Recently, we have isolated a novel cadherin from myogenic mouse cells which was named M-cadherin (M for muscle). It is encoded by a gene different from the known N-, E- and P-cadherin genes but shares with them a significant degree of homology at the amino acid level. During myotube development in culture, M-cadherin mRNA is upregulated. Analysis of RNA from mouse tissues such as skeletal muscle, brain, spleen, kidney and liver revealed that M-cadherin appears to be expressed in an almost skeletal muscle-specific manner. Furthermore, first stainings of sections from normal and regenerating skeletal muscle with anti-M-cadherin peptide antibodies indicate that M-cadherin protein changes its pattern of expression during regeneration processes. Taken together, our results suggest that M-cadherin is involved in morphogenesis of skeletal muscle.

Actin-activated ATPase of copolymeric myosin-rod filaments

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One possible reason for the complex kinetics of actomyosin ATPase is the assembly of myosin into filaments which interact with actin filaments at many sites simultaneously. In an attempt to avoid these kinetically complex cooperative effects, synthetic filaments have been studied in which the myosin molecules are separated from each other by copolymerization with rod fragments of myosin which can not bind actin. Copolymer filaments were formed by dilution of high salt solutions containing myosin mixed with rod. The structure of the copolymer filaments was examined in the electron microscope at molar ratios of rod to myosin of 1:1, 4:1 and 12:1. The copolymer filaments were intermediate in general appearance between control filaments of myosin or rod alone. The distribution of myosin heads on the filaments was studied by adding a low concentration of actin so that contacts between myosin heads and actin could be visualized by negative staining. At low rod to myosin ratios, arrowhead structures were evident in places where actin filaments were lying between myosin-rod filaments, but arrow heads were not seen when the rod to myosin ratio exceeded 4:1. It appears, therefore, that the myosin heads are distributed approximately randomly along the filaments. However, by our methodology, we could not rule out the possibility that dimer or trimer clusters of myosin may have been present. The actin-activated ATPase of these mixed filaments per unit myosin (at pH 7, ionic strength approximately 0.12) decreases with increasing rod content, and at a ratio of 4:1 rod to myosin, it reaches a plateau of about 30% of the control ATPase in the absence of rod. The actin dependence of the ATPase of copolymer filaments shows the same biphasic character as control myosin, but the apparent affinity of actin for myosin is lower. In summary, the activating effect from multi-site interactions of myosin and actin filaments seems to be removed by 'diluting' the myosin with rod, but the complex kinetics remain, suggesting that this complexity is not caused solely by multi-site filament interactions but involves other factors such as, possibly, a non-equivalence of myosin heads or myosin molecules in the filament environment.

Are contractile and regulatory protein properties under similar neural activity influence?

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It is generally accepted that physiological and biochemical properties of skeletal muscles are under neural control. Particularly, it has been well established that innervation had a significant role in determining and maintaining the characteristics of muscle contractile properties. The aim of our study was to see whether the suppression of the nervous influence affected differently the functional properties of two groups of myofibrillar proteins: the contractile and the regulatory proteins. We examined the effects of 15-day denervation on the contractile properties of skinned fibres from a rat muscle, the slow soleus. Denervation had an atrophic effect on the fibre size of the soleus (SOL) as the fibre diameter was reduced to 82% of the control SOL value. The maximal tension (Po in mg) was significantly decreased but this decline did not persist when expressed per surface unit (kg cm^{-2}). This indicated that the force decrease could be directly correlated to the muscle atrophy and also suggested that after denervation the amount of cross-bridges was reduced. As regulatory protein isoforms are generally associated with differences in the Ca activation of tension development, the relationship between tension and calcium concentration (T/pCa) was established. After denervation, the T/pCa curve was shifted towards higher Ca concentrations by 0.23 pCa units, indicating a reduced Ca affinity of the troponin C (TnC) and the steepness of the curve was significantly decreased. As the shape of the T/pCa was sharply influenced by the type of TnC and/or tropomyosin isoforms, we suggested that, after denervation, the SOL acquired regulatory protein properties that changed it into an even slower muscle. However, the tension responds to strontium (T/pSr) revealed that after denervation, the SOL fibres were less sensitive to Sr than the control SOL fibres, and thus were identified as fast fibres. The maximal velocity of shortening (V_{max}) was measured and indicated a much greater diversity among the denervated SOL fibres. Indeed, after denervation, the SOL fibres had either intermediate or higher values of V_{max} when compared with the control SOL. Finally, the analysis of the composition in myosin heavy and light chains revealed the emergence of fast types of isoforms. In conclusion, we suggested that (1) there was a heterogeneous response to denervation among the contractile and regulatory proteins, (2) in our study, the changes in Sr activation characteristics could be better correlated to the myosin properties, and (3) the maintenance of the normal muscle contractile properties was strongly dependent on the neural activity.

Localization of divalent cation- and nucleotide-induced conformational changes in G-actin

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Conformational changes in G-actin that might be responsible for modulation of its polymerization properties by the tightly bound divalent cation and nucleotide have been detected by a variety of methods. Little information is, however, available on the location of these changes in the actin polypeptide chain. Using proteolytic susceptibility as a probe for conformational changes, we were able to identify four regions of the actin polypeptide chain where metal ion- and nucleotide-dependent structural rearrangements take place: the segment spanning residues 42–48, the regions around Arg-62 and Lys-68, and the C-terminal segment. Replacement of the tightly bound Ca^{2+} by Mg^{2+} in ATP-actin most strongly affected the regions around Arg-62 and Lys-68 as judged from a complete inhibition of tryptic cleavage at these residues. It also significantly diminished the rate of removal by trypsin of the three C-terminal residues. The rates of specific cleavages within the 42–48 segment by substilisin, chymotrypsin and a novel protease from *E. coli* were only slightly lower in Mg-actin than in Ca-actin. In contrast, conversion of ATP-actin to ADP-actin (with Mg^{2+} as the tightly bound cation) strongly inhibited the cleavages within the 42–48 segment, whereas its effects on tryptic cleavages at Arg-62, Lys-68 and in the C-terminal segment (partial reversal of the inhibitory effect of Mg^{2+}) were small. These data suggest that the tightly bound cation and nucleotide regulate the polymerization by changing the structure of different regions of the monomer. In terms of the atomic models of G- and F-actin proposed by Holmes and colleagues (*Nature* 347, 37–44, 44–49 (1990)), the Mg^{2+}-induced change in folding of the loop region adjacent to Lys-68 is likely to alter the relative orientation of the subdomains 1 and 2 of the monomer. One can suppose that this structural alteration facilitates formation of the intermonomer bonds involving these two subdomains. The ADP-induced burying of surface residues within the 41–51 loop which, in its N-terminal part, is thought to be one of the monomer-monomer interfaces, may be among changes responsible for the weakening of monomer-monomer interactions by this nucleotide.

Histochemical and immunohistochemical characterization of muscles in different breeds of chicken

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Three strains of 28-day-old chickens: fast-growing broiler (Cornish-White) and slow-growing layers (Polbar and Leghorn H_{22}) were investigated. The aim of our study was to compare histochemical (myofibrillar ATPase and SDH activity), immunohistochemical (parvalbumin), and morphometrical characteristics of muscle fibres in pectoralis and sartorius of these three genetically different lines of chickens.

According to mATPase activity I (two subpopulations), IIA and IIB fibre types were identified in the red region which forms the deep cranial edge of pectoralis. In the rest of this muscle one type of fibres (IIB) was observed. Sartorius was composed of four fibre types in the medial part. The frequency of type I and IIA gradually decreased towards lateral part of the muscle whereas the proportion of IIB type increased. Morphometrical analysis revealed interlinear variability of proportions and sizes of the fibre types. The most notable results obtained were as follows. (1) Broiler had approximately 50% more muscle mass than two rest of the same age lines of chicken. The fibre diameters correlated with the differences of weight in certain extention. It means that the greater muscle mass in broiler is from a higher total number of fibres. (2) The ratio of type I to type II fibres decreased: broiler > Leghorn > Polbar. The amount of type IIA fibres increased and type IIB fibres decreased: broiler < Leghorn < Polbar. The amount of high oxidative fibres increased: broiler < Leghorn < Polbar. (3) These results were postively correlated with morphometric analysis of muscle fibre volume density (Vvf), surface density (Svf) and numerical density (Naf) of muscle fibres with regard to their differences in sizes and proportions. (4) The muscles investigated did not show immunoreactivity with anti-parvalbumin monoclonal antibody (antibody 235) (Celio *et al.* (1988), *Cell Calcium* **9**, 81–6).

On the basis of many studies of avian myogenesis, the number of fibres is genetically determined and established before birth and formation of all fibre types depends on the properties of myoblasts from which the fibres were formed. The differences we found between chicken lines belonging to the same species (*Gallus domesticus*) seem to be determined by expression of different genes in phase of primary myogenetic cell lineages. We suggest that these differences are mainly caused by animal selection to increase muscularity.

Effects of intracellular alkalinization on force production of depolarized uterine smooth muscle

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In many smooth muscles intracellular pH (pH_i) has profound effects on active force production (Wray (1988) *Am. J. Physiol.* **254**, C213). In the isolated rat uterus, alkalinization of the cytoplasm at constant external pH by the application of weak bases NH_4Cl, TMA (trimethylamine hydrochloride) or DEA (diethylamine hydrochloride), resulted in an increased frequency of spontaneous contractions (Heaton and Wray (1991) *J. Physiol.* (in press)) and an increase in amplitude. Removal of the base transiently abolished spontaneous force production. Furthermore, application of the weak acids butyrate or propionate resulted in an acidification of the cytoplasm and an abolition of spontaneous contractile activity (Duggins *et al.* (1989) *J. Physiol.* **417**, 125P). We now report the effects of application of weak bases to uterine smooth muscle activated by depolarisation with KCl.

Small strips (4 mm long by 1 mm wide) of longitudinal smooth muscle of non-pregnant and late-pregnant (19–21 day) rat uteri were superfused at 35 °C with HEPES-buffered Krebs continuously bubbled with 100% oxygen. Perfusion with 40 mM KCl-Krebs resulted in a sustained contraction. Application of 30 mM NH_4Cl in KCl-Krebs (substituted isomolarly for NaCl), reduced the tension in 12 of 13 non-pregnant (20% ± 8.9 relaxation (mean ± SD); $p < 0.05$ paired t-test) and all six pregnant tissues (22% ± 11 relaxation). Removal of the base transiently raised force above that previously observed in KCl-Krebs in all non-pregnant (15% ± 9.2 increase) and five of six pregnant (18% ± 8.9 increase) tissues. At 30 mM TMA, similar results were produced to NH_4Cl in 9 of 11 non-pregnant tissues. Preliminary experiments simultaneously monitoring tension and pH_i (with the pH-sensitive fluorophore carboxy-SNARF-1) have confirmed that application of 30 mM NH_4Cl or TMA to these tissues raises pH_i and removal of the base produces a transient rebound acidification. Thus procedures to alkalinize the cytoplasm affect spontaneous and fully activated force production of the uterus in opposite manners and suggests a complex relationship between pH_i and uterine contractility.

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Myosin isoforms in cross-reinnervated rat soleus muscles

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For normal rat hind limb muscles (slow-twitch soleus, fast-twitch peroneus longus (PL) and extensor digitorum longus (EDL) a good correlation exists between experimentally determined maximum shortening velocity (V_{max}) and that predicted on the basis of histochemically determined muscle fibre type composition (Ranatunga and Thomas (1990) *J. Muscle. Res. Cell Motil.* **11**, 240–50). The shortening velocities for individual fibre types used in the model were based on data from skinned rat muscle fibres (Sweeney *et al.* (1988) *J. Biol. Chem.* **263**, 9034–9)) as no data from rat fibres were available at that time. Shortening velocities since reported for rat skinned muscle fibres (Bottinelli *et al.* (1990) XIXth European Conference on Muscle Contraction and Cell Motility, Brussels, Belgium) indicate similar differences between fibre types. V_{max} values predicted using the rat fibre data were similar to those obtained previously. In soleus muscles examined 15–35 weeks after cross-reinnervation by either PL (X-SOL(p.l.)) or EDL (X-SOL (e.d.l.)) nerves, however, the predicted V_{max} was significantly higher than that determined experimentally. This was especially so with respect to X-SOL (p.l.) muscles (Ranatunga and Thomas (1989) *J. Physiol.* **418**, 53P; Thomas (1990) PhD Thesis, University of Bristol) similar shortening velocities were measured in normal soleus (N-SOL) and X-SOL (p.l.) muscles although the latter contained significantly more Type 2A (fast) fibres. A study of the myosin heavy chain (MHC) isoforms in these muscles was therefore undertaken using SDS-polyacrylamide gel electrophoresis. It was found that normal and cross-reinnervated soleus muscles contained both Type 1 and Type 2A MHCs while self-reinnervated soleus contained only Type 1 MHC. The MHC isoform composition of X-SOL (p.l.). muscles was similar to that of N-SOL while the Type 2A isoform became predominant in X-SOL (e.d.l.) muscles. V_{max} values predicted using MHC isoform compositions were consistent with values from both N-SOL and X-SOL (p.l.) muscles.

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External ATP induces cAMP-dependent phosphorylation on the outer side of cilia membrane

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The ciliary excitable membrane from higher organisms has received little attention. However, it would play a main role in the stimulation of ciliary beat by external Ca, as it has been shown that Ca^{2+} ions interact wtih the axoneme constituents inside cilia to produce cilia bending (Verdugo (1980), *Nature* **283**, 764–765; Girard and Kennedy (1986) *Eur. J. Cell. Biol* **40**, 203–9). Besides, external ATP equally

provokes an acceleration of cilia beat (Ovadyahu *et al.* (1988) *Biorheology* **25**, 489–501). We investigated the possibility of ATP facilitating Ca binding and the location of ATP action in cilia from the quail oviduct. Ca movements and cilia phosphorylation were followed by incubating pure isolated cilia in presence of either ^{45}Ca or ^{32}P-ATP. Samples were removed at 5-s intervals, filtered on Millipore filters and radioactivity bound to the cilia recovered was measured. The non-permeation of external ATP was assessed by using ^{14}C-ATP, and the possibility of internal cAMP intervention was assayed with dibutyryl-cAMP. In presence of ATP, Ca movements extended over short repetitive periods separated by long resting periods. They were preceded by successive phosphorylation-dephosphorylation cycles. Both Ca movements and cilia phosphorylation were inhibited by protein kinase A inhibitor, stimulated by Forskolin (adenylate cyclase stimulator), anticipated by cAMP, and prolonged by NaF (protein phosphatase inhibitor). Exclusively, one protein of 60 kDa was found phosphorylated and dephosphorylated in a cyclic manner during phosphorylation-dephosphorylation steps. The calcium channel blocker, nifedipine, inhibited both ^{45}Ca movements and cilia phosphorylation. ^{14}C-ATP failed to significantly label cilia, and dibutyryl-cAMP was unable to anticipate Ca movements. The results indicate that in presence of ATP: (1) Ca movements are strictly correlated with cAMP-dependent phosphorylation of a 60 kDa ciliary protein, and (2) the cAMP-dependent phosphorylation takes place at the outer side of the ciliary membrane, suggesting that the phosphorylable 60 kDa protein would be a membrane component.

'Anti-rigor' crossbridges may reflect altered state of actin

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We reported earlier that in insect muscle, myosin heads could form either rigor bridges or 'anti-rigor' bridges, with reversed angle. When rigor develops at constant fibre length, the bridge angle is typically 45° – the rigor bridge. When rigor develops isotonically, so that the specimen can shorten, regularly arranged anti-rigor bridges are also observed (Trombitás *et al.* (1988) *J. Ultrastruct. Mol. Struct.* **98**, 267).

To investigate anti-rigor bridges further, bumblebee-flight muscle fibres were prepared. Fresh fibre samples were stretched to 5–15% beyond their natural length, and then chemically skinned by a 2-hour soak in a relaxing solution containing 0.5% Triton X-100. The stretched muscles were then alternately transferred and kept for 20 min in relaxing and rigor solutions until some of the fibres broke as a result of the developing rigor tension. Samples were then fixed in rigor, and the broken fibres were separated from the intact fibres.

Micrographs of the intact fibres revealed the conventional rigor-bridge angles. 'Chevrons' formed by bridge pairs pointed away from the Z-line in a regular manner. Bridges forming the chevrons were oriented at about 45° to the filament axis. By contrast, the broken fibres, which had shortened considerably, generally contained a mixed population of bridges. Two distinct orientations were found within the same half-sarcomere. Along some thin filaments, chevrons pointed toward the M-line, while along others, the chevrons pointed away from the M-line. Bridge angles were always symmetrical about the thin filament, not the thick.

At least two interpretations seem plausible *a priori*: (1) As specimens were extended during fibre preparation, some actin filaments may have broken off their anchor points on the Z-line. As these fibres developed rigor tension, the freed filaments might then have been propelled past the M-line to the opposite side of the sarcomere. Thus, anomalously polarized actin filaments on either half of the sacromere might account for bridges with reversed angle (Reedy *et al.* (1989) *Nature* **339**, 481). (2) A second possibility is that the different bridge orientations represent different states of actin. This interpretation implies that the actin conformation may change during rigor development. Thus, an actin filament in one state might show the rigor angle, while an actin filament in another state might show the anti-rigor angle (Trombitás and Pollack, (1990) *Proceedings of XII ICEM*, Vol. 3, 480). If this explanation is correct, it should be possible to find single actin filaments along which both states might be observed simultaneously. This situation could indeed be found, though less commonly than the situation in which only a single state could be detected. Filaments could be found where the bridges were transformed progressively from one state to the other: from the rigor configuration to the anti-rigor configuration. In the transition zones between rigor and anti-rigor, bridge regularity appears to be disturbed.

The presence of such regular transitions along single actin filaments militates against the first explanation. On the other hand, the second explanation is consistent with this observation. As bridges are symmetrical about the thin filament axis irrespective of their particular angle, something in the thin filament appears to be the agent responsible for angle determination. The fact that the bridge angle can change along the single thin filament implies that constituent actins can undergo local structural changes.

Nature and origin of gap filaments in striated muscle

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Immunoelectron microscopy was used to study the nature and origin of 'gap' filaments in frog semitendinosus muscle. Gap filaments are fine longitudinal filaments observable only in sarcomeres stretched beyond thick/thin filament overlap: they occupy the gap between the tips of thick and thin filaments. To test whether the gap filaments are part of the titin-filament system, we employed monoclonal antibodies to titin (T-11, Sigma) and observed the location of the epitope at a series of sarcomere lengths in single, mechanically skinned fibres. At resting sarcomere length, the epitope was positioned in the I-band approximately 50 nm beyond the apparent ends of the thick filament. The location did not change perceptibly with increasing sarcomere length up to 3.6 μm. Above 3.6 μm, the span between the epitope and the end of the A-band abruptly increased, and above 4 μm, the antibodies could be seen to decorate the gap filaments. Between 5 and 6 μm, the epitope remained approximately in the middle of the gap. Even with this high degree of stretch, the label remained more or less aligned across the myofibril. The abrupt increase of span beyond 3.6 μm implies that the A-band domain of titin is pulled free of its anchor points along the thick filament, and moves toward the gap. Although this domain is functionally inextensible at physiological sarcomere length, the epitope movement in extremely stretched muscle shows that it is intrinsically elastic. Thus, the evidence confirms that gap filaments are clearly part of the titin-filament system. They derive not only from the I-band domain of titin, but also from its A-band domain.

Joule temperature jump in skinned muscle fibres with compressed myofilament lattice

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The temperature jump (T-jump) from 4–8 to 28–35 °C initiates a biexponential tension rise in fully activated fibres (Bershitsky and Tsaturyan (1986, 1989); Goldman *et al.* (1987). The first (fast) phase reflects the force-generating step of attached crossbridges while the second (slow) one is accompanied by the crossbridge reattachment (Tsaturyan and Bershitsky (1988)). The lateral shrinkage of the filament lattice in skinned fibres from rabbit psoas muscle was obtained using 30 g l^{-1} of either polyethylenglycol (MW 20 000) or Ficoll

(MW 400 000). This led to an increase in the fibre stiffness (Goldman and Simmons (1986)). The polymers induced an increase in fibre stiffness from 12.2 ± 3.3 to 24.3 ± 7.4 MN m^{-2} (mean ± SD, $n = 7$), i.e. to the value more than that without polymers in rigor (17.5 ± 3.2 MN m^{-2}). The shrinkage enhanced not only the number of the attached crossbridges, but also the crossbridge stiffness. In compressed fibres, the tension response to the T-jump was also biexponential. The rate constant k_1 (860 ± 220 s^{-1}) and the amplitude of the first phase were less than those in polymers-free solution ($k_1 = 560 \pm 190$ s^{-1}). The rate constant of the second phase was invariable. It shows that the increase in the crossbridge stiffness hampers the crossbridge to making the force-generating step. This result agrees with the Huxley-Simmons model (1971). Also, in the compressed fibres, the stiffness rise after the T-jump was less pronounced than that without compression. It should be noted that the rabbit fibres under compression were similar to those in the uncompressed frog fibres. An increment of the tension to stiffness ratio (i.e. force per attached crossbridge) during the second phase of the response to the T-jump was independent of the compression.

Oxygen uptake of single muscle fibres from Xenopus during and after fatiguing, intermittent stimulation

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Oxygen uptake was measured to determine the contribution of phosphocreatine (PCr) and oxidative phosphorylation to ATP production during fatiguing stimulation of skeletal muscle fibres.

Single, intact fibres, dissected from the iliofibularis muscle of *Xenopus laevis* (type 1) (Lännergren and Smith 1966) *Acta Physiol. Scand.* **68**, 263–74) were simulated intermittently (40 Hz) at 20 °C to produce one 250 ms tetanus every 5 s. The rate of oxygen consumption (VO_2) of the fibre was determined as described previously (Elzinga and van der Laarse (1988) *J. Physiol.* **399**, 405–18).

During the first 1.5–2 min of intermittent stimulation, force fell to 80 ± 2 (mean ± SD, four fibres) % of original, while VO_2 increased to its maximum (VO_{2max}). VO_{2max} equalled 22 ± 8 pmol O_2 mm^{-3}, s^{-1} and was maintained for 3.5–5 min, during which force fell slowly to 62 ± 14% of the original. During the subsequent 4–6 min, force as well as VO_2 decreased: force to 37 ± 3% of the original and VO_2 to 72 ± 9% of VO_{2max}. After this phase, it seemed that a steady state was reached. The amount of oxygen taken up in recovery was 3.8 ± 1.9 nmol O_2 mm^{-3}, which, assuming $P/O_2 = 6.3$, is just sufficient to resynthesize all PCr present in fully rested type 1 fibres (25 ± 3 nmol mm^{-3}) (Nagesser *et al.* (1990) *J. Physiol.* **426**, 32P).

Because VO_{2max} in type 1 fibres is reached when the PCr store is fully depleted (van der Laarse and Elzinga (1991) *J. Physiol.* **438**, 47 P), it is concluded that the PCr store is depleted during the first 2 min of intermittent stimulation. This indicates that accumulation of inorganic phosphate can at most explain a force reduction to 80% of original, and that buffering of ATP by PCr has ceased after 2 min of intermittent stimulation.

Sarcomere length distribution in relation to isometric force in skinned fibres of the human rectus abdominis muscle

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During current surgery, human biopsies of the rectus abdominis muscle (RAM) were frozen in liquid nitrogen and dried for at least 3 weeks. To compare force-sarcomere length relations between single fibres, biopsies were frozen while shortening was allowed to an adjustable extent against a springlike holder. Fibres from these biopsies could cover the desired range of sarcomere lengths including the expected range of maximal overlap of the actin and myosin filaments. After drying, fibres of 2–5 mm could be easily dissected under a microscope and stored further on at −70 °C for >6 month without loss of their force-generating capacity up to 250 kPa. Human fibres of the RAM on which this freeze-drying skinning technique (Stienen and Blange (1985) *Eur. J. Physiol.* **405**, 5) was applied and used throughout a period of >6 month thereafter, also revealed normal Hill-curves with parameter values of $pCa_{50} = 5.9 \pm 0.2$ and $n = 2.2 \pm 0.4$, independent of the sarcomere length above 1.8 μm (Van Kaam *et al.* (1990) *J. Muscle Res. Cell Motil.* **12**, 93; Blangé *et al.* (1991) *Biophys J.* **59**, 45).

After each isometric contraction, sarcomere length distribution was measured by laser diffraction by hand along the entire fibre length (2–8 mm), including the fixed ends (typically 0.4 mm, maximally 0.6 mm). SEM of the sarcomere length distribution ranged from 1 to 20%, which reduced to <10% when the SL value was averaged for three isometric contractions. At a particular fibre length, three activations were performed after which the fibre length was measured. Subsequently the fibre was stretched during the relaxed state. Another measure of sarcomere length distribution was used by means of the fibrelength to average sarcomere length ratio (FL to SL) representing the amount of sarcomeres which remain constant during the experiment. However, owing to inhomogeneous sarcomere length distribution, the FL to SL ratio found experimentally is not constant. For each set of three contractions at different fibre lengths the FL to SL ratio revealed a SEM value of <5%.

Normalized forces developed 1 min after the start of activation were plotted against the sarcomere length, both taken as the average of each set of three contractions. The maximum force was found within the range of 2–2.4 μm and the descending limb intersected the abscissa at 3.8–4.0 μm.

Freeze-dried single fibres of the human rectus abdominis muscle thus far appear to give results similar to those obtained from freeze-dried frog fibres (Stienen *et al.* (1985) **405**, 19).

Functional change in guinea pig urinary bladder smooth muscle after partial urethral obstruction

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Gradual partial urethral obstruction was created in 20 immature male guinea pigs using silver jeweller's jump rings (*J. Urol.* (1991) **145**, 854–8). After 4 or 8 weeks obstruction the animals were killed and contractile responses of muscle strips to electrical field stimulation of intramural nerves, direct electrical muscle stimulation, 0.1mM carbachol and high K^+ solution were sampled in Baltimore. Files were sent by electronic mail (BITNET) to Rotterdam for analysis using plots of the rate of change in force as a function of the force, i.e. phase-plots (*Am. J. Physiol.* (1986) **251**, R978–83). For the greater part this phase-plot could be characterized by a straight line of which the horizontal intercept, i.e. maximum extrapolated force, was referred to as Fiso and the negative reciprocal value of the slope of this line was referred to as C. This C represents the time constant in isometric force development. Following 8 weeks obstruction, the value of Fiso, indicative of the number of contractile muscle units, was reduced to 60% of the control response to nerve stimulation ($p < 0.05$) and to 77% of the control response to carbachol stimulation ($p < 0.05$). The parameter Fiso was reduced to 68% of the control response for direct muscle stimulation, but this result was not significant ($p = 0.10$). The timeconstant C was unchanged for all forms of stimulation. It has been shown that in intact smooth muscle cells C describes the influx of extracellular calcium (Van Koeveringe (1991) *Am. J. Physiol.* (in press)). The results

indicate that this process is not affected by obstruction. Obstruction seemed to affect responses to nerve and muscle stimulation similarly suggesting that muscle change may possibly be a common denominator of dysfunction. It is concluded that obstruction reduces the number of active muscle units (Fiso) per gram of smooth muscle tissue but leaves the limiting rate constant in the excitation contraction coupling (C) unaffected.

Time-resolved X-ray diffraction studies of muscle during activation by double stimulus

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The approach of using the second stimulation (ss) to muscle during twitch reveals the connection between force increase and structural changes. Frog sartorius muscle was stimulated by double pulses with intervals of 50–300 ms. The time course of intensity changes ($I(t)$) of meridional $I_{440\,Å}(t)$, $I_{214\,Å}(t)$, $I_{143\,Å}(t)$ and equatorial $I_{10}(t)$ and $I_{11}(t)$ reflections with time resolutions of 5–15 ms has been studied. The ss in the final twitch phase results in two similar maxima on $P(t)$ and two identical in depth minima on $I_{143\,Å}(t)$, but the amplitude of the second minima is almost twice smaller than the first one. The ss in the active twitch phase results in a marked increase of $I_{143\,Å}(t)$. $I_{214\,Å}(t)$ and $I_{440\,Å}(t)$ have a monotonous character. $I_{10}(t)$ and $I_{11}(t)$ follow the $P(t)$. These results suggest that crossbridges (cb) in the active phase in the vicinity of thin filaments are not incorporated into the actin helix range: they form a short-living mesophase with a period of 143 Å in the triangular channel of neighbouring thick filaments. Our calculations of diffraction patterns from such a structure are in agreement with the experiment. Thus, the increase of force proceeds upon interaction of incommensurable structure. The quick release applied soon after ss results in a force decrease and a sharp drop of $I_{143\,Å}(t)$ to a value much lower than that before ss. $I_{214\,Å}(t)$ and $I_{440\,Å}(t)$ change monotonously. This can be interpreted as a formation of a specific rigor-like complex cb with actin. The structure of fresh muscles quickly recovers to the initial state. The experiments with fatigued muscles during twitch have shown that $I_{10}(t)$ and $I_{11}(t)$ do not return to the original level, the time of structural relaxation increases. The application of pairwise stimulation have normalized the structure of tired muscle to the original state.

Coordinated changes in myofibrillar properties, mitochondrial creatine kinase functional state and performance of isolated perfused rat heart in hypothyroidism and long-term calcium channel- and beta-blockade

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We studied the relationship between cardiac contractility, properties of myofibrils and mitochondrial creatine kinase (CKmit) activity in hypothyroidism as well as after long-term (6–8 weeks) treatment with verapamil (V) or propranolol (P) in rats. The pump function of the isolated heart was decreased in hypothyroidism (as compared with control), but increased after V or P treatment. Likewise, myofibrillar ATPase activity measured in isolated Triton-skinned cardiomyocytes in the absence, as well as in the presence, of Ca^{2+} was considerably lower in hypothyroidism and higher after long-term V or P administration. Calcium sensitivity of skinned ventricular fibres was increased in all experimental groups. Functional activity of CKmit was estimated in saponin-skinned cardiac fibres by percentage of mitochondrial respiration rate enhancement after the addition of creatine in the presence of low ADP concentration. This parameter was severely depressed in hypothyroidism, but increased after chronic V and P treatment. Activities of extracted creatine kinase isoenzymes were similar in all experimental groups.

The data obtained show that changes in inotropic contractile status due to chronic influences are followed by a coordinated modulation of myofibrillar ATPase activity and the functional state of CKmit, but not in biochemical activity of extracted CKmit. The modulation of the CKmit functional state is hypothesized to be related to the intensity of energy turnover in cardiac cells.

Electrolyte content of human muscle during fatigue from repeated submaximal contractions

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During repeated contractions MVC force declines gradually, without significant changes in energy substrates or metabolites (Vøllestad *et al.* (1988) *J. Appl. Physiol.* **64**, 1421–7). Loss of K^+ from the muscle has been proposed as a mechanism for fatigue (Sjøgaard (1990) *Acta Physiol. Scand.* **140** (Suppl. 593) 1–63). We examined whether the muscle content of electrolytes changes during repeated 30% MVC contractions carried out until exhaustion (65 ± 8 min). Two-legged isometric quadriceps contractions were held for 6 s with 4 s rest between. The MVC force fell almost linearly to $58 \pm 4\%$ of control at exhaustion. Muscle biopsies were taken from vastus lateralis at rest and after 5, 15 and 30 min of exercise and at exhaustion. The mean ($\pm$SE) muscle content of K^+, Ca^{2+} and Mg^{2+} were 400 ± 10, 3.8 ± 0.4 and 38 ± 1 $\mu mol\,g^{-1}$ dry weight and did not change significantly during exercise. At exhaustion the respective values were $103 \pm 4\%$, $100 \pm 21\%$ and $107 \pm 5\%$ compared with control values. Muscle lactate and P_i concentration increased from resting values of 0.5 ± 0.2 and 4 ± 1 $mmol\,kg^{-1}$ wet weight to 1.3 ± 0.6 and 6 ± 1 $mmol\,kg^{-1}$ after 5 min. From 5 to 30 min of exercise lactate and P_i remained stable, before a rise to 4.8 ± 1.1 and 15 ± 5 $mmol\,kg^{-1}$ w/w was seen at exhaustion. Studies of arteriovenous concentrations show that the exercising muscle looses a small amount of K^+ with a small increase in extracellular K^+ concentration (Vøllestad *et al.* (1991) *J. Physiol.* 89P). However, the lack of detectable changes in muscle K^+ content suggest that this ion is of little importance for fatigue under the present conditions. Moreover, fatigue is not related to changes in muscle Ca^{2+} or Mg^{2+} content. The increase in lactate and P_i concentrations were modest and did not change linearly as MVC did. It is concluded that muscle fatigue cannot be ascribed to changed cellular content of any of the measured electrolytes.

Titin: a template for sarcomere formation in differentiating human myoblasts?

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A characteristic of many muscle diseases is a failure to develop or maintain structural and functional intensity of the skeletal muscle cells. To understand better the normal processes, we have studied the pattern of differentiation of normal human skeletal muscle cells *in vitro* by analysing cytoskeletal and sarcomeric proteins using immunohistochemical methods. Satellite cells were isolated from human muscle biopsies by trypsin and collagenase treatment, and grown in high-nutrition culture medium (Benders *et al.* (1991) *Exp. Cell. Res.* (in press)). Differentiation of the satellite cells occurred at confluence, but could also be induced by changing to the low-nutrition culture medium.

After such a medium-switch, the satellite cells fused forming polynucleated and elongated syncytia which occasionally contracted rhythmically. Successive stages of differentiation were studied by immunofluorescence microscopy using antibodies against contractile and structural proteins. Desmin, the muscle specific intermediate filament protein, was found in the prefusion stages as well as in all stages of differentiating cells in a diffuse or distinctly filamentous pattern. Antibodies to titin and myofibrillar myosin, both specific skeletal muscle markers and components of the sarcomere, did not show reactivity in undifferentiated cells. Within a few hours after a medium switch, titin was found in a punctate pattern in mononucleated cells, young polynucleated cells and myotubes. Further differentiation was accompanied by the appearance of longitudinally oriented titin and myosin filaments, which eventually became striated. From these findings we conclude that during *in vitro* myofibrillogenesis in non-diseased muscle cells, titin and myosin are organized in a cross-striated pattern before desmin. Therefore, the function of desmin in the formation of the sarcomere seems questionable (Gard *et al.* (1980) *Cell* **19**, 263–275). The results suggest that titin itself may constitute at least in part, a template for sarcomere-formation. Further studies are being directed to the investigation of the appearance and distribution patterns of functional markers of differentiation in satellite cells derived from patients suffering from congenital myopathies.

Caldesmon-phospholipid interaction: the role of protein kinase C-catalysed phosphorylation

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Earlier we have demonstrated the Ca^{2+}-independent interaction of smooth muscle caldesmon (CaD) with the mixture of soybean phospholipids (azolectin) (Vorotnikov and Gusev (1990) *FEBS Lett.* **277**, 134). Continuing this investigation we found that CaD preferentially interacts with acidic phospholipids (phosphatidylserine) and is practically unable to interact with phosphatidylcholine. When the mixture of chymotryptic peptides of CaD and azolectin vesicles was subjected to ultracentrifugation, the pellet contained only the peptides with M_r 40, 23, 22 and 19 kDa. Ca^{2+}-saturated calmodulin prevents the interaction of these peptides with phosholipids. The data presented indicate that the sites involved in CaD-phospholipids interaction are located in the C-terminal part of CaD in close vicinity to the sites of CaD-calmodulin interaction. The data of literature indicate that Ca-phospholipid-dependent protein kinase preferentially phosphorylates Ser-587 and Ser-600 (Adam and Hathaway (1990) *Biophys. J.* **57**, 180), i.e. the sites located close to calmodulin-binding site of CaD. In our hands phosphorylation by protein kinase C has no effect on the CaD-calmodulin interaction, but significantly decreases the affinity of CaD to phospholipids. Thus, the data presented indicate that the interaction of CaD with phospholipids is controlled by calmodulin and depends on CaD phosphorylation. Taking into account the perimembrane localization of both calmodulin and protein kinase C we suppose that CaD-phospholipids interaction is important in certain processes of the cell motility.

Role of pH in fatigue of isolated mouse muscle fibres

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Acidosis caused by lactic acid accumulation may contribute to skeletal muscle fatigue. We have now used the pH indicator BCECF to measure intracellular pH (pHi) in mouse single muscle fibres during fatiguing stimulation. Single muscle fibres were dissected from the flexor brevis muscle of the mouse and loaded with BCECF in the AM form. Fatigue was produced by repeated 350 ms tetani which were given until tension was reduced to about 30% of the original. In the rested state the mean pHi was 7.3 ($n = 14$), similar to earlier measurements in mouse muscle (Aickin and Thomas (1977) *J. Physiol.* **267**, 791–810). During fatiguing stimulation pHi initially went alkaline reaching a maximum after about ten tetani (mean pH increase 0.03 units). Subsequently pHi became acid so that in the fatigued state pHi was 0.06 pH units lower than control. This acidification is much smaller than that observed in whole mouse muscle fatigued in a similar way (Juel (1988) *Acta Physiol. Scand.* **132**, 363–71). This difference may arise because in the present experiments the extracellular fluid was kept constant, whereas in whole muscles lactate and hydrogen ions will accumulate in the restricted extracellular space. To test this hypothesis, rested fibres were exposed to a solution containing 20 mM lactate and with pH reduced from the normal 7.3 to 6.8. This resulted in an acidosis of 0.3 pH units ($n = 3$). Furthermore, fibres fatigued in the presence of the lactate transport inhibitor cinnamate showed a sizable acidification (mean pHi decline 0.4 pH units, $n = 5$); these fibres also fatigued more rapidly.

In conclusion, fatigue in isolated mouse muscle fibres is accompanied by a pHi reduction which is too small to explain the tension reduction. The very small pHi decline in fatigue can be explained by an effective extrusion of lactate and hydrogen ions. If this acid extrusion is inhibited, tension falls faster during fatiguing stimulation.

Effect of diabetes and insulin treatment on heart actomyosin ATPase activity and contractile properties of rat skeletal muscle

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I. Actomyosin ATPase activity. Previous experiments showed that a decrease in contractile function of rat hearts during reperfusion after ischemia could partly be explained by a decrease in actomyosin ATPase activity (Westra and Achterberg (1990), *J. Muscle Res. Cell Motil.*). We also found a decrease in contractile function during perfusion in hearts from diabetic rats. In the present study we investigated whether this decrease could also be explained by a decrease of actomyosin ATPase activity. The results showed that (1) in the absence of calcium heart actomyosin ATPase activity was not significantly different in control, diabetic and insulin treated hearts (79 ± 5 ($n = 6$), 78 ± 3 ($n = 7$) and 83 ± 5 ($n = 6$) nmol Pi per mg protein per min, respectively), and (2) calcium stimulated actomyosin ATPase activity in diabetic hearts was significantly lower than in control hearts (89 ± 4, 167 ± 6 and 139 ± 5 nmol Pi per mg protein per min). Insulin treatment partly restored the activity (139 ± 5 nmol Pi per mg protein per min). From the preliminary results we conclude that loss of contractile function in perfused diabetic rat heart can only partly be explained by a decrease in actomyosin ATPase activity. Insulin treatment has a beneficial effect on actomyosin ATPase activity.

II. Contractile properties of skeletal muscle. We investigated further whether diabetes affected contractile properties and/or energy metabolism of fast-twitch skeletal muscle. Medial gastrocnemius muscles performed a series of 15 dynamic contractions (duration 134 ms) within 6 s. Energy consumption was calculated from the changes in high-energy phosphates ($\sim$P) (de Haan *et al.* (1986) *Pflügers Archiv.*). Total work output and energy consumption were not different between the diabetic and control group. This resulted in similar efficiencies (14.25 ± 2.99 and 13.94 ± 1.77 mJ $\mu mol^{-1} \sim$ P, respectively).

The results suggest that diabetes has a greater effect on slow-twitch heart than on fast-twitch skeletal muscle.

Patterned growth of neonatal rat skeletal muscle cells in culture

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Analysis of electrophysiological and polarization-optical signals of cultured skeletal muscle cells is limited by the circumstances that myotubes fuse into randomly orientated strands in such preparations. A technique described by Rohr and colleagues (1991) for cardiac muscle was adapted to produce well defined oriented growth of myotubes in cultures of skeletal muscle. Lithographic fabrication of patterned coverslips was performed using the techniques described in Rohr and colleagues (1991). Skeletal muscle cells were dissociated according to conventional culture technique. For comparison, both types, patterned and control coverslips (without photoresist coating) were used. Our experiences and results with this preparation are as follows: (1) As judged from morphological and electrophysiological criteria, the photoresistive material had no toxic effects on the muscle cells in culture. (2) The width of the growth channels on the coverslips was varied between 70 to 200 µm and their length ranged from 2 to 18 mm. (3) The myocytes grew only in the grooves resulting from the development procedure. Myotubes of up to 15 mm total length were frequently obtained. (4) On control coverslips without photoresistive coating, the fused myotubes were randomly orientated. On patterned coverslips, the orientation of the myotubes was dependent on the width of the particular channel. In narrow channels (width below 100 µm) all the myotubes were aligned along the longitudinal axis of the channel. In wider channels (width above 100 µm) the orientation of the myotubes deviated to various degrees from the axis of the channel. (5) Fusion of myocytes to myotubes could be observed after day 4 and cross-striation became apparent after day 10 *in vitro*. (6) Spontaneous, synchronous contractions of all the myotubes within a particular groove could be observed after 5 days *in vitro*. After 7 days *in vitro* membrane potentials in the range of −70 to −80 mV were measured.

The method allows to produce myotubes in tissue cultures which exhibit a defined and desired two-dimensional pattern. The longitudinal axis of myotubes on patterned coverslips is parallel to the axis of each growth channel, such a preparation is suitable for orientation sensitive optical experiments such as measuring of intrinsic polarization optical signals. On the other hand, upon using specifically arranged patterns, this technique might be used as well to obtain defined neuromuscular contacts in cocultures with neuronal tissue.

Evidence for two kinds of myosin in insect flight muscles

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Myosin has been extracted from different insect flight muscles by high ionic strength solutions (40mM $Na_4P_2O_7$, 5mM $MgCl_2$, 5mM ATP, 1mM EGTA, 10mM Tris HCl, pH 8.0) in the presence of PMSF, leupeptin and pepstatin A. The crude extracts were applied to a FPLC Mono Q HR 5/5 column. Myosin of the locust (*Locusta migratoria*) eluted at 0.15M NaCl (in 40mM $Na_4P_2O_7$, 10mM Tris HCl, pH 8.0 with protease inhibitors), fleshfly (*Phormia terrae-novae*) and bee (*Apis mellifica*) myosin at 0.21M NaCl. SDS-gels showed differences in the light chain composition of locust (2 LCs; MW 18, 28 kDa) and fleshfly myosin (3 LCs; MW 18, 30, 31 kDa). By isoelectric focusing (3% polyacrylamide gels (the acrylamide methylenebisacrylamide ratio being 12:1) with 10% glycerol and 5% ampholyte) either myosin, the myosin of the fleshfly and the myosin of the locust, could be separated into close bands at about pH 6.1 (2 for locust, 3 for fleshfly myosin). Rotary shadowing of the whole myosin fractions yielded two populations of molecules in both cases. Diagrams of rod lengths plotted *versus* number of molecules showed maxima at 149 nm and 164 nm for locust and at 143 nm and 167 nm for fleshfly rods. Double peaks could be obtained by Mono Q chromatography if the myosin of the fleshfly was eluted by a slowly rising Na_2SO_4 (0 to 0.2M) gradient. The myosin molecules of peak 1 and peak 2 were rotary shadowed separately. Measurements of the rod lengths of fleshfly myosin showed, that the rods of the molecules of peak 1 had a length of 151 ± 15 nm, those of peak 2 had a length of 161 ± 27 nm (maxima at 149 nm respectively 167 nm). Obviously, the myosins with the shorter rods have been eluted first, the myosins with the longer rods at slightly higher ionic strength.

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Alternative model for a 'Multi-ion' channel such as potassium

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It is customary to assume that the charge structure of an ion channel remains unchanged as the ion passes through. However, owing to the low electrical polarizability of the protein and lipid environment, the electric field of the transient ion has a long range, and this may cause transient changes in the effective pK and thus the charged state of nearby ionizable channel-protein residues (Edmonds (1989), *Eur. Biophys. J.* **17**, 113–19). If two single-ion channels interact with the same ionizable residues then the motion of ions through the two channels become correlated. It will be shown that all the properties which at present are attributed to single channels which are occupied by more than one ion simultaneously, may be reproduced by models of correlated single-occupancy channels. The behaviour predicted by the alternate model of a 'multi-ion' channel will be compared with those of the presently accepted Hille and Schwarz (*J. Gen. Physiol.* (1978) **72** 409–42) model.

Promiscuous expression of myosin heavy and light chains in the human masticatory muscles

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The pattern of expression of the myosin heavy (MHC) and light chains (MLC) in the human limb muscles have now been well documented. The muscle fibres form as two distinct generations in man as in other mammals with the primary generation fibres being formed in a relatively synchronous manner between 7 and 10 weeks of gestation and the second generation fibres forming asynchronously between 10 and 18 weeks *in utero*. Embryonic and fetal MHCs together with MLC1fast, MLC2fast and the embryonic MLC (MLC1emb) are predominantly expressed during early fetal development. As the muscle fibres differentiate and mature the myosin isoforms characteristic of development are eliminated and are replaced by the adult fast and slow isoforms such that by birth the muscle has almost attained its mature phenotype. However, this is not true for the masticatory muscles which seem to have evolved different intrinsic programmes of muscle fibre differentiation and maturation. This has resulted in a promiscuous expression of various myosin heavy and light chains in the adult muscle with the persistence of the developmental isoforms (MLC1emb, MHCemb, MHCfetal), particularly in the small diameter type II muscle fibres. In addition a MHC (α-cardiac) previously described as being present exclusively in the heart is expressed in some masticatory muscle fibres.

Interaction of myosin subfragment 1 with monomeric actin

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The interaction of myosin subfragment 1 (S1) with filamentous (F-) actin is regarded as a paradigm of the force generating crossbridge action in muscular tissues. In the absence of ATP S1 forms stable complexes with F-actin and in the presence of ATP the Mg^{2+}-dependent S1-ATPase is greatly stimulated. In contrast, the interaction of S1 with monomeric actin is still enigmatic. Results have recently been presented indicating that only one isoform (S1A2) can form a stable complex with monomeric actin, whereas S1A1 rapidly induces actin polymerization. The effect of the latter was attributed to the N-terminal extension of the A1 light chain (Chaussepied and Kasprzak (1990) *Nature* **342**, 950–3). We reinvestigated this reported difference in actin polymerizing ability of two S1 variants and found that both S1 isoforms are able to induce actin polymerization. The observed different rates of induced actin polymerization could be attributed to differences in their ATPase activity, as it was found that the polymerizing effect of S1 can only become operative after depletion of the ATP. It thus appears that the S1 has to form strongly interacting links with actin to induce its polymerization.

This analysis prompted us to further investigate the interaction of S1 with actin fixed in a quasi-monomeric state by complex formation with DNase I. Actin-DNase I complex was found unable to stimulate the SI-ATPase. By a number of different techniques, however (chemical crosslinking, cosedimentation and affinity adsorption to immobilized actin-DNase I complex), we could demonstrate interaction and complex formation of both variants of S1 with actin in complex DNase I and define the conditions of this interaction more accurately.

To define the regions of the actin molecule involved in S1-binding we used subtilisin-treated G-actin (cleavage after Meth 47) freed from the N-terminal peptide by ion exchange chromatography. This actin is still able to bind the DNase I and to form filaments in the presence of phalloidin or subfragment 1. After immobilization to DNase I-Sepharose this core-actin is still able to retain S1A1-like untreated G-actin indicating that removal of the 47 residues-long N-terminal peptide does not exclude S1-binding.

Phosphorylation domain of cardiac troponin I. Structural investigations by NMR and CAD using a synthetic peptide as a model

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It is known that phosphorylation of cardiac troponin I by cAMP-dependent protein kinase changes the affinity of tropinin C for Ca^{2+} (Robertson *et al.* (1982) *J. Biol. Chem.* **257**, 260–4). Which of the possible phospho-forms, non-, mono- and bisphospho- troponin *I* (Swiderek *et al.* (1990) *Eur. J. Biochem.* **190**, 575–82), affects the Ca^{2+}-binding properties of troponin C is not known. For a better understanding of function and signal transduction it is necessary to characterize the phosphorylation domain on a molecular basis for example by ^{31}P-NMR spectroscopy and CAD. As a model a decapeptide was synthesized, whose amino acid composition PVRRRSSANY was identical to the phosphorylation domain of cardiac troponin I. Both serine residues can be phosphorylated by cAMP dependent protein kinase resulting in the two monophosphorylated or the bisphosphorylated peptide depending on assay conditions (Mittmann *et al.* unpublished data). ^{31}P-NMR-spectra showed two signals for the bisphosphorylated peptide. For the bisphosphorylated troponin I only one signal could be observed at lower p.p.m. values. All signals had small line width indicating a relative free mobility of the phosphate groups. Tryptic digestion of the phospho- and dephospho- peptide showed that arginino groups might interact with the phosphate groups bound to the serine residues: NMR measurements do not favour this type of salt bridge formation and weak interaction with acidic groups is more likely. Simulation of molecular dynamics at 300 K using the program MOBY also showed a very high mobility (rotation) of the arginyl- and phosphoseryl-residues leading to changing distances (1.5–10 Å) between the residues. Energetically the most favourable conformation seemed to be an interaction of only one phosphoseryl residue with one arginyl residue: a weak interaction between the phosphates may occur. ROSY experiments will allow to measure interactions more accurately. Furthermore, we will try to identify those groups within the troponin molecule which may interact with the phosphorylation domain.

Molecular approaches to the study of TnC function

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To investigate the role of the various Ca^{2+} binding sites on skeletal muscle troponin C (STnC), a variety of mutants have been prepared that are unable to bind Ca^{2+} at one or more of the Ca^{2+}-binding sites and the structural and functional effects of these changes have been studied. To study the role of the Ca^{2+}-specific sites (I and II) of STnC in the regulation of contraction, two TnC mutants have been produced which have lost the ability to bind Ca^{2+} at either site I (VG1) or at site II (VG2). Results from C.D. measurements suggest that the structure of these mutants is not grossly different from wild type TnC (WTnC). Both mutants were able to partially restore force to TnC-depleted skinned muscle fibres (approximately 25% for VG1 and approximately 50% for VG2). In contrast, bovine cardiac TnC (BCTnC), which like VG1 binds Ca^{2+} only at site II, could fully reactivate the contraction of TnC-depleted fibres. Higher concentrations of both mutants were required to restore force to the TnC-depleted fibres than with WTnC or BCTnC. VG1 and VG2 substituted fibres could not bind additional WTnC, indicating that all of the TnC-binding sites were saturated with the mutant TnCs. The Ca^{2+} concentration required for force activation was much higher for VG1 and VG2 substituted fibres than for WTnC or BCTnC substituted fibres. Also, the steepness of force activation was much less in VG1 and VG2 *versus* WTnC and BCTnC substituted fibres. These results suggest cooperative interactions between sites I and II in WTnC. In contrast, BCTnC has essentially the same apparent Ca^{2+} affinity and steepness of force activation as does WTnC. Thus, cardiac TnC must have structural differences from WTnC which compensate for the lack of site I, while in WTnC, both Ca^{2+}-specific sites are probably crucial for full functional activity. The double mutant ($A_I + A_2$) which lacks Ca^{2+} binding to sites I and II was totally unable to reactivate Ca^{2+}-dependent force and in addition could not bind to TnC depleted fibres. To study the role of the Ca^{2+}-Mg^{2+} sites (III and IV) a variety of mutants have been prepared which lack Ca^{2+} binding to one or more of these sites. Two of these mutant TnCs that lacked Ca^{2+} binding to either site III (A_3) or IV (A_4) could fully reactivate force, but A_4 had a lower affinity for its binding site on the fibre and dissociated in relaxing solution.

Ca^{2+}-induced Ca^{2+} release from the SR of barnacle myofibrillar bundles, triggered by the photorelease of calcium ions from caged calcium

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We have triggered Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) of isolated myofibrillar bundles of barnacle striated muscle fibres using the rapid photorelease of Ca^{2+} from the

caged calcium molecule, nitr-5 (Adams *et al.* (1988) *J. Chem. Soc.* **110**, 3212–20), thus overcoming diffusional delays normally inherent in such experiments.

Myofibrillar bundles of about 100 μm diameter were mounted for isometric tension recording and were first equilibrated in a conventional 'skinned' muscle solution containing 0.1mM nitr-5 as the only Ca^{2+} buffer, at an initial pCa of 6.8–6.6. A pulse of UV light of 25 ns duration from a frequency-doubled ruby laser was used to photolyse the nitr-5 in the bundle, thus releasing free Ca^{2+} within 1 ms. This resulted in a phasic contraction which in some experiments reached the maximum Ca^{2+}-activated tension amplitude (P_0). The mean rise time to half amplitude of the responses was 2.3 s at 12°C ($n = 12$).

The main source of activating Ca^{2+} in these responses was attributed to CICR from the SR since pretreatment with ryanodine (0.1mM) or Triton X-100 (1%) abolished a large part of the response. The small tonic response remaining was attributed to direct activation of the myofibrils by the Ca^{2+}, which was released photolytically from the nitr-5. By reference to a pCa-tension curve, obtained with EGTA-based activating solutions, this residual tension could be used to estimate the post-photolysis pCa in these experiments, i.e. the trigger pCa for the CICR. This varied from pCa 6.7 to 6.0 over the range of laser pulse energies used; the CICR response increased in amplitude with increasing photorelease of Ca^{2+}.

The CICR responses, obtained using the photorelease of Ca^{2+} as a trigger, were significantly slower than the fastest tetani which have been recorded from electrically stimulated fibres of the barnacle muscle; these had a mean half rise time of 177 ms at 12°C (Griffiths *et al.* (1990) *Pflügers Arch.* **415**, 554–65). The isolated myofibrillar bundle could be made to contract at rates comparable to this, when the photorelease of Ca^{2+} was made sufficiently large so as to fully activate the myofibrils directly, using 2.0 mM nitr-5 at an initial pCa 6.5. These results indicate that either (1) the physiological release of calcium ions from the SR is by some mechanism other than CICR in this crustacean muscle, or (2) if CICR is involved then its efficiency is impaired under the conditions used with the isolated myofibrillar bundles.

Ordered phosphorylation of a duplicated minimal recognition motif, RRRSS, present in cardiac troponin I by cAMP-dependent protein kinase

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Multiple phosphorylation of a protein can be carried out principally by more than one protein kinase; alternatively, all sites are phosphorylated by only one protein kinase. Cardiac troponin I contains two adjacent serine in sequence to three arginine residues thus building up a minimally duplicated recognition motif for the cAMP-dependent protein kinase. Indeed, in the test tube cAMP- and cGMP-dependent protein kinase as well as protein kinase C are capable of phosphorylating both these serine residues in troponin I yielding the bisphosphorylated product (Swiderek *et al.* (1990) *Eur. J. Biochem.* **190**, 575–82). However, it is unknown how monophosphorylated species are generated which have been shown to be present in troponin I isolated from intact heart.

To study the phosphorylation pattern of this domain we synthesized the corresponding peptide: PVRRRSSANY. The catalytic subunit of cAMP-dependent protein kinase phosphorylates subunit of cAMP-dependent protein kinase phosphorylates both serine residues. Detailed kinetic analysis shows that phosphorylation occurs in an ordered manner according to the following reaction sequence:

$$\text{Peptide} \xrightarrow{k_1} \text{Peptide-P} \xrightarrow{k_2} \text{Peptide-P}_2$$

The calculated rate constants are: $k_1 = 4400\text{M}^{-1}\,\text{min}^{-1}$ and $k_2 = 400\text{M}^{-1}\,\text{min}^{-1}$. The 11-fold slower phosphorylation of the monophosphorylated to the bisphosphorylated form explains generation of the intermediate monophosphorylated state.

Effect of hypothyroidism on the expression of some developmental isoforms of troponin T in rat striated muscles

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Our recent studies using antibodies to fast troponin T have identified four developmental isoforms of this protein in developing rat skeletal muscles. Two of these called fetal, FF1 and FF2 and the other two called neonatal isoforms, NF1 and NF2 were detected during early development of all rat skeletal muscles investigated. The presence of these isoforms was first detected during the late fetal to early neonatal period and these were generally undetectable in a large majority of the muscles after 1–2 months of age. These developmental isoforms were gradually replaced by a number of adult isoforms of fast troponin T (AF1-AF5). Most adult skeletal muscles expressed different amounts of these five major isoforms. The changes in the troponin T isoforms also occur in the cardiac muscle during development. The major isoform detected in the fetal rat cardiac muscle is an embryonic isoform of cardiac troponin T. This is gradually replaced by the adult isoform during late fetal to neonatal period. The study of transitions of embryonic or fetal and neonatal to adult isoforms in both skeletal and cardiac muscles therefore can be used to compare the degree of differentiation in normal and experimental muscles to get some insight into the factors affecting the process of differentiation. The present study was undertaken to determine whether hypothyroidism affects the transitions in the development isoforms or leads to the re-expression of development isoforms in the adult striated muscles.

The induction of hypothyroidism reduced the rate of suppression of fetal and neonatal isoforms of troponin T in developing rat skeletal muscles. While the suppression of fetal and neonatal isoforms of troponin T was reduced in developing skeletal muscles, the expression of adult isoforms of fast troponin T although much retarded nevertheless proceeded during the neonatal period. The induction of hypothyroidism at the age of 3 months for a period of 2 months in contrast did not result in any changes in the isoforms of troponin T in the rat skeletal muscles studied. Hypothyroidism also did not induce any changes in the pattern of expression of adult and embryonic cardiac troponin T in either the adult or developing cardiac muscles.

Rate and mechanism of the assembly of tropomyosin with actin filaments

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The rate of assembly of tropomyosin with actin filaments was measured by stopped flow experiments. Binding of tropomyosin to actin filaments was followed by synchrotron radiation X-ray solution scattering and by the change of the fluorescence intensity of a dimethylaminonaphthalene label covalently linked to tropomyosin. The experimental conditions were 2mM $MgCl_2$, 100mM KCl, pH 7.5, 25° C. At the higher concentrations used for time resolved X-ray synchrotron radiation (24 μM actin filaments, 3.4 μM tropomyosin) most of the assembly reaction has taken place during the mixing time (50–100 ms). At low concentrations of actin filaments (2.5–5 μM) and tropomyosin (<1.4 μM) the half-life time was in the range from 0.2 to 0.6 s. The results were analysed quantitatively by a model in which tropomyosin initially binds to isolated sites. Further tropomyosin molecules bind contiguously to bound tropomyosin along the actin

filaments. Good agreement between the experimental and theoretical time course of assembly was obtained by assuming a fast pre-equilibrium between free and isolatedly bound tropomyosin. The rate constant for contiguous binding was found to be in the range of 10^7 to $10^8\,M^{-1}\,s^{-1}$.

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Efficiency of the respiratory and physical work measured by 'Szeged' Kayak-simulator

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The mechanical efficiency of water sports has been investigated by our team. It was found that the speed of many kayak-canoe competitor's boat decreased during the second-half of the distance. This results from the unsatisfactory endurance. For the investigation of this problem a special ergometer, the 'SZEGED' mechanical ergometer, kayak-simulator, was developed by the authors. The subjects consisted of 22 young male elite kayak-competitors, aged 20–24 years. The ergometer simulates the effort, the pattern of movement and the energy cost of canoeing in the laboratory. Along with the biomechanical parameters (force, distance, velocity, work, power), selected physiological parameters (heart rate, blood pressure, ventilation volume, oxygen uptake, respiratory quotient) were also registered. These cardiological and respiratory parameters were measured by a Jaeger-type spiro-ergometer.

It was found that the propulsive power decreased during 8 min of exhaustive kayaking. The mechanical efficiency is:

$$\eta = \frac{P_2}{P_1}$$

where P_2 is the propulsive power and P_1 is the aerobic power. The aerobic power was calculated from the oxygen uptake. In this calculation oxygen was assumed to be equal to 5 kcal, and 1 kcal is equal to 4185 joule (Asami *et al.*). The mechanical efficiency generally decreased during work-load when the P_1 and P_2 simultaneously increased.

In the case of increasing work-load, the activity of the respiratory systems uses up the aerobic power in higher and higher proportion than the energy supply of the musculature performing external mechanical work.

Structure and analysis of human vinculin gene

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Vinculin is one of the cytoskeletal protein associated with cell-cell and cell-extracellular matrix adherens-type junctions (Geiger (1979) *Cell* **10**, 193–205; Burridge and Feramisco (1980) *Cell* **19**, 587–95). There is at least one additional isoform of vinculin, termed metavinculin, which has a higher apparent molecular weight and is expressed specifically in muscles (Glukhova *et al.* (1986) *FEBS Lett.* **207**, 139–141).

In this study we have obtained the data on the structure of human vinculin gene. We have obtained, practically the complete human vinculin gene, excluding some gaps in introns on the very 5′ end of gene and between exons 7 and 8, 10 and 11. At present we have determined sequences of nine exons from 22 and its flanking introns including branch points. The sequence of metavinculin was located in separate exon 19 and contained 204 bp. Identification of consensus sequences of donor and acceptor of human vinculin gene by comparison with the similar sites of other genomic genes showed high ratio of homology: 5′ donor sites were usually GTPuAGT (Pu = purine) and 3′ acceptor sites are Py_nNPyAG (Py = pyrimidine, N = any nucleotide) as it was identified for published genes (Andreadis *et al.* (1987) *Ann. Rev. Cell Biol.* **3**, 207–42). However, the 3′ acceptor sites for metavinculin exon was different: GACAAAG. Besides, the last exon of vinculin gene contained the part of 3′ end and untranslated tail of human vinculin cDNA. As far as it is established that vinculin is coded by a single gene (Weller *et al.* (1990) *Proc. Natl. Acad. Sci.* **87**, 5667–71) and there are two different mRNAs for vinculin and metavinculin (Ogryzko, unpublished data), consequently vinculin and its muscle-specific isoform metavinculin are generated by the alternative splicing of one gene.

Purification and physical-chemical properties of invertebrate mini-titin and its identification as twitchin

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In invertebrate skeletal muscle we found a doublet of proteins with high molecular masses (HMP I, 700 kDa, HMP II, 600 kDa; also called mini-titin). Native HMP II was purified from *Locusta migratoria* flight muscle following essentially the same protocol as used for vertebrate titin. Rotary shadowing revealed a highly asymmetrical structure of the protein: individual molecules have a diameter of 3–4 nm and an average length of 260 nm. Immunoelectron microscopy using a polyclonal antibody to HMP II located the polypeptide to the I-band and a short proportion of the A-band. Immunoblotting and immunofluorescence microscopy confirmed that HMP is present in the sarcomeric muscles of different invertebrates. HMP, purified from the nematodes *Ascaris lumbricoides* and *Caenorhabditis elegans*, also reacted with these antibodies, as well as with antibodies raised against peptides contained in the sequence predicted for twitchin, the product of the *C. elegans* unc-22 gene (Benian *et al.* (1989) *Nature* **342**, 45–50).

HMP-specific antibodies strain in immunofluorescence microscopy both the body muscles and the pharynx of wild type *C. elegans*, while in the twitchin mutant E66 only the pharynx is stained.

We conclude that the invertebrate muscle HMPs, which we have described as 'mini-titins', are twitchin.

Localization of cytoskeletal proteins in insect flight muscle

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Insect flight muscle has structural proteins in addition to the contractile and regulatory proteins. Proteins of 35 kDa (zeelin-1) and 23 kDa (zeelin-2) are found in preparations of isolated Z-discs. The zeelins are very hydrophobic proteins which are insoluble in aqueous solutions (Sainsbury & Bullard (1980) *Biochem. J.* **191**, 333–9). Zeelins were isolated from Z-disc preparations by ion exchange chromatography. Two-dimensional electrophoresis showed that zeelin-2 has three isoforms with different isoelectric points.

Monoclonal antibodies were raised to pure zeelins (in collaboration with Dr G. Butcher, AFRC, Babraham, UK) and the proteins were localized in the sarcomere by immunoelectron microscopy on thin sections of frozen fibres and fibres freeze-substituted and embedded in Lowicyrl. Sections were immunolabelled using Protein A gold. Antibodies to zeelin-2 were of two types with different labelling patterns. One type of antibody labelled the Z-disc predominantly, the other labelled the Z-disc and a limited region in each half of the A-band. The labelling pattern in oblique thin sections suggested that zeelin-2 is between thick and thin filaments in the A-band. Antibodies

to zeelin-1 also gave two labelling patterns: one type labelled Z-disc and A-band and the other the A-band only. The epitopes on zeelins in different parts of the sarcomere may be in different orientations or masked by other proteins. All monoclonal antibodies to zeelin-2 reacted with all three isoforms of the protein on two-dimensional blots, therefore the two types of immunolabelling were not from different locations of isoforms.

A cDNA library was constructed from *Lethocerus* flight muscle. The library was cloned in the lambda gt11 vector, expressed in *E. coli* and screened with zeelin antibodies. Positive clones were obtained for zeelin-1 and the partial amino acid sequence deduced corresponds to a 10 kDa peptide which has weak homology with the variable immunoglobulin domain and with vertebrate myosin LC-1 and *Drosophila* myosin LC-2.

These results show that zeelins are in the A-band as well as in the Z-disc. They probably stabilize the lattice of thick and thin filaments in the flight muscle, although it is not known if the proteins are in the form of filaments *in vivo* or bound to thick and thin filaments.

Time resolved spectroscopy study of tryptophan residues in filamentous actin structures

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Most globular proteins possess the tryptophan residue. To demonstrate that domain movement in proteins may be monitored by quantification of time-resolved tryptophan fluorescence emission, a Lifetime Resolved Fluorescence Anisotropy (LRFA) decay study was performed on the glycolytic enzyme hexokinase. When substrate or substrate inihibitor is bound to hexokinase, the resulting conformational change gives rise to domain movement within the enzyme. Small angle X-ray scattering (SAXS) was employed to monitor the corresponding changes in the radius of gyration for the hexokinase glucosamine/OTG complexes.

The role of inorganic phosphate (Pi) and the chloride anion (Cl^-) on the dynamic properties of the actin molecule in the thin filament of striated muscle was investigated *in vitro* using tryptophan LRFA. This preliminary study: (1) confirms the presence of anisotropy rotation relaxation times (ϕ) for actin filaments ($\phi > 8$ ns) that are greater than tryptophan residue decay times in solution (0.04 ns $> \phi >$ 2 ns), and (2) indicates a loss of actin domain mobility, within the myosin binding domain of the actin subunit of filamentous actin structures, on binding inorganic phosphate (Pi).

This study was supported by a project grant (Wellcome Trust) and the provision of an SRS beam-time grant (SERC). The advice and assistance of Drs M. Anson, R. H. Gigg (NIMR, Mill Hill) and D. McKillop (Biochemistry, Bristol University) in the preparation of S1-A1, the synthesis of OTG and purification of the tropomyosin-troponin complex is gratefully acknowledged.

Calcium transients and the effect of a photolytically released calcium chelator during electrically-induced contractions in rabbit rectococcygeus muscle fibres

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The cellular events regulating actin and myosin interaction during contractions of smooth muscle are not fully understood. We have used a highly ordered smooth muscle, the rabbit rectococcygeus, to study the relation between variations in intracellular Ca^{2+} (measured with Fura-2) and active force at 22 °C. In the presence of extracellular Ca^{2+} (2.5mM) the rectococcygeus is relaxed. Electrical stimulation gives reproducible contractions with durations of about 20 s. Fura-2 was loaded into the preparations using the ester form (Fura-2/AM). Using a microscope based recording system and a rotating excitation filter wheel, a time-resolution of 10 ms was achieved. After electrical activation an increase in [Ca^{2+}] preceded the increase in force. Ca^{2+} decayed before the peak of force. The fluorescence properties of Fura-2 were determined using time-resolved spectroscopy using synchrotron light at the MAX-laboratory, Lund, Sweden. The fluorescence decay of free Fura-2 was best described by two exponential decays (time constants 0.4 and 1.4 ns) at pCa 9. At high Ca^{2+}, fluorescence decay became slower. Time-resolved anisotropy of free Fura was characteristic of free rotational diffusion (correlation time 0.3 ns). Motion of Fura-2 in solution could be markedly inhibited by high concentrations of creatine kinase. Time-resolved anisotropy measurements of rectococcygeus preparations loaded with Fura-2 showed that the probe in these muscle fibres was highly mobile. The Ca^{2+}-dependence of contraction and relaxation was further studied using a photolabile calcium chelator Diazo-2 (Adams *et al.* (1989) *J. Am. Chem. Soc.* **111**, 7957) which could be loaded into the muscle cells in an esterified form in a similar manner as Fura-2. Photolysis of Diazo-2 leads to an increase in its Ca^{2+}-affinity and a fall in free Ca^{2+}. When the muscle was illuminated with a uv-light flash to photolyze Diazo-2 during the rising phase of contraction (i.e. at or before the peak of intracellular Ca^{2+}) the rate of contraction was reduced, suggesting a tight coupling between intracellular Ca^{2+} and crossbridge interaction possibly through the degree of Ca^{2+}-dependent light chain phosphorylation. In contrast, photolysis of the Ca^{2+}-chelator during the relaxing phase did not influence the rate or extent of force-decay, which suggests that relaxation from a phasic contraction in this smooth muscle is not determined by the rate of Ca^{2+}-removal, but instead is limited by other deactivation processes such as dephosphorylation or detachment of tension-bearing cross-bridges.

Measurement of ATP turnover by single, immobilized myosin filaments

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Recent *in vitro* motility assays have detected the interaction between single myosin heads with an actin filament during repetitive action of the crossbridge cycle (Uyeda *et al.* (1991) *Nature* **352**, 307–11). At present, quantitative analysis of such experiments to determine the crossbridge step-size relies on steady-state ATPase measurements which assume that the myosin population is homogeneous. In order to determine the ATPase properties of immobilized myosin molecules more directly, we have initiated a study using an ATP analogue which can be detected by fluorescence microscopy. Transient kinetic analysis, in solution, has shown that methylanthraniloyl ATP (mant-ATP) is a good analogue of ATP with rabbit skeletal myosin (Woodward *et al.* (1991) *Biochemistry* **30**, 422–30) and with scallop striated adductor myosin. Mant-ATP fluorescence ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 440$ nm) can be detected by video-enhanced microscopy, although the signal-to-noise ratio is relatively poor because of the unfavourable transmission properties of the epifluorescence filters and objective lens at these wavelengths. This analogue supports movement of rhodamine-phalloidin-labelled actin on rabbit skeletal HMM in an *in vitro* motility assay, although the velocity of sliding is about half that observed with ATP. When rabbit skeletal synthetic myosin filaments are adhered to the coverslip, the bound nucleotide fluorescence during mant-ATP turnover can be detected above the background because of the increased quantum yield and local high concentration of myosin heads. Addition of excess ATP to the immobilized filaments causes a loss of fluorescence with a half-time of several seconds. Thus, this assay provides a direct measure of the mant-ATP turnover rate by individual

myosin filaments. Advances in technology may allow a corresponding measurement to be made in the presence of actin. We have also explored the properties of clam (*Mercenaria*) thick filaments from the striated and catch muscles and have shown that mant-ATP supports movement of actin filaments with a bimodal velocity distribution (Sellers & Kachar (1990) *Science* **249**, 406–8). These filaments are easy to observe by light microscopy because of their size, but as yet we have not found conditions where the mant-ATP fluorescence is significantly enhanced. If this is achieved, this methodology should allow the heterogeneity problem of molluscan preparations (Jackson & Bagshaw (1988) *Biochem. J.* **251**, 527–40) to be investigated at the level of individual filaments.

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Phosphorylation of sheep aorta caldesmon

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Caldesmon was phosphorylated by using an endogenous kinase. The crude kinase fraction was obtained from the ATP-soluble extract of sheep aorta, after thin filaments were pelleted of high speed centrifugation. The phosphorylation was carried out in the presence of 1mM EGTA and reached 1 mol phosphate per mol caldesmon. We were also able to phosphorylate caldesmon of both natural and synthetic thin filaments. Phosphorylation does not seem to effect actin binding or ATPase inhibition. However, phosphorylation seems to decrease the affinity of caldesmon to both phosphorylated and unphosphorylated myosin. Native thin filaments bind smooth muscle HMM with high affinity in the presence of ATP. The maximum binding depends on the Ca^{2+} concentration: it is the higher in the presence of 0.1mM Ca^{2+} than with 1mM EGTA, while the binding affinity seems to be very similar. If the thin filaments are phosphorylated, the tight binding becomes independent from the Ca^{2+} concentration, reaching only the lower level of the maximum binding.

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