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Calcium sensitivity modulation of myofilaments in cardiac and smooth muscle

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INVITED LECTURES

Changes in Ca^{2+} sensitivity in skeletal muscle during fatigue; consequences for relaxation

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Ca^{2+} sensitivity can be measured in intact fibres of mouse skeletal muscle which have been injected with indo-1 to measure intracellular calcium ($[Ca^{2+}]_i$). A series of tetani with different stimulus frequencies is elicited and this allows measurement of the $[Ca^{2+}]_i$ at various force levels. Using this method the $[Ca^{2+}]_i$ which give 50% maximum force (Ca_{50}) is 386 ± 46 nM under control conditions. During fatigue caused by short tetani continued until force has fallen to about 50%, Ca_{50} rises by 77 ± 19 nM. A likely cause of this desensitization is the rise in [Pi] which occurs in fatigued muscle.

An important feature of fatigued muscle is the slowing of relaxation which occurs. $[Ca^{2+}]_i$ falls more slowly in fatigued muscle and this would be expected to slow relaxation. However the reduced Ca^{2+} sensitivity will tend to oppose this effect because the force will be lower for any given $[Ca^{2+}]_i$. To quantify this effect we have plotted the Ca^{2+}-derived force in which the $[Ca^{2+}]_i$ throughout relaxation is converted to force using the appropriate force-$[Ca^{2+}]_i$ relationship. This analysis shows that the Ca^{2+}-derived force falls at about the same rate in control and fatigued muscle. Thus the reduced $[Ca^{2+}]_i$ sensitivity offsets the slower fall of $[Ca^{2+}]_i$ and the slower relaxation seems to be due to changes in cross-bridge kinetics.

Cross-bridge kinetics and structure in smooth muscle fibres

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Structure and mechanics of the contractile system in smooth muscle were investigated using chemically skinned preparations. In the relaxed state with ATP, X-ray diffraction revealed reflections from organized actin and myosin. In the absence of ATP, changes in actin reflections consistent with rigor cross-bridge binding were observed (Arner *et al.*, 1990, In *Basic and Applied Research Prospects for the 90's* (edited by CARRARO) pp. 699–704 Padova: Unipress). When ATP was released from caged-ATP in rigor muscles a rapid relaxation of rigor force was observed. The rate of this relaxation was increased in the presence of phosphate (Pi). (Arner *et al.*, 1987, *J. Muscle Res. Cell Motil.* **8**, 377–85). When ATP was released in activated (thiophosphorylated) muscle fibres, the presence of Pi increased the rate of force development but decreased the resulting isometric force. (Österman & Arner, 1995, *J. Physiol.*, in press). These data suggest co-operative attachment of cross-bridges after detachment from rigor. Also, Pi-release is associated with force generation in smooth muscle fibres. The maximal shortening velocity (V_{max}) was dependent on the level of myosin light chain phosphorylation. At maximal activation V_{max} was not influenced by Pi whereas in submaximally activated fibres V_{max} was increased in the presence of Pi (Österman & Arner, 1995, *J. Physiol.*, in press). These data suggest that cross-bridge interaction during isotonic shortening is regulated by phosphorylation and that Pi-sensitive cross-bridge states constitute an internal load reducing V_{max} at low levels of activation.

Activation and relaxation mechanisms in muscle

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The effect of Ca^{2+} on the time course of force generation in skeletal skinned muscle fibres and cardiac trabeculae has been investigated using laser flash photolysis of the caged-calcium. Gradations in the rate and extent of contraction could be achieved by changing the energy of the laser pulse, which varied the amount of caged Ca^{2+} photolysed and hence the amount of calcium released. The half-time for force development at 12° C was noticeably calcium-sensitive when small amounts of calcium were released (low energy pulses) but did not change appreciably for calcium releases which produced a final tension of more than 50% of the maximal tension at pCa 4.5, although this value was increased in trabeculae by the Ca^{2+} sensitiser, EMD 57033. Our experimental results show that force develops relatively rapidly at intermediate Ca^{2+} which produce only partial activation (i.e. 50% P_{max} or greater). The kinetics of calcium exchange with the regulatory sites may be much more rapid than cross-bridge cycling, so that if calcium binding to a particular functional unit induces cross-bridge attachment and force production, the force producing state may be maintained long after calcium has dissociated from that particular functional unit. The relaxation of skinned muscle fibres has also been successfully studied following the rapid uptake of Ca^{2+} by a photolabile chelator Diazo-2, a photolabile derivative of BAPTA, which is rapidly (>200 s^{-1}) converted from a chelator of low Ca^{2+} affinity (K_d 0.073 μM). This procedure has enabled us to examine the effects of the intracellular metabolites, ADP, P_i and H^+ upon the rate of relaxation. The rate of relaxation was not affected in skeletal or cardiac preparations by EMD 57033, but was slowed

by ADP and H^+. The support of the MRC and Dr Inge Lues, Merck is acknowledged.

Evaluation of thiadiazinone Ca^{2+}-sensitizers: from subcellular preparations to conscious animals

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In 1981, Herzig, Feile, and Rüegg introduced the novel therapeutic concept of augmenting myocardial contractility by increasing the Ca^{2+}-responsiveness of the contractile elements. Since then, many compounds have been described as being active at the myofibrillar level. However, these compounds are far from ideal as prototypes for evaluating the new concept *in vivo*: first, they have very low potencies at the myofibrillar level; and, more importantly, almost all of them have another inotropic mode of action, namely inhibition of cyclic nucleotide phosphodiesterase III.

The new thiadiazinone compounds EMD 57033 and EMD 58328 seem to be more suitable. These compounds potentially increase the Ca^{2+}-responsiveness of myocardial contractile proteins in both subcellular and multicellular systems, increase contractile force in skinned ventricular fibres in low micromolar concentrations, and increase contraction in intact myocytes or multicellular preparations without augmenting the Ca^{2+}-transient. Results from functional and binding studies indicate that the compounds act downstream from the Ca^{2+}-regulatory system at the level of the cross-bridges. Due to both their high potencies for Ca^{2+}-sensitization and their favourable ratio of Ca^{2+}-sensitization to PDE inhibition, these compounds were highly suitable as tools for the evaluation of the novel concept *in vivo*.

In anaesthetized rats, EMD 57033 produced a pronounced increase in left ventricular dP/dt_{max}, without affecting heart rate (HR) or mean arterial pressure (MAP). The hemodynamic profile of EMD 58328 was investigated in conscious dogs and compared with that of an equieffective dose (50–60% increase in $LVdP/dt_{max}$) of the pure PDE III inhibitor EMD 57439. The profiles of the two compounds were strikingly different. Characteristic for EMD 58382 was an increase in stroke volume and cardiac output, while TPR and MAP remained unchanged and HR was slightly decreased. In contrast, the PDE III inhibitor decreased TPR and increased HR as is typical for a cAMP-mediated effect. As a result, the double product increased after PDE-inhibition, but was hardly changed by EMD 58328 indicating an improved economy of myocardial performance by the Ca^{2+}-sensitizer. Moreover, LVEDP and the rate constant of isovolumetric relaxation (τ) was not affected by EMD 58328, while LVEDP and τ decreased after PDE inhibition. The latter results clearly indicate that Ca^{2+}-sensitization did not impair diastolic function in healthy animals.

Thus, the profile of the thiadiazinones can be taken as proof of the novel concept to strengthen myocardial contractility and increase its economy by enhancing the Ca^{2+}-responsiveness of the myofilaments.

Regulation at the cross-bridge level. Recruitment vs rate modulation

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Based on the rate constant of force redevelopment after a period of unloaded shortening, k_{redev}, measured at different activation levels, we previously proposed that movement of tropomyosin upon binding of calcium to TnC regulates muscle contraction not through control of the number of cycling cross-bridges ('recruitment', Podolsky & Teicholz, *J. Physiol.*, 1970) but rather through speeding up of the transition from weakly bound cross-bridge states to the strongly bound force-generating states ('rate modulation', Brenner, *PNAS*, 1988). The use of k_{redev} as a probe for cross-bridge cycling kinetics at partial activation was questioned because of possible changes in thin filament activation during high-speed shortening when most cross-bridges are weakly bound to actin. We now exchanged IANBD-labelled TnI into skinned fibres and monitored IANBD-TnI fluorescence as a probe for the activation level of the thin filament. At both high and low activation levels no change in fluorescence is seen during unloaded shortening and force redevelopment. At intermediate activation levels we find a small change (<16% of the maximum change observed upon full activation) indicating slightly smaller activation during high-speed shortening. Apparently contribution of force-generating cross-bridges to activation of thin filaments in muscle is small and only little correction is required for k_{redev} at intermediate activation levels. Such correction, however, does not alter our previous proposal about regulation of muscle contraction through 'rate modulation' and implications for modulation of force-pCa relation.

Inositol trisphosphate induced Ca^{2+} release from A7r5 smooth muscle cells

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Inositol 1,4,5-trisphosphate ($InsP_3$) releases Ca^{2+} from intracellular stores by binding to the $InsP_3$ receptor ($InsP_3R$) and on prolonged exposure to $InsP_3$ a fast and slow release component can be distinguished. Low [$InsP_3$], even when added for a long time period, is unable to release the same amount of Ca^{2+} as a short-lasting stimulation with a higher [$InsP_3$]. The slow phase is very pronounced when Ca^{2+} release occurs in the presence of functional Ca^{2+} pumps and could therfore be much more important in intact cells than hitherto appreciated. The slowing down of the release has been explained by three phenomena: (1) control of Ca^{2+} release by luminal Ca^{2+}, (2) heterogeneity of the $InsP_3Rs$, and (3) intrinsic inactivation of the release.

The $InsP_3R$ is controlled by luminal Ca^{2+} because stores with a high Ca^{2+} content bound [^{3}H]$InsP_3$ and released Ca^{2+} with higher affinity than less filled stores. Effects of luminal Ca^{2+} were still present for $InsP_3$ challenges lasting 20 min, indicating that also the extent and not only the rate of release are controlled from within the store. We recently identified a Ca^{2+}-binding site in the luminal loop of $InsP_3R$-I after expressing this part of the $InsP_3R$ as a fusion protein in bacteria. Steady-state Ca^{2+} release controlled by luminal Ca^{2+} significantly contributes to 'quantal' release: as Ca^{2+} is released, the decrease in luminal [Ca^{2+}] is unable to keep the channel open.

$InsP_3R$ heterogeneity can also explain 'quantal' Ca^{2+} release. There is an $InsP_3R$-I, -II, -III and -IV. We recently described a type V. We used RT-PCR to determine the different mRNAs coding for these various isoforms in a variety of rat, mouse and human cell types. Seventy-three per cent of the messengers in A7r5 cells corresponded to type I, 26% to type III and less than 1% to the other isoforms. These findings were confirmed at the protein level with isoform-specific antibodies, indicating that there is $InsP_3R$ heterogeneity in A7r5 cells. [^{3}H]$InsP_3$-binding experiments of $InsP_3R$ isoforms in identical conditions revealed that their affinity for $InsP_3$ differed by a factor of 35. $InsP_3R$ heterogeneity may therefore cause 'quantal' Ca^{2+} release.

Evidence for intrinsic inactivation in A7r5 cells was obtained but was of minor importance.

Cytosolic Ca^{2+} (<1 μM) potentiates $InsP_3$-mediated Ca^{2+} release. This potentiation of Ca^{2+} release by cytosolic Ca^{2+} in A7r5 cells is not an artifact caused by the use of Ca^{2+} chelators.

An evaluation of the inhibition of actomyosin ATPase activity by caldesmon

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The mechanism by which caldesmon inhibits the actin-activated ATPase activity of myosin in the presence of tropomyosin is controversial. We recently observed that: (1) competition of binding between S1-AMP-PNP and caldesmon to actin-tropomyosin occurred under conditions where each should bind with high affinity to actin; (2) caldesmon inhibited the binding of the S1-ATP like state, ρPDM-S1, to actin-tropomyosin even when ρPDM-S1 was in excess over actin; (3) the inhibition of ATPase activity increased to a greater extent than did the binding of ρPDM-S1 suggesting that an inactive S1-actin-caldesmon or actin-caldesmon-S1 complex can exist; (4) the change in fluorescence when caldesmon-NBD binds to actin-tropomyosin is reversed by the addition of S1; (5) the change in fluorescence when fluorescein-S1 binds to actin is reversed by the addition of caldesmon; (6) the rate of S1 binding to actin decreased with increasing caldesmon concentrations. These results confirm that S1 and caldesmon greatly weaken the interaction of each other with actin-tropomyosin. Caldesmon-tropomyosin and troponin-tropomyosin have different effects on the actin-myosin interaction. The mechanism of caldesmon function in the cell is largely uncertain because of the possible involvement of other actin binding proteins.

Functional properties of calponin

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Calponin is a protein associated with thin filaments constituting both cytoskeleton and contractile apparatus of smooth muscle cells. *In vitro* it inhibits actomyosin ATPase activity and this effect can be released either by specific Ca^{2+}-receptor protein or by phosphorylation. These findings imply a role of calponin in actin-linked regulation of smooth muscle contraction. With regard to the question of the basis for this regulation we have studied the calponin-actin interaction using low- and high-speed sedimentation, electron-microscopy, ATPase and *in vitro* motility assays. All these techniques led us to identification of two types of calponin-actin complexes: one, isoluble, containing a lower ratio of calponin to actin (1:2), the another soluble, with a higher ratio of calponin to actin (1:1). The former complex consisted of paracrystalline bundles of actin filaments, while in the latter case separate filaments were observed. Calponin-induced formation of actin bundles was independent of the presence of tropomyosin and myosin subfragment 1, but strongly dependent on ATP and, in the presence of Ca^{2+}, on calmodulin. At a molar ratio to calponin of 3:1 calmodulin dissociated the bundles, when added at higher excess it caused complete release of calponin from the filaments. Reversible bundling of actin filaments by calponin observed here, and demonstration that it increases force production by smooth muscle myosin (Haeberle, 1994, *J. Biol. Chem.* **269**, 12424–31) may suggest its dual cytoskeleton- and contractile machinery-linked role in maintenance of force in smooth muscle.

Receptor activated increases in $[Ca^{2+}]_i$, genistein, and tyrosine phosphorylation (TP) of *ras*GAP in vascular smooth muscle cells

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Our studies are guided by a novel hypothesis suggesting that the signalling pathway between receptor activation of smooth muscle and increases in $[Ca^{2+}]_i$ may involve receptor-activated increases in protein TP. Therefore, we studied the effects of genistein, a potent tyrosine kinase inhibitor, on increases in $[Ca^{2+}]_i$, evoked by receptor activation of cultured canine femoral arterial vascular smooth muscle cells (VSMC) and two VSMC lines (A10/A7r5). Stimulation of femoral VSMC with serotonin or phenylephrine induced a biphasic Ca^{2+} response characterized by a rapid transient increase in $[Ca^{2+}]_i$, followed by a lower sustained increase in $[Ca^{2+}]_i$. Oscillations in $[Ca^{2+}]_i$ occurred in about half of the cells. Stimulation of A10 cells with endothelin of A7r5 cells with vasopressin evoked similar biphasic Ca^{2+} responses but oscillations were seldom observed. In most of the cells >70% of the Ca^{2+} transient was due to influx of extracellular Ca^{2+} whereas 20–30% was due to release of intracellular Ca^{2+}. Surprisingly, in about 30% of the A10 or A7r5 cells virtually all of the transient was due to only intracellular release, and in about 80% of the femoral cells >90% of the transient was due to only influx. Preincubation of the cells with genistein always blocked both the influx and release components of the transient evoked by each agonist. Receptor activation of femoral VSMC was associated with enhanced TP of several substrates including *ras*GAP, the GTPase activating protein for the small monomeric G-protein, *ras*. The time course for the TP of *ras*GAP was similar to the time course for the Ca^{2+} transient. Phosphorylation of *ras*GAP, like the Ca^{2+} transient, was blocked by genistein. These results suggest that receptor activation of VSMC induces TP of several substrates including *ras*GAP and that its phosphorylation may be coupled to mechanisms which regulate receptor activated increases in $[Ca^{2+}]_i$.

Functional expression of the transient receptor potential-like (trpl) protein from *Drosophila* and identification of its mammalian equivalents

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Comparison of the various membrane-spanning segments forming the ion-conducting pores in voltage-activated calcium channels with protein data bases revealed homologies to trpl from invertebrates (A. M. Phillips *et al.*, 1992, *Neuron* **8**, 631–42). This protein supposed to be involved in phototransduction has been expressed as a cation channel in sf9 insect cells (Y. Hu *et al.*, 1994, *BBRC* **201**, 1050–6). Here we report the functional expression of trpl in the human embryonic kidney (293) cell line and the identification of its equivalents in mammalian tissues. Transient expression of dipterian trpl short circuited the equilibrium potential for potassium in 293 cells and shifted the resting potential from about −35 mV to values close to 0 mV. Intracellular dialysis with calcium chelators reversed the membrane potential to resting levels within 5–10 min. The expression of trpl was confirmed by single cell PCR. Using degenerate oligonucleotide primers synthesized on the basis of the amino acid sequence of trpl, cDNAs were amplified from various mammalian tissues and had 60% nucleotide (65% amino acid) sequence identity to trpl. Screening of a specifically primed cDNA library with one of the amplified cDNAs yielded several cDNA clones between 230 and 4830 bp in length that could be subdivided

at least into three groups. Obviously, trpl homologous proteins, which may represent voltage-insensitive cation channels regulated by intracellular calcium, appear to be expressed in mammalian tissues.

Phosphorylation-dephosphorylation of smooth muscle myosin

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In the cell the level of myosin phosphorylation reflects the balance of two activities, myosin light chain kinase and myosin phosphatase. Kinase activity is controlled by Ca^{2+}-calmodulin, but it is not known if phosphatase activity is regulated. Recent experiments with permeabilized smooth muscle preparations have suggested that following certain regimes the phosphatase activity is inhibited. One suggestion to account, at least partially, for the regulation of phosphatases involves the concept of target molecules. These recognize the substrate and provide a target for the catalytic subunit. Several laboratories have shown that the gizzard and bladder phosphatase involved in myosin dephosphorylation is composed of 3 subunits: 130 kDa, 38 kDa and 20 kDa. The 38 kDa component is the catalytic subunit and is the PP-1δ isoform. The holoenzyme binds to myosin and to myosin-affinity columns but the catalytic subunit does not bind. Interaction with myosin is thought to be a property of the 130 kDa subunit and thus is considered the target molecule. The cDNA for the gizzard 130 kDa has been cloned and a derived sequence obtained (Shimizu *et al.*, 1994, *J. Biol. Chem.* **269**, 30407). Two isoforms are found of 963 and 1004 residues, differing by a central inset region, 512 to 552. The N-terminal third of the molecule is composed of eight repeat sequences, similar to the cdc10/SW16 or ankyrin repeat. Using Western and Northern blots it was shown that the 130 kDa subunit is present in many tissues: all smooth muscles tested plus lung, kidney, heart, brain, spleen. It was not detected in skeletal muscle and liver. The 58 kDa component isolated previously, is the N-terminal part of the 130 kDa molecule. The function of the 20 kDa subunit is unknown. (Supported by grants from NIH to DJH, HL-23615 and HL-20984).

Pattern formation on cardiac troponin I by consecutive phosphorylation and dephosphorylation

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Two serine residues located adjacently in the heart-specific N-terminus of cardiac troponin I can be phosphorylated *in vivo*. Both residues are phosphorylated and dephosphorylated sequentially by cAMP dependent protein kinase and protein phosphatase 2A. Dependent on the concentration ratio of active protein kinase and protein phosphatase four different troposin I species can be generated: one nonphosphorylated, two monophosphorylated and one bisphosphorylated species. Rate constants for these transitions have been determined separately for the phosphorylation and the dephosphorylation reaction. Combination of these rate constants allows to calculate the concentration of each species during the time course of this reaction sequence. Thus patterns are generated which are observed only in proteins phosphorylated and dephosphorylated by a single protein kinase and phosphatase on more than one site. Such pattern formation is a new principle inherent in signal cascades.

Calcium sensitivity modulation as an inotropic concept

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Myocardial calcium sensitivity, i.e. the dependence of myofibrillar force and/or ATPase activity upon the cytosolic free calcium ion concentration, is not a constant but subject to physiological as well as pathophysiological modulation. For instance, the increase in stroke volume with increased cardiac filling on the ascending limb of the Frank-Starling-Diagram is based on a stretch induced sensitization of the myofibrillar apparatus to calcium. On the other hand, accumulation of acidic metabolites or inorganic phosphate in, e.g., the hypoxic heart, leads to a reduction of calcium sensitivity and, hence, of myocardial contractility. Likewise, phosphorylation of troponin I under beta adrenergic stimulation induces a decrease in calcium sensitivity. As, concomitantly, the cytosolic free calcium increases due to cAMP dependent phosphorylation of calcium channels and phospholamban, an overall positively inotropic (and chronotropic) effect results, calcium densensitization contributing to accelerated relaxation of the myocardium. A profile virtually identical to beta adrenergic stimulation results from other agents increasing intracellular cAMP levels, like forskolin or phosphodiesterase III (PDE III) inhibitors.

All clinically established positively inotropic agents, like, e.g., digitalis glycosides, beta adrenoceptor agonists, dobutamine, or PDE III inhibitors, exert their inotropic actions via an increase in cytoplasmic free calcium. Unwanted side effects like cardiac necrosis, tachycardia, arrhythmia and other consequences of Ca overload, though, limit their clinical use. With rare exceptions like hypocalcaemia, congestive heart failure (CHF) is not due to reduced availability of activator calcium. Therefore, an inotropic regimen not involving an increase in cytoplasmic calcium should be much safer. Such considerations have led to the concept of pharmacological modulation of calcium sensitivity. An increasing number of Ca sensitizing agents were published during the last years, virtually all of them including in their profiles an inhibition of PDE III. Only recently, with CGP 48506, has an agent become available that exerts considerable Ca sensitizing activity in the total absence of PDE III inhibition.

Common to nearly all known Ca-sensitizing agents is a deceleration of contractility paramenters, especially relaxation speed. This appears to go along with an increase in isometric holding economy, i.e., under the influence of Ca-sensitizing agents like EMD 53998 or CGP 48506, any given myocardial force is produced at a reduced consumption of ATP. Further work will be needed to determine whether this energetic advantage will outweigh the reduced relaxation rate, especially in cases of heart failure associated with diastolic dysfunction where relaxation is impaired.

cGMP increases and decreases cytosolic calcium

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NO is formed in most tissues and acts as a local hormone. In many tissues, NO activates the soluble guanylyl cyclase and increases cGMP. cGMP affects cellular functions through at least three different receptors: cAMP phosphodiesterases, cGMP gated cation channels and cGMP kinase. The activation of cGMP kinase leads to a decrease in cytosolic calcium ($[Ca]_i$) in smooth muscle and platelets. The mechanism of the $[Ca]_i$ decrease is controversial. We have stably transfected cGMP kinase Iα into CHO cells (Ruth *et al.*, 1993, *PNAS* **90**, 2623). Activation of cGMP kinase prevents thrombin, Il-1 and Il-2, but not CCK induced increases in $[Ca]_i$. $[Ca]_i$ increases by the same peptides are also blocked or unaffected by pertussis toxin pretreatment. *In vitro* experiments show that

cGMP kinase phosphorylates the α-subunit of G_i. These results suggest that cGMP kinase interferes with $[Ca]_i$ by attenuating specific hormone transduction pathways. cGMP regulates a non-specific cation channel in the rod outer segment and olfactory cells. We have cloned a new member of the cGMP regulated cation channel which is expressed in kidney heart, intestine and cones (Biel *et al.*, 1994, *PNAS* **91**, 3505). The channel is permeable to calcium and may mediate cGMP stimulated increases in $[Ca]_i$. An additional possible mechanism of cGMP mediated increase in $[Ca]_i$ is the activation of cADPR synthase and release of calcium from intracellular stores (Gallione *et al.*, 1993, *Nature* **365**, 456). These results suggest that cGMP may not only decrease but also increase $[Ca]_i$.

Probing the calcium-dependent regulation of muscle contraction with synthetic peptides and antipeptide monoclonal antibodies

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We have utilized an anti-TnI peptide (104-115) monoclonal antibody, B4 (which binds specifically to TnI) to mimic actin, including the ability to activate the S1 ATPase activity and the TnI-mediated regulation of the S1 ATPase. Interestingly, B4 binds to the native protein receptors, TnI and S1, with relative affinities of 100- and 25 000-fold higher than to the 12-residue peptide immunogen. The antibody has enabled the identification and purification of the N-terminal actin binding domain(s) on myosin and TnI. The anti-peptide antibody results emphasize the importance of structural homology rather than sequence homology for actin binding to different target proteins. Delineation of the functional domains of TnI using synthetic peptides has allowed the study of each binding site with TnC or actin independent of other sites of interaction, and has allowed the systematic combination of the TnI binding sites in order to understand cooperativity. For example, the N-terminus of TnI in conjunction with the TnI inhibitory region, TnT and TnC, potentiates the calcium-dependent acto-S1 ATPase activity. The C-terminal region of TnI (which interacts both with actin and TnC), in conjunction with the inhibitory region of TnI, affects the calcium sensitivity (pCa_{50}) of the ATPase activity. In addition, a single amino acid mutation in the TnI inhibitory region alters the pCa_{50} value both in solution ATPase assays and, when reconstituted with TnC, in the TnI-TnC extracted skinned cardiac muscle fibres. This indicates that the N-terminus and C-terminus of TnI modulate the activity of the inhibitory region to control the biological function of the thin filament.

Drug-induced modulation of cardiac economy

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Different pharmocological modes of action of positive inotropic compounds may influence excitation-contraction coupling and basal contractile mechanisms of the actomyosin system differently thereby altering myocardial energetics in different ways. We used the following methods to analyse myocardial energetics: (1) heat measurements in guinea pig papillary muscles; (2) the aequorin method for measuring calcium transients of human failing and nonfailing myocardium; (3) skinned fibre experiments to evaluate the force-pCa relations; and (4) rapid length perturbation analysis for evaluation of cross-bridge kinetics of human failing myocardium. A synopsis of the data obtained by these four different methods indicates that at least three different types of inotropic compounds exist with respect to myocardial economy: (1) compounds that increase cyclic AMP like epinine, isoprenalin (β_1-antagonist) and phosphodiesterase inhibitors have an energy wasting effect by increasing calcium turnover excessively, desensitizing contractile proteins and changing cross-bridge kinetics unfavourably. (2) The second family of inotropic compounds has only a moderate effect on calcium turnover and leaves the cross-bridge kinetics unchanged. To this group of cardiotonic compounds belong hight extracellular calcium, ouabain and BAY K 8644. UDCG-115 and EMD 53998 have complex pharmacological modes of action, but belong also to this group of compounds from an energetic point of view. (3) EMD 57033 is a pure calcium sensitizer; it does not alter the calcium transient, sensitizes the contractile proteins for calcium and prolongs the on-time of the cross-bridges. Therefore, EMD 57033 is the most economical compound. However, further studies have to deal with the possible disadvantage of this type of compounds, i.e., impaired relaxation and, thereby, unfavourable effects on the diastolic myocardial properties.

Function, structure, chemistry and genetics in muscle research

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These are four approaches to the question, how does a muscle work? It is now universally recognized that all are important, but this has not always been so: many physiologist contemporaries of mine used to dismiss anatomical information as irrelevant, and early this century the excellent knowledge of muscle structure gained by the 19th century microscopists was lost through premature concentration on chemical events. The only chemical change then known was the production of lactic acid, which was therefore assumed to be the essential event. All chemical studies were in fact out of contact with the actual process of contraction until (1) myosin was shown (in 1939) to be an ATPase, (2) the separation (in 1943) of 'myosin' into actin and what we now call myosin, and (3) sliding filaments (1953–54).

Caspar Rüegg has been exceptional among those originally trained in biochemistry in that much of his work has been on the mechanical behaviour of the muscles he has studied; he is exceptional also in the range of muscle types that he has worked on: skeletal, cardiac and smooth muscles of vertebrates, asynchronous insect muscle, and molluscan catch muscle. Both these aspects of his breadth are highly commendable.

Ca^{2+} release in heart muscle

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Potentiated guinea-pig ventricular myocytes respond to 4 or 180 ms clamp steps to 0 mV with similar full-sized Ca^{2+} transients. That the response is not graded but 'regenerative' is attributed to the positive feedback of Ca^{2+}-induced Ca^{2+} release (CICR) that amplifies the Ca^{2+} influx with high gain. In the same cell, pulses shorter than 4 ms induce graded Ca^{2+} release, gradation correlating with the waiting time to first opening of L-type Ca^{2+} channels. The result points to the importance of colocalization of channels for Ca^{2+} entry and Ca^{2+} release at the 12 nm junctional gap between sarcolemma and SR ('Ca^{2+} synapse'). During Ca^{2+} release, Ca^{2+} diffusion from the SR Ca^{2+} release channels builds up steep Ca^{2+} gradients that were measured with fluorescent indicators (fura-2 or

Ca-green 2) using high speed, 3-D digital imaging microscopy. For 15 ms of initial systole, $[Ca^{2+}]$ peaked at the t-tubule region and a fell to the centre of the sarcomere. The steepness of the Ca^{2+} gradients varied with treatments that altered Ca^{2+} release from the SR. The functional importance of a spatial separation of Ca^{2+} release, binding and Ca^{2+} reuptake is discussed.

Relaxation induced by β-adrenoceptor activations in vascular smooth muscle cells

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In vascular smooth muscle (VSM), cAMP activated the Ca^{2+}-dependent large conductance (maxi-) K^+ channel and hyperpolarized the membrane, thus inducing the relaxation through inhibition of voltage-dependent Ca^{2+} channels. However, it was observed that the relaxation of VSM, as induced via the β-adrenoceptor, was preserved following TEA-induced suppression of Ca^{2+}-dependent (maxi-) K^+ channel. Thus it was hypothesized that hyperpolarization may not be the sole mechanism responsible for induction of vascular relaxation. Activations of β-adrenoceptor was also evaluated in β-escin treated skinned and intact rat mesenteric arteries. Increased $[Ca^{2+}]_i$ as induced by noradrenaline (NAd) via the α-adrenoceptor were consistently counter reduced by β-adrenoceptor agonists, cAMP and db-cAMP. It is known that α-adrenoceptor activation is mediated via synthesis of $InsP_3$ and consequent release of Ca^{2+} from the Ca^{2+} store. Cyclic AMP did not modify the amount of $InsP_3$ synthesized nor the amount of $[Ca^{2+}]_i$ level. However, cyclopiazonic acid attenuated the inhibitory action of db-cyclic AMP on the NAd-induced increase in $[Ca^{2+}]_i$. Cyclic AMP inhibited phosphorylation of myosin light chain, MLC_{20} (suppression of Ca^{2+}-sensitization). Increase in cAMP induced via the β-adrenoceptor may inhibit the $[Ca^{2+}]_i$ through accelerated Ca^{2+} uptake by Ca^{2+}-ATPase at the SR. It is probable that cAMP induces the hyperpolarization of most but not all of VSMs, yet this agent consistently relaxed all VSMs. Thus, reduction in the $[Ca^{2+}]_i$ by acceleration of Ca^{2+} pump in the SR may play a central role in the β-adrenoceptor-induced vasodilation.

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Modulation of calcium sensitivity in intact cardiac muscle: a useful inotropic mechanism?

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After previous unsatisfactory experiences with conventional inotropic agents, especially in relation to arrhythmogenesis, many clinicians now doubt whether positive inotropic agents will be beneficial in the treatment of heart failure. However, enhancement of myofilament calcium sensitivity is attractive as an inotropic mechanism since it should avoid the arrhythmogenic problem of calcium overload and be associated with a more advantageous energetic profile. On the negative side, this mechanism might prolong the twitch and impair diastolic filling. In order to be clinically useful a calcium sensitizing inotrope should: (1) increase force in diseased heart muscle – i.e. overcome the effects of ischaemia and hypoxia; (2) not cause arrhythmias; (3) be effective in intact animals with heart failure. We have studied the prototype calcium sensitizer EMD 57033. In intact cardiac muscle it produces positive inotropy predominantly by increasing calcium sensitivity (1993, *Circ Res* **73**, 61). It is very effective at reversing the negative inotropic effect of acidosis (1993, *Clin Sci* **84**, 141), but is relatively ineffective (along with conventional inotropes) at reversing the decreased force caused by hypoxia (1994, *Cardiovasc Res* **28**, 1209). In an isolated working heart model, wall stress induced arrhythmias were increased by this compound. However, in a canine model of cardiac failure EMD 57033 produced marked short-term improvement of cardiovascular parameters without increases in arrhythmias. Overall the results are encouraging for the continued development of calcium sensitizers for clinical use.

Protein kinase C and smooth muscle contraction

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A significant fraction of the contractile response of ferret aorta cells to the α-agonist, phenylephrine, persists in the absence of extracellular calcium. This response occurs in the absence of increases in $[Ca^{2+}]_i$ myosin light chain phosphorylation and is not significantly inhibited by ML9, an inhibitor of myosin light chain kinase. Conversely, this contraction is significantly inhibited by calphostin C, staurosporine, and the pseudosubstrate inhibitory peptide of PKC. A contractile response to phorbol esters, purified, activated, ε-PKC, PKM, or phenylephrine can be demonstrated even when permeabilized cells are clamped at pCa7. Based on quantitative image analysis of kinase trafficking, it is suggested that this pathway involves PKC-dependent targeting of MAP kinase to the plasmalemma followed by lyrosine kinase-dependent targeting of MAP kinase to the cytoskeleton. At the cytoskeleton, it has been suggested that phosphorylation of caldesmon results in disinhibition and contraction; however exogenously added calponin also inhibits this contraction. Ferret portal vein cells also contract in response to phorbol esters and α-agonists, but this contraction is abolished in the absence of calcium. Measurements of $[Ca^{2+}]_i$, however, indicate that resting levels of $[Ca^{2+}]_i$ are both sufficient and necessary for the contraction as well as translocation of the α-PKC in this cell type. PKC-dependent contraction of portal vein cells appears to be associated with a redistribution of calponin amongst different actin isoforms. These results suggest that different types of smooth muscle may recruit different PKC-dependent signal transduction pathways in initiating contractile activation.

Energy cost of cross-bridge cycling and regulation in smooth muscle

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Our objective was to partition oxygen consumption between basal metabolism, activation, and cross-bridge cycling in the swine carotid media. A media ring preparation was employed that could be stretched to 1.8 L_0 (the optimal length for force production). F_0 was not impaired on return to L_0 (116 ± 9 mN mm^{-2}). Length had no effect on basal ($5.4 \pm 1.7\%$) or potassium-depolarized ($24.4 \pm 1.8\%$) myosin regulatory light chain (MRLC) phosphorylation values at $L \geq L_0$. Assuming that MRLC phosphorylation is the only mechanism regulating cross-bridge cycling, the fall in force at

$L \geq L_0$ (force = −1.36 length + 2.53; R = 0.997: as in vertebrate striated muscle) was due to reduced cross-bridge cycling. Suprabasal oxygen consumption fell linearly with force as a function of isometric length ($L_0 < L < 1.65\ L_0$). A linear extrapolation to zero force of suprabasal oxygen consumption as a function of active force yields an estimate of the ATP cost of activation (calcium metabolism and MRLC phosphorylation). While activation was a significant element in ATP consumption, it was small in absolute terms. The extraordinary economy of force maintenance ($(0.5 \pm 0.3\ mN\,mm^{-2})$/(nmol ATP per min × g tissue)) was attributable to extremely low rates of ATP consumption by cross-bridge cycling. Supported by NIH grants PO1 HL19242 (RAM), HL23240 (RJP) and an American Heart Assoc., Virginia Affiliate fellowship (CJW).

Fibroblast contractility studied with reconstituted fibres: regulation independent of myosin LC_{20} phosphorylation

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Fibroblasts grown in a collagen matrix reconstitute into a fibre-like structure in which mechanical properties, including force-velocity relations and stiffness could be directly measured. Calf serum (CS), thrombin and nocodazole all elicited reversible contractures suggesting that both myosin- and microtubule-based motile systems are likely to be involved. Cytocholasin D reduced all forces to near or below baseline, indicating that the mechanical properties were attributable to the 3T3 cells. Calf serum elicited a dose-dependent contraction with a time to peak force of 21.5 ± 1.3 min at 37° C and the largest isometric forces of 101.8 ± 15.1 μN, (~0.2–1 $mN\,mm^{-2}$). Force-velocity relations were well fit by the hyperbolic Hill equation, V_{max} was $0.037 \pm 0.008\ L_0\,s^{-1}$ and the curvature parameter, $a/F_0 = 1.09 \pm 0.11$. V_{max} is similar to those for tonic smooth muscles, but a/F_0 is higher than most muscles. Stiffness, measured by imposition of rapid (<1 ms) shortening steps, averaged $0.0181 \pm 0.0019\ L_0/F_0$, a lower value than intact smooth muscle, but comparable to that reported for isolated cells.

We tested the role of phosphorylation of the 20 kDa myosin light chain (LC_{20}-P_i) in the regulation of non-muscle contractility by comparing wild type NIH fibroblasts to fibroblasts expressing the constitutively active, catalytic domain of myosin light chain kinase (tMK), which show a 5.7-fold increase in LC_{20}-P_i relative to the wild type. Calf serum elicited a dose-dependent response in which maximum force and ED_{50} were similar in both types. LC_{20}-P_i was not increased above basal levels by CS in either preparation. Moreover, V_{max}, force-velocity relations and stiffness were also similar, despite the higher level of LC_{20}-P_i in the tMK fibroblasts. Our results indicate that (1) force generation in serum-stimulated fibroblasts, in contrast to smooth muscle, can occur without an increase in LC_{20}-P_i, and (2) high levels of LC_{20}-P_i do not alter contractile parameters. Thus phosphorylation of LC_{20} does not appear to be a major mechanism for regulating contractility in fibroblasts. Supported by NIH HL23240 (RJP) and HL35808 (PdL).

Studies on the role of cardiac TnI phosphorylation in the inotropic response of the heart to catecholamines

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The phosphorylation of cardiac muscle troponin I (CTnI) at two adjacent N-terminal serine residues by cAMP-dependent protein kinase (PKA) has been implicated in the inotropic response of the heart to β-agonists. In this study we found that phosphorylation of a cardiac skinned muscle preparation (CSM) by PKA, mainly at CTnI, results in a decrease in the Ca^{2+}-sensitivity of muscle contraction. The pCa_{50} decreased by ~0.27 ± 0.06 pCa units upon phosphorylation. To determine the role of the two PKA phosphorylation sites in mouse CTnI (serine 22 and 23), serine 22 or 23, or both were mutated to alanine. The wildtype and the mutated CTnIs were expressed in *E. coli* and purified. Using these mutants, we found that serine 23 was phosphorylated more rapidly than serine 22 and that both serines are required to be phosphorylated in order to observe the characteristic reduction in Ca^{2+}-sensitivity of force development seen in skinned cardiac muscle preparation. The results also suggest that one of the serines (23) may be constitutively phosphorylated and that serine 22 may be more functionally important. To study cardiac muscle relaxation, we have used diazo-2, a photolabile Ca^{2+} chelator with a low Ca^{2+} affinity in its intact form, that is converted to a high affinity form after photolysis. We found that the rate ($t_{0.5}$) of cardiac muscle relaxation increased from 110 ± 10 ms to 70 ± 8 ms after CTnI phosphorylation. Model calculations show that increased dissociation of Ca^{2+} from CTnC, coupled with the faster uptake of Ca^{2+} by the sarcoplasmic reticulum stimulated by PKA phosphorylation of phospholamban, can account for the faster relaxation seen in the inotropic response of the heart to catecholamines.

Small GTP-binding proteins and Ca-sensitivity of smooth muscle contraction

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Stimulation of β-escin permeabilized intestinal smooth muscle fibres from guinea-pig with the muscarinic agonist, carbachol, reversibly increases force of constant submaximal [Ca^{2+}]. The response to carbachol declines with repeated stimulations. Loading the fibres with Val14RhoA-GTP reverses this rundown: in fibres incubated with RhoA for 15 min at pCa 6.27, the response to the fifth challenge with carbachol was 104% of the initial response which compared to 38% in fibres not incubated with RhoA. Unlike Ras, RhoA had no significant effect on Ca^{2+}-activated force in the absense of the agonist. Further evidence that RhoA participates in the signalling cascade leading to an increase in Ca-sensitivity was obtained by inactivation of endogenous RhoA with botulinum C3 exoenzyme which ADP-ribosylates Rho. In the presence of C3 the response to carbachol was completely and irreversibly abolished. Ca-sensitivity in the absense of the agonist was not affected, however, there was an ~20% inhibition of maximal force. The inhibitory effect of C3 was reversed by loading the fibres with Ile41RhoA which is not a substrate for C3. In conclusion, these experiments suggest that RhoA is a necessary but not sufficient component of the signalling cascade leading to Ca-sensitization of smooth muscle filaments.

Remodelling of adult rat cardiomyocytes in long-term culture

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The effects of basic fibroblast growth factor (bFGF) and of insulin-like growth factor-I (IGF-I) on remodelling of actin cytoskeleton and myofibrillar apparatus have been followed in adult rat ventricular cardiomyocytes during the hypertrophy reaction up to 3 weeks in culture. Cells attach to the substratum, spread into polygonal shapes with pseudopodia and resume contractile function after 1 week. A well-structured actin cytoskeleton with stress fibre-like structures fills the cell bodies and the extensions. In controls and with IGF-I cells grow to the double volume while

bFGF induces a fourfold increase. The myofibrillar apparatus follows the actin stress fibre-like structures as a scaffold in growing out into the cell periphery. The foetally occurring α-smooth muscle actin (α-sm-actin) is re-expressed in stress fibre-like structures but not in myofibrils. IGF-I down-regulates α-sm-actin and promotes myofibrillar growth. bFGF in contrast, up-regulates α-sm-actin 3–8 times (immunoblotting) more than in controls. In addition, bFGF restricts myofibrillar growth with a sharp boundary in the perinuclear region. The most dense packing of α-sm-actin in the cytoskeleton is localized just beyond the myofibrillar boundary. So α-sm-actin inhibits myofibrillar assembly and growth. These cells are nevertheless beating vigorously like the controls. This represents a functional diversity between different actin isoforms in the cytoskeleton.

Myosin light chain phosphorylation and mechanical control of cross-bridge kinetics in smooth muscle

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In order to learn how myosin light chain phosphorylation and mechanical state control the kinetics of cross-bridge cycling, single turnover of ADP bound to myosin was determined in permeabilized smooth muscle. Thiophosphorylation of the light chains appears to mediate the distribution of myosin between two activated pools: phosphorylated cross-bridges (fast cycling) and co-operatively activated unphosphorylated cross-bridges (slow cycling). (Vyas *et al.*, 1992, *Am. J. Physiol.* **263**, C210–19) (Vyas *et al.*, 1994, *J. Biol. Chem.* **269**, 7316–22). In other experiments, the myosin light chain phosphatase activity was determined from the time course of exchange of phosphate in the light chain. At pCa 4.5, this rate is quite high (0.37 s^{-1}), suggesting the potential for repeated phosphorylation and dephosphorylation of myosin during a single cross-bridge cycle (Butler *et al.*, 1994, *Am. J. Physiol.* **267**, C1160–6). The effect of kinase and phosphatase activity on the relationship between turnover of myosin-bound ADP and light chain phosphorylation was determined in calcium-mediated contractions. For a given phosphorylation level, the time course of ADP exchange on myosin was very similar to that seen under conditions of thiophosphorylation when the phosphatase was inactive. The results suggest that a fast kinase and phosphatase does not cause the equilibration of the phosphorylated and unphosphorylated intermediates in the cross-bridge cycle in this preparation. We also investigated the mechanical control of cross-bridge kinetics. Compared to isometric conditions, there is about a tenfold increase in the rate constant for turnover of myosin-bound ADP when the muscle shortens at V_{max}, and an intermediate increase at an intermediate shortening velocity. In contrast, stretch has little effect. This suggests that the rate constant for ADP release from AM · ADP is very sensitive to the axial displacement of the cross-bridge in the shortening direction, but much less so in the stretching direction. (Supported by NIH Grant HL-50586).

Use of mutagenesis and transgenic animals in the study of modulation of myofilament response to calcium in heart muscle

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Regulation of the myofilament response to Ca most likely involves alterations in the reaction of troponin C (TnC) with TnI as well the reaction of TnI with actin-tropomyosin(Tm)-TnT. Our hypothesis is that special structural features of cardiac TnI are of significance in the unique modulation of Ca sensitivity of heart myofilaments by protein phosphorylation, pH and length. Our approach to this hypothesis involves deletion and site-directed mutagenesis of TnI sites for protein kinase (PK) A and PKC dependent phosphorylation. The mutant proteins are exchanged into myofibrils of fully reconstituted preparations. By use of a cardiac specific promoter, we have also generated transgenic mice which express muscle specific beta-Tm in the heart. Our results demonstrate localized regions of functionally significant sites of PKA and PKC phosphorylation, which involves both altered sensitivity to Ca and the extent to which the thin filament is turned on at saturating Ca. In the case of over expression of beta-Tm, isoform switching occurs in the myofibrils and affects the myofilament response to Ca, activation by strong cross-bridges, and the desensitizing effect of PKA-dependent phosphorylation. Our results also indicate that over expression of mutant proteins or isoforms of thin filament proteins is a useful method for exchanging myofilament proteins *in situ*.

Flash photolysis studies of the cross-bridge cycle and its modulation by MgADP

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We will summarize flash photolysis studies, with caged ATP and a caged Ca^{2+}-chelator (diazo 2), of the kinetics of the cross-bridge cycle in smooth muscle (sm.m.). Earlier studies revealed that detachment of rigor (AM) bridges by ATP, in the presence and in the absence of Ca^{2+}, is rapid and not rate limiting the cycle, and that MgADP reduces the amplitude of the rapid phase of detachment from rigor and prolongs the latter, slow phase of relaxation. The (high) affinity of rigor bridges for MgADP is highest in tonic smooth muscles. In more recent studies, we determined the effects of MgADP during relaxation, induced by photolysis of diazo 2, from isometric contraction. MgADP (<250 μM) had no effect on the phasic (rabbit bladder) sm.m. In contrast, in femoral artery, MgADP (K_i 15–25 μM vs MgATP) significantly slowed both the initial and the late phase of relaxation. The effect of MgADP on the late phase of relaxation during which MLC_{20} is already dephosphorylated suggests that: (1) the high affinity of myosin for MgADP in tonic smooth muscle prolongs the life-times of strongly bound AM.D states and so contributes to force maintenance at low levels of MLC_{20} phosphorylation and (2) the slower time course of relaxation than dephosphorylation is rate-limited by the slow off-rate of MgADP. Supported by NHLBI PO1 HL19242.

Potential messengers of calcium sensitizaton signalling to smooth muscle myosin LC_{20} phosphatase, SMPP-1M

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Agonist-receptor-G-protein activation leading to Ca sensitization in smooth muscle inhibits SMPP-1M, resulting in increased levels of phosphorylation of MLC_{20} and force. Some of the messengers (arachidonic acid (AA), diacylglycerol (DAG), protein kinase C, p21 Rho) that have been suggested to couple the membrane events to inhibition of the myosin-bound SMPP-1M will be discussed. Agonists in intact muscle and agonists plus GTP, GTPγS or PDBu in α-toxin permeabilized femoral artery or ileum significantly increased AA and DAG. Increases in AA and DAG release

preceded force development. GTPγS increased AA mass to 61–88 μM, a concentration which, if added exogenously, inhibits purified SMPP-1M activity towards myosin and sensitizes the contractile response of permeabilized fibres to calcium. These results are consistent with a role of AA and DAG as second (co-) messengers of calcium sensitization, and suggest that the increase in AA and DAG induced by PDBu may contribute to some cellular effects of phorbol esters. Characterization of purified mammalian heterotrimeric SMPP-1M on skinned fibres showed that the relaxant effect of the catalytic subunit is markedly potentiated by the addition of the 130 kDa regulatory subunit, supporting the hypothesis that myosin dephosphorylation is regulated *in vivo* by targeting subunits that specifically alter substrate specificity of the catalytic subunit of protein phosphatase 1 to myosin (Alessi *et al.*, 1992, *Eur. J. Biochem.* **270**, 1023–35; Shirazi *et al.*, 1994, *J. Biol. Chem.* **269**, 31598–606). Supported by NHLBI PO1 HL19242.

Calmodulin, phosphate and skinned smooth muscle fibres

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Skinned smooth muscle fibres have had a longstanding role in elucidating the regulatory pathways of contraction – since the introduction of glycerinated fibres (Filo *et al.*, 1965, *Science* **147**, 1581). The skinning procedure of Gordon (1978, *Proc. Natl. Acad. Sci. USA* **75**, 3527) using Triton X-100 was markedly improved by Ruegg and co-workers. Replacement of the calmodulin lost during skinning greatly increased the Ca^{2+}-sensitivity and the speed of contraction (Sparrow *et al.*, 1981, *FEBS Lett.* **125**, 141). The inclusion of inorganic phosphate greatly promoted the speed of relaxation (Schneider *et al.*, 1981, *Experientia* **31**, 980) so that now a series of reproducible contractions as well as cumulative pCa^{2+}-force curves could be obtained. The inhibition of force generation by cAMP-dependent protein kinase on myosin light chain kinase could be demonstrated as well as that of trifluoperazine acting via the calmodulin. The relaxing effect of inorganic phosphate could be described in terms of it increasing the rate of breaking of slowly cycling cross-bridges. On the other hand, vanadate, an analogue of phosphate, inhibited both actively cycling and slowly cycling cross-bridges. The relative contribution of the two heavy chain isoforms of myosin to the maximum velocity of shortening was evaluated. In the pig trachealis $V_{(unloaded\ shortening)}$ was highly correlated with the change in percentage of MHC1 but in pig duodenum circular muscle there was none.

Calcium-induced calcium desensitization in contracting smooth muscle

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Smooth muscle Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) is phosphorylated *in vitro* and *in vivo* by the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II at a regulatory site near the calmodulin-binding domain. This phosphorylation increases the Ca^{2+} concentrations required for MLCK activity in airway, vascular, and myometrial smooth muscles. However, $[Ca^{2+}]_i$ required for MLCK phosphorylation is greater than that required for myosin light chain phosphorylation. Myosin light chain kinase phosphorylation and dephosphorylation occur at rates sufficient to modulate the Ca^{2+} sensitivity of light chain phosphorylation. Increases in $[Ca^{2+}]_i$ precede light chain phosphorylation, but the rate of increase in light chain phosphorylation is significantly greater than the rate of increase in $[Ca^{2+}]_i$. The onset of MLCK phosphorylation coincides with a diminished rate of light chain phosphorylation. Thus, MLCK is sensitive to small increases in $[Ca^{2+}]_i$ during the initiation of contraction with the kinase subsequently becoming desensitized to Ca^{2+}, thereby limiting the extent of light chain phosphorylation and contraction.

Basic properties of individual cross-bridges as revealed by experiments with force-movement assay systems

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When a myosin-coated glass microneedle is made in contact with actin filament bundles (actin cables) in giant algal cells in the absence of ATP, the needle stays in position due to rigor actin myosin linkages. On iontophoretic application of ATP, the needle moves along actin cables for a distance until it stops due to reformation of rigor linkages as the applied ATP diffuses away. By applying constant amount of ATP to the needle at various initial baseline forces, the amount of work done by the ATP-induced actin-myosin sliding was found to increase with increasing baseline force from zero to a certain value. As the time course of the needle movement was almost independent of the initial baseline force, the results suggest that the increase in the apparent efficiency of actin-myosin sliding in converting chemical energy into mechanical work is primarily determined by the force on the cross-bridges rather than the velocity of actin-myosin sliding. If the amount of iontophoretically applied ATP was sufficiently reduced, the distance of the needle movement was distributed around integral multiples of 10 nm, suggesting the unitary distance of actin-myosin sliding of about 10 nm.

Enhancement and inhibition of smooth muscle contraction by okadaic acid, a protein phosphatase inhibitor

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Okadaic acid (OA) is a protein phosphatase inhibitor which acts on type 1 and type 2A protein phosphatases (PP1 and PP2A) with the dissociation constants (Kd) of 150 nM and 30 pM respectively. In skinned smooth muscles, OA (>0.5 μM) causes a marked enhancement of contraction in the presence of submaximal concentrations of calcium/calmodulin, accompanied by elevation of the phosphorylation level of 20 kDa myosin light chains (MLC). The MLC phosphatase activity in the extract of skinned fibres is dose-dependently inhibited by OA (1 mM–10 μM). Okadaic acid strikingly slows down relaxation and MLC dephosphorylation induced by calcium removal after prior activation with calcium. These observations are in agreement with the phosphorylation theory of smooth muscle activation. In smooth muscle fibres with intact plasma membrane, however, OA (1–10 μM) exhibits an irreversible inhibitory effect on the contractility. In contrast with OA, tautomycin (10 μM), another phosphatase inhibitor having a higher affinity to PP1 (Kd = 0.4 nM) than to PP2A (Kd = 30 nM), produces a marked increase of contraction in intact as well as skinned muscles. The contractile effect of tautomycin on intact muscles is slowly reversed by adding 10 μM-OA. The inhibitory effect of OA on smooth muscle contractility may be mediated by inhibition of intracellular PP2A activity which is lost by skinning.

Growth-dependent modulation of receptor-effector coupling for intracellular Ca^{2+} (Ca^{2+}_i) handling in vascular smooth muscle cells (VSMCs)

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Vascular smooth muscle cells (VSMCs) modify the phenotype expression both *in vitro* and *in vivo*, as might be important for the pathogenesis of atherosclerosis. Previously, we have reported that Ca^{2+}_i responses in VSMCs are heterogeneous in agonist stimulation (high K^+ depolarization, caffeine and angiotensin (I) and dependent on the cell growth (Shin *et al.*, 1991, *Circ. Res.* **69**, 551–6) (Masuo *et al.*, 1991, *Circ. Res.* **69**, 1327–36). As is similar to most vasoactive peptides, endothelin-1 (ET_1) employs two signal transduction systems; (I) receptor to transient Ca^{2+} release from the sarcoplasmic reticulum and (II) receptor to sustained Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the sarcolemma. Using 2-dimensional Ca^{2+}_i image analysis (Shin *et al.*, 1992, *J. Biol. Chem.* **267**, 20377–82) of cloned VSMCs (A7r5), we have identified two cell populations showing both transient and subsequent sustained phases or the sustained phase alone after sufficient dose (100 nM) of endothelin-1 (ET_1) stimulation. To elucidate how ET_1 selects two Ca^{2+} signalling pathways, we stopped or promoted the cell growth in serum-free medium or by PDGF and found that lack of the transient response is caused not by a defect in ET_1-receptors or effectors (IP_3-sensitive Ca^{2+} channels or voltage-dependent Ca^{2+} channels), but by uncoupling between the ET_1-receptor and effector during cell growth. If time is available, we will present evidence that the heterogeneity of VSMCs were histologically confirmed *in vivo*; intimal hyperplasia induced by endothelial denudation in rat carotid artery, which is an animal model of human arteriosclerosis.

Peptide mimetics as a probe of the contractile cycle

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The various sites by which actin and myosin contact each other during the contractile cycle have been identified using synthesized peptides to mimic each individual site in turn and employing the partner protein to act as the molecular template. Specific binding has been established by using NMR techniques, by showing they were competitive inhibitors of the acto-S1 Mg.ATPase activity and by direct binding studies. The structure of each peptide in solution has been investigated by 2D-NMR techniques and was found to possess the same elements of secondary structure as in the parent protein. In some cases, the structure of the peptide bound to its partner has been determined by the transferred NOESY experiment which revealed subtle changes in the peptide structure on binding, suggesting how the allosteric response between different sites might be communicated. The sites on actin that have been identified as binding to S1 are contained within the N-terminal region, residues 338–349, 77–94 and the loop around residue 40. The sites on S1 identified as containing residues binding to actin are 400–416, 528–552, and 568–579. Experiments monitoring the displacement of S1 from actin (± ATP) have allowed contacts that occur in the weakly attached acto-S1 complex to be distinguished from the strongly attached states and have suggested an ordered and sequential docking of the two proteins.

Colocalization of Ca^{2+} transporting proteins and functional Ca^{2+} microdomains in smooth muscle

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Functional intracellular Ca^{2+} microdomains were studied by X-ray microanalysis (EPMA) in isolated urinary bladder myocytes shock-frozen either before or 500 ms after SR Ca^{2+} release induced by depolarization and I_{Ca}. Ca^{2+} release is indicated by less numerous spots of high total [Ca] close to surface membrane while [Ca] spots close to the nucleus did not change significantly. It is concluded that Ca^{2+} release through ryanodine-receptors occurs predominantly at the peripheral SR close to the surface membrane. Three dimensional immuno-fluorescence with antibodies labelled by fluorescein or texas red reveal colocalization of Na^+-Ca^{2+} exchanger, L-type Ca^{2+} channels and ryanodine-sensitive Ca^{2+} release channels within the same voxel ($200 \cdot 200 \cdot 700\ nm^3$) close to the surface membrane. The above Ca^{2+} transporting proteins co-localized with caveolin but not with vinculin suggesting a close spatial relation to the caveolae. Immuno gold labelling localized the $InsP_3$-receptors mostly in the inner regions of the cell.

POSTERS

Tyrosine kinase inhibitors potently inhibits calcium-induced tension in small arteries

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It has been suggested (Di Salvo *et al.*, 1993 *Biochem. Biophys. Res. Commun.* **190**, 968) that tyrosine phosphorylation is of importance for agonist induced modulation of calcium sensitivity in smooth muscles. It has also though been demonstrated that ionomycin induces tyrosine phosphorylation in vascular smooth muscles, which is not dissimilar to that induced by an agonist (Tsuda *et al.*, 1991 *FEBS Letts* **285**, 44). We therefore tested a range of tyrosine kinase inhibitors (TKIs) for their effect on calcium and calcium + GTP + noradrenaline (NA) induced isometric tension in α-toxin skinned rat mesenteric small arteries and on the 125 mM K^+ and NA-induced tension of denervated arteries. In skinned arteries, genistein, daidzein, tyrphostin A-23 and A-47 all potently (pIC_{50} 4.74–5.23) inhibited both calcium and calcium–GTP + NA induced tension. Tyrphostin A-1 and bis-tyrphostin had no effect in concentrations up to 100 μM. Genestein was significantly more potent towards calcium + GTP + NA compared to calcium induced tension; for the other drugs no significant difference was seen. In denervated arteries, all TKIs, except bis-tyrphostin inhibited tension and there was no difference in potency towards NA and K^+ induced tension. The potent TKI mediated inhibition of tension induced by calcium is consistent with the possibility that the ionomycin induced (presumably calcium induced) tyrosine phosphorylation modulates the contractile response to calcium.

Ca^{2+}-modulate interaction phosphorylated and dephosphorylated heavy meromyosin with actin in ghost fibre

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The changes in actin structure under the influence of phosphorylated and dephosphorylated heavy meromyosin (HMM) binding was followed by the measurements of the polarized fluorescence of rhodamine-phalloidin complex attached to F-actin. The results showed that HMM caused phosphorylaton- and Ca^{2+}-dependent alteration of actin conformation in ghost fibres. Thus, phosphorylated HMM in the presence of Ca^{2+} induced the conformational changes of actin typical for 'on' state of actin monomers in thin filaments, the change specifically caused by 'strong-binding' of myosin heads. This effect was markedly inhibited in the absence of Ca^{2+} and when HMM light chain was dephosphorylated. We suggested that both phosphorylation of myosin regulatory light chains and Ca^{2+} switch myosin heads from 'weak-binding' to a 'strong-binding' conformation and the binding of myosin heads switches actin monomers from 'off' to 'on' state. Supported by ISF and RBRF and grants from USPHS.

Diadenosine pentaphosphate-induced contractions of mesenteric microarteries are inhibited by tyrosine kinase inhibitors

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Diadenosine pentaphosphate (AP5A) is released from platelets. AP5A induces phasic contractions of intact mesenteric microarteries (internal diameter 80–100 μm). The dose-response relation is biphasic, threshold concentration is 1 μM and maximal force is produced at about 10 μM. The contractions induced by 3 μM AP5A are inhibited by verapamil with an IC50 of about 3 μM. Complete inhibition is observed at 100 μM verapamil. This indicates that AP5A-induced contractions are mediated by a Ca^{2+} influx. AP5A-induced contractions are also inhibited by two structurally unrelated inhibitors of tyrosine kinase activity, tyrphostin and genistein. Both, tyrphostin and genistein inhibit concentration dependently contractions induced by 3 μM AP5A. Maximal inhibition is obtained at 20 $\mu g\,ml^{-1}$ genistein and 100 μM tyrphostin, the IC50 values are approximately 4 $\mu g\,ml^{-1}$ (genistein) and 50 μM (tyrphostin). In β-escin permeabilized mesenteric microarteries, 1 μM AP5A increases force at constant pCa of 6.17 by about 24%. These results suggest that AP5A induces contractions of mesenteric microarteries by increasing intracellular Ca^{2+} as well as by increasing Ca^{2+} sensitivity of smooth muscle myofilaments and may require phosphorylation of proteins on tyrosine residues.

Increased phosphorylation of the 20 kDa MLC in the resting airway smooth muscle at reduced concentrations of extracellular calcium

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It has been shown by Palant and colleagues (*Hypertension* 1989, **14**, 549–55) that removal of extracellular calcium will result in depolarization of the membrane potential in the resting aortic smooth muscle. This phenomenon was not linked to any significant gain in intracellular calcium, which would support the assumption that none of the pathways mediated by intracellular calcium should be magnified. To corroborate this hypothesis for the resting airway smooth muscle (rASM) we exposed rat-ASM to different lower concentrations of extracellular calcium ($[Ca^{2+}]_e$: nominally zero, 0.05, and 0.6 mM) and analysed the proportion of myosin-light-chain phosphorylation (MLCP) by protein quantification on 2D electrophoresis gels. MLCP became significantly increased under resting conditions after 1 h of incubation at reduced $[Ca^{2+}]_e$ (0.05 and 0.6 mM). Phosphorylated fractions were elevated to 53% and 42%, respectively, compared to 20% in nominally Ca^{2+}-free Tyrode solution. Increase in phosphorylation was time dependent and saturable at each concentration, but showed an initial boost of approximately 10%. While time dependent increase in MLCP could be suppressed by use of the 4-bromo derivative of Ca^{2+}-ionophore A23187 (related to ionophore concentration: 0.01–10 $\mu m\,l^{-1}$), it turned out to be impossible to impede the first raise. None of the experimental conditions resulted in development of tonic contraction, although about one third of the assays revealed a transient force at the beginning of incubation. Possibly, the membrane potential in 'rASM' is on a lower level at reduced concentrations of extracellular calcium. This may cause the phosphorylation and/or dephosphorylation mechanisms to be changed without affecting the force generating pathways.

Resistance artery growth induced by organ hypertrophy does not change the relative content or the isoforms of contractile and structural proteins

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Growth (remodelling) of resistance arteries is important in hypertension but the characteristics of microvascular growth under normotension have not yet been described. To this purpose, rat urethras were partially obstructed to induce bladder hypertrophy (from 68 mgr to 380 mgr in 10 days). This hypertrophy caused the bladder resistance arteries to grow (diameter from 150 μm to 256 μm) without altering blood pressure or heart rate (Boels *et al.*, 1994, *Pflüger's Arch* **426**, 506–15). Microvascular samples (≈0.5 mgr) were taken from control rats and rats with obstructions, subjected to one-dimensional SDS-PAGE and the ratio of actin-myosin and desmin/actin was determined. Growth did not influence these ratios. One 20 kDa light chain and two 17 kDa myosin light chains were shown with IEF followed by SDS-PAGE. The ratio between the LC17-isoforms was not influenced by growth. Thus, in this model of microvascular growth, it is hypothesized that: (1) the newly synthesized contractile proteins will not acquire altered cross-bridge kinetics (Malmqvist *et al.*, 1991, *Flüger's Arch* **418**, 523–30); (2) the mode of growth is by hyperplasia rather than by hypertrophy as no relative increases of desmin were detected.

Comparison of the effect of calponin and 38 kDa fragment of caldesmon on 'strong' binding formation in a ghost muscle fibre

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The effect of calponin and 38 kDa actin-binding fragment of caldesmon on the structural changes in actin caused by decorating thin filaments with myosin subfragment-1, modified by NEM and phosphorylated smooth muscle HMM was investigated by polarized fluorimetry. Myosin-free F-actin in a ghost fibre was labelled

with fluorescent probes, fluorescein-maleimide and TRITC-phalloidin. It was found that both of the actin-binding regulatory proteins inhibit the conformational changes in actin typical for the formation of 'strong' binding between actin and myosin head. Tropomyosin reduces the effect of calponin and increases that of 38 kDa caldesmon. These regulatory proteins inhibit actin filament sliding over immobilized surface of skeletal myosin in an 'all or non' fashion. We suggested that inhibition of the formation of 'strong' binding is very important for the mechanism of regulation of smooth muscle contraction. Supported by RBRF and grants from USPHS.

Molecular mechanisms of force generation in muscle: isometric force vs quick tension recovery

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Analysing quick tension recovery in response to rapid stepwise length releases, Huxley and Simmons (*Nature*, 1971) proposed that quick tension recovery results from rapid re-equilibration of cross-bridges between several stable configurations in which cross-bridges generate increasing amount of tension. Isometric force was proposed to arise from cross-bridges occupying the second (or a subsequent) of the postulated force generating states. Thus, in the concept of Huxley and Simmons, isometric force is assumed to originate from the structural changes associated with transitions among the various stable positions; i.e. isometric force and quick tension recovery result from the same reaction steps and the same structural changes within the cross-bridge cycle. In disagreement with the concept of Huxley and Simmons, we find that during isometric steady state contraction only the first of the series of stable force generating states is significantly occupied, and that cross-bridges in this first state are responsible for generation of isometric force. Thus, isometric force appears to result from a structural change of the actin attached cross-bridge prior to and different from that responsible for quick tension recovery. Based on our previous work we propose that it is the structural change associated with the transition from a weakly bound to a strongly bound cross-bridge configuration that is responsible for generation of isometric force. Occupancy of force generating states subsequent to the first, apparently becomes relevant only in response to rapid releases (quick tension recovery) and during fibre shortening.

Is the calcium sensitivity of the cardiac contractile apparatus *in vivo* accurately estimated using skinned fibres?

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We estimated intracellular [Ca^{2+}] in myocytes from normal embryonic chick ventricle with fura-2. The peak [Ca^{2+}] in a twitch is about 500 nM. Twitch force of ventricular strips is about 40% of the maximal. However, Triton-skinned preparations require greater than 1 μM [Ca^{2+}] for 40% activation. The discrepancy is even greater in embryos following ablation of neural crest, which induces structural heart defects and decreased contractility. The intracellular calcium transient in experimental myocytes is markedly reduced and cannot be discriminated from background noise, which is around 100 nM. This is due to a decrease in L-type Ca^{2+} current and inhibition of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum. Nevertheless, these hearts contract. In skinned preparations, the force-pCa relation is similar in control and experimental hearts, and predict no force production at 100 nM Ca^{2+}. One possible explanation is erroneous calibration of the fura signal in the myocytes. On the other hand, the calcium sensitivity of the contractile apparatus *in vivo* may differ from that in skinned fibres due to differences between the composition of the intracellular milieu and that of the skinned fibre solutions. We find the force and [Ca^{2+}] relation in normal ryanodine-treated preparations is similar to skinned fibre data. If skinned fibre solutions mimic intracellular conditions, e.g. by decreasing [Mg^{2+}]. (Support NIH HL36059).

Activation dependence of tension redevelopment kinetics is modulated by cardiac TnC at submaximal activation of skinned rabbit psoas muscle

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Isometric force redevelopment rate (kTR) is dependent on the level of activation of individual thin filament regulatory units. We have found that this dependence is influenced by the properties of troponin C (TnC), indicating that kTR may be determined by the dynamics of protein interations within each unit (Chase *et al.*, 1994, *Biophys. J.* **67**, 1994–2001). with endogenous skeletal TnC (sTnC), kTR increased ~tenfold when force increased from <20% (pCa 6.6) to maximum (pCa 4.0). At submaximal Ca^{2+} activation, kTR was elevated ~2–3-fold after replacement of sTnC with purified rabbit cardiac TnC (cTnC). Although the entire kTR-force relationships differ, at submaximal activation (~20% force) kTR was nearly maximal when sTnC was replaced by either a modified, constitutively activating cTnC (aTnC) or a recombinant cTnC mutant (F77W) that exhibits enhanced calcium binding sensitivity (Pan *et al.*, *Biophys. J.* **66**, A308). Supported by US NIH HL52558, HL51277 and American Heart Association, WA Affiliate.

Effects of polyamines on excitation-contraction coupling in smooth muscle

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The polyamines spermidine and spermine (0.1–1 mM) decrease excitability in intestinal smooth muscle by inhibiting inflow and intracellular release of calcium, but also increase the calcium sensitivity after permeabilization (Swärd *et al.*, 1994, *AJP* **266**, C1754). The decreased excitability is caused by inhibited spike activity. Whole-cell voltage clamp revealed decreased inward current through voltage-dependent calcium channels. Steady-state activation and inactivation relationships were not shifted along the voltage axis, but their amplitudes reduced, causing reduced window current. Consistent with this, the calcium-force relationship in depolarized rat portal vein was shifted to the right by extracellular spermine, showing decreased calcium inflow, even though maximal contracture amplitude was unaffected. Using reversible permeabilization for intracellular loading, the spermine contents of portal veins were increased by 2–3-fold. Depolarized spermine-loaded veins showed increased sensitivity to calcium. The degree of sensitization was consistent with effects in permeabilized portal veins, considering that the calculated normal intracellular concentration of spermine was found to be almost 1 mM. Spontaneous activity and response to α-adrenergic stimulation were unchanged, suggesting that the inhibitory effect on calcium handling in this preparation was saturated at normal intracellular polyamine levels. The results indicate a physiological role of polyamines in determining the contractile response to excitation.

Calcium sensitization induced by receptor agonists may be superimposed on a sensitization level set by endogenous polyamines. Moreover, the role of polyamines in cellular proliferation and differentiation suggests that they may link growth and contractile properties.

TnI inhibitory peptide competes with caldesmon fragments for the binding to actin

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The C-terminal domains 3 and 4 of caldesmon are interacting with actin through several sites. While domain 3 (residues 483-579) contains a weak binding site, further binding sites with higher affinity to actin have been defined in domain 4a (residues 580-657) and domain 4b (residues 658–756) (Mornet *et al.*, 1995, *Biochem.*, in press). Both C- and N-termini of the actin molecule have been shown to be involved in the interaction with caldesmon. Two regions of the N-terminus of actin are also interacting with the inhibitory peptide of Troponin I (residues 96-115). We have assessed the affinity of several C-terminal actin binding fragments and found that the binding sites are very salt sensitive when separated in fragments, i.e. caldesmon fragments H2 (residues 626-710) H7 (residues 565-710) and 658C (residues 658-756) bind with an affinity of around 10^{-6} M at low salt (I = 5 mM). The affinity decreases however sharply to values below 10^{-5} M when the ionic strength is increased to 50–70 mM KCl. This interaction is abolished when the TnI inhibitory peptide is titrated into the complex of the caldesmon fragments with actin. Nuclear magnetic resonance (Mornet *et al.*, as above) has shown that 658C does not share the same actin site with TnI peptide, but the actin binding sites occupied by the caldesmon fragments seem to be sufficiently close to the sites occupied by the TnI peptide to result in binding interference. Further studies assessing the salt dependence of this interference and the dependence on the caldesmon fragment location are in process.

Effects of dibutyryl-cGMP on Ca^{2+}-sensitivity and unloaded shortening velocity in skinned cardiac fibres

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In order to clarify the inhibitory effects of cGMP on cardiac contractility, Ca^{2+}-sensitivity and unloaded shortening velocity (V_{max}) in response to dibutyryl-cGMP were determined in chemically skinned cardiac muscle fibres. Ca^{2+}-sensitivity was assessed by determining the $[Ca^{2+}]$ required for half maximum isometric tension (pCa_{50}) and V_{max} was measured by the slack-test method. pCa_{50} was reduced from 5.61 (5.59–5.63) under control conditions to 5.54 (5.52–5.55; means and 95% confidence limits, $n = 4$) in the presence of dibutyryl-cGMP (100 $\mu mol\,l^{-1}$). This effect could be blocked by KT 5823 (3 $\mu mol\,l^{-1}$), a specific inhibitor of the cGMP-dependent protein kinase (PKG). V_{max}, which is thought to be determined by the rate of cross-bridge detachment from actin, was increased by dibutyryl-cGMP from $0.174 \pm 0.034\ L\,s^{-1}$ to $0.215 \pm 0.027\ L\,s^{-1}$ (means ± SD, $n = 3$). It is therefore concluded that cGMP affects myocardial contractility and relaxation by reducing the response of myofilaments to Ca^{2+}. This effect is obviously related to an increase in the rate of cross-bridge release from actin possibly via a PKG-dependent phosphorylation of myofibrillar proteins.

Regulation of the rate of force development in chemically skinned cardiac muscle fibres

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Calcium regulation of cardiac contraction is mainly mediated via the troponin/tropomyosin system on the thin filaments. Binding of calcium to troponin-C is thought to cause cross-bridge interaction and force generation by relieving a steric inhibition of cross-bridge attachment or by altering kinetic steps associated with attachment and force generation. We have investigated the calcium regulation of the force generating reactions in chemically skinned porcine ventricular fibres. A low-tension rigor state was induced in the presence of the compound 2,3-butanedione monoxime (BDM) to inhibit force development. The rate of force development was determined following rapid photolytic release of ATP from caged-ATP at varied calcium concentrations. Release of ATP caused a rapid force development with an apparent rate-constant for force development of about 2.6 s^{-1} at saturating calcium concentrations (pCa 4.3). At low Ca^{2+} both force and the rate of force development were reduced. The slower rate of tension development at low Ca^{2+} is consistent with the concept that calcium regulation of cardiac contraction is partially due to a direct modulation of the rates of attachment and force generation.

EMD 53998 decreases nucleotide and phosphate association constants of cross-bridges in porcine myocardium

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Several studies have shown that positive inotropic agent EMD 53998 enhances contractility of myocardium by increasing the myofilament responsiveness to Ca^{2+} and by inhibiting the phosphodiesterase III activity. To further understand the mechanism of force enhancement, we studied the effect of EMD on elementary steps of the cross-bridge cycle in skinned myocardium activated at pCa 4.4. We studied the effect of MgATP on exponential process (C) and the effect of Pi on exponential process (B) with sinusoidal analysis in the presence of 50 μM EMD and in its absence. Our results show that the isometric tension increases by twofold with 50 μM EMD and saturates with a further increase in EMD. The rate constants and the equilibrium constants of cross-bridge detachment and attachment steps are not significantly affected by EMD, whereas the association constants of MgATP and Pi decrease 9 and 3 times, respectively. These results are consistent with tension enhancement with EMD, because the Pi release step is accelerated by EMD. We conclude that EMD increases the resistance to phosphate accumulation in myocardium.

Partial extraction of troponin C and elementary steps of the cross-bridge cycle in rabbit psoas

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Troponin C (TnC) was partially extracted from rabbit psoas muscle fibres, and elementary steps of the cross-bridge cycle were investigated by sinusoidal analysis under full Ca^{2+} activation. When ~60% of the TnC was extracted, the remaining tension was 7–14%. The TnC extraction caused a 10 × increase in the ATP-association constant (K_1), presumably because the condition of the

regulatory unit (Tn-TM-actin) modified the nucleotide binding site. With TnC extraction, the equilibrium constant of the cross-bridge detachment step (K_2) did not change much. The equilibrium constant of the force generation step (K_4) decreased sixfold. Our results of a large decrease in isometric tension (10 ×) and a small decrease in K_4/K_5 (2.6 ×) are consistent with the all-or-none hypothesis of cross-bridge activation by thin filament proteins. Our result of the decrease in K_4 is consistent with the cooperativity hypothesis of the thin filament regulatory units. The Pi-association constant (K_5) changed slightly (2 ×) with the TnC extraction. The present results demonstrate that the cross-bridge kinetics are under the influence of thin filament regulatory proteins.

Contractile properties of single fibres from human soleus muscle

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As basis for investigating the effects of mutations in the β-myosin isoform seen in familial hypertrophic cardiomyopathies and also expressed in slow skeletal muscle, we studied several mechanical parameters of chemically skinned slow twitch fibres of human soleus muscle. These studies allow to characterize both, actin binding kinetics and kinetics of active cross-bridge cycling. At 5, 10 and 20° C relaxed and active fibre stiffness, isometric force and fibre ATPase activity, the rate constants for force redevelopment, and maximum isotonic shortening velocity (V_{max}) were determined.

We found that force increases significantly with temperature and that the rate constant for force redevelopment is severalfold larger at high temperature compared to 5° C. In contrast, active fibre stiffness seems to be already rather high at low temperature (at 5° C more than 50% of its value at 20° C). Surprisingly, between 10 and 20° C, the Q_{10} for the increase in V_{max} is approximately fivefold larger than the Q_{10} of tension cost (ATPase/isometric force). This may indicate that g_{app} for strained and unstrained cross-bridges (g_1 and g_2 of A. F. Huxley, 1957) are dominated by different reaction steps.

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Effects of EMD 57033 in skinned fibres of rabbit psoas muscle at different levels of calcium-activation

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Several studies on cardiac muscle demonstrated that the calcium-sensitizer EMD 57033 (E. Merck, Darmstadt) increases the calcium sensitivity of myocardium and the maximum calcium-activated force without an equivalent increase in fibre ATPase. Previously we showed (1994, *Biophys. J.* **66**) that EMD 57033 also increases force in rabbit psoas muscle at maximum calcium activation. Apparently, only a small part of this increase is due to changes in cross-bridge cycling kinetics, i.e. a larger fraction of cycling time spent in the force generating states. Instead, increase in force seems to mainly result from a higher force contribution of the cross-bridges in each of the force generating states which is possibly due to a higher actin affinity of the force generating cross-bridges with EMD 57033.

We now extended our studies of EMD 57033-effects to partial activation levels and found that also in rabbit psoas, similar to myocardium, the force/pCa relation is shifted to lower calcium-concentrations. In principle, such leftward shift with EMD 57033 could result from (1) direct changes in cross-bridge cycling kinetics or (2) increased calcium sensitivity of troponin C or (3) larger contribution of force generating cross-bridges to thin filament activation at low calcium-concentrations because of a larger actin affinity of the force generating cross-bridges. To differentiate these possibilities, we measured force, force redevelopment and isometric fibre ATPase in parallel.

Vanadate oxidation elicits a contracture in skinned guinea-pig taenia coli independent of myosin light chain (LC_{20}) phosphorylation

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We previously showed (1993, *Biophys. J.* **64**, A257) that pre-incubation in high concentrations (mM) of vanadate (Vi) elicits a contracture in the absence of Ca^{2+} upon washout of Ca^{2+}. We further characterized this contraction by measuring LC_{20} phosphorylation, velocity of shortening, reversibility of contracture, and response of fibres to Vi after thiophosphorylation. Control forces in 6.6 μM Ca^{2+} were 0.5–2 mN for fibres with dimensions 5 mm in length, ~100 μm in width. Fibres pre-incubated in 4 mM Vi, then transferred to Ca^{2+}-free solution developed ~50% of the control force, but showed little LC_{20} phosphorylation (<9% of control). Subsequent transfer to 6.6 μM Ca^{2+} increased force, but the total never exceeded the initial control force in Ca^{2+}. Moreover, after maximal phosphorylation of LC_{20} with ATPγS, treatment with Vi did not increase force. Fibres had a control velocity of shortening (V_{max}) of 0.15 $L_0\ s^{-1}$ in Ca^{2+}. After pre-incubation in Vi, V_{max} increased to 1.85 × control with addition of 6.6 μM Ca^{2+}. After ATPγS, V_{max} was 1.6 × control, and neither Vi pre-incubation nor the addition of Ca^{2+} after Vi had any effects on V_{max}. Fibres pre-incubated in 4 mM Vi and 25 mM DTT did not produce force. Vi contracted fibres could be returned to the initial control state with sustained exposure to DTT, indicating no irreversible damage or protein extraction. After Vi pre-incubation in rigor solution, and transfer to Ca^{2+}-free solution, fibres did not contract. Thus, Vi could not affect fibres in rigor. Vi is known to be an oxidizing agent of -SH and -OH groups, and thus, could be important in understanding oxidant injury to smooth muscle. Supported by NIH HL23240, HL22619 and TG-HL07571.

Interpretation of the desensitizing effect of 2,3-butanedione monoxime on myocardium by a mathematical approach

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Low 2,3-butanedione monoxime (BDM) concentration is known to inhibit myocardial contraction at nearly unchanged intracellular Ca transients. To analyse how desensitization of the myofilaments for Ca could be involved, isometric twitches of guinea-pig papillary muscle were related to typical Ca transients measured in isolated cardiomyocytes. 2,3-butanedione monoxime at concentrations of up to 4 $mmol\,l^{-1}$ produced a clear negative inotropic effect, whereas the Ca transients were only slightly affected. Based on a reaction scheme for the cross-bridge cycle, a mathematical model using a Ca transient and typically modified mechanograms as input data has been developed. Kinetic parameters describing steps of the cross-bridge cycle were estimated by fitting the model to various time courses of force obtained at rising concentrations of BDM. 2,3-butanedion monoxime decreased the ratio of rate constants for cross-bridge attachment and detachment in a concentration dependent manner: the formation of cross-bridges was inhibited, whereas the dissociation was promoted to a less extent. Above 4 $mmol\,l^{-1}$ BDM the more marked alterations of the time course of the mechanogram indicate an additional suppressing effect on intracellular Ca supply. The results suggest how the

cellular mechanism of the BDM-induced negative inotropic effect due to desensitisation is reflected in the time course of the mechanogram.

Limits of cardiac titin extension as studied in single isolated myofibrils

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Single myofibrils were isolated from chemically skinned rabbit ventricle. We used a sensitive force transducer (resolution, ~5 nN) to measure passive force and stiffness (from the response to 500 or 1300 Hz sinusoidal oscillations) of relaxed myofibrils (T ~20° C, ionic strength 200 mM, pH 7.1) over a wide range of sarcomere lengths (SLs), from 2 to 5 μm. Stretch of cardiac myofibrils up to SLs of ~3 μm resulted in a steady increase of both tension and stiffness. With further stretch, however, the slope of the passive force-length curve was greatly reduced and the length-stiffness curve even became flat. Clearly, an elastic limit had been reached at ~3 μm SL. In addition, between 3.0 and 4.2 μm SL, the curves often showed a series of 2–3 inflections, which we assume to result from disruption of titin-thick filament anchorage and addition of previously bound A-band titin segments to the elastic I-band titin portion. Beyond ~4.2 μm, both curves became steeper again, likely due to contribution of endosarcomeric intermediate filaments to passive tension and stiffness. We conclude that (1) both myofibrillar passive force and stiffness are determined by the properties of the titin filament; (2) the segmental titin extension model of passive tension proposed for skeletal muscle (Wang *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**, 7101–5) appears to be valid for cardiac muscle as well, with an elastic limit occurring at shorter SLs than in skeletal muscle; (3) at the elastic limit, the I-band segment of cardiac titin is extended by ~7 times its slack length. This is a value higher than that suggested for skeletal titin, but it is in agreement with the maximally possible extension ratio of the elastic domains (e.g., fibronectin III domains) comprising I-band titin.

Purification and characterization of subtilisin cleaved actin void of a pentapeptide in the DNase I binding loop

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The protease subtilisin cleaves skeletal muscle G-active between Met 47 and Gly 48 generating a core fragment of 33 kDa and a small N-terminal peptide, which remains attached to the core fragment. Aminoacid sequencing and mass spectroscopy of subtilisin cleaved actin revealed two cleavage sites, one between Met 47 and Gly 48 and a second between Gln 41 and Val 42. Here we describe a procedure to purify the actin core fragment and the attached N-terminal peptide from a pentapeptide comprising aminoacid residues 42-47 under native conditions by anion exchange chromatography. After removal of the pentapeptide the induced polymerization of actin was abolished. However, the purified fragments could be polymerized by addition of salt plus myosin subfragment 1 or salt plus phalloidin as shown by cosedimentation and fluorometry using N-(1-pyrenyl)iodoacetamide labelled actin. These results confirm earlier reports proposing that the site associated with the ion induced polymerization of actin is located within its N-terminal region. Monomeric and filamentous subactin exhibited a reduced ability to inhibit deoxyribonuclease I (DNaseI) and to stimulate the myosin subfragment 1 ATPase activity, respectively. Direct binding of subactin to DNase I was verified by gelfiltration and to myosin subfragment 1 by affinity chromatography and chemical crosslinking.

A common mechanism for Ca^{2+} regulation of skeletal and smooth muscle thin filaments

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In comparative studies skeletal muscle troponin and smooth muscle caldesmon share the following functional characteristics. (1) Troponin and caldesmon are inhibitory at physiological ratios to actin (1:7 or 1:14) provided tropomyosin is also present. (2) Inhibition does not involve any changes in the affinity of M.ADP.Pi (i.e. weak binding) complexes for actin-tropomyosin. (3) Inhibition is associated with blocking the strong binding actomyosin interaction as determined by direct assay of strong complex (S-1.ADP or S-1.AMP.PNP) binding to regulated actin-tropomyosin. (4) Inhibition is reversed by NEM-S-1, an agent that acts as a strong-binding complex in the presence of ATP. (5) In the *in vitro* motility assay both caldesmon and troponin reduce the number of thin filaments that are motile whilst not affecting the velocity of motile filaments or the number of filaments attached to myosin.ADP.Pi.

We therefore believe that caldesmon and troponin regulate the thin filament by the same mechanism thus: Actin-tropomyosin exists in two states, OFF and ON, in equilibrium. In the OFF state the strand of tropomyosin is located on the actin monomer in such a way that it prevents binding of myosin to actin in the 'strong' conformation but allows binding in the 'weak' conformation. Caldesmon or troponin I bind preferentially to actin-tropomyosin when it is in the OFF state thus the actin-tropomyosin unit to which they bind is inhibited. Troponin C or calmodulin cause a conformational change which changes the preference of caldesmon or troponin I to binding to actin-tropomyosin in the ON state.

Desensitization of glycerinated rabbit psoas fibres by calgon

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Strauss and Ruegg (*FEBS Lett.* **310**, 229–34) showed that 10 mM vanadate desensitized glycerinated cardiac muscle fibres to Ca^{2+} by selectively removing troponin I and C. Since it is believed that vanadate acts in a polymeric form we investigated the properties of polymeric phosphates: ('phosphate glass' – $Na_{n+2}P_nO_{3n+1}$). Calgon is a crude phosphate glass mixture with $n = 13–18$. Treatment of glycerinated rabbit psoas muscle fibres for 1 h in rigor buffer containing 10 mg ml^{-1} calgon desensitized the fibres. On incubation in ATP/EGTA a strong contraction was obtained (>50% of pretreatment ATP/Ca contraction) and subsequent incubation in ATP/Ca buffer produced only a small additional isommetric contraction. Tension in ATP/EGTA was on average 80% of tension in ATP/Ca. Tests with a series of phosphate glasses showed that $n = 15$ (i.e. similar to calgon) was optimal. Fifteen per cent PAGE-SDS showed that the troponin I content of calgon-desensitised psoas muscle fibres was much reduced. Contractions in EGTA could be maintained for up to 3 h, but the rate of tension development was reduced. The contraction in EGTA could be reduced by about 70% in 2 h by infusing high concentration of pure troponin I.

Calgon treatment may be generally useful for removing highly basic regulatory proteins. It offers advantages over vandate since polymer length is well defined, there is no possibility of oxidation reactions and the technique is not restricted to cardiac muscle.

The effect of N- and C- terminal deletions of cardiac troponin I on the regulation of cardiac myofilaments

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Cardiac troponin I (cTnI) is a key element in the molecular switching mechanism by which thin filaments regulate the actin-myosin interaction. We have generated a deletion mutant ($cTnI_{53-211}$), using the polymerase chain reaction, in which an additional 20 AA at the N-terminus, spanning one of the TnC binding domains, are removed. Interaction of $cTnI_{53-211}$ with cTnC was studied using sepharose-TnC as an affinity ligand and a fluorescently (IAANS) labelled TnC. Unlike native cTnI, the $cTnI_{53-211}$ mutant did not bind to cTnC in the presence or absence of Ca. The ability of $cTnI_{53-211}$ to inhibit myofibrillar ATPase in a Ca-dependent manner was examined using myofibrillar preparations from which endogenous cTnI and cTnC were extracted by incubation in the presence of excess bovine cardiac troponin T. In contrast to the inhibition of ATPase activity by native cTnI, which is readily reversed by the addition of cTnC in a Ca-dependent manner, the inhibition by the mutant $cTnI_{53-211}$ was not affected by the presence of cTnC. Our results indicate that the presence of the N-terminal cTnC binding domain of cTnI is essential for the interaction of cTnI with cTnC in the presence of Ca. We have also generated two C-terminal deletion mutants which we expect to modify the inhibitory properties of cTnI, one ($cTnI_{1-199}$) in which amino acids 200-211 are removed and a second ($cTnI_{1-188}$) in which amino acids 189-211 are deleted. Supported by NIH Grant HL-49934.

Altered muscular Ca currents in the calcium channel disease hypokalaemic periodic paralysis

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Hypokalaemic periodic paralysis (HypoPP), a dominantly inherited skeletal muscle disease, is linked to the gene of the α1-subunit of the dihydropyridine receptor (DHP receptor). Point mutations replace arginines in S4 segments of either repeat II or IV. As a model system we investigated cultured myotubes derived from muscle biopsies of HypoPP patients with an R528H (IIS4) or an R1239H (IVS4) mutation. mRNA resulting from transcription of both the normal and the mutant allel could be found in myotubes at a developmental stage used for whole cell patch clamp experiments. Two Ca current components were altered in HypoPP myotubes: (1) a rapid DHP-insensitive inward current was found to be increased in its maximal average density by a factor of about 2.5; (2) the slow, DHP-sensitive L-type Ca current was decreased in density to one-third of the control value (IVS4) or its voltage at half-maximum steady state inactivation was shifted from −4.2 mV to −41.5 mV (IIS4). Since the mutations suppress the activity of the DHP receptors they may also downregulate EC coupling.

Measurement of the elastic force generated by single kinesin molecules

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We used the kinesin *in vitro* motility assay as a model system to study the molecular mechanism of chemo-mechanical transduction and directly measured the force single motor molecules can exert. To make such recordings we attached one end of a microtubule to the tip of a tiny glass fibre that served as force transducer and manipulated the glass needle such that the free end of the microtubule could interact with kinesin molecules adsorbed to the surface of a small silica bead and transmit force to the glass needle. The position of the glass needle was tracked opto-electronically with a spatial resolution of about 1 nm. The glass fibres were calibrated from the thermal motions of their tips and had stiffnesses from 0.02 to 0.63 $pN\,nm^{-1}$ and time constants between 0.33–5.7 ms.

At low kinesin density (<30 μm^{-2}) the interactions between motors and microtubules were stereotyped: the translocation speed of the attached microtubule was initially high (~0.8 $\mu m\,s^{-1}$), and decreased approximately linearly as the maximum force was reached. The repeatability, the low kinesin density on the spheres and the constant maximum forces (range 4–8 pN) suggest that the events are due to single motors. The maximum force was 5.4 ± 1.0 pN (mean ± SD, $n = 16$), independent of the stiffness of the glass fibre, the damping from the fluid, and on whether the ATP concentration was high or low. Supported by the AHA (EM), and NIH (AR40593) and Pew grants to JH.

The gens of the calcium channel β3 and γ subunits

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The β subunits of voltage activated calcium channels are encoded by four different genes (β1, β2, β3 and β4). Their expression seems to be controlled by different mechanisms. β3 is expressed in most of the tissues examined so far including smooth muscle and brain whereas the others are primarily expressed in skeletal muscle (β1), heart (β2), brain (β1 and β4) and kidney (β4). In contrast to β subunits the γ subunit of voltage activated calcium channels is encoded by a single gene which is expressed exclusively in skeletal muscle. As a step toward the elucidation of difference in the mechanism in the β and γ gene expression, the genomic organization of mouse β3 and γ subunits were investigated. Mouse genomic DNA fragments were cloned by screening a genomic library with the complete protein coding regions of β3 and γ. Two λ clones were isolated with the β specific probe and the hybridizing restriction fragments were sequenced. Comparison of the genomic clone DNA and cDNA sequences revealed that the mouse β3 gene contains at least 13 exons and 12 introns. The mouse γ gene contains four exons and has a similar genomic structure like the recently cloned human γ gene. The determination of the overall organization of the subunit genes provides important frame work and information for clarifying the differential regulatory mechanisms controlling transcription of these calcium channel subunits.

Myogenic reaction and intracellular free calcium in kidney vessels

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Variations of perfusion pressure in isolated perfused kidneys of hydronephrotic mice cause changes of the vessel diameters. In many experiments, an initial dilatation was followed by vasoconstriction. Very often a segmental constriction upon pressure stimulation was observed, indicating a weak coupling of cells under our experimental conditions. The degree of these constrictions were strongly modulated by the endothelium. In the

glomerula, pressure variations caused transient rise of $[Ca^{+2}]_i$, indicating the presence of a stretch sensitive mechanism that may use the same channels that have been described in glomerular mesangial cells.

Method: The walls of the vessels (media cells and/or endothelial cells) were loaded with the fluorescent dye fura 2. Simultaneous measurements of intracellular calcium during all experiments were performed using fast ratio imaging: a maximum of 12.5 ratios s^{-1} can be recorded from a specially designed microscope. This high-speed ratio imaging is needed for studying changes of $[Ca^{+2}]_i$ in moving tissue and dilating and/or constricting vessels. Due to a limited intensity of fluorescent light, measurements at maximal time resolution are possible at the price of higher signal noise and a limited accuracy.

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The low molecular mass GTPase rhoa may be involved in pharmacomechanical coupling of smooth muscle

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Activation of smooth muscle with an excitatory agonist increases both intracellular Ca^{2+} and Ca^{2+} sensitivity of myofilaments. Exoenzyme C3 from *C. botulinum* which ADP ribosylates RhoA thereby inactivating it inhibits the agonist-induced increase in Ca^{2+} sensitivity in β-escin permeabilized smooth muscle. RhoA may also be inactivated by toxin B from *C. difficile* (Just *et al.*, 1994, *J. Biol. Chem.* **269**, 10706) which unlike C3 permeates intact cells. Intact intestinal smooth muscle strips were incubated with 20–40 μg ml^{-1} toxin B for several hours and were then permeabilized with β-escin. Treatment with toxin B inhibited carbachol-induced contractions completely while the response to K-depolarization was barely affected. In permeabilized fibres, the carbachol-induced increase in Ca^{2+} sensitivity was completely inhibited, the response to GTPγS which also increases Ca^{2+} sensitivity was significantly reduced in the toxin treated tissues. In conclusion these experiments suggest that RhoA is involved in the agonist-induced increase in Ca^{2+} sensitivity of smooth muscle contraction.

Effects of cardiotonic thiadiazinone, EMD 57033, on cardiac troponin C, actomyosin ATPase and skinned fibres

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The effects of EMD 57033 on recombinant human cardiac troponin c (cTnC), actomyosin ATPase and skinned rabbit ventricular trabeculae were investigated. EMD 57033 ((+) enantiomer) and EMD 57439 ((−) enantiomer) caused concentration-dependent quenching of tyrosine fluorescence of cTnC at pCa 4 and had little effect on the fluorescence at pCa 9, indicating that the drugs bind cTnC in a Ca^{2+}-dependent manner. kDa for the cTnC-drug complex, estimated from Tyr fluorescence titrations, was approximately 40 μM for 57033 and 160 μM for 57439. Thus, EMD 57033 binds cTnC more strongly than 57439. Ca^{2+}-dependent binding of EMD 57033 to cTnC was also demonstrated by an equilibrium dialysis-based direct binding assay and by experiments showing that 57033 interfered with binding of hydrophobic probe bis-ANS to hcTnC. The effects of EMD 57033 on the relations between $[Ca^{2+}]$ and Tyr fluorescence of cTnC and between $[Ca^{2+}]$ and bis-ANS fluorescence in the presence of cTnC suggested strongly that the EMD 57033 binding site was induced by Ca^{2+}, but not Mg^{2+}, binding to the Ca^{2+}/Mg^{2+} sites of cTnC. Bovine cardiac myosin S-1 MgATPase was measured at low ionic strength (−15 mM) as a function of [actin]. EMD 57033 stimulated actoS-1 MgATPase while 57439 did not. V_{max} increased from 5.3 s^{-1} to 5.9 s^{-1} in the presence of 12 μM 57033. The data suggest that 57033 accelerated the rate-limiting step of the ATPase cycle. Supra-pharmacological concentrations (>10 μM) of 57033 induced isometric tension production in skinned trabeculae in a relaxing solution (pCa 9.0, 5 mM MgATP). The tension-stiffness ratio in the drug-induced contractions was similar to that measured during Ca^{2+} activation in the absence of the drug. This suggests that EMD 57033-induced tension was not caused by rigor-like cross-bridges. The effect of EMD 57033 is most likely mediated by a direct effect on cross-bridges.

Accelerated smooth muscle contraction kinetics after protein kinase C activation

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The time constant of force recovery after cessation of short (1 s) periods of force-inhibiting length vibrations (amplitude 5% of the muscle length, sinusoidal length changes of 100 Hz) was set as a parameter to describe contraction kinetics in the activated (K depolarization) isolated rat tracheal muscle. The addition of 32 μM DB-cAMP or 26.4 μM Indolactam V (=IL) decreased the force developed from 7.2 ± 0.2 mN (K depolarization) down to 5.2 ± 0.1 mN (cAMP) or to 5.1 ± 0.8 mN (IL). The time constant of postvibration force recovery, however, remained almost unchanged at 19.7 ± 0.8 s during cAMP treatment, but decreased down to 12.4 ± 0.6 s during IL treatment. As force development of the depolarized smooth muscle is strongly correlated with the sarcoplasmic calcium concentration, lower force reflects lower sarcoplasmatic calcium. The acceleration of the contraction kinetics is therefore not the result of increasing activity of the calcium-linked myosin light chain kinase, but probably the result of phosphatase inhibition controlled by the protein kinase C activity.

PKA-induced activation of K_{Ca} channels sets the level of vascular myogenic tone and mediates vasodilation

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Application of the catalytic subunit of PKA together with MgATP to the K_{Ca} channel of rat tail artery smooth muscle cells in the inside-out configuration of the patch-clamp technique produced a large increase of channel activity. Neither MgATP nor cAMP nor the catalytic subunit of PKA alone altered channel activity suggesting that the channel activation is due to phosphorylation of the K_{Ca} channel by PKA. The myogenic tone of pressurized rat tail resistance arteries was enhanced by Rp-8-CPT-cAMPS and H-89, specific inhibitors of PKA, as well as by iberiotoxin and TEA, specific blockers of K_{Ca} channels. After preincubation of these vessels with iberiotoxin the response to Rp-8-CPT-cAMPS was considerably reduced. These results, together with the patch-clamp data, suggest that the PKA-induced activation of K_{Ca} channels sets the level of the myogenic tone. The dilation of pressurized rat tail resistance arteries produced by iloprost was largely inhibited by Rp-8-CPT-cAMPS and H-89 as well as by iberiotoxin and TEA. Iberiotoxin also inhibited the dilation produced by Sp-5,6-DCl-cBIMPS, a specific activator of PKA. In cell-attached patch-clamp experiments iloprost and Sp-5,6-DCl-

cBIMPS activated K_{Ca} channels. These results, together with the inside-out patch-clamp data, suggest that the PKA-induced activation of K_{Ca} channels mediates the vasodilation to iloprost.

Endothelial modulation of cardiac myofilament response

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Recent work indicates that agents released by endocardial and vascular endothelial cells (EC), e.g. nitric oxide (acting via cGMP), endothelin, and other factors, modulate myocardial contraction. We studied the subcellular action of some of these factors. In indo-1 loaded rat cardiac myocytes, the cGMP analogue 8-br-cGMP decreased myocyte shortening, induced earlier relaxation and increased resting length without altering cytosolic Ca^{2+} transients; i.e., it reduced the myofilament response to Ca^{2+}. Similar reversible effects were observed with a stable low molecular weight factor(s) (myofilament desensitizing factor 'MDF') found in the superfusing effluent of cultured EC. 8-br-cGMP effects were mediated via cGMP-dependent protein kinase but those of MDF were not attributable to cGMP, cAMP or PKC. Endothelin increased both cytosolic Ca^{2+} and myofilament response to Ca^{2+}, thereby augmenting myocyte shortening and delaying relaxation. In a single myocyte model of brief hypoxia-reoxygenation where altered Ca^{2+}-myofilament interaction markedly delays post-hypoxic relaxation, 8-br-cGMP abolished relaxation abnormalities. Acutely hypoxic EC released another factor that profoundly but reversibly depressed myocardial contraction via direct inhibition of actomyosin ATPase. Paracrine modulation of myocardial contraction by EC factors, mediated largely via changes in myofilament properties, appears to be a potentially important regulatory pathway.

Syndecan, an anionic biopolyelectrolte, senses blood flow

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The mechanism of flow-dependent dilatation of blood vessels is so far unknown. Indications, however, are growing that a sensor macromolecule anchored in the membrane of endothelial and smooth muscle cells initiates vascular relaxation possibly via membrane hyperpolarization (G. Siegel *et al.*, 1991, *Polymer J.* **23**, 697–708). Since the Na^+ dependence of flow-induced dilatation in resistance arteries was reported, such a sensor molecule has to fulfill certain mechano-chemical and mechano-electrical requirements. It should possess viscoelastic and cation binding properties capable of undergoing conformational changes caused both mechanically and electrostatically. Moreover, the latter should be ion-specific (Na^+, Ca^{2+}). Two classes of polyanionic macromolecules, proteoglycans and acidic glycoproteins, exhibit such characteristics. These are polyanions as a result of their sulfated, carboxylated or sialic acid containing residues. The properties of the proteoglycan syndecan appropriate to its possible role as a blood flow sensor were studied using $^{23}Na^+$ nuclear magnetic resonance techniques. With an external strain, such a compound can go from a randomly coiled state to an oriented state (coil-helix conversion). The intensity of the blood flow can cause such a conformational transition of proteoheparan sulfate.

Based on its viscoelastic properties, haparan sulfate proteoglycan is proposed to be present as a random coil under 'no flow' conditions and as an unfurled filament structure with increasing flow. The conformational change produces additional anionic binding sites to which Na^+ ions of the blood are bound. Membrane hyperpolarization could be directly initiated by this Na^+ binding via a configurational transition of the protein fraction within the macromolecule or via a change in ζ-potential leading to signal transduction of the flow effect resulting in vasodilatation. Decrease in flow is followed by a structural change of the macromolecule towards coil conformation, a release of Na^+ ions and, thus, an interruption of the signal chain. Native proteoheparan sulfate, adsorbed at a hydrophobic surface, proved to be extremely Ca^{2+}-sensitive in adsorption, conformation and Na^+ binding. While Ca^{2+} ions modulate the adsorption (promotion with increasing Ca^{2+} concentrations) by changing the conformation of the sensor molecule, the adsorbed amount is determined sensitively by the Na^+ concentration. K^+ and Mg^{2+} ions showed slightly desorbing properties with increasing concentrations. Thus, it may be concluded that Na^+ ions play the role as 'first messenger' in flow-dependent vasodilatation.

Dense innervation of the developing bronchial tree in foetal and neonatal pigs revealed with SV2 synaptic vesicle protein

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The growing airways of the bronchial tree of the foetal lung display bronchial tone and a functional cholinergic innervation early in gestation (Sparrow *et al.*, 1994, *Eur. Resp. J.* **7**, 1416–24). We therefore sought to map the distribution of the nerves to the bronchial tree. The parenchyma and vasculature were removed from the entire bronchial tree of 8–12 g foetal pigs (first trimester) and from the periphery of the neonate lung (4 kg pigs). Sections of the bronchial tree were then prepared as whole mounts for antibody staining. A dense innervation was seen at both ages stained with an antibody to SV2 synaptic vesicle protein; the airway wall was simultaneously stained from smooth muscle myosin. Loosely packed SV2 stained nerves gave off branches to the smooth muscle of the distal airways with a dense network of fibres extending all the way to the cylindrically arranged muscle cells of the terminal airways. A plexus of fine nerves was distributed over the surface of the smooth muscle with an abundance of prominent varicosities 1–3 μm in diameter and 3–10 μm apart. We conclude that the airways are densely innervated. Nerves accompany the newly forming smooth muscle of the distal airways and functionally innervate this tissue.

Actin sub-domain and tropomyosin movements during Ca^{2+}-regulation

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Low-angle X-ray diffraction patterns from resting and activated vertebrate skeletal muscles, and differences in the actin pattern observed when relaxed, overstretched frog muscles were activated, have been analysed (Squire *et al.*, 1993, *J. Chem. Soc. Faraday Trans.* **89**, 2717–26, and ms in preparation) in terms of the known sub-domain structure of the actin monomer (Kabsch *et al.*, 1990, *Nature* **347**, 37–44) and its probable configuration in actin filaments (Holmes *et al.*, 1990, *Nature* **347**, 44–9). It is suggested that when tropomyosin/troponin binds to F-actin in the absence of Ca^{2+} there is a small movement of actin sub-domain 2 towards tropomyosin

with other sub-domains moving little. In non-overlap muscle, when the full thin filament is activated by Ca^{2+}-binding to troponin, there is a shift of the tropomyosin from its off position between sub-domains 3 and 1, away from sub-domain 1 and across the face of sub-domain 3. At the same time sub-domain 2 moves back towards its position in F-actin. Since the major binding site of the myosin head on actin is on sub-domain 1, the original idea of tropomyosin sterically hindering myosin attachment in the off state remains a possibility. However, even in the on state, the tropomyosin position is still adjacent to the myosin binding site where it could well regulate the transition of the bound myosin head from a weak to a strong state. Supported by the BBSRC.

Myosin cross-bridges in defined states of rat cardiac muscle

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Freshly dissected rat papillary muscles, with sarcomere order and length monitored by laser diffraction, were fixed in glutaraldehyde, cryoprotected with 2.3 M sucrose, rapidly frozen by plunging into liquid cryogen and cryo-sectioned for electron microscopy. Sections were usually negatively-stained with ammonium molybdate. Studied states of intact muscle were quiescent and iodoacetate-poisoned rigor; states in triton-skinned muscles were relaxed and rigor. Differences were observed between the A-band structures in relaxed, quiescent and rigor musles consistent with a gradually varying population of myosin cross-bridges attached to actin. Rigorized papillary muscles previously treated with N-ethylmaleimide, which has been shown to be effective at producing improved structural regularity in rigorised skeletal muscles, probably by preventing cross-bridge turnover on actin, were found to be in a state very similar to relaxed. Longitudinal cryo-sections of rat cardiac muscles in different states were analysed by Fourier and other methods and were found in some cases to show clear diffraction peaks to the tenth order of the A-band 43 nm repeat, a resolution of about 4.3 nm. In addition to changes in the cross-bridge region in different states, M-band appearance also changed between states, suggesting that in cardiac muscle the M-band might have an active physiological role. Supported by the British Heart Foundation.

Heparin inhibits proliferation without inhibiting the spontaneous de-differentiation of airway smooth muscle cells in culture

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The anionic glycosaminoglycan, heparin, is released from pulmonary mast cells with other mediators in response to antigen exposure. Heparin inhibits smooth muscle cell (SMC) proliferation and has been reported to inhibit vascular SMC de-differentiation. The effects of heparin on airway smooth muscle cell (ASMC) proliferation and phenotype have not known. We measured ASMC proliferation in the presence (+h) or absence (−h) of heparin using canine tracheal SMC cultures. Growth rate (+h) was 40% slower than (−h). Confluence cell number (+h) was only 50% of that (−h) suggesting that heparin inhibits cell division but not cell growth. Expression of SMC phenotypic markers (SM-myosin heavy chain [SM-MHC], α-actin, myosin light chain kinase [MLCK], non muscle-MHC [NM-MHC] and vimentin) was assessed by protein blotting. Mitotic cells (day 6–10) (−h) expressed ≈10% of SM-MHC, MLCK and α-actin compared to isolated cells. NM-MHC and vimentin expression increased fourfold in mitotic cells. NM-MHC at day 20 remained elevated, while vimentin decreased ≈75%. Expression profiles (+h) mimicked those of (−h), except: (1) at confluence, (+h) levels of vimentin were higher, and (2) MLCK was greater in mitotic (−h) cells than (+h). These data indicate that heparin inhibits ASMC division without inhibiting de-differentiation. Heparin released by pulmonary mast cells may play a role in regulating airway SMC growth associated with chronic asthma. (Supported by the Medical Research Council of Canada and the Manitoba HSC Foundation).

Phenotypic markers of airway smooth muscle contractility

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Smooth muscle cells (SMCs) exist in a range of phenotypes between a contractile cell and a proliferative/non-contractile cell. Phenotypic modulation of airway SMCs associated with airways remodelling in chronic asthma has not been reported. We used Western and Northern blot analysis to identify protein markers of freshly dispersed, contractile canine tracheal SMCs and of cultured, de-diferentiated/non-contractile canine tracheal SMCs. Tropomyosin, myosin light chain kinase, smooth muscle myosin, calponin, desmin and *h*-caldesmon were expressed at lower levels ($p < 0.05$) in de-differentiated cells (day 7 in culture). Conversely, non-muscle myosin, vimentin, *l*-caldesmon and CD44 were higher in 'non-contractile' cells. Changes were blocked by the DNA synthesis inhibitor 5-fluoro-2'-deoxyuridine whereas the SMC mitogens, histamine and TPA had little effect. We also compared the protein profiles of a relatively fast smooth muscle (SM), the canine trachealis (TSM), with that of a relatively slow one, the canine pulmonary artery (PASM). Protein markers of 'contractile' SMCs were 10–50% higher in TSM tissues compared to PASM. The slower PASM also had higher levels of 'non-contractile' cell-associated proteins than TSM. The protein composition of a smooth muscle reflects the tissue's mean cellular phenotype and appears to be a correlate of its contractile characteristics. Such markers may be useful as tools for assessing the phenotype and functional characteristics of airway SMC's in chronic asthma. (Supported by the Medical Research Council of Canada).

Effects of heparin on cell cycle progression of cultured airway smooth muscle cells

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Heparin inhibits serum-stimulated replication of canine tracheal smooth muscle cells (SMCs) in culture. Heparin reportedly, inhibits cell cycle entry and progress through G_1 into S phase. However other reports suggest heparin releases TGF-β_1 from serum α_2 macroglobulin and prolongs G_2 and M phases of the vascular SMC cell cycle. We used flow cytometry to examine heparin effects on cell cycle progression of cultured canine tracheal SMC. DNA was measured by propidium iodide staining, DNA synthesis was assessed by measuring bromo-deoxyuridine (BrdU) incorporation using a fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody, and cellular protein was measured by staining cells with FITC. Heparin reduced by 40%, the number of growth arrested tracheal SMCs entering the cell cycle after serum stimula-

tion. Progress time through S-phase, of cells which responded to serum stimulation, was not affected. However, transit time from S through M phase was doubled by heparin. Forward angle light scatter and FITC-staining data confirmed that mean cell size was increased in the presence of heparin. Western blot analysis showed that the levels of several tyrosine phosphorylated proteins (116, 73, 70 and 68 kDa) were reduced in cells in G_2 phase in the presence of heparin. Hence, the anti-proliferative effects of heparin on tracheal SMC's is consistent with those described for TGF-β_1 on vascular SMCs. Understanding of the mechanism of inhibition of SMC replication by heparin will be of benefit in elucidating the role of the proteoglycan in determining the chronic response of the airways to inflammatory mediators. (Supported by the Medical Research Council of Canada).

Protein kinase A does not influence the rate of cross-bridge cycling in skinned cardiac trabeculae from the rat

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Recent experiments have suggested that catecholamines may increase the rate of cross-bridge cycling in cardiac muscle, independent of changes in intracellular calcium caused by β-adrenergic stimulation. To test this hypothesis we measured the rate of ATP utilization as a function of the developed force in thin right ventricular trabeculae of the rat permeabilized by Triton X-100. ATP hydrolsis was enzymatically coupled to the breakdown of NADH. The NADH concentration was determined photometrically from the absorption of near UV-light. Experimental conditions: sarcomere length: 2.2–2.3 μm, temperature 20° C, 0.9 mM NADH, 10 mM PEP, 5 mM MgATP, 1 mM free Mg^{2+}, ionic strength 200 mM (adjusted with K-propionate), pH 7.1 (adjusted with KOH).

β-adrenergic stimulation was mimicked by incubation of the skinned preparation in a solution containing 3 μg ml^{-1} Protein Kinase A (PKA) for 40 min. PKA treatment caused a significant increase in the [Ca^{2+}] required for half-maximal force development. The steepness of the force-[Ca^{2+}] relation was not affected. The rate of ATP utilization was linearly related with force development both without and with PKA treatment. PKA did not affect either the maximum rate of ATP utilization nor the economy of force maintenance. This suggests that β-adrenergic stimulation does not alter the rate limiting step of cross-bridge cycling during isometric contraction in myocardium.

Histamine-dependent desensitization of depolarization-induced contractions in porcine carotid smooth muscle

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Rabbit femoral artery smooth muscle exhibits a long lasting (~2 h) heterologous desensitization of depolarization-induced contractions after phenylephrine incubation (Ratz & Lattanzio 1994, *Biophys. J.* **66**, A410). The authors attributed this desensitization to modulation of post-receptor signal transduction mechanisms. We have confirmed this phenomena in porcine carotid medial ring preparations using the contractile agonist histamine. Small carotid segments, stripped of adventitia, were contracted with KCl (40 mM) before and after treatment with histamine (1–30 μM). The post-histamine stimulation with KCl resulted in a slower rate of force development relative to control, less isometric tension (up to 40% decrease), reduced rate of oxygen consumption, and reduced shortening velocity and was associated with reduced myosin light chain phosphorylation. The degree of desensitization, which lasted <1 h, was dependent upon histamine concentration, duration of histamine incubation, and the amount of time between removal of histamine and subsequent KCl depolarization. Desensitization was not observed with concurrent incubation with histamine and the specific H1 antagonist pyrilamine but was unaffected by the specific H2 receptor antagonist, cimetadine. Although incubation with 100 μM histamine for 1 h could inhibit subsequent contractions under maximal depolarizing conditions (109 mM KCl), these contractions were less affected by treatment with histamine, suggesting perhaps that the phenomena involves altering the voltage dependence of calcium influx. Supported by NIH P01 HL 19242 (RAM) and AHA VA-93-F-13 (JDS).

In vitro motility assay with myosin isolated from a single skinned muscle fibre

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To directly correlate mechanical parameters recorded from single skinned muscle fibres with sliding velocity of actin filaments observed in an *in vitro* motility assay (e.g. Kron & Spudich, *Methods in Enzymology*, 1991) we attempted to modify the standard assay such that myosin isolated from an approximately 10 mm segment of a single skinned fibre is sufficient to support ATP-driven sliding of fluorescently-labelled actin filaments. Myosin was extracted from the single fibres by a high ionic strength buffer in the presence of 2.5 mM MgATP and infused into a flow cell that was miniaturized such that the small quantity of extracted myosin was sufficient to yield a functional motility assay. TRITC-phalloidine labelled actin filaments were prepared according to Harada and colleagues (*JMB*, 1990).

Having established such a micro assay allows us to directly correlate *in vitro* sliding velocity with various mechanical parameters (e.g. unloaded fibre shortening, rate constant of force redevelopment etc.) for identical myosin populations under different experimental conditions, for myosin of different sources like fast and slow twitch muscle fibres of the rabbit (heavy chains IIa, IIb, IId and I; Aigner *et al.*, 1993, *Eur. J. Biochem.*) and for comparison of wild-type β-myosin heavy chain from normal human soleus muscle with β-myosin heavy chain mutations found in some kindreds with hypertrophic cardiomyopathy.

Low concentrations UD-CG 212, the metabolite of pimobendan, increase myocardial force via calcium sensitization in skinned fibres as well as intact myocytes

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Pimobendan (ACARDI®) is a novel positive inotropic agent which increases myocardial force via calcium sensitization. *In vivo*, pimobendan is demethylated to UD-CG 212 which is the main metabolite of pimobendan. In this study, we measured the effects of UD-CG 212 on contractile force generated by chemically skinned cardiac fibres and cell length changes of intact myocytes in the presence of 5 mM inorganic phosphate (P_i) to mimick myocardial ischemia.

UD-CG 212 ($10^{-10} - 10^{-6}$ M) caused significant ($p < 0.05$) additional increases in tension of chemically skinned canine myocardial fibres at pCa = 5.72. A maximal increase by 17.5% was observed at 10^{-7} M UD-CG 212. The changes in tension induced by 10^{-8} M of

UD-CG 212 amounted to +22.7% at pCa = 6.23, +13.7% at pCa = 5.99 and +1.5% ($p > 0.05$) at pCa = 4.36. Myofibrillar ATP-ase activity was not affected by 10^{-8} M UD-CG 212 at these pCas.

Cell shortening of isolated guinea-pig ventricular myocytes electrically stimulated at 1 Hz was significantly increased by UD-CG 212. An increase of 37% was observed at 10^{-9} M of UD-CG 212, 90.5% at 10^{-8} M UD-CG 212 and 164% at 10^{-7} M UD-CG 212. Peak INDO-1 fluorescence ratios measuring the free intracellular calcium concentrations were not significantly influenced at 10^{-9} and 10^{-8} M UD-CG 212, but increased by 19.4% at 10^{-7} M.

The results indicate that low concentrations UD-CG 212 increase myocyte contractility selectively by calcium sensitization whereas at higher (>10^{-7} M) concentrations additionally an effect on intracellular calcium probably mediated by cAMP is observed.

Bistheonellide A, a marine sponge metabolite, induces marked shape changes of 3Y1 fibroblasts accompanying nuclear division but no cytokinesis

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Bistheonellide A (BT-A), an antitumor macrodiolide isolated from a marine sponge *Theonella* sp., was introduced at a concentration of 0.1 μM into rat 3Y1 fibroblasts of 2.5×10^4 cells ml^{-1} in an MEM medium containing 10% FCS and these were incubated at 37° C for 24 h under 5% CO_2. While untreated cells having normal extended shapes increased to over twofold concentration, the growth of cells treated with BT-A was completely inhibited. In association with cell growth inhibition, the shape of treated cells changed markedly, resulting in the formation of several spines with the cell body rounding up. When treated cells were stained with a rhodamine/phalloidin solution, no stress fibre was observed, in contrast to normal cells which clearly showed many stress fibres. Furthermore, cells treated with BT-A showed two nuclei per one cell by staining with DAPI, suggesting that the cell cycle was stopped with no cell division even after nuclear duplication. Then, cells treated with BT-A were washed with the medium containing no BT-A and incubated for 48 h. Washed cells regenerated stress fibres, resulting in the formation of a cell shape close to that before the BT-A treatment. In addition, this regeneration process was accompanied by the alteration of multinuclear cells into mononuclear ones.

Influence of calcium sensitizers on sarcomere dynamics of rat cardiomyocytes

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This study aimed at the investigation of effects of calcium sensitizers on the interval shortening relation as well as post rest staircase of electrically paced unattached cells of the rat ventricle.

Mean sarcomere shortening, whether determined by laser diffractometry or by Fast Fourier Transform of quickly captured frames, increased in Pimobendan (100 μM) treated cardio-myocytes when paced at intervals between 0.3 and 10 s with a significant optimum at 2 s. Contractility was slightly diminished at intervals beyond 10 s. The negative staircase after a rest period of 2 min was significantly flattened in comparison with the controls (beat interval 2 s).

EMD 57033 (2 μM) caused similar effects with respect to the interval sarcomere shortening relation. Contractility was enhanced at intervals between 0.3 and 20 s but did not change in the range between 20 and 120 s. Further, the post rest staircase was found to be flatter compared to control cells.

The enhancement of contractility was accompanied by a decrease in the diastolic sarcomere length (interval 2 s): controls 1.82 ± 0.03 μm, Pimobendan treated cells 1.70 ± 0.07 μm, EMD treated cells 1.68 ± 0.03 μm (mean ± SD). There was no effect with respect to Ca^{2+} transients measured by confocal laser scanning microscopy with the fluorescent probe Fluo-3/AM.

Modifications of the sarcomere dynamics of Ca^{2+}-tolerant rat cardiomyocytes under the influence of calcium sensitizers are due to the direct action on the contractile proteins and may be understood, too, in terms of a two compartment model that simulates calcium movements between the uptake and release compartment of the sarcoplasmic reticulum.

Non-linear velocity of propagating calcium waves in rat cardiomyocytes

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Spontaneous Ca^{2+}-waves were investigated by confocal laser scanning microscopy using a CCD camera to follow local fluorescence light changes. Enzymatically isolated cardiomyocytes of adult rats were loaded with the fluorescent Ca^{2+}-indicator fluo-3 AM.

As recently shown, the origin of a spreading Ca^{2+}-wave is a spark-like focus in the membrane of the sarcoplasmic reticulum (Cheng *et al.*, 1993, *Science* **262**, 740). The propagation velocity was found to be nearly constant (76.1 ± 18.6 μm s^{-1}, mean ± SE, 20° C) when determined by conventional fluorescence microscopy at different points along the cell (Wussling *et al.*, 1994, *Pflügers Arch. Suppl.* **426**, R 80). More precise measurements with a confocal laser scanning microscope (Meridian) resulted in a non-linear propagation of Ca^{2+}-waves with respect to time. The velocity was low near the focus (about 60 μm s^{-1}) and increased (to about 110 μm s^{-1}) in a hyperbolical manner. This result was surprising inasmuch as for geometrical reasons a decrease of the propagation velocity might be expected if the confocal plane is not identical with the plane where the focus of the calcium wave was located.

It is supposed that the observed nonlinearity of the propagation velocity is due to the calcium induced calcium release mechanism that becomes more effective with increasing cytocolic Ca^{2+}-concentration during the propagation of the spontaneous calcium wave along the cell.