The maximum sustainable duty cycle of single xenopus muscle fibers related to succinic dehydrogenase an myofibrillar ATP-ase activities.

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EXCITATION-CONTRACTION COUPLING

Activation and relaxation mechanism in single muscle fibres
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The effect of Ca$^{2+}$ on the rate course of force generation in frog skinned muscle fibres has been investigated using laser flash photolysis of the caged-calcium, either nit-5 or DM-Nitrophen or diazo 2. 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$^{2+}$ photolyzed 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 maximal tension at pCa 4.5.

Our present model has two calcium-binding sites per functional unit and therefore each functional unit can be in any one of six different states. It also differs from the model proposed by Hill (1983) as the affinity of the regulatory sites for calcium is not altered by crossbridge formation. Our experimental results show that force develops relatively rapidly at intermediate Ca$^{2+}$ which produce only partial activation (i.e. 50% Pmax). This would not be the case if the affinity of the regulatory sites changes slowly with crossbridge attachment.

We have used single skinned muscle fibres from both frog (actin regulated) and scallop striated muscle (myosin regulated), to study the rate course of muscle relaxation using diazo 2. This procedure has enabled us to examine the effects of the intracellular metabolites, ADP, P, and H$^+$ upon the rate of relaxation.

Time course of fibre shortening and its temporal correlation to calcium binding to troponin C in skeletal muscle fibres
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Cut skeletal muscle fibres of the hog mounted into a single Vaseline gap and voltage clamped to −100 mV were loaded intracellularly with the calcium sensitive dyes Antipyrpylazo III (APIII) and fura-2. The tendon at the intact segment was attached to a force transducer. Depolarizing pulses were applied and the changes in fibre absorbance at 720 and 850 nm, in fibre fluorescence at 510 nm and tension were recorded simultaneously. The parameters describing the calcium binding properties of fura-2 were set so that the calcium transient calculated from APIII absorbance and fura-2 fluorescence became identical (Klein et al., Biophys. J. 55, 971-88, 1988). When APIII was not present in the intracellular solution the absorbance changes showed two components one of which was also present below the contraction threshold and could be identified with an intrinsic absorbance change described previously (Melzer et al., J. Physiol. 372, 261-92, 1986) whereas the other component had a time course identical with the tension transient (movement artifact). Fura 2 fluorescence on the other hand showed no signs of a movement artifact.

The rate constants for the calcium binding to the regulatory sites on troponin C were determined from the calcium transients measured at different pulses belonging to the strength-duration curve for just detectable contraction assuming that the saturation of troponin C should be equal for all these pulses. These rate constants were then used to calculate the calcium bound to troponin C ([CaTn]) at and slightly above the contraction threshold. To avoid possible distortion due to movement artifacts on the absorption records, the calcium transients calculated from the fura-2 fluorescence were used. ([CaTn]) directly involved in the initiation of contraction was calculated as the difference between that measured above and at the threshold. The time course of this difference in [CaTn] closely resembled that of the contraction the latter having a 9 ms longer latency. This latency should reflect the time necessary from the conformation change of troponin C to the appearance of physical shortening.

Measurements of K$^+$ efflux from myometrium, during hypoxia and intracellular acidification, and also during application of K$^+$ channel agonists and antagonists
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The spontaneous contractions of isolated rat uteri are greatly reduced or abolished by the inhibition of oxidative phosphorylation (Wray et al., Expt. Physiol. 77, 307-19, 1992). The mechanism of this decrease is still unresolved. The causation may, in part, be metabolic inhibition inducing both pHi, and [ATP], the resultant effects on the contractile machinery (Wray, J. Physiol. 423, 411-23, 1990). However, another possible explanation is that as [ATP], falls, the surface membrane conductance of K, increases. We have therefore measured potassium ($^8$Rb) efflux from strips of isolated rat myometrium.

Cyanide (2 mM) reduced contraction. This was accompanied by a large, reversible increase in $^8$Rb efflux from the tissue. This was the case for both pregnant and non-pregnant uteri where the increase was 30 ± 5% (n = 10) and 33 ± 7% (n = 15) respectively. The increase in K$^+$ efflux was not due to alterations in membrane electrical activity as the increase still occurred in both high-K and 0-Calcium solutions.

Glibenclamide (20 μM), an antagonist of KATP channels, reduced the cyanide-evoked increase in efflux by about 50%. The Glibenclamide-insensitive component of the efflux still persisted in a Ca$^{2+}$-free environment. Despite its action on K$^+$ efflux, glibenclamide did not restore contractions. Preliminary experiments using the K$^+$ channel opener lemakalim (>20 μM), showed that it both relaxed the tissue, presumably by hyperpolarization, and that it also increased the rate of $^8$Rb efflux within 5 min by 10 ± 2% (n = 3). Previous experiments had shown lemakalim at lower concentrations (<10 μM) not to produce any measurable alterations in $^8$Rb efflux from rat uterus (Hollingsworth et al., Br. J. Pharmac. 91, 803-13, 1987).

Intracellular pH falls during metabolic inhibition. Reducing pH, (in the absence of cyanide) by application of the weak acid butyrate (60 mM at constant external pH 7.4) had no significant effect on the
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Phosphorylation of the skeletal muscle ryanodine receptor/Ca2+ release channel complex and its effect on single channel activity
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The ryanodine receptor of rabbit skeletal muscle was phosphorylated by endogenous protein kinase(s) associated with the membranes of the triads. For this phosphorylation exogenously added ATP or endogenously synthesized ATP (Han et al., 1991, Biochemistry 30, 577) could be utilized.

To study the effect of phosphorylation on single channel activity, the receptor protein from phosphorylated and non-phosphorylated triads was solubilized with CHAPS and the solubilate was separated on a linear sucrose/CHAPS gradient. Reconstitution of the isolated receptor into planar lipid bilayers showed that the phosphorylated channel displayed an enhanced open probability in presence of micromolar cytoplasmic (cis) Ca2+. Also, its sensitivity to cis ATP was shifted to submillimolar concentrations. These effects could partially be reversed by dephosphorylation with phosphatase 2a.

Excitability of muscle: ion channel genes, their characterization, development control and significance for disease
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Properly regulated, nerve-induced contraction of adult mammalian muscle depends on a cascade of signals mediated or regulated by muscular ion channels: Acetylcholine receptor (ACHR), voltage dependent Na+ channels (SKM1 in the adult), a number of K+-channels, the chloride channel CIC-1, and the slow Ca2+ channel or DHP-receptor (DHPR) in the plasma membrane, as well as the Ca2+-release channel or ryanodine receptor (RyR) in the sarcoplasmic reticulum. The genes for these channels have been identified via cDNAs and chromosomally assigned Na+- and K+-channel genes, their characterization, development control and significance for disease processes under observation by the newly available methods might be delayed relative to the processes occurring in intact tissue.

In single frog muscle fibres, activated in normal Ringer solution by field stimulation of 200 μs duration the delay from the onset of the stimulus to the onset of the birefringence signal is below 800 μs at room temperature (20–23°C). The beginning of latency relaxation follows with an additional delay of 300 μs. Upon increasing the toxicity of the holding medium the delays are increased. Furthermore in twice normal tonicity latency relaxation is preceded by a short increase in muscle tension. This increase in tension starts at the same time as the birefringence signal and is hypothetically considered to be caused by the same basic process. Taking into account the compliance of the transducer and the amplitude of the tension increase preceding latency relaxation the corresponding change in sarcomere length might be as small as about 0.1 nm per half sarcomere.

Kinetics of dihydropyridine-sensitive Ca2+ channels in skeletal muscle
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In skeletal muscle, the binding protein for Ca2+ channel antagonists (the dihydropyridine receptor = DHP receptor) is believed to exhibit two functions: forming a pore to allow Ca2+ influx into the muscle fibre and controlling Ca2+ release from the sarcoplasmic reticulum. However, Ca2+ release activation does not require the Ca2+ influx; in addition, Ca2+ release is maximally activated within only a few milliseconds after depolarizing the membrane while the Ca2+ inward current shows a considerably slower time course. The Ca2+ channel may simultaneously work as a control device for Ca2+ release if it 

Temporal relationships of processes involved in excitation contraction-coupling in skeletal muscle
H. Oetliker

In the recent past it became possible to visualize structural correlates of processes leading to contractile activation of skeletal muscle with the resolution of the electron microscope by ultra rapid freezing and stimulation of the muscle fibre at μs intervals before the single fibre touches the copper block cooled by liquid helium (Sommer, this meeting). Furthermore changes in muscle tension and length can be followed upon fast changes of ATP and calcium concentration (Ashley, this meeting) initiated by photolysis of caged compounds. These methods eliminate diffusion delays and allow precise measurements of reaction times. In addition it is possible to follow changes in elementary content of various cellular compartments (Somlyo et al., J. Cell. Biol. 90, 577–94, 1981). The measurements mentioned before have the advantage of following partial aspects of contractile activation rather precisely. But on the other hand, they must be performed under quite unphysiological conditions. It is therefore of interest to compare the time information derived from such experiments with the time information obtainable from measurements considered to be non invasive on intact single fibres of frog skeletal muscles, such as the earliest changes in muscle tension and optically observable intrinsic changes in optical retardation. The latter are thought to reflect changes in myoplasmic calcium concentration. Such intrinsic optical measurements are barely interfering with the physiological processes and can therefore be used as time markers to compare the direct structural and chemical changes with them to judge if and to what extent the processes under observation by the newly available methods might be delayed relative to the processes occurring in intact tissue.
possesses a rapid voltage-sensing reaction in addition to the slow opening reaction. A rapid step participating in the gating of the slow Ca\(^{2+}\) channel could be identified when applying strong depolarizing prepulses. Shortly after a sufficiently strong depolarization the current could be activated considerably more rapidly by a second depolarization (Feldmeyer et al., J. Physiol. 425, 347–67, 1990; J. Physiol. 457, 639–55, 1992). Flash photolysis showed that the fast gating mode of the channel was also reached when the slow Ca\(^{2+}\) current was blocked during the prepulse by the light-sensitive DHP compound nifedipine. The conditioning pulse apparently removes the rate limitation by the slow reaction and uncovers a channel gating reaction which seems sufficiently rapid to account for the speed of activation of Ca\(^{2+}\) release.

A challenge in structure-function correlation: structural correlates of the coupling of excitation–Ca\(^{2+}\) release (ECR) in skeletal muscle at high temporal and spatial resolution

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The translation of an action potential into intracellular calcium release for contraction is a fundamental event in muscle physiology. Its time course is central to understanding the mechanism of excitation-contraction coupling in striated muscle. Following the development of new methods for cryofixation with high temporal and spatial resolution for structural and quantitative elemental analysis, including x-ray imaging (Nassar et al., Som. Electron Microsc. 1, 309, 1986; Nassar & Sommer, Scanning Microsc. 6, 745, 1992; LeFurquey et al., J. Microsc. 165, 191, 1992), we have measured changes in [Ca] in junctional SR (JSR) following electrical stimulation of single, intact skeletal muscle fibres. Our preliminary results show that calcium is released within about 0.5 ms after a single stimulus. After cryofixation and heavy metal staining, granular material inside the JSR of resting fibres is very electron dense and surrounds unstained core cylinders. This is never observed after chemical fixation. Cryofixation of the calcium-depleted tetanic state produces images similar to those seen after chemical fixation. These structural images, observable only after cryofixation, are consistent with a calcium-loaded state of ca lsquo;eseque\rqnos in which exposed hydrophobic groups are strongly contrasted with heavy metal EM stains, and a calcium-depleted state in which these groups are buried and, thus, less accessible to stains. In light of the 0.5 ms ECR time, an earlier chance observation of a significant increase in E-face pits in free SR and JSR in a freeze-fracture preparation cryofixed during the first ms may now be re\dash viewed as another possible structural correlate of the instant of calcium release: conformational changes in channel proteins during calcium release may result in the momentary creation of protein-lipid boundaries more suitable as cleavage planes for freeze-fracture than might exist in the resting state. Our discovery of an extended JSR (EJSR) and its ryanodine receptors at a far distance from plasmalemma further stresses the need for contemplating structural and geometric features when formulating hypothetical mechanisms for ELC.

Optimal force-length relationship is dependent upon calcium concentration

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The isometric force-length relationship of fibres from the Musculus extensor carpi radialis of the horse was measured. The optimal sarcomere length was investigated at different calcium concentrations. De Beer and colleagues (Basic Res. Cardiol. 83, 410–23, 1988) have already shown the calcium dependency of the optimum of the force-length relation at Gracilis muscles of the rabbit.

Fibres were skinned by means of freeze drying. Single fibres were activated at different calcium concentrations. Tension was recorded using a microforce transducer (Scientific Instruments, KG3). Sarcomere length, varying from 1.8 to 4.0 mm, was set and controlled using laser diffraction. The optimum of the isometric force-length relation was calculated using a parabolic fit. The calcium sensitivity was calculated.

The optimal sarcomere length proves to be dependent on the [Ca\(^{2+}\)] at low pCa (high [Ca\(^{2+}\)]) the optimal sarcomere length seems to be constant at 2.4 mm. But at high pCa (low [Ca\(^{2+}\)]) the optimal sarcomere length shifts to 3.2 mm. At a pCa of 6.34 little tension was measured and therefore no exact optimal length could be determined.

The development of tension is also plotted against pCa values. The sensitivity of the fibres to calcium increases when sarcomere length is increased. This sensitivity change has already been extensively described.

The increased calcium sensitivity at increased length is in agreement with shifting the optimal sarcomere length in the force-length relation when the calcium concentration is decreased. It is concluded that the increased calcium sensitivity at longer lengths governs these effects in combination with change in lattice spacing.

Early stages of channel development in L6 myoblasts

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In order to elucidate a possible role of membrane ionic channels in the myogenesis itself, if any, we are concentrating on correlation of muscle subsystems development by means of electron microscopy and patch clamp methods. We present here the results of the inquiry into the channel development in the muscle cell line L6. At the very early stages of development, the membrane mosaics in these cells is fairly simple; the action potential mechanisms are absent and the resting membrane potential is dominated exclusively by potassium ions (Kidokoro, J. Physiol. 244, 129-43, 1975). The first channel to appear belongs to the category of voltage sensitive chloride channel of large conductance and shows the following characteristics. The conductance of the channel in symmetrical 120 mmol l\(^{-1}\) choline chloride is 331 ± 4pS. The probability of the channel being in the open state decreases with the increasing imposed voltage. The bell-shaped steady state channel conductance-voltage relationship is asymmetric and can be fitted by two Boltzmann equations with different \(V_0\) and \(k\) constants: \(-25.6\) mV and \(-6.8\) respectively for the negative side and \(+50.0\) mV and \(+13.7\) for the positive side. The stilbene derivatives (DIDS and SITS) blocked the channel in \(\mu\)mol concentrations (10–100 \(\mu\)mol l\(^{-1}\)). The channel shows multiple conductance levels that may develop in a step-like manner. The channel is assumed to serve to some basic functions of the cell as it appears very early in myogenesis and is common in a great variety of cells.

ADAPTATION, FATIGUE AND REPLACEMENT

Contributions of molecular myology to development of skeletal muscle-cardiac assists

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Biomechanical cardiac assist involves the use of a skeletal muscle to assist the cardiac function. Both systolic (dynamic cardiomyoplasty) and diastolic assistance (skeletal-muscle-driven counterpulsation) stand on a conditioned muscle which may or may not withstand the
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increased demand. Several problems which limit the clinical application of these promising approaches may be solved studying the conditioned muscle at molecular level. In particular muscle damage (lipidosis and fibrosis) which may accompany fibre necrosis and regeneration seems to emerge as a limiting factor whose causes need to be clarified further. We are developing biochemical micromethods to complement quantitative morphology of biopsied muscle in both experimental (studying the biological interactions between a glutaraldehyde treated porcine left ventricular ventricle, used as Venticular Assist Device chamber, and a sheep lattissimus dorsi muscle powering it) and clinical (studying for reverting inactivity-induce hydropotism of patients' skeletal muscle by a light, muscle specific, physiokinesiologic training may improve the pump performance) settings. We will give details of a new peptide mapping approach to reveal ubiquitin and embryonic myosin heavy chains based on hydroxilamine digestion and immunochemical analysis (as markers of actual muscle damage) and of our biochemical approaches to fibrosis quantitation (as marker of ongoing muscle damage) in cryostat sections serial to those used for histological and histochemical analyses. We will stress the contribution to these approaches of our new method to concentrate and quantitate polypeptides after SDS solubilisation, a method which takes advantage of the differential precipitation power of KDS (Carraro et al., Electrophoresis 12, 1005-10, 1991; Sandri et al., Basic Appl. Myol. 2, 107-14, 1992; Sandri et al., Anal. Biochem. 213, 34-9, 1993).

Analysis of structure-function relationship on soleus atrophied fibres
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The aim of the present study was to determine how the structure of fibres isolated from rat atrophied soleus could be correlated with the modifications observed in the functional properties. The atrophy of the slow soleus was induced by two weeks of hindlimb suspension (2 week HS). We found that 2 week HS induced slow-to-fast changes as well in the contractile properties as in the protein isoform expression. The contractile properties were characterized by different parameters: the calcium and strontium activation characteristics (T/PCA and T/SP relationships), two kinetic indicators of the tension development (P/\( \Delta P \)/\( \Delta t \)) and the maximum shortening velocity (V\( _{\text{max}} \)). The analysis of all these functional parameters after HS permitted us to observe in the slow soleus the emergence of fibres exhibiting fast characteristics. More particularly there was an increase in the values of V\( _{\text{max}} \), P/\( \Delta P \) and P/\( \Delta t \). Moreover, the evolution of the V\( _{\text{max}} \) parameter was strongly coordinated with that of the relative calcium/strontium affinity. This suggested the existence of a correlation between V\( _{\text{max}} \) and the regulatory proteins, especially that responsible for the calcium (or strontium) affinity, the TnC. The electrophoretic analysis of MHC, MLC and TnC isoforms, performed on the same fibres, revealed that three types of populations were present after HS: slow, fast and hybrid fibres. Our results indicated that there was a relationship between the protein composition of a fibre and the transformation of its functional characteristics in a disuse situation.

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Efficiency of energy turnover during contraction of skeletal muscle
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Efficiency is the fraction of energy used by the muscle that is converted into useful mechanical work. It is a muscle property that directly influences animal locomotion and it is directly related to crossbridge function.

We have measured efficiency of shark fibres during sinusoidal movement and intermittent stimulation chosen to mimic muscle performance in vivo. The ratio of work to total energy turnover (sum of work and heat) was taken as an estimate of efficiency. With optimal values of sinusoidal frequency and stimulus phase, the maximum observed efficiency was 0.41 (± standard error, n = 13). This value is higher than that found during shortening at constant velocity. The difference in efficiency may be related to the stimulation patterns: stimulation was intermittent during sinusoidal movement, but continuous during constant velocity shortening.

The efficiency during sinusoidal movement represents 23 × 10^-3 J of work done for each molecule of ATP split, and may involve more than one crossbridge 'power stroke' per molecule of ATP split.

The active transport and the reactivity of the electrogenic pump in soleus muscle after chronic stimulation
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The membrane potential in rat chronic denervated soleus muscle for 60 days and after electrical stimulation also for 60 days was recorded. The resting membrane potentials of the denervated muscle were less than normal and correlated inversely with the frequency of chronic electrical stimulation. The tension of the denervated muscles was found to have the same rank order as the normal soleus. The chronic stimulation may reduce atrophy and hence tension loss of denervated muscle. The parameters of the membrane potential were greatly affected by the chronic denervation. The transient depolarization was almost abolished by tetrodotoxin (5 \( \mu \)M) while tetraethyl ammonium (25 \( \mu \)M) increased the duration and amplitude of the transient depolarization. This suggests a prolonged depolarization to positive membrane potentials is accompanied by an increase of the specific membrane conductance. After chronic electrical stimulation the results show that the denervated soleus almost completely recovers.

Transformations in mouse soleus induced by chronic stimulations
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The alteration of the morphological and physiological state of chronic denervated muscle, evolves progressively, which is evidenced among other procedures, by diminishing of its functional capacity. In the first series of experiments, we have followed the reactivity of the soleus muscle after 60 days chronic excitation. The chronic stimulation was produced by using a magnetic field from a Magnetodiathux stimulator (0.2 T). The results show that, in spite of the fact that the denervated soleus was stimulated for a long period, the biomechanical activity diminished significantly. The twitch and the tetanus contraction showed that this slow twitch muscle becomes slower after the chronic denervation. In a second series of experiments on the same muscle, we compared the protein composition of the sarcolemma both of the normal and denervated soleus, to test if the low mechanical output of the denervated muscle could be correlated with a deficiency in the synthesis or intracellular proteins or secondary messengers. In a final series of experiments, we have studied comparatively, by the SDS-PAGE method, the composition of total soluble and insoluble proteins found in denervated muscles. The results show that, in denervated muscle, protein metabolism is strongly modified. Chronic stimulation,
To what extent can we engineer the properties of skeletal muscle for cardiac assistance?

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We have become accustomed to viewing skeletal muscle cells as a kit of interchangeable parts. Most of the constituent proteins are members of families of isoforms. the particular mix of isoforms determines the mechanical performance of the whole cell. Whereas molecular differences in the various isoforms of the contractile proteins are sometimes subtle, the same cannot be said for differences in their functional characteristics. Fast fibres have a 10-fold greater maximum power output per gram than slow, and a three-fold greater maximum speed of shortening.

It would be arrogant to pretend that we could engineer all the properties of the adult muscle cell. We can, however, elicit responses from some of the regulatory mechanisms built into the cell by overload or chronic electrical stimulation. Continuous stimulation at 10 Hz transforms fast muscle fibres to slow. Such a change involves re-expression of the genome and the substitution of newly synthesized proteins into the contractile structure.

We used a combination of continuous stimulation at 2.5 Hz, 5 Hz or 10 Hz to investigate how the ultimate mechanical state of a chronically stimulated muscle depends on the amount of imposed activity. Transformation took place in a dose-related way, with no evidence of sharp transitions between states. The finding that moderate stimulation can produce a stable state of muscle with intermediate mechanical properties allows us to investigate this state in more detail. Do all the fibres have intermediate properties because they have a mixture of isoforms, or is the change in the whole muscle simply due to a shift in the percentage distribution of the classical fibre types?

In order to develop successful strategies for cardiac assistance from skeletal muscle grafts we must continue to work towards a better understanding of the relationship between the pattern of activation of a skeletal muscle and its ultimate mechanical performance.

Human muscle energetics: analysis of mitochondrial function

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The chemical changes during contractile activity were separated from recovery metabolism in the forearm flexor musculature in normal human subjects using 31P nuclear magnetic resonance spectroscopy. Percutaneous, supramaximal twitch stimulation of the motor nerve provided a measure of oxidative ATP synthesis activity. The small PCr breakdown rate after 250 seconds of ischemia provided an oxidative measure of human muscle ATPase rate and ATP synthetase rate, the separation of which is necessary for assessment of a biochemical energy balance.

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Contractile inactivation in fatigue-resistant frog skeletal muscle fibres (structure-function characteristics)

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The fatigue-resistant single twitch muscle fibres of the frog lowered and/or lost their contractility by the combined action of the repetitive tetanic stimulation (at a rate 70 s-1 for 0.5 s every 3 s) and low Ca2+ (0.02 mM per l) with verapamil (0.02 mM per l) Ringer's solution (RI). Electron micrographs reveal a large amount of vacuoles. It is obvious that the vacuoles primarily originate from swollen, damaged and disrupted mitochondria. The T-tubular system and the SR appeared to be normal. These functional and structural effects were rapidly and fully reversible in the rest and being exposed to normal Rj. In Rj, fatigue-resistant properties of these fibres also recovered. They were able to contract for a 10 min stimulated in the same way and the final tetanic tension was reduced to about 80% of its initial value. Under these conditions swollen T-tubules and vacuoles located in vicinity of Z-bands are present. The tetanic tension and fatigability also survived in low Ca2+ Rj alone as well as verapamil Rj alone, though much less than in normal Rj. Now it is known that: (1) several types of Ca2+-active drugs cause a use-dependent block of contraction; (2) the voltage-sensor of the T-SR is a high affinity receptor for these drugs (Rios & Pearson, Physical Rev. 71, 849–901, 1991); (3) there may be some functional contacts between T-system and mitochondria (Lehringer, Supp. Circ. Res. 34–5(III) 83–90, 1974). Our results suggest that muscle fatigue occurs at the transmission phase of excitation-contraction. T-SR signal has been changed by the repetitive tetanic stimulation in low Ca2+ and verapamil Rj solution. It appears that mitochondria in some way accept this failure and this leads to their structural changes. There is some correlation between the degrees of contractile inactivation and the change in structure of skeletal muscle fibres due to repetitive tetanic stimulation.

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min x kg) had 2-fold higher mitochondrial densities compared to untrained subjects (VO_{\text{max}} = 35 ± 4 ml per min x kg) in biopsies from m. vastus lateralis. Correspondingly, cytochrome oxidase (COX) mRNA (mitochondrially encoded) as well as cytochrome oxidase (COX) IV mRNA (nuclear encoded) were both present in higher concentrations in the athletes (1.65 ± 0.2 and 1.83 ± 0.2-fold, respectively). The concentration of mitochondrial DNA was found to be constant in the two groups.

No differences between the trained and untrained subjects were found in the expression pattern of myosin alkali light chain (MLC) mRNAs as judged from in situ hybridizations. The mRNA of MLC 1sb was found in all type I fibres. A minor proportion of them also contained the mRNA of MLC 1sa. These type I fibres were characterized by very low glycolytic capacity (ë-GBP/H) and high content of carbonic anhydrase III mRNA as well as protein. The fast MLC 1f/3f mRNA was preferentially localized in type II fibres and also to a lower and variable extent in type I fibres.

Other muscles such as m. deltoideus, m. teres major and m. pect. maior showed a slightly different pattern, in as far as all fibres expressing 1sb mRNA also express 1sa mRNA.

Mechanical and biochemical modifications induced by hibernation state in skinned m. psoas from ground squirrel Z. E. Rogdestvenskaya, A. S. Khromov, L. K. Srebniatskaya and V. V. Lednev Institute of Theoretical and Experimental Biophysics, Pushchino, Russia

To understand the adaptation mechanisms of skeletal muscle contractile apparatus to the functional changes during hibernation, we studied the Ca-activated isotropic force and protein isoforms profile of skinned fibres from m. psoas of hibernating and summer-active ground squirrels (Citellus undulatus). Each preparation was exposed to a range of activating solutions (pCa 8.4) at room temperature. A significant shift of the Ca-sensitivity towards the higher pCa was observed with all mitochondrial substrates investigated (glutamate + malate, pyruvate + malate, octanoylcarnitine + malate, succinate + rotenone). The reason for this is that the Ca-sensitivity of the mito- chondria decreased with lower Ca++ concentration. A 50% reduced oxidation rates were observed with all mitochondrial substrates investigated (glutamate + malate, pyruvate + malate, octanoylcarnitine + malate, succinate + rotenone). The reason for this behaviour was found to be caused in the case of B.K. by a 30-50% reduced content of cytochrome c oxidase as determined independently by the cytochrome aa3 and cytochrome c1, content and diminished activity of the enzyme. The activities of other mitochondrial enzymes were proven to be identical to the controls. In the second myopathy case (L.H.) no significant shift of the Ca-sensitivity was observed. A marked effect was observed on the rates of respiration of muscle fibres of a patient (B.K.) suffering from a rare myopathy with elevated lactate levels (exercise intolerance with elevated lactate levels) with ptosis were compared to controls (orthopaedic patients). In both myopathy cases approximately 50% reduced oxidation rates were observed with all mitochondrial substrates investigated (glutamate + malate, pyruvate + malate, octanoylcarnitine + malate, succinate + rotenone). The reason for this behaviour was found to be caused in the case of B.K. by a 30-50% reduced content of cytochrome c oxidase as determined independently by the cytochrome aa3, content and diminished activity of the enzyme. The activities of other mitochondrial enzymes were proven to be identical to the controls. The second myopathy case (L.H.) showed no enzyme defect in oxidative phosphorylation but an approximately 50% reduction in mitochondria. This was verified by measurements of citrate synthase, glutamate oxaloacetate transaminase and adenylate kinase. The activity of pyruvate kinase was increased in the muscle homogenate and decreased levels of flavoprotein and pyridine nucleotide fluorescence. Therefore, functional investigations of mitochondrial oxidative phosphorylation in saponin-skinned muscle fibres allow a reliable detection of mitochondrial defects (mitochondrial myopathies and encephalomyopathies) in biopsy specimens containing less than 50 mg muscle tissue.
Myotonic dystrophy kinase is localized at neuromuscular and myotendinous junctions


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The manifestation of myotonic dystrophy (DM), a multisystemic inherited disease characterized by myotonia, muscular weakness and abnormalities in several other tissues, is correlated to a mutational expansion of an unstable [CTG]DNA-repeat. This repeat is in the 3' non-coding exon of a gene encoding a protein kinase (Myotonic dystrophy kinase, DM-PK), the most likely candidate to be causally involved in disease manifestation. In the DM-PK protein the following putative distinct domains can be distinguished: (i) a N-terminal signal peptide, (ii) a serine/threonine kinase catalytic domain, (iii) a glycosaminoglycan attachment site, (iv) an a-helical domain and (v) a C-terminal hydrophobic stretch. However, due to alternative splicing, multiple DM-PK isoforms may exist. We have raised and affinity purified specific rabbit antisera against synthetic DM-PK oligopeptides, and studied its subcellular localization in rodent and human muscle fibres as well as in cultured skeletal muscle cells using indirect immunofluorescence assays. In vitro, DM-PK shows a perinuclear, probably intracellular membrane-related localization in proliferating satellite cells, while the diffuse distribution pattern seen in young myotubes changes to a co-localization with clustered AChR upon further differentiation. A homogeneous reactivity was also found in immature muscle fibres in mouse embryos and regenerating fibres in human tissue specimens. Muscle fibre maturation was accompanied by a conspicuous concentration of DM PK in neuromuscular and myotendinous junctions. Furthermore the sarcoplasm of type I fibres and sites at or near the sarcolemma were weakly stained in cryosections of adult skeletal muscle. In cardiac tissue DM-PK was found to be localized in intercalated discs, while in smooth muscle cells dense plaques were stained. Remarkably, the distribution pattern of DM-PK in skeletal muscle sections from congenital and adult DM patients did not obviously differ from that in normal skeletal muscle. Our results and earlier classification of congenital DM as a disease characterized by an attenuated fibre development due to a responsiveness to innervation, point to a function for DM-PK in the regulation of synaptic signal mediation or the assembly of the junctional architecture. Further progress is dependent on the availability of better tools for in situ quantification of DM-PK fluorescent signals and the generation of more specific reagents for any of the different DM-PK isoforms.
Mechanisms of slowing of relaxation in fatigue

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Slowing of relaxation is an important feature of the decline in performance which occurs in skeletal muscle during repeated activity. This slowing may in principle be due to a reduced rate of crossbridge cycling or slowed Ca²⁺ handling. To distinguish between these two mechanisms, we have developed a method to study changes of the time-course of Ca²⁺ handling. For this purpose the intracellular free Ca²⁺ concentration ([Ca²⁺]i) was measured with indo-1 and changes of [Ca²⁺]i during tetani were converted to force by means of the steady-state [Ca²⁺]i-force relation. In this way a calcium-derived force record was created which represents a situation where force responds to changes in [Ca²⁺]i without delays due to crossbridges. This method has been applied to results from single muscle fibres from mouse and Xenopus which were fatigue by repeated, short tetani. During fatigue the fibres displayed a marked slowing of relaxation and this was accompanied by a reduced rate of [Ca²⁺]i decline. In mouse fibres the slowed [Ca²⁺]i decline was fully counteracted by a reduced myofibrillar Ca²⁺ sensitivity and hence the relaxation of calcium-derived force remained virtually unchanged during fatigue. Thus, in this preparation the slowing of relaxation during fatigue appears to reflect slowed cross-bridge kinetics. In Xenopus fibres, on the other hand, the relaxation of calcium-derived force was slowed during fatigue, which indicates that the slowing of relaxation in this preparation reflects impaired Ca²⁺ handling.

Investigations of control of oxidative phosphorylation in saponin-skinned muscle fibres

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Mitochondrial oxidative phosphorylation in skeletal muscle was functional investigated in saponin-skinned muscle fibres from rat M. soleus. After 30 min of incubation of muscle tissue in a saponin containing permeabilization medium more than 85% of mitochondria were accessible to functional investigations. Oxidative phosphorylation in these fibres can be stimulated in the presence of ATP by the addition of 15 mM caffeine. This effect was attributed solely to the activation of actomyosin ATPase due to the caffeine induced calcium efflux from sarcoplasmic reticulum (Kunz et al., FEBS Lett. 323, 166–90, 1993). More directly, the respiration of saponin-skinned muscle fibres can be stimulated in the presence of ATP approximately 3-fold by the addition of 2 μM free calcium. This calcium-dependent fibre respiration is sensitive to the action of the inhibitor of actomyosin ATPase, vanadate, to the inhibitor of the mitochondrial adenine nucleotide translocase, carboxyatractyloside and to the inhibitor of mitochondrial H⁺-ATPase, oligomycin. Therefore, using these inhibitors the control coefficients of the mentioned enzymes on mitochondrial oxidative phosphorylation flux can be determined. The titration of fibre respiration with carboxyatractyloside and oligomycin resulted in sigmoidal titration curves from which the flux control coefficients of the adenine nucleotide translocase and H⁺-ATPase were calculated to be equal to 0.25 and 0.16, respectively. Using vanadate the flux control coefficient of actomyosin ATPase was determined on the basis of titrations of respiration and of fibre actomyosin ATPase activity. Its value was found to be equal to 0.51. Therefore, the control of mitochondrial oxidative phosphorylation in skeletal muscle seems to be distributed mainly between phosphorylation reactions and actomyosin ATPase.
splice variants of the L domain in porcine skeletal and cardiac muscle. After first strand synthesis using primers antisense to sequences in exon 4 or 12 in muscle total RNA, PCR amplification using two further sense primers chosen from predicted exon 1 in the 5' untranslated region and exon 2 was carried out. Skeletal muscle RNA showed a single PCR product of 146bp with the first primer pair and 713 with the second, as predicted for the exon 3-deleted variant. However cardiac RNA showed in addition to this further products of 223 and 90bp with the corresponding primer pairings, which indicated the full-length targetted calpastatin version. RT PCR products from heart were always more intense than from skeletal muscle, reflecting increased abundance of calpastatin mRNA in line with inhibitory activity. The identity of the PCR products was confirmed by sequencing, and the implications of this difference between the exon usage in the two muscle types for the translation of mRNA and for processing and function of the protein are currently being investigated.

**In vivo expression and molecular characterization of the porcine slow-myosin heavy chain**

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Muscle growth in agricultural animals, such as the pig, is of major scientific and commercial interests. Since the final determinant of muscle growth is the laying down of myofibrillar proteins, one approach to elucidating the molecular processes of growth is to determine the regulation of muscle-specific genes. To this end, the porcine slow-myosin heavy chain (HC) β gene and its 5' end cDNA were isolated and characterized. *In vivo expression study, by in situ hybridization and histochemistry, revealed a highly regular rosette pattern of fibre arrangement, with a slow fibre occupying the central core, in all skeletal muscles examined. This feature can be advantageous in the distinction of primary and secondary fibres in myogenic lineage studies. In the neonatal heart, β isoform expression is diffuse, with higher expression occurring in the ventricle than in the atrium. Transient transfection assays showed the porcine promoter functions in a muscle- and differentiation stage-specific manner. In the 5' regulatory region are several putative positive and negative regulatory elements, including a positive and a negative element in close proximity to each other in intron 1.**

A molecular genetic and cell biological analysis of neuromuscular remodelling in the kyphoscoliotic mouse (ky)


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The autosomal recessive murine muscle disease, kyphoscoliosis (ky), has muscle fibre degeneration in tonic muscles (eg. soleus) sometimes from different nerves sometimes one junction is supplied by a sprout from another nearby. We have also localized several junction-associated proteins and found that 43 kDa was associated with AChR in ky muscles but AChR could be found without 43 kDa. We have also shown that the soleus muscles of older ky mice are significantly weaker and express only the slow myosin heavy chain, presumably as a long term consequence of the abnormal neuromuscular interaction. The long term outcome of degenerative muscle disease is not simply a consequence of the relative efficiency of muscle regeneration but is a consequence of the complex interaction of genes regulating nerve and muscle differentiation and stability.

Using an interspecific backcross segregating the ky mutation we have mapped the ky locus to chromosome 9 and it is non-recombinant with the microsatellite marker D9Mit24. We have eliminated most obvious candidate proteins (AChR's, AChE, myosin, actin, 43 kDa protein, dystrophin, DRRP etc) which might otherwise have been implicated in the development of the ky disease. Based on current evidence the ky mutation would appear to lie in a gene coding for an as yet unidentified junction-associated protein.

The ky mouse is a valuable experimental system for the investigation of the molecular and cellular processes which precipitate abnormal gene expression following initial muscle fibre death.

**Mouse-twist is an inhibitor of muscle differentiation**

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The twist gene codes for a protein with a HLH domain which is conserved from insects to vertebrates. In *Drosophila* twist plays an important role in gastrulation and is expressed in mesodermal derivatives. The mouse twist gene also is expressed in mesodermal cells as well as in neural crest cells which develop into mesoderm-like structures. During mouse embryogenesis twist can be detected in the somite. In the process of somite differentiation into derma-myotome and sclerotome twist expression becomes restricted to the sclerotome. It is not expressed in the myotome where the myogenic HLH gene *myf5* is active. This is a striking similarity to *Drosophila* where twist is expressed in muscle precursor cells and disappears at the time of expression of β-tubulin, an early myogenic marker. Therefore a common function of twist might be to prevent premature muscle differentiation in cells which are already committed to form muscle.

To test whether twist can act as an inhibitor of muscle differentiation we transfected myogenic mouse cells (C2C12) with a twist expression vector. Almost 10% twist expressing C2C12 clones analysed were greatly impaired in their ability to differentiate and to form myotubes when transferred to differentiation medium (2% HS). In the clones tested so far the degree of 'differentiation incompetence' correlates with the level of twist expression.

If one function of twist is to inhibit premature muscle differentiation, this inhibition should be reversible. Therefore we tested whether the muscle differentiation capacity could be restored by neutralizing twist with antisense oligonucleotides. Following transfer to differentiation medium containing twist antisense oligonucleotides, several clones tested differentiated normally. The effect of antisense oligos was concentration dependent, random sequenced and sense oligos had no effect.

These experiments show that the mouse twist gene can act as an inhibitor of muscle differentiation and that this inhibition is reversible.
Transcriptional regulation of two mouse myosin alkali light chain genes in vitro and in vivo

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Tissue and cell-type specific transcription is determined by the interaction of trans-acting factors with several regulatory modules of a gene. (i) the TATA box and/or the initiator region. (ii) the proximal promoter region and (iii) enhancer(s). We have chosen two mouse myosin light chain (MLC) genes, MLC1A and MLC1F/3F, to dissect different levels of transcriptional regulation during myogenesis in vitro and in vivo. For the MLC1A promoter we show the physical interaction of a general transcription factor, TFII-I, with the initiator region. This protein/DNA interaction is impeded by addition of myogenin or El2, respectively. Heterodimerization of myogenin and El2 however rescues TFII-I binding to the initiator region. Immuno-precipitation experiments show that physical interactions between TFII-I, a 120 kDa protein containing several HLH motifs, and myogenin and El2, respectively, could be responsible for the observed regulation of initiator binding activity. Implications for multiple regulatory pathways will be discussed. The complex regulation of temporal and spatial gene expression seems to require the involvement of one or several enhancers in addition to the proximal promoter. In the case of the MLC1F/3F gene, which shows differential regulation during muscle development, an evolutionarily conserved enhancer 3' to the locus is required for muscle specific expression from the MLC1F promoter in vitro and in vivo. We have analysed the role of this enhancer on expression from the MLC3F promoter in muscle cells in culture. A second regulatory element has been identified in the large first intron of this gene which enhances MLC3F expression to an extent equivalent to the downstream enhancer in differentiated myotubes. Furthermore, the intron and downstream activating elements interact cooperatively to augment reporter gene expression in vitro. The relative roles of these two elements on transcription from the MLC3F promoter during myogenesis in vitro is being analysed in lines of transgenic mice.

Transcriptional regulation of the mouse gene encoding the muscle-specific isoform of the glycolytic enzyme enolase (β-enolase)

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Enolase (2-phospho-D-glycerate-hydrolyase) is a dimeric enzyme of the glycolytic pathway. We have previously shown that the expression of the gene encoding the β subunit of enolase ('β-gene') is early marker of striated muscle development in the mouse. β-gene transcripts are first detected in the embryo, in the cardiac tube and in newly formed myotomes, and further accumulate at selected stages of foetal and post-natal muscle development (Keller et al., M.O.D. 38, 41–54. 1992. Lucas et al., Differentiation 51, 1–7, 1992). Our current studies are directed towards an understanding of the genetic and epigenetic mechanisms that confer to β-gene its specific spatial and temporal patterns of expression. The results reported here concern the regulation of β-gene during myogenic differentiation in cultured cell lines. Contrary to most muscle-specific structural genes, β-gene is already transcriptionally active in dividing myoblasts. β-gene expression is accompanied by an increase in the level of β-transcripts. It was therefore of particular interest to assess the metabolic stability of β-mRNA in myoblasts and myotubes. Newly-synthesized RNA was pulse-labelled in the presence of thiouridine, isolated by mercured agarose affinity chromatography and analyzed by RNA-blot hybridization. The data indicate that the accumulation of β-mRNA during terminal differentiation is primarily transcriptionally regulated. With the aim of characterizing the cis-acting DNA sequences and cognate transacting factors involved in the regulation of β-gene transcription, we have cloned and sequenced the mouse gene and flanking regions. A single transcription start site has been found. The promoter region as well as the first intron show putative binding sites for trans-acting regulatory factors and their functional significance has been assessed by transient transfection analysis in C2 myogenic cells and in fibroblasts. The transcriptional activity of the β-gene in C2 myotubes was found to depend on a genomic region present into the first intron and showing the properties of a muscle-specific enhancer. The characterization of the DNA sequences involved in the specific binding of nuclear proteins is underway.

In vitro transfection of β-gal gene in satellite cells from adult rat muscle and their incorporation in regenerating myofibres

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We have explored the use of satellite cells as somatic tissue for the introduction of foreign genes in adult muscles. Satellite cells were isolated from Bupivacaine-damaged Tibialis Anterior muscles of 200 g Wistar rats by the procedure described by Funanage and colleagues (Funanage et al., J. Cell. Physiol. 150, 251–7, 1992). After 4–5 days in culture the cells were transfected by ß-Gal gene (Sanes et al., EMBO J. 11(12), 3133–42, 1992). After additional 24 h the cells were resuspended in PBS and injected into Bupivacaine-treated EDL muscle (about 3 000 000 cells). After ten days the animals were killed and the EDL muscles were fixed and reacted for ß-Gal. Transfected satellite cells were also cultured in vitro for 10 days and then processed for β-Gal. Neonatal rat myoblasts, transfected in vitro by β-Gal gene and injected into Bupivacaine-treated EDL muscle, were also used as control. Major achievements of our study are: (1) the treatment of muscle with Bupivacaine results in an increased (more than five times) yield of satellite cells; (2) the satellite cells transfected with the β-Gal gene and injected into the muscle fuse with the regenerating cells to form mature myofibres. These results indicate that muscle precursor cells isolated from adult muscle of a patient can be transfected in vitro and then reintroduced in vivo as a means of genetic therapy.
Control of the caldesmon isoform expression by differential mRNA splicing

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SDS-PAGE has shown there to be three isoforms of the caldesmon protein in all the species we have examined. One isoform corresponding to the non-muscle isoform (537 amino acids in humans) has an apparent molecular weight of 80 kDa. The other two isoforms, one of which corresponds to the 793 amino acid protein sequenced by Humphrey and colleagues (Gene 112, 197–204, 1992) migrate closely, have an apparent molecular weight of 120 kDa and are expressed in contractile smooth muscle cells.

A recent publication by Hayashi and colleagues (Proc. Natl. Acad. Sci. (USA) 89, 12122–6, 1992) has revealed the various isoforms are derived from differential splicing of mRNA transcribed from a single gene. The large isoforms contain exons 1, 2, 3a, 3b, 5, to 13, the small isoform contain exons 1, 2, 3a, 5, to 13. The two large isoforms we believe are formed by the presence or absence of exon 4 which codes for 26 amino acids at the C terminus of the central helix domain (chain A, 1991, Marston & Redwood, Biochem. J. 279, 1–16).

Hayashi also found evidence for the substitution of the establish first exon (exon 1) and for alternative exon 1 in the mRNA of some cell lines.

mRNA expression of the isoforms has been detected using RT-PCR amplification in various tissues from a variety of species using primers that specifically amplify exons 1, 11, 3a, 3b and 4. To date CDh with and without exon 4 and CDh without exon 4 have been detected in most tissues from all the species tested. CDh with exon 4 has only been detected on amplification of CDh clones from a human aorta lambda gt10 library. Exon 1 has not been detected in any sample derived from untransformed tissue.

The two heavy isoforms can be separated on long 6% acrylamide SDS-PAGE gels. Equal amounts of each isoform were seen on extraction of protein from vascular smooth muscle tissue as determined by Coomassie Blue staining. We have raised antibodies specific to exon 1, exon 3b and a non specific antibody. These are being used to investigate the isoforms expressed in a variety of tissues. We have found that there are indeed at least two CDh isoforms expressed at the protein level, however both CDh bands are anti-exon 4 serum immuno reactive. There appears to be two populations of CDh protein, one containing exon 4 and one not, each population is composed of two proteins of different sizes. Each of these two proteins has a similar Mr in each population. We plan to examine these populations more closely for sequence differences and post translational modification.

The additional exons expressed by the two CDh isoforms are not involved in the regulatory function of the caldesmon however the exon 3b amino acids are known to bind tropomyosin. We hypothesize that since nonmuscle tropomyosin is shorter than smooth muscle tropomyosin, this may define the different sizes of caldesmon isoforms. It could also be hypothesized that an equimolar ratio of CDh with and without exon 4 is required to accurately bind tropomyosin in the smooth muscle thin filament.

Muscle mutants - what can they tell us about muscle development and function?

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In recent years Drosophila muscles, especially the indirect flight muscles (IFMs), have been increasingly used to investigate muscle structure, function and development because of the opportunities this organism provides for genetic studies. Drosophila IFMs are asynchronized striated muscles, similar in structure and function to those extensively studied in the water-bug, Lethocerus (Peckham et al., J. Muscle Res. Cell Motil. 11, 203–15, 1990). Most of the major muscle protein genes from Drosophila have been cloned, sequenced and their expression characterized (reviewed Bernstein et al., Int. Rev. Cytol. 143, 63–152, 1993). While this has also been achieved in a number of other species, including various vertebrates, only in the nematode Caenorhabditis elegans and the fruitfly Drosophila melanogaster have the amenable genetics permitted recovery of mutants in the major myofibrillar protein genes and other genes involved in myogenesis. However, with the development of a number of genetic, physiological and biochemical techniques it is now also possible to perform both mechanical (Drummond et al., Nature 348, 480–2, 1990) and biochemical experiments on mutant Drosophila muscles despite their rather small size (largest IFMs are 1.5 mm in length). By combining such approaches with biochemical studies of the mutant muscle proteins one can investigate the molecular biology of muscle contraction and its regulation.

Mutants affecting Drosophila IFMs are easily recovered because of their effect on flight ability. The technique of germine transformation also permits the in vivo study of site-directed mutations induced in cloned genes by in vitro mutagenesis (reviewed in Sparrow et al., J. Cell Sci. 114, 73–8, 1991). Using mutants of the major muscle proteins insight can also be gained into processes involved in myofibrillogenesis (Sparrow et al., S.E.B Symposium 46, 111–29, 1992).

Inhibition of neuritogenesis by antisense arrest of the expression of a specific isoform of brain myosin II

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Sequence data is now available for at least three brain myosin II heavy chain isoforms (Sun & Chantler, J. Cell Biol. 115, 329a, 1991; J. Mol. Biol. 224, 1165–95, 1992), made possible by screening a rat brain cDNA expression library with a polyclonal antibody against neuroblastoma myosin II, characterized previously (Miller et al., Neuron. 8, 25–44, 1992). We have attempted to determine the specific function performed by each of these isoforms through an approach involving the transfection and intracellular expression of antisense oligonucleotides. Isoform-specific sequences from all three brain myosin II heavy chains were inserted in appropriate orientation onto the directional cloning site of the eukaryotic expression vector, RECMV, so as to express either sense or antisense probes upon transfection into cells of the mouse neuroblastoma cell line, Neuro-2A. Neuritic process outgrowth was completely inhibited upon transfection of these cells with the construct expressing an antisense probe complementary to
the message for one particular isoform, the neuronal myosin heavy chain characterized previously (Sun & Chantler, J. Mol. Biol. 224, 1185–95, 1992). Neither transfection in the absence of construct, sense probes specific for this isoform, nor antisense or sense probes complementary to message for the two other brain myosin heavy chain isoforms considered, abolished process outgrowth. Cell division was not disrupted by the expression of any of the above probes. All antisense probes used in these experiments were shown to hybridize to the expected message and were able to arrest translation. Sense probes did not interfere with the translation process. These data implicate a specific isoform of non-sarcomeric myosin II as a key player in the mechanism of neuritogenesis.

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**Effect of mechanical work load on expression of muscular collagens pre- and post-translational regulation**

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Decreased (Savolainen et al., Am. J. Physiol. 283–8, 1987) and increased (Mylllyla et al., Pflugers Arch. 407, 04–70, 1986) rate of collagen synthesis seems to occur in skeletal muscle after immobilization and a single bout of prolonged running, respectively. In the present study we investigated the effect of running and cast immobilization on the expression of different collagen types and prolyl 4-hydroxylase (PH), the key up- and post-translational modulator of collagen chains, in rat hind limb muscles. The concentrations of mRNAs for type I and III collagen measured by Northern and slot blot hybridizations were decreased after immobilization for 3 days in soleus, gastrocnemius, plantaris and tibialis anterior muscles. The level of mRNA for α-subunit of PH was decreased already after 1 day of immobilization and PH activity 2 days later. mRNA levels for non-fibrous type IV collagen and β-subunit of PH were unaltered or slightly increased through the one week experimental period. mRNA levels for type I, III and α-subunit of PH were increased 2 and 4 days after running at a speed of 17 m min⁻¹ for 90 min in rectus femoris muscle, in which the exercise induced muscle damage was most pronounced. mRNA for type I collagen, but not for type III collagen, was increased in soleus muscle and in red part of gastrocnemius muscle after exercise. No change in any mRNA measured was observed in white part of gastrocnemius or in tibialis anterior muscle. mRNA for non-fibrous type IV collagen was unaltered in all muscles studied. The increased expression of mRNAs for the fibrous collagens may be a part of the reparative process after exercise induced muscle damage and/or physiological response to increased force transmission. The expression of PH α-subunit is known to be the limiting factor in the formation of active PH, which correlates with the biosynthesis rate of collagen.

The results suggest that decreased or increased physical activity of skeletal muscles causes respective coordinated pretranslational down regulation or upregulation in the expression of PH and the two force transmitting fibrous collagens in skeletal muscle.

**Regulation of the expression of myogenic regulatory factors during mouse development**

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Four myogenic regulatory factors have been identified, each able to trigger myogenic conversion in a variety of cultured cells. They are called myf-5, myogenin, herculin and myoD1, and are members of the superfamily of basic-helix-loop-helix (bHLH) transcription factors. Each will bind, most efficiently, as heterodimers with the products of the ubiquitously expressed E2 genes, to the consensus sequence (CANNTG) known as the E-box. Such binding leads to activation of the transcription of muscle-specific genes, to autoregulation of the genes encoding the regulatory factors themselves, and, in some cases, to cross-regulation of the genes encoding other regulatory factors. During muscle development the first gene to be activated in the trunk is myf-5, at 8.0 days post coitum (dpc), followed half a day later by myogenin. In the limbs activation occurs 2.5 days later. We have used transgenic mice in order to define DNA sequences required for proper temporal and spatial regulation of myogenin expression. Our data show that only 133bp of 5′ flanking DNA are necessary. These sequences contain a single E-box which is essential, showing that there is a bHLH protein upstream of myogenin. We presume that this is myf-5. If this E-box is mutated and transcription restored by providing another E-box upstream, we observe normal expression in the trunk but an effect on expression in the fore limbs. This is of interest given that trunk and limb muscles have different embryological origins. The 133bp also contains a binding site for the RSRF family of transcription factors which comprise the DNA binding activity called MEF-2. Mutation of this site does not affect activation of expression in the trunk or limbs. However, it perturbs the trunk expression pattern at 10.5 dpc with some cells expressing the transgene and some not, indicating that there are at least two classes of cells. We have furthermore shown that a single E-box and a single RSRF site are probably sufficient for muscle-specific expression. We are presently attempting to define the regulatory elements that control myf-5 expression and to ascertain exactly where and when the various RSRF genes are expressed. Our objective is to identify the transcription factor which first triggers myogenic differentiation and, by studying its regulation, to identify the extra-cellular signals which control muscle formation.

**Development and differentiation**

M-cadherin in rat skeletal muscle

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The integral membrane protein M-cadherin is supposed to be involved in cell-cell adhesion mechanisms in skeletal muscle tissue. It was expressed at low levels in myoblasts and was upregulated in myotubes when a mouse cell line was investigated with a polyclonal antibody and immunofluorescence staining (Donalies et al., PNAS 88, 8024–8, 1991). The aim of this study was to find out in which cell types within normal rat muscle M-cadherin is expressed in vivo; whether its expression changes in denervated or regenerating fibres; and where M-cadherin is localized at the ultrastructural level. Denervation of the soleus muscle was performed by removing a segment from the sciatic nerve. Aneurysmal regenerates were produced by devascularization of the soleus muscle and by denervation as described above. Cryostat sections of the muscle tissue were used in order to gain information about the distribution of M-cadherin-containing cells within the tissue; thick and thin sections were prepared in order to evaluate the distribution of M-cadherin at the cellular level. In both normal and denervated muscle, M-cadherin was localized in satellite cells. In aneurysmal regenerates, it was found in mononuclear myogenic cells which were attached to myotubes but not in myoblasts in isolation. In myotubes, staining was equivocal. It was, however, present in satellite cells attached to normal muscle fibres which had remained intact at a distance from the necrotic zone.

M-cadherin was always localized at the plasma membrane of the respective cell. The presence of M-cadherin may parallel the readiness of a mononuclear myogenic cell to fuse with a myotube or a muscle fibre.
ATP splitting among skeletal muscle fibres is related to the presence of different myosin heavy chain (MHC) and/or alkali myosin light chains and Tibialis anterior muscles of the rat were maximally conducting and gating properties replaces the foetal AChR. Previous experiments indicated that the motor nerve induces trophically at the junction of synaptic AChR clusters, indicating that it was caused by proteolysis of BL components. AChR clustering and induction of e-mRNA are not related, however: AChR clustering in cultured myotubes by recombinant agrin, which is thought to mediate nerve-induced AChR clustering at the synapse (rev. in McMahan et al., *Carr. Op. Cell Biol. 4*, 869-74, 1992), left e-mRNA levels unaffected.

### Basal lamina controls the expression of the epsilon-subunit of the acetylcholine receptor at the neuromuscular junction

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Skeletal muscle fibres express two functional subtypes of acetylcholine receptor channels (AChRs), termed fast and slow, that are composed in different stoichiometries of five subunits α, β, γ, δ and ε (Mishina et al., *Nature 321*, 406-11, 1986). Before innervation, the foetal AChR containing α, β, γ and δ-subunits is expressed constitutively throughout the muscle membrane. When muscle fibres are innervated, expression of AChRs becomes restricted to the site of the neuromuscular contact and then the ‘adult’ e-type composed of α, β, ε- and δ-subunits and showing different ion conducting and gating properties replaces the foetal AChR. Previous experiments indicate that the motor nerve induces trophically at the site of the synaptic contact the activity-resistant expression of subunit mRNAs encoding the adult AChR subtype, while in the extrasynaptic fibre segments, their synthesis is down-regulated by muscle activity (Witzemann et al., *J. Cell Biol. 114*, 125-41, 1991). The neural signal involved is not known. It is very stable, however, as after denervation, expression of e-mRNA remains localized to synaptic sites for as long as 130 days. We have now obtained several lines of evidence that it is associated with the synaptic portion of the basal lamina (BL): (1) When muscle fibres were destroyed and left to regenerate inside vacated original basal lamina sheaths from satellite cells in the absence of the nerve, synapse-specific expression of AChR e-subunit gene was observed where the old synapses had been. (2) When rat myotubes were cultured on BL isolated from adult rat muscle, they expressed AChR e-subunit mRNA at increased levels at sites where they contacted synaptic BL. (3) Treatment of adult rat flexor digitorum brevis (fDB) muscle with collagenase/trypsin produced a specific decrease in e-mRNA in the synaptic region of fDB fibres; the same treatment caused dispersal of synaptic AChR clusters, indicating that it was caused by proteolysis of BL components. AChR clustering and induction of e-mRNA are not related, however: AChR clustering in cultured myotubes by recombinant agrin, which is thought to mediate nerve-induced AChR clustering at the synapse (rev. in McMahan et al., *Carr. Op. Cell Biol. 4*, 869-74, 1992), left e-mRNA levels unaffected.

### A carp myosin heavy chain gene that is expressed in small muscle fibres

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Whilst the expression of different isoforms of both the heavy and light chains of myosin are well characterized in mammals (EL Ha), Molecular Biology of Muscle. SEB publication, Company of Biologists Ltd., Cambridge UK, 1992), little is known about the myosin isoforms present in fish. Work in our laboratory has focused on the genes coding for the myosin heavy chain protein in carp (*Cyprinus carpio*).
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We have constructed a carp genomic library and isolated 28 different lambda clones which contain MHC gene sequences (Gerlach et al., Mechanisms of temperature acclimation in the carp: a molecular approach. Am. J. Physiol. 259, R237–48, 1990). We present here the partial characterisation of one of the carp MyoHc isoforms we have isolated. The genomic clone FG2 was restriction mapped and partially sequenced to reveal the location of various exons. The clone contains a complete MyoHc gene which is approximately 12.0 kb long, from transcriptional start site to polyadenylation signal, and transcribes to a mRNA of approximately 6000 nucleotides. Analysis of sequence data generated from the exons of this clone revealed a high degree of homology with published mammalian skeletal muscle MyoHc genes. Intron sequences however are approximately 50% shorter than in mammalian MyoHc genes. The peculiarities of vertebrate skeletal muscle regeneration

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The regeneration of skeletal muscle tissue from mammals (rodents) and fishes (sturgeons) was studied under different conditions (age, laser, irradiation, training exercises, pollution of environment). There is opinion that regeneration of tissues declines with the animals' age. Our data showed that during early postnatal period, the regenerative capacity of skeletal muscle tissue in rats and guinea pigs was worse than in adult and even old animals. Most newborn muscle grafts did not recover their contractile function. The age peculiarities of skeletal muscle regeneration were the same both in immature-born and in mature-born animals. Local irradiation in high doses suppresses the capacity of skeletal muscle for posttraumatic regeneration. Laser rays improved the recovery of muscle organ by means of stimulation of inflammatory reaction and proliferative activity of myogenic cells. Many unfavourable factors of environment result in degeneration of muscle tissue both in mammals and fishes. Our observations suggest that the destruction of myofilament apparatus of sturgeon was correlated with direct and indirect influence of various water pollutants. The regeneration process of sturgeon muscle tissue is impossible under existing conditions (pollution). Experimental data showed that myogenesis of young fishes was disturbed by such factors as heavy metals, nitrates and others. Intensification of functional activity of rat skeletal muscles caused destructive-regenerative processes in muscle tissue. At the ultrastructural level lesions of myofilaments, myofibrils, mitochondria, focal denervation of some red muscle fibres in m. quadriceps femoris were revealed in the endurance-trained rats (regular run sessions on treadmill from 5 min till one hour a day for 6 weeks at a speed of 35 m min\(^{-1}\)). Regeneration was characterized by the increased number of satellite cells, hyperplasia of muscle fibres, renewal of axon terminals. Thus, the regenerative process in skeletal muscle tissues depends on various factors which may stimulate or decrease it.

Myosin heavy chain changes in cat hemidiaphragm reinnervated by the ipsilateral recurrent laryngeal nerve

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Progressive changes in myosin heavy isoforms and in energy-generating enzyme activities were followed up in the hemidiaphragm of six cats subjected to experimental denervation and selective reinnervation by the recurrent laryngeal nerve (RLN). Under deep Nembutal anesthesia, through an intercostal approach the right hemidiaphragm was denervated by severing the phrenic nerve; after partial removal of the thyroid cartilage, the right RLN was dissected down to its two terminal divisions (aductor and adductor branches) and anastomosed to the ipsilateral superior or inferior roots of the phrenic nerve. The animals were tested electrophysiological from 173 to 363 days after the anastomosis to assess the functional rehabilitation of the paralyzed hemidiaphragm. After completion of the final recording session, the cats were killed by Nembutal overdose; the whole diaphragms were then removed and immediately frozen in isopentane cooled at −190°C in liquid nitrogen. Myosins were extracted from each diaphragmatic quadrant (A + B = normal hemidiaphragm; C = reinnervated by the RLN; adductor branch; D = reinnervated by the adductor branch). ATPase of myofibrill/myosin proteins was evaluated histochemically on representative cryostat sections taken from each quadrant and processed with the method of Padykula and Herman (1958). Monodimensional gel electrophoresis of myosin heavy chains was carried out in the presence of SDS. A 40–90% increase in the type 1 heavy chains was noticed in both reinnervated quadrants. All myosins were of the adult type, confirming the previous results of one of us (Carraro et al., Muscle & Nerve 5, 515–24, 1982) on the ability of chronically denervated diaphragm to maintain its constitutional structure independently of the continuous presence of neural control. The shift observed between type I and type 2 heavy chains in the quadrants reinnervated selectively by either one of the RLN branches might be the expression of the adaptation the reinnervated hemidiaphragm undergoes as a consequence of the different workload it has to bear because of the smaller number of axons the RLN can regrow into the phrenic stump.

Fibre type characteristics and meat quality in longissimus muscle of normal and halothane sensitive pigs

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The current pig breeds show remarkable differences in the adaptation to environmental conditions. The reasons are alterations in the anatomy and physiology of the animals which entail increased losses during transportation and poor meat quality. Practical methods for determining stress susceptibility are the two halothane tests, i.e. the traditional halothane mask test as well as the recently used halothane gene test. In response to these tests stress susceptible pigs react with intensive muscle contracture (halothane positive pigs), whereas stress resistant ones do not show such reaction (halothane negative pigs). The halothane reaction corresponds with the malignant hyperthermia syndrome. Biopsy samples from longissimus muscle of pigs were taken at the age of 200 days (n = 90; German landrace) for histological and histochemical investigations of muscle fibre characteristics (and traits of capillarity). Furthermore, the serum CK-activity and parameters of meat quality post mortem were determined. Compared with the halothane negative group, halothane positive pigs showed a greater area of longissimus muscle, indicating an increased growth rate. The diameters of red, intermediate and white fibres were increased. There
was a difference in the fibre type composition showing a tendency higher frequencies of white, glycolytic fibres in the halothane positive group. Fibre hypertrophy in muscle of the halothane positive group was not significantly connected with a greater number of capillaries per fibre, indicating an insufficient supply of the cell with oxygen and nutrients substances. The meat quality of the halothane positive group was characterized by increased CK-activity; relatively high water loss during storage (drip loss), low pH value and pale meat. The results suggest that the decreased adaptability of the halothane positive pigs is in relationship with altered microstructural and physiological conditions, which are connected with more frequently occurring cellular defects in muscle cells.

Effects of thyroid hormone and increased neuromuscular activity on the expression of carbonic anhydrase III and parvalbumin in fast-twitch muscle of the rat

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Chronic low-frequency stimulation (CLFS) induces in rat fast-twitch muscle fibre type conversions in the order of types IIB → IID → IIA. As compared to the rabbit, where CLFS ultimately converts fast into slow-twitch fibres (type D), the fast-to-slow conversion is incomplete in the rat. Small increases in slow myosin HCl or in the percentage of type I fibres occur only after stimulation periods longer than 60 d. This is in agreement with our previous observation that carbonic anhydrase III (CAIII), which is thought to be expressed only in type I fibres, does not increase, neither at the mRNA nor at the protein level in fast-twitch muscles of euthyroid rats subjected to CLFS for up to 56 d (Jeffery et al., FEBS Lett. 263, 225–7, 1990). In view of our finding that the fast-to-slow transformation of rat muscle is greatly enhanced at reduced thyroid hormone levels (Kirschbaum et al., J. Biol. Chem. 265, 13974–80, 1990), it was of interest to investigate the combined effects of hypothyroidism and CLFS on the expression of CAIII. Parvalbumin (PA) is present at high concentrations in type IIB and type IID fibres (Schmitt & Pette, Histochemistry 96, 159–65, 1991) and represents an early marker of fast-to-slow fibre transformation (Leberer et al., Biofam., J. 239, 295–300, 1986). Therefore, PA was included in the present study. Hypothyroid rats were subjected to CLFS (10 Hz, 10 h/d) and analysed after various stimulation periods for up to 40 d. Using specific digoxigenin-labelled cRNA probes, we followed the expression of CAIII and PA at the mRNA level. Unstimulated, hypothyroid tibialis anterior (TA) muscle displayed markedly elevated levels of CAIII mRNA, as well as highly reduced levels of PA mRNA. CLFS led to further increases in CAIII mRNA, concomitant with rapid decreases in PA mRNA. CAIII mRNA in 17-d-stimulated TA was 150% above its level in slow-twitch soleus muscle. Longer stimulation periods (up to 40 d) did not yield higher increases in CAIII mRNA. Conversely, PA mRNA decayed rapidly after the onset of stimulation. PA mRNA was no longer detectable in 8-d-stimulated muscles. These results confirm and extend the previously observed antagonism between thyroid hormone and enhanced neuromuscular activity. Thus, the expression of CAIII, which is not inducible by CLFS in the euthyroid rat, is drastically increased in the hypothyroid state. Furthermore, the repression of PA by CLFS is greatly enhanced under the same conditions.

Selective changes in myofibre differentiation with temperature and nutrition suggest a key role for rhomboideous muscle in thermoregulation

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The proportion of oxidative fibres in longissimus dorsi (LD) muscle has been shown previously to be greater in young pigs living at a low (10°C) compared with a high (35°C) ambient temperature, under conditions of controlled energy intake (Dauncey & Ingram, Eur. J. Appl. Physiol. 58, 239–44, 1988). The present investigation has determined whether this is a specific effect of low temperature and whether the proximity of the source of heat production to the thermosensitive neurones of the cervical spinal cord may influence the extent to which myofibre type is affected. Six litters each of four 3-week-old male pigs were kept either close to thermal neutrality (26°C) or in the cold (10°C), and fed a high (H) or low (L) energy intake (where H = 2L), thus establishing four treatment groups: 26H, 26L, 10H and 10L. At 7 weeks of age animals were killed humanely, 24 h after feeding. The LD and rhomboideous (RH) muscles, lumbar/thoracic and cervical respectively, were assessed histochemically for myosin ATPase and succinate dehydrogenase activity, to evaluate fibre contractile (fast/slow) and metabolic (oxidative/glycolytic) properties. Cold-acclimation had a small but significant effect on the distribution of fibre types in LD muscle, with the proportion of Type I slow oxidative fibres being 8.5 and 11.6% at 26 and 10°C respectively (p < 0.05). By contrast, there were considerably more Type I fibres in RH muscle at 10 compared with 26°C (72 and 42% respectively; p < 0.001). Furthermore, in RH muscle there was a significant interaction between thyroid status and contractile activity associated with shivering predominated. These changes in myofibre differentiation of RH muscle would tend to conserve energy in animals at a low energy intake, was of major importance. By contrast, at 10°C compared with 26°C, the effects of increased contractile activity associated with shivering predominated. These changes in myofibre differentiation of RH muscle would tend to conserve energy in animals at a low food intake at 26°C, since Type I have a greater contractile efficiency than Type II (fast-twitch) fibres, and will also facilitate the prolonged low frequency muscular contraction required for shivering in the cold. The present results, taken together with the interscapular location of RH muscle near to the cervical temperature sensitive neurones, suggest a key role for this muscle in thermoregulation.
Expression of hepatocyte growth factor in rat skeletal muscle during growth and regeneration

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Hepatocyte growth factor (HGF) is a polypeptide growth factor originally isolated from serum of rats subjected to partial hepatectomy (Nakamura et al., Biochem. Biophys. Res. Comm. 122, 1450–9, 1984). It has since been cloned and sequenced in rats and humans. Rat HGF consists of two subunits, α and β, with 440 and 233 amino acids, respectively. Hepatocyte growth factor is expressed in a number of rat organs such as lung, kidney, thymus, spleen and brain. An increased expression of HGF has been demonstrated after tissue injury in liver and kidney (Matsumoto & Nakamura, J. Gastroenterol. Hepatol. 6, 509–19, 1991). Hepatocyte growth factor does not appear to be expressed in mature striated muscle. Hepatocyte growth factor’s possible involvement in muscle growth has not been investigated. The aim of this study was to investigate if HGF is expressed in rat skeletal muscle during normal postnatal growth and during regeneration after ischemic injury. A cRNA probe (Tashiro et al., Proc. Nat. Acad. Sci. 87, 3200–4, 1990) labelled with digoxigenin was used for Northern blots and in situ hybridization experiments. By Northern blot analysis no signal could be detected in RNA preparations from adult skeletal muscle, whereas in newborn rats two mRNA transcripts with estimated sizes of about 6 and 3 kb were demonstrated. These bands were prominent also in RNA preparations from regenerating muscle. By Northern blot analyses no signal could be detected in RNA preparations from adult skeletal muscle, whereas in newborn rats two mRNA transcripts with estimated sizes of about 6 and 3 kb were demonstrated. These bands were prominent also in RNA preparations from regenerating muscle. By Northern blot analyses no signal could be detected in RNA preparations from adult skeletal muscle, whereas in newborn rats two mRNA transcripts with estimated sizes of about 6 and 3 kb were demonstrated. These bands were prominent also in RNA preparations from regenerating muscle. 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with fish raised at ambient river temperature (Stickland et al., Anat. Embryol. 178, 253–77, 1988). In contrast, faster growing juvenile and adult salmon show an increase in muscle fibre hyperplasia. During embryonic growth until first feeding the fish are fully dependent on the nutrient supply of their yolk and growth during this period is an important factor for later survival. Additionally, protein synthesis at the expense of nuclear proliferation may represent a more efficient way to acquire an appropriate size. In mammals, for example, energy restriction while maintaining an appropriate protein supply leads to a reduction in nuclear proliferation in muscle with the fibre size remaining unchanged (Cheek & Hill, Fed. Proc. 29, 1503–9, 1970). One factor distinguishing the embryos from older fish is the restriction of physical space within the chorion. We therefore removed the chorion from embryos reared at ambient river temperature at Gorodilov stage 25 and incubated the fish in physiological saline at 5°C and 11°C until stage 31 or stage 33. It was found that by stage 31 and, more pronouncedly, by stage 33 the salmon exhibited a statistically significant reduction in average fibre size at the elevated temperature. The total muscle area was also significantly reduced. There was no difference in total muscle fibre number at stage 31 as determined from fibre numbers per unit area. However, at stage 33 the higher temperature fish exhibited a larger white muscle fibre number than their 5°C counterparts. These findings are very much in contrast to the situation found in chorionated salmon embryos. It therefore seems that the absence of a chorion radically alters the effect of temperature on the development of muscle cellularity. This work was supported by the NERC.

Defective maturation of viral glycoproteins in multinucleated L6 muscle cells

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Fusion of L6 myoblasts into multinucleated myotubes involves reorganization of the Golgi complex from a juxtanuclear position into a perinuclear ring structure (Ralsdon, J. Cell Biol. 120, 399–409, 1993). We found that during the fusion of L6 myoblastic cell line this conversion involved dispersion of a considerable portion of the Golgi into the cytoplasm. Electron microscopy studies revealed that a major part of the myotube Golgi complexes were disassembled, showing no stacked structure and existing as tubulovesicular structures reminiscent of the situation during mitosis. We analysed the functionality, i.e. exocytic processing of proteins in the morphologically changed Golgi apparatus, by utilizing enveloped viral glycoproteins as models. The myotubes could be readily infected by vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV), indicating that an acidic endocytic route existed. Pulse-chase labelling studies of myotubes infected with VSV showed that about half of the viral glycoprotein remained endoglycosidase H sensitive, indicating that half of the viral glycoprotein is not carbohydrate processing in the Golgi occurred. We performed double immunofluorescence studies using Golgi-specific antibodies, under conditions when protein synthesis was inhibited. Such analyses showed that all the viral glycoprotein arrived the Golgi complex. Cell surface labelling studies showed that only the terminally processed VSV glycoprotein was found on the surface. Thus the endoglycosidase H sensitive glycoprotein remained in the Golgi vesicles. When similar experiments were performed for cells infected with SFV, we found that about half of the SFV glycoprotein was blocked in the Golgi while the other half arrived at the cell surface. The SFV glycoprotein on the cell surface was fully matured while that remaining in the Golgi was immature. Our results suggest that the vesiculated form of the Golgi complex lost functionality. Since the morphology and functionality of the myotube Golgi did not recover upon ageing, it seems likely that the differentiation of the L6 myotubes is blocked at a stage where most of the Golgi complex has disassembled and lost functionality.

The expression of the fast-type isoform of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA1) in the L6 muscle cell line is stimulated by thyroid hormone and insulin-like growth factor-I through different mechanisms

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Thyroid hormone (T₃) is a major determinant of fast-type sarcoplasmic reticulum Ca²⁺-ATPase (SERCA1) levels in vivo. We previously showed that in the L6 muscle cell line, SERCA1 levels are greatly increased by T₃, while insulin-like growth factor-I (IGF-I) elevates SERCA1 levels to a small extent. However, the effect of simultaneously added IGF-I and T₃ proved to be more than additive (Muller et al., Biochim. J. 275, 35–40, 1991). To gain further insight in the mechanism of action of T₃ and IGF-I, we investigated the corresponding SERCA1 mRNA levels in L6 myotubes. Northern blot analysis showed that the SERCA1 mRNA level was strongly elevated by T₃ (240%), while IGF-I induced a moderate increase of 50%. In the presence of both T₃ and IGF-I, SERCA1 mRNA content increased by 140%. SERCA1 protein and mRNA increased proportionally in the presence of T₃ (protein/mRNA ratio: 0.80), while a significantly larger increase of SERCA1 protein vs mRNA was observed in IGF-I treated cultures (protein/mRNA ratio: 1.40). Addition of both T₃ and IGF-I resulted in an intermediate ratio of 1.06. The discrepancy between SERCA1 protein and mRNA increase by IGF-I may be explained by enhanced SERCA1 protein stability, since addition of IGF-I to T₃-treated cultures increased the half-life of SERCA1 protein from 15.2 ± 2.1 h to 29.0 ± 6.0 h (mean ± SEM, n = 4). The increase of SERCA1 mRNA levels by T₃ and IGF-I could result from regulation at transcriptional or post-transcriptional level or both. Results showed that the half-life of SERCA1 mRNA was not different in controls and T₃-treated cultures (17.0 ± 4.3 and 13.5 ± 2.6 h respectively), but addition of IGF-I to T₃-treated cultures increased the mRNA half-life to 32.5 ± 7.9 h. On the other hand, nuclear run-on assays showed a three-fold stimulation of SERCA1 transcription by T₃. In support of this it was shown that transient transfection of L6 cultures using SERCA1 promoter fragments coupled to the CAT gene gave a maximal 2.5-fold stimulation of transcription by T₃. We conclude that the synergistic stimulation of SERCA1 expression by T₃ and IGF-I results from regulation at different levels by these agents: T₃ primarily increases the transcription frequency of the SERCA1 gene, while IGF-I seems to act primarily at posttranscriptional levels by enhancing SERCA1 protein and -mRNA stability.

Assignment of two cDNA clones to biochemically and histochemically classified type IIb and IId fibres of rabbit muscle – a single fibre study

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Fast-twitch muscles of the rabbit contain fast fibre types IIb, IId and IIA. These can be distinguished by histochemical staining for myosin heavy chain (HC) isoforms (Aigner et al., Eur. J. Biochem. 211, 367–72; 1993; Hämäläinen & Pette, J. Histochem. Cytochem. 41, 733–43, 1993). We have previously established methods for analyzing specific myosin HC mRNA isoforms in fragments of single fibres. Thus, we were able to assign pMHC20-40 cDNA to type IIb fibres in rabbit adductor magnus muscle (Uber & Pette, J. Muscle Res. Cell Motil. 4, 253, 1993). The identity of an additional pFG cDNA clone, pMHC24-79 (Maeda & Wiltinghofer, unpublished), was investigated in the present study. Total RNA was extracted from freeze-dried fibre fragments, microdissected from 120 μm thick muscle cross sections. After reverse transcription, the assay mixture was transferred
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Prenatal and postnatal development of skeletal muscle is marked by changes of the sarcoplasmic reticulum (SR), both in membrane molecular composition (Yuan et al., J. Cell Biol. 112, 289-301, 1991; Damiani et al., Dev. Biol. 153, 102-14, 1992) and morphological architecture (Schiaffino & Margreth, J. Cell Biol. 41, 855-75, 1969; Franzini-Armstrong, Dev. Biol. 146, 353-63, 1991). Postnatal differentiation of SR is characterized by proliferation of terminal cisternae (TC), and the accumulation of specific functional SR proteins, calcequastrin (CS) and ryanodine receptor (RyR)/Ca²⁺ release channel, as an early event. In mammalian species, such as mouse and rabbit, SR attains the adult organization, i.e., TC in register with the skeletal CS isoform and RyR with a specific Ca²⁺ release channel. Western blot data on skeletal muscle membranes, purification of two CS isoforms by phenyl-Sepharose chromatography, and their immunolocalization in muscle fibres, all show that both CS isoforms are coexpressed in neonatal muscle. Our results, at the protein level, indicate that the disappearance of cardiac CS fibers from rabbit developing fast-twitch skeletal muscle is time-correlated with changes in the respective mRNA (Arai et al., Am. J. Physiol. 262, C614-20, 1992) and that it also follows the steady accumulation of both the RyR and skeletal CS. These findings seem to imply that myogenic factors, rather than the neural input to the muscle, might be responsible for the switching-off of the cardiac CS gene during postnatal differentiation of mammalian fast-twitch fibres.

Changes in shape of mitochondrion during differentiation of L6 cells

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Onset of muscle specific gene transcription is a characteristic phenomenon during myoblast differentiation. Changes in the transcription of proteins involved in muscle contraction and energy metabolism appear during the differentiation.

In adult muscle high energy demand during muscle contraction is covered by high mitochondrial volume and high potential for aerobic metabolism.

The purpose of this study was to assess changes in the shape and distribution of mitochondrion during myoblast fusion.

We cultured rat L6 myoblasts in DMEM supplemented with 10% foetal calf serum. The myoblasts were induced to differentiate by medium supplemented with 1% horse serum and 4 IU/ml of insulin. Mitochondrial distribution and shape was evaluated by using a mitochondrion specific antibody (34 kDa ~ CA V). A vital dye, Rhodamine 123, was used to confirm the distribution of the antigen. Transmission electron microscopy was used to further confirm the distribution of mitochondrion in myoblasts and myotubes.

In myoblasts mitochondrion have a rod-like shape and are localized with tubulin. During differentiation few large round mitocondrion are localised at the perinuclear zone. In multieucleated myotubes mitochondrion have regained their rod-like shape and they are evenly distributed in the myotube.

These results show that changes in mitochondrial shape and localization during myoblast differentiation

Muscle fibre types in rat laryngeal muscle

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The muscle fibres of the posterior cricoarytenoid (PCA) muscle and thyroarytenoid (TA) muscle of young adult rats were characterized by histochemical and immunohistochemical staining and SDS-PAGE. These muscles were compared to rat extrinsic muscles, extensor digitorum longus (EDL), diaphragm and atrial muscles. The PCA had 10-15% type I fibres and about 1% type IIC fibres, as detected by myosin-specific antibodies and ATPase histochemistry. The majority of the remaining fibres could be characterized as type II since they stained positively for a fast MHC antibody (sigma clone MY-32), and retained myofibrillar ATPase activity after alkali buffer preincubation. Yet these fibres could be distinguished from types IIA, IIB and IIB fibres of the EDL and soleus muscles by their ATPase activity after acid buffer incubation, and the lack of reactivity for IIA antisera in most fibres. The TA muscle contained almost exclusively fast fibres similar to those in the PCA. Antiserum specific for atrial and IIM MHC also did not stain laryngeal muscles, and an antiserum specific for neonatal MHC stained only a very few TA/PCA fibres in some rats. Glycerol-enhanced SDS-PAGE with 5% acrylamide isolated MHC isoform bands from the laryngeal and control muscles with the following mobilities from fastest to slowest: IIA, IIB and IIB fibres of the EDL and soleus muscles by their ATPase activity after acid buffer incubation, and the lack of reactivity for IIA antisera in most fibres.
Myogenesis of intrafusal fibres in rat muscle spindles

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We have studied the development of rat muscle spindles by analysing the ultrastructural differentiation and myosin heavy chain (MHC) expression of their intrafusal fibres in relation to their sensory and motor innervation. Neonatal deinnervation leads to the disintegration of muscle spindles, whereas adult spindles survive denervation, their intrafusal fibres increase in number and their nuclear bag fibres continue expressing their characteristic slow tonic (STO) MHC, which appears to be induced and maintained by sensory terminals during normal development. Neonatal deinnervation does not reverse the differentiation of intrafusal fibre types, but prevents or reduces the development of regional differences in the MHC expression along the bag fibres; moreover, after the fourth week it leads to neogenesis of supernumerary bag and chain fibres, which either originate from 'bag' or 'chain' satellite cells, or appear to arise by division or branching of original intrafusal fibres. Neonatal deinnervation followed by nerve section 3–4 weeks after birth allowed differentiation of supernumerary intrafusal fibres, many of which eventually displayed STO MHC-like immunoreactivity despite denervation. Our results suggest that those supernumerary intrafusal fibres which do not arise by division develop from satellite myoblasts of different types, presumably determined by their origin derived from parent nuclear bag or chain fibres. This is consistent with the idea that regional heterogeneity in the MHC expression is due to nuclear domains related to sensory and motor innervation and muscle cell lineage.

Fibre transformations in neck muscles of patients with dysfunction of the cervical spine

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Biopsies of ventral (sternocleidomastoid, omohyoid, longus colli) and dorsal neck muscles (rectus capitis posterior major, obliquus capitis inferior, splenius capitis, l. capitis) were taken from 54 patients who underwent spondylodesis for cervical dysfunction. Muscle fibres were classified histochemically as type I, IIA, IIB or IIC (transitional fibres) according to the pH lability of their myofibrillar ATPase. Additionally reactions for cytochrome c oxidase and alpha glycero-phosphate dehydrogenase were performed. In all muscles investigated muscle fibre transformations were observed. The observations occurred independently of (i) the type of muscle (i.e. more 'postural' or more 'phasic'), (ii) the sex and age of the patients, and (iii) the type of affection. Thus, the muscular reaction pattern was the same in patients with rheumatoid arthritits as in patients with soft-tissue injuries of the neck (e.g. 'whiplash injury'). This strongly suggests that neck pain which is the most common symptom in these patients is the main stimulus for the uniform muscular reactions. From experiments it is known that muscles can also respond to a variety of unspecific stimuli, e.g. pain (Müntener, Exp. Neurol. 77, 666–78, 1982). The transformations were of limited duration generally terminating within two years despite the persistence of the chronic neck pain and the other symptoms. Patients with transformations ceased displaying an increased percentage of fast IIB fibres. This demonstrates that the transformations proceeded in the direction from 'slow oxidative' to 'fast glycolytic'.

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Age related changes of the diameter of immunocytochemically classified fibre types of rat extraocular muscles

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Monoclonal antibodies specific for different myosin heavy chains (MHC) were used to classify the muscle fibre types of the extraocular muscles (EOM) – the six ocular motor (OM), the levator palpebrae (LP) and the retractor bulbi (RB) muscles – of rats of different ages: 5, 10, 15, 20, 30, 90, 180, 360 and 1100 days old. Serial sections were stained using antibodies with a nominal specificity for slow-twitch (type I), fast twitch (type IIA and IIB), fast-twitch oxidative (type IIA), neonatal/embryonic, slow-twitch and cardiac α MHC. In adult rats (3–12 months old) all OM exhibit the well known layer organization with an orbital (OR) and a global (GR) region. A high number of the fast fibres in the OR express neonatal MHC. The proportion of these fibres declines somewhat with age, but does not disappear entirely in old age. A small number of the slow fibres of the OR shows positive reactions with antibodies against avian slow-tonic or cardiac α MHC. About 10% of the fibres in the GR express slow-twitch MHC, and about 25% of the fibres show positive reactions with antibodies against fast-twitch MHC. However, the majority of the muscle fibres of both regions of adult rat OM do not react with any antibody normally sufficient to characterise fibre types in skeletal muscles. These fibres should express an isoform of MHC specific to OM (Saito et al., J. Muscle Res. Cell Motil. 8, 161–72, 1987). The adult RB contains about 40% fibres expressing fast-twitch MHC, but also in this muscle most of the fibres do not react with any antibody used and should contain a specific MHC. There is no expression of neonatal, embryonic, slow-twitch or cardiac α MHC. The LP contains 15% type I, 25% type IIA and 60% type IIB fibres; there is no sign for a specific MHC in this muscle. There is no expression of neonatal, embryonic or cardiac α MHC in the adult RB. At the age of 3 days all muscle fibres contain neonatal MHC but also the expression of the other MHC has yet started. At that age the fibres of all muscles are small (4–7 μm). During the following days the neonatal MHC disappears gradually and the fibres become thicker. The final pattern of myosin expression is reached around the twentieth day of life, but the fibres grow thicker up to 6 months. In old age there is a remarkable atrophy of all fibres of the LP, the RB and the GR of the OM, but not of the fibres of the OR of the OM.

A reliable and inexpensive system for the cultivation and longterm observation of dissociated nerve-muscle cocultures

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The mechanisms of muscle cell recognition by nerve cells and the dynamic changes of nerve and muscle cells during contact formation are of general interest. In this paper a method is presented, which simplifies the observation of the process of contact formation in dissociated nerve-muscle cocultures. The method combines standard culturing techniques with a new approach to growing cocultures within spatial patterns. It allows the continuous observation of cell growth and of the formation of cell to cell contacts over a time span of 2–3 weeks.

Nerve muscle cocultures are prepared from dissociated cells of newborn wistar rats. The cultures are kept under stable conditions by means of a two shell incubator. An outer shell surrounds most of the inverted microscope and an inner shell accommodates a multivwell plate containing the cell culture on the stage of the microscope. The outer shell is designed to maintain the whole system at a stable temperature whereas the inner shell keeps the culture in a high humidity atmosphere and at a defined carbon dioxide partial pressure. This is regulated externally by pumping the atmosphere of an ordinary
Although the method of recording and analysis applied here has been specifically designed to investigate nerve-muscle contacts it is suitable as well to study other dynamic processes or even tropic and trophic effects of chemicals on tissue or cell cultures.

Sexual dimorphism between muscle fibre types of hind-limb in the toad (Bufo bufo)
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Composition of muscle fibre types in male forelimb during breeding season in Rana temporaria were investigated (Faber & Zawadowska, Gegenbaur's morphol. Jahrb. 134, 877-84, 1988) and compare with female forelimb muscles. Those studies showed, according to expectation, that muscle mass of male forelimbs increased significantly until amplexus and after seasonal breeding a slow diminution of their mass was observed (Jadwiga Faber, personal communication). In the present study sexual differences possibly existing in other muscles which do not take such a clear role in breeding behaviour were investigated. For these studies one of the hindlimb muscles - sartorius in a toad (Bufo bufo L.) were chosen. Muscles of four males and four females were frozen and analysed histochemically for myofibrillar ATPase (mATPase) (pH 10.4, 10.5, 4.2, 4.35, 4.5, 4.6), cytochrome c oxidase and SDH activity. Moreover, serial sections were put on a cold slide glass and kept in vapour of acetone at -20°C for 5 days. After that time slides were immunoreacted for parvalbumin (PV) (antibody 235, Celio et al., Cell Calcium 9, 81-6, 1988). In the control, immunoincubation with primary antibody was omitted. Sartorius muscle in male contained 55.3% of type I fibres, 19.7% of type IIa and 25.0% of type IIB fibres (J. Muscle Res. Cell Motil. 14, 253, 1993). In sartorius muscle of female type I fibres were not found. There were notice only type IIa - 56.0% and IIb - 44%. Most of these fibres there were transitional forms containing probably both type of myosin - IIa and IIB. In conclusion I suggest that the existence in the female sartorius of exclusively 'fast' muscles and fatigue resistant IIA fibres can be connected (i) with relatively higher weight (female weights average 2.5 more than male, after the breeding season this discrepancy is even higher), (ii) Bufo belongs to species having short breeding season and females sometimes have to migrate a long distance to the place where they are attracted by male vocalization, (iii) in breeding sites density of males searching for females is very high and female has to use her hindlimbs to push away additional males which are not in amplexus with her.

Clones of human satellite cells can express in vitro both fast and slow myosin heavy chains
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Satellite cells were isolated from human quadriceps and masseter muscles. The growth kinetics, morphological features and programmes of differentiation of these cells were examined in vitro. The expression of myogenin was used to measure the commitment and fusion of these cells. The expression of the different myogenin heavy (embryonic, foetal, fast and slow) and light chain isofoms was used to assay myotube diversification. In addition to embryonic and foetal isoforms we found that fused cultures of human satellite cells express both adult fast and slow MHC's. Only the four fast type light chains (MLC1emb, MLC1F, MLC2F and MLC3F) were synthesized and no slow MLC's were ever detected in these cultures. In order to determine if the human satellite cells were committed to distinct fast and slow cell lineages a clonal analysis was also carried out on both cell populations. This analysis was first carried out on clonal populations and was confirmed by the analysis of isolated clones. All myogenic clones expressed both fast and slow MHC's suggesting that there is no evidence for different fast and slow satellite cell lineages in human skeletal muscle.

Heat-dependent structural transitions of myosin subfragment-1
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Structural transitions in myosin and especially in its active fragment, subfragment-1 (S1), are relevant to function of this molecule in that it is a perfect contractile system (Vibert & Cohen, J. Muscle Res. Cell Motil. 9, 256-305, 1988). In the present work the native temperature-dependent transitions of skeletal muscle myosin S1, the process of its melting and some features of its proteolytic degradation were studied. To reveal small conformational changes in proteins we used the two-wave
length fluorescent method developed in our laboratory which measured the tryptophan fluorescence intensity ratio $I_{350}/I_{300}$, the so-called parameter B (Filenko et al., Ukr. Biokhim. Zhurn. 59, 2–9, 1987).

A variety of reversible structural transitions was discovered in myosin and S1 in narrow intervals of native temperatures: 10–12, 20–21 and 32–35°C. The origin of these native transitions may be explained in the terms of domain organization of the S1. We consider that these reversible transitions represent the changes in mutual arrangement of domains in native protein structure.

Analysis of heat dependence of parameter B and light scattering of S1 (1.3 μm) at denaturative temperatures reveals three structural transitions at 40–46, 46–50 and 50–60°C corresponding probably to three structural units. Those may be identified as the three structural domains, corresponding to the proteolytic fragments of the S1 heavy chain, namely 50, 20 and 23 kDa. Prominent melting of the three structures was demonstrated only in high ionic strength solutions suggesting an essential role of charges in the interaction of contact locuses of individual domains.

The three-dimensional structure of a molecular motor, myosin subfragment-1


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Directed movement is one of the hallmarks of a living organism and involves the transformation of chemical energy into mechanical energy. Myosin is one of three families of molecular motors that are responsible for cellular motility. The three-dimensional structure of the head portion of myosin, or subfragment-1, that contains both the actin and nucleotide binding site will be discussed. This structure represents the first molecular motor to be determined by single crystal X-ray diffraction and thus provides a framework for understanding the molecular basis of motility.

The structure of F-actin and the actomyosin complex

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The structure of monomeric actin (G-actin) has been solved by X-ray crystallography as a complex with DNase I (Kabsch et al., Nature 347, 37, 1990). Using the X-ray fibre diagram from oriented F-actin it has been possible to determine the orientation of the G-actin monomer in the F-actin polymer (Holmes et al., Nature 347, 44, 1990). The fit has been refined to yield an atomic model of F-actin showing the nature of the inter-subunit bonding (Lorenz et al., J. Mol. Biol., submitted, 1993). This shows a strong longitudinal bond between neighbouring subunits into which a hydrophobic plug from the opposing strand is inserted. Apart from two loops, differences between the G- and F-forms are small.

The structure of the myosin head (S1) has been determined by X-ray crystallography (Raymnt et al., Science 261, 50, 1993a) and by combining the results with cryo-electronmicroscopic images of decorinated actin (Milligan et al., Nature 346, 217, 1990) it has been possible to identify the residues involved in the actin-myosin interaction (Rayment et al., Science 261, 58, 1993b). This appears to have three elements: a non-specific electrostatic part which could be responsible for the ionic-strength dependent weak interaction; a contact involving hydrophobic residues which is probably the initial stereospecific weak interaction and also part of the rigor interaction; and a third interaction which produces the full strong (tiger) actomyosin interaction. The nature of the weak-strong conformational change can be postulated. This appears to be accompanied by the closing of a cleft in S1. The closing of the cleft in S1 in turn may lead to a reduction of the affinity for phosphate at the active site.

Skeletal muscle myosin regulatory light chain conformation affects the positioning of A1 light chain

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The existence of at least two different states of structural organization of myosin heads complexed with actin seems evident. Changes of myosin regulatory light chains conformation influences the alterations of the myosin head induced by actin (Babykhuk et al., FEBS Lett. 295, 55–6, 1991). Miiles and Reiseler (J. Mol. Biol. 182, 271–9, 1985) have shown that actin binding to heavy meromyosin accelerates the cleavage of the A1 light chain by papain. In the present studies the influence of magnesiu-calcium exchange and phosphorylation of regulatory light chain (RLC) on the accessibility of the myosin and heavy meromyosin light chains (A1 and papain digestion) was investigated. Exchange of magnesium ions bound to RLCs by calcium ions accelerates the digestion of A1 in the presence of ATP both in dephosphorylated myosin and heavy meromyosin and acto-myosin and acto-heavy meromyosin complex. Thus, it may be assumed, that the alteration of RLC conformation due to calcium concentration increase in excited muscle lead to changes of A1 N-terminal positioning on myosin head. The cooperation of RLC with heavy chains in the changes of structural organization of myosin head during muscle contraction is suggested.

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

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The regulatory light chain (RLC or LC2) of rabbit fast skeletal muscle myosin is a member of the intracellular Ca-binding protein family. It is associated non-covalently with the heavy chain (HC). It contains 169 amino acid residues and two unique Cys (Cys-128 and Cys-157). With the bifunctional thiol reagent bis-(maleimidophenyl)methane (bisMPM) the RLC can be crosslinked to the HC as we demonstrated by immunodotting. Digestion of such myosin with papain produces papatic subfragment-1 (pap-S1) still containing the crosslinked RLC. Pap-S1 prepared from native myosin comprises both the RLC and LC1/3. The endogenous RLC can be removed from pap-S1 and replaced by radioactively labelled exogenous RLC. In pap-S1 the RLC can also be crosslinked to the HC by bisMPM. Pap-S1 with its crosslinked RLC can then be further digested by arginase-C between Lys-626 and Lys-637. The resulting 25 kDa fragment of pap-S1-HC contains the crosslinked RLC. Its sequence between Lys-642 and its C-terminus at Leu-842 possesses 5 Cys. Our results indicate that the two reactive Cys-697 and Cys-707 are not involved in crosslinking the RLC.

Determination of C.terminus and peptide sequencing indicates that chymotrypsin cleaves the myosin-HC between Phe-814 and Cys-815 and thrombin splits the HC between Arg-821 and Ala-822, producing chyme-S1 and throm-S1, respectively. Chymo-S1 and throm-S1 only contain the LC1/3 but no RLC. RLC does bind neither to native chyme-S1 nor to native throm-S1. We have therefore restricted the binding region of the HC for the RLC to a stretch of 21 amino acid residues running from Ala-822 down to Leu-842: A F M N V K H W P W M K L Y F K I P L L. The last Leu-842 represents the C-terminus of the pap-S1-HC. Pro-840 is the last Pro in the HC sequence before subfragment-2 follows downstream. This
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binding region contains 14 out of the 21 amino acid residues that are hydrophobic. Removal of the RLC causes pap-S1 to aggregate as demonstrated by electron microscopy. The sequence of the binding region for the RLC is highly conserved among different sarcomeric myosin-HC and lies right at the end of the pap-S1 moiety.

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New mapping of the actomyosin interfaces: three contiguous recombinant fragments spanning the entire myosin subfragment-1 interdependently interact with actin.

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Recombinant DNA methods were used to obtain native and soluble myosin subfragment-1 heavy chain (S1-HC) fragments of any desired length, thus allowing a novel examination of HC regions potentially interacting with actin and ATP. We observed actin binding in three isolated contiguous HC segments spanning the entire S1-HC and ATP binding by an HC segment spanning the N-terminal half of S1-HC only. The electrostatic nature of the interactions between the myosin fragments and actin was studied. The central actin-binding S1-HC fragment (residues 249-524) is probably a key factor for communication within the myosin head. The successive and/or concomitant involvement of all three myosin segments, during each contractile cycle, could constitute the molecular basis for an efficient generation of force involving multiple and sequential interactions with actin molecules.

Calorimetric studies on the stable complexes of myosin subfragment-1 with ADP and vanadate or beryllium fluoride.

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The thermal unfolding of the myosin subfragment-1 (S1) in its complexes with ADP and vanadate (S1·ADP·V,) or beryllium fluoride (S1·ADP·BeF3) was studied by differential scanning calorimetry (DSC). These complexes are stable analogues of the S1"·ADP·P transition state of the S1-catalysed ATP hydrolysis. It has been previously shown by DSC that the formation of the S1·ADP·V complex causes the global change of S1 conformation which is reflected in a pronounced increase of S1 thermal stability (Levitsky et al., Eur. J. Biochem. 209, 829-35, 1992). Recently we have shown that the structure of S1 in the S1·ADP·BeF3 complex is similar to that of S1 in the S1·ADP·V complex. Thus, the DSC method allows to register the conformational changes of the whole S1 molecule caused by formation of the S1·ADP·V, or S1·ADP·BeF3 complexes. We have studied the properties of these complexes obtained from specifically modified S1. It was found that the nucleotide-induced tryptic cleavage of the N-terminal part of the S1 heavy chain between Arg-23 and Ile-24 decreased the S1 thermal stability in the complexes S1·ADP·V and S1·ADP·BeF3 (the maxima of the heat sorption curves shifted by about 4°C towards lower temperatures). Trinitrophenylation of Lys-83 as well as the specific modification of the SH1-group of Cys-707 decreased the S1 thermal stability in the S1·ADP·V, complex but had practically no influence on the temperature of the thermal transition of S1 in the S1·ADP·BeF3 complex. In the case of SH1 modification the effect depended on the reagent employed; NEM was the most effective. Ihe stability of the S1·ADP·V, and S1·ADP·BeF3 complexes obtained from S1 modified at Lys-83 or at Cys-707 was significantly less than that of the complexes obtained from unmodified S1. When excess reagents were removed by dialysis following the formation of the S1·ADP·V, or S1·ADP·BeF3 complexes, almost all the modified preparations showed the peaks characteristic for nucleotide-free S1, while the decomposition of the complexes obtained from unmodified S1 was negligible.

The high resolution structure of the profilin:β-actin complex and its functional implications.

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The three-dimensional structure of the bovine profilin: β-actin complex has been solved to 2.55 Å resolution by X-ray crystallography. Actin monomers in the crystal are organized into ribbon-like structures which appear to preserve the 1-start helical contacts found in filamentous actin. Profilin has structural features in common with Src homology 3 (SH3) domains. In addition to a direct structural homology, it has a surface-exposed, hydrophobic patch of aromatic amino acids reminiscent of the site in SH3 domains that recognizes proline-rich motifs in SH3-binding proteins. We have shown that acidic amino acid replacements in this region in profilin abolish its poly(L-proline)-binding capacity. This strengthens the view that profilin links signal transduction and actin filament assembly.

Crystal structure of the actin:gelsolin segment 1 complex at 2.5 Å and the orientation of the actin monomers in the gelsolin:actin, complex.

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Gelsolin is a 62 kDa globular protein present in almost any cell and in blood plasma, that severs F-actin in a Ca2+-dependent fashion. The amino acid sequence of gelsolin shows marked repeats (six segments in all) that exhibit the same conserved pattern of mostly hydrophobic residues. We have grown crystals of the 1:1 complex of skeletal muscle G-actin and the N-terminal segment (segment 1) of human gelsolin and solved the structure to 2.5 Å. Segment 1 binds with high affinity to G-actin and inhibits its polymerization. The structure of the actin:segment 1 complex comprises 3921 non-hydrogen atoms and 32 water molecules and the chain trace for segment 1 is complete (R-factor = 0.19 for all data to 2.5 Å). Segment 1 has a three layer structure composed of a central β-sheet sandwiched between α-helices. It binds between subdomains 1 and 3 of actin. The longer α-helix of segment 1 forms the actin binding site. The contact area is composed of a number of central hydrophobic interactions surrounded by 13 intermolecular hydrogen bonds. A calcium ion has been identified that is coordinated by residues of both actin and segment 1. A second calcium has been located that is bound to the surface of segment 1 alone and shows similarities to proteins of the annexin family which bind phospholipids. This site might represent the PIP2 binding site. A number of structural changes (in complex with DNase I and segment 1) did not show any gross changes in the relative positions of the four subdomains (the Ca2-positions superimpose to 0.75 Å). There is little evidence for density of residues 40-50 (the DNase I binding loop) in the complex with segment 1. The orientation of the actin monomers within gelsolin:actin, (GA1) and ternary complexes of two actins with other gelsolin fragments was analysed by chemical
cross-linking. The data obtained indicate that the two actin monomers are in an antiparallel orientation different from their orientation in the F-actin filament, since cross-linking of GA, with phenylenebis-maleimide only generates the 'lower' actin dimer of 86 kDa in which the Cys374 of both actins are cross-linked.

Digestion of actin with gly-C and the localization of the epitope for the monoclonal antibody NH3

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The monoclonal antibody NH3 was derived from a fusion using spleen cells from a mouse immunized with human monocytes and the human monocyte-like cell line U937 (Dransfield et al., Biochem. Soc. Trans. 16, 163-4). The epitope on actin that is recognised by NH3 is preserved across isoforms, the antibody cross-reacting with actin isolated from rabbit skeletal (psoas), beef cardiac and chicken smooth (gizzard) muscle. In contrast, complexes of DNA-se 1-actin were not recognised by the antibody.

To localize the epitope for NH3, we have digested actin with CNBr, glu-C and the novel protease gly-C. We have found that gly-C has a restricted specificity towards actin, digestion being confined primarily to glycines 46, 48 and 150, with some cleavage also being found at alanine 230. Western blotting & sequence analysis of actin fragments derived from gly-C digestion and from glu-C digestion indicate that the epitope for NH3 is localized between residues 151 and 200 of actin. Preliminary data obtained using partial products from CNBr digestion of actin suggest that the epitope is located between gly 151 and met 190 of actin.

Perturbation of actin structure by beryllium fluoride

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Beryllium fluoride (BeF,) has been widely used as a phosphate analogue in nucleotide-binding proteins. It was shown to bind tightly to F-actin, but not to G-actin (Combeau & Carlier, J. Biol. Chem. 263, 17429-36). The binding of BeF, to F-actin was found to affect the 3D structure of the filament by stabilizing the subdomain 2 region of the actin protomer (Orlova & Edelman, J. Mol. Biol. 227, 1043-53). In this work we examined the BeF,-induced structural and functional changes in G- and F-actin using proteolysis, chemical modifications, ATPase and in vitro unfolding assays. According to the results of the proteolysis studies BeF, binds also to MgADP-G-actin and transforms its structure to a similar one to that of MgATP-G-actin. This is manifested in enhanced subtilisin and decreased trypsin digestions in the subdomain 2 region of G-actin. BeF, had a strong effect on the proteolysis of MgADP-F-actin both the trypsin and subtilisin digestion in subdomain 2 were completely inhibited. Significant protection against proteolysis was observed even at 3:1 molar ratios of BeF, to actin indicating cooperative effects on the structure of the actin filament. The finding that BeF, affects the proteolysis of MgADP-F-actin stronger than that of MgADP-G-actin is probably related to the tighter binding of BeF, to the polymer than to the monomer form of actin. Phosphatase has a similar although milder effect on the proteolysis of F-actin than BeF, which suggests that BeF, acts as a phosphate analogue in this system. The reduced rate of Cys374 alkylation with 7-diethylamino-3-[4'-(maleimidylphenyl)-4-methylcumarin and an increased subtilisin cleavage near the C-terminus of actin in the presence of BeF, indicates that the structure of the subdomain 1 region of F-actin is also perturbed by BeF,.

Finally, the binding of BeF, to actin does not change the sliding velocity of actin filaments in the in vitro motility assay. The relatively minor effect of actin bound BeF, on myosin-actin interaction is not due to an S-1 induced dissociation of BeF, since F-actin-ADP, BeF, remains resistant to trypsinolysis even after incubation with S-1. The beryllium fluoride-induced specific and distinct changes in G- and F-actin point to the dynamic nature of actin structure and to local differences between monomeric and polymeric forms of actin.

Unfolding/refolding studies of myosin subfragment-1 and myosin rod

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The effect of guanidine hydrochloride (GdnHCl) on the stability of myosin subfragment 1 (S-1) and myosin rod was studied under equilibrium and kinetic conditions. The enzymatic activity of S-1 is at first lost at very low concentrations of GdnHCl (lower than 0.5 M) and its light chains dissociate at slightly higher GdnHCl concentration (about 0.5 M). This dissociation is closely followed by the formation of aggregates between the naked heavy chains of S-1 molecules in the GdnHCl range of concentrations 0.5-1 M. Above 1 M, aggregates gradually disappear and S-1 loses its secondary and tertiary structures. The head fragment of 20 kDa was suggested to be implicated in the binding of light chain to heavy chain and in the self-association of free heavy chains (Nozais et al., Biochem. 31, 1210-15, 1992). The kinetics of S-1 denaturation in GdnHCl at the concentration of 0.5 to 1 M were usually found to be biphasic with an initial fast phase (a few seconds) and a slow phase (a few minutes or hours). The rates of enzyme inactivation were found to be faster than the rates of unfolding determined by circular dichroism measurements and than those of aggregation which occurred in a complex polyphasic process.

The unfolding and refolding of myosin rod was studied under equilibrium conditions. Different probes were used and their changes with the concentration of guanidine suggested that the loss of helix content and the dissociation of the two chains were not tightly linked. A step-wise process is proposed as a model for the unfolding of the large-sized myosin rod.

Substitution of Mg++ with Fe++ in stable myosin subfragment-1-nucleotide complexes

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The predominant intermediate of the myosin-catalysed ATP hydrolysis is the M**-MgADP-Pi transition complex whose dissociation is accelerated by actin during the cross-bridge cycle. We studied the role of divalent cations in the transition complex and tried to localize the metal binding site in the primary structure of the myosin head by substituting Mg++ with Fe++. This cation has been found to be a good substitute for Mg++ in the S1 ATPase, since the observed ATPase activity in the presence of Fe++ is at first lost at very low concentrations of GdnHCl (lower than 0.5 M) and its light chains dissociate at slightly higher GdnHCl concentration (about 0.5 M). This dissociation is closely followed by the formation of aggregates between the naked heavy chains of S-1 molecules in the GdnHCl range of concentrations 0.5-1 M. Above 1 M, aggregates gradually disappear and S-1 loses its secondary and tertiary structures. The head fragment of 20 kDa was suggested to be implicated in the binding of light chain to heavy chain and in the self-association of free heavy chains (Nozais et al., Biochem. 31, 1210-15, 1992). The kinetics of S-1 denaturation in GdnHCl at the concentration of 0.5 to 1 M were usually found to be biphasic with an initial fast phase (a few seconds) and a slow phase (a few minutes or hours). The rates of enzyme inactivation were found to be faster than the rates of unfolding determined by circular dichroism measurements and than those of aggregation which occurred in a complex polyphasic process.

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found. Upon addition of $H_2O_2$ to $Fe^{3+}$-containing stable $S_1$ complexes, $Fe^{2+}$ is oxidized to $Fe^{3+}$, a process which is accompanied by formation of reactive short lived oxygen radicals (Fenton reaction). These radicals can cleave the polypeptide chain in the vicinity of the transition metal-binding site. We observed that $S_1$ is cleaved by the radicals at two specific sites – at 23 kDa and 58 kDa from the N-terminus – in the $S_1-FeADP-Vi$ and at only one of these sites – 58 kDa from the N-terminus – in the $S_1-FeADP-BeF_2$ complex. We assume that these sites, which are probably proximal to each other in the tertiary structure, take part in the formation of the metal binding sub-site of the nucleotide-binding site in $S_1$, because no cleavage has been observed in the presence of $Fe^{2+}$, when either ADP or the phosphate analogue was absent. This assumption is supported by the findings that (1) a reciprocal correlation exists between the extent of the decomposition of the $Fe^{2+}$-containing stable complexes – measured by the recovery of the ATPase activity – and the extent of the $H_2O_2$-induced $S_1$ cleavage; (2) the slow exchange of $Fe^{2+}$ with $Mg^{2+}$ in the $Fe^{2+}$ containing stable $S_1$-nucleotide complexes inhibits the cleavage of $S_1$ caused by the Fenton reaction.

**ASSEMBLY OF CONTRACTILE STRUCTURES**

**The structural basis of actin filament polymerization and dynamics**

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We have compared our 2.5 nm resolution 3-D reconstructions of negatively stained F-actin filaments with an X-ray diffraction data-based atomic model of the actin filament (Holmes et al., Nature 347, 44–9, 1990) and found a high degree of similarity. By comparison with the model, many structural features of our reconstructions can be explained in atomic terms, e.g., the cleft that separates the larger outer domain, and the overall size and shape of the subunit. In addition, the mass density that connects the two long-pitch helical strands in our reconstructions could be identified as the ‘hydrophobic’ loop of the actin molecule that is believed to extend macroscopically-ordered pellets of F-actin with and without HMM, caldesmon and/or tropomyosin. The filament bundles that connect the myotubus to the terminal membrane have a double function. In addition to a role in sarcomere formation, they must play a role in force transmission. As they surround new, incomplete sarcomeres, they will act as a bypass transmission for these sarcomeres in force transmission.

**Orientation distribution of spin labels in actin filaments: evidence for modification by actin-binding proteins**

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Earlier EPR studies on the interaction of spin-labelled F-actin with myosin fragments, caldesmon, and/or tropomyosin have shown that all these proteins strongly reduce actin motions and these proteins affect the static ordering of probe molecules on actin (Galazkiewicz et al., Eur. J. Biochem. 186, 233–8, 1989). To gain more information about the conformational changes, we performed additional experiments on the macroscopically-ordered pellets of F-actin with and without HMM, caldesmon, and/or tropomyosin.

A simplified direct calculation of the orientational distribution of spin labels in uniformly-aligned actin filaments (actin pellets and glycerinated muscle fibres) showed that there are two populations of spin labels on actin that have different angles of tilt between the z-axis of the molecular reference system of the label and the longer axis of actin filaments. The most probable values of angles $\Theta_1$ and $\Theta_2$ were estimated to be $36^\circ$ and $65^\circ$, respectively, and agreed well with the former and recently published data on F-actin filaments (Ostap et al., Biophys J. 63, 906–75, 1992). The values of $\Theta_1$ and $\Theta_2$ remained constant, independent of binding of HMM, caldesmon and tropomyosin, but the proportions between the two populations of spin labels were significantly affected. Thus, caldesmon and tropomyosin induced a significant increase of the $\Theta_2$ population, while HMM significantly decreased it. The resulting EPR spectrum of the F-actin–HMM complex resembled spectra of randomly distributed spin labels and did not significantly change upon the binding of caldesmon and tropomyosin. On the basis of these data we conclude that the binding of HMM to actin is accompanied by both conformational change of actin protromers in the filament and distortion of intersubunit bonds.
myosin heads, demonstrated here in myofibrils, may have a possible physiological role in the cooperative behavior of the actin-activated ATPase activity.

Dissection of dystrophin actin binding site using recombinant proteins

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Dystrophin is a 427 kDa protein encoded on the Xp21 gene which is present in a wide range of tissue. Its absence or altered presence cause Duchenne or Becker muscular dystrophies respectively. This protein is an elongated cytoskeletal protein which contains an actin binding domain in its N-terminal part (Hammonds, Cell 51, 1, 1987). This part was shown to contain two actin binding sites in NMR experiments named ADB1 (residues 80-117) and ADB2 (residues 128-156) whose counterparts on actin have been also elucidated (Levine et al., FEBS Lett. 298, 44-6, 1992). Published reports have also shown that a N-terminal dystrophin fragment fused to α-actinin devoid of its actin binding site, is able to bind actin in COS cells (Hemmings et al., J. Cell Biol. 116, 1309-80, 1992). Here we present that an enriched preparation in native dystrophin interacted with actin specifically in cosedimentation assays. We used also genetic engineering to produce different N-terminal dystrophin fragments containing either ADB1 or ADB2 site to characterize their potential ability to bind actin. Several experiments show that these sites have different characteristics. These results support the concept of a multiple actin binding contact in the N-terminal region of dystrophin.
Confocal microscopy used to follow diffusion and spatial distribution of fluorescently-labelled proteins in skeletal muscle fibres

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Diffusion of various substances including macromolecules like peptides and proteins into cells is a widely used method. One example is the study of elementary mechanisms of muscular contraction, which frequently requires diffusion of proteins or protein-fragments into demembranated muscle fibres. Diffusion of molecules into muscle fibres might well be limited by factors such as size, shape and charge of the molecules and the space available between the lattice of fibre proteins. If the molecules bind to specific sites of the muscle fibres, the accessibility of these binding sites and the affinity of the molecules for the binding sites might dominate diffusion. Furthermore, since the architecture of the sarcomeres is not homogeneous, characteristic binding patterns are expected depending on distribution of binding-sites for the proteins within each sarcomere.

We studied the diffusion of several proteins into chemically skinned skeletal muscle fibres in three dimensions using a BioRad MRC-600 confocal microscope which allowed optical sectioning with 0.3-0.4 μm thickness such that background fluorescence coming from fibre parts out of the focal plane was minimized. It was possible (1) to follow the time course of diffusion of fluorescently labelled proteins into the fibres, (2) to determine the time-dependent distribution of some proteins within the different regions of each sarcomere, which might reflect their binding properties and (3) to investigate the exchange characteristics of native proteins for externally added proteins. In the present study confocal microscopy was used to follow diffusion of e.g. creatine-phosphokinase, pyruvate-kinase, caldesmon-fragments, antibodies and N-ethylmaleimide-modified myosin S-1 (NEM-S1) into rabbit psoas fibres. It was found that equilibration for some proteins into the fibres was surprisingly slow (t½ of antibodies takes several hours, NEM-S1 takes several days, IgG that binds specifically takes several days, IgG without specific binding takes only a few minutes to reach homogeneous distribution), and that there is no simple correlation between the time required for complete diffusion and the molecular weight. It is concluded that the time needed to equilibrate muscle fibres with molecules can be dominated by their binding-characteristics to specific binding sites within the fibres.

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Effects of proteolytic removal of the C-terminal residues of actin on ATP hydrolysis associated with polymerization

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Based on the atomic model of F-actin, it has been suggested that the C-terminal phenylalanine participates in the intersubunit interactions along the two-start F-actin helix (Holmes et al., Nature 347, 44-9, 1990). Consistent with this conclusion, we have recently shown that proteolytic removal of the three C-terminal residues results in destabilization of the actin filament (Mossakowska et al., Biochem. J. 289, 897-902, 1993). Here we compare polymerization properties of actins devoid of the last three (actin-) or only two residues (actin-). The modified actins were obtained by limited digestion with trypsin of Mg-actin in the monomer and filamentous form, respectively.

The critical concentrations for 0.1 M KCl-induced polymerization, determined by the DNase-I-inhibition assay, were 3.5, 2.7, and 1.3 μM for actin-, actin- and intact actin, respectively. In agreement with earlier studies, the hydrolysis of the bound ATP was associated with KCl-induced spontaneous polymerization of intact Mg-actin closely followed the polymer formation. In contrast, ATP hydrolysis on actin- was largely delayed relative to the increase in the light scattering intensity; only about 0.3 mol ATP per mol actin was dephosphorylated at the time when the light scattering intensity reached the plateau value. This unusually strong uncoupling of ATP hydrolysis from the polymer growth did not seem to be simply due to the enhanced rate of polymerization of actin- , since the hydrolysis on this actin was significantly delayed also in relation to that on intact actin. The hydrolysis of ATP on actin- , also lagged behind the increase in the light scattering intensity, but to a smaller extent than that on intact actin. At steady state, the filaments of both truncated derivatives of actin were less stable than the filaments of control actin, which manifested itself in the enhanced rates of their depolymerization in the presence of DNase I.

In the light of these data, the enhanced rate of polymerization of actin-, seems to be related to stabilization of growing filaments by an ATP-cap rather than to their increased fragmentation as we have previously suggested. Thus, the removal of the C-terminal residues appears to result in filament stabilization at the initial stages of polymerization, and destabilization of the steady-state polymers with all or nearly all subunits in the ADP-state. The observed effects cannot be solely due to elimination of the monomer–monomer interaction involving the C-terminal phenylalanine because this residue was absent from both preparations of truncated actin. The behaviour of actin-, intermediate between that of actin- and of intact actin, suggests that sequential removal of two and of the third residue from the C-terminus gradually changes the structure of the C-terminal domain and that these alterations are transmitted to the environment of the gamma-phosphoester bond of the nucleotide.
Donnan potentials in rabbit psoas muscle and the effect of temperature change

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We study the polyelectrolyte nature of skeletal muscle and the association between the net fixed charge on the contractile proteins and order–disorder transitions in the filament lattice. By infusing different solutions into glycinated muscle, changing the electrical regime between the proteins, changes between the centre to centre filament distances can be observed (e.g. Rome, J. Mol. Biol. 27, 331–44, 1968). The electrical properties of the proteins, and the state of order of the lattice, are of importance in the contractile mechanism.

Donnan potentials in the A- and I-bands of glycinated rabbit psoas muscle can be measured using microelectrodes, and the net fixed electrical charge on the contractile proteins can then be calculated (Naylor et al., Biophys. J. 48, 47–59, 1985). There is a dramatic difference in the A-band potentials between the relaxed and rigor states (Bartels & Elliott, Biophys. J. 46, 61–76, 1985). In rigor the A-band charge is 40% higher than that in the I-band (ie 112–126 electrons (e) per molecule). In the relaxed state however the charges are about equal (ie 80–90 e). A charge amplification effect is taking place, as the increase of the A-band charge in the rigor state can not be due to the binding of one ATP molecule per active site on the myosin head alone (3–4 e). Elliott (Biophys. J. 32, 95–7, 1980) suggested that the increase in the net fixed charge may be due to the immobilization of ions, on the electrically-effective surface of the myosin filament. Ion binding (or ion association) may occur at Saroff sites (by weak hydrogen bonds onto networks of charged side chains).

Temperature has a profound effect on the visibility of the myosin layer lines in relaxed rabbit muscle (Widy, Muscle Res. J. 6, 62, 1967). We measured A- and I-band potentials as a function of temperature. Anions bound to the electrically-effective surface of the myosin filaments are governed by Boltzmann’s theorem, and should be released at higher temperatures. This charge release may increase the order of the filament lattice.

Both the A- and I-band potentials fall off sharply as the temperature is increased, in rigor and in relaxed muscle. Since the temperature reduction of the A-band potential in relaxed muscle appears to coincide with the appearance of myosin layer lines, it seems likely that the two observations are connected.

Amoeboid motility without actin: structure and function of the major sperm protein (MSP) of Ascaris suum

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Nematode sperm crawl like amoeba but contain little actin and myosin; instead their cytoskeleton is based on a 14 kDa major sperm protein (MSP). The numerous characteristics shared by MSP-based and actin-based systems suggest that they have similar mechanisms driving locomotion. Moreover, the apparent simplicity of the sperm cytoskeleton may facilitate the identification of molecular mechanisms for migration and general principles of amoeboid motility.

MSP assemblies into 10 nm filaments in the pseudopod of Ascaris sperm. These filaments are grouped together into long, branched fibre complexes that extend from the leading edge of the pseudopod to the cell body. As sperm crawl, the fibre complexes treadmill rearward due to continuous production of filaments along the leading edge. This vectorial assembly of MSP, which is governed by a pH gradient within the cell, is a key element of sperm motility.

We have purified and sequenced two isoforms of Ascaris MSP (King et al., J. Cell Sci. 101, 847–57, 1992). In vitro, either isoform forms 10 nm filaments indistinguishable from native filaments isolated from extracted sperm. Electron microscopy shows these filaments are formed from two helical subfilaments that coil round one another. We have obtained MSP crystals that contain subfilaments identical or very similar to those in filaments. Solving these crystals should allow the structure of the MSP molecule as well as the macromolecular assemblies it forms to be studied to high resolution. In this way we hope to obtain an understanding of amoeboid motility in nematode sperm at the molecular level.

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Glycation of myofibrillar proteins in vivo and in vitro

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Glycation (nonenzymatic glycosylation) of myofibrillar proteins was studied in vivo (galactosemic rats) and in vitro (protein sugar incubation) to see, how ATPase activities are influenced and what is the relation between glycation and enzymatic activity.

When myofibrillar proteins were incubated in the presence of eleven sugars, a concomitant decrease of ATPase activity and increase in the extent of glycation occurred. Glyceraldehyde was the most efficient, pentoses were more efficient when compared with hexoses. β-mercaptoethanol, when present in incubation mixture, partially blocked myofibrillar glycation and ATPase activity was less inactivated.

Electrophoretic studies were performed to illustrate the polymerization and/or fragmentation of myofibrillar proteins due to glycation.

Insect paramyosin and projectin: simple and rapid purification and their binding to myosin

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Insect paramyosin, projectin and myosin were purified by Mono Q-FFLC and Superose 12 chromatography from the synchronous flight muscles of the moth (Acherontia atropos) or the locust (Locusta migratoria). The procedure yields about 3 mg projectin and 6 mg paramyosin which are essentially free of actin and myosin. The interactions of myosin with projectin and paramyosin were studied on microtiter plates using monospecific antibodies to myosin or monoclonal antibodies to LMM fragments for detection in an indirect ELISA. Competition experiments showed the specificity of the interactions and/or fragmentation of paramyosin and projectin binding of dissolved proteins to immobilized substrates is essentially reduced by increasing amounts of competing dissolved substrates but not by other proteins in solution. In contrast to projectin and paramyosin, myosin shows considerable amounts of unspecific binding to various blocking agents (BSA, gelatin, casein, DNA). In the linear range of the binding curves, however, the ascent (ng myosin bound/ng myosin per well) is essentially increased by immobilized paramyosin and projectin when compared to the unspecific binding in solutions of various ionic strengths. Lineweaver-Burk plots (1 ng⁻¹ myosin bound versus 1 ng⁻¹ myosin per well) show that saturation of either protein with myosin molecules is independent of the ionic strengths in binding solutions (0.05–0.6 M NaCl) but the molecular ratio myosin/projectin exceeds the myosin/paramyosin ratio by about 70% when saturation is achieved. With increasing ionic strengths, however, the ascent of myosin binding decreases essentially in the case of binding to paramyosin and projectin as well as in the case of unspecific binding. Studies on the binding of recombinant fragments of LMM (Drosophila melanogaster) to projectin and paramyosin suggest that the presence of sequences located at the very C-terminus of the myosin molecule
may promote the interaction of the myosin rod with both proteins. With regard to the saturation characteristics, results obtained with synthetic fragments seem to confirm the finding that more molecules can be bound by projectin than by paramyosin.

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**ACTIN-MYOSIN INTERACTION**

**In vitro motility at nanomolar ATP levels**

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In the *in vitro* motility assay, actin-filament velocity has been shown to depend strongly on the free ATP concentration, but at 1–2 μM ATP, filament movement is consistently abolished. Here we report that following a brief exposure of actomyosin to 1 mM ATP, actin-filament motility persists at nanomolar ATP concentrations. Actomyosin was pretreated with 1 mM ATP. Subsequently, the ATP level was reduced by multiple rigor-solution washes. By the final rigor-solution wash, the ATP concentration, monitored by the luciferase assay, dropped to 8 nM. Actin-filament movement persisted even at such low ATP levels. This was in marked contrast to the situation where ATP concentration was gradually increased from zero; in this case, filament movement began only at 1–2 μM ATP.

The difference indicates that potential energy may be stored during the initial ATP treatment, and this energy is utilized as the free ATP falls to nanomolar levels.

Motility at nanomolar ATP levels was supported by both skeletal and cardiac myosins. Although movement supported by cardiac myosin was four times slower than by skeletal myosin, motility persisted down to similar ATP concentrations with both. The similarity of the two myosins in their response to ATP concentration changes implies a similar degree of potential energy storage. Because cardiac and skeletal myosins have significantly different specific ATPase activities, the molecular mechanism involved in the storage of potential energy is most likely to be independent of the factors determining the myosin ATPase activity.

**Actin-myosin interaction in fast and slow muscles of mouse**

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Slow-twitch and fast-twitch muscles contain several myosin isozymes in various proportions. Isomyosins have three kinds of heavy chains: myosin heavy chain 1 (MHCl), myosin heavy chain 2A (MHCl2A) and myosin heavy chain 2B (MHCl2B), which are combined with several forms of light chains. These three chains can control the force-velocity mechanical parameters of muscles (Beckers-Bleukx & Maréchal, Eur. J. Physiol., in press). It is interesting to know whether actin-myosin interaction in a muscle is modified by myosin heavy chains. In this work we have studied the conformational changes of F-actin in single glycercinated muscle fibres prepared from soleus (SOL) or extensor digitorum longus (EDL) during transformation from relaxation to rigor by polarization fluorosence techniques (Borovikov et al., Gen. Physiol. Biophys. 10, 441–59, 1991). F-actin in muscle fibres was stained with 0.6 μM rhodamine-phalloidin (RP). The myosin heavy chains of the single fibres were separated by electrophoresis on glycerol-SDS-polyacrylamide gels and the zones were quantified by computerized densitometry: MHCl was 65.43 ± 7.26% (n = 15) in SOL and was absent in EDL. Myosin heavy chain 2A was 34.57 ± 7.26% in SOL and 4 ± 2.56% (n = 9) in EDL, and MHCl2B was 90 ± 2.56% in EDL. During transformation of the muscle fibres from relaxation to rigor the polarization of fluorescence of RF F-actin changed for SOL and EDL differently. The anisotropy of fluorescence of RF F-actin noticeably increased for EDL, while for SOL the same parameter practically did not change. These results are interpreted in terms of changes in actin monomer conformation induced by different myosin heavy chains in SOL and EDL.

**Effects of temperature and inorganic phosphate on rapid dissociation and reassociation of force generating cross-bridges**

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We previously demonstrated that fibre stiffness during active contraction is sensitive to speed of stretch or release used for the stiffness measurement. This suggested that (i) even during force generation crossbridges can dynamically interact with actin, i.e., detach and reattach from and to actin rapidly on the time scale of active crossbridge cycling, and (ii) at our low experimental temperature (5°C), force-generating crossbridges mainly occupy the first of a series of force-generating states among which crossbridges may redistribute during and after sudden length changes (Huxley & Simmons, Nature 233, 553–6, 1971).

In this study we examined the response of activated fibres to stretches and releases at different temperatures (T) to see whether (i) rapid dissociation/reassociation occurs at all temperatures and (ii) redistribution of crossbridges among different force-generating states contributes to the increase in force with T. The stiffness-speed relations recorded under isometric conditions are little affected by T, and the effects of T on plots of force vs. imposed length change imply that redistribution among force-generating states is not a major factor for the increase in force with T. Neither is redistribution among preforce- and force-generating states. Instead, it appears that at least in the first of the force-generating states the average strain sustained by attached crossbridges increases with temperature while the association/dissociation kinetics are little affected.

No effect of inorganic phosphate (P) on stiffness-speed-relations recorded during isometric contraction was found when P, was minimized enzymatically. This suggests that dissociation/reassociation during force generation occurs without a change in bound nucleotide or products, and apparently does not involve a state with P, at the active site.

**Effects of antibody fragments against the N-terminal residues of actin on active force and relaxed stiffness of skinned rabbit psoas fibres**

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F2(1–7), antibody fragments against the first seven N-terminal residues on actin, were previously shown to inhibit binding of myosin subfragment-1 (S-1) to actin both in the absence and presence of nucleotides including MgATP (DasGupta & Reisler, Biochim. 1991, 92). F2(1–7), antibody fragments against the first seven N-terminal residues on actin, were previously shown to inhibit binding of myosin subfragment-1 (S-1) to actin both in the absence and presence of nucleotides including MgATP (DasGupta & Reisler, Biochim. 1991, 92). In the presence of ATP, acto S 1 ATPase was inhibited but to a larger extent than binding of S-1 to actin. It was concluded that F2(1–7) can act to inhibit both the binding of S-1 to actin and a catalytic step in the ATP hydrolysis cycle.

We diffused the F2(1–7) into skinned rabbit psoas fibres to examine whether the F2(1–7) can interfere with crossbridge binding to actin and force generation. Fibre stiffness observed under relaxing conditions was used to probe crossbridge attachment to actin in preforce-generating states. It was found that inhibition of active force is much
Crossbridge cycling kinetics during isotonic contraction of skinned rabbit psoas fibres

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To analyse crossbridge cycling kinetics during fibre shortening, we extended the approach of recording the rate constant of redevelopment of force or stiffness, \( k_{\text{max}} \), to isovelocity contraction conditions: after some 200 ms of lightly loaded or unloaded shortening fibres are restretched to their initial (isometric) sarcomere length and redevelopment of force and stiffness is then followed either while sarcomere length is held constant (isometric) or reduced with constant velocity (isovelocity contraction). For isovelocity contraction, \( k_{\text{max}} \) of force or stiffness increased with shortening velocity and approached more than 10-fold higher values than observed during isometric contraction. Fibre ATPase, however, increased to only about 150% of its isometric value. Assuming that redevelopment of force and fibre stiffness reflects crossbridge cycling kinetics, fast redevelopment with low ATPase during high-speed shortening could be due to fast return of force-generating crossbridges to the weak-binding states via rebinding of inorganic phosphate \( P_i \) (e.g. effect of Ca\(^{2+}\) on stiffness-speed-relation in presence of MgATP\(\psi \)), and also interferes with activation of the thin filament by strong crossbridge binding to actin (e.g. when [MgATP] is reduced).
but also for the observation that there is no difference between the cross-bridges of vertebrate striated muscle. It seems reasonable to divide all existing models into two groups: the crossbridge models and the other ones. Because there are some inconsistencies of the crossbridge theory and some difficulties in answering the following fundamental questions:

(1) Which part of the myosin molecule does the crossbridge consist of?
(2) Does a redistribution of mass between the thick and thin filaments exist in the case of transition from the relaxed state to the rigor state?
(3) If it does, how can the S1 with the length of 16-19 nm and the S2 with the length of 43 nm act to cover the 6-17 nm distance between the thick and thin filaments?
(4) What kind of symmetry is there around the thick filament in the relaxed state and around both thick and thin filaments in the rigor state?
(5) What are two heads of the myosin molecule needed for?
(6) What is the role of the thin and connecting filaments in muscle contraction?
(7) In what way is the metabolic energy of ATP converted into mechanical work of muscle contraction?

The mentioned above questions can be resolved on the basis of the model proposed previously (Skubiszak, Technology and Health Care 1, 133-42, 1993; Biochem. Biophys. Res. Commun. 13, 1993 (in press)) without rejection of the main idea of the crossbridge theory.

Is the crossbridge theory correct?

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There are numerous conceptions concerning the mechanism of contraction of vertebrate striated muscle. It seems reasonable to divide all existing models into two groups: the crossbridge models and the other ones. Because there are some inconsistencies of the crossbridge theory with experimental data, more and more scientists reject this conception. In my opinion, the lack of a correct model of myosin molecule packing into thick filament is the cause. According to generally accepted models of the thick filament, the tail part of myosin molecule lies parallel or near parallel to the filament axis, and the filament has 3-fold rotational symmetry. From the point of view of such packing scheme, there are some difficulties in answering the following fundamental questions:

(1) Which part of the myosin molecule does the crossbridge consist of?
(2) Does a redistribution of mass between the thick and thin filaments exist in the case of transition from the relaxed state to the rigor state?
(3) If it does, how can the S1 with the length of 16-19 nm and the S2 with the length of 43 nm act to cover the 6-17 nm distance between the thick and thin filaments?
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CYTOSKELETAL PROTEINS

The immunolocalization of the 43K dystrophin-associated glycoprotein in relation to the location of dystrophin in skeletal muscle

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Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is associated with a large oligomeric complex of novel sarcolemmal glycoproteins (Campbell & Kahl, Nature 328, 259-62, 1989). The glycoprotein complex consists of a cytoskeletal protein (59K), three transmembrane glycoproteins (50K, 43K, 35K), a transmembrane protein (25K) and an extracellular glycoprotein (150K). The binding of dystrophin to the complex is thought to be via the 43K glycoprotein.

A monoclonal antibody was raised against the 43K dystrophin-associated glycoprotein (DAG) using a synthetic peptide representing 15 of the last 16 C-terminus amino-acids (Ibraghimov-Beskrovnaya et al., Nature 355, 696-702, 1992). This antibody was used to localise the 43K DAG in eight control and four DMD biopsies by immunogold (EM) and immunofluorescence (LM) labelling. In the control muscles 43K DAG was localized to the plasma membrane of the myofibres. Histograms of the values for nearest neighbour spacing showed modes at approximately 120 nm and were closely similar to histograms of nearest neighbour spacings of dystrophin in the same biopsies. This is strong evidence for a close interdependence of DAG and dystrophin positioning at the membrane, as predicted by the biochemical data (Ervasti et al., Nature 345, 315-19, 1990).

In each of the DMD biopsies there was positive 43K DAG labelling despite the lack of dystrophin. We were interested to know whether, in the absence of dystrophin, the spacing of the 43K DAG changed, as the molecules became more dispersed or alternatively more clustered, or whether it remained the same. Nearest neighbour measurements show that the 43K DAG molecules become more dispersed.

In regenerating rat muscle, dystrophin is first detected at the plasma membrane four days after the necrosis-inducing exposure to toxin (Vater et al., Acta Neuropath. 83, 140-8, 1992). Labelling of 43K DAG in the same muscles shows that it can be first detected at the plasma membrane at two days; considerably in advance of dystrophin. This implies that the insertion of 43K DAG into the plasma membrane is independent of the expression of dystrophin, but that the presence of 43K DAG may be necessary for the normal localization of dystrophin.
Identification and localization of an abundant novel high molecular mass (300 kDa) I-band protein which is immunologically related to the smooth muscle actin-binding protein filamin

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This laboratory recently undertook a series of experiments designed to investigate the cytoskeletal protein composition of mammalian slow-twitch (soleus: SOL) and fast-twitch (vastus lateralis: SVL) skeletal muscle. The cytoskeletal fraction prepared from purified rat skeletal muscle myofibrils was resolved by SDS gel electrophoresis. Two polypeptides referred to as B₁ and B₂ with molecular masses of 500 kDa and 300 kDa respectively were identified as major components of the myofilibrillar cytoskeleton. Electrophoretic analysis of whole SOL muscle tissue homogenates demonstrated that B₂ was present in quantities similar to nebulin. The protein B₂ was electrophoretically preparative gels and used to produce polyclonal antibodies in rabbits. Immuno blot analysis revealed that B₂ is immunologically related to rat aortic and uterine smooth muscle filamin. Immunofluorescence experiments indicated that B₂ was localized in the I-band in both the SOL and SVL muscles of the rat. Comparison of stained gels and immunoblots of whole tissue homogenates and the myofilibrillar cytoskeletal fractions revealed that the protein B₂ is approximately two-fold more abundant in the SOL compared to the SVL. It is concluded that the protein B₂ represents a newly identified and major component of the myofilibrillar cytoskeletal lattice. The immunological relation to filamin and the localization to the I-band suggest that B₂ may be a member of the actin-binding protein family. Should B₂ prove to be an actin-binding protein with a specificity for sarcomeric actin, this protein may serve to stabilize the thin filament lattice and to maintain the order of lattice in highly stretched muscle. The higher content of B₂ in the slow-twitch SOL muscle is not clear at the present time but may indicate a structural correlate to the different mechanical properties of slow- and fast-twitch skeletal muscle.

Titin elasticity and energetics of unfolding

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Denaturation of the giant muscle protein titin following gradual addition of guanidino hydrochloride has been studied by tryptophan fluorescence and circular dichroism spectroscopy. These methods should respectively monitor local and global changes during denaturation. Plots of purified titin fluorescence (exciting at 285 nm and monitoring at 345 nm) nd negative ellipticity (at 213 nm) both showed evidence of two distinct transitions. There was an initial transition at 0.1 M GuHCl, followed by second transition at approximately 1.5 M GuHCl. The CD spectra showed that the second transition was accompanied by a complete loss of β-structure and therefore probably involved unfolding of domains. Analysis of the co-operative nature of this transition indicated solvation of 60 internal amino acid residues/domain. This value is consistent with the known structure of the transition indicated solvation of 60 internal amino acid residues/domain. This value is consistent with the known structure of fibronectin ( Fn) and immunoglobulin (Ig)-like domains respectively. Titin shows a unique N-terminal head domain followed by 11 repeats in the order II-II-II-II-I-I-I-I-I-I-I-I-II where I and II describe fibronectin (Fn) and immunoglobulin (Ig)-like domains respectively. The first two domains are degenerate. The calculated molecular weight of 127 806 is somewhat lower than the 140 000 estimated by SDS PAGE. A 120K protein of muscle has been defined by an incomplete cDNA clone as human fast C-protein. We have now studied these proteins by cDNA cloning and characterized some of their molecular properties.

We have established a fast method for the isolation of homogeneous C-protein from bovine skeletal muscle. Electron micrographs reveal a uniform population of short rods with a contour length of about 50 nm. In vitro binding assays using radiolabelled C-protein show strong and specific decoration of myosin rods and purified titin II. A complete cDNA clone for human fast C-protein extends the information previously derived from a partial chicken cDNA clone as human fast C-protein.

Insertin, an actin-binding protein that allows insertion of actin molecules between the barbed ends and barbed end-bound insertin

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Insertin is a 35 kDa protein that can be isolated from chicken gizzard smooth muscle. Substoichiometric amounts of insertin retard actin polymerization 5-fold (100 mM KCl, 2 mM MgCl₂, pH 7.5, 37°C). Insertin binds strongly to the barbed ends of actin filaments. 10 nM insertin are sufficient to bring about maximal retardation of 1 μM polymeric actin. Insertin does not cause polymerization and depolymerization at the barbed ends in contrast to capping proteins. According to a quantitative kinetic analysis two insertin molecules bind cooperatively to the barbed end of an actin filament and remain bound to the terminal filament subunit during polymerization (Ruhnau et al., Mol. Biol. 210, 141-8, 1999). Any mechanism can be excluded in which insertin hops off to allow attachment of a new terminal actin subunit and then rebinds (Gaertner & Wegner, J. Muscle Res. Cell. Motil. 12, 27-36, 1991). Great parts of the amino acid of insertin have been determined. The primary structure of insertin turned out to be almost identical to amino acid residues 862–1212 of the actin binding protein "tensin" (Weigt et al., J. Mol. Biol. 227, 593–5, 1992). It is suggested that insertin allows polymerization and depolymerization at the plasma membrane-bound filament and in the cell without detachment of actin filaments from the plasma membrane.

Titin-associated proteins: intracellular members of the immunoglobulin superfamily


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Previous work from this laboratory raised the possibility that titin may interact with thick filaments via C-protein and described a tight complex between titin and two M-line proteins, called 165K and 190K protein. We have now studied these proteins by cdNA cloning and characterized some of their molecular properties.

We have established a fast method for the isolation of homogeneous C-protein from bovine skeletal muscle. Electron micrographs reveal a uniform population of short rods with a contour length of about 50 nm. In vitro binding assays using radiolabelled C-protein show strong and specific decoration of myosin rods and purified titin II. A complete cDNA clone for human slow C-protein extends the information previously derived from a partial chicken cdNA clone. C-protein shows a unique N-terminal head domain followed by 11 repeats in the order II-II-II-II-I-I-I-I-I-I-I-I-II where I and II describe fibronectin (Fn) and immunoglobulin (Ig)-like domains respectively. The first two domains are degenerate. The calculated molecular weight of 127 806 is somewhat lower than the 140 000 estimated by SDS PAGE. A 120K protein of muscle has been defined by an incomplete cdNA clone as human fast C-protein.

Human 165 and 190K proteins have unique N-terminal domains followed by the repeat pattern II-II-I-I-I-I-II-II-II-II-II where both proteins share 50% sequence homology. The 105K protein corre-
Ultrastructure of the subsarcolemmal cytoskeleton of mouse skeletal muscle fibres
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The subsarcolemmal cytoskeleton (SCS) is a hypothetical structure, which connects the contractile apparatus, the myofibrils, through membrane protein complexes to the extracellular matrix. As suggested by the distribution of its putative comprising proteins, these links occur predominantly at the Z-lines. Absence of dystrophin, a protein demonstrated to possess the features of a linking protein, often leads to fibre necrosis, suggestive for the importance of the SCS. However, detailed knowledge of the spatial relations among proteins comprising the SCS is still lacking, but would contribute substantially to a better understanding of its function. Therefore, this study was undertaken to expose the SCS and study it at high resolution with scanning electron microscopy (SEM). To this end, flexor digitorum brevis muscles from NMRI mice were treated with collagenase and a protease mixture to isolate fibres and remove their basement membrane. Then they were treated with Triton X-100 to the presence or absence of glutaraldehyde at low concentration. Results indicate that after the protease treatment, the sarcolemma appears intact, but subsequent treatment with triton X-100 exposes a filamentous network in the absence of glutaraldehyde, and a denser network with globular structures in the presence of glutaraldehyde. One hypothesis is that the SCS is better preserved by the presence of glutaraldehyde, the globular structures representing proteins or membrane protein complexes by which the filaments are anchored to the sarcolemma and extracellular matrix. In the absence of glutaraldehyde, a superficial layer is removed by mechanical shearing during the Triton treatment, so that a deeper layer of the SCS is exposed. Initial results of immuno-SEM show that the deeper filamentous SCS contains desmin at the Z-lines, and desmin filaments at the myotendinous junctions.

Myocardial tagging for non-invasive quantification of myocardial motion by magnetic resonance imaging
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Myocardial tagging is a new magnetic resonance imaging technique which allows to assess non-invasively the motion of the heart wall. Prior to an ECG triggered magnetic resonance multiphase imaging procedure in the enddiastolic heart phase, the muscle tissue is labelled by a spatially periodic modulation of the magnetization. In the first image acquired immediately after this modulation procedure a periodic grid of modulated spins appears as a grid of dark stripes. They are fixed with respect to the muscle tissue and disappear with the relaxation of the spins. The motion of the wall between this first and subsequently acquired images is extracted from the displacement, the rotation, and the distortion of the grid.

However conventional tagging techniques suffer from the rapid fading of the grid which restricts its application to the contraction phase of the ventricle. Furthermore the motion orthogonal to the imaging plane falsifies the results of the analysis. With more sophisticated tagging techniques the tagging contrast for later heart phases is strongly improved. In combination with a slice following imaging procedure they allow highly accurate motion analysis throughout the entire cardiac cycle.

First examinations indicate a wringing motion of the left ventricle with a clockwise rotation at the base and a counterclockwise rotation between TnT1 and TnT3 were markedly affected in disease: compared with control, we measured 65 ± 4% (n = 15) shift in mass from TnT1 → TnT3 in disease. The results manifest a novel correlation between the altered Frank-Starling mechanism and the regulatory proteins in cardiomyopathy of streptozotocin-induced diabetes.

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Alteration of calcium sensitivity by troponin substitution in skinned atrium muscle fibres: effect of EMD 53998
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As previously described by J. D. Strauss and colleagues (FEBS Lett. 310, 229, 1992) it is possible to extract troponin-I (and some troponin-C) from skinned cardiac muscle independently. It is possible, however, to reconstitute calcium sensitivity by incubating the fibre bundle with cardiac or skeletal muscle troponin-I or with recombinant troponin-I. We used this method to study the effects of different isoforms on Ca-sensitivity of pig atrial fibres. These fibres require a lower Pca for 50% activation and have thus a lower calcium sensitivity than ventricle skinned fibres. The difference may well be due to differences in the regulatory protein troponin. Thus, reconstitution with tropominis (Tnl, Tnc, TnT) from bovine ventricles (kindly donated by Dr N. Beier, Co. E. MERCK, Darmstadt) increase the calcium sensitivity significantly. A similar increase was observed when skeletal muscle troponin was used for reconstitution. The calcium sensitiser drug EMD 53998 (E. MERCK, Darmstadt) caused a large increase in calcium sensitivity before troponin extraction. But there was little effect of EMD after reconstitution with skeletal muscle troponin. However, the drug still had a calcium sensitizing effect after reconstitution with bovine cardiac troponin. We conclude (1) that calcium sensitivity of skinned atrial fibres may be altered by troponin replacement and (2) that the calcium sensitiser EMD 53998 has a different effect on the calcium responsiveness depending on the troponin isoform.

HEART AND CARDIOMYOCYTES IN CULTURE
TnT hand shifts in cardiomyopathy correlated with sarcocemere length-dependent myocardial contractile dysfunction in the diabetic rat
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Despite the emergent evidence on the isoform switching in cardiac muscle regulatory proteins during cardiomyopathy, the pathophysiological correlations have remained tenuous. We here resolve these uncertainties by investigating the length-dependent contractile alteration during heart disease, to assess the modifications in the Frank-Starling mechanism. The sarcomere length dependent pCa-tenion responses were measured on right ventricular skinned trabeculae from the diabetic rat with myocardial abnormalities. The maximal force levels (P0 in pCa4 medium) were unaltered in disease. Also, at 2.4 μm, the Ca-sensitivity (pCa0) was 5.75 ± 0.03 for controls and 5.77 ± 0.03 in cardiomyopathy (n = 22). But, contrarily, the pCa0 at 1.9 μm was 5.57 ± 0.03 for controls, and 5.43 ± 0.04 in disease, which indicates a 2-fold greater effect in cardiomyopathy. Three TnT bands (putative TnT1, TnT2 & TnT3) were evident in the Westerns of normal and myopathic tissue. But mass intensity distribution
Cardiac gene expression in growth and hypertrophy

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Cardiac growth is primarily characterized by an increase in myocyte mass or hypertrophy except in the focal stages where hyperplasia is common. Throughout the growth process whether due to normal developmental patterns or hemodynamic overload a number of quantitative and qualitative changes in gene expression have been well described which can account for, at least partially, some of the physiological characteristics of cardiac contraction and relaxation. From in situ hybridization analyses of cardiac tissues during embryonic development, both the sarco(endo)plasmic reticulum ATPase (SERCA) and phospholamban (PLB) are present throughout the ventricles and atria. Surprisingly, in late development the relative signal intensities for these transcripts vary from atria to ventricle and provide a working model to explain how the embryonic heart is able to contract without valves. In the adult, their levels of expression are rather uniform but are subject to change following imposition of a hemodynamic overload, resulting in an effective down-regulation of expression which can partially explain the increase in calcium transient times seen in hypertrophy.

Myosin heavy chain (MHC) isoform expression is intimately associated with the contractile properties of rat myocardium. Recently, we have described the presence of a naturally occurring antisense RNA to β myosin heavy chain whose expression is transcriptionally regulated and whose presence may play an active role in destabilizing the nascent β MHC transcript with normal growth and with thyroid hormone manipulations, suggesting a new and complex mechanism of regulating gene expression. Recent results obtained from nuclear run-on assays on the transcription of SERCA, PLB and the MHCs will be discussed. Finally, data concerning the regulation of translation will be presented.

Differential steroid hormone regulation of myosin subunit expression in cardiac and smooth muscle cells in the rat

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Two genes coding for myosin heavy chains (MHC, α-MHC and β-MHC) are expressed in the cardiac muscle. Smooth muscle cells (SMC) express one specific MHC gene exist which is alternatively spliced producing two MHC isoforms (SM1 and SM2). In addition, non-muscle MHC (NM-MHC) is expressed in smooth muscle cells. In the cardiac muscle MHC expression is regulated by steroid hormones on the transcriptional level but nothing is known about steroid hormone regulation of smooth muscle myosin expression. We investigated MHC expression in the left ventricle and uterus and 17 kDa myosin light chain (MLC) isoforms in the uterus of non-operated and sham-treated (N), ovariectomized and sham-treated (S;), estradiol- (E; 0.4 mg d⁻¹ substituted rats. Eight-week-old animals were operated and treated daily with hormones starting at ten weeks for two weeks (n = 6 per group). Values are means ± SD. Interestingly, all steroid hormones investigated decreased α-MHC expression in the heart: α-MHC expression in the left ventricle was 89.6 ± 5.9%, 97.3 ± 6%, 74.3 ± 11%, 79.2 ± 8.5%, and 78.4 ± 5.5% in N, S, E, T, and P rats, respectively. Uterus of virgin rats expressed SM1 and non-muscle but no SM2 could be detected. T and P, but not E enhanced SM1 expression: SM1 expression decreased from 66.4 ± 7.3% in N to 53.3 ± 7.2 in S rats (p < 0.05); substitution with T and P increased SM1 expression above the normal level to 86.0 ± 6% and 71.0 ± 8.3%, respectively. E rats remained at 57.5 ± 10.1%. In contrast, E and T but not P participate in the expression regulation of the MLC17: MLC17a expression in the uterus decreased significantly upon ovarectomy from 52.8% ± 11.5% (N) to 31.0 ± 6.8% (S). Treatment with E and T normalized MLC17a expression to 74.6 ± 6.2% (E) and 62.6 ± 11% (T) but remained at 39.1 ± 20% in P rats.

Abnormal function of β-myosin in hypertrophic cardiomyopathy

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Hypertrophic cardiomyopathy (HCM), a primary cardiac disease inherited in an autosomal dominant pattern, is an important cause of sudden death in otherwise healthy young individuals such as athletes. The phenotype has been linked, in some kindreds, to the β-myosin heavy chain (β-MHC) gene. The β-MHC gene, however, has been excluded as the disease locus in other kindreds. Thus, there is allelic and nonallelic heterogeneity in HCM. We have previously reported that the mutant β-myosin protein is expressed, together with the wild type, not only in cardiac, but also in skeletal muscle of individuals carrying the 403⁴⁰³C→G⁴⁰³ mutation. In this study, the enzymatic activity of soleus muscle β-myosin purified from 32 members of 11 unrelated kindreds linked to seven distinct missense mutations in the β-MHC gene (residues 12⁴¹²⁴⁰⁳%, 16²⁵⁰⁴⁰³-, 25⁰⁴⁰³-, 40³⁴⁰³-, 60⁰⁴⁰³-, 80⁰⁴⁰³-, 100⁰⁴⁰³-) is analysed using the actin sliding assay in vitro motility assay, in which rhodamine-phalloidin labelled actin filaments are translocated by β-myosin bound to a nitrocellulose-coated surface. Since soleus muscle contains both the fast- and the slow-β-myosin isoforms, an anti β-MHC specific antibody (Ab), raised against a unique sequence of the C terminus of the β-MHC gene, was used to selectively retain the β-myosin isoform on the surface of the motility assay chamber. SDS-PAGE analysis confirmed that only β-myosin is bound to the surface. The presence of the Ab did not interfere with the rate of sliding of actin filaments as demonstrated by control studies using purified β-myosin from cardiac tissue. All HCM samples showed a slower rate of translocation of actin filaments compared to that of 22 normal controls. Some β-MHC mutations (16²⁵⁰⁴⁰³-, 25⁰⁴⁰³-, and 40³⁴⁰³-) led to a very dramatic decrease in velocity, interestingly, the aminoacid (AA) 162 is localized near to the ATP binding site and the AA 403 is involved in the actin binding velocity: interestingly, the aminoacid (AA) 162 is localized near to the ATP binding site and the AA 403 is involved in the actin binding site. In the heart specific N-terminal region of troponin I two phosphorylatable serine residues are located adjacent - ser 23,24 in bovine (Swiderek et al., Eur. J. Biochem. 176, 335--12, 1988), see 22,23 in rabbit and rat heart (Mittmann et al., FEMS Lett. 273, 41--5, 1990). Thus freshly isolated troponin I is a mixture of four different species containing a non-, two mono- and a bisphosphorylated form of

Analysis of different troponin I phosphoforms in trabeculae isolated from rabbit heart

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In the heart specific N-terminal region of troponin I two phosphorylatable serine residues are located adjacent - ser 23,24 in bovine (Swiderek et al., Eur J. Biochem. 176, 335--12, 1988), see 22,23 in rabbit and rat heart (Mittmann et al., FEMS Lett. 273, 41--5, 1990). Thus freshly isolated troponin I is a mixture of four different species containing a non-, two mono- and a bisphosphorylated form of

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troponin I. Incorporation of about 1 mol phosphate per mol troponin I after β-adrenergic stimulation is correlated with Ca"²⁺-affinity decrease and a contraction force increase (England, Biochem. J. 160, 295–304, 1976). However, which of the three possible phospho forms is responsible for the observed effects is not known yet. Therefore troponin I is isolated from trabeculae from rabbit heart equilibrated in relaxing or contracting solution. The phosphorylation state of troponin I is analysed by IEF combined with immunoblotting. With troponin as isolated from bovine heart, three distinct bands are observed for troponin I, which can be assigned to the non-, mono- and bisphosphorylated forms.

The difficulty in determining the phosphorylation state of troponin I in trabeculae is the low content of troponin I. A muscle fibre of 500 µm diameter and 2 mm lengths theoretically contains about 20 ng troponin I. Thus, the troponin I species can not be visualized by gel staining but only by using monoclonal antibodies directed against cardiac troponin I (Professor Dr Cummins, Birmingham, Alexander Dahm, Bochum). Maximaly 5 ng troponin I could be detected using a dot blot. From trabeculae troponin I was isolated using DEAE sephadex in a micro system and analysed by IEF and immunoblotting. This troponin I contained two bands corresponding to the bis- and monophospho forms. The nonphosphorylated form could not be detected. It is not known until now if the nonphosphorylated form observed in troponin isolated from hearts is due to an artefact in preparation.

Formation of intercalated disc (ID)-like structure in adult rat cardiomyocytes in culture

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Cell adhesion is the result of a homotypic interaction of cadherins at the extracellular domain which depend on the ability to bind Ca²⁺ (Takeichi, Annu. Rev. Biochem. 59, 231–52, 1990; Geiger & Ayalon, Annu. Rev. Cell Biol. 8, 302–32, 1992). Adult rat cardiomyocytes (ARC) in culture undergo morphological and physiological changes and grow by cellular hypertrophy without cell division (Claycomb et al., FEMS Lett. 169, 2, 261–6, 1984; Eppenberger et al., The dynamic state of muscle fibres, 193–204, edited by PETTE, D. 1990). It is known that gap junctions are internalized after isolation of ARC (Severs et al., Circulation Research 65, 1, 22–42, 1989) and reappear in culture to finally restore electric coupling.

Cell-cell contact might involve various mechanisms and factors. In ARC two neighbouring cells trigger the extension of pseudopodia like structures where the N-cadherin is shown to be targeted to the membrane and vectorially driven to the sites of contact to establish cell adhesion and regenerate intercalated disc-like structures. The formation of the ID-like structure in ARC in culture has been followed by video time-lapse recording and immunostaining of N-cadherin and connexin 43, two proteins characteristic of the adherens and gap junctions respectively. On the other hand this process can be disrupted in "differentiated" ARC in culture upon treatment with EGTA, a chelator of Ca²⁺, that leads to the degradation of the cadherins and the concomitant loss of contact. N-cadherin as well as connexin 43 are membrane glycoproteins that can be arrested in golgi and their transit to the membrane has been studied. We show the spatial and temporal expression of these two proteins in ARC during rebuilding of ID-like structure in ARC.

A related phenomenon to cell-cell contact is that cadherin-mediated cytoskeleton interaction provides the mechanism for subsequent remodelling of the membrane. We report that overexpression of a mutated cadherin impaired the normal localization and function of the endogenous cadherin, thus hinders the formation of ID and therefore the tissue like structures in culture.

Synthetic peptides: models for the phosphorylation domain of troponin I

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Phosphorylation of troponin I occurs upon β-adrenergic stimulation and changes the Ca²⁺-affinity of troponin C (England, Biochem. J. 160, 295–304, 1976). Two phosphorylation states of troponin C (England, Biochem. J. 160, 295–304, 1976) two phosphorylatable serines (23, 24) are located within the N-terminal region following three arginines, thus showing a minimal duplicated recognition motif for protein kinase A in several mammals (Mittmann et al., Fels Lett. 302, 133–7, 1992). Freshly isolated troponin contains a mixture of two mono-, one bis- and a nonphosphorylated form of troponin I (Swidercek et al., Eur. J. Biochem. 176, 353–42, 1996). Each form gives rise to one 19F-NMR signal, respectively. Upon isolation of troponin I from the complex two signals are observed for the bisphospho form identical to those of the monophospho forms. To restore the three signal spectrum reconstitution of the complete complex T/I/C is necessary. Thus, the bisphospho form interacts with at least one of the other troponin subunits. The occurrence of different signals for each species might be due to changes in the structure of the N-terminus of troponin I. For structural investigations synthetic peptides are used. The bisphosphorylated peptide (PVR/RSIP/SIP/ANT) from the troponin I N-terminus shows two 19F-NMR signals identical to those of isolated troponin I. Corresponding monophospho peptides were obtained by the exchange of one phosphorylatable serine with an alanine. The pKa-value of each phosphate group within the bisphosphorylated peptide is identical to those of the corresponding monophospho monophospho and those of the monophospho species of troponin I. It could be shown that phosphoser-24 interacts with basic groups, probably the neighbouring arginine residues. Phosphoser 23 is not involved in interactions. In future NMR and CD spectra will help to elucidate the structure of peptides deriving from the N-terminal region of troponin I.

Isomorph switching of the sarcolemmal Ca²⁺-ATPase during myogenic differentiation

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The function of the calmodulin-dependent plasma membrane Ca²⁺-ATPase (PMCA) – as opposed to the sarcoplasmic reticulum Ca²⁺-pump – in muscle is unknown. To assess its potential role in muscle differentiation we characterized isomorph patterns of the PMCA in various muscle and non-muscle cell types. Expression of the different isomorph of the pump (from four genes) and their subisomorphs (derived by alternative splicing in the calmodulin binding region) was detected in several rat cell types by reverse transcription polymerase chain reaction using isomorph specific primer pairs.

The ubiquitous PMCA isoforms 1b and 4b formed the typical isoform pattern of rat L6 myoblasts, the heart derived cell line 1H9c2(2-1), different rat fibroblast cell lines (FR and NRK-49F), smooth muscle cells and endothelial cells. In addition to these two enzymes novel expression of the splicing variants 1c, 1d, and 4a was induced during myogenic differentiation of 1H and 1H9c2(2-1) cells. To determine whether expression of a differentiation specific isoform pattern may be under the control of a myogenic determination factor, rat fibroblasts were transfected with myogenin. Fibroblasts overexpressing this muscle specific transcription factor converted to multinucleated myotubes which displayed the PMCA isomorphs 1c, 1d, and
4a, typical of differentiated muscle cells additional to the constitutively expressed splicing variants 1b and 4b. Thus, the distribution of the various PMCA isoforms is cell type- and differentiation-specific. Overexpression of the myogenic determination factor myogenin is sufficient to direct transcription and alternative splicing of muscle specific PMCA isoforms. PMCA isoforms may play a role for the function of terminally differentiated muscle cells and possibly during the differentiation process itself.

SR-independent spontaneous oscillations in single cardiac myofibrils: quantitation and mechanism

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Spontaneous oscillations observed in various heart-muscle preparations are widely thought to be triggered by spontaneous release of Ca^{2+} from the SR. Here, we report undamped, propagated oscillations that occur in the absence of SR. Single myofibrils were prepared from glycerinated rabbit cardiac tissue and further skinned in 1% Triton X-100-containing buffer. SR removal was confirmed by electron microscopy, and in control experiments using ryanodine (known to interfere with the SR function). Myofibrils were mounted between two glass needles and partially activated (pCa 6.0 to 5.5). The length of each sarcomere was measured to a resolution of 50 nm.

Upon activation, all myofibrilar sarcomeres exhibited spontaneous, periodic length oscillations persisting up to one hour. In an individual sarcomere, the oscillation cycle generally consisted of a slow shortening phase, followed by a phase of rapid lengthening. Oscillations usually propagated along the myofibril in a wave-like fashion (average velocity 12.3 µm s^{-1} at 10°C; Q_{sl} x 1.3). The oscillation period was periodic length oscillations persisting up to one hour. In an individual prepared from glycerinated rabbit cardiac tissue and further skinned length of each sarcomere was measured to a resolution of 50 nm.

Early Growth Response Gene-1 (Egr-1) in muscle differentiation

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Recent evidence has suggested a role for the immediate-early gene Early Growth Response Gene-1 (Egr-1) in muscle differentiation and/or proliferation (Kurabayashi et al., Circulation 86(4), 1-10, 1992). Neyses et al., ibid. 1-20). Kurabayashi and colleagues showed that the promoter of Id, the gene which inhibits differentiation by binding to the myogenic determination factors, contains an Egr-1 consensus binding site (CGCCCCCGC). We therefore investigated the role of Egr-1 in the regulation of muscle growth by various growth factors that are able to induce proliferation or differentiation. Angiotensin II (AII), insulin (I), norepinephrine (NE), transforming growth factor β (TGFβ), endothelin (E), basic fibroblast growth factor (bFGF), and platelet derived growth factor BB (PDGF BB) were investigated. bFGF, PDGF BB, and I increased total protein after 24 h by 80%, 50%, and 40%, respectively. bFGF and PDGF BB increased DNA synthesis - as assessed by thymidine incorporation - by 100% and 50% (proliferation, confirmed by cell count), whereas I decreased DNA synthesis by 50%, parallelled by morphologic differentiation. E, AII, TGFβ, and NE showed no significant effect on either protein or DNA synthesis. OnNorthern blots, AII, E, and NE induced Egr-1 mRNA 5-fold, insulin and PDGF BB 10-fold, bFGF 20-fold at 30 min. TGFβ showed no induction. On Western blots, PDGF BB showed 10 fold, bFGF 20-fold stimulation of the Egr-1 protein at 30 min. None of the other factors stimulated Egr-1 protein.

In addition to known growth factors, angiotensin II and endothelin lead to transcription of muscle genes suggesting that Sox8 cells contain receptors and a functional signal transduction pathway for these substances. Egr-1, however, is not translated upon the actions of all and endothelin; therefore, the action of these factors in Sox8 cells remains to be determined. Egr-1 mRNA is induced by both proliferative and differentiative stimuli, while translation of the message is restricted to proliferative stimuli. These results suggest a role for Egr-1 in blocking myogenic differentiation and potentially in the induction of cell division by endo-/paracrine stimuli. Recently, Trouche and colleagues (Nature 363, 79-82, 1993) have shown that transcription of the immediate-early gene c-fos is blocked by MyoD in muscle differentiation after 24 h. To this late transcriptional regulation, our results add translational regulation of an immediate-early gene as a very early event in muscle differentiation.

Reorganization of cytoarchitecture in cultured adult cardiomyocytes

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Freshly isolated adult rat cardiomyocytes (ARC) undergo a stereotype sequence of changes when cultured in the presence of foetal calf serum (Eppenberger et al., Dev. Biol. 130, 1-15, 1988). The ARC cell culture system has proven to be very useful for studies on the cytoskeleton and myofibrils for the following reasons. (a) ARC degenerate the myofibrilla apparatus when cultured in the presence of serum and subsequently reassembled new myofibrils. (b) The first myofibrils emanate in the perinuclear region and new sarcomeres are added in concentric ring shaped regions around this zone. In the outermost region non-striated stress fibre-like filaments predominate. This layout permits the observation of several stages of myofibrillar assembly in one cell. Furthermore it lends itself to studies on isoprotein sorting (Soldati & Perriard, Cell 66, 277-89, 1991) since myofibrillar and non-myofibrillar regions are present in a topological well-defined pattern. A model describing the putative growth regions of myofibrils in vitro is presented. Several described assembly stages are identified in individual cells. Using 3D confocal microscopy it could be shown that the growth regions are close to the membrane proximal to the culture substrate. This can be shown most effectively by comparing computed views from above and below the cell (across the culture dish).

Rate of active tension development from rigor in skinned atrial and ventricular cardiac fibres from swine following photolytic release of ATP from caged-ATP

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We investigated the rate of tension development (k_{1}) after photolysis of ATP from P-1-(2-nitrophenyl)ethyladenosine-5'-triphosphate ('caged ATP') of atrial and ventricular fibres from pig. Contraction was initiated from high tension (HT) and low tension (LT) rigor at maximal Ca^{2+} activation (pCa 4.5). The k_{1} of atrial fibres was 6.8 s^{-1} from LT-rigor and 6.9 s^{-1} from HT-rigor. The rate of tension development of ventricular fibres was significantly lower (p < 0.001) being 1.06 s^{-1} and 0.94 s^{-1} from LT- and HT-rigor, respectively. The
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k_d of skinned ventricular fibres incubated in cardioplegic solution prior to the skinnig procedure decreased significantly (p < 0.05) to 0.73 s^{-1} and 0.63 s^{-1} from LT- and HT-rigor, respectively, whereas that of atrial fibres remained at 7.1 s^{-1} and 6.9 s^{-1} from LT and HT-rigor, respectively. Phosphorylation levels of the myosin light chain 2 isoforms of directly skinned atrial and ventricular fibres were 15.6 ± 2.7%, 31.2 ± 0.4%, and 25.1 ± 2.3% of the ALC-2, VLC-2, and VLC-2', respectively. Phosphorylation levels of fibres incubated in cardioplegic solution prior to skinnig were 11.6%, 18.9%, and 15.4% of the ALC-2, VLC-2, and VLC-2', respectively. The results show that the rate of tension development is more than 7-fold higher in atrial compared to ventricular fibres. These results correlate with the differences in ATPase activity of the contractile proteins in solution and, most likely, reflect differences in the myosin isoform composition. In ventricular fibres the increased levels of light chain phosphorylation increased the rate of contraction.

Interaction between type 1 myosin light chain (MLC-1) and actin and the expression of different MLC-1 isoforms modulates force of contraction of skinned human heart fibres

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The amino terminus of type 1 myosin light chains (MLC-1) binds to the carboxy terminus of actin. We studied the functional role of this interaction by monitoring isometric force of contraction of chemically skinned left ventricular fibres prepared from terminally failing human hearts using two approaches: (1) incubation with a synthetic peptide corresponding to the sequence 5-14 (P5-14) of the human ventricular MLC-1 (VLC-1) in order to saturate actin binding sites, and (2) incubation with a monoclonal antibody (mAbVLC-1) raised against ventricular MLC-1 amino terminal region in order to deteriorate VLC-1 binding to actin. Taking force prior incubation period as 100%, incubation with the maximal effective peptide dose (6.4 × 10^{-13} M) increased isometric tension by 24.55 ± 5% (31) at maximal (pCa 4.5) and 58 ± 9% (31) at submaximal (pCa 5.5) Ca^{2+} activation level (means ± SD; number of fibres in parenthesis). Threshold concentration of P5-14 was 10^{-13} M. VLC-1 peptides corresponding to the sequence 1-10 were without effect up to 10^{-12} M suggesting that the effective sequence was between 10-15. Incubation with maximal effective antibody concentration (10^{-9} M) increased tension by 3.5%, 17%, and 27.5 ± % at pCa 6.5, 6.0, and 5.5 respectively. Threshold concentration of mAbVLC-1 was 5 × 10^{-13} M. Primary sequence of the atrial MLC-1 (ALC-1) differs from the sequence of VLC-1 in the N-terminal region which binds to actin. Forty-one percent of patients investigated expressed detectable amounts of ALC-1 in the ventricle. Skinned fibres which expressed the ALC-1 were significantly more Ca^{2+}-sensitive than fibres without ALC-1: pCa_{50} (log, free [Ca^{2+}] at half-maximal activation) of fibres with 7.6, 3.4% and 0.8% ALC-1 was 6.0 ± 0.8 (5), 5.97 ± 0.05 (5), and 5.87 ± 0.08 (6), respectively (means ± SD; number of fibres in parenthesis; p < 0.05). In conclusion, interaction between MLC-1 and actin seems to be a very effective modulator of force of cardiac contraction and may be a target for a new positive inotropic intervention.

Molecular approaches to the study of cardiac muscle regulation by troponin

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Troponin I (TnI), the inhibitory subunit of the troponin complex, has three isoforms: fast and slow skeletal, and cardiac muscle. Two important properties of cardiac TnI (C'TnI) are that it contains an extra 26-33 NH2-terminal amino acids and it has two serines (SER22 and SER23) which can be phosphorylated by protein kinase A (PKA) in vitro, or as the result of beta adrenergic stimulation in vivo. It is thought that since this phosphorylation lowers the Ca^{2+} affinity of TnC (the Ca^{2+} binding subunit of Tn) in the Tn complex in vitro, that it may contribute to the relaxation of the heart in vivo using catecholamine stimulation. To study this we have isolated a full length cDNA clone for mouse cardiac TnI and have expressed C'TnI as well as several mutants of it in E. Coli. Using porcine cardiac muscle fibres, we have shown that when they are phosphorylated by PKA (mostly C'TnI and C protein) that the pCa_{50} decreases by 0.3 pCa units, indicating a decrease in Ca^{2+}-sensitivity. These fibres were treated with vanadate (Strauss et al., FEBS Lett. 310, 229-34), which selectively removes C'TnI and C'TnC, making the fibres develop Ca^{2+}-insensitive force. Ca^{2+}-sensitive force can be subsequently reconstituted with various C'TnC C'TnI complexes. Vanadate treated fibres restored with native C'TnC C'TnI complex behaved the same as unextracted fibres when they were treated with PKA. When vanadate treated fibres were restored with a complex of C'TnI and a C'TnI mutant where both SER22 and 23 had been converted to ALA, the fibres no longer responded to PKA treatment, indicating that C'TnI phosphorylation is responsible for the change in the observed Ca^{2+}-sensitivity. We are currently studying whether SER22 and/or SER23 are required for this effect. We also studied whether PKA phosphorylation altered the kinetics of force relaxation in these fibres using the photolabile Ca^{2+}-chelator, Drazo-2. The time for force relaxation dropped from 110 ms in untreated fibres to 60 ms in PKA treated fibres, indicating that the decrease in Ca^{2+}-sensitivity seen with PKA treatment is probably due to a faster dissociation of Ca^{2+} from C'TnC. In another study we examined the developmental expression of the TnI isoforms in mouse heart using mouse C'TnI and rat slow skeletal muscle (SS'TnI) cDNA clones. Total RNA from cardiac muscle from three stages of development, 3-day embryos, 1-2-day newborns and adults, were used for Northern blots. SS'TnI was expressed in the embryo but was absent in the adult. C'TnI began to be expressed during the embryonic stage and it gradually replaced SS'TnI in the adult heart. Previous studies by Solano and colleagues suggested that the relative pH-insensitivity of the Ca^{2+}-dependence of force development in neonatal versus adult hearts was caused by the difference in the expression of these two TnI isoforms. To test this, we have reconstituted vanadate-treated fibres with C'TnC C'TnI and C'TnC SS'TnI complexes and studied the pH sensitivity of the force/pCa relationship. Since this relationship was the same in both cases, it appears that the observed developmental isoform switch cannot account for the differences seen between adult and neonatal hearts.

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Biophysical aspects of cardiac contractility in hyperthyroid rats

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Our previous results (Revnic et al., Rom. J.G.G. 4, (II), 1990) have pointed out an increase in active shortening capacity of heart sarcomeres from thyroxine treated rats. In order to obtain new data concerning muscle contraction at the molecular level we have studied the ionic behaviour of contractile apparatus from glycinated rat heart in contraction (Co), relaxation (Re) and rigor (Ri) media with different ionic strengths (10 mM, 50 mM, 150 mM NaCl) using a radiotrophic method of C^{4+}H_{3}CO_{3} Na uptake. An increase in C^{4+}H_{3}CO_{3} Na uptake in contraction state has been recorded in comparison with rigor and relaxation which accounts for an increase in charge density on contractile filaments. Also, we have been
Characterization of cardiotin, a structural component in the myocard

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Cardiotin, a recently described component in the cardiovascular system, is characterized by the monoclonal antibody R2G. Application of immunofluorescence assays, revealed that cardiotin is expressed in the myocard of several species and to a lesser extent also in skeletal muscle. Cardiotin is not found in smooth muscle tissues, or any other type of mesenchymal, epithelial or neural tissue. The cardiotin distribution pattern is different from that of other sarcomeric components, such as desmin, myosin, actin, titin, nebulin, and desmoplakins, and shows a longitudinal filamentous localization between the myofilaments. Using confocal scanning laser microscopy, an average distance of 2.3 μm was measured between parallel running cardiotin filaments in human cardiac muscle sections. The cardiotin filamentous staining reaction is oriented perpendicularly to the typical cross-stria tions observed with antibodies to desmin, and was normally found spanning several sarcomeres. This localization pattern suggests a possible link with the sarcoplasmic reticulum. Immunoelectron microscopy studies are in progress to determine its exact distribution and possible association with other structures. Immunoblotting and immunoprecipitation experiments have shown that cardiotin is a high molecular weight protein restricted to cross-striated muscle. Cardiotin subunits have a molecular weight over 300 kDa, but migrate below titin in SDS-polyacrylamide gels. Cardiotin cannot be solubilized from cardiac muscle tissue by nonionic detergents or high concentrations of KCl and KL suggesting a structural role in the myocard.

Future studies at the molecular level will have to reveal the basic structure of the cardiotin molecule, which should then also allow us to draw up a more precise model for cardiotin assembly.

Transient expression of keratin during early myocard development in the rabbit: the association with desmosomal proteins of the intercalated disc

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Cardiac muscle cells are interconnected by spot-desmosomes, which are found in the intercalated disc, connecting the cardiomycocytes. The major components of the cytoplasmic plaque of these spot desmosomes are desmoplakin I (MW: 250 kDa) and desmoplakin II (MW: 215 kDa). A direct and close association between desmosomes and intermediate filament proteins (IFP) of the keratin type is already proven in embryonic and in adult epithelial tissues. As described before (Viebahn et al., Cell Tiss. Res. 253, 553, 1988), in rabbit, keratin is not expressed in accordance with germ layer origin of tissues normally seen in the mammalian embryos. Rather the expression of these proteins seems to be related to cellular function during embryonic development. In this study we show an interaction of keratin filaments with desmoplakin at certain stages of rabbit embryonal cardiogenesis. We found that in the very early stages in the heart anlage desmoplakins are much more abundant than in the adjacent intra- and extraembryonic mesoderm. In the epicard desmoplakins are gradually lost and are found to be confined at the intercellular desmosome-like junctions in later stages. Keratin expression in the developing myocard of the rabbit heart decreases with the age of the embryo. Keratin filaments are gradually lost via dot-like aggregates which are also positive for desmoplakin. Our results suggest a role for...
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keratins in the developmental rearrangement of desmoplakin into the intercalated discs. There does not seem to be a direct relation between desmoplakin organization and the rearrangement of titin and desmin in rabbit cardiogenesis.

Molecular changes in chronic hibernating myocardium
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The morphologic and molecular counterparts of chronic hibernating myocardium (CHM) are not well documented. Cardiomyocytes (CM) in hypoxic contractile segments are affected by partial to complete loss of sarcomeres, accumulation of glycogen as well as disorganization and loss of sarcoplasmic reticulum (SR). The space left by the vanished sarcomeres becomes occupied by glycogen. Glycogen accumulation was quantified in a group of patients with chronic ischemic myocardium. Our data strongly suggest a direct relationship between the existence of hibernating cells loaded with glycogen and a supranormal glucose extraction pattern seen with positron emission tomography.

With light- and electron microscopic procedures we have examined the expression and organization of tubulin in patients with CHM. Microtubules were absent in the regions where glycogen replaced the sarcomeres, but still present in the sarcromere containing regions. The expression and organization pattern of other contractile and cytoskeletal molecules such as desmin, titin, actin, myosin were also studied on biopsies of patients with CHM. Immunofluorescence studies performed on biopsies with different degrees of sarcomere loss showed an altered expression of some of the muscle specific proteins. Titin, which is the first sarcomere protein expressed during rabbit and mouse heart development, is downregulated in CMH. Titin expression varied in patients with CMH from practically normal to virtually absent. With these immunofluorescence assays we have also studied the distribution pattern of cardiotin in CHM, a high molecular weight binding-protein for Z-line stability. Fifteen patients with CM were studied. As controls for immunocytochemistry normal human myocardium and isolated myocytes from adult rat hearts were used. Titin was stained blood vessels (BC), produced a faint cross striation (CS) and showed large perinuclear granules in eight of 15 patients, that correspond to 'storage bodies' in the endoplasmic reticulum. 10-12 μm long and 4-6 μm wide as seen in the electron microscope (EM). With EA 53 (against rabbit skeletal muscle) BC and α-A-bodies were only slightly stained but the cross striation was evident. The Z-line staining in the myocytes was nearly identical to that seen with the T antibody showing a cross striation sometimes reduced with empty myocytes and a rest of Z-line material at the membrane. These structures could also be observed by electron microscopy. A polyclonal α-A antibody stained BC, CS and α-A-bodies. Controls only rarely showed α-A-bodies and were normal for T. Thus, in chronic CM the expression of T and α-A is disturbed which may lead to disturbances in sarcomerogenesis and sarcomere function. Additionally, a 'smooth muscle' α-A is accumulated in myocytes indicating the occurrence of a rare isof orm of α-A in CM.

Inotropic effect of angiotensin I and II in human auricular and ventricular myocardium
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We investigated the inotropic effects of angiotensin I and II (AI, AII) in human auricular and ventricular myocardium (isolated muscle strips, isometric contraction, 60 beats min-1, 37°C). In auricular trabeculae (n = 69, bypass surgery, EF = 57 ± 12%), AI and AII had a dose dependent positive inotropic effect (PIE) which was maximal at 10-6 M (AI: 128 ± 8%; p < 0.05) and 10-7 M (AII: 132 ± 6%; p < 0.05). Neither propranolol (10-5 M) nor prazosin (10-4 M) did affect the PIE. AII-receptor blockade with saralasine (10-4 M) abolished the PIE of AI and AII completely. With enalapril (10-5 M), no PIE of AI could be detected. Removal of the endocardium did not change the PIE of AI: the intracellular Ca2+-transient, as detected with aeroin, increased to 130 ± 5% (p < 0.05) at 10-7 M. Compared to the standard Ca2+-dose-response curve, this finding does not indicate a Ca2+-sensitization of the myofibrils by A II.

In contrast to auricular myocardium, neither AI nor AII yielded any PIE in left-ventricular (LV) muscle strips from nonfailing (n = 10), end-stage failing hearts (n = 22; EF = 19 ± 2) or right-ventricular infants myocardium (n = 10). Correspondingly, in LV myocardium, no increase in Ca2+-transient after AII could be detected.

In conclusion, AI and AII exert a PIE in human auricular, but not in ventricular myocardium. The PIE is due to specific AII-receptor-stimulation after cleavage of AI to AII by local ACE and is mediated by an increase in the Ca2+-transient.

Interaction between ionic changes and electrical function in acute myocardial ischemia
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Acute myocardial ischemia is associated with rapidly developing electrical dysfunction which leads to disturbances of impulse conduction (unidirectional conduction block, conduction slowing, circulating excitation) and associated ventricular arrhythmias. At a cellular level the electrical changes show a typical time course: within seconds after interruption of flow there is a depolarization of the resting membrane and subsequent shortening and reduction of amplitude and upstroke rate of rise of the action potential. After 4-7 min cells in the centre of the ischemic zone are depolarized to approximately −50 mV and inexitable. Electrical cell-to-cell uncoupling shows a rapid onset after approximately 10-15 min and is fully developed after 30-40 min. The major causes underlying the acute changes in excitability are the intra- and extracellular acidification and the cellular loss and extracellular
accumulation of K+. The cellular loss of K+ appears to be related to the intracellular acidification, whereas opening of ATP-dependent K+ channels seems to play a minor role. Further mechanisms which contribute to extracellular K+ loss are the shunt of the extracellular space and partial inhibition of Na+/K+ pumping. Electrical uncoupling occurs concomitantly with an increase in free cytosolic Ca2+, reduction in [ATP], and development of ischemic contracture. It is delayed by application of Ca2+ entry blockers, inhibitors of acylcarnitine-transferase and accelerated by acidification. Comparison of the changes in intracellular and extracellular concentrations of K+, Na+, Ca2+ and H+ in ischemia reveals numerous interactions in a complex system which makes it particularly difficult to delineate the initiating events.

Calcium signalling in cardiac myocytes
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In cardiac myocytes the influx of Ca2+ through voltage-dependent Ca2+ channels is the initial event in excitation-contraction (e-c) coupling. This trigger signal is amplified several fold by additional Ca2+-induced Ca2+ release (CICR) from the sarcoplasmic reticulum (SR). At present, it is largely unknown whether and how the signal amplification provided by CICR is regulated or modulated. But several experimental results have indicated that the degree of positive feedback in the CICR mechanism may be quite variable. In order to reveal features of Ca2+ signalling attributable to the proposed modulation, we investigated the subcellular Ca2+ distribution in heart muscle cells during triggered and spontaneous Ca2+ release. Cells were loaded with two fluorescent Ca2+ indicators (Fluo-3 and Fura-Red) to allow ratiometric imaging of intracellular Ca2+ with laser scanning confocal microscopy (Lipp & Niggli, Cell Calcium 14, 359–72, 1993). In cultured neonatal rat myocytes two types of spontaneous Ca2+ release were identified: (i) focal Ca2+ release with limited or without subcellular propagation and (ii) Ca2+ release propagating through the entire cell as a Ca2+ wave. In myocytes isolated from adult guinea-pigs the same fundamental patterns of Ca2+ release were found. In addition, we also observed striking deviations from the simple linear propagation pattern. Sequences of confocal optical sections revealed circular waves and spiral waves spinning around a subcellular core. Changes in the Ca2+ load of the cells and pharmacological tools which modify the function of the SR revealed that the probability, extent and pattern of Ca2+ release is modulated on the subcellular level. The Ca2+-dependent variability of the release patterns can be explained by a space- and time-dependent variability in the degree of positive feedback of the CICR mechanism within an individual myocyte. The degree of positive feedback may be modulated locally by the loading state of the SR. Subcellular variability also implies the existence of functionally separate SR elements, possibly corresponding to the 'cluster bombs' proposed in a mathematical model of cardiac e-c coupling (Stern, Physiological and Clinical Research, Department of Experimental Cardiology, Bad Nauheim, Germany)

Glycerinated cardiac and skeletal muscle activity following heat stress
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Reperfusion of the ischemic myocardium is often accompanied by diminished cardiac function. The explanation is oriented towards the calcium overload or the free radical release which take place immediately after the restoration of the flow. Both phenomena affect the membrane components (fatty acid chains and thiol groups on proteins). Heat stress is demonstrated to reduce the damage induced by ischemia/reperfusion phenomenon. There are new observations concerning the role played by the myofibrils in this domain. They seem to be affected directly by superoxide anion (MacFarlane & Miller, Circ. Res. 121–74, 1992), by the hydrosyl radical (Robert et al., Am. J. Physiol. 261, H1785–90, 1991), and by the ischemic phenomenon itself (Westfall & Solaro, Circ. Res. 70, 302–13, 1992).

Taking all into account we tried to verify if heat stress protection, which is supposed to be due to heat stress proteins or to an increase of cellular antioxidants, it is not acting directly on myofilaments. In our experiments we have measured the calcium uptake and the ATPase activity of the glycerinated muscular tissue (myocardium and skeletal) from control and heat stressed animals, collected in normal conditions or subjected to ischemic conditions in vitro.

Ischemia induces early changes in contractile and cytoskeletal proteins in diseased human myocardium but not in healthy rabbit or porcine hearts
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We tested the hypothesis that during myocardial ischemia in addition to damage of subcellular organelles various cytoskeletal and contractile proteins undergo destructive changes. Total global ischemia was induced by incubation in buffer of tissue samples from human left ventricles removed during transplantation surgery, and of normal pig and rabbit hearts. Incubation temperatures were 0°C, 4°C and 20°C. Samples were frozen after different time intervals and investigated by immunocytochemistry using monoclonal antibodies against myosin, actin, tropomyosin, troponin T, myomesin, desmin, and tubulin. The degree of ischemic injury was determined by electron microscopy (EM). As compared to control myocardium human cardiac tissue showed disturbances of the localization pattern of myosin, actin, tropomyosin and troponin T as early as 10 min after onset of ischemia. Reversible ischemic injury was present by EM. In contrast, myocardium from rabbits and pigs showed late ischemic changes. Contractile proteins were altered earlier than the cytoskeleton. The degree of injury depended also on the temperature of incubation. At 20°C the alterations occurred at earlier time points than at lower temperatures.
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However, 0°C caused more severe damage than incubation at 4°C. It is concluded that in diseased human hearts damage to the contractile proteins occurs rapidly after the onset of ischemia and earlier than subcellular injury, that the cytoskeletal proteins are more resistant to ischemia and that vinculin is the least sensitive. Normal rabbit and pig hearts show a high ischemic tolerance. The early destruction of the proteins in human hearts may have been caused by the increased amount of lysosomes and their proteolytic enzymes. These findings are important for the situation of induced cardiac arrest in heart surgery and for preservation of donor hearts for transplantation.

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Estimate of Na–Ca fluxes during systole in rat ventricular cells
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The aim of this study was to compare the fluxes of calcium resulting from the calcium current and Na–Ca exchange in cardiac muscle. We have measured the Na–Ca exchange during and after depolarizing pulses. The experiments were performed on single cells from the rat ventricle which were loaded with the fluorescent indicator Indo-1. The whole-cell patch clamp technique was used. The procedure used was as follows: (i) The Ca-dependence of the electrogenic Na–Ca exchange current was measured by rapidly applying caffeine and measuring both [Ca$^{2+}$] and current (Varro et al., Pfuiigers Arch. 423, 159–60, 1993). (ii) The derived relationship between current and [Ca$^{2+}$] was then used to estimate the Na–Ca exchange currents activated by depolarizing pulses of various durations from ~40 to 0 mV. The extrusion of calcium by Na–Ca exchange could then be compared with the entry via the Ca current. We find that there is a net loss of calcium with short duration depolarizing pulses (less than about 100 ms) and a gain with longer pulses. This is correlated with a negative staircase on starting stimulation with short pulses and a positive one with long pulses.

In conclusion this method allows changes of the systolic Ca transient to be correlated to estimated changes of net cell Ca content.

Microheterogeneity of subsarcolemmal Na-concentrations: an X-ray microprobe study
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Steady gradients of intracellular sodium concentration, from the sarcolemma to the centre were measured in ventricular myocytes of the guinea-pig after Ca$^{2+}$ influx and contraction were potentiated to an optimum with a train of 18 paired voltage-clamp pulses (2 mM [Ca$^{2+}$]), 36°C). Potentiation reversibly enlarged and prolonged the diastolic tail currents due to Na$^{+}$,Ca$^{2+}$-exchange, reversibly increased the current at +50 mV and made it more noisy. Cell attached recordings with a second electrode, attributed this noise to the activation of K$^{+}$ (Na) channels. This result suggests that potentiation can explain intracellular sodium concentration to 40 mM. The gradients indicate that sodium cannot freely diffuse. Electron-probe microanalysis (EPMA) measured $\Sigma \text{Na}$ in a volume within 20 nm from the inner side of the sarcolemma. In unstimulated cells subsarcolemmal Na was 17 ± 5 mM. Potentiation reversibly increased subsarcolemmal sodium to 40 ± 7 mM. When stimulation was terminated, subsarcolemmal sodium fell within 8 s to 37 ± 8 mM and within 3 min to 19 ± 6 mM. From the sarcolemma to the centre, $\Sigma \text{Na}$ fell with a space constant of 28 nm, at 1 µm distance $\Sigma \text{Na}$ was 12 ± 3 mM. The steep gradient suggests that sodium is not freely diffuse and that subsarcolemmal sodium is controlled by transmembrane fluxes rather than by cell dialysis. $\Sigma \text{Na}_{20\text{min}}$ data were distributed with peaks at 5 mM, 30 mM and 60 mM. Quantitative elemental digital imaging demonstrated patches with 60–80 mM subsarcolemmal sodium alternating with others of 0–15 mM $\Sigma \text{Na}_{20\text{min}}$. This 'Na-microheterogeneity' suggests that Ca$^{2+}$ influx at low subsarcolemmal Na and K$^{+}$ (Na) channels activation at high $\Sigma \text{Na}_{20\text{min}}$ can operate simultaneously.

Na$^{+}$-current induced calcium release in cardiac myocytes
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In cardiac muscle the electrical excitation is linked to mechanical activity by an influx of Ca$^{2+}$ through voltage-gated Ca$^{2+}$-channels and Ca$^{2+}$-induced Ca$^{2+}$ release. Based on indirect experimental evidence it has been suggested that subsarcolemmal Ca$^{2+}$-concentration gradients play an important role during the process of dc-coupling (Lipp et al., J. Physiol. 454, 321 J6, 1992). Recently, similar short-lived concentration gradients were also proposed to exist for Na$^{+}$ (Leblanc & Hume, Science 248, 372–6, 1990). This hypothesis was based on the observation, that $I_{\text{Ca,L}}$ (in the absence of $I_{\text{Na,L}}$) was able to trigger Ca$^{2+}$-release in ventricular myocytes, presumably by activating Na–Ca exchange in the Ca$^{2+}$-influx mode. However, the significant increase of [Na$^+$] required for this activation was only feasible if Na$^{+}$ accumulated (at least transiently) in a restricted space between sarcolemma and SR membrane (Lederer et al., Science 248, 283, 1990). We used ratemetric laser-scanning confocal microscopy (Fluo-3 and Fura-Red) to record [Ca$^{2+}$] in guinea-pig cardiac myocytes while the whole-cell current was simultaneously measured with the patch-clamp technique (Lipp & Niggli, Cell Calcium 14, 359–72, 1993). The high spatial and temporal resolution of the confocal instrument enabled us to resolve striking differences in the kinetics between $I_{\text{Na,L}}$ and $I_{\text{Ca,L}}$-induced Ca$^{2+}$-transients, both in the absence and presence of ryanodine (20 µM). In addition, we were able to detect a residual $I_{\text{Ca,L}}$-induced Ca$^{2+}$-transient in the presence of ryanodine and verapamil (10 µM). This Ca$^{2+}$-transient represents the missing link between $I_{\text{Ca,L}}$ and the release of Ca$^{2+}$ from the SR and probably reflects Ca$^{2+}$ influx via Na–Ca exchange. Control experiments showed that $I_{\text{Na,L}}$-current through Na$^{+}$-channels was not able to induce Ca$^{2+}$ influx or release. This result indicates that uncontrolled activation of Ca$^{2+}$-channels due to loss of voltage-control as well as Ca$^{2+}$ influx through Na$^{+}$ channels were negligible. Our findings confirm and directly visualize the existence of $I_{\text{Na,L}}$-induced Ca$^{2+}$ release from the SR after activation of the Na–Ca exchange and thus support the notion of a subsarcolemmal restricted space. Moreover, the fast kinetics of the $I_{\text{Na,L}}$-induced Ca$^{2+}$-transients revealed by confocal microscopy suggest a significant contribution of the Na$^{+}$ influx to the early phase of the Ca$^{2+}$-transient during an action potential.

SMOOTH MUSCLE AND NON-MUSCLE MOTILITY

Modulation of vascular smooth muscle spontaneous activity by alteration of intracellular pH (pH$_{i}$)
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Spontaneous contractile activity of portal venous smooth muscle is reduced during hypoxia when intracellular pH may be expected to fall (Lovgren & Hellstrand, Acta Physiol. Scand. 123, 484, 1985). We have therefore investigated the functional effects of directly altering pH$_{i}$ in rat portal vein by simultaneously measuring force and pH$_{i}$ (using carboxy SNARF). pH$_{i}$ was changed, whilst maintaining external pH at 7.4, by isosmotic substitution for sodium HCO$_3^{-}$ in the Krebs buffer (gassed with 100% CO$_2$). The resting pH$_{i}$ in the preparations was 7.06 ± 0.03. Addition of butyrate (20 mM) reduced pH$_{i}$ by 0.18 ± 0.01 pH unit (n = 8). This was accompanied by an initial and transient, increase in force followed by...
Expression of desmin in cultured human smooth muscle uterine cells

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The smooth muscle of the uterus contains a large amount of the cytoskeletal protein, desmin, which is known to be expressed only in differentiated muscle cells. Desmin expression was researched in human myometrial cells in order to assess if they keep a muscular phenotype after subculturing. Moreover, we tested the influence of steroid hormones (17β-estradiol, E2, and progesterone, P) and epidermal growth factor (EGF) upon desmin expression because these factors are known to influence myometrial cells hyperplasia and/or hypertrophy. Myometrial cells were obtained by the explant method and studied until seven passages. The cells were grown to confluence in media supplemented with serum. Cell content in desmin was tested and frequency of contraction.

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Influences of certain changes in extracellular ion concentrations upon α1-adrenergic effects in the rat aorta

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Several classifications of α1-adrenoceptors have been proposed so far. The large amount of data originating from various experimental approaches still lacks an integrative description. Reconsideration of previous results, as well as new attempts, may therefore be useful. We studied the contractile responses evoked by α1-adrenergic agonists in de-endothelised rat aortic rings, along with their alteration upon changes in the extracellular concentration of K⁺, Na⁺ or Ca++. High K⁺ (40 mM). Precontracted rings display a sustained superimposed contraction with phenylephrine challenge (PHE, 10⁻³ M), but a transient one with norepinephrine (NE, 10⁻⁵ M). Along with the differences observed in the D600-sensitivity, such results support the idea that PHE is α1B-, while NE is α1A-selective (Filipeanu et al., Pharmacol. and Toxicol., submitted, 1993). Extracellular NaCl was replaced (25, 50, 75 and 100% of the initial 118 mM) with either UCl, CholineCl or sucrose. The tonic contractions observed were studied in terms of their dependence upon the degree of extracellular Na⁺-removal and their sensitivity to a number of relaxing agents was tested. Superimposed PHE-induced contractions were significantly reduced with substitution degrees higher than 50% (Toma et al., Naunyn Schmiedeberg. Arch. Pharmacol., submitted, 1993). We applied a classical protocol for the depletion of intracellular Ca²⁺-stores. We observed differences between high K⁺- and PHE-induced contractions upon return to normal extracellular Ca++. The differences observed for the proposed mechanisms of depletion-repletion of intracellular Ca²⁺-stores (Toma et al., Romanian Physiol. Conf., abstract, 1993). Other studies were performed in connection with these and some of them brought useful new insights. We studied the calmodulin-sensitivity of high K⁺- and PHE-induced contractions. The differences observed could be related to the involvement of calmodulin in different mechanisms and to the variable Ca²⁺-sensitivity of the contractile element (Filipeanu et al., Life Sci., in press, 1994). Interspecies difference in α1A- and α1B-dependent relaxing mechanisms seems to change with various contractile stimuli (Serban et al., unpublished), and so do the relations between pathways involving phosphodiesterases and guanylate cyclase (Serban et al., Romanian Physiol. Conf., abstract, 1993).

Searching for precursor cells of the vascular wall

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Endothelial and smooth muscle cells are well characterised components of the vascular wall. In vitro and in vivo they are easily distinguished by their phenotype as well as by their expression of specific proteins. For a number of years, a third cell type, distinct from either family has been postulated (Taggart & Wray, Pfugers Arch. 423, 597, 1993), and may contribute to the fall in force observed during hypoxia in these tissues.

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by a decrease or abolition of contraction. Decreasing external pH lowered pH, and decreased force, although not to same extent as pH, but transient effects on force were absent. Addition of the weak bases TMAB (n = 7) or NH₄Cl (n = 4) (20 mM) resulted in a elevation of pH, (0.23 ± 0.03 and 0.19 ± 0.02 pH unit respectively). The intracellular alkalization was initially associated with a transient decrease in contraction which was followed by an increase in both the magnitude and frequency of contraction.

Thus alterations in pH, result in profound and complex changes in mechanical performance of spontaneous vascular smooth muscle. The impairment of contractile activity with continually reduced pH, is similar to that seen in the myometrium, a spontaneously active non-vascular smooth muscle (Taggart & Wray, Pfugers Arch. 423, 597, 1993), and may contribute to the fall in force observed during hypoxia in these tissues.

Influences of certain changes in extracellular ion concentrations upon α1-adrenergic effects in the rat aorta

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Searching for precursor cells of the vascular wall

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Kinetic determination of filamin binding to monomeric actin
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Filamin—a highly flexible homodimer—is found in large quantities in smooth muscle with actin binding sites at the N-terminal ends (Hartwig & Stossel, J. Biol. Chem. 250, 5696–705, 1975). Although filamin is known as a filamentous (F) actin binding protein and the affinity has been determined, we have measured the interaction of filamin to monomeric actin. We have used various kinetic techniques to determine the binding parameters of filamin and monomeric actin in solution.

Filamin prepared from chicken gizzard binds to skeletal muscle monomeric actin with an overall affinity of ~0.6 μM. The stoichiometry of 1:1.7–1.3 for filamin to fluorescently labelled monomeric actin was measured by steady state continuous titration. The association rate constant of filamin and fluorescently labelled monomeric actin of ~1.8 × 10^6 M^-1 s^-1 and the dissociation rate constant of ~0.7 s^-1 were ascertained by the stopped flow method. Furthermore, recent thermodynamic studies suggest hydrophobic binding of filamin with phospholipid vesicles (Goldmann et al., Eur. J. Biochem. in press, 1993).

Chicken gizzard myoglobin: a fatty acid binding protein
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Chicken gizzard appears to be the only vertebrate smooth muscle containing myoglobin (Gröschel-Stewart et al., Experientia 27, 512–13, 1971). We now present evidence that it not only stores oxygen in the tissue, but also functions as a fatty acid binding protein (FABP).

Highly purified oxy-myoglobin from chicken gizzard binds sulfobromophthalein = BSP (Ockner et al., Science 177, 512–13, 1972) and fluorescent fatty acids. Both ligands can be displaced by unlabelled long chain fatty acids, unsaturated acids being more effective than saturated ones. The molar binding ratio was calculated to be 1.1. Fatty acid binding properties correlate with the degree of oxygenation of myoglobin, the met-form being unable to bind. Although we were able to isolate an established 14.5 kDa fatty acid binding protein from gizzard with distinct biochemical and immunological properties, we feel that the cytoplasmic hemoprotein may also have an important physiological role in fatty acid binding and -transport in the gizzard, and maybe in other tissues as well.

Enhanced contractility of the pregnant human uterus correlate with different myosin subunit expression
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We investigated mechanical performance (force-velocity relation of chemically skinned fibres) and in vivo expression of myosin heavy chains (MHC) and 17 kDa myosin light chain (MLC) isoforms in the uterus of late pregnant (P) (septo) and non-pregnant (N) (hysterectomy) patients. Values are means ± SEM with number of patients/fibres per patient investigated. Mechanical parameters were measured at pCa 4.5 with 1 μM calmodulin. Resting tension curves and normalized length-tension ratios of active contractions (pCa 4.5) were the same in both groups. Maximal force of isometric contraction of skinned fibres (slack length) was 2.85 ± 0.3 (5/2) mN/mm^2 and 5.6 ± 1.1 (5/2) mN/mm^2 in N and P, respectively (p < 0.01). Maximal shortening velocity as extrapolated from the force-velocity relation rose from 0.134 ± 0.011 muscle lengths s^-1 (ML s^-1) in N patients to 0.243 ± 0.015 ML s^-1 (5/5) in pregnant women (p < 0.001). Phosphorylation levels of the 20 kDa MLC isozymes upon Ca^2+ activation of the fibres increased to the same extent in both N and P. Three MHC are expressed in the human uterus with decreasing MW as SM1, SM2, and NM. SM1 and SM2 reacted with an antibody specific for smooth muscle MHC, NM with an antibody specific for non-muscle MHC. N patients expressed 31.7 ± 0.7%, 34.7 ± 1.3%, and 33.6 ± 1.3% of SM1, SM2, and NM-MHC, respectively (4/9). P had 35.1 ± 1.2%, 40.9 ± 0.7, and 24 ± 0.9% SM1, SM2, and NM-MHC, respectively (4/4). The increased SM2 and NM-MHC expression in P patients was statistically significant (p < 0.01). MLC1α expression was increased from 23.5 ± 1.6% (4/9) in N to 44.2 ± 0.8% (4/9) in P (p < 0.001). Thus, similar to the pregnant rat uterus enhanced mechanical properties correlated with changes in myosin expression especially with increased amounts of MLC1α.

Contractile and cytoskeletal compartments in the smooth muscle cell
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Differentiated smooth muscle cells typically contain a mixture of muscle (α and γ) and cytoplasmic (β and γ) actin isoforms. Of the cytoplasmic actins the β-isomorph is the more dominant, making up from 10–30% of the total actin complement. Employing an antibody raised against the N-terminal peptide specific to β-actin, which labels only the β-cytoplasmic isoform, we have shown that this isoform has a restricted localization in smooth muscle. In longitudinal, super-spread ultrathin sections of gizzard smooth muscle, β-actin was localised in the dense bodies and in longitudinal channels linking consecutive dense bodies that were also occupied by desmin. It was also found at the membrane-associated dense plaques, but was excluded from the acymyosin-containing regions of the contractile apparatus. Taken
together with earlier results these findings identify a cytoskeletal compartment containing intermediate filaments, cytoplasmic actin and the actin cross-linking protein filamin. Smooth muscle γ-actin was localized to the regions containing myosin filaments and was excluded from the cores of the dense bodies, that contain β-actin. If the dense bodies couple the contractile actin filaments with those of the cytoskeleton then anchorage of the contractile actin would appear to occur only at the surface of the dense bodies.

Additionally double labelling experiments confirmed that caldesmon was restricted to the contractile apparatus but that calponin, another actin- and calmodulin-binding protein of smooth muscle, was present in both the contractile and cytoskeletal domains, as well as in the dense bodies. Calponin would thus appear to function as a regulator in both domains.

Molluscan striated and smooth muscle myosin heavy chain isoforms are produced by alternative splicing of a single gene

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Muscle myosin heavy chain (MHC) isoforms are encoded by either a multigene family (vertebrates and nematodes) or by a single gene (Drosophila). Here we show that the smooth and striated muscle MHC isoforms of scallop are produced by alternative RNA processing.

Scallop catch (smooth) muscle cDNA and genomic DNA were amplified by PCR using primers based on the sequence of striated adductor muscle MHC (Nyitray et al., J. Biol. Chem. 266, 18469, 1991). Mapping and sequencing revealed that the 24 kb gene encodes the MHC in 27 exons, and, moreover, that two sets of Isolated exon pairs are alternatively spliced into the striated and smooth MHC mRNA, respectively. The striated muscle-specific isoform is not expressed in other tissues, while the catch muscle-specific isoform was detected in different smooth muscles but not in striated muscle.

Exon 8a,b (residues 176-211 in the striated MHC sequence) and -6a,b (212-243) encodes part of the ATP-binding site. Exon 5 contains the consensus P-binding site (GESGAGKT) and the highly divergent 23 kDa/50 kDa proteolytic junction. Residues in exon-5 are thought to be involved in the Ca2+ regulation of scallop myosin (Kerwin & Young, Proc Natl Acad Sci USA 90, 35, 1993; Biophys J. 64, A181, 1993). Exon-20a,b (1214-1239) encodes the middle of the rod hinge region. Exon-26 in the striated-specific sequence starts with the stop codon, while the catch-specific exon codes for an additional ten residues. The longer non-helical tailpiece of the catch MHC may explain why it is only the catch muscle which is phosphorylated at Ser-1926 but not the striated one by an endogenous kinase (Castellani & Cohen, Science 235, 334, 1987). Differences between the alternative exons presumably determine the lower ATPase activity of molluscan smooth muscle myosins, contribute to the different structure of the smooth and striated muscle thick filament and may also be important in the molecular mechanism of the catch phenomenon. Finally, our results demonstrate that the invertebrate smooth and striated muscle myosins (at least in molluscs) are closely related unlike their vertebrate counterparts.

Passive tissue mechanics and contractile force in guinea-pig portal vein

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It is known that the force generated by muscle cells is modified by the mechanical properties of the passive tissue. This study was carried out to determine the variation of the passive tissue stiffness with the frequency of the length perturbation and to study quantitatively the effect of the passive tissue characteristics on the force generated in the guinea-pig portal vein. To analyse the contractile properties of the portal vein rhythmic spontaneous contractions were recorded. Also tension responses to sudden stretches were measured during the development of its spontaneous contractions. To assess the passive tissue mechanics, the muscles were left in a passive solution and tension responses to stretches were measured. After the experiments spontaneous contraction patterns and the tension responses were analysed in time and frequency domains. It was found that spontaneous contraction patterns of the guinea-pig portal vein had rhythmic components within the frequency range of 0.01-0.2 Hz. The tension responses of the contracting portal vein displayed a characteristic feature consisting of four phases. Initially there was a rapid change in tension in the direction of the length perturbation. Then tension reversed its direction (early recovery). This phase was followed by a delayed tension increase. Thereafter tension reached steady-state. In the responses, the early recovery was observed at 1-4 s and the delayed tension response was obtained at 9-14 s. The stiffness and phase characteristics evaluated from the tension responses of the contracting portal vein had maxima and minima. The work characteristics displayed a prominent positive peak at 0.04-0.06 Hz which indicated that the muscle strips were using oscillatory work on the mechanical system. On the contrary, the tension responses of the relaxed muscles displayed a behaviour similar to the responses of viscoelastic materials. Besides the stiffness characteristics of the relaxed muscles lacked maxima and minima of the contracting muscles. Instead they exhibited a linear relationship between the stiffness and the logarithm of the frequency and the slope of the stiffness characteristics was 0.21 dB per octave. This means that, for a given preload, the stiffness of the passive tissue increases with the frequency of the length perturbation. From these results we deduced that the passive tissue mechanics could enhance the contractions at high frequencies.

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Prevention of high-calcium induced retardation of contraction kinetics by phosphatase inhibition

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The smooth muscle contraction kinetics were analysed by using the vibration method (Klemt et al., J. Physiol. 312, 281, 1981); after cessation of the force-inhibiting 1 s length vibration (100 Hz, sinus, 5% of ML), an exponential function was fitted to the time course of force recovery. The extent of force generation reflects the number of attached crossbridges, the time constant of force recovery depends on the mechanical system. On the contrary, the tension responses of the relaxed muscles displayed a behaviour similar to the responses of viscoelastic materials. Besides the stiffness characteristics of the relaxed muscles lacked maxima and minima of the contracting muscles. Instead they exhibited a linear relationship between the stiffness and the logarithm of the frequency and the slope of the stiffness characteristics was 0.21 dB per octave. This means that, for a given preload, the stiffness of the passive tissue increases with the frequency of the length perturbation. From these results we deduced that the passive tissue mechanics could enhance the contractions at high frequencies.

The isolated rat portal vein, was activated either by electrical field stimulation (50 Hz, sinus, 7 volts), or by K-depolarization. Prolongation of the activation period prior to vibration decreased the contraction kinetics as indicated by an increase of the time constants from 0.7 to 1.8 s (=crossbridge downregulation, Siegmam et al., J. Muscle Res. Cell Motil. 7, 39, 1986). For the force development, the ED50 of extracellular calcium was calculated to be 0.73 mmol L-1. After both the depletion of the intracellular calcium stores and the depolarization in calcium-free potassium-rich bath solution, the ED50 shifted to 10.8 mmol L-1 CaCl2. Maximum force was developed in a 51.8 mmol L-1 CaCl2 containing depolarizing bath solution. Under these experimental conditions, the time constant of postvibration force recovery was prolonged up to 12.31 ± 1.35 s. In contrast to the high intracellular calcium, this increased time constant may reflect less phosphorylation of the 20 kDa myosin light chains due to either a low activity of the protein kinase or a high activity of the protein phosphatase. The addition of the phosphatase inhibitor okadaic acid (10 μmol L-1) partly prevented the increase of the time constants which averaged only 8.04 ± 0.86 s under these conditions. We conclude that the pronounced retardation of the contraction
kinetics (seen after calcium-depletion in the depolarized rat portal vein activated by high extracellular calcium) is probably due to an activation of the myosin light chain phosphatase.

**Energetics of the contractile process of bacteriophage T4**

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The tail sheath of phage T4 is the most simple motile system known in living nature. We showed that one molecule of GTP is bound to each subunit of the extended sheath. We also found that structural changes of the protein during contraction are accompanied by hydrolysis of GTP, and the protein itself possesses GTPase activity. By analogy with the role of ATP in muscle contraction we inferred that the energy required for contraction of the phage sheath may be provided by the breakdown of GTP.

Arisaka and coworkers (1981) showed by calorimetric measurements that the contraction of the tail sheath is an exothermic process which is accompanied by a release of energy amounting to 44 kcal mol\(^{-1}\) contractile protein. It is, however, known that only 7 kcal mol\(^{-1}\) is liberated on splitting of a phosphatase group from one molecule of GTP. Taking into account that each molecule of the contractile filament of phage T4 is bound to one molecule of GTP, we may conclude that the amount of the energy liberated on contraction of the sheath is virtually six times more than that released by the breakdown of GTP. This holds even in the case if all energy is dissipated in the form of heat and not used for the motile process. From these considerations we may conclude that hydrolysis of GTP does not supply the energy for motion.

**Effects of inorganic phosphate on contraction in skinned smooth muscle**

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We have investigated the effects of inorganic phosphate (Pi) on skinned smooth muscle fibre bundles from the guinea-pig taenia coli. In maximally activated (thiophosphorylated) fibres, Pi gave a dose-dependent inhibition of active force. At 20 mM Pi, force was decreased by about 20%. In Ca\(^{2+}\) activated fibres the relative inhibition of active force (about 20% at 20 mM Pi) was similar at all investigated Ca\(^{2+}\) levels. Thus the Ca\(^{2+}\) sensitivity of contraction was not altered by 20 mM Pi. Force-velocity relations were determined at different Ca\(^{2+}\) levels. The maximal shortening velocity (\(V_{\text{max}}\)) varied almost in parallel with active force when the level of activation was varied with altered [Ca\(^{2+}\)] in the absence of Pi. Addition of 20 mM Pi decreased force at all [Ca\(^{2+}\)] whereas [Ca\(^{2+}\)] was unaltered at higher Ca\(^{2+}\) and slightly increased at lower [Ca\(^{2+}\)]. In maximally thiophosphorylated fibres 20 mM Pi decreased force (by about 20%) without any significant effects on \(V_{\text{max}}\). The ATPase/force (tension cost) increased significantly. The rate of active tension development following photolytic release of ATP in activated fibres was increased in the presence of 20 mM Pi. The results show that Pi interacts directly with the crossbridge cycling in smooth muscle fibres. The decreased force in the presence of Pi suggests a reduction in the number of attached force-generating crossbridges without affecting the flux through crossbridge cycle since ATPase was not reduced. This can be associated with an increase in the backward rate of the Pi, release reaction, since the reduction of force was associated with an increased rate of force generation. The unaltered sensitivity of force to [Ca\(^{2+}\)] in the presence of Pi suggests that the Pi\(^{2-}\) induced alteration in attached crossbridge states does not affect the Ca\(^{2+}\)-regulation. In maximally activated muscle, Pi does not affect the rate limiting steps for the isotonic shortening. However, at low levels of activation, Pi increased \(V_{\text{max}}\) which might suggest that the isotonic shortening under these conditions is rate-limited by crossbridge reactions that can be affected by Pi.

**REGULATORY PROTEINS**

**Identification of functional domains in smooth muscle caldesmon**

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To better characterize the functional organization and binding surfaces of smooth muscle Caldesmon (CaD) with Actin, Calmodulin (CaM) and Myosin, we used a mapping procedure that employs partial chemical cleavage with NTCB of Caldesmon (Fo) at its two cysteine residues (153 and 580). The cleavage yields a series of polypeptides termed F1 to F5. F1 (1–152) and F3 (580–756) represent the N- and C-term fragments, respectively; F2 contains the central part (153–579) whereas F4 represents the fragments F1 + F2 (1–579) and F5 contains fragments F2 + F3 (153–756). Only peptides F2, F3 and F5 are retained by Ca\(^{2+}\)/Calmodulin-Sepharose column. Furthermore, both fragments F3 and F5 co-sediment with F-actin and inhibit the actomyosin Mg\(^{2+}\)ATPase in a Ca\(^{2+}\)/Calmodulin dependent manner. Comparison with uncleaved CaD and with the 10 kDa CNBr C-terminal fragment (Bartegi et al., J Biol Chem 265, 15231–6, 1990) suggests that the C-terminal fragment F3 is responsible for the strong inhibitory activity of the actomyosin ATPase. The extent of this inhibition was almost identical to that displayed by native CaD. F3 could be crosslinked by Carbodiimide to calmodulin in the presence of Ca\(^{2+}\) because it contains the 10 kDa fragment. Unlike the 10 kDa fragment which did not undergo any carbodiimide crosslinking reaction with F-actin, F3 could be crosslinked to the N-terminal acidic residues of actin. This implies the presence of an additional actin-binding site intervening in this specific recognition by carbodiimide which is located upstream of the 10 kDa fragment and sparing the residues 580 to 658. Since only the F3 or F5 fragments of CaD interact with F-actin, our data rules out an elongated end-to-end alignment of CaD on the actin filament in vitro, but instead, the N-terminal part of CaD which has no actin binding property may project out from the filament. Smooth muscle myosin affinity column retains native CaD and all its fragments F1 through F5. Whereas CaD can be crosslinked to smooth muscle myosin by carbodiimide none of its individually purified fragments do so. This latter result provides evidence that the myosin binding domain of CaD is formed by separate regions along the molecule of CaD. Although separated in the primary sequence, these regions may well interact with each other in the folded structure.

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**Effect of calcimedin on caldesmon functioning**

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Calcimedin, a 67 kDa Ca\(^{2+}\) and phospholipid binding protein, was isolated from duck gizzard and some of its properties were investigated. In the presence of Ca\(^{2+}\), calcimedin interacted and cosedimented with vesicles of mixed phospholipids (azolectin) or with F-actin. Different Ca\(^{2+}\)-binding proteins (calmodulin, troponin C, S-100 protein and calcimedin) were tested for their ability to reverse the inhibition of actomyosin ATPase caused by caldesmon. Calcimedin...
was found to be more effective than all other Ca-binding proteins in reversing of caldesmon action. In the presence of Ca\(^{2+}\) calmodulin, troponin C and S-100 protein interacted with isolated caldesmon and the complex formed was detected by native gel electrophoresis and cross-linking techniques. Addition of EF-hand proteins to actin filaments resulted in partial dissociation of caldesmon and can by this means (at least partially) reverse caldesmon-induced inhibition of actomyosin ATPase. Calcineurin was unable to interact with isolated caldesmon at any Ca\(^{2+}\) concentration and did not cause dissociation of caldesmon from thin filaments. Under the conditions used caldesmon and calcineurin can coexist on actin filaments. Thus the mechanism of calcineurin action seems to be different from that of other EF-hand proteins. Calcineurin was much less effective in reversal of caldesmon inhibition of acto-HMM ATPase than of actomyosin ATPase therefore we suppose that the effect of calcineurin is at least partly due to the bundling of actin filaments. Taking into account that the content of calcineurin in smooth muscle is significantly less than that of caldesmon calcineurin cannot be directly involved in regulation of smooth muscle contraction. This does not exclude the possibility that in certain cell compartments (i.e. perimembrane regions) calcineurin in the presence of Ca\(^{2+}\) can reverse the inhibitory action on caldesmon.

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Myosin light chain kinase expression during smooth muscle development
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The pattern of expression of smooth muscle myosin light chain kinase (MLCK) was investigated by high-performance liquid chromatography (HPLC), by immunoblotting and by amino acid sequence in embryonic, post-hatching and adult chicken gizzard. In this study, a polyclonal antibody raised to adult chicken gizzard smooth muscle was used. Western blot analysis showed that this antibody recognized a 130 kDa band of MLCK in all stages of gizzard development. HPLC analysis of 4-day-old and adult gizzard MLCK revealed different patterns of elution for the two proteins. A partial amino acid sequence of 4-day-old gizzard MLCK failed to find differences in the primary sequences of the two proteins. The results suggest the presence of a unique form of MLCK characteristic of the same primary sequence in all stages of gizzard development. However, our findings cannot rule out the possibility that different post-translational modifications (phosphorylation) of the protein take place during development.

Functional site mapping within the C-terminal 288 residues of human caldesmon
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A cDNA clone, encoding the C-terminal 288 amino acids of human caldesmon, was used to produce a range of DNA fragments by the polymerase chain reaction. Fragments were subcloned into the vector pMW172 and peptides were expressed in E. coli representing regions of domains 3, 4a and 4b (Marston & Redwood, Biochem. J. 279, 1–16, 1991). The whole clone (H1) encodes all these domains, equivalent to amino acids 506–793 of human caldesmon (Humphrey et al., Gene 112, 197–204, 1992; Huber et al., J. Muscle Res. Cell Motil. 14, 385–91, 1992). There is a strong smooth muscle myosin binding site in H1. However, of the expressed subfragments, only weak myosin binding was found in H2 (683–767, domain 4a) and in H3 (622–680, junction of domains 3 and 4a) and H7 (622–767, 3/4a junction + domain 4a). Thus it would appear that no expressed fragment fully represents the myosin binding site and that binding is not mediated by a distinct site in our H1 fragment. The presence of two calmodulin binding sites in the C-terminus of caldesmon has been demonstrated using native caldesmon, the expressed subfragments H2, H8 (715–793, domain 4b) and H9 (726–793, domain 4b less N-terminal 11 amino acids) and a synthetic peptide M73 (714–723). These have been termed site A and site B. Site A corresponds to the sequence 714MWEKGNVFS723, which was identified by Zhan and colleagues, J. Biol. Chem. 266, 21810, 1991 and Hayashi and colleagues, J. Biol. Chem. 266, 355, 1991), whilst site B is located nearer the C-terminus in the sequence common to H2 and H9 (726–793). It is apparent that binding of calmodulin at site B is associated with reversal of caldesmon inhibition, whilst calmodulin binding at site A, represented by M73, has no functional consequences. The smallest region of caldesmon which is fully functional as a tropomyosin dependant, Ca\(^{2+}\)-calmodulin regulated inhibitor is H9 (726–793), just 68 amino acids at the C-terminus. This confirms earlier predictions that the inhibitory site is in domain 4b. However, H7 (622–767), which overlaps the N-terminal half of H9, inhibited just as well whereas H2, containing the sequence common to H7 and H9, was not inhibitory. This would suggest that H2 (N-terminal half of domain 4b) contains the inhibitory and B calmodulin binding sites, but that the site only has its native structure and function in the presence of a flanking sequence. H9, H7 and H2 all bind to actin-tropomyosin with affinities of the same order of magnitude (10\(^{-4}\) weaker than H1 or native caldesmon), whilst two fragments from domain 3 did not bind actin-tropomyosin and were not inhibitory. Tropomyosin binding was previously predicted to be in domain 3. However neither of our fragments from this region were found to interact with tropomyosin, whereas fragments containing domain 4a (622–715, H2, H4, H5, 506–680, domain 3 + 3/4a junction) and H7, did interact. Preliminary NMR measurements have been carried out with 658C, a fragment constituting domain 4b of gizzard caldesmon. Broadening of the signals of tryptophan 692 (human 749) and 722 (human 779) has been observed upon adding actin and/or calmodulin, suggesting involvement of these residues within binding sites.

Ultrastructure of the contractile apparatus in triton-extracted smooth muscle and intact ultrarapidly frozen smooth muscle
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Abstracts

The structure (in particular the lengths) of some of the components of the contractile apparatus in smooth muscle and thus their exact relationship to each other is not fully understood. This is because of its relative disorder compared to cardiac or skeletal muscle. Electron microscopy studies require a chemical fixation step which, by its very nature, induces artefacts in the muscle tissue. Using quick-freeze, deep-etch techniques, we eliminate the use of chemical fixatives whilst allowing the visualization of the ultrastructure in three dimensions. Three dimensional visualization offers an opportunity to shed some light on the structure of the contractile apparatus. Freeze fracture replicas of Taenia coli smooth muscle tissue reveal actin filaments that are very well preserved. The 5.4 nm repeat of the actin sub-units along the roughly parallel filaments is clearly visible. Filaments appear to run for only short lengths in the plane of fracture and run roughly parallel to the long axis of the cell but are not highly ordered. Possible lateral links to the cell membrane are sometimes observed. Freeze fracture of fixed and cryoprotected, triton-extracted tissue (using a method which preserves the contractility of the muscle) reveals the contractile apparatus more clearly. Filaments in extracted tissue appear more deeply etched and myosin thick filaments and dense bodies are more easily observed. Filaments appear to have many cross links some of which seem to arise from thick filaments. Myosin thick filaments are most easily distinguished from thin filaments in transversely fractured tissue; here their distribution is comparable to that seen in thin, transverse sections from freeze substituted samples. In extracted tissue the face polar nature of the filaments is indicated, especially in longitudinal sections from freeze substituted samples. In intact tissue where all the filaments appear less distinct and less deeply etched, thick filaments are often difficult to distinguish in longitudinal replicas. Preliminary data comparing relaxed and rigorised extracted muscle indicate a more ordered structure in rigor state and it appears that there may be more cross links between filaments.

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Comparison of the properties of dimeric and monomeric calponin

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Calponin is a component of smooth muscle thin filaments. Demonstrated in vitro inhibition of actin-activated ATPase activity of myosin and sliding of actin filaments over myosin by calponin, as well as the reversal of this inhibition by Ca2+ and calmodulin or by Ca2+-dependent phosphorylation of calponin, make it a likely candidate for a role in actin-linked regulation of actin-myosin interaction (Makuch et al., Biochem. J. 280, 53-6, 1991; Shirinsky et al., J. Biol. Chem. 267, 15886-92, 1992; Winder & Walsh, J. Biol. Chem. 265, 10146-55, 1990).

In the present studies we focused our attention on some properties of calponin that can be related to its function. Solubility of calponin is very low and varies minimally over a wide range of NaCl concentration and pH. The absence of reducing agents from the solution does not affect calponin solubility, despite the formation of oligomers (preferentially dimers) due to intermolecular sulphurdry cross-linking.

Upon interaction with actin, about 50% of actin complexed with calponin (at a molar ratio of 2:1) precipitates. Light microscopy and electron microscopy of negatively stained samples reveal the presence of paracrystalline bundles of actin in the precipitate. Dimers of calponin are much more effectively incorporated into the bundles than are monomers. Ultracentrifugation of the supernatant, after removal of bundles by low speed centrifugation, showed that calponin also binds to the remaining pelleted actin.

Interaction of calponin dimers with calmodulin is stronger than that of monomers, since buffer containing 2 mM EGTA and 0.2 mM NaCl which elute monomers from calmodulin conjugated with Sepharose 4B column is unable to dissociate dimers. The dimers are eluted upon elevation of salt concentration to 2 M.

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Weak magnetic fields affect Ca-calmodulin dependent myosin phosphorylation in suspension myofibrils

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A number of observations indicate that cellular functions and metabolism may be affected by the application of weak magnetic fields (modulated or static) with intensities comparable to that of Earth’s magnetic field. The observed effects may be possibly explained by the influence of magnetic fields on the binding of Ca to the calmodulin-binding sites in calmodulin when the reaction of myosin phosphorylation is going on (Lednev, Bi electromagnetics 12, 71-5, 1991). We examined the effect of modulated magnetic field on the myosin phosphorylation in suspensions of myofibrils from chicken gizzard. Suspension was prepared and UREA PACE was performed according to Habets & Jentsch (Electrophoresis 7, 417-23, 1986). The bathing solution contained (in mmol l-1): MgCl2 10, ATP 7, KCl 85, EGTA 4, imidazole 20 (pH 6.7). The control experiments remain in the local field while the experimental ones were exposed to the modulated field. The reaction of myosin phosphorylation was initiated by addition of calcium-activating solution (pCa 6.8) simultaneously to the control and experimental samples and was stopped with 8 M urea after 5 min. The combined magnetic field was tuned to the ‘cyclotron’ resonance conditions (constant component of 209 mG plus the sinusoidal 418 mG peak-to-peak, 16 Hz). Quantitative determination of the phosphorylation levels of myosin light chains was obtained from the densitometric traces of the myosin electrophoresis patterns.

We have found that with pCa 6.8, the degree of phosphorylation increased from 20% in the local field to 50% in the combined field (p < 0.05). At the same time there was no significant difference in the level of phosphorylation with pCa 4.0. So our results appear to suggest that the combined magnetic fields significantly influence the myosin phosphorylation.

Caldesmon regulation of smooth muscle thin filaments is functionally analogous to striated muscle troponin

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We studied the mechanism by which caldesmon regulates actomyosin ATPase. We found that the C terminal 99 amino acids contain all the regulatory functions of the whole protein and that this region, domain 4b, is functionally homologous to troponin I. Both inhibit by the same tropomyosin mediated mechanism (Marston & Redwood, J. Biol. Chem. 266, 12317-20, 1993). Both bind actin strongly but tropomyosin weakly or not at all, both are associated with an extended tropomyosin binding peptide not essential for regulatory function (troponin T and the rest of caldesmon). In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin. In this model tropomyosin-actin interaction determines the ‘on’ and ‘off’ states of tropomyosin.
similar in the two states. We propose that troponin I and caldesmon both act as allosteric effectors by binding preferentially to the weak (off) state, thus shifting the equilibrium so that most of actin-tropomyosin is in the weak, low ATPase state. If the calcium binding proteins change the interactions such that troponin I and caldesmon do not discriminate between strong and weak states in the presence of Ca\(^{2+}\), the ATPase activity will be restored to uninhibited levels.

We tested this hypothesis by measuring the binding of S1.ADP (i.e. strong binding) to actin smooth muscle tropomyosin in the presence and absence of inhibitory concentrations of caldesmon. In the presence of caldesmon S1.ADP binding became very weak at low S1.ADP concentration and the binding curve overall was highly cooperative. In the absence of caldesmon, S1.ADP binding was strong and roughly hyperbolic. This confirms that caldesmon, like troponin, switches actin-tropomyosin from strong to weak state.

We have investigated the location of the inhibitory actin-tropomyosin binding sites within the C-terminal domain 4 of caldesmon using expressed fragments of the human caldesmon. The smallest active fragment (H9) consisting of the N-terminal 2/3 of domain 4 was inhibitory whilst a fragment (H2) representing the usual C-terminal 1/3 of domain 4 bound actin but was not inhibitory. Thus inhibition can be observed if a site in the middle 1/3 of domain 4 (site B, common to H2, H7 and H9) is continuous either with a site at the N-terminus of domain 4 (site C) or a site at the C-terminus (site B'), whilst in native caldesmon all the sites are present (C-B-B'). This idea is supported by measurements of actin-tropomyosin binding affinity which are in the order caldesmon-domain 4 > H7(C+B) > H2(B) > H9(B+B') and by the observation that H2 can antagonise caldesmon inhibition by occupying site B.

### Disruption of the actin-cytoskeleton in living nonmuscle cells by microinjection of the antibodies to the tropomyosin-binding domain-3 of caldesmon

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Caldesmon, an elongated protein present in all non-muscle tissues and smooth muscle cells, is thought to play a pivotal role in the regulation of muscle and cell motility. The protein exists as several isoforms. The substructure of the muscle specific members of the family is made up of four domains designated domains-1, -2, -3 and -4 from the amino- to the carboxyl-terminal end of the protein, compared to the smaller nonmuscle isoform which has lost domain-2. Caldesmon interacts with F-actin and calmodulin, inhibiting in a calcium modulated fashion, the actomyosin ATPase activity. It also binds tropomyosin but the biological significance of this interaction is not well understood (Marston & Redwood, Biochim. J. 279, 1–16, 1991). Microinjection into cultured fibroblasts of affinity purified polyclonal antibodies specifically directed against different domains of gizzard caldesmon was employed to probe the functional importance of the interaction of caldesmon with tropomyosin within living nonmuscle cells. Low concentration of the antibody specifically directed against gizzard caldesmon 15 kDa thrombin fragment which encompasses the entire structure of domain-3 (residues 483-578) caused a rapid, severe and reversible disassembly of the microfilament network. In contrast, the antibodies to the remaining domains-1, -2 and -4 were ineffective. The properties of the former antibody were demonstrated to result not from an alteration of the association of caldesmon with F-actin but rather from the specific blocking of the interaction of tropomyosin with caldesmon domain-3, known to harbor a putative tropomyosin binding site within residues 508-565. The data provide the first direct evidence for the in vivo involvement of this domain in the attachment of nonmuscle caldesmon to tropomyosin. They also offer an experimental support to the direct participation of the caldesmon-tropomyosin complex in the cytoskeletal organization of the microfilaments.

### Precise identification of the actomyosin ATPase inhibitory sequence in the 10 kDa carboxyl-terminal domain of Caldesmon

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The COOH-terminal segment of gizzard caldesmon between residues 659-756 was previously isolated as a 10 kDa CNBr fragment (Bartegi et al., J. Biol. Chem. 265, 15231, 1990) and as a recombinant protein (Redwood & Marston, J. Biol. Chem. 267, 10796, 1992) both expressing two major functions of caldesmon: the inhibition of the actomyosin ATPase and the regulatory binding to Ca\(^{2+}\)-calmodulin. While a recognition site for the latter protein has been assigned to the NH\(_2\)-terminal stretch W659-F665, the exact location of the F-actin binding and inhibitory region is unknown. In this study we have characterized this critical component by investigating the limited chymotryptic digestion of the 10 kDa CNBr fragment in the absence and presence of F-actin, its influence on the peptide functions, the F-actin binding and inhibitory effects of the chymotryptic fragments identified by HPLC-chromatography, NH\(_2\)-terminal sequencing, gel electrophoresis and mass spectrometry. We also assessed the properties of three synthetic peptides which cover the well conserved sequence between N675 and K729 and corresponding to N675-K695 (NK21), L693-W722 (LW30) and R711-K729 (RK19), respectively. Only LW30 and chymotryptic fragments including its sequence cosedimented with F-actin and inhibited the actomyosin ATPase in a tropomyosin-specific manner. The inefficiency of RK19 indicates that the structural integrity of the peptide chain at L710-R711 is required for actin binding and is consistent with the lack of inhibition previously observed for fragments terminating at L710. Thus, the essential elements reside within the minimum sequence L693-W722 which also harbors residues phosphorylated by proline-directed protein kinases thought to regulate in vivo the caldesmon-actin interaction.

On the other hand, NK21, but neither LW30 nor RK19, bound to immobilized calmodulin in a calcium-dependent manner. The corresponding sequence N675-K695 is just adjacent to the actin-binding stretch L693-W722 and should represent the actual regulatory calmodulin-binding site recently reported to occur in the caldesmon segment of residues 609-737 (Redwood & Marston, J. Biol. Chem. 268, 10999, 1993).

### Caldesmon isoforms as phenotypic markers in arterial smooth muscle

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Caldesmon (CD) exists as two major isoforms, 'CD', found in smooth muscle thin filaments and 'CD', which occurs in certain non-muscle cells. Smooth muscle cells undergo phenotypic modulation between a differentiated, 'contractile' form and a 'synthetic' form which can divide or migrate and which may not be contractile. These changes are accompanied by changes in the expression of contractile protein isoforms. We investigated whether CD isoforms might be good markers for phenotypic modulation. We studied both human and animal arteries using our isoform-specific anti-CD sera. The ratio of CD,CD is approximately 5:1 in vascular or in visceral smooth muscle tissues, and the CD content of rabbit carotid arteries (n = 28) is 245 ± 6 nmol CD,CD/g-tissue protein and 68 ± 4 nmol CD,CD/g-tissue protein. This is compatible with a function for CD in the regulation of F-actin in smooth muscle.
Abstracts

Intimal thickening was induced in rabbit carotid arteries by fitting a silastic 'collar', and CD measured by immunoblotting. Intimal thickening was negligible four days later but the CD content had already fallen to 56% of control, implying that medial smooth muscle cells lost CD. At 14 days when intimal thickening was maximal the CD content was 30% higher than control. By 28 days, intimal thickening in collared arteries was reduced, and CD had fallen below control levels, while CD levels had not increased.

In atherosclerotic human coronary arteries. CD-specific immunofluorescence was strong in medial cells, but less intense in the intima, decreasing towards the lumen; staining was weaker in layers of medial smooth muscle underlying the plaque core and was seen only in scattered cells in the plaque cap. Staining by non-isotopically-selective anti-CD serum was more uniform. CD-specific staining was weak with no clear gradient across the wall.

Our results indicate that CD isoforms are rapidly-responding and sensitive markers of smooth muscle cell phenotype.

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Interaction of the cytoplasmic domain of phospholamban with calmodulin

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Phospholamban (PLB) is a 52 residue pentameric membrane protein involved in transducing CaM signalling to the Ca++ ATPase in cardiac and smooth muscle. We have shown by 1H NMR spectroscopy that the N-terminal 20 residue segment of PLB binds to calmodulin. We have followed, in particular, the response of methionine S-resonances from calmodulin's hydrophobic binding pockets to the binding of PLB residues 1-20, 1-13, and 8-20. In this way we are beginning to define the residues that determine the specificity of the interaction with PLB.

Paramagnetic spin-label probes, at residues 27 and 143 from the N- and C-terminal domains of calmodulin respectively, help define the orientation of PLB when bound, significantly broadening many PLB resonances. The results are discussed, with the view that PLB residues 1-25 has little structure when free in solution, and that peptide ligands for calmodulin often form amphiphilic helices on binding.

Casein kinase II-catalysed phosphorylation of caldesmon

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Smooth muscle caldesmon is an elongated protein (74 x 1.9 nm) which contains an actin-binding site near the N-terminus and a myosin-binding site near the N-terminus. The isolated protein is capable of cross-linking actin and myosin filaments suggesting that it may serve to organize the contractile filaments so that they have the proper spatial orientation and relationship for efficient contraction in response to stimulation. Caldesmon is phosphorylated in intact muscle upon stimulation by a variety of agonists and several kinases phosphorylate the isolated protein in vitro. One such kinase is casein kinase II. We have partially purified casein kinase II from smooth muscle. It contains three distinct subunit types: a(43 kDa), x(59 kDa) and (72 kDa). The smooth muscle kinase phosphorylated caldesmon to (6 molP/mol)

The sites of phosphorylation, located in the N-terminal peptide obtained by cleavage at cys 153 with 2-nitro-5-thiocyanobenzoic acid, were identified as ser 73 and the 63 (underlined) in the sequence 70 AQNSVAEEETKRDSTDEAA 88. This phosphorylation had no effect on the binding of caldesmon to actin but it completely abolished binding to myosin, consistent with the myosin-binding site being located near the N-terminus. These results suggest that casein kinase II may play a role in regulation of contractile filament organization in smooth muscle.

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Phosphorylation and dephosphorylation of smooth muscle calponin

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Calponin is a 34 kDa smooth muscle-specific protein which has been implicated in the regulation of contraction. Characterization of native thin filaments, in vitro binding studies and confocal immunofluorescence microscopy of saponin-permeabilized single smooth muscle cells indicate that calponin is localized on the actin filaments. Purified calponin inhibits the actin-activated MgATPase activity of smooth muscle myosin without affecting myosin phosphorylation. The isoforms of calponin is phosphorylated in vitro by protein kinase C or Ca++/calmodulin-dependent protein kinase II and dephosphorylated by a type 2A protein phosphatase. The principal site of phosphorylation by either kinase is ser 175 (the amino acid sequence of one of the several isoforms of calponin was deduced from the sequence of its cDNA cloned by reverse transcriptase-PCR). Phosphorylation abolishes actin-binding and inhibition of the actomyosin ATPase and these properties are restored following dephosphorylation. Calponin phosphorylation has been confirmed in intact smooth muscle strips metabolically labelled with radiolabelled inorganic phosphate and stimulated to contract with carbachol or okadaic acid. These observations suggest that calponin functions in the regulation of the contractile state of smooth muscle and that this function is in turn regulated by phosphorylation-dephosphorylation. The concerted regulation of smooth muscle contraction by two mechanisms, myosin phosphorylation and calponin phosphorylation, which are independently controlled by Ca++ permits the actomyosin ATPase (cross-bridge cycling rate) to be very precisely set; providing a degree of flexibility necessary to explain the physiological properties of smooth muscle contraction.

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Oligomerization of smooth muscle myosin light chain kinase revealed by zero-range cross-linkage and SDS-electrophoresis

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It has recently been shown that smooth muscle myosin light chain kinase (MLCKase) is activated by calmodulin (CM) in a cooperative manner. This positive cooperativity could possibly be explained by kinase oligomerization (Sobieszek, Mol. Biol. 220, 947-57, 1991). We have subsequently demonstrated that the cooperativity results from a Ca/CM-dependent possibly oligomeric modification of the kinase (Sobieszek et al., Biochem. J. 295, in press). The oligomeric properties of turkey gizzard MLCKase have now been investigated using the zero-range cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and SDS-PAGE. Under normal assay conditions (60 mM KCl and pH 7.0) cross-linking by 2-5 mM EDC for 30 min resulted in relatively low losses of kinase activity, amounting to a 5-15% reduction in the absence and a 15-25% reduction in the presence of Ca and CM. After the SDSPAGE cross-linked kinase exhibited, in addition to the 125 kDa monomer band, a strong band of M, 190 kDa, representing a dimer and a weak broad band in the range of
550–600 kDa most likely corresponding to kinase oligomers. Significantly, increasing the salt concentration from 60 up to 150–200 mM resulted in a gradual disappearance of the dimer band. This demonstrates that the monomeric, dimeric and oligomeric forms of the kinase exist in an equilibrium with intermolecular apparent affinities in the micromolar range, in contrast to the nanomolar affinities observed for the CM-kinase interaction. Similar dynamic equilibrium was formed during gel filtration chromatography of the native kinase. This equilibrium was not significantly affected by ionic strength, therefore the distances between the monomers (within the dimers) and not the oligomerization per se appeared to be modified by ionic condition. If CM and Ca were present during cross-linking the ‘oligomer’ band became weaker or absent, indicating that the equilibrium is shifted into direction of the dimers and monomers. The cross-linked kinase was also fractionated by gel filtration chromatography in order to obtain partial separation of the different forms. We examined ascending and descending fractions of the kinase peak for their activation by CM. Significantly, we could demonstrate that the descending tubes contained kinase activated by CM in a cooperative manner while activation of the ascending kinase was uncooperative. Correspondingly, our SDS-gel revealed that the kinase from ascending fractions was enriched in the oligomers while the descending fractions contained mainly the dimers. Thus, cooperativity of MLCKase relative to CM results from the reduced affinity of kinase dimers for CM. Upon binding of CM to the dimeric form the oligomer-monomer equilibrium is shifted in the monomer direction with the affinity of kinase for CM increasing to a normal level. The following abstracts, which were presented at the meeting, have recently been published as full papers in the Journal and therefore have been omitted.


