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 time course of force generation in frog skinned muscle fibres has been investigated using laser flash photolysis of the caged-calcium, either nitr-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 time course of muscle relaxation using diazo 2. This procedure has enabled us to examine the effects of the intracellular metabolites, ADP, Pi 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
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Cut skeletal muscle fibres of the frog mounted into a single vaseline gap and voltage clamped to -100 mV were loaded intracellularly with the calcium sensitive dyes Antipyrylazo III (APIII) and fura-2. The tension 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 505 nm and tension were recorded simultaneously. The parameters describing the calcium binding properties of fura-2 were set so that the calcium transient was 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 (Mezter 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.

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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 uterus 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 reducing both pH, and [ATP], and the resultant effects on the contractile machinery. (Wray, J. Physiol. 423, 411-23, 1990). However, another possible explanation is that at ATP, falls, the surface membrane conductance of K+ increases. We have therefore measured potassium ($^{86}$Rb) efflux from strips of isolated rat myometrium.

Cyanide (2 mM) reduced contraction. This was accompanied by a large, reversible increase in $^{86}$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 K$_{ATP}$ 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 hyperpolarisation, and that it also increased the rate of $^{86}$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 $^{86}$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
Phosphorylation of the skeletal muscle ryanodine receptor/Ca" 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 solubilisate 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) Ca". 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 Ca"-channel or DHP-receptor (DHPR) in the plasma membrane, as well as the Ca"-release channel or ryanodine receptor (RyR) in the sarcoplasmic reticulum. The genes for these channels have been identified via cDNAs and chromosomally mapped in mouse and man. Except for K"-channel genes, hereditary muscle diseases have been associated with these genes. Such 'ion channel diseases' are muscular dysgenesis (MDG, DHPRα2 defect) of the mouse, paramyotonia and related diseases in man (SKM1 defects), and true myotonias = CIC-1 diseases in mouse and man. Furthermore, malignant hyperthermia in man and the stress syndrome of pigs have been associated with the RyR gene. MDG is the muscle disease with the earliest known manifestation: MDG mice are born without skeletal muscle and are therefore non-viable. This demonstrates the significance of an ion channel for normal development and the structural maintenance of the muscle fibre. In the mouse, myotonias have been found to be caused by either a retroposon insertion or a nonsense or a missense mutation in the CIC-1 gene. Physiologically, all these may be classified as recessive Cl-" mutations whereas the homologous Thomsen myotonia in man is dominant. Myotonias are relatively mild diseases. However, the CIC-1 channel defect not only leads to hyperexcitability but also to drastic changes in the expression of a whole battery of muscle-specific genes, including that of the Cl-1 gene itself and its response to denervation. Na"- and K"-channel diseases are being searched for in the mouse, on the basis of recently obtained chromosomal mapping data, in order to define new animal models for human diseases.

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Temporal relationships of processes involved in excitation contraction-coupling in skeletal muscle
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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 ms 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 extra 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 occur during 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 360 μs. Upon increasing the toxicity of the bathing 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 as 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 μm per half sarcomere.

Kinetics of dihydropyridine-sensitive Ca"- channels in skeletal muscle
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In skeletal muscle, the binding protein for Ca" channel antagonists (the dihydropyridine receptor = DHP receptor) is believed to exhibit two functions: forming a pore to allow Ca" influx into the muscle fibre and controlling Ca" release from the sarcoplasmic reticulum. However, Ca" release activation does not require the Ca" influx; in addition, Ca" release is maximally activated within only a few milliseconds after depolarizing the membrane while the Ca" inward current shows a considerably slower time course. The Ca" channel may simultaneously work as a control device for Ca" release if it...
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, 639–53, 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; LeFurqey et al., J. Microsc. 165, 191, 1992), we have measured changes in \([\text{Ca}^{2+}]\) 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 calsequestrin in which exposed hydrophilic 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 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 avian 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 EK.

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 sarcormere length was investigated at different calcium concentrations.
increased demand. Several problems which limit the clinical application of these promising approaches may be solved by 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 Ventricular Assist Device chamber, and a sheep latissimus dorsi muscle powering it) and clinical (studying if reverting inactivity-induce hypotrophyism 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 isolated 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/pSr relationships), two kinetic indicators of the tension development (dP/dt) and the maximal shortening velocity (V_{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_{max}, P/t and dP/dt. Moreover, the evolution of the V_{max} parameter was strongly coordinated with that of the relative calcium/strontium affinity. This suggested the existence of a correlation between V_{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 (±SEM 0.02, 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^-12 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 an 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 μM) while tetraethyl ammonium (25 mM) 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 Magnetostimulus 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 of 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,
seems to restore the protein content to levels before denervation, but has little effect on the biomechanical activity.

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 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 was used in combination with temporary ischemia of the forearm to characterize the ATPase activity. The recovery following restoration of blood flow provided a measure of oxidative ATP synthesis activity. The small PCR breakdown rate after 250 seconds of ischemia provided a measure of the basal metabolic rate (0.008 mmol s\(^{-1}\) ± 0.002 SD, n = 5). The PCR breakdown rate during twitch stimulation of the oxygen-depleted muscle was constant at 1 Hz at a rate of 0.15 mmol PCR per s or per twitch (± 0.03 SD, n = 8). A constant cost per twitch (0.15 mmol) was found from 0.5 to 2 Hz stimulation. No net anaerobic recovery of PCR was found during a 2 min post-stimulation ischemia. Upon restoration of blood flow, PCR recovery followed an exponential time course with a time constant of 0.1 s (± 14 SD, n = 8). pH changed less than 0.1 unit during the stimulation-recovery cycle in contrast to experiments using voluntary contraction. From these recovery rates, the capacity for oxidative phosphorylation was estimated to be 0.4 mmol ~ P per s. This recovery is described by a negative feedback regulation of mitochondrial oxidative phosphorylation by ADP. From these measures estimates of apparent Kt for ADP in respiratory control and of V_{max} are obtained. These estimates correspond to estimates in vitro and of mitochondrial volume density. The experimental approach defined here provides a non-invasive and quantitative measure of human muscle ATPase rate and ATP synthetase rate, the separation of which are 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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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 Ca\(^{2+}\) (0.02 mmol per l) with verapamil (0.02 mmol 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 RI. In RI, 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 fatigue resistance also survived in low Ca\(^{2+}\) RI alone as well as verapamil RI alone, though much less than in normal RI. Now it is known that: (1) several types of Ca\(^{2+}\)-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; (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 Ca\(^{2+}\) and verapamil RI 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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mRNAs of Cox I, Cox IV and myosin alkali light chains in skeletal muscles of untrained and endurance trained subjects
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Human muscle adapts to altered load with changes in the expression of enzymes involved in energy metabolism and isoform switches of contractile proteins. We use RT-PCR to quantitate RNAs in single cryostat sections and in situ hybridization to localize specific RNAs in individual fibres.

A group of highly trained runners (V_{O,max} = 71 ± 5 ml per
Abstracts

min × kg) had 2-fold higher mitochondrial densities compared to
untrained subjects (VO₂max = 35 ± 4 ml per min × kg) in biopsies
from m. vastus lateralis. Correspondingly, cytochrome oxidase (Cox
I) mRNA (mitochondrially encoded) as well as cytochrome oxidase
IV mRNA (nuclear encoded) were both present in higher concen-
trations 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 propor-
tion of them also contained the mRNA of MLC 1sa. These
type I fibres were characterized by very low glycolytic capacity
(α-GPDH) 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.
oralis 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
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To understand the adaptation mechanisms of skeletal muscle contrac-
tile apparatus to the functional changes during hibernation, we
studied the Ca-activated isometric 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 [Ca²⁺] were
observed in the hibernating preparations as in the case of B.K. in the
summer-active ones.

To correlate the observed changes with the contractile and
regulatory protein composition, a SDS-PAGE analysis was performed
with the same specimens. A marked effect was observed on the
myosin heavy chains (MHC). A slow-type MHC appeared in m.
psoas from hibernating animals. Changes in myosin light chains
(MLC) also occurred but to a less extent: LCl and LCZ were
unchanged and only the LC3 amount was decreased in hibernating
animals. There were also the corresponding modifications in tro-
opin-tropomyosin regulatory complex.

In conclusion, the biochemical and contractile properties of m.
psoas were modified during hibernation in such a way that initially
fast twitch fibres acquired properties of slow-twitch ones.

The transformation revealed could be caused mainly by a
change in the level of the muscle activity in the condition of
hypometabolism when the glycolytic flow is considerably
depressed and the hypothyrosis took place (Wang, Hibernation
and torpor in mammals and birds, Acad. Press: NY, London: 200–36,
1982).

The molecular basis of hereditary excitation disturbances
in human skeletal muscle – a review
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Molecular biology has provided the methods to define the
genetic defects of most of the human hereditary diseases character-
ized by myogenic hyperexcitability (resulting in myotonia) and/or
hypoeexcitability (resulting in episodes of paralysis). According to the
present knowledge, these diseases are best classified in the following
way:

1. Myotonic dystrophy. Caused by an expansion of a trinucleotide
(CTG) repeat at the 3' end of a transcript encoding a protein
kinase family member (Brook et al., Cell 68, 799–808, 1992). The
genome is localized to chromosomes 19q13.3.

2. Chloride channel diseases (Thomsen & Becker myotonia). Caused
by point mutations in the chloride channel gene (HUMCLC)
localized to chromosome 7q35. Two allelic mutations have been
reported for the recessive (Koch et al., Science 257, 797–800,
1992) and dominiant (George et al., Nature Genetics 3, 305–9,
1993) forms.

3. Sodium channel diseases. Since SCN4A, the gene encoding the α
subunit of the sodium channel in adult human skeletal muscle,
was localized to chromosome 17q23 and sequenced, molecular
biology has revealed that the diseases belonging to the 'ady-
namia-paramyotonia complex', i.e. paramyotonia congenita, hy-
perkalemic and normokalemic periodic paralysis and some rare
forms of myotonic disease are caused by point mutations in this
genome. So far 13 different mutations and six polymorphisms have
been detected (Review: Lehmann-Horn et al., Neuromusc. Disorders
3, 1993). The mutations causing a particular form of sodium
channel disease are not distributed over the gene in an easily
understandable manner.

4. Hypokalemic periodic paralysis. Myotonia is absent in this
disease. The molecular defect has not yet been detected, but
linkage to the gene encoding the skeletal sodium channel α
subunit has been excluded (Fontaine et al., Neuromusc. Disorders
1, 235–8, 1991).

The use of saponin-skinned muscle fibres for the detection
of mitochondrial myopathies and encephalomyopathies
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Saponin skinnning of muscle fibres allows a functional investigation of
mitochondrial oxidative phosphorylation in skeletal muscle biopsy
specimens containing 30–50 mg of muscle tissue. After 30 min of
incubation of muscle tissue in a saponin containing permeabilization
medium more than 85% of mitochondria were accessible. Using this
technique the rates of respiration of muscle fibres of a patient (B.K.)
developing mild myopathy in connection with ptosis and depression
with psychotical symptoms (progressive external ophthalmoplegia)
and a patient (L.H.) developing mild myopathy in connection with ptosis
and depression with elevated lactate levels 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, ac-
tanoylcamitine + 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 a, 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
enzyme defect in oxidative phosphorylation was detected but an
approximately 50% reduction in mitochondria. This was verified by
measurements of citrate synthase, glutamate oxaloacetate transamin-
ase and adenylate kinase (decreased activities in the muscle homogen-
ate) 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.
The maximum sustainable duty cycle of single Xenopus muscle fibres related to succinate dehydrogenase and myofibrillar ATPase activities

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Different types (1, 2 and 3; cf. Lännergren & Smith, Acta Physiol. Scand. 68, 263-74, 1966) of single, intact muscle fibres, dissected from the iliofibularis muscle of Xenopus laevis, were stimulated intermittently (40 Hz at 20°C in phosphate-buffered Ringer solution, pH 7.2) to determine the maximum sustainable duty cycle (MSDC) at a sarcomere length of 2.3 μm. The smallest observed MSDC was 0.5% of total time (type 1; one 250 ms tetanus required 50 s recovery) and the largest was 19% (type 3; one 250 ms tetanus required 1.5 s recovery). An imposed duty cycle was assumed to be sustainable when force did not drop by more than a few percent during 15 min of intermittent stimulation. The maximum sustainable duty cycle’s (mean ± SD(n)) were significantly different for the three fibre types (F1,2 = 144, p < 0.001): type 1: 1.6 ± 0.6% (4); type 2: 7.6 ± 3.9% (4); type 3: 18 ± 8.6% (7). Fibres fatigued when the duty cycle was increased further. They recovered completely when the imposed duty cycle was decreased. Steady isometric force production at the MSDC was about 75% of maximum force. After determination of the MSDC the fibres were processed for quantitative histochemical determinations of SDH activity. The maximum sustainable duty cycle was independent of the imposed duty cycle/ATPase activity for fibres with low to intermediate oxidative capacity, whereas activation failure is a likely determinant in high-oxidative muscle fibres.

Myotonic dystrophy kinase is localized at neuromuscular and myotendinous junctions

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Myotonic dystrophy kinase (DM) is an abundant enzyme catalysing the reaction MgADP + P(O)3 + H++ → MgATP + Cr. Different DM 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 tissues 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 human tissue specimens. Muscle fibre maturation was accompanied by a conspicuous concentration of DM PK in neuromuscular and myotendinous junctions. Furthermore the sarcolemma 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 the earlier classification of congenital DM as a disease characterized by an attenuated fibre development due to an unresponsiveness 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 the in situ quantification of DM-PK fluorescence signals and the generation of more specific reagents for any of the different DM-PK isoforms.

Creatine kinase isoenzymes and muscle energetics: ultrastructural localization, functional compartmentation and structure of Mi-CK

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In tissues and cells with high and fluctuating energy requirements, e.g. in excitable cells like skeletal and cardiac muscle, brain cells, photoreceptor cells, electrocytes and spermatozoa, creatine kinase (CK) is an abundant enzyme catalysing the reaction MgADP + P(O)3 + H++ → MgATP + Cr. Different CK isoenzymes are expressed tissue-specifically and are associated with subcellular structures in an isoenzyme specific manner. In muscle, most of the 'cytosolic' fraction of CK is localized at the I-band in association with glycolytic enzymes. Approximately 10% of total CK, however, is rather firmly bound to the sarcomeric M-band, and another fraction of the enzyme is bound to the sarcoplasmatic reticulum (SR) membrane. A further fraction of CK, the mitochondrial isozyme Mi-CK, is specifically located within the mitochondria where it is bound to the outer face of the inner mitochondrial membrane (IM), along the cristae as well as peripherally at the mitochondrial contact sites between IM and outer membrane. Based on these specific localizations of the enzyme at cellular sites of energy consumption (myofibrillar ATPase, SR-Ca2+-pump etc.) or energy production (glycolysis, oxidative phosphorylation), and based on the fact that functional coupling of CK with the above processes has been demonstrated, we propose the CK-PCr system to be involved in: (1) temporal energy buffering, (2) spatial energy buffering, (3) preventing a rise in intracellular [free ADP], thus avoiding inactivation of cellular ATPases and a net loss of adenine nucleotides, (4) buffering of protons and thus prevention of tissue acidification, (5) providing appropriate local ATP/ADP ratios at subcellular sites where CK is functionally coupled to ATPases and thus increasing the thermodynamic efficiency of ATP hydrolysis. The proposed CK-PCr circuit represents an intricate energy distribution network connecting intracellular sites of ATP production with sites of ATP consumption. A considerable amount of experimental support is provided from recent data on the biochemical properties and oligomeric structure of Mi-CK which has been thoroughly characterized in our laboratory over the past years. An update on the
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 be due to a reduced rate of crossbridge cycling or slowed Ca$^{2+}$ handling. To distinguish between these two mechanisms, we have developed a method to study changes of the time-course of Ca$^{2+}$ handling. For this purpose the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) was measured with Indo-1 and changes of [Ca$^{2+}]_i$ during tetani were converted to force by means of the steady-state [Ca$^{2+}]_i$ force relation. In this way a calcium-derived force record was created which represents a situation where force responds to changes in [Ca$^{2+}]_i$ 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 [Ca$^{2+}]_i$ decline. In mouse fibres the slowed [Ca$^{2+}]_i$ decline was fully counteracted by a reduced myofibrillar Ca$^{2+}$ 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 Ca$^{2+}$ 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 calcium. 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. 325, 186–90, 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, carb oxyatracyloside 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 carb oxyatracyloside 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.

GENE EXPRESSION AND ITS REGULATION

Analysis of the muscle specific promoter region of the human dystrophin gene in transgenic mice

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Duchenne muscular dystrophy (DMD) is a lethal X-chromosome linked recessive disorder affecting about 1 in 3500 boys. The major cause of pathology is the lack of the gene product dystrophin resulting in a distinctive pattern of progressive muscle wasting. There is evidence that functional expression of dystrophin in dystrophic muscles of mdx mice (Wells et al., Hum. Mol. Gen. 1 (1): 35–40, 1992) may be a useful strategy for assessing possible gene therapy regimes. As part of a broader gene therapy investigation by the transgenic approach, we are assessing the human dystrophin promoter region (Klamut et al., Mol. Cell. Biol. 10 (1): 193–205, 1990) for correct temporal and spatial expression as well as relative promoter strength in vivo, using the E. Coli lacZ gene as a reporter. Preliminary results indicate expression in muscle fibres with no expression in non-muscle tissues. Compared to the rat α-skeletal actin promoter (Asante et al., in press, Transgenic Research, 1993), the dystrophin promoter appears to be weak. However, when the effects of over-expression of the dystrophin minigene under the control of stronger promoters such as the muscle creatine kinase and human skeletal actin (work in progress) are known, the natural dystrophin promoter may eventually be the most attractive for future gene therapy protocols.

Alternative splicing of calpastatin in porcine skeletal and cardiac muscle

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In muscle, the three isoforms of the calcium dependent proteinases, μ-calpain, m-calpain and muscle-specific P94 probably act at the myofibrillar Z-line, membrane/cytoskeletal interface and in the nucleus respectively. Together with calpastatin, the specific inhibitor, the calpain system is believed to participate in muscle cell differentiation and to regulate myofibrillar turnover in adult muscle. In skeletal muscle, expression of calpastatin is highly sensitive to β-adrenergic stimulation, which in turn is closely linked to muscle hypertrophy (Parr et al., Eur. J. Biochem. 208, 333–9, 1992). The calpastatin protein is comprised of four inhibitory domains and a leader (L) domain, which is sometimes incomplete in the mature protein. The structure of the L domain of the human calpastatin gene indicates alternative exon usage in different cell types (Lee et al., J. Biol. Chem. 267, 8437–42, 1992). In particular, exclusion of exon 3 from the mRNA transcript would mean the absence of a highly basic peptide sequence which may control intracellular targeting of the inhibitor to membranes and/or the nucleus.

In the present study, calpastatin activity in porcine cardiac muscle was found to be 9323 ± 2846 U·kg$^{-1}$ compared to 1869 ± 304 U·kg$^{-1}$ in L. dorsi, a 5-fold higher level of activity in heart. Reverse Transcriptase (RT)-PCR was used to compare the mRNA
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 123 and 706bp with the corresponding primer pairings, which indicated the full-length targeted 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 *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 neuro-muscular remodelling in the kyphoscoliotic mouse (ky)

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The most striking histopathological differences between ky and other mutants such as mdx mice are the extreme motor axon sprouting in ky as well as the grossly abnormal distribution of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) in affected muscles, reminiscent of myasthenic syndromes. There are also multiple endplates on single regenerated muscle fibres 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 genes (AChR, AChE, myosin, actin, 43 kDa, dystrophin, DSRP 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 50% twist expressing C2C12 clones analysed were greatly impaired in their ability to differentiate and to form myotubes when transferred to differentiation medium (2% HS). 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 vivo 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 an 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, but terminal differentiation 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 mercurated 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 β-Gal gene (Sanes et al., EMBO J. 11(12), 5333–42, 1990). 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 β-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.

P 20. A P-element induced Drosophila mutant. shows defects in the fusion of myoblasts to myotubes during the embryonic muscle formation

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Mesoderm formation in Drosophila is a well characterized process. After the separation of somatic and visceral mesoderm the muscle differentiation program begins. Using a combined genetic and molecular approach we want to isolate and characterize genes involved in this differentiation process. As an excellent marker for the differentiating muscle system we used our antibody against β3-tubulin (Leiss et al., Development 104, 525–31, 1986) which allows the detection of mutants with defects in the embryonic mesoderm differentiation program.

Here we present in detail a phenotypic characterization of one P-element induced mutant, named P 20. This mutant shows disturbances in the fusion of myoblasts to myotubes in the somatic musculature, sometimes parts of the musculature are deleted. The development of the visceral musculature as well as of the heart is not markedly affected, showing a specific effect of this mutation on the
differential program of the somatic musculature. Using 'plasmid-rescue' procedure and subsequent screening of genomic libraries we cloned the genomic region surrounding the P-insertion site. With these genomic clones we isolated two different cDNAs. One cDNA maps 8 kb left of the P-element and shows an open reading frame. The second cDNA with an open reading frame localizes 1 kb to the right of the P-element. Our first sequence comparison, based on the deduced protein sequences, shows no significant homologies to genes of the vertebrate MyoD family or other genes. Parallel to the sequence analysis we try to rescue the mutant phenotype by P-element injection with genomic sequences to decide which of the cDNAs is relevant for the muscle phenotype. More information about the function of the affected gene will be given by embryonic whole mount in situ hybridization experiments to determine the cytological distribution of the transcript. In a next step we will produce antibodies for localization of the protein. With the combination of cytological and molecular genetic methods we hope to get an insight in the function of this new gene and its developmental relevance.

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 (domain 9, 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 exon 1, 2, 3a, 3b, 5, to 13. To date CDh with and without exon 4 and CDh without exon 4 have been detected in various tissues from all the species tested. CDh with exon 4 has only been detected on amplification of cDNA 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 4, 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 both 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 equilibrium 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 asynchronous 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 345, 530-2, 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 germline transformation also permits the in vitro 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. 118, 329a, 1991). J. Mol. Biol. 224, 1185-95, 1992), made possible by screening a rat brain cDNA expression library with a polyclonal antibody against neuroblasto trash myosin II, characterized previously (Miller et al., Neuron. 8, 203-14, 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, RC/CMV, 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. RS83-8, 1987) and increased (Myllylä et al., J. Physiol. 407, 1185-95, 1992) 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 α1-subunit of PH was decreased already after 1 day of immobilization and PH activity 2 days later. mRNAs for non-fibrous type IV collagen and β-subunit of PH were unaltered or slightly increased through the one week experimental period. mRNAs levels for type I, III and α1-subunit of PH were increased 2 and 4 days after running at a speed of 17 m/min 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 mouse 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 limbs 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. Aneural 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 neural 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.

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ATP splitting among skeletal muscle fibres is related to the presence of different myosin heavy chain (MHC) and/or alkali myosin light chain isoforms. Single, chemically skinned fibres from Soleus, Plantaris and Tibialis anterior muscles of the rat were maximally activated in isotonic conditions at 12°C. The hydrolysis of ATP inside the fibre was coupled to the oxidation of NADH to NAD⁺. ATPase activity was determined from the NADH breakdown evaluated from the absorbance at 340 nm of W-light which passed beneath the preparation. MHC content of the fibres was determined by a combined use of monoclonal antibodies against MHCs and SDS-PAGE. On the basis of MHC composition five groups of fibres were identified. Mean ATPase activities (±SE) and ranges of variability of ATPase activity (in brackets) in mmol·mm⁻²·s⁻¹ were: 0.050 ± 0.008 (0.020–0.104) for type 1 fibres (n = 13), 0.168 ± 0.026 (0.053–0.264) for type 2A (n = 9), 0.178 ± 0.023 (0.068–0.291) for type 2X (n = 11), 0.227 ± 0.013 (0.160–0.335) for type 2B fibres (n = 15), and 0.188 ± 0.013 (0.067–0.243) for fibres containing more than one fast MHC (n = 10). ATPase activity was clearly dependent on MHC content. However, the variability in ATPase activity of fibres with the same MHC content was large (3-5-fold). For fast fibres, the ratio between MLClf/MLC2f content was determined by densitometric scanning of SDS-PAGE runs. MLClf/MLC2f ratio is a good index of the ratio between the two alkali MLCs. MLClf and MLClf. The rate of ATP splitting of fibres of homogeneous MHC composition was not related to MLClf/MLC2f ratio. The latter result suggests that alkali MLC isoforms are not likely involved in determining ATPase activity.

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 types of acetylcholine receptor channels (AChRs), termed foetal and adult, 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 e-mRNA in the synaptic region of fast 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., J. Cell Biol. 4, 869–74, 1992), left e-mRNA levels unaffected.

An experimental model to study functional properties of different fibre populations within the same skeletal muscle

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Comparisons of functional characteristics between slow fatigue-resistant (FR) and fast fatigueable (FF) fibres are often made using the mammalian Soleus muscle (FR) and a FF muscle such as the Extensor Digitorum Longus. However, the Soleus muscle properties are probably not a good representation of slow FR fibres in other muscles with a more mixed fibre population. In order to study functional properties of FR and FF fibres within the same muscle, an in situ model was developed. In medial gastrocnemius muscle-tendon complexes (GM) of anaesthetized male Wistar rats (body mass 250–300 g), tiny electrodes were placed on the most proximal and distal primary nerve branches, while intermediate nerve branches were cut. Glycogen depletion of the two activated compartments showed that the proximal nerve branch innervated a deep proximal area of the GM predominantly consisting of fibres with a high oxidative capacity. In contrast the distal nerve branch innervated superficial distal glycolytic fibres.

Functional properties of the proximal fibres were compared with those of the distal fibres (n = 6). Maximal isometric force of the activated compartments was 20–30% of the maximal force generated by the whole muscle. Optimum force production of the distal fibres was at a longer length of the passive muscle complex than for the proximal fibres (A length = 1.65 ± 0.041 mm; n = 5). The proximal fibres appeared to be slower than the distal fibres as indicated by the 70 Hz fn 200 Hz isometric force ratios of 73.1 ± 10.1% and 59.1 ± 12.9%, respectively. Moreover the shortening velocity with the highest power production was lower for the proximal compartment (47.5 ± 3.5 vs. 55.8 ± 4.1 mm s⁻¹ for the distal compartment; n = 4). Differences in fatigue sensitivity are indicated by the finding that sixty seconds of 100 Hz stimulation (200 ms trains at 1 Hz) decreased force to 70.6 ± 6.4% in the proximal and 39.7 ± 5.0% in the distal part of the muscle (n = 6). It is concluded that the presented model can be used to study functional properties (and fatigue) of different regions, which have different fibre populations, within the same skeletal muscle.

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 in well characterised myotubes (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).
Abstracts

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, R337-48, 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, explaining why at the genomic level the gene is about half the size of the mammalian MyoHc genes. Expression of the FG2 isoform was only observed in the white muscle of adult carp which had been subjected to an increase in environmental temperature. In situ hybridization studies showed that expression of the FG2 isoform was limited to small budding like processes from larger white muscle fibres. Muscle growth in fish occurs by both fibre hypertrophy and fibre hyperplasia (Stickland, Growth and development of muscle fibres in the rainbow trout. J. Anat. 137, 323-33, 1983) whereas mammals are restricted only to fibre hypertrophy with no increase in fibre number once embryonic development is complete. We hypothesize that this isoform of the myosin heavy chain is expressed in the cells responsible for muscle fibre hyperplasia in fish. Supported by the NERC and SERC (UK).

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 halothane negative group, halothane positive pigs showed a greater
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

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 Nembutil 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 (abductor and adductor branches) and anastomosed to the ipsilateral superior or inferior roots of the phrenic nerve. The animals were tested electrophysiologically from 172 to 365 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 abductor 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 (1985). 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 1 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.
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 detects 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 (CLSFS) induces in rat fast-twitch muscle fibre type conversions in the order of types IIb → IIα → Iα. As compared to the rabbit, where CLSFS ultimately converts fast into slow-twitch fibres (type B), 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 the 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 (Lebere 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 cDNA probes, we followed the expression of CAIII and PA at the mRNA level. Unstimulated, hypothrophic 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 of type I fibres occurring only after stimulation periods longer than 60 d. In the recovery of the damage. To investigate this, rats of two different ages, 6 and 16 weeks were forced to run for two hours on a level motor driven treadmill at a speed of 19 m min⁻¹ and killed at different time intervals. The temporal profile, up to 3 weeks, of muscle damage was investigated by quantification of the focally disturbed fibre area in longitudinal sections of the m. soleus. The S-phase marker Bromodeoxyuridine (BrdU) was injected at two hours before death to determine the temporal profile of the labelling index of satellite cells. Labelled and unlabelled satellite cells, myonuclei and fibres were counted in cross sections of the belly part of the muscles. The effects were most pro-

**Satellite cell activation and muscle damage after exercise in young and adult rats**

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Exercise leads to damage of muscle fibres, evidenced by alterations of the normal structure and an increase of muscle specific enzymes like CK. We questioned whether it also induces activation of muscle satellite cells towards proliferation and whether these cells play a role in the recovery of the damage. To investigate this, rats of two different ages, 6 and 16 weeks were forced to run for two hours on a level motor driven treadmill at a speed of 19 m min⁻¹ and killed at different time intervals. The temporal profile, up to 3 weeks, of muscle damage was investigated by quantification of the focally disturbed fibre area in longitudinal sections of the m. soleus. The S-phase marker Bromodeoxyuridine (BrdU) was injected at two hours before death to determine the temporal profile of the labelling index of satellite cells. Labelled and unlabelled satellite cells, myonuclei and fibres were counted in cross sections of the belly part of the muscles. The muscle damage differed in both amount and temporal profile between young and older animals. Damage was already visible immediately after running. However while in the younger animal the amount of damage increased gradually in time till maximum value and food intake, due to the control levels at 1 week after running, in the older animals the amount of damage was lower but remained present for at least 2 weeks. The cell kinetic data on both groups showed a proliferation response of satellite cells throughout the muscle. The effects were most pro-

**Selective changes in myofibre differentiation with temperature and nutrition suggest a key role for rhomboides 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 rhomboideus (RH) muscles, lumbar/thoracic and cervical respectively, were assessed histochemically for myosin ATPase and succinate dehydrogenase activities, 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 11.8% 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 temperature and food intake (where H = 2L), thus establishing four treatment groups: 26H, 26L, 10H and 10L respectively. Two dominant regulators of muscle differentiation are thyroid status and contractile activity. It is suggested that in RH muscle of the 26L group, the effect of reduced thyroid function, due to the low energy intake, was of major importance. By contrast, at 10°C, the effects of increased contractile activity associated with shivering predominated. These changes in myofibre differentiation of RH muscle will tend to conserve energy in animals on 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.
nounced in the older rats in which normally only a small fraction of the cells is involved in proliferation. In these rats a statistically significant increase of the labelling index was found between 24 h and 1 week while the total number of satellite cells was consistently higher from 2 days on till 2 weeks after running. In the younger animals, in which a greater fraction of the satellite cells was proliferative, roughly the same time pattern was observed. Since the damage differed in amount and time between the two age groups, we conclude that it is the exercise itself rather than the amount of muscle damage induced by it that evokes a proliferation response of satellite cells.

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 both 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. Natl. 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. In situ hybridization using a DIG-labeled probe confirmed the results from the Northern blots in that no signal could be demonstrated in normal adult muscle cells whereas in both growing and regenerating muscle HGF mRNA expression was evident in immature muscle cells. The results show that HGF mRNA is transiently expressed in growing skeletal muscle. Expression of HGF could be a part of the developmental programme of muscle cells. Further studies are necessary to elucidate the precise role of HGF in muscle growth.

The expression of an additional slow myosin heavy chain isoform in transforming skeletal muscle of the rabbit

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Two myosin heavy chain (HC) isoforms are expressed in cardiac muscle, HCA₉₉ and HC₉₃. They exist as three isomyosins V₁ (αα), V₂ (αβ), and V₃ (ββ). HCA₉₉ is thought to be identical with the slow HCl and that this fully accounts for the isoform present in slow-twitch skeletal muscle fibres. The HCS₃₂₃ isoform, uniquely found in cardiac muscle, is also expressed in chronically stimulated and denervated fast-twitch hindlimb muscles, as well as in adult diaphragm of the rabbit. Its presence in stimulated and denervated muscles could indicate an additional step in the sequential fast-to-slow transition of myosin isoforms, and furthermore, suggests that the expression of this isoform is under neural control.

Hyperplasia of lateral muscle during normal growth of the sea-bream, Sparus aurata

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The contribution of hyperplasia to lateral muscle growth was studied in the sea-bream Sparus aurata, a commercially farmed teleost fish. Epaxial quadrant fibres were identified as superficial monolayer (SM) fibres, slow-red (SR), fast-white (FW), or pink (P) fibres as appropriate on morphological grounds and by mATPase staining, and their diameters measured with a VIDAS-V image analysis system. Changes in the number of fibres per quadrant, and the distribution of fibre diameters across quadrants in subjects of increasing ages revealed the following processes:

(a) Only SM and FW fibres were present at hatching. Their number increased several-fold from hatching to 60 days, and during this period the smallest diameter fibres predominated in the far epaxial portions of the quadrant, indicating that this was the principal site of new fibre formation. Hyperplasia in this zone ceased between 45–60 days. A minor hyperplastic zone for FW fibres lay just deep to the SM layer.

(b) Very small diameter fibres then began to re-appear throughout the FW portion of the quadrant after about 60 days (later in smaller, slower-growing individuals), indicating the start of a second, and longer-lasting, hyperplastic process for FW fibres.

(c) Hyperplastic processes also contributed to the increase in numbers of SR fibres (first detected at six days) and P fibres (present from about 45 days).

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Does the chironomid have an effect on the development of muscle cellularity in pre-hatch atlantic salmon (Salmo salar L)?

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Muscle development in embryonic salmon is dependent on temperature, and embryos reared at increased temperatures show an increase in muscle fibre hypertrophy and a reduced rate of hyperplasia from Gorodllov stage 25 (GosNIORKh 22, 107–26, 1983) when compared
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 charotoned 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 (Ralston, *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 was not carbohydrate processed in the Golgi. 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.

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 histochemically distinct myosin heavy chain (HC) isoforms (Aigner et al., *Eur. J. Biochem.* 211, 367–72; 1993). We have previously established methods for analysing 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 (Über & Pette, *J. Muscle Res. Cell Motility* 14, 253, 1993). The identity of an additional VH-HC clone, pMHC24-79 (Maeda & Wittinghofer, 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

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., *Biochem. 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 ± 2.3 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 mRNA stability.
Abstracts

into PCR medium and divided into three separate PCR assays. Two assays were run with primers for pMHC20-40, and pMHC24-79 and one assay for α-skeletal actin. After 36 cycles, aliquots of the assay mixture were analysed electrophoretically for reaction products of defined length. PCR run without reverse transcription and one assay for α-skeletal actin. After 36 cycles, aliquots of assays were run with primers for pMHC20-40, and pMHC24-79 into PCR medium and divided in three separate PCR assays. Two specific to pMHC20-40 or pMHC24-79. Because positive signals for level. Interestingly, type IID fibres were identified lacking the signals same fibres, the possibility could be largely excluded that RNA isoforms was shown for the same (hybrid) fibres also at the protein level. Interestingly, type IID fibres were identified lacking the signals specific to pMHC20-40 or pMHC24-79. Because positive signals for actin mRNA were obtained in parallel PCR assays performed on the same fibres, the possibility could be largely excluded that RNA extraction or reverse transcription were insufficient. Taken together, we are able to assign the pMHC20-40 cDNA to type IID fibres and the pMHC24-79 cDNA to type IIB fibres. The finding that not all type IID fibres reacted positively, points to the existence of type IID subpopulations. To date, these subpopulations (IID/IXD) can not be distinguished by mATPase histochemistry or by single fibre electro-physiology.

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-1 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 localised with tubulin. During differentiation few large round mitochondrion are localised at the perinuclear zone. In multinucleated myotubes mitochondrion have regained their rod-like shape and they are evenly distributed in the myotube.

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

Postnatal development of rabbit fast-twitch skeletal muscle: accumulation, isoform transition and fibre distribution of calsequestrin

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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, 263-301, 1991; Damiani et al., Des. 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, calsequestrin (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 A-I boundaries and longitudinal SR at the A band level, at about 2-4 weeks after birth (Flucher, Dev. Biol. 154, 245-60, 1992).

The time-course of disappearance of slow-cardiac calsequestrin (CS) and that of appearance of the skeletal CS isoform were investigated in developing fast-twitch skeletal muscle of the rabbit between postnatal days 1 and 60, along with changes in density of the ryanodine receptor (RYR/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 from rabbit developing fast-twitch muscle is time-correlated with changes in the respective mRNA (Araki et al., Am. J. Physiol. 262, C214-20, 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 the neural input in the muscle, might be responsible for the switching-off of the cardiac CS gene during postnatal differentiation of mammalian fast-twitch fibres.

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 muscle fibres were compared in rat extracardiac, sphenoid, posterior 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 alcali buffer preincubation. Yet these fibres could be distinguished from types IIA, IIX and IIB fibres of the EDL and sphenoid 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. Antiseria 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 isoforms bands from the laryngeal and control muscles with the following mobilities from fastest to slowest: Type I, atrial, neonatal, IIB and IIA/IID. An additional band migrating between the atrial and neonatal isoform bands could be isolated from both the PCA and TA. On densitometric scans this laryngeal muscle MHC represented 30-32% of MHC in the TA and 15-20% in the PCA.
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 defferentiation 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 defferentiation does not arrest 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 supernumery 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 defferentiation followed by nerve section 3-4 weeks after birth allowed differentiation of supernumery intrafusal fibres, many of which eventually displayed STO MHC-like immunoreactivity despite denervation. Our results suggest that those supernumery 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 coli) and dorsal neck muscles (rectus capitis posterior major, obliquus capitis inferior, splenius capitis, (capitis, (aponeurosis) were taken from 54 patients who underwent spondylodesis for cervical dysfunction of different etiologies. 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 transformations 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 arthritis 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'.

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 ocularorotatory (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 IIB), neonatal/embryonic, slow-tonic and cardiac 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 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 characterize fibre types in skeletal muscles. These fibres express an isoform of MHC specific to OM (Sartore 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 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 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 long-term 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 cultures. 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 multivell 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

Abstracts
laboratory incubator through the inner shell. The muscle and nerve cells are seeded into a defined spatial pattern made up by means of Sylgard masks on coated glass cover slips. This results in distinct nerve and muscle cell patches with diameters of 6 and 15 mm respectively. A gap of less than 1 mm between the two compartments is obtained with this method. Later on the Sylgard masks are removed without detaching the nerve and muscle cultures from the cover slips. The behavior of the cultures is observed by means of a CCD video camera coupled to a time lapse video recorder. At playback the sequences of interest are digitized on a Macintosh computer and analysed by means of imaging software. This allows for a detailed analysis of the time history of morphometric cell parameters and of contact formation. 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) B. Zawadowska Zoology Institut, Jagiellonian University, Krakow, Poland Composition of muscle fibre types in male forelimb during breeding season in Rana temporaria were investigated (Faber & Zawadowska, Gegenbaur 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 muscle 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 three females were frozen and analysed histochemically for myofibrillar ATPase (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 II and 25.0% of type IIIB fibres. (J. Mus. Res. Cell Motil. 14, 253, 1993). In sartorius muscle of female type I fibres were not found. There were notice only type II - 55.0% and IIIB - 47%. 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 IIB fibres in sartorius of exclusively 'fast' muscles and fatigue resistant IIB fibres. In conclusion I suggest that the existence in the female sartorius of exclusively 'fast' muscles and fatigue resistant IIB fibres. In the control, immunoincubation with primary antibody was omitted. Sartorius muscle in male contained 55.3% of type I fibres, 19.7% of type II and 25.0% of type IIIB fibres. (J. Mus. Res. Cell Motil. 14, 253, 1993). In sartorius muscle of female type I fibres were not found. There were notice only type II - 55.0% and IIIB - 47%. 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 IIB fibres. (J. Mus. Res. Cell Motil. 14, 253, 1993). In sartorius muscle of female type I fibres were not found. There were notice only type II - 55.0% and IIIB - 47%. 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 IIB fibres. (J. Mus. Res. Cell Motil. 14, 253, 1993).

Clones of human satellite cells can express in vitro both fast and slow myosin heavy chains F. Eom1, V. Mouly1, J. P. Barbet1, M. Y. Fiszman1 and G. S. Butler-Browne1 1URA CNRS 1448, UFR Biomedicale des St-Pères, 45, rue des St-Pères, 75720 Paris cedex 06, 2Hôpital St Vincent de Paul, 74 au DenfertRochereau, 75014 Paris and 3Département de Biologie Moléculaire, Institut Pasteur, 25 rue de Dr Roux, 75724 Paris cedex 15, France 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 myosin heavy (embryonic, foetal, fast and slow) and light chain isoforms 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 MHC'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.

STRUCTURE OF CONTRACTILE PROTEINS

Second site mutations to probe muscle structure in Dro sophila J. D. Clayton, A. Harrison and J. C. Sparrow Department of Biology, University of York, York, UK The striated indirect flight muscles (IDMs) of Dro sophila melagaster provide a useful model system in which to study the genetics of muscle structure and function. As the IDMs are dispensable for viability, mutations with severe effects may be maintained in the laboratory as homozygotes. Furthermore, as the IDMs are large enough to be dissected intact, genetic manipulation and mechanics can be used to complement one another (Drummond et al., Nature 348, 440-2, 1990). Large numbers of mutants have been isolated which affect the IDMs. Unfortunately, the majority of these mutants have muscle structures too abnormal for mechanical analysis. To generate mutants more suitable for mechanics, and to probe functional interactions between muscle proteins we have selected for flighted revertants or suppressors of existing muscle mutants. Act88F A'38V is a dominant flightless allele of the IFM specific actin gene (Act88F). It contains a single conservative substitution (Alanine 138 to Valine) in the putative hinge region of the monomer. Act88F A'38V flies accumulate little mutant actin, yet in Act88F tripleoids, Act88F A'38V/-/+ this small amount of mutant protein abolishes flight. One flighted revertant of Act88F A'38V has been recovered from two mutant screens, totalling 60 000 flies. The revertant maps to 57.5 ± 0.6 cm on chromosome 3, coincident with the Act88F gene, and the two tropomyosin genes (Tml and Tm2). The Act88F and Tm genes are too close to be separated genetically. The revertant actin gene has been amplified by PCR for sequencing. A suppressor mutation of the troponin I mutant, hdp1 has been obtained and maps to the 88F region of chromosome 3 (Prof. A. Ferrus, Madrid). We are currently investigating the Act88F allele from this strain and characterising the functional effects of the suppressor on both flight and muscle physiology.

Heat-dependent structural transitions of myosin subfragment-1 V. M. Danilova, A. M. Filenko, V. S. Omelyaniuk and E. B. Babiyhik Institute of Physiology, Kiev University, Kiev, Ukraine Structural transitions in myosin and especially in its active fragment, subfragment-1 (S1), are relevant to function of this molecule in the contractile system (Vibert & Cohen, J. Muscle Res. Cell Motil. 9, 577-80, 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 \( I_{365}/I_{295} \), the so-called parameter B (Filenko et al., Ukr. Biochim. Zhurn. 59, 5-9, 1997).

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 third 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 domain 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 (Babijchuk et al., FEBS Lett. 295, 55-60, 1991). Müllers and Reissers (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 magnesium-calcium exchange and phosphorylation of regulatory light chain (RLC) on the accessibility of the myosin and heavy meromyosin alkali light chains (A1) for 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 myosin 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 (bis-MPM) the RLC can be crosslinked to the HC as demonstrated by immunodotting. Digestion of such myosin with papain produces papain 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 bis-MPM. Pap-S1 with its crosslinked RLC can then be further digested by arginase-C between Lys-632 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 chymo-S1 and throm-S1, respectively. Chymo-S1 and throm-S1 only contain the LC1/3 but no RLC. RLC does bind neither to native chymo-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 acids residues running from Ala-822 down to Leu-842: AMNKVHPWPKLYFKKPL. 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
Abstracts

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 nucleotides.

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 S1·ADP·BeF3) was studied by differential scanning calorimetry (DSC). These complexes are stable analogues of the S1(ADP·V)2·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, 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 switch 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 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 sever's 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 comparison of the actin structures (in complex with DNase I and segment 1) did not show any gross changes in the relative positions of the four subdomains (the Cα-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 monocotyes and the human monocyte-like cell line U937 (Drussel 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 (psos), boar heart 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 CBNBr, 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, 17429-50). 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 (Orlou & 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 motility assays. According to the results of 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 tryptic digestions in the subdomain 2 region of G-actin. BeF, has 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 1:14 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. Phosphate 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 Cys 374 alklylation 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,. By probing the influence of BeF, on actin's function we found that BeF, binding inhibits slightly the actin-activated ATPase activity of S-1 by decreasing V∞,sat without affecting kcat.

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 trypsinolyis 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 (GuHCl) 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 GuHCl (lower than 0.5 M) and its light chains dissociate at slightly higher GuHCl 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 GuHCl range of concentrations 0.5-1.0 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 GuHCl 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 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 Mg2++ with Fe3++ in stable myosin subfragment-1-nucleotide complexes

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The predominant intermediate of the myosin-catalysed ATP hydrolysis is the M2+-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 Mg2+ with Fe3+. This cation has been found to be a good substitute for Mg2+ in the S1 ATPase, since the observed ATPase activity in the presence of Fe3+, as in the presence of Mg2+, was very low and became substantially activated by addition of actin. Fe3+ was found to substitute for Mg2+ in the stable ternary S1 complexes containing ADP and phosphate analogues, either vanadate or beryllium fluoride. In these Fe3+-containing complexes, like in those containing Mg2+, the ATPase activity of S1 was abolished. However, the lost activity was recovered during the decomposition of the complex, which was very fast upon addition of actin, and much slower upon dialysis or addition of EDTA. By carefully dialyzing the S1 FeADP Vi and S1 FeADP BeF complexes, one can remove the excess of free Fe3+ without causing significant decomposition of the complexes. In these cases a nearly 1:1 Fe3+/S1 molar ratio was
found. Upon addition of H$_2$O$_2$ to Fe$^{2+}$-containing stable S1 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 S1 is cleaved by the radicals at two specific sites— at 23 kDa from the N-terminus in the S1-FeADP-Vi and at only one of these sites—58 kDa from the N-terminus—in the S1-FeADP-BeF$_3$ 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 S1, because no cleavage has been observed in the presence of Fe$^{3+}$, 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$_2$O$_2$-induced S1 cleavage; (2) the slow exchange of Fe$^{2+}$ with Mg$^{2+}$ in the Fe$^{2+}$ containing stable S1-nucleotide complexes inhibits the cleavage of S1 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 inner from the smaller 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 across the filament axis. Also, the major mass density connecting adjacent subunits along the two long-pitch helical strands contains the 'Gly$_{14}$' loop connecting Gly$_{14}$ with Val$_{15}$ on subdomain 4, which according to the atomic model is implicated in the major long-pitch helix intersubunit contact. Consistent with the location of this loop at an intersubunit contact is the previous observation that mutation of Gly$_{14}$ to Asp$_{14}$ causes a polymerization defect of the corresponding actin (Taniguchi et al., J. Biochem. 103, 707–13, 1988; manuscript in preparation), which when expressed in the Drosophila indirect flight muscle leads to myofibrillar disruption with a flightless phenotype (Sakai et al., J. Biochem. 107, 499–505, 1990). It has also been shown that transfection of cultured fibroblasts with such mutated β-actin genes causes serious alterations of cell morphology including neoplastic transformation (Leavitt et al., Mol. Cell. Biol. 7, 2427–66, 1997).

Currently, we are analysing the 3-D structure of actin filaments that differ in the salt(s) used for polymerization, in the state of hydrolysis of the bound nucleotide, or that have been polymerized in the presence of small effector molecules. An example for such an effector molecule is the mushroom toxin phalloidin, a heptapeptide that specifically binds to actin filaments. It eliminates the lag phase of the polymerization reaction, most likely by stabilizing nuclei. The molecular basis for the stabilization of F-actin filaments upon stoichiometric binding of phalloidin is an increased strength of the intersubunit contact between the two long-pitch helical strands in a region that is close to the phalloidin binding site as determined with 3-D reconstructions (Bremer et al., J. Cell Biol. 115, 689–703, 1991). Comparison of 3-D reconstructions of F-actin filaments formed under different conditions for polymerization among each other as well as with atomic models is used to arrive at molecular and atomic explanations for structural differences, and to correlate these with kinetic and thermodynamic data.

Sarcomere formation at the muscle tendon junctions of carp (Cyprinus carpio L.) muscle fibres


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We studied the interaction of force transmission and sarcomere formation at the muscle tendon junction (MTJ) of adult growing carp (Cyprinus carpio L.) of 18–25 cm standard length. At the MTJ, new sarcomeres are assembled in bundles of thin filaments that connect the last sarcomere to the terminal membrane. Sarcomeres are formed by addition of thick filaments to these bundles. The end zone of the fibres (where new sarcomeres are formed) contains titin. This is in accordance with the proposed role of titin in integrating thin and thick filaments into sarcomeres (Fürst et al., J. Cell Biol. 109, 517–27, 1989). Terminal Z lines appear late; they are not always fully developed when the next sarcomere appears. α-actinin is not present in the filament bundles. A relatively high amount of desmin at the junctional region probably is located between the filament bundles in a network interconnecting all muscle fibre organelles. In addition to the titin filaments this network also may have a function in positioning new A bands.

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 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 molecule reference system of the label and the longer axis of actin filaments. The most probable values of angles Θ$_1$ and Θ$_2$ were estimated to be 36° and 65°, respectively, and agreed well with the formers and recently published data on F-actin filaments (Ostap et al., Biophys J. 63, 960–75, 1992). The values of Θ$_1$ and Θ$_2$ 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 Θ$_1$ 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 protomers in the filament and distortion of intersubunit bonds.
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, 1987). This part was shown to contain two actin binding sites in NMRI experiments named ABS1 (residues 86-117) and ABS2 (residues 128-156) whose counterparts on actin have been also elucidated (Levine et al., FEBS Lett. 298, 44-8, 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, 1309-60, 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 ABS1 or ABS2 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.

‘Postmortem’ disassembly of myofibrils
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We report some changes in the contractile apparatus of the mechanically treated ‘postmortem’ skeletal muscle, and evaluate the mechanical resistance of such a muscle.

Pig muscles (m. biceps femoris) either fresh or conditioned for 48 h at a temperature of 0 ± 1°C were used. The muscles were treated mechanically in various ways subjected to pressure combined with stretching and mincing in a ‘meat activator’, minced with a small pressure component in a ‘meat grinder’ with a kidney plate, or stretched and cut in a ‘knife tenderiser’ (Tyszkiewicz & Olikiewicz, Annuals IMPM 128, 17-31, 1991). For controls two intact muscles were used: a fresh one and a 48 h ‘postmortem’ one. Samples were taken for the electron microscope examination from the biceps femoris muscles after the above mentioned treatments and from the controls. The samples were fixed from the deeper muscle regions (not touched directly by the apparatus) and prepared as described earlier (Jakubiec-Puka, J. Muscle Res. Cell Motil. 6, 385-401, 1985).

In the ultrastructure of the control 48 h ‘postmortem’ muscle several changes were found (familiar from the study of such a muscle such as: disruption of sarcotema, damaged mitochondria, loss of the Z-line, and myofibrils disrupted at the Z-line level. In the 48 h ‘postmortem’ muscle mechanically treated samples all the anomalies were present and they were even more pronounced than in the controls. Additionally, some other changes were observed: kinked fibres, fractures across the fibres, expansion of the contractile structure (leading to breaking between the A- and I-band), and irregular damage of the contractile structure. In the mechanically treated fresh muscle some from the above-mentioned damages and irregularities of the contractile structure and kinking of fibres were present, however without loss of continuity of the contractile structure.

T-tubule-associated mono(ADP-ribose)transferase of rabbit skeletal muscle
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Microsomal preparations from rabbit skeletal muscle have been shown to contain arginine-specific mono(ADP-ribose)transferase activity (mADP-RT) (Peterson et al., J. Biol. Chem. 265, 17062-9, 1990). This mADP-RT seems to be a unique enzyme existing only in skeletal and cardiac muscles (Zolkiewska et al., Proc. Natl. Acad. Sci. (USA) 89, 11352-6, 1992). For elucidating the function of this enzyme and its target protein(s), a precise intracellular localization is desirable. For this purpose, crude SR preparations were separated by two successive sucrose density gradient centrifugations into several membrane fractions. The identity of these fractions was defined by selected marker enzymes and measuring ligand-binding to specific ion channels (ryanodine and the dihydropyridine analogue PN200-110). The by far highest mADP-RT activity was found in fractions enriched in vesicles derived from transverse tubules. In addition, autoradiography with 14C-PNAD, using a reaction system for endogenous arginine-specific mono(ADP-ribose)lysylation produced labelling only of proteins in the transverse tubule fractions. The orientation of the catalytic centre of the mADP-RT was determined by proving the sidedness of our transverse tubule vesicles to be mainly right-side-out. Treatment of the vesicles with the detergent lysophosphatidylcholine or polyoxyethylene-9-lauryl ether yielded a marked increase in mADP-RT activity, indicating an alignment of the catalytic centre of the enzyme in the transverse tubules towards the sarcoplasm. SDS polyacrylamide gel electrophoresis and autoradiography showed that endogenous mADP-RT activity induced labelling of a distinct protein pattern. The following proteins were labelled exclusively in the transverse tubule fraction: 15 KDa, 26 KDa, 37 KDa, 39 KDa, 43 KDa, 55 KDa, 60 KDa, 69 KDa, 102 KDa, 160 KDa, and 250 KDa. These
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 is surprisingly slow (F₄₅-fragments of antibodies take 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 determined by their binding-characteristics to specific binding sites within the fibres.

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The structure of the myosin filament

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Myosin is a major protein in muscle and is specifically located in the thick filament structure. We suggest a model of the thick filament which is based on the biochemical, X-ray data and calculations. The myosin heads are set in three rows on the cylinder surface of the thick filament and run parallel to the filament axis, forming the three-stranded helix. The order of myosin molecules in each row is determined by the DNase-I-inhibition assay, were 3.5, 2.7, and 1.3 μM for actin-, , and intact actin, respectively, in their Ca-bound form. The polymerization rates, examined by the light scattering method, were higher for actin-, than for the two other preparations. In agreement with earlier studies, the hydrolysis of the bound ATP associated with KC1-induced spontaneous polymerization of intact Mg-actin in the monomer and filamentous form, respectively.

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 μM KC1-induced polymerization, determined by the DNase-I-inhibition assay, were 3.5, 2.7, and 1.3 μM for actin-, , and intact actin, respectively, in their Ca-bound form. The polymerization rates, examined by the light scattering method, were higher for actin-, than for the two other preparations. In agreement with earlier studies, the hydrolysis of the bound ATP associated with KC1-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 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.

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.
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-FPLC 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 in the cases 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. Lineeweaver-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 mM 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 luciferin-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 (MHCI), myosin heavy chain 2A (MHC2A) and myosin heavy chain 2B (MHC2B), which are combined with several forms of light chains. These three chains can control the force-velocity mechanical parameters of muscles (Beckers-Bleukx & Marechal, 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 glycерinated muscle fibres prepared from soleus (SOL) or extensor digitorum longus (EDL) during transformation from relaxation to rigor by polarized florescence techniques (Borovikov et al., *Gen. Physiol. Biophys.* 10, 441–59, 1991). F-actin in muscle fibre was stained with 0.6 μM rhodamine-phalloidin (RP). The myosin heavy chains of the single fibres were separated by electrophoresis on glyceral-SDS-polyacrylamide gels and the zones were quantified by computerized densitometry: MHCI 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 MHC2B was 96 ± 2.56% in EDL.

During transformation of the muscle fibres from relaxation to rigor the polarization of florescence of RP F-actin changed for SOL and EDL differently. The anisotropy of florescence of RP 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 pre-force- 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/disassociation kinetics are little affected.

No effect of inorganic phosphate (P_i) on stiffness-speed-relations recorded during isometric contraction was found when P_i 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_i 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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F_{1,7} (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 (Das Gupta & Reissler, *Biochem.*, 1991, 1992). 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 F_{1,7} (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 F_{1,7} (1–7) into skinned rabbit psoas fibres to examine whether the F_{1,7} (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
more sensitive to the $F_p(1-7)$ than relaxed fibre stiffness. Both at high (170 mM) and low (30 mM) ionic strength active force could be fully abolished by 0.05–0.1 mg ml$^{-1}$ of the $F_p(1-7)$, while relaxed fibre stiffness was reduced by only 20–30%. When at low ionic strength active force was inhibited to <2% of original force, stiffness in the activating solution was still comparable to that in the relaxing solution at the same ionic strength. This suggests that the more effective inhibition of active force compared to the relaxed fibre stiffness does not originate from a more effective inhibition of the weak crossbridge binding to actin at high Ca$^{2+}$-concentrations. Instead, at least part of the observed inhibition of active force by $F_p(1-7)$ apparently occurs without inhibition of weak crossbridge attachment to actin. We are currently testing whether the $F_p(1-7)$ also inhibits other Ca$^{2+}$-effects (e.g. effect of Ca$^{2+}$ on stiffness-speed-relation in presence of MgATP$\gamma$S) and also interferes with activation of the thin filament by strong crossbridge binding to actin (e.g. when [MgATP] is reduced).

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, $k_{rde}$, to isovelocity contraction conditions: after some 200 ms of lightly loaded or unloaded shortening fibres are restrretched to their initial (isometric) sarcomere length and redevelopment of force and fibre stiffness is then followed either while sarcomere length is held constant (isometric) or reduced with constant velocity (isovelocity contraction). For isovelocity contraction, $k_{rde}$ 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 reactivation of the thin filament after deactivation during the preceding period of unloaded shortening but instead reflects crossbridge activation kinetics during fibre shortening.

Actin-filament motion in the in vitro motility assay is periodic


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Interaction between actin and myosin can be studied in the in vitro motility assay, where fluorescent actin filaments move over a lawn of myosin heads. Crossbridges are thought to interact independently of one another—and without memory of past events. To test this, we measured actin-filament motion with high precision. Spatial coordinates of the trajectories of the front end, rear end, and centroid of the actin filament were numerically differentiated to obtain the velocity. Velocity proved to be periodic. Peak-to-peak variation was ~5 μm$^{-1}$, relative to an average velocity of ~4 μm$^{-1}$. Auto- and cross-correlation functions of the motions of the three regions exhibited clear waves, indicating that the observed periodicity contains a limited number of independent frequencies. The correlation functions could be well-fitted with a single sine wave (periodicity 380 ± 80 ms) in seven out of 11 filaments, the remainder with two sine waves. The lifetime of the waves was on the order of seconds at least, implying long-term memory. Clear phase shifts between the motions of the three parts of the actin filament were observed. The issues of the independence of force generators and the absence of memory are challenged by our observations: (1) motion of actin filaments is not steady, but periodic; and (2) periodic waves persist on a long (second) time scale. These observations can be understood if either: binding of one crossbridge enhances formation of adjacent crossbridges; or, the driving force for motion is a propagated wave along the actin-filament.

Mechanism of activation of the contractile system of skeletal muscle fibres by NEM-SI compared to calcium-activation

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It is well known that binding of myosin heads to actin with high affinity (strong-binding crossbridge states) activates the acto-myosin ATPase activity even in the absence of calcium. It was proposed that for complete activation of the contractile system of muscle fibres not only calcium, but also the presence of strongly bound crossbridges is required. According to this hypothesis one might expect differences between activation by strongly bound crossbridges and activation by calcium alone. In order to study activation by strong binding cross bridges in the presence of MgATP, N-ethylmaleimide-modified myosin-subfragment-1 (NEM-SI) was used as an analogue for strong-binding crossbridge states. We diffused rhodamine labelled NEM-SI (Rh-NEM-SI) into single skinned muscle fibres of rabbit psoas and monitored its binding to actin and its distribution within the fibres by X-ray diffraction and confocal laser microscopy. To ensure only partial occupancy of actin sites by Rh-NEM-SI, the concentration of Rh-NEM-SI had to be <3 μM. At this concentration it took four days of incubation of the fibres to reach homogeneous distribution over the whole cross-section of the fibres.

Once diffusion is complete, isometric force generation and the rate constant of force redevelopment ($k_{rde}$) activated by NEM-SI can be determined and compared to calcium-activation. Apparently, binding of Rh-NEM-SI to actin activates force and $k_{rde}$ to the same level as calcium alone, and addition of calcium to the Rh-NEM-SI activated fibres had no further effect on both parameters. The differences in isometric force and $k_{rde}$, between pCa 4.5 and pCa 8 at high P$_i$-concentrations shown previously were found to be due to incomplete diffusion. When the distribution of Rh-NEM-SI is homogeneous, at all $P_i$-concentrations isometric force and $k_{rde}$ of Rh-NEM-SI activated fibres are the same as pCa 4.5 and pCa 8.

Since at full activation force redevelopment is independent of the presence or absence of Rh-NEM-SI bound to the actin filaments, we conclude that force redevelopment does not reflect the time course of reactivation of the thin filament after deactivation during the preceding period of unloaded shortening but instead reflects crossbridge activation kinetics during fibre shortening.
cycling kinetics. A model for regulation of the crossbridge cycle will be discussed which not only accounts for the calcium effect on k_{rev} but also for the observation that there is no difference between Rb NEM S1 activation and activation by high calcium alone.

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Attachment-detachment behaviour of crossbridges on the surface of reconstituted myosin filaments: effect of pH, Ca^{2+}-ions and myosin light chains phosphorylation

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Muscle contraction is thought to involve structural rearrangement of myosin heads and of the regions connecting these heads to the thick filament backbone. Here we describe the effects of pH, Ca^{2+} and myosin light chain phosphorylation on structural behaviour of myosin crossbridges on the surface of reconstituted rabbit skeletal muscle myosin filaments. Myosin filaments with dephosphorylated light chains or fully phosphorylated ones formed by dialysis against the solution containing 120 mM KCl, 1 mM MgCl₂, 10 mM imidazole-HCl (pH 7.0), 0.1 mM CaCl₂ or 1 mM EGTA have been negatively stained for electron microscopic observations. In the absence of Ca-ions the filaments of dephosphorylated myosin exhibit an ordered arrangement of myosin heads and the axial period of 14.5 nm, corresponding to the so-called 'crowns' of the crossbridges (State 1). This 'compact' state is formed by the crossbridges attached to the filament backbone and is typical for thick filament in the relaxed muscle. In the presence of Ca^{2+}-ions, myosin light chains phosphorylated crossbridges are oriented randomly on the surface of the filament due to their detachment from the filament backbone (State 2). Decreasing pH up to 6.5–6.0 independently of Ca^{2+}-level returns the crossbridges to the 'compact' state. Increasing pH up to 8.0 leads to the appearance of a 'spread' structure of myosin filaments with numerous 'whiskers' due to head and myosin rod portions moving away from the backbone (state 3). Substitution of Ca^{2+} by EGTA (pH 8.0) has no marked effect on the filament appearance. The filaments formed from phosphorylated myosin (pH 8.0) exhibit also a 'spread' structure. However, at pH 7.0 both in the presence and in the absence of Ca^{2+} they show the distinct periodic crossbridge array (state 1). Transitions between the above states occur rather readily: treatment of the myosin filaments on the electron microscope grid with corresponding solutions results in the change of structural behaviour of the crossbridges. The above myosin filament states are discussed as possible starting points for the study on dynamics of crossbridges behaviour during ATP-hydrolysis by rapid freezing electron microscopy.

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?
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; Biochm. Biophys. Eng. 13, 1993 (in press)) without rejection of the main idea of the crossbridge theory.

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 immuno-gold (EM) and immunofluorescence (LM) labelling. In the control muscles 43K DAG is 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 set skeletal muscle myofilibrils 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 B1 was present in quantities similar to nebulin. The protein B2 was electrophoretically resolved from preparative gels and used to produce polyclonal antibodies in rabbits. Immunoblot analysis revealed that B1 is immunologically related to rat aortic and uterine smooth muscle filamin. Immunofluorescence experiments indicated that B1 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 B1 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 B1 may be a member of the actin-binding protein family. Should B1 prove to be an actin-binding protein with a specificity for α-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 B1 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 guanidine 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) and 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 titin which is composed predominantly of domains containing ~100 residues. The free energy associated with the second transition was approximately 10 kcal per mole per domain. If, as seems likely, there are ~300 domains in titin each containing ~100 residues, the free energy of titin unfolding is ~3000 kcal per mole. Single molecules of titin span between the M- and Z-lines in muscle, a distance of ~1 μm. The I-band region of the molecule is thought to form an elastic connection between thick filaments and the Z-line. There are a number of muscles in which extensions to sarcomere lengths ~4 μm appear fully reversible. This implies the I-band region of titin can be extended reversibly to ~4 times its rest length. The A-band region of titin is thought to be attached to thick filaments under normal circumstances but can also exhibit elasticity. This occurs when titin becomes detached from the thick filament, either at sarcomere lengths >4 μm or following solubilisation of myosin. The molecular mechanism of titin elasticity is not known; however, since the molecule runs parallel to the muscle fibre axis in situ, large extensions may involve domain unfolding. From length vs. tension data in the literature, the work required to extend individual titin I-band domains in situ can be estimated and is of a similar order of magnitude to the free energy of unfolding in the second transition referred to above.

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

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Inserin is a 35 kDa protein that can be isolated from chicken gizzard smooth muscle. Substoichiometric amounts of inserin retard actin polymerization 5-fold (100 mM KCl, 2 mM MgCl2, pH 7.5, 37°C). Inserin binds strongly to the barbed ends of actin filaments. 10 nM inserin are sufficient to bring about maximal retardation of 1 μM polymeric actin. Inserin does not cease polymerization and depolymerization at the barbed ends. Inserin is immunologically related to the smooth muscle actin-binding protein 'tensin' (Weigt et al., J. Mol. Biol. 227, 593-5, 1992). It is suggested that inserin 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 proteins. 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 site 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 derived previously 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-II-I-I-I-II-I-II-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 127 800 is somewhat lower than the 140 000 estimated by SUS 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-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 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.

HEART AND CARDIOMYOCYTES IN CULTURE

TnT band shifts in cardiomyopathy correlated with sarcomere 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 alterations during heart disease, to assess the modifications in the Frank-Starling mechanism. The sarcomere length dependent pCa-tension responses were measured on right ventricular skinned trabeculae from the diabetic rat with myocardial abnormalities. The maximal force levels (Pm in pCa4 medium) were unaltered in disease. Also, at 2.4 μm, the Ca-sensitivity (pCa50) was 5.75 ± 0.03 for controls and 5.77 ± 0.03 in cardiomyopathy (n = 22). But, contrarily, the pCa5o 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. These TnT bands (putative TnT1, TnT2 & TnT3) were evident in the Westerns of normal and myopathic tissue. But mass density distribution between TnT1 and TnT3 were markedly affected in disease: compared with control, we measured 56 ± 4% (n = 15) shift in mass from TnT1 to TnT3 in disease. The results manifest a novel correlation between the altered Frank-Starling mechanism and the regulatory proteins in cardiomyopathy of streptoloztocin-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 skinned 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 troponins (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 sensitizer 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 that calcium sensitivity of skinned atrial fibre may be altered by troponin replacement and that the calcium sensitizer EMD 53998 has a different effect on the calcium responsiveness depending on the troponin isoform.

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 the 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 warping motion of the left ventricle with a clockwise rotation at the base and a counterclockwise rotation
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 fetal 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 phosphorylamin (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.

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 43036→G6 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 12434→G; 16234→G; 25634→G; 40334→G, 60634→G, 69034→G, 86434→G) 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 (16234→G and 40334→G) 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 domain in the tertiary structure of the β-myosin heavy chain head. We conclude that, in individuals with β-MHC gene-linked HCM, single amino acid changes in the β-myosin sequence, resulting in an abnormal acto-myosin interaction, may be the cause of the disease in these patients.

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 to ser 23,24 in bovine (Sviderek et al., Eur J. Biochem., 176, 335-12, 1988), see 22,23 in rabbit and rat heart (Mittmann et al., FEBS 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
Abstracts

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 Ca\(^{2+}\)-affinity of troponin C (England, Biochem. J. 160, 295–304, 1976). Two phosphorylation sites of troponin C (England, Biochem. J. 160, 295–304, 1976) 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., FEMS Lett. 302, 133–7, 1992). Freshly isolated troponin contains a mixture of two mono- and a nonphosphorylated form of troponin I (Swidercek et al., Eur. J. Biochem. 176, 335–42, 1988). Each form gives rise to one 3P-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 (PIRARRSPF/S/PANT) from the troponin I N-terminus shows two 3P-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 and monophospho species of troponin I. It could be shown that phosphoser-24 interacts with basic groups, probably the neighboring arginine residues. Phosphoser-24 is not involved in interactions. In future NOESY and CD spectra will help to elucidate the structure of peptides deriving from the N-terminal region of troponin I.

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 Ca\(^{2+}\) (Takeichi, Annu. Rev. Biochem. 59, 237–52, 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 EDTA, a chelator of Ca\(^{2+}\) 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.

Isoform switching of the sarcosommal Ca\(^{2+}\)-ATPase during myogenic differentiation
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The function of the calcium-dependent plasmamembrane Ca\(^{2+}\)-ATPase (PMCA) – as opposed to the sarcoplasmatic reticulum Ca\(^{2+}\)-pump – in muscle is unknown. To assess its potential role in muscle differentiation we characterized isoform patterns of the PMCA in various muscle and non-muscle cell types. Expression of the different isoforms of the pump (from four genes) and their subisoforms (derived by alternative splicing in the calmodulin binding region) was detected in several rat cell types by reverse transcription polymerase chain reaction using isoform specific primer pairs.

The ubiquitous PMCA isoforms 1b and 4b formed the typical isoform pattern of rat L6 myotubes, the heart derived cell line H9c2(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 in myogenic differentiation from H9c2(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 isoforms 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 glycinerated 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 cytochalasin (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 50nm.

Upon activation, all myofibrillar 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 \(\mu\)m s\(^{-1}\) at 10°C; Q\(_{10}\) 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 of Ca\(^{2+}\) from the SR. Here, we report undamped, propagated oscillations where a length-dependence of myofibrillar Ca\(^{2+}\) sensitivity, as well as a dynamic Z-line structure are essential.

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 myofibrillar 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 protein 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 computerized 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 (\(k_{\text{tension}}\)) after photophysical release of ATP from P1-(2-nitrophenyl)ethyladenoxy-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 \(k_{\text{tension}}\) of atrial fibres was 0.8 s\(^{-1}\) from LT-rigor and 0.9 s\(^{-1}\) from HT-rigor. The rate of tension development of ventricular fibres was significantly lower (\(p < 0.001\)) being 0.6 s\(^{-1}\) from LT- and HT-rigor, respectively. The
k_d of skinned ventricular fibres incubated in cardioplegic solution prior to the skinning 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 skinning 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^{-3} 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^{-11} M. VLC-1 peptides corresponding to the sequence 1-10 were without effect up to 10^{-11} M suggesting that the effective sequence was between 10-15. Incubation with maximal effective antibody concentration (10^{-7} M) increased tension by 3.5%, 17%, and 27.5 ± % at pCa 6.5, 6.0, and 4.5 respectively. Threshold concentration of mabVLC-1 was 5 × 10^{-12} 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 7.5%, 3.4% and 0% ALC-1 was 6.01 ± 0.08 (5), 5.97 ± 0.03 (5), and 5.87 ± 0.08 (8), 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 (TnI) are that it contains an extra 26-33 NH-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 the may contribute to the relaxation of the heart following catecholamine stimulation. To study this we have isolated a full length CDNA clone for mouse cardiac TnI and have expressed CTnI 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 CTnI 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 TnI and CTnC, making the fibres develop Ca\(^{2+}\)-insensitive force. Ca\(^{2+}\)-sensitive force can be subsequently reconstituted with various CTnC CTnI complexes. Vanadate treated fibres restored with native CTnC-CTnI complex behaved the same as unextracted fibres when they were treated with PKA. When vanadate treated fibres were restored with a complex of CTnI and a CTnT mutant where both SER22 and 23 had been converted to ALA, the fibres no longer responded to PKA treatment, indicating that CTnT 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, Diazo-2. The t_1/2 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 CTnC. In another study we examined the developmental expression of the TnI isoforms in mouse heart using mouse CTnI and rat slow skeletal muscle (SSnI) cDNA clones. Total RNA from cardiac muscle from three stages of development, 17-day embryos, 1-2 day newborns and adults, were used for Northern blots. SSnI was expressed in the embryo but was absent in the adult. CTnI began to be expressed during the embryonic stage and it gradually replaced SSnI in the adult heart. Previous studies by Solaro 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 CTnC-CTnI and CTnC-SSnI 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 glycercinated rat heart in contraction (Co), relaxation (Re) and rigor (Ri) media with different ionic strengths (10 mM, 50 mM, 150 mM NaCl) using a radioisotopic method of C\(^{14}\)H\(_4\)CO\(_3\) Na uptake. An increase in C\(^{14}\)H\(_4\)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

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**Abstracts**

**ALC-1:** pCa\(_{-}\), (-log free [Cal\(^{+}\)] at half-maximal activation) of fibres amounts of ALC-1 in the ventricle. Skinned fibres which expressed Forty-one percent of patients investigated expressed detectable actin seems to be a very effective modulator of force of cardiac regulation by trösnin.
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 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 immuno
duprecipitation 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 KI suggesting a structural role in the myocard.

Future studies of 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 cardiomyocytes. The major components of the cytoplasmic plaque of these spot des
somes 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 embry
conic 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 desmoplakin are much more abundant than in the adjacent intra- and extracardiac 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
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. Cardiomycocytes (CM) in hypocontractile segments are affected by partial to complete loss of sarcomeres, accumulation of glycerogen as well as disorganization and loss of sarcomeric reticulum (SR). The space left by the vanished sarcomeres becomes occupied by glycerogen. 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 glycerogen 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 existence of hibernating cells loaded with glycogen and a supranormal glucose extraction pattern seen with positron emission tomography. The expression of T and u-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 isoform 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 (10⁻⁶ M) nor prazosin (10⁻⁷ M) did affect the PIE. AII-receptor blockade with saralasin (10⁻⁸ M) abolished the PIE of AII completely. With enalaprilat (10⁻⁶ M), no PIE of AI could be detected. Removal of the endocardium did not change the PIE of AII: the intracellular Ca²⁺-transient, as detected with aerquinin, increased to 130 ± 5% (p < 0.05) at 10⁻⁷ M AII. Compared to the standard Ca²⁺-dose-response curve, this finding does not indicate a Ca²⁺-sensitization of the myofilbrils by A II. In contrast to auricular myocardium, neither AII 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 AII at 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 inexcitable. 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 relate 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 passive shrinking of the extracellular space and partial inhibition of $Na^+/K^+$ pumping. Electrical uncoupling occurs concomitantly with an increase in free cytosolic $Ca^{2+}$, reduction in [ATP], and development of ischemic contracture. It is delayed by application of $Ca^{2+}$ entry blockers, inhibitors of acylcarnitine-transferase and accelerated by acidification. Comparison of the changes in intra- and extracellular concentrations of $K^+$, $Na^+$, $Ca^{2+}$ 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 $Ca^{2+}$ through voltage-dependent $Ca^{2+}$ channels is the initial event in excitation-contraction (e-c) coupling. This trigger signal is amplified several fold by additional $Ca^{2+}$-induced $Ca^{2+}$ 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 $Ca^{2+}$ signalling attributable to the proposed modulation, we investigated the subcellular $Ca^{2+}$ distribution in heart muscle cells during triggered and spontaneous $Ca^{2+}$ release. Cells were loaded with two fluorescent $Ca^{2+}$ indicators (Fluo-3 and Fura-Red) to allow ratiometric imaging of intracellular $Ca^{2+}$ with laser scanning confocal microscopy (Lipp & Niggli, Cell Calcium 14, 359–72, 1993). In cultured neonatal rat myocytes two types of spontaneous $Ca^{2+}$ release were identified: (i) focal $Ca^{2+}$ release with limited or without subcellular propagation and (ii) $Ca^{2+}$ release propagating through the entire cell as a $Ca^{2+}$ wave. In myocytes isolated from adult guinea-pigs the same fundamental patterns of $Ca^{2+}$ 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 $Ca^{2+}$ load of the cells and pharmacological tools which modify the function of the SR revealed that the probability, extent and pattern of $Ca^{2+}$ release is modulated on the subcellular level. The $Ca^{2+}$-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, Biophysical J. 63, 497–517, 1992).

The mechanism of cardiac protection following sublethal ischemia and sublethal heat stress
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Restoration of the flow in the ischemic myocardium is essential for the survival but often it is accompanied by negative effects. Calcium imbalance and/or free radicals release are considered to be the causes of the damages which appear. It is known that heat stress improves myocardium recovery after an ischemic insult. It is not clearly demonstrated if this beneficial effect is due to heat stress proteins or/and to the cellular antioxidants (as superoxide dismutase, catalase, glutathione) or to other cellular mechanisms. Meanwhile it is necessary to find new ways of inducing this protection as heat stress has also damaging effects upon the organisms. In this respect we have measured the levels of heat stress proteins, especially 72 kDa one, and of the superoxide dismutase, catalase and glutathione in the hearts of the heat stressed rats and rabbits and also after a short repeated period of sublethal local ischemic, in situ, in rabbits. The data were compared with the recovery potential of the treated myocardium expressed as infarct/risk ratio.

The heat stress proteins and the antioxidants we have measured seem to play an active role in the mechanism of cardiac protection but the degree in which they intervene depend not only on species but on the kind of stress we have used as well.

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 myotubins in this domain. They seem to be affected directly by superoxide anion (MacFarlane & Miller, Circ. Res. 1217–24, 1992), by the hydroxyl 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 $^{45}Ca^{2+}$ 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.
Abstracts

However, 0°C caused more severe damage than incubation at 4°C. It is concluded that in diseased hearts heart 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., Pflügers Arch. 423, 159–66, 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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Steep 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 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 $\Sigma$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, $\Sigma$Na fell with a space constant of 28 µm, at 1 µm distance $\Sigma$Na was 12 ± 3 mM. The steep gradient suggests that sodium does not freely diffuse and that subsarcolemmal sodium is controlled by transmembrane fluxes rather than by cell dialysis. $\Sigma$Na$_{20}$ 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 $\Sigma$Na$_{20}$. This 'Na-microheterogeneity' suggests that Ca$^{2+}$ influx at low subsarcolemmal Na and K$^{+}(Na)$ channel activation at high $\Sigma$Na$_{20}$ 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 EC-coupling (Lipp et al., J. Physiol. 454, 321–36, 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 $I_{Na}$ (in the absence of $I_{Ca}$) 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 $[Na^+]_i$ 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 284, 283, 1999). We used ratiometric laser-scanning confocal microscopy (Fluo-3 and Fura-Red) to record $[Ca^{2+}]_{i}$ in guinea-pig cardiac myocytes while the whole-cell current was simultaneously measured with the patch-clamp technique (Lipp & Niggl. 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 $I_{Na}$ and $I_{Ca}$-induced Ca$^{2+}$-transients, both in the absence and presence of ryanodine (20 µM). In addition, we were able to detect a residual $I_{Na}$-induced Ca$^{2+}$-transient in the presence of ryanodine and vera-pamil (10 µM). This Ca$^{2+}$-transient represents the missing link between $I_{Na}$ and the release of Ca$^{2+}$ from the SR and probably reflects Ca$^{2+}$ influx via Na–Ca exchange. Control experiments showed that Na$^{-}$ 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 $I_{Na}$-induced Ca$^{2+}$ release from the SR after activation of the Na–Ca exchange and thus support the notion of a subsarcolem- minal restricted space. Moreover, the fast kinetics of the $I_{Na}$-induced Ca$^{2+}$-transients revealed with 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, 1098). 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 isomotic substitution for NaCl of weak acids (butyrate) or bases (trimethylamine (TMA) or ammonium chloride (NH$_4$Cl)) in HEPES buffered superfusing Krebs solution (gassed with 100% O$_2$ at 37°C). 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...
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 in confluent in media supplemented with serum. Cell content in desmin was tested five days after confluence has been reached, the cells being maintained in the presence of serum, or in serum depleted media (SDM) supplemented or not with steroid hormones and/or EGF. A specific monoclonal antibody was used to check the presence of desmin in the cells. At the beginning of the culture (two passages), desmin expression in the cells was found to be comparable to that in the tissue. After five passages, it was reduced by three in cells maintained with E2 or with E2 plus progesterone or EGF (and not in cells maintained in SDM, with serum or with EGF alone). In the presence of EGF plus steroid hormones, the myometrial cells are able to incorporate 3H thymidine, it is hypothesized that smooth muscle cell quiescence is not obligatory for desmin synthesis. The study of the interactions of steroid hormones and growth factors in the regulation of desmin synthesis appears as an interesting new way of research for the understanding of uterine growth during pregnancy.

Search 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 vivo and in vitro 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 (Gordon et al., Circ. Res. 59, 633–44, 1986). Ascribed with stem cell properties, these cells are thought to reside in the subendothelial layer and have been implicated in the pathogenesis of arteriosclerosis. Although they show expression of smooth muscle actin and myosin isoforms they seem to be partially deficient in specific regulatory proteins like h-caldesmon and calponin (Frid et al., Dev. Biol. 153, 185–93, 1992).

To explore in more detail the heterogeneity of smooth muscle, we have established several aortic smooth muscle cell lines using tissue at various developmental stages from H-2Kb-tsA58 transgenic mice (Jat et al., Proc. Natl. Acad. Sci. U.S.A. 88, 5096–100, 1991). These mice carry a temperature sensitive mutant of the simian virus 40 large T antigen that is under the further regulation of the mouse histocompatibility promoter (H-2Kb), inducible by interferon-γ. Depending on the gestational age, stable smooth muscle cell lines of two different phenotypes can be distinguished. Adult organs give rise to cell lines of a fibroblast-like morphology, whereas aortic cells from foetal or newborn mice show an epithelioid growth pattern reminiscent of endothelial cells. However, simultaneous expression of α-smooth muscle actin and smooth muscle myosin clearly classify even the latter cell type as smooth muscle-derived although additional marker proteins are scarce (h-caldesmon) or not expressed at all (metacvinclutin). Judging by their smooth muscle specific protein composition, these cells correspond to the presumed 'stem cell population' of blood vessels. In contrast to adult smooth muscle cells, they show an increased metabolism of low density lipoproteins, characteristic of cells involved in atherogenesis.

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

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Several classifications of α1-adreceptors 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-endothelialised rat aortic rings, along with their alteration upon changes in the extracellular concentration of K+, Na+ or Ca2+. High K+ (40 mM). Precontracted rings display a sustained superimposed contraction with phenoxyline challenge (PHE, 10–8 M), but a transient one with norepinephrine (NE, 10–6 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 LCl, 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., Nannym 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 Ca2+, and discuss the significance of these observations 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 manners and to the variable Ca2+-sensitivity of the contractile apparatus (Filipeanu et al., Life Sci, in press, 1993). Interference between cAMP- and cGMP-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).
Abstracts

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 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 1:17-13 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 x 10^7 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 (Göschel-Stewart et al., Expression 21, 5:12-15, 1975) where myoglobin is similar to that in whale skeletal muscle. 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, 36-6, 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 net-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.

Myofibrillar deoxyribonuclease from smooth muscle, characterization and interaction with actin
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We have shown previously the presence of DNase I-like activities in the cytosol of smooth, striated and heart muscle. Similarly to DNase I from bovine pancreas, they were inhibited on the gels by skeletal muscle actin. The aim of our present work was the isolation and characterization of myofibrillar (MYF) DNase from smooth muscle of turkey gizzard. Our initial source material was gizzard MYF-like preparations which represented well fragmented and extensively washed muscle fragments. The extract obtained at 25 mM MgCl\(_2\) and fractioned with ammonium sulphate was subjected to further purification steps on an anion exchange, gel filtration and oligo d(T) affinity columns. DNase was isolated with about 2000-fold purification. The smooth muscle MYF-DNase, similarly to DNase I hydrolyzes native as well as denatured DNA with optimum activity in alkaline pH, in the presence of divalent cations (Mg\(^{2+}\), Ca\(^{2+}\). Its molecular mass was found to be 38 kDa and isoelectric point close to pH = 6.63. Myofibrillar DNase was inhibited more efficiently by endogenous turkey gizzard G-actin (K_i = 40 nM) than by rabbit skeletal muscle actin (K_i = 270 nM). Myofibrillar DNase from smooth muscle affecting actin polymerization.

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 (MLC17) isoforms in the uterus of late pregnant (P) and non-pregnant (N) 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) μN mm^-2, and 5.6 ± 1.1 (5/2) μN mm^-2 in N and P, respectively (p < 0.01). Maximal shortening velocity as extrapolated from the force-velocity relation rose from 0.134 ± 0.01 ML s^-1 (5/5) 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 isosforms 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 designated 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%, 40.0 ± 0.7, and 24 ± 0.9% SM1, SM2, and NM-MHC, respectively (4/9). The increased SM2 and NM-MHC expression in P patients was statistically significant (p < 0.01). MLC17a expression was increased from 23.5 ± 1.0% (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 MLC17a.

Contractile and cytoskeletal compartments in the smooth muscle cell
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Differentially 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-isofrom 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 actomyosin-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.

Additional 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 (Nyitrai 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 tandem 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 5a,b (residues 176–211 in the striated MHC sequence) and -a,b (212–243) encodes part of the ATP-binding site. Exon 5 contains the consensus P-binding site (GSEGAKTT) 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). 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 codons 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 stripped 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 stripped muscle myosin (at least in molluscs) are closely related unlike their vertebrate counterparts.

Passive tissue mechanics and contractile force in guinea-pig portal vein* N. Öztiirk and P. Ungan
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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 myosin solution and tension responses to stretches were measured. After the experiments 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 contraction kinetics. 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 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 are liberated on splitting of a phosphate 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 chemically 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 Ca²⁺-activated fibres the relative inhibition of active force (about 20% at 20 mM Pᵢ) was similar at all investigated Ca²⁺ levels. Thus the Ca²⁺ sensitivity of contraction was not altered by 20 mM Pᵢ. Force-velocity relations were determined at maximally activated fibres. The effects of 20 mM Pᵢ were investigated at the maximal activation level in the presence of 20 mM ATP. In this case the maximum rates of force development Vₘₐₓ and V₄₃ₕ₃ were decreased by about 20% at 20 mM Pᵢ whereas V₄₃ₕ₃ was unaltered at higher Ca²⁺ and slightly increased at lower Ca²⁺. In maximally thiorphosphorylated fibres 20 mM Pᵢ decreased force (by about 20%) without any significant effects on V₄₃ₕ₃. 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 cycle in smooth muscle fibres. The decrease in 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 unchanged sensitivity of force to [Ca²⁺] in the presence of Pᵢ suggests that the Pᵢ-induced alteration in attached crossbridge states do not affect the Ca²⁺-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 Vₘₐₓ 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 contains the fragments F1 + F2 (1-579) and F5 contains fragments F2 + F3 (153-756). Only peptides F1, F3 and F5 are retained by Ca²⁺-Calmodulin-Sepharose column. Furthermore, both fragments F3 and F5 co-sediment with F-actin and inhibit the actomyosin Mg²⁺ ATPase in a Ca²⁺/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 Ca²⁺ 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 spanning 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 retain 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 Ca²⁺ and phospholipid binding protein, was isolated from duck gizzard and some of its properties were investigated. In the presence of Ca²⁺, calcimedin interacted and co-sedimented with vesicles of mixed phospholipids (azolecin) or with F-actin. Different Ca²⁺-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, tropomyosin 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. Calcedin 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 calcemin can coexist on actin filaments. Thus the mechanism of calcemin action seems to be different from that of other EF-hand proteins. Calcemin was much less effective in reversal of caldesmon inhibition of acto-HMM ATPase than of actomyosin ATPase therefore we suppose that the effect of calcemin is at least partly due to the bundling of actin filaments. Taking into account that the content of caldesmon in smooth muscle is significantly less than that of caldesmon calcemin 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) calcemin in the presence of Ca$^{2+}$ can reverse the inhibitory action of caldesmon.

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Myosin light chain kinase expression during smooth muscle development

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In striated (skeletal and cardiac) muscles the polymorphism of many contractile proteins is well documented. In smooth muscles, however, the heterogeneity of contractile proteins has not been defined until recently. Myosin light chain kinase (MLCK) is an enzyme that phosphorylates the regulatory light chain of myosin (LC20) in a Ca/Caldesmon-dependent manner, thus giving origin to smooth muscle contraction. Since the target of MLCK, namely LC20 subunit of myosin, is expressed as a different isoform in neonatal stages compared to adult situation (Inoue et al., Eur. J. Biochem. 183, 645–51, 1991). The whole clone (H1) encodes all those 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), H7 (622–680, junction of domains 3 and 4a) and H9 (726–767, domain 4b, non-tissue specific isoform). 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$^{-10}$ weaker than H1 or native caldesmon), whilst two fragments from domain 3 did not bind actin-tropomyosin. 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 (506–600, domain 3 + 3/4a junction) and H7), did interact. Preliminary NMR measurements have been carried out with 658K, a fragment constituting domain 4b of gizzard caldesmon. Broadening of the signals of tryptophans 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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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 encodes all those 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), H7 (622–680, junction of domains 3 and 4a) and H9 (726–767, domain 4b, less N-termiinal 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$^{-10}$ weaker than H1 or native caldesmon), whilst two fragments from domain 3 did not bind actin-tropomyosin. 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 (506–600, domain 3 + 3/4a junction) and H7), did interact. Preliminary NMR measurements have been carried out with 658K, a fragment constituting domain 4b of gizzard caldesmon. Broadening of the signals of tryptophans 692 (human 749) and 722 (human 779) has been observed upon adding actin and/or calmodulin, suggesting involvement of these residues within binding sites.
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. Electron microscopy studies require a chemical fixation step which, by its very nature, induces artefacts in the muscle tissue.

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 come from the actin 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 tissue. In extracted tissue the face polar nature of the filaments is indicated, especially in transverse 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 Ca\(^{2+}\) and calmodulin or by Ca\(^{2+}\)-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, 10148–55, 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 sulphhydril 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 eluted 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, Bionanotechnics 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 PAGE was performed according to Sobieszek and Jeremic (Electrophoresis 7, 417–25, 1986). The bathing solution contained (in mm01 l–1): MgCl2 10, ATP 7, KCl 85, EGTA 4, imidazole 20 (pH 6.7). The control specimens 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. 268, 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 can exist in two extreme states, described as 'on' and 'off' or 'Strong' and 'Weak', which are in equilibrium. The Weak state has a low rate of activation of Myosin ATPase whilst the strong state has a high rate, although affinities of MADP/Pi (weak crossbridges) for actin are
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 conforms 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) was just 68 amino acids long (C-terminal 1/2 of domain 4). However, there is not a single discrete site since another inhibition can be observed if a site in the middle 1/3 of domain 4 (site +B') is used. A larger fragment (H7) consisting of the N-terminal 2/3 of domain 4 was inhibitory whilst a fragment (H12) representing the usable 1/3 of domain 4 bound actin and calmodulin 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 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.
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 58% 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 SMC's underlying the plaque core and was seen only in scattered cells in the plaque cap. Staining by non-isomor-specific 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 cAMP signalling to the Ca2+ 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 C-terminus and a myosin-binding site near the N-terminus. The isolated protein in vitro. One such kinase is casein kinase II. 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.

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 Ca2+/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-dimethylamino-propyl) 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 SDS-PAGE 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. Signifi-
cantly, increasing the salt concentration from 60 up to 150–200 mM
resulted in a gradual disappearance of the dimer band. This demon-
strates 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 con-
tained kinase activated by CM in a cooperative manner while
activation of the ascending kinase was uncooperative. Correspon-
dingly, our SDS-gel revealed that the kinase from ascending fractions
was enriched in the oligomers while the descending fractions con-
tained 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.
Trombitás, K. et al., Contraction-induced water movements in
single fibres of frog skeletal muscle. J. Muscle Res. Cell Motil. 14,
573–84.
Bartoo, M. L. et al., Active tension generation in isolated skeletal
Trombitás, K. and Pollack, G. H. Elastic properties of the thin filament
in the Z-line region of vertebrate striated muscle. J. Muscle Res. Cell
Motil. 14, 416–422.
Vorotnikov et al. Aortic caldesmon phosphorylation by endogenous