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MYOSIN AND MYOSIN-LINKED REGULATION

Phosphorylation of a second light chain site by myosin light chain kinase: effects on enzymatic activity and conformation of smooth muscle myosin

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Smooth muscle myosin is phosphorylated by myosin light chain kinase (MLCK) at a second site on the 20 kDa light chain [Cole *et al., FEBS Lett.* **180**, 165 (1985); Tanaka *et al., J. Biochem.* **97**, 1823 (1985); Ikebe & Hartshorne, *J. biol. Chem.* **260**, 10027 (1985)]. This was identified as threonine 18 (Ikebe *et al., J. biol. Chem.* **261**, 36 (1986)]. Evidence indicating that MLCK phosphorlyates the second site is as follows: the MLCK preparations do not contain obvious contaminants; during purification of MLCK activity towards both sites copurify; many kinases reported to phosphorylate light chains do not phosphorylate intact myosin (e.g. casein kinase II, cAMP-dependent protein kinase); the phosphorylation of thr 18 is Ca^{2+}- and calmodulin-dependent and the calmodulin stoichiometry (with MLCK) is unity for the phosphorylation of both sites, a position equivalent to thr 18 is phosphorylated by MLCK in synthetic peptide substrates [Kemp & Pearson, *J. biol. Chem.* **260**, 3355 (1985)], proteolysis of MLCK by trypsin causes initially an inhibition of activity followed by an activation and this pattern is repeated for the phosphorylation of both sites, the Ca^{2+}-independent form of MLCK phosphorylates both sites, neither ser 19 nor thr 18 is phosphorylated by protein kinase C. Phosphorylation at both sites increases actin-activated ATPase activity: for 2 mol P per mol myosin (2P) actin-activated ATPase ~ $0.47\,s^{-1}$; for 4 mol P per mol myosin (4P) actin-activated ATPase ~ $1.25\,s^{-1}$. Phosphorylation at the second site also increases the rate of superprecipitation. In order to determine if the alteration in enzymatic activity is reflected by conformation the following experiments were carried out. The relative viscosity of 2P-myosin decreased at KCl concentrations below 0.2 M KCl (in 1 mM $MgCl_2$, 1 mM ATP) and this is paralleled by a reduction in Mg^{2+}-ATPase activity. In contrast, 4P-myosin did not show a reduction of viscosity under similar conditions and ATPase remained constant. Sedimentation velocity measurements show that under the conditions used for viscosity double phosphorylation favoured the formation of 6S myosin. Filament formation also is influenced by phosphorylation of thr 18. At 1 mM $MgCl_2$, 1 mM ATP and 85 mM KCl dephosphorylated myosin (0P) myosin does not form filaments and 2P-myosin forms only small aggregates. Phosphorylation of thr 18 induces the formation of larger filaments. To eliminate the possibility that myosin aggregation was responsible for the alteration of ATPase activity, experiments were carried out with HMM. Double phosphorylation altered the V_{max} of actin-activated ATPase ($4.3\,s^{-1}$ for 2P HMM, $8.5\,s^{-1}$ for 4P HMM) and had no effect on K_a. As the ionic strength is reduced the Mg^{2+} ATPase of HMM also is reduced. This effect is most marked for 0P HMM, 2P HMM is more resistant (i.e. the reduction of activity occurs at lower ionic strength) and 4P HMM is the most resistant. Limited papain proteolysis was used as a conformational probe. It was shown previously [Ikebe & Hartshorne, *J. biol. Chem.* **259**, 11639 (1984)] that dephosphorylated (inactive) HMM is more resistant to proteolysis than phosphorylated (active) HMM (2P) and that one of the regions affected is in the S1–S2 junction. Using 4P, 2P and 0P HMM (at low salt concentrations; 50 mM KCl), it was found that the susceptibility to papain digestion decreased in the same order. The dephosphorylated HMM was most resistant to proteolysis and 4P HMM was not susceptible to proteolysis. The difference in digestability was found only in the presence of ATP and not in its absence. At higher salt concentrations (0.5 M KCl) where phosphorylation does not affect Mg^{2+}-ATPase activity of HMM the proteolysis profiles for 0P, 2P and 4P HMM were similar. The results presented above offer further support to the idea that the enzymatic activity of myosin is determined by the conformation at the S1–S2 region of the molecule. Supported by grants HL 23615 and HL 20984 from NIH.

Two-site phosphorylation of the 20 kDa myosin light chain of glycerinated porcine carotid artery smooth muscle

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A number of laboratories have now reported two-site phosphorylation of the 20 kDa myosin light chain of purified gizzard myosin. In all cases, these reports have described phosphorylation of a threonine residue in addition to phosphorylation of ser 19 by myosin light chain kinase. We have recently observed significant two-site phosphorylation of the 20 kDa myosin light chain (LC20) of glycerinated porcine carotid arteries. Porcine carotid arteries were glycerinated for 48 h at 5°C and frozen at −70°C as previously described [Haeberle *et al., J. Musc. Res. Cell Motility* **6**, 347 (1985)]. Myosin light chain phosphorylation was measured using urea–glycerol gel electrophoresis followed by radioimmunoblotting [Hathaway & Haeberle, *Am. J. physiol.* **249**, C345 (1985)]. Three LC20 bands were present on urea–glycerol gels of extracts from contracted glycerinated porcine carotid arteries. Using [γ^{32}P]ATP-labelled skinned muscles, it was determined that the two faster migrating bands were phosphorylated forms of LC20. The specific activity of the faster migrating phospho-LC20 band was found to be 1.8 times that of the slower migrating phospho-LC20 band. This suggested that the faster migrating band was a doubly phosphorylated form of LC20. After 10 min of contraction in the presence of saturating calcium and calmodulin, 70% of the total LC20 was doubly phosphorylated. Tryptic peptide mapping of the LC20 bands from urea–glycerol gels demonstrated the presence of two major phosphopeptides. Both the monophosphorylated and diphosphorylated bands yielded the same two tryptic phosphopeptides. However, the relative amount of the two phosphopeptides differed for mono- and diphosphorylated LC20. Phosphoamino acid analysis of the mono- and diphosphorylated

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LC20 bands indicated that phosphoserine was the predominant phosphoamino acid present. These results demonstrate that a second site on the 20 kDa myosin light chain of porcine carotid artery smooth muscle can be phosphorylated by an endogenous kinase. In contrast to previous reports of second-site phosphorylation of a threonine residue in gizzard LC20, these studies suggest that two serine residues are phosphorylated in carotid artery LC20 during contraction of glycerinated muscles.

Mapping the actin and light chain binding sites of myosin S1

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Tryptic digestion of skeletal muscle myosin S1s generated by papain (+ Mg^{2+}) or chymotryptic digestion produced C-terminal fragments having molecular masses of 24 kDa or 20 kDa respectively. It has previously been shown by a variety of techniques that a major binding site for the regulatory light chain is present in the 4 kDa difference peptide between these fragments. Using a gel overlay technique we have shown that the light chains (regulatory and essential) and actin bind to the C-terminal 24 kDa fragment. The regulatory light chain binds to the 24 kDa fragment much more strongly than to the 20 kDa fragment whereas the essential light chain binds equally strongly to both fragments. We observed that actin binding to the 24 kDa fragment was unaffected by MgATP, whereas its binding to the intact S1 heavy chain was abolished by MgATP. The 24 kDa fragment was isolated from a tryptic digest of papain-S1 by CM–cellulose chromatography in the presence of 8 M urea and was shown to contain the reactive SH_1 thiol residue. The amino acid composition, *N*-terminal and C-terminal sequence analyses of this isolated fragment compared favourably with the known myosin heavy chain sequence in the C-terminal region of S1 [see Karn *et al.*, in *Cell and Muscle Motility*, Vol. 6 (edited by SHAY, J.). New York: Plenum Press. (1985)] The 24 kDa fragment was further digested with *S. aureus* V-8 protease to yield a 10–12 kDa peptide, derived from the region between the SH_1 thiol residue and the C-terminus of the fragment, which retained the ability to bind the light chains and actin. To try to identify the residues in the heavy chain involved in light chain and actin binding, a protein engineering approach is being used with the plasmid pmyo561 which contains the entire head sequence of the *C. elegans unc54* myosin heavy chain gene (residues 1–844). *E. coli* cells containing this expression vector synthesize a 90 kDa protein that cross reacts with an anti-nematode myosin polyclonal antiserum and binds light chains (regulatory and essential) and actin (in an ATP-dependent manner) using the gel overlay technique. Attempts are being made to purify this synthesized S1 in a biologically functional state. In addition, protein engineering/site-directed mutagenesis and a deletion mapping analysis of subclones of the 24 kDa coding region are being used together with the gel overlay technique to map the residues in the heavy chain involved in actin and light chain interactions.

10S conformation of chicken gizzard myosin

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Gizzard myosin filaments in 0.15 M KCl (and 10 mM $MgCl_2$) disassembled into myosin monomers with a sedimentation coefficient of approximately 10S upon addition of ATP, whereas increasing the KCl concentration to 0.3 M dissociated the filaments into 6S-myosin monomers. Electron microscopy revealed that the rod of 10S-myosin was folded at two distinct regions located approximately 500 Å and 1000 Å away from the neck, and the latter region was attached to the neck, thus forming an intramolecular loop. The MgATPase activity of 10S-myosin was only about one-tenth that of 6S-myosin. We have now reported two findings. (1) 6S-Myosin and 10S-myosin had different susceptibilities in the proteolytic digestion and different reactivities in the chemical modification. For example, with 10S-myosin, the cleavage by papain was strongly inhibited at a junction between S1 and rod and at two junctions in the S1 moiety (the junction between the 72 kDa and 22 kDa fragments of S1 heavy chains and the one between the 3 kDa and 17 kDa fragments of 20 kDa light chains). The 22 kDa fragments produced by the papain digestion contained the SH_1 type of thiol groups. When 10S-myosin was allowed to react with *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine (abbreviated as IAEDANS), the modification of the thiol groups was also strongly inhibited. All of these observations suggest that the 22 kDa peptide regions located adjacent to the myosin neck participate in the 6S–10S transition of gizzard myosin. (2) The 22 kDa fragments were isolated from the papain digest of IAEDANS-modified gizzard myosin. The amino acid sequence of the fragments was determined: KGMFRTVGQLYKE-QLTKLMTTLRNTNPNFVRCIIPHNEKRAGKLDAHLVLE-QLRCNGVLEGIRIC*RQGFPNRIVFQEFRQRYEILAANAIPK-GFMDGKQACILMIKALELDPNLYRIGQSKIF-FRTGVLAHLEEERDLKITDVIIAFQAQCRGYLARKAFAKRQQ-QLTAMKVIQRNCAAYLKLRNWQWWRLFTKVKPLL ↓ Q ↓ VTR ↓ Cysteine which was modified with IAEDANS (*) was only of the SH_1 type. The 22 kDa fragments were the mixture of three fragments with different COOH-terminal amino acids (↓), suggesting that three adjacent sites are cleavable in the S1–rod junction. Assuming that the loose alpha-helix begins with the last proline of 22 kDa fragments as proposed by Mclacklan & Karn [*Nature, Lond.* **299**, 226 (1982)], these sites are very close spatially.

Conformation of smooth muscle myosin: relationship to enzymatic activity, phosphorylation, proteolysis, actin binding

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Monomeric smooth muscle myosin can exist in two conformations, the folded (10S) and extended (6S) forms. Based on the correlation of conformation with various enzymatic activities it was suggested that some component of the 6S–10S transition is a determinant of enzymatic activity. At physiological ionic strength, phosphorylation of myosin converts the 10S to the 6S conformation and it is this transition, rather than a direct effect of phosphorylation, that is thought to be responsible for the activation of actin-activated ATPase activity. The 10S–6S transition involves conformation changes in several parts of the myosin molecule and one obvious objective is to identify the region of the molecule responsible for alteration of enzymatic activity. Using limited proteolysis as a probe of myosin conformation, it was shown [Ikebe & Hartshorne *Biochemistry* **24**, 2380 (1985)] that two sites of the myosin heavy chain are influenced by the 6S–10S transition, namely sites A and B. Both sites are protected by the formation of 10S myosin. Site B is located at the head–neck, or S1–S2, junction and this part of the molecule is proposed as the determinant of ATPase activity. Site A in 6S myosin is blocked also by the binding of actin. This suggests that site A could be located at, or close to, the actin-binding site, and if this is accepted then the affinity of actin for 6S and 10S myosins should differ. Binding of actin to myosin was measured in

the presence of AMPPNP, and it was shown that for 6S myosin the dissociation constant (K_3) of actin from the actin–myosin–nucleotide complex is the same for phosphorylated (1.9 mol P per myosin) and dephosphorylated myosin (1.3 and 2.4 μM respectively). However, for 10S dephosphorylated myosin, K_3 is 42 μM. Thus the conformation of myosin can influence both enzymatic activity, via site B, and actin-binding, via site A. Recently it was found that the 20 kDa light chain of gizzard myosin can be phosphorylated by myosin light chain kinase at a second site identified as threonine 18 [Ikebe *et al.*, *J. biol. Chem.* **261**, 36 (1986)]. Phosphorylation of both serine 19 and threonine 18 increases actin-activated ATPase and causes rapid superprecipitation (both compared to phosphorylation only at serine 19). Double phosphorylation also influenced myosin conformation and favoured 6S myosin. This was demonstrated by viscosity measurements, sedimentation velocity, limited proteolysis with papain and filament formation. These data add additional support to the idea that the enzymatic activity of myosin is directed by the conformation of myosin. Supported by grants from the National Institutes of Health HL 23615 and HL 20984.

Folding of smooth muscle myosin traps ADP.P_i

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In vitro, addition of ATP to smooth muscle myosin filaments under apparently physiological conditions dissociates them to folded monomers, in which the tail of the myosin molecule bends back on itself and contacts the neck region of the heads. The molecular folding is associated with an inhibition of the myosin ATPase. In an attempt to throw light on the mechanism of the filaments to folded myosin transition, and thereby on its relevance to the contractile mechanism, we followed the fate of the ATP which drives the formation of monomer.

Using an airfuge centrifugation assay, it was shown that the solubilization from filaments to molecules required the binding of two molecules of ATP per myosin. There was strong positive cooperativity in the binding of the second molecule of ATP. Parallel experiments using Penefsky [*J. biol. Chem.* **252**, 2891 (1977)] columns confirmed this stoichiometry, and indicated an apparent affinity of ~6 μM, with a Hill coefficient of 2.6, consistent with strong positive cooperativity. The bound ATP was cleaved quantitatively to ADP.P_i within the resolution time of a manual assay (2 s). Thereafter the products, ADP.P_i were held in a non-exchangeable form, such that FPLC gel filtration under appropriate conditions did not remove them. Under physiological conditions the halftime for P_i release was more than 1 h at 18° C, and independent of the presence of excess substrate in the environment. The finding that 10S myosin 'traps' ADP.$^{32}P_i$ offers the possibility to assay the proportion of 10S myosin in any population of myosin molecules. We have been unable to pellet ADP.$^{32}P_i$ with myosin filaments, and therefore feel that a 10S configuration is not attainable for filamentous myosin.

Role of tropomyosin, myosin conformation and myosin phosphorylation state in Ca-activation of vertebrate smooth muscle actomyosin

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In earlier studies we established that the presence of smooth muscle tropomyosin on action results in a two-fold amplification of the actin-activated ATPase activity of gizzard actomyosin [Sobieszek & Small, *J. molec. Biol.* **112**, 559 (1977)]. This amplification was interpreted in terms of a reduction of the average number of undissociated actin–myosin complexes via acceleration of product release [Sobieszek, *J. molec. Biol.* **157**, 275 (1982)].

We have now investigated the tropomyosin amplification effect in relation to the conformational state of myosin and to its phosphorylation level. As a model system we have been using tropomyosin- and MLCKase-free actomyosin as well as actomyosin reconstituted from purified gizzard actin and myosin. The phosphorylation levels were varied by addition of different concentrations of purified myosin light chain kinase (MLCKase) for constant incubation times.

For both model systems, the tropomyosin amplification of the actin-activated ATPase activity was proportional to the extent of myosin phosphorylation. In a related manner, the binding of tropomyosin to both actomyosins became two to three-fold weaker following phosphorylation. Thus, phosphorylation of myosin has a negative effect on the dramatic increase in the affinity of tropomyosin for actin that takes place after binding of myosin heads to actin.

Measurements of the ATPase activities as a function of pH and KCl concentration for myosin alone, for reconstituted and for native actomyosin, indicated that the actin-activation is strongly dependent on myosin conformation. Optimal actin-activated ATPase occurs at 40–80 mM KCl with a rapid decay above 80 mM KCl resulting from myosin folding. The tropomyosin amplification was exceptionally high under conditions where myosin forms table filaments in the presence of ATP (pH ~ 6.6) while for folded myosin, actin-activation was very low with a negligible tropomyosin amplification effect.

In addition to amplifying the actomyosin ATPase, tropomyosin also reduced the rate of phosphorylation of the myosin component. The inhibition was greater at the lower Ca^{2+}-concentration. As a result, Ca^{2+}-activation of phosphorylation in the presence of tropomyosin occurs with increased cooperativity.

The combined action of the various effects of tropomyosin is to sharpen the response of the system to Ca^{2+} during activation. Thus, in smooth muscle, tropomyosin appears to act as an allosteric effector. At low Ca^{2+} tropomyosin inhibits phosphorylation rate and at high Ca^{2+}, it amplifies the existing level of the ATPase activity.

Structure and function analysis of brush border myosin with monoclonal antibodies

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We have produced, purified and characterized a family of mouse and rat monoclonal antibodies against chicken brush border myosin. All these antibodies bind to the myosin rod portion, as determined by electron microscopy of rotary shadowed myosin antibody complexes and by western blots of myosin peptide maps. The crossreactivities of the antibodies against a variety of myosins from different tissues and organisms were determined by ELISA and immunofluorescence techniques.

We examined the effects of these antibodies and their Fab fragments on (1) brush border myosin filament formation and (2) brush border myosin actin activated Mg^{2+}-ATPase activity. Three antibodies which bound to different sites along the myosin rod allowed only partial filament assembly, i.e. in the presence of antibodies the myosin filaments were shorter, and showed a decoration with a characteristic periodicity of ~14 mm, and a central 17 mm bare zone. These antibodies did not appear either to decrease or increase dramatically the myosin actin-activated Mg^{2+}-ATPase activity. Two antibodies binding to the tip of the myosin tail, however, completely inhibited myosin assembly into filaments, and also reduced the myosin actin activated Mg^{2+}-ATPase activity by 70%. This effect did not appear to result from

conformational changes in the myosin head induced by antibody binding at the tip of the tail, since the digestion patterns of the myosin head in the presence or absence of these antibodies were identical. Because of their effects on myosin filament formation and actin activated Mg^{2+}-ATPase activity, these antibodies should be useful as probes to investigate the organization of brush border myosin *in vivo*, under resting and activating conditions.

The sticky patch responsible for intramolecular and intermolecular aggregation of skeletal myosin devoid of both regulatory light chains is comprised entirely in the head heavy chain portion

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Both myosin regulatory light chains (RLC) were removed from rabbit fast skeletal actomyosin followed by dissociation of the myosin from actin [Häusermann *et al., J. Musc. Res. Cell Motility* **6**, 73 (1985)]. Isolated myosin rods, light meromyosin (LMM) and head portions (S1) were obtained after digestion with chymotrypsin from myosin. All molecular species were examined hydrodynamically by gel permeation in 0.7 M KCl for determination of their Stokes radii and their maximal axial ratios (MAR). Intact myosin has a MAR of 53. Myosin devoid of RLC elutes in two positions. One corresponds to its monomeric form which is more asymmetric with a MAR of 66. The second peak contains mainly dimers as visualized by electron microscopy. In myosin devoid of RLC the head portions were significantly shorter by 18% ($P < 0.001$) and closer together than in intact myosin, but they were of the same width. The association between the two heads devoid of RLC may explain the higher MAR value. The dimers, and some trimers, all associated together in the head region, the tails stretching apart individually. Isolated rods and LMM eluted as homogeneous peaks with MAR values of 68 and 64, respectively. They never formed aggregates. Isolated heads, like myosin, devoid of RLC, eluted also in two positions, one monomeric with a MAR of 5 and the second dimeric with a MAR of 13. The formation of head-dimers was confirmed by electron microscopy. These intramolecular and intermolecular interactions are non-covalent and hydrophobic in nature and reside entirely in the head heavy chain portion. The results imply that one function of the RLC may be to prevent hydrophobic interactions between adjacent heads in the myosin filament.

V-Type down regulation of skeletal muscle myosin light chain kinase by rapid autophosphorylation at a site in the *N*-terminal half of the molecule

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Rabbit skeletal muscle myosin light chain kinase (MLCK) is isolated in an unphosphorylated form. It exhibits a kinetically biphasic intermolecular autophosphorylation in presence of Ca^{2+}-calmodulin (CaM); in its absence, no significant autophosphorylation occurs. The dependence on the CaM stoichiometry indicates that CaM·MLCK may be a better substrate for autophosphorylation than apo-MLCK. At 0.5 μM enzyme, about 1 mol per mol of phosphate is incorporated with a half time of 1–2 min; another up to 0.5 mol per mol are incorporated with a much lower rate ($t_{\frac{1}{2}} < 30$ min). Phosphoamino acid analysis indicates that all phosphate is bound to serine. Upon incorporation of more than 1 mol per mol of phosphate the apparent M_r of MLCK increases detectably on sodium dodecyl sulphate-containing gels. Comparison of [^{32}P]-autoradiograms with the stained gels indicates that a monophosphorylated species (migrating like unphosphorylated MLCK) and species with about two and three phosphates per molecule (increasing in M_r by about 1.5 and 3 kDa), respectively, are generated. Apparently, minimally three autophosphorylation sites exist. MLCK containing close to 1 mol per mol of rapidly incorporated phosphate was subjected to a steady state kinetic analysis. All kinetic parameters are unchanged except that V_{max} is significantly decreased by about 50% as compared with an unphosphorylated control. Limited tryptic digestion in presence of CaM of [^{32}P]-labelled MLCK containing only the rapidly incorporated phosphate results in two labelled peptides only slightly differing in M_r (about 15 and 16 kDa). 90% of the radioactivity is equally distributed among these fragments which cannot be separated by HPLC. *N*-terminal-gas phase sequencing yields two sequences in parallel. No alignment with the known sequence of the *C*-terminal half of MLCK is possible. Consequently, both fragments, either resulting from two internally homologous segments or from one segment with two *N*-terminal cleavage sites, must come from the *N*-terminal 'tail' of MLCK [Mayr & Heilmeyer, *Biochemistry* **22**, 4316 (1983); Edelman *et al., J. biol. Chem.* **260**, 11275 (1985)].

Rapid autophosphorylation in this segment of MLCK of no other assigned function leads to a down regulation of MLCK activity which may play a role in dampening the Ca^{2+}-induced increase of myosin phosphorylation in skeletal muscle.

Melting of myosin rod as revealed by electron microscopy I. Effects of glycerol and anions on length and stability of myosin rod

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The length of the rods of intact myosin molecules and of isolated myosin rods were determined under a variety of conditions by electron microscopy. In all experiments, except for freeze-drying, the temperature and pH were kept at 20° C and 7.0.

Glycerol was found to have a marked effect on the stability of myosin especially for air-dried molecules. In the presence of 0.3 M of volatile buffer salts, e.g. ammonium acetate, -formate, -benzoate, -bicarbonate, -carbonate, 30–50% of glycerol were needed to get average lengths of myosin rods comparable to published values and to the values of freeze-dried molecules (145–149 nm). Below 10% glycerol the average length of rods was shorter by about 10 and 20 nm in intact myosin and isolated rods, respectively. Chloride caused a significant concentration-dependent shortening of myosin rods due to destabilization of the α-helical double coiled rod structure. Similar or higher concentrations of volatile salts, not containing chloride as an anion, had no shortening effect.

Thus, subtle influences depending on the composition of the dispersion solution on the final appearance and lengths of myosin rods have to be considered, before studying temperature- and pH-dependent changes of myosin rod structure.

Melting of myosin rod as revealed by electron microscopy II. Effects of temperature and pH on length and stability of myosin rod and its fragments

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Effects of temperature and pH on intact rabbit and chicken myosin,

isolated myosin rods, rabbit subfragment-2 (61 kDa, 53 kDa, and 34 kDa) and chicken LMM fragments were tested to induce a phase transition from α-helix to coil conformation, within the hinge region. The influence of temperature and pH were studied directly with length determination by electron microscopy. An increase of temperature to 50° C yielded a shortening of 16 nm, 8–9 nm and 7–11 nm for intact myosin, isolated rods and S2 fragments, respectively. The length of the 34 kDa short S2 and LMM fragments were unchanged. An increase of pH from neutral to pH 8.0 yielded values that were somewhat smaller, e.g. 12 nm, 6 nm and 6–8 nm for intact myosin, isolated rods and S2 fragments, respectively, whereas the 34 kDa short S2 and LMM fragments were also unaffected. Thus, melting and subsequent shortening is confined to the region between LMM and short S2 segment, that is the hinge region. Alteration of temperature had a stronger shortening effect than alteration of pH, and shortening of long S2 was more pronounced under physiological salt conditions as compared with high (0.3 M) salt. The shortening of rods in intact myosin amounted to twice the value observed with isolated rods. The amount of contraction was somewhat smaller in rods than in the 61 kDa and 53 kDa S2 fragments.

ACTIN, ACTIN-ASSOCIATED PROTEINS AND ACTIN GENES

Ca^{2+} regulation of the smooth muscle actin–myosin interaction mediated by cytocalbin (cytoskeleton-related calmodulin-binding protein)

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It has been generally accepted that calmodulin is an intracellular Ca^{2+}-mediator in many biological phenomena. During the search for the major acceptors of calmodulin, we have isolated or identified ten species of calmodulin-binding proteins. These proteins are found to be interacted with cytoskeletal elements and regulated their functions. Caldesmon, calspectin (fodrin, non-erythroid spectrin), MAP2, tau factor, 135 kDa protein (myosin light chain kinase) and 340 kDa, 129 kDa, 105 kDa and 94 kDa proteins bind to actin filaments as a target cytoskeleton. MAP2 and tau factor are also calmodulin-binding and tubulin-binding proteins. Therefore, we propose that these proteins are collectively termed 'Cytocalbin', derived from cytoskeleton-related calmodulin-binding proteins. The binding of cytocalbin to calmodulin or target cytoskeletal elements alters depending upon the concentration of Ca^{2+} ('flip-flop' binding). At lower Ca^{2+} concentration (< 1 μM), cytocalbins tend to bind to cytoskeletal elements. Whereas at higher Ca^{2+} concentration (higher than 1 μM of free Ca^{2+}), there is a formation of calmodulin–cytocalbin complexes, which then release from cytoskeletal elements.

Among these cytocalbins, caldesmon and the 135 kDa protein (MLCK) are found to be most important regulators of the actin–myosin interaction in smooth and nonmuscle tissues. The actin–myosin interaction is dually controlled by Ca^{2+} and calmodulin via the caldesmon-dependent actin-linked and 135 kDa protein-dependent myosin-linked systems.

Caldesmon phosphorylation blocks its inhibitory effect on the actomyosin Mg^{2+}-ATPase of smooth muscle

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Caldesmon, a major actin- and calmodulin-binding protein of smooth muscle, has been implicated in the regulation of actin–myosin interaction and therefore the contractile state of smooth muscle. For example, isolated caldesmon inhibits the actin-activated myosin Mg^{2+}-ATPase in a system reconstituted from purified contractile and regulatory proteins [Ngai & Walsh, *J. biol. Chem.* **259**, 13656–9 (1984)]. Isolated thin filaments contain actin, tropomyosin and caldesmon and when reconstituted with thiophosphorylated smooth muscle myosin, confer Ca^{2+}-dependence on the Mg^{2+}-ATPase [Marston & Lehman, *Biochem. J.* **231**, 517–22 (1985)]. Caldesmon purified by calmodulin–Sepharose affinity chromatography contains endogenous Ca^{2+}, calmodulin-dependent caldesmon kinase activity. Phosphate incorporation is primarily onto serine residues (83.2%) with a small amount (16.6%) of threonine and no tyrosine phosphorylation. Caldesmon can be completely dephosphorylated by a partially purified preparation of myosin phosphatase of smooth muscle. Unphosphorylated caldesmon inhibits the actin-activated Mg^{2+}-ATPase of smooth muscle myosin whereas phosphorylated caldesmon does not. Several lines of evidence suggest that caldesmon itself is responsible for this kinase activity. (1) Caldesmon kinase activity could be separated into two peaks by ion-exchange chromatography which corresponded to caldesmon and a 93 kDa proteolytic fragment of caldesmon. (2) Caldesmon and caldesmon kinase activity were bound to an affinity column of anti-caldesmon coupled to AffiGel-15. (3) Caldesmon was bound tightly to an AffiGel Blue affinity column. (4) Caldesmon was photoaffinity labelled with 8-azido-ATP-α^{32}P; labelling was inhibited by the non-hydrolysable ATP analogue, AMPPNP. A model is therefore developed to explain the possible physiological role of caldesmon in the regulation of smooth muscle actin–myosin interaction and its own regulation by reversible phosphorylation.

Caldesmon is an elongated actin-gelating protein present in the actomyosin domains

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Caldesmon can be rapidly purified from either avian or mammalian smooth muscles using a KI-extract of myosin-depleted myofibrils. Analytical gel filtration indicates a Stokes radius of ~ 90 nm at 60 mM KCl as previously noted (Bretscher, 1984). With increasing salt concentration, up to physiological levels, the molecule showed a strong tendency towards dimerization that was insensitive to calmodulin and Ca^{2+}. In the electron microscope caldesmon exhibited an elongated highly flexible morphology, the length distribution showing two maxima corresponding respectively to the monomer and dimer forms. The dimer length was similar to that of filamin dimer, consistent also with the identical mobility of both molecules on gel filtration. The gelling or bundling activities of caldesmon are currently controversial. In line with earlier studies (Sobue, 1985) we found that the method of purification and storage of caldesmon affects its interaction with F-actin. Nonetheless caldesmon had a dramatic effect on actin viscosity in both high and low shear assays. The effect decreased with increasing salt concentration. At 120 mM salt, caldesmon at a 1:90 molar ratio (M_r for caldesmon 300 kDa) increased the apparent viscosity of F-actin ~ 2.5 fold. Filamin produced a similar effect at a six-fold less molar ratio to actin (M_r for filamin 500 kDa).

We suggest that caldesmon and filamin belong to a group of actin gelation proteins that could contribute to tension maintenance in smooth muscle.

Studies on structure–function relationship of caldesmon by its limited proteolysis

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Caldesmon is a component of the smooth muscle thin filament which may serve as a regulator of actin–myosin interaction. Its binding to F-actin results in the inhibition of actomyosin ATPase that in the presence of Ca^{2+} can be released by calmodulin.

In present studies the structure–function relationship of caldesmon has been probed by limited proteolysis to locate its calmodulin- and actin-binding sites as well as those sites responsible for the inhibition of actomyosin ATPase activity in the presence of tropomyosin. Digestion of chicken gizzard caldesmon by chymotrypsin (6 min, at an enzyme to substrate weight ratio of 1:1000) yielded 110 kDa, 80 kDa, 54 kDa, 40 kDa, 29 kDa, 23 kDa, 18 kDa and occasionally 16 kDa polypeptides. The 110 kDa, 80 kDa, 54 kDa and 29 kDa polypeptides did not bind to calmodulin and had no ability to inhibit actomyosin ATPase. 40 kDa, 23 kDa and 18 kDa polypeptides were retarded on a calmodulin-affinity column and, in pelleting experiments, bound to F-actin. They also had the ability to inhibit the actomyosin ATPase. From the sequence of generation of the polypeptides during caldesmon degradation it could be deduced that the 23 kDa and 18 kDa polypeptides were derived from the 40 kDa fragment, and the 54 kDa from the 110 kDa fragment.

The pattern of digestion was very little affected by either calmodulin binding, F-actin binding or the presence of tropomyosin. F-actin–tropomyosin complex altered the digestion pattern by the generation of an additional 120 kDa polypeptide.

The results show that the sites responsible for calmodulin binding, actin-binding and for the inhibition of actomyosin ATPase are located in the same fragment(s) of the caldesmon molecule, possibly at its terminal part. That it might be the carboxy-terminus is suggested by a decrease of the ability of caldesmon to inhibit actomyosin ATPase upon its treatment with carboxypeptidase A.

Actin–caldesmon interaction measured by electron paramagnetic resonance

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In previous studies we have discussed some aspects of interaction of caldesmon with G- and F-actin [Gałazkiewicz *et al.*, *FEBS Lett.* **184**, 144–9 (1985); Dabrowska *et al.*, *Biochim. biophys. Acta* **842**, 70–5 (1985)]. In this report the interactions of these two actin forms with caldesmon have been further characterized by measurements of electron paramagnetic resonance (EPR) using a spin label attached to the thiol group of actin (Cys 374).

EPR spectral changes accompanying polymerization of G-actin by caldesmon indicated a biphasic, cooperative character of this process. Saturation transfer (ST) EPR measurements showed that the average mobility of spin labels in the caldesmon-polymerized actin (at a 1:5 molar ratio of caldesmon to actin monomer) was the same as that observed in F-actin polymerized by KCl. Caldesmon increased the immobilization of spin labels in F-actin at least by a factor of two ($\tau_2 > 200\,\mu s$). The orientation dependence of ST–EPR spectra with respect to the actin filament axis showed that caldesmon increased the degree of order of the spin label. This could be attributed to a reduced flexibility of actin filaments and/or to a decreased angle of tilt of the molecular z-axis in spin labels induced by caldesmon in the internal region of the actin monomers holding the label.

ST–EPR spectra indicated that the rotational mobility of the actin-bound label was neither affected by adding caldesmon to a preformed F-actin–HMM complex nor by adding HMM to a preformed F-actin–caldesmon complex. Beside that, caldesmon evoked an opposite alteration in the hyperfine splitting of the probe molecules in the F-actin–HMM complex to that caused by HMM in the F-actin–caldesmon complex (demonstrated by increasing and decreasing, respectively, the $2A'_{ZZ}$ parameter). These observations provide evidence that the binding sites of caldesmon and HMM on actin filaments are different.

The structural basis for caldesmon regulation of vascular smooth muscle contraction

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Native vascular smooth muscle thin filaments show Ca^{2+}-dependent regulation of myosin Mg^{2+}-ATPase activity and are composed mainly of actin, tropomyosin and caldesmon in molar ratios of 1:1/7:1/28. A fully functional Ca^{2+}-dependent thin filament can be reconstituted from these proteins with the addition of brain calmodulin. Inhibition of actin–tropomyosin activation of myosin is relieved when caldesmon binds to a Ca^{2+}-calmodulin complex. The stoichiometry of high affinity binding, inhibition of Mg^{2+}-ATPase activity and the native occurrence of caldesmon in thin filaments all suggest that caldesmon interacts with actin at a molar ratio of 1:28. Such stoichiometry could be accounted for either by filament (e.g. bundling) or intrafilament (e.g. comparable to the skeletal muscle troponin–tropomyosin system) structural mechanisms.

A recent study [Moody *et al.*, *FEBS. Lett.* **191**, 107–12 (1985)] showed that whereas binding of caldesmon to actin and caldesmon-inhibition of myosin Mg^{2+}-ATPase were closely correlated, levels of caldesmon-induced actin bundling were too low to account for the inhibitory mechanism. Therefore possible interfilament mechanisms are being studied. Two types of model could explain the observed stoichiometry: if caldesmon is an elongated molecule like tropomyosin it could extend along the actin filament, or if globular, it could bind at the unique point along the filament where the ends of four tropomyosin molecules meet.

Isolated thin filaments do not show structural components that can be identified as caldesmon. Under certain preparative conditions, thin filaments can form bundles, yet these show no signs of the periodicity expected should caldesmon occur at discrete intervals along the actin filament. Thin filaments aggregated by anti-caldesmon antibody are also characterized by a lack of periodicity (cf. periodicity of skeletal muscle thin filaments aggregated by anti-troponin antibody). This evidence indicates that caldesmon may be an elongated molecule. In order to locate caldesmon more precisely in reconstituted thin filaments a number of specific markers have been investigated. *N*-hydroxysuccinamide biotin was covalently linked to caldesmon. Biotinylated caldesmon retains actin-binding activity and streptavidin binding to an actin–caldesmon–biotin preparation has been shown. We plan to visualize the biotin–caldesmon using electron microscopy via the attachment of bulky markers. Gold–streptavidin, avidin–ferritin, and streptavidin oligomers have been tested. Experiments to determine the optimal method and conditions for caldesmon visualization in reconstituted and negatively stained thin filament preparations are in progress.

The mechanism of Ca^{2+}-dependent control of vascular smooth muscle thin filaments by caldesmon

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Native thin filaments extracted from smooth muscle mainly consist

of actin, tropomyosin and caldesmon in molar ratios 1:1/7:1/28. They fully activate skeletal or smooth (thiophosphorylated) myosin at 10^{-5} M Ca^{2+} but activate only slightly in the absence of Ca^{2+} (Ca^{2+} sensitivity 4–30 fold). Ca^{2+} switches on thin filaments rapidly (within 5 s) and this is not associated with any phosphorylation of caldesmon, therefore Ca^{2+} does not control the thin filament via kinase activation.

Pure caldesmon binds tightly to actin and actin tropomyosin (binding constant around 10^7 M^{-1}); there is also additional weak binding ($K = 2 \times 10^5$ M^{-1}). The tight binding sites saturate at 0.039 ± 0.11 ($n = 26$) caldesmon per actin both in the presence and absence of tropomyosin. Tight binding affinity decreases with increasing [KCl] (3.5 fold between 60 and 120 mM KCl) and temperature ($Q_{10} = 1.35 \pm 1$ ($n = 5$)]. It is not changed by substituting skeletal for smooth actin or tropomyosin. Tight binding of caldesmon to actin–tropomyosin is always 3.7 ± 1.1 ($n = 4$) times the affinity for actin alone. Caldesmon also binds to tropomyosin with an affinity of at least 10^5 M^{-1}.

Caldesmon inhibits actin tropomyosin activation of smooth or skeletal myosin MgATPase activity but has little effect on actin alone. MgATPase activity is inhibited to a basal level by saturating concentrations of caldesmon. The basal level is $26 \pm 12\%$ ($n = 29$) of the actin-activated MgATPase activity, measured over a fifty-fold range of activity, and is independent of the initial uninhibited actin–tropomyosin activation. Under all conditions, inhibition to the basal level is linearly proportional to the binding to the tight sites [full inhibition at 0.034 ± 0.005 ($n = 26$) caldesmon–actin].

Caldesmon binds to IAEDANS-labelled Ca^{2+}–calmodulin inducing a 10–40% increase in dansyl fluorescence. The binding constant is $0.8–1.0 \times 10^6$ M^{-1} at 60 mM KCl, 25°C, and does not change greatly with increases of [KCl] and temperature. When Ca^{2+}–calmodulin is added to actin–tropomyosin–caldesmon the caldesmon inhibition is released. The amount of Ca^{2+}–calmodulin required is highly variable; it depends on [KCl], temperature and source of proteins. In general Ca^{2+}–calmodulin is more potent at releasing inhibition at higher temperatures and [KCl]. At 120 mM KCl and 37°C, 50% release of inhibition requires only 2 μM Ca^{2+}–calmodulin (3·5 μM caldesmon, 10 μM actin–tropomyosin), but at 60 mM KCl and 25°C it requires > 60 μM. Concentrations of Ca^{2+}–calmodulin sufficient to fully reverse inhibition reduce the affinity of caldesmon for tight actin–tropomyosin sites by only 2–8 fold, consequently a complex actin–tropomyosin–caldesmon–Ca^{2+}–calmodulin exists when inhibition has been released. The relationship between release of inhibition by Ca^{2+}–calmodulin and the formation of the various complexes between Ca^{2+}–calmodulin and caldesmon is complicated and may be influenced by factors not yet studied.

Smooth muscle caldesmon in an extended flexible monomeric protein in solution that can readily undergo reversible sulphydryl crosslinking

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The solution properties of smooth muscle caldesmon, purified by a minor modification of our earlier procedure [*J. biol. Chem.* **259**, 12873–80), were examined. Under reducing conditions, caldesmon has a sedimentation coefficient of 2.54 S and an apparent Stokes radius of abut 91Å as determined by gel permeation chromatography. Since caldesmon has an apparent polypeptide molecular weight of about 140 000, these data suggest that it exists in solution as an extended monomeric protein. Low angle rotary shadow images of caldesmon confirmed its extended morphology and indicated that it is a highly flexible molecule.

Caldesmon is easily oxidized in buffers containing either low concentrations or no reducing agent to form intermolecular and intramolecular disulphide bonds. When these species are anlaysed by non-reducing SDS–PAGE, a series of distinct higher molecular weight species are observed. Chemical crosslinking experiments performed in the presence of excess reducing agent failed to produce any higher molecular weight species. Gel filtration analysis of the reduced and disulphide crosslinked species indicated that the reduced protein exists as a monomer in solution. Amino acid analysis indicated that caldesmon contains few (≤ 4) cysteine residues.

The effect of caldesmon disulphide links on the interaction with F-actin was investigated. Oxidized caldesmon formed massive bundles with F-actin, with bundle formation being abolished by the presence of high levels of DTT. Since such a vast molar excess of DTT is required to reduce the caldesmon disulphide links, the disulphide crosslinked species, and therefore the F-actin bundling activity, may be of some physiological significance.

Effect of caldesmon on the acto–HMM ATPase and binding

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Caldesmon was purified to homogeneity from both chicken gizzard and bovine aortic smooth muscles. Caldesmon purified from bovine aorta was slightly larger ($M_r = 145\,100$) than caldesmon purified from chicken gizzards when the two were compared electrophoretically. Caldesmon could be chemically crosslinked to form a major oligomeric species ($M_r = 280\,000$), suggesting that caldesmon can form dimers. Caldesmon bound tightly to actin saturating at a molar ratio of one caldesmon dimer per 13.3 actin monomers. Ca^{2+}–calmodulin appeared to reduce the affinity of caldesmon for actin. Caldesmon was also a potent inhibitor of acto–HMM ATPase activity producing a maximal effect at a ratio of one caldesmon dimer per 20 actin monomers. This effect was also antagonized by Ca^{2+}–calmodulin. While caldesmon inhibited acto–HMM ATPase activity, it greatly enhanced binding of both unphosphorylated and phosphorylated HMM to actin in the presence of MgATP, reducing the K_d for binding by a factor of 40 for each form of HMM. Although we did identify a Ca^{2+}–calmodulin-stimulated 'caldesmon kinase' activity in caldesmon preparations purified under non-denaturing conditions, we observed no effect of phosphorylation (2 mol P0/mol caldesmon monomer) on the capacity to inhibit acto–HMM ATPase activity. Our results suggest that caldesmon could serve some role in smooth muscle function by enhancing crossbridge affinity while inhibiting actomyosin ATPase activity.

Quantitative determination of the striated muscle isoactin proteins: comparison with corresponding mRNA levels reveals absence of translational control. The phalloidin binding-site of actin

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Skeletal muscle and cardiac actin differ only by a permutation of Glu and Asp at positions 2 and 3 by two additional neutral amino acid exchanges. These minor differences do not allow separation of these isoforms by current techniques including ion exchange chromatography or two-dimensional polyacrylamide gel electrophoresis. In order to evaluate the expression of the two isoactins at the protein level, we have developed a procedure to distinguish and to quantify these isoforms. The technique makes use of the observed Glu-Asp permutation. Thus, a different set of tripeptides

can be generated after partial acid hydrolysis of the isolated NH_2-terminal tetrapeptides: Asp-Glu-Asp and Glu-Asp-Glu from skeletal muscle actin and predominantly Asp-Glu-Glu from cardiac actin. Full hydrolysis of the *o*-phthaldialdehyde NH_2-terminally blocked tripeptides yields a Glu:Asp ratio which is a hyperbolic function of the percentage of cardiac actin. Using this method we demonstrate that both striated muscle isoactins are simultaneously expressed in a ratio similar to that previously reported for the corresponding mRNAs, excluding the existence of a significant control at the level of translation. Thus adult cardiac mucle contains up to 20% skeletal muscle actin, while skeletal muscle tissue is essentially free of cardiac actin (less than 5%). Fusing myotubes contain equal amounts of the two striated muscle isoforms in addition to beta and gamma nonmuscle actin. In early embryonic muscle (12 day old chicken embryo muscle) we found predominantly the cardiac type, but later in development, (18 day old embryo) the skeletal actin form becomes the dominant isotype.

In a separate study we have determined the phalloidin-binding site of F-actin. Therefore we have used an alkylating iodoacetyl (I) and a carbene generating photoactivated diazirine derivative (II) of phalloidin. Actin was affinity labelled at positions 119 and 355 by I and predominantly at position 117 by II. The regions around these binding sites are conserved among all actins sequenced so far, except for the actins from soybean [Shah *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **79**, 1022–6 (1982)]. The latter actins may thus be expected to exhibit altered phalloidin-binding activities. Position 355 is the neighbouring residue of Trp 356 which is probably a part of the ATP-binding site, located at the interdomain cleft [Hegyi *et al.*, *J. Musc. Res. Cell Motility* **5**, 74 (1985)]. It is therefore suggested that phalloidin also binds in the vicinity of the interdomain cleft, probably after translocation of the nucleotide.

FILAMENT ORGANIZATION

Three-dimensional organization of contractile cytoskeletal components in smooth muscle cells: present state and prospects for the future.

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The contractile system of smooth muscles presents unique problems. The apparently non-planar organization makes it difficult, if not impossible, to visualize all interacting components of a contractile unit in any given section plane. Also, it is uncertain whether certain components, such as dense bodies, are part of the contractile system, the cytoskeleton, or both. Enzymatically isolated smooth muscle cells, introduced by Bagby *et al.*, [*Nature, Lond.* **234**, 351 (1971)], have been essential to many three dimensional imaging techniques because they allow viewing whole cells which reasonably approximate the *in vivo* condition. Several laboratories, including our own and those of Fay and Small, have made extensive structural studies of living cells during contraction and immunofluorescent studies of isolated cells from several smooth muscles which provide evidence for fibrils being the contractile unit, and for some type of segregation of different proteins along the fibrils. Many studies also reported an apparently helical arrangement of fibrils. Speculative models have been proposed to explain these findings, but such models have their limits. [For a review of early work and the resultant models see: Bagby, in *Biochemistry of Smooth Muscle*, Vol. 1, (edited by STEPHENS, N.), Boca Raton: CRC Press (1983)].

the 'New Wave' of structural studies emphasizes three-dimensional analysis and reconstructions. Currently, the most sophisticated studies (e.g. Fay *et al.*, *J. Cell Biol.*, **96**, 783 (1983)] use serial optical sections (SOS) of immunofluorescently stained cells for CAR (computer-aided reconstruction). We have chosen an optical method, holographic reconstruction for our SOS, using a new technique called 'multiplexing' which was performed by Dover & Wright of King's College, London [for details, see Bagby, *Int. Rev. Cytol.*, in press (1986)]. Future improvements in technique should make it competitive with CAR. Another technique we have applied to smooth muscle is axial rotation where we view critical-point dried cells with 200 kV transmission electron microscopy after aligning the cell's axis with the tilt axis of the specimen holder, allowing viewing through 60° of rotation [Frierson & Bagby, *J. Cell Biol.* **101**, 167a (1985)]. It is clear that structural studies of isolated smooth muscle cells have an exciting future.

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Contractile and cytoskeletal domains in vertebrate smooth muscle

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Recent immunocytochemical analysis has revealed the existence of two distinct fibrillar domains in smooth muscle cell: contractile domains containing actin and myosin and 'cytoskeletal' domains containing intermediate filaments, dense bodies and actin. In the actomyosin domains no periodic distribution of actin or myosin label could be detected in the longitudinal direction in the light microscope, indicating a continuous distribution of actin and myosin filaments within these fibrils. For myosin, this feature was particularly evident in isolated cells whose structure was loosened by partial removal of actin using an actin-fragmenting protein purified from smooth muscle.

Two actin-binding proteins that occur in considerable amounts in smooth muscle, caldesmon and filamin, are spatially segregated from each other in the two separate domains: caldesmon in the actomyosin domains and filamin in the cytoskeletal domains. Both proteins show an extended and flexible molecular morphology, are dimeric and are capable of crosslinking actin filaments into gelled arrays. It is proposed that such crosslinking activity contributes to tension maintenance, or tone, in vertebrate smooth muscle.

Spatial and temporal relationships between vinculin and talin in the developing chicken gizzard smooth muscle

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The spatiotemporal relationships between vinculin and talin in developing chicken gizzard smooth muscle were studied. Immunofluorescent and immunoelectron microscopic labelling of vinculin and talin indicated that both proteins are associated with the membrane-bound dense plaques of smooth muscle cells. However, immunocytochemical localization in embryonic chicken gizzard, showed that both proteins are mostly cytoplasmic during the first two weeks of embryo development. Between days 16 and 18 talin becomes associated with the plasma membrane, concomitantly with the appearance of distinct, myofilament-bound, dense

plaques. Vinculin, on the other hand appears in the plaque only one day after hatching. Comparative statistical analysis of the distances measured between immunogold particles and the plasma membrane, showed that the peak of vinculin labelling was somewhat closer to the membrane than that of talin. Therefore it is proposed that the interaction of talin with the membrane does not depend on the presence of vinculin and that the binding of vinculin to the membrane is not strictly directed by talin. It is suggested that other structural proteins may be involved in the independent incorporation of both proteins into the dense plaque structure.

Vinculin and meta-vinculin from smooth muscle: bulk purification and a reevaluation of their interaction with actin filaments

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Vinculin and meta-vinculin are two membrane-associated proteins in smooth muscle, thought to be involved in actin-membrane attachment. However the relative roles of these proteins, as well as their mode of interaction with actin is currently unclear.

We have now developed new procedures for the purification of both of these proteins in high yield from avian and mammalian smooth muscle utilizing a KI-extract of myosin-depleted myofibrils. This has allowed a reevaluation and comparison of the *in vitro* properties of these two molecules.

In accordance with earlier studies the extinction coefficient of vinculin was 4.6; the corresponding $E^{1\%}_{280}$ for meta-vinculin was 3.6. As for vinculin, meta-vinculin also showed at least four isoelectric variants but in a more basic position than for vinculin.

By rotary shadowing and negative staining both molecules showed a globular morphology with the suggestion of a tail on shadowed vinculin. Evidence has previously been presented that indicates the presence in purified vinculin preparations of a contaminant that affects the low shear viscosity of F-actin. We found that both vinculin and meta-vinculin purified by this new procedure affected the low shear viscosity of F-actin in a Ca^{2+}-insensitive manner, causing a dose-dependent decrease (reduction of F-actin viscosity by 50% for molar ratios of vinculin and meta-vinculin of 1:30 actin and 1:50 actin respectively). Since vinculin and meta-vinculin showed very different elution characteristics on ion exchange media, these effects are considered unlikely to arise from the presence of an associated contaminant. In line with earlier work [Evans *et al., J. biol. Chem.* **259**, 3916–24 (1984)], evidence for an active low molecular weight contaminant (~35 kDa) in crude vinculin preparations was found. This contaminant, which had a similar but much more potent effect on actin viscosity, could, however, be separated from vinculin by refined purification procedures. No sign of this contaminant was found in crude meta-vinculin preparations.

From these findings it is concluded that vinculin and meta-vinculin both bind to actin and promote actin–actin interactions *in vitro*. The precise functions and relative roles of these two proteins remain to be established.

Plectin and other intermediate filament binding proteins of muscle

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Plectin, an acidic phosphoprotein of $M_r = 300\,000$, was originally identified as a major component of Triton X-100/high salt-resistant cell residues (intermediate filaments) from cultured cells. In fibroblasts (CHO cells), where plectin amounts up to 1% of the total cellular protein, the distribution of intermediate filament-bound to soluble plectin is about 3:7. Plectin isolated from a variety of cell lines including rat glioma C6, human HeLa and baby hamster BHK-21 cells is chemically and immunologically hardly distinguishable indicating conserved evolution. Purified plectin samples assume different shapes in solution, such as thin rods and rods with globular structures at one or both ends. These plectin oligomers show a strong tendency for aggregation into more complex structures consisting of globular centre pieces with numerous spike-like extensions. The binding of plectin structures to vimentin filaments reconstituted *in vitro* from purified protein preparations was demonstrated using a variety of techniques including ultracentrifugation and electron microscopy of negatively stained, rotary shadowed and immunogold-decorated specimens. Plectin's association with vimentin filaments *in situ* was demonstrated by immunoelectron microscopy of whole mount cytoskeletons and immunofluorescence microscopy of fibroblast cells. Plectin is ubiquitously distributed in tissues of man and rodents, as shown by immunofluorescence microscopy and immunoelectron microscopy of frozen tissue sections. Conspicuous locations are desmosomal structures of several cell types and various locations in muscle cells, such as Z-discs in striated muscle (skeletal and cardiac) and intercalated disc regions (cardiac muscle). In cross-sections of smooth muscle (urinary bladder) plectin was enriched at regularly spaced dots along the cell periphery. In grazing or longitudinal sections these dots appeared as long wavy bands running from one end of the cell to the other. Probably these zones represented intracellular attachment sites of myofilaments. The occurrence in muscle, particularly smooth muscle, of several other intermediate filament binding proteins reported to date will be discussed and the relationship of high M_r intermediate filament binding proteins such as synemin, paranemin and IFAP-300, to plectin will be clarified.

Study of myosin from gravid and nongravid human, monkey and rat uteri

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Electrophoretic analysis of uterine myosin in nondissociating conditions shows that a single band of similar mobility is obtained in the three animal species studied (human, monkey and rat) and whatever the physiological states. Migration of uterine myosin in dissociating conditions (5% acrylamide) leads to the appearance of two very close bands (205 and 201 kDa) for the primate uterus myosin heavy chains, while the rat bears only one band of 205 kDa. No modification during pregnancy in the heavy chains bands was observed. Migration of uterine myosin in focusing and in two-dimensional gel electrophoresis shows that its 17 kDa light chain exists in two forms, different in their isoelectric points, and which are both found in all the uteri studied and in the three animal species. In the two primates, the more basic form of LC17 predominates in the nongravid uteri and at the beginning of pregnancy. There is a shift during pregnancy, the more acidic form becoming predominant at the end. In the rat uteri, where the more acidic form is always predominant, there is no evolution during the short (22 days) gestation.

From these electrophoretic data and a previous peptide analysis, it appears that, in primates, myosin modification occurs during pregnancy and is localized at the LC17 level. Functional repercussions of this phenomenon remain to be elucidated.

Two properties of uterine myosin were investigated: its ATPase activity and its *in vitro* filament behaviour. Purified human uterus myosin ATPase activity was found to be similar for both pregnant and nonpregnant uteri. When filament formation was studied in

various conditions (pH, ATP, LC20 phosphorylation), it appeared that pregnant uterus myosin consistently gives longer, thicker or more numerous filaments than non pregnant uterus myosin in strictly similar conditions. The physiological meaning of such differences is evidently to be investigated.

'Isoforms of myosin' in uterine smooth muscle comprise both filamin and myosin

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The evidence for isoforms of myosin in visceral and vascular smooth muscle is based largely on the appearance of two (or more) bands on pyrophosphate gel electrophoresis of tissue extracts run under non-denaturating conditions. The difference in mobility of the 'isoforms' has been ascribed to the presence of two heavy chains of myosin of molecular weight 200 and 230 kDa in the bands [Beckers-Bleukx & Maréchal, *Eur. J. Biochem.* **152**, 207–11]. We submit evidence that the bands contain a myosin heavy chain (200 kDa) and also filamin (approximately 240 kDa).

Protein was extracted from the myometrium of pregnant and nonpregnant rabbits and rats using pyrophosphate or ATP salt solution. After electrophoresis under non-denaturating conditions two distinct bands of approximately equal proportions were present which migrated at about 1.5 times the rate of ventricular myosin. Markers of myosin and filamin from chicken gizzard run concurrently, migrated with the fast and slow bands respectively. On mixing filamin with the rat or rabbit myometrium extracts, it co-migrated with the slower migrating band. On elution of the protein from both bands from the pyrophosphate gels, re-electrophoresis on denaturing 6% SDS gels revealed two well separated proteins which corresponded to the myosin heavy chain (mol. wt 200 kDa) and filamin markers. Filamin was the predominant constituent of the bands from rabbit uterus.

We conclude that the two bands seen on native gels of smooth muscle myosin extracts comprise two proteins, myosin and filamin, and as a consequence suggest that these bands are not necessarily manifestations of myosin isoforms.

Filamin was a gift from P. Ngai and M. Walsh, Department of Medical Biochemistry, University of Calgary, Alberta, Canada.

The Z-line structure, intermediate filaments and the regulation of protein biosynthesis in skeletal and cardiac muscle: a hypothesis

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Ultrastructural analysis of mammalian skeletal muscle has revealed that the Z-line of slowly contracting fibres is significantly thicker than in rapidly contracting fibres. We have recently shown that the cellular content of the intermediate filament protein desmin, which is located at the periphery of the Z-line, is two-fold higher in slow fibres compared with fast fibres. The functional significance of this difference in Z-line thickness and desmin content with respect to contractile properties however, remains to be elucidated. Our hypothesis is that the intermediate filament system is the major site of protein synthesis in skeletal and cardiac muscles and is based on the following observations. (1) Autoradiographic studies of newly synthesized proteins in skeletal and cardiac muscles indicate that an unexpectedly high proportion of radioactivity is associated with the Z-line despite the fact that the two principle Z-line proteins, α-actinin and desmin, are minor products of *in vitro* translations containing mRNA or polyribosomes. (2) Fibres with thick Z-lines and higher relative desmin content also have significantly higher concentrations of polyribosomes, mRNA and total RNA. (3) During the transformation of fast fibres to slow fibres the increase in Z-line thickness and RNA content occurs very early in the process and these changes are complete prior to detectable changes in myosin phenotype. (4) During hypertrophy of cardiac muscle the density of the intermediate filaments increases significantly and these are found associated with polyribosomes. (5) Experiments with other tissues have demonstrated that initiation factors, polyribosomes, mRNA and CAP binding proteins are all associated with the cytoskeletal framework. (6) Certain types of muscular dystrophies are characterized by a lack of Z-line integrity and a failure to accumulate protein. Finally, it is suggested that further studies on the intermediate filament system of skeletal muscle include consideration of fibre type and the physiological implications of cytoskeletal structures.

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Rigor crossbridges and other structural aspects of smooth muscle contractility

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Earlier ultrastructural studies of smooth muscle are consistent with a sliding filament mechanism of contraction [Somlyo, in *The Handbook of Physiology: The Cardiovascular System*, Vol. 2, *Vascular Smooth Muscle* (edited by BOHR, D. F. *et al.*) pp. 33–67. Bethesda: American Physiological Society. (1980)]. Actin filaments emerge from membrane-bound and cytoplasmic dense bodies and, when decorated with S1 subfragments of myosin, show a polarity similar to the Z-lines of skeletal muscle (Bond & Somlyo, *J. Cell Biol.* **95**, 403–13 (1982)]. Myosin filaments are stable structures *in situ* and are present in both relaxed and contracted smooth muscle [Somlyo *et al.*, *Nature, Lond.* **294**, 567–70 (1981)]. In the present study, we determined the presence and arrangement of rigor crossbridges in guinea pig and rabbit portal vein. Longitudinal strips approximately 200 μm wide were permeabilized with saponin or with freeze-glycerination followed by incubation in 0 calcium rigor (ATP-free) or in ATPγS containing, followed by calcium rigor solution. Muscles were fixed in glutaraldehyde and 0.1–0.2% tannic acid, followed by osmium and uranyl acetate. Clearly visible chevron patterns of crossbridges were evident in longitudinal views of alternating actin and myosin filaments, in muscles fixed in rigor. Preliminary measurements of stereo views of the most regular regions showed that the number of crossbridges attached to actin (0.5 μm) thick filament was 18 ± 1.7 S.D. ($n = 22$) in low rigor and 18 ± 3.6 S.D. ($n = 4$) in calcium rigor following thiophosphorylation. Our results provide structural support of the existence of a rigor state due to crossbridge attachment in smooth muscle.

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INTACT AND SKINNED MUSCLE

Structure and function of the 20 kDa myosin light chain in smooth muscle

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The 20 kDa myosin light chain of ^{32}P-labelled rat uteri or porcine carotid arteries exhibited four spots on two-dimensional gel electrophoretograms; the corresponding autoradiograms revealed that three spots were radioactive. Complete dephosphorylation of the light chain by 2 h treatment of muscle homogenate with 1 mM EGTA, or three-day storage at 4° C of intact muscle in physiological salt solution without glucose, showed three and two spots on the electrophoretograms for the uterine and arterial light chains, respectively. The following arguments suggest that the multiple forms of smooth muscle light chain are isoforms: (1) the two forms of dephosphorylated arterial light chain had similar tryptic peptide maps; (2) the four spots of arterial light chain coelectrophoresed with the four spots of uterine light chain; (3) preelectrophoresis of the gels in the presence of thioglycolate, as described by Haeberle *et al.* [*Biochim. biophys. Acta* **790**, 78 (1984)], had no effect on the four spots of either arterial or uterine light chain; thus the four spots are not caused by charge modification. The same conclusion was reached from results of two-dimensional isoelectric focusing (Gagelmann *et al., Biochem. Biophys. Res. Commun.* **120**, 933 (1984)] which did not reveal any off-diagonal spots of arterial light chain; (4) the same two-dimensional gel electrophoretic technique which gave four uterine or arterial light chain spots showed only two spots for the phosphorylatable light chain from frog, turtle, rat, or chicken skeletal muscle. In addition to monophosphorylation, diphosphorylation of light chain was also observed in intact smooth muscle; serine and threonine residues were identified as the two phosphorylation sites. The ratio of phosphoserine to phosphothreonine was 5.7:1 in uterus and 11.4:1 in arteries. These ratios did not change upon drug-induced contractions of the muscles, however, the ratio decreased upon stretching the arteries. Smooth muscle contraction was invariably associated with light chain phosphorylation. Nevertheless, the same extent of light chain phosphorylation was produced by stretching the muscle. From determination of light chain phosphorylation of muscles in various functional states, it is concluded that this phosphorylation is necessary for the activation of smooth muscle.

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Contributions of intracellular (sarcoplasmic reticulum) and extracellular (plasma membrane) Ca^{2+} pools to myoplasmic $[Ca^{2+}]$ and activation of vascular smooth muscle

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It is now clear that the sarcoplasmic reticulum (SR) is an important source of activator Ca^{2+} in smooth muscle, although extracellular Ca^{2+} (Ca_e^{2+}) must be present for sustained contractions. Tonic contractions require precise regulation of myoplasmic $[Ca^{2+}]$, a property not associated with the SR membranes of striated muscle. We sought to test the hypothesis that (1) the SR in smooth muscle serves as an agonist releasable Ca^{2+} pool associated with initial transients in myoplasmic Ca^{2+}, crossbridge phosphorylation, and rapidly cycling crossbridges and (2) stress (S) maintenance associated with low myoplasmic $[Ca^{2+}]$, low phosphorylation, and low cycling rates ('latch') requires Ca_e^{2+} and precise regulation of myosplasmic $[Ca^{2+}]$ by the plasma membrane (PM). The role of the SR was explored using various protocols in which contractions were induced by histamine and phenylephrine in the absence of Ca_e^{2+} (zero $CaCl_2$ + 1 mM EGTA) or in the presence of the Ca^{2+} influx blocker, La^{3+}. Transient contractions were obtained which were comparable to the initial response in normal physiological salt solution (PSS) in respect to initial dS/dt, peak S, peak V_0, and peak phosphorylation. These results are consistent with the hypothesis that agonist-induced Ca^{2+} release can provide initial Ca^{2+} transients associated with high levels of phosphorylated, rapidly cycling crossbridges. The role of the PM and Ca_e^{2+} was explored using protocols which depleted intracellular Ca^{2+} pools. When Ca^{2+} was added to PSS containing histamine or phenylephrine, contractions were elicited whose steady-state levels of S and phosphorylation were equal to those of control tissues without Ca^{2+}-depletion. Initial transients in phosphorylation were absent (phenylephrine) or reduced (histamine) and the rates of attainment of steady-state S were slow, reflecting low initial levels of phosphorylation. Likewise, activation of PM Ca^{2+} channels independent of SR Ca^{2+} release with Bay K 8644 produced slow S development to high steady-state values without a transient in phosphorylation. These experiments suggest that: (1) initial transients in myoplasmic Ca^{2+} and phosphorylation are not required for high S development; (2) myoplasmic $[Ca^{2+}]$ is tightly regulated, presumably by the PM; and (3) the role of the SR produced Ca^{2+}-transient is to initially provide high levels of phosphorylated, rapidly cycling crossbridges which enhance the attainment of steady-state S. In summary, our results suggest that the SR is not required for agonist-induced contractions and may lack any capacity to regulate myoplasmic $[Ca^{2+}]$. However, the SR plays an important role in initiating crossbridge phosphorylation which greatly accelerates the development of tonic S ('latch').

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ATP regeneration in chemically skinned smooth muscle of guinea pig taenia coli

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In smooth muscle, with its small phosphagen pools and close coupling of oxidative metabolism and contraction, phosphocreatine (PCr) is not a major energy store but could be important for the flow of high-energy phosphate to the contractile system, as has been proposed in striated muscle [for a review see Bessman & Carpenter, *A. Rev. Biochem.* **54**, 831–62 (1985); also Meyer *et al., Am. J. Physiol.* C365–77 (1984)]. An important aspect of this function is the localization of creatine kinase (CK) in close vicinity of the contractile proteins. This was investigated in guinea pig taenia coli skinned in 1% Triton X-100 and subsequently stored in 50% glycerol at −18° C until use. After an initial control contracture at pCa 4.5, 3.2 mM MgATP (22° C), taenia strips were incubated in solutions containing various concentrations (0–3 mM) ADP, 2 mM free Mg^{2+}, but no added ATP or CK. Sodium azide (1 mM) and the myokinase inhibitor AP_5A (0.2 mM) were present throughout. At all ADP levels the strips remained relaxed at pCa 4.5. When PCr (12 mM) was added the strips contracted depending on the amount of added ADP up to a maximum at 1 mM of 70% of the control force (EC_{50}: 0.3 mM). Addition of CK (15 U ml^{-1}) increased force at all ADP levels, and caused a leftward shift of the concentration–response relationship (EC_{50}: 0.1 mM). Analysis of the incubation solutions showed no occurrence of ATP outside the strip after PCr addition and contraction, whereas after the further addition of CK most of the ADP was converted to ATP. The force development in the ADP–PCr system thus results from ATP produced and utilized locally within the strip. The submaximal force and higher EC_{50} in the absence of added CK could be an effect of a higher ADP:ATP ratio at the site of ATP utilization. After myosin light chain

thiophosphorylation, EC_{50} for ADP in the absence and presence of added CK were 0.1 mM and 0.02 mM, whereas maximal force relative to the control was the same as in untreated strips. EC_{50} values for ADP with added CK were slightly lower than those for ATP, with phosphoenolpyruvate (PEP, 5 mM) and pyruvate kinase (PK, 20 U ml^{-1}) as ATP-regenerating system [Hellstrand & Arner, *Pflügers Arch.* **405**, 323–8 (1985)]. Like PCr, PEP was able to support contraction in the presence of ADP but no added ATP or ATP-regenerating enzyme (PK). In conclusion, skinned taenia coli cells contain enzyme systems capable of formation of ATP close to its site of utilization by the contractile proteins. Both myosin light chain kinase and the crossbridge ATPase are accessible to the formed ATP, but their sensitivities differ.

Crossbridge cycling can be re-accelerated in rat tracheal smooth muscle when tonically activated by ACh or 5-HT

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The contraction kinetics of tonically activated smooth muscle show an initial acceleration followed by a time-dependent decline to a lower steady-state level. This time course has been interpreted as corresponding changes in the activity of the MLCK [Aksoy *et al.*, *Am. J. Physiol.* **242**, C109 (1982)]. The question arises as to whether maximum crossbridge cycling rates are restricted to the very first period of tonic activation or whether they can be re-accelerated to initial peak rates during sustained stimulation. All the experiments were carried out with the rat tracheal smooth muscle by using the post-vibration technique of Peiper [*Pflügers Arch.* **399**, 203 (1983)]. Control contractions of the isolated preparation were induced by electrical square wave pulses (30 Hz; 0.17 ms; 1 min) known to cause neurogenic release of ACh. Subsequently, the preparation was activated for 45 min either by 100 or 2 μmol l^{-1} acetylcholine (ACh) or by 10 μmol l^{-1} serotonin (5-HT). The electrical stimulus was also applied repetitively in the presence of ACh or 5-HT as an extra activation. The vibration (100 Hz; sinus; 8% of muscle length; 1.8 s) was performed to inhibit force development during all types of activation. After 45 min of tonic 5-HT activation, additional neurogenic ACh-release induced a pronounced increase in both the rate and the extent of post-vibration tension recovery. During tonic activation by maximum effective ACh doses, however, additional electrical stimulation produced only minimal extra tension increase and had virtually no effect on the time constants. During stimulation by ACh in concentrations creating half the maximum tension increase, the electrical pulse stimulation re-accelerated the contraction kinetics to an extent similar to that seen under maximally effective 5-HT concentrations. The time constants of post-vibration tension recovery were as follows: 30 s EL only $= 5.1 \pm 0.1$ s; 45 min 5-HT $= 11.0 \pm 0.2$ s; 45 min 5-HT + 30 s EL $= 6.5 \pm 0.3$ s; 45 min 100 μmol ACh $= 11.5 \pm 0.2$ s; 45 min ACh + 30 s EL $= 11.0 \pm 1.0$ s; 2 μmol l^{-1} ACh $= 11.1 \pm 0.5$ s; 45 min ACh + 30 s EL $= 6.2 \pm 0.3$ s. Our findings show that during tonic activation the contractile proteins remain ready to respond to further stimulation by a transient increase in contraction kinetics.

Effects of substrate and hypoxia on metabolism and contraction of smooth muscle from rabbit urinary bladder

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An unusual feature of the metabolism of some smooth muscles is a substantial contribution of aerobic glycolysis to total metabolic energy production. This is generally observed, however, when glucose is present as substrate and in the present study I have examined the metabolic and functional consequences of providing a smooth muscle with an exogenous substrate that it was unable to utilize for glycolysis. Strips of longitudinal smooth muscle from rabbit urinary bladder were mounted isometrically in the appropriate apparatus to allow measurement of force production and either heat production, oxygen consumption or lactate production. The muscles were bathed in Krebs–Henseleit Ringer solution containing 10 mmol l^{-1} of either glucose or pyruvate. Under aerobic conditions ($pO_2 > 640$ mmHg), with glucose as substrate, the net suprabasal energy flux in a 30 s contraction, as represented by the total heat liberated, was 123 ± 24 sd mJ g^{-1} ($n = 10$). Suprabasal oxygen consumption amounted to 0.18 ± 0.04 μmol g^{-1} ($n = 9$), equivalent to 81 ± 16 mJ g^{-1}, whilst suprabasal lactate production was 0.36 ± 0.09 μmol g^{-1} ($n = 10$) providing 35 ± 9 mJ g^{-1} of energy. When pyruvate replaced glucose as substrate under aerobic conditions the total heat liberated in a 30 s contraction was unchanged, being 119 ± 26 mJ g^{-1} ($n = 9$), whilst oxygen consumption rose to 0.25 ± 0.04 μmol g^{-1} ($n = 9$) providing 113 ± 19 mJ g^{-}. Force development was unchanged and it was assumed that no energetically useful lactate production arose from the pyruvate. Interestingly, the pattern of spontaneous contractions differed markedly depending upon the substrate. The frequency of these contractions being much reduced in the pyruvate Ringer. Under a regime of regular stimulation (3 s contraction every 30 s) and with glucose as substrate force was well maintained under anaerobic conditions ($pO_2 < 2$ mmHg), remaining at $98 \pm 3\%$ ($n = 5$) of the steady state level previously established under aerobic conditions after 15 min hypoxia. In contrast, when pyruvate was provided as substrate, force declined substantially under anaerobic conditions being reduced to $11 \pm 3\%$ ($n = 5$) of the aerobic level by 15 min. In the presence of glucose the total heat liberated in a 30 s contraction under anaerobic conditions was 101 ± 24 mJ g^{-1} ($n = 8$) and associated lactate production was 0.89 ± 0.22 μmol g^{-1} ($n = 5$) accounting for 87.6 ± 22 mJ g^{-1}. The results indicate that under aerobic conditions and with glucose as substrate smooth muscle of rabbit urinary bladder generates around one third of its active energy requirements through glycolysis, and that glycolysis can be further accelerated under anaerobic conditions to provide sufficient energy to sustain contraction. If pyruvate replaces glucose as substrate the metabolism shifts to being virtually totally oxidative and contraction can no longer be supported in the absence of oxygen.

The use of prestimulation and variable relaxation times to probe the regulation of crossbridge states in mammalian smooth muscle

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Previous experiments using a consecutive-tetanus paradigm in which the rabbit taenia coli was stimulated for 25 s, allowed to relax for 30 s and restimulated showed that there was a large decrease in maximum velocity of shortening (V_0) and chemical energy usage, and significantly decreased myosin light chain phosphorylation (MLCP) during the second tetanus even though active force redeveloped to $90 \pm 4\%$ of the initial tetanus. Further energetics experiments showed no evidence for an increased dissipation of work against an internal load during the second response, in contrast to the predictions of the 'latchbridge' hypothesis. Thus, the condition of low energy input, slow velocity of shortening, low degree of MLCP gives rise to high force maintenance through a general slowing of crossbridge cycling rate.

As an extension of these studies, we have begun to study the characteristics of the regulation of crossbridge slowing during consecutive contractions. We found that the down-regulation of crossbridge cycling during the second tetanus measured after 30 s of relaxation is independent of the duration of the initial stimulus

within the range 5–25 s. Therefore, once contraction is activated, a process is promptly initiated through which crossbridge cycling rate in the second tetanus is constrained. Varying the duration of relaxation after 10 or 20 of initial stimulation prior to restimulation shows a marked decrease in the maximum rate of force redevelopment ($\dot{P}$) which partially recovers during 30 s of relaxation. From an analysis of the time course of recovery of $\dot{P}$ and force, conditions were found where equivalent force production was associated with approximately a five-fold difference in $\dot{P}$. An in-depth study of the mechanical properties showed that a 10 s stimulation followed by 20 s relaxation and 10 s restimulation gave $121 \pm 7\%$ force and $117 \pm 6\%$ stiffness, but only $51 \pm 6\%$ V_0 compared to the initial 10 s stimulation. Corresponding values after 60 s relaxation were $100 \pm 8\%$ force, $104 \pm 9\%$ stiffness and $92 \pm 10\%$ V_0. These experiments showed that there was a marked slowing of crossbridge cycling rate that was almost totally reversed after 60 s of relaxation. After 10 s stimulation, the degree of MLCP was $31 \pm 3.7\%$. In the consecutive tetanus design, the MLCP was reduced to $23.6 \pm 2.6\%$ and $23.3 \pm 2.5\%$ after 10 s of restimulation following 20 s and 60 s of relaxation, respectively. Thus, the velocity of crossbridge cycling rate recovered under conditions where the phosphorylation transient did not. Further, a two-fold change in velocity occurred with no significant change in MLCP. Therefore, upon activation of contraction, a regulatory process is set in motion that slows the rate of crossbridge cycling. This inhibitory process is long-lived, and its effectiveness is markedly evident even after 20 s of relaxation, but not at 60 s of relaxation. The identity of the inhibitory process is not known, but it is independent of MLCP.

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Photolysis of caged nucleotides (ATP and CTP) for kinetic studies of vascular smooth muscle contraction

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Photolysis of photolabile precursors of adenine nucleotides [Goldman *et al., J. Physiol., Lond.* **354**, 577–604 (1984)] and inositol triphosphate [Goldman *et al., J. Physiol., Lond.*, in press (1986)] has been succesfully used to overcome diffusional delays in studies of striated and smooth muscle. We have now used photolabile (caged) ATP and CTP to quantitate the relaxation accompanying detachment of rigor crossbridges, and to determine the effect of thiophosphorylation with ATPγS on the rate of activaton of vascular smooth muscle. All studies were done on guinea pig portal veins permeabilized by freezing in glycerol [Peterson, *J. gen. Physiol.* **79**, 437–52 (1982)] and with 5 μM calmodulin present. Stiffness was measured with 500 Hz 0.05% sinusoidal length changes. In the presence of Ca^{2+}, the liberation of approximately 0.5 mM ATP from caged ATP initiated active tension. In muscles initially in 'high rigor' [Kawai & Brandt, *J. gen. Physiol* **68**, 267–80 (1976)], tension developed with a latency of less than 60 ms, and with an exponential phase of $0.5\,s^{-1}$. Thiophosphorylation of myosin light chains with ATPγS increased the rate constant to approximately $1.6\,s^{-1}$. In the absence of Ca^{2+}, tension relaxation from 'high rigor' upon photolysis of caged nucleotides showed a plateau or slight convexity during the initial (average) 390 ms. As in skeletal muscle [Goldman *et al., J. Physiol., Lond.* **354**, 577–604 (1984)], the 'bump' could be accentuated by decreasing muscle length prior to photolysis. Stiffness transients contained an early, faster (< 100 ms) component of decay than the tension records. The delayed time course of tension relaxation was similar when caged CTP was photolysed. Since CTP is not a substrate for smooth muscle myosin light chain kinase [Walsh *et al., Fedn Proc. Fedn Am. Socs exp. Biol.* **42**, 45–50 (1983)], this plateau or 'bump' was not due to reattachment caused by light chain phosphorylation by the liberated nucleotide.

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Agonist and calcium dependence of myosin phosphorylation and contraction of airway smooth muscle

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The role of myosin phosphorylation in regulating contraction of intact airway smooth muscle was investigated by comparing the dose dependence and calcium dependence of active stress, phosphorylation and shortening velocity in muscle stimulated with carbachol, potassium depolarizing solution or serotonin. Myosin phosphorylation and shortening velocity both increased transiently at the onset of contraction with carbachol suggesting a shift to dephosphorylated crossbridges during tonic contraction. Dephosphorylation coincided with decay of isotonic shortening velocity during rapid relaxation following washout of carbachol. However, dephosphorylation was significantly slower than decay of shortening velocity during slow relaxation induced by Ca^{2+}-free physiological salt solution (PSS) containing 1 μM carbachol. Carbachol dose–response curves for isometric stress development and myosin phosphorylation were superimposable, but shifted to the left of the shortening velocity dose–response suggesting a difference in the Ca^{2+} dependence of shortening velocity and myosin phosphorylation. Myosin phosphorylation and active stress development also appeared to have different Ca^{2+} sensitivities. Extracellular Ca^{2+} was depleted by exposure to Ca^{2+}-free PSS (0.1 mM EGTA). Muscles were stimulated with 0.1 or 1 μM carbachol, 10 μM serotonin or 60 mM K^+ in Ca^{2+}-free PSS, and a Ca^{2+} dose–response curve constructed by increasing $CaCl_2$ in the bath. Significant phosphorylation (0.2–0.3 moles P_i/mole myosin light chain), but no active stress, was observed in muscles stimulated with carbachol or serotonin in Ca^{2+}-free PSS. K^+ depolarization in Ca^{2+}-free PSS (60 mM KCl + 1 μM atropine) yielded phosphorylation not significantly different from basal levels. All agonists induced active stress following readmission of calcium. The Ca^{2+} dose–response curve for myosin phosphorylation in muscles stimulated with carbachol was shifted up and to the right of the curve for contraction. Atropine (1 μM) significantly reduced phosphorylation induced by carbachol in Ca^{2+}-free PSS, as did 30 μM nifedipine and 10 mM EGTA. Muscarinic activation in low Ca^{2+} solution promotes phosphorylation via a Ca^{2+} dependent mechanism. Methylene blue (1 or 10 μM) did not potentiate active tension in Ca^{2+}-free solutions in the presence of carbachol, suggesting that increased guanylate cyclase activity was not inhibiting contraction. Phorbol 12-myristate, 13-acetate or phorbol 12, 13-dibutyrate did not increase basal phosphorylation or phosphorylation in low Ca^{2+} solutions, suggesting that protein kinase C did not phosphorylate myosin in this case. Stimulation of airway muscle with carbachol or serotonin, but not potassium induces significant phosphorylation in Ca^{2+}-free PSS. Myosin phosphorylation under these conditions is not sufficient to support active stress, and is reduced by treatments that decrease Ca^{2+} entry. The results are consistent with dual Ca^{2+} regulation of the contractile element in intact tracheal muscle, and suggest independent control of myosin phosphorylation and development of active stress during muscarinic or serotonin stimulation.

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Effects of phosphate and magnesium on rigor and ATP induced relaxation in chemically skinned guinea-pig taenia coli

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The mechanical properties of chemically skinned smooth muscle in rigor were investigated at varied levels of inorganic phosphate (P_i), Mg^{2+} and ionic strength. A rigor state was obtained by transferring the fibres, at the plateau of a maximal active contraction to a rigor solution (composition in mM: EGTA 4, $MgCl_2$ 2.3, imidazole 30, DTE 0.5 and ionic strength 90; pH 7.0; 22° C), supplemented with an ATP depleting system (glucose, hexokinase). This induced a slow decrease in force (P) to a level of 30–40% of the maximal tension. From the force response (ΔP) to rapid (0.8 ms) length steps (ΔL), stiffness relations ($\Delta P/\Delta L$) were obtained. Compared with the active contraction the rigor was characterized by an increase in relative stiffness ($\Delta P/(\Delta L.P)$). No change in force or stiffness was observed when ionic strength was increased to 150 mM or when 10 mM P_i was added. An increase in $[Mg^{2+}]$ to 10 mM reversibly decreased force in rigor by about 15% and increased relative stiffness. These results may reflect an effect of Mg^{2+} on the conformation or organization of the contractile filaments. The rate of relaxation from rigor following photolytic release of ATP from caged-ATP was investigated. The preparations were incubated in rigor solution with 12 mM caged-ATP and 10 mM DTE, which had no effect on rigor force. With a light flash from a Xenon lamp 1–2 mM ATP was liberated. This resulted in a rapid relaxation (rate constant $0.6\,s^{-1}$) which was accelerated in the presence of P_i (3 mM, $1.3\,s^{-1}$; 10 mM, $2.2\,s^{-1}$). The rate of relaxation was faster at low $MgCl_2$ concentrations (0.5 mM) and when ionic strength was increased by 60 mM. A markedly slower relaxation was observed at high $MgCl_2$ concentrations (5–12 mM). The effect of P_i was seen at high and low ionic strength and at $MgCl_2$ concentrations in the range 0.5–12 mM. These results may reflect effects of P_i, Mg^{2+} and ionic strength on detachment of rigor crossbridges. However, the effects of P_i are qualitatively similar, although with slower rate constants, to those described for striated muscle where the ATP induced relaxation is proposed to involve crossbridge detachment and a phase of reattachment affected by P_i [Hibberd *et al.*, *Science, N.Y.* **228**, 1317–19 (1985)]. If the present results are interpreted according to this model, the relaxation from rigor at low $[Ca^{2+}]$ in smooth muscle is associated with detachment and reattachment of dephosphorylated crossbridges.

The effects of Ca^{2+} and Mg^{2+} on the activation of actomyosin ATPase in thiophosphorylated skinned taenia coli smooth muscle fibres

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Skinned taenia coli smooth muscle fibres activated either by a Ca^{2+}-calmodulin (CaM)-insensitive myosin light chain kinase (MLCK) or by Ca^{2+} showed a similar behaviour in the contraction, ATPase activity and phosphorylation (~53%) of the regulatory myosin light chains (LC). After activation by the Ca^{2+}-insensitive MLCK addition of Ca^{2+} caused a slight increase of 10–15% in ATPase and tension [Mrwa *et al.*, *Experientia* **41**, 1002 (1985)]. In order to investigate the effect of Ca^{2+} and Mg^{2+} on activation of the myosin ATPase, skinned fibres were thiophosphorylated to obtain thiophosphorylated myosin which is considered to be a poor substrate for phosphatases (1 mM adenosine-5′-*O*-(thiotriphosphate), 1 μM CaM, 2 mM $MgCl_2$, pCa 4.5). The fibres were contracted either at pCa 4.5 or pCa 8 in the presence of 7.5 mM MgATP, 2 mM Mg^{2+}, 2 mM EGTA and an ATP regenerating system. Protease inhibitors (1 μM antipain, 0.5 μM leupeptin, 50 μM E-64) were added during skinning and were present in all solutions. The ATPase activity was determined in the fibres by an NADH-coupled optical assay [Güth & Junge, *Nature, Lond.* **300**, 775 (1982)] and LC phosphorylation by two-dimensional-gel electrophoresis. As shown in the table the ATPase activity at pCa 4.5 increased after thiophosphorylation of the LC to 80–90% on raising the free Mg^{2+} concentration.

	ATPase (%) ± S.E.		
pCa	*0.05 mM* Mg^{2+}	*0.5 mM* Mg^{2+}	*2.0 mM* Mg^{2+}
4.5	90 ± 16	185 ± 26	467 ± 39
8.0	119 ± 7	124 ± 9	100

However, at pCa 8.0 the actin-activated ATPase activity was not dependent on the Mg^{2+} concentration. It was demonstrated earlier that Ca^{2+} has a direct effect on actin-activated ATP hydrolysis and that the Ca^{2+}-sensitivity of the actomyosin ATPase depends on the free Mg^{2+} concentration [Chacko & Rosenfeld, *Proc. natn. Acad. Sci. U.S.A.* **79**, 292 (1982)]. Using thiophosphorylated skinned muscle fibres we observed a similar result. The actin-activated myosin ATPase of skinned fibres is also modulated by Ca^{2+} and Mg^{2+} and Ca^{2+} may act not only via phosphorylation but also by a direct effect on actin–myosin interaction.

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Antibodies to turkey gizzard myosin light chain kinase inhibit contraction and myosin phosphorylation in skinned guinea pig taenia coli.

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We have used an immunological approach to investigate the relation of myosin phosphorylation (MLC-P_i) to other putative Ca^{2+}-regulatory mechanisms in the control of contractility in smooth muscle. Our aim is to inhibit selectively myosin light chain kinase (MLCK) so that the dependence of other Ca^{2+}-regulatory systems may be unmasked. Anti-MLCK antibodies were produced by immunizing goats with MLCK purified from turkey gizzards. An IgG fraction was prepared and Fab fragments were generated by digesting the IgG fraction with papain. The anti-MLCK Fab were purified by applying the digest to an affinity resin made by coupling purified turkey gizzard MLCK to Sepharose 4B. The anti-MLCK Fab were eluted from the column, concentrated and dialysed in the appropriate buffers. Anti-MLCK Fab inhibit the activity of purified turkey gizzard MLCK and interact monospecifically with MLCK in various mammalian smooth muscles as demonstrated by a western blot analysis. Guinea-pig taenia coli were made permeable to proteins (skinned) as described by Rüegg & Paul [*Circ. Res.* **50**, 394–9 (1982)]. Small fibres approximately 100 μm in diameter and 4 mm long were connected to transducers and maintained at 25° C. The fibres were relaxed ($pCa^{2+} > 8$) or contracted ($pCa^{2+} = 5.18$) by immersion in calcium–EGTA buffers.

The fibres were also incubated with 6×10^{-8} M anti-MLCK Fab dialysed in relaxing or contracting solution. Myosin phosphate content was quantitated by first separating the muscle proteins by IEF PAGE. The proteins were then transferred to nitrocellulose paper and incubated with anti-LC_{20} antibodies followed by peroxidase labelled and ^{125}I-labelled second antibodies. The immunoreactive material was visualized by developing the peroxidase reaction. Two types of experiments were performed. First, the fibres were incubated with anti-MLCK Fab in relaxing solution and then contracted. The post-Fab contraction, after 75 mins, developed about 25% of the force of a test contraction performed prior to Fab. Fibres incubated with Fab for shorter or longer times and then stimulated with Ca^{2+} generated proportionately more or less tension. Second, contracted fibres incubated with Fab relaxed completely in about 90 mins despite the presence of Ca^{2+}. No significant effect on isometric force was seen in the presence of affinity-purified mouse Fab against the Fc region of human IgG. MLC-P_i, which was increased from basal values of 0.13 ± 0.03 mol P_i per mol LC_{20} to 0.62 ± 0.01 in pCa 5.18 solutions, was lowered to 0.36 ± 0.01 after 90 min in the presence of Fab. Moreover, Fab relaxed ATPγS-induced contraction without any apparent decrease in MLC-P_i. These data suggest that anti-MLCK Fab may relax taenia coli by both inhibiting MLCK activity and by another undefined mechanism.

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Changes in molecular distribution underlying contraction of single isolated smooth muscle cells

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Single smooth muscle cells have been studied to understand their mechanism of contraction. Cells were obtained by enzymatic disaggregation of the stomach muscularis of *Bufo marinus*. Immunocytochemical analysis of molecular distribution in single cells was carried out in three dimensions using the digital imaging microscope. They revealed that α-actinin is present in cytoplasmic (CDB) and plasma membrane dense bodies (PMDB). The PMDB act as membrane attachment areas for strings of CDB with a regular centre-to-centre mean distance of 2.2 μm in relaxed cells. Similar strings are seen in unfixed skinned cells with phase contrast optics. The spacing of elements in these strings diminishes in cells that have actively shortened. Actin is found in cables that can be followed for over 25 μm. It is packed most densely within the cytoplasmic dense bodies. Myosin is found in filaments many of which in thin sections of intact cells are over 1.5 μm long. Thus they cannot be positioned in line with the strings of CDB, but instead must interact with CDB-associated actin at the periphery of the strings. Negative staining of thick filaments isolated from skinned cells reveals crossbridge projections with a 14 nm repeat that continues along the whole filament length. Analysis of tilt pairs rotated around the longitudinal axis supports a side polar model of the thick filament.

$[Ca^{2+}]$ was measured in single living cells with Fura-2. Cytoplasmic $[Ca^{2+}]$ at rest was 140 nM and rose to 800–1000 nM with excitatory stimuli. The rise in $[Ca^{2+}]$ preceded the onset of contraction. Ca^{2+} imaging revealed a region of high Ca^{2+} adjacent to the periphery of the cell that is retained when cells are skinned with saponin but not with Triton suggesting that it reflects intra-sarcoplasmic reticulum Ca^{2+}. Intranuclear $[Ca^{2+}]$ was higher than cytoplasmic $[Ca^{2+}]$ at rest and appeared to be regulated independently of that in the cytoplasm. A model is presented of the contractile process in smooth muscle in which $[Ca^{2+}]$ is the primary determinant of contractile activity which comes about through sliding of elements in adjacent strings demarcated by α-actinin rich CDBs due to interaction with side-polar myosin containing filaments positioned between adjacent strings.

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Polycation-modulable phosphatase suppresses Ca^{2+}-dependent actin–myosin interaction in detergent-skinned smooth muscle

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Recently, we purified a holoenzyme form of protein phosphatase from bovine aortic smooth muscle. SDS electrophoresis of the purified enzyme revealed three polypeptides of 76, 53, and 35 kDa. This enzyme dephosphorylates native myosin from a variety of smooth muscles, phosphorylase *a*, myocardial myosin light chains, inhibitor 1, and several other phosphoproteins. Moreover, dephosphorylation of phosphorylase *a* is stimulated 6–15 fold by low concentrations of polylysine (0.01–0.1 μM) or lysine-rich histone-H, (< 4 μg ml^{-1}), whereas dephosphorylation of isolated light chains is virtually abolished. Accordingly, we have called this enzyme Polycation-Modulable (PCM) phosphatase (*Proc. Soc. exp. Biol. Med.* **177**, 24 (1984); *Adv. Prot. Phosphatases* **I**, (1985)].

Since the Ca^{2+}-regulatory mechanism for actin–myosin interaction in smooth muscle involves Ca^{2+}-dependent phosphorylation of the 20 kDa myosin light chains, we studied the influence of PCM-phosphatase on contraction and relaxation of detergent-skinned fibres bundles from the smooth muscle of chicken gizzard. The results were as follows.

(1) Ca^{2+}-sensitivity for isometric contraction was decreased: the concentration of Ca^{2+} required for half maximal contraction (0.26 μM) was increased two-fold in the presence of PCM-phosphatase ($n = 10$).
(2) Relaxation in low Ca^{2+} ($< 10^{-9}$ M; $t_{\frac{1}{2}} = 12 \pm 2$ min) was accelerated four-fold when PCM-phosphatase was included in the incubation medium.
(3) Unloaded shortening velocity in the presence of an intermediate Ca^{2+}-concentration (0.5 μM; 0.06 ± 0.01 L s^{-1}) was decreased six-fold (0.01 L s^{-1}; $n = 5$).
(4) Addition of PCM-phosphatase to fibre bundles which had been contracted with 0.4 μM Ca^{2+} promptly relaxed.
(5) Each of the effects of PCM-phosphatase was associated with parallel decreases in Ca^{2+}-dependent phosphorylation of the 20 kDa light chains.
(6) All effects were reversed when PCM-phosphatase was removed from the incubation medium.

These results show that PCM-phosphatase suppresses actin–myosin interaction and that such suppression is correlated to reduced phosphorylation of the regulatory myosin light chains. Since protein phosphatases are regulated enzymes the results also suggest that modulation of phosphatase activity may participate in control of smooth muscle contractility.

Free calcium changes and Na^+-Ca^{2+} exchange indicated by the fluorescent dye fura-2, in smooth muscle cells isolated from guinea-pig taenia coli

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Excitation–contraction coupling in smooth muscle cells may be mediated by an increase in cytosolic free Ca^{2+} concentration. Guinea-pig taeniae were dissociated into individual cells and 'loaded' with either quin2 or fura-2 [Tsien *et al.*, *Cell Calcium* **6**, 145–57 (1985)] or with 20 μM quin2 or 4 μM fura-2 AM in DMSO [*Grynkiewicz et al.*, *J. biol. Chem.* **260PB**, 3440–50 (1985)] Quin2-

loaded taenia smooth muscle cells had a resting Ca_i^{2+} of 93 ± 39 nM ($n = 14$, seven preparations) with 1 mM Ca_0^{2+}, similar to reported values for other cells. Quin2 fluorescence signals were not affected by treatment of the cells with 100 μM carbachol or 80 mM K_0^+, or electrical stimulation.

Addition to carbachol to fura-2-loaded smooth muscle cells rapidly decreased 510 nm fluorescence (380 excitation) corresponding to an increase in Ca_i^{2+} to approximately 1.4 times resting levels. Ca^{2+} was elevated (4 min), so that excitation of resting and stimulated cells could be compared. Cells were then lysed with 50 μM digitonin (pH 8.3) to determine Ca^{2+}-free dye fluorescence. Fluorescence emission (510 nm) was reproducibly enhanced at 340 nm and diminished at 380 nm. Resting Ca^{2+} of fura-2 loaded smooth muscle cells was estimated as 158 ± 72 nm ($n = 6$) using 340:380 excitation ratio. Carbachol will reduce the median cell length of smooth muscle cells by 40% but does not affect the fluorescence of dye-free cells.

The existence of Na^+–Ca^{2+} exchange in smooth muscle is controversial. In further studies using fura-2, we found that a resting Ca_i^{2+} of 101 ± 14 nM ($n = 9$) was elevated to 306 ± 189 nM ($n = 6$, $t = 2.68$, $P > 0.05$), after incubation with 10^{-4} M ouabain for 15 min. This was detected by an increase in 510 nm fura-2 fluorescence excited at 340 nm and a decrease in 510 nm emission excited at 380 nm. Similarly, when Na_0^+ was reduced below 10 nM (substituting bis-2 hydroxyethyl dimethyl ammonium chloride for Na_0^+), Ca_i^{2+} increased to $276 + 24$ nM ($n = 5$), $t = 8.37$, $P > 0.01$). Elevation of Na_i^+ by ouabain blockade of the Na^+–K^+ ATPase, or reduction of Na_0^+-Na_i^+ should have enhanced Na^+ efflux, and hence Ca^{2+} influx, via an Na_i^+–Ca_0^{2+} exchanger [Pritchard & Ashley, *FEBS Lett.* **195**, 23–7 (1986)].

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