6 Hellstrand, P., and Paul, R.J., Vascular smooth muscle: relation between energy metabolism and mechanics, in: Vascular Smooth Muscle: Metabolic, Ionic and Contractile Mechanisms, pp. 1–36. Eds M.F. Crass III and C.D. Barnes. Academic Press, New York 1982.
7 Marston, S.B., The regulation of smooth muscle contractile proteins. Prog. Biophys. molec. Biol. *41* (1982) 1–41.
8 Murphy, R.A., and Mras, S., Control of tone in vascular smooth muscle. Archs intern. Med. *143* (1983) 1001–1006.
9 Nonomura, Y., and Ebashi, S., Calcium regulatory mechanism in vertebrate smooth muscle. Biomed. Res. *1* (1980) 1–14.
10 Paul, R.J., Doerman, G., Zeugner, C., and Rüegg, J.C., The dependence of unloaded shortening velocity on Ca^{2+}, calmodulin, and duration of contraction in 'chemically skinned' smooth muscle. Circulation Res. *53* (1983) 342–351.
11 Portzehl, H., Caldwell, P.C., and Rüegg, J.C., The dependence of contraction and relaxation of muscle fibers from the crab *Maja squinado* on the internal concentration of free calcium. Biochim. biophys. Acta *79* (1964) 581–591.
12 Rüegg, J.C., and Paul, R., Vascular smooth muscle. Calmodulin and cyclic AMP-dependent protein kinase alter calcium sensitivity in porcine carotid skinned fibers. Circulation Res. *50* (1982) 394–399.
13 Siegman, M.J., Butler, T.M., Mooers, S.U., and Davies, R.E., Calcium-dependent resistance to stretch and stress relaxation in resting smooth muscles. Am. J. Physiol. *231* (1976) 1501–1508.
14 Siegman, M.J., Butler, T.M., Mooers, S.U., and Davies, R.E., Cross-bridge attachment, resistence to stretch, and viscoelasticity in resting mammalian smooth muscle. Science *191* (1976) 383–385.
15 Solaro, R.J., Di Salvo, J., and Paul, R.J., Symposium: Coordination of Metabolism and Contractility by Phosphorylation in Cardiac, Skeletal, and Smooth Muscle. Fedn. Proc. *42* (1983) 7–71.
16 Sparrow, M.P., Mrwa, U., Hofmann, F., and Rüegg, J.C., Calmodulin is essential for smooth muscle contraction. FEBS Lett. *125* (1981) 141–145.
17 Walsh, M.P., Bridenbaugh, R., Hartshorne, D.J., and Kerrick, W.G.L., Phosphorylation-dependent activated tension in skinned gizzard muscle fibers in the absence of Ca^{2+}. J. biol. Chem. *257* (1982) 5987–5990.
18 Walsh, M.P., Dabrowska, R., Hinkins, S., and Hartshorne, D.J., Calcium-independent myosin light chain kinase of smooth muscle. Preparation by limited chymotryptic digestion of the calcium ion dependent enzyme, purification and characterization. Biochemistry *21* (1982) 1919–1925.
19 Walsh, M.P., and Hartshorne, D.J., Actomyosin of smooth muscle, in: Calcium and Cell Function, Vol. III, pp. 223–269. Eds. W.Y. Cheung. Academic Press, New York 1982.
20 Walsh, M.P., Hinkins, S., Dabrowska, R., and Hartshorne, D.J., Smooth muscle myosin light chain kinase. Methods Enzymol. *99* (1983) 279–288.

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The role of myosin phosphorylation in the contraction-relaxation cycle of smooth muscle[1]

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Summary. Considerable evidence from a variety of experimental procedures indicates that the phosphorylation of myosin is involved in the regulation of contractile activity in smooth muscle. Phosphorylation of the 20,000-dalton myosin light chains is required to initiate crossbridge cycling and this is consistent with the observation that the actin-activated Mg^{2+}-ATPase activity of myosin is phosphorylation-dependent. In the simplest interpretation of this process it may be proposed that phosphorylation acts as an 'on-off' switch. Clearly this cannot explain the observed complexity of smooth muscle contractile behavior and such may imply either that additional mechanisms are involved or that the role of myosin phosphorylation is not fully appreciated. Recently it has been shown that monomeric smooth muscle myosin can exist in a 'folded' and an 'extended' conformation and that each form is characterized by distinct enzymatic properties. Under appropriate solvent conditions phosphorylation of myosin favors the extended conformation. It is tentatively suggest that this, or an analogous, transition might be involved in the regulation of the smooth muscle contractile apparatus, and this possibility is discussed.
Key words. Smooth muscle; regulation; phosphorylation; myosin; conformation; myosin light chain kinase.

The role of myosin phosphorylation in the contraction-relaxation cycle of smooth muscle still is not established. From in-vitro studies, originating with that of Sobieszek[21], it has been shown that phosphorylation of myosin is required for the expression of actin-activated ATPase activity and this has been confirmed in many subsequent reports[2,28]. In addition, numerous investigators have found that tension development in skinned or intact smooth muscle fibers is accompanied by an increase in the level of myosin phosphorylation. Other evidence from a variety of experimental approaches[28] offer further support for the phosphorylation theory. Thus it is reasonable to conclude that phosphorylation of myosin forms at least part of the regulatory mechanism in smooth muscle and the controversial, or unknown, aspects of the regulatory process are concerned with the possible function of alternative or complementary mechanisms.

Evidence cited in favor of an additional process is given by Murphy and co-workers[6] (see article in this volume) who found that the load-bearing capacity of strips of carotid artery was maintained despite a reduction in the level of myosin phosphorylation. From these and other data it was suggested that the phosphorylation of myosin light chains initiates crossbridge cycling (which would be predicted from the phosphorylation-dependent activation of ATPase activity) and that a second mechanism is involved in the maintenance of tension. The latter is

thought to reflect non-cycling attached crossbridges which could maintain a given tension with very little energy cost. These rigor-like bonds dissociate on relaxation of the muscle and therefore the attachment-detachment process would be expected to be Ca^{2+}-dependent. Since the range of Ca^{2+} concentrations over which the rigor-like bonds are controlled is lower than that required for myosin phosphorylation it would be expected that the affinity for Ca^{2+} of the putative Ca^{2+}-binding protein is higher than the affinity of Ca^{2+} for calmodulin. This Ca^{2+}-binding site has not been identified and the two most likely candidates are Ca^{2+} interactions with either myosin or a component of the thin filament. Both possibilities have literature precedents. Chacko and Rosenfeld[3] suggest that the binding of Ca^{2+} to myosin is involved in the regulatory process of smooth muscle and Walters and Marston[29] have proposed a thin filament-based control system.

Another mechanism that must be resolved and evaluated with respect to its role relative to myosin phosphorylation is the leiotonin system [12] (see article in this volume). Its mechanism of action is unknown but is thought to be independent of myosin phosphorylation and it is important to establish whether or not leiotonin constitutes an independent and alternative regulatory mechanism.

Thus, there are several options for additional regulatory mechanisms and the verification and elucidation of their in-vivo role(s) is clearly of the highest priority. But before this can be achieved the role of myosin phosphorylation must be more precisely defined. This is being attempted in several laboratories and some preliminary results and speculations are discussed below.

Effect of phosphorylation on the properties of myosin

a) ATPase activity

The critical aspect of the phosphorylation theory is that phosphorylation of the light chains allows activation by actin of the Mg-ATPase of myosin. This has been known for many years and these original in-vitro results formed the basis of the phosphorylation theory. It is known that myosin has two active sites (two heads) each associated with one of the two 20,000-dalton light chains and a more recent concern was to determine the stoichiometry of phosphorylation in relation to the activation of the two active sites. It was found that phosphorylation of both light chains is required to activate ATPase activity[9,17] (i.e., phosphorylation at one site does not achieve activation of either head). It was also suggested[9,17,18] that the phosphorylation of myosin is sequential in that the phosphorylation of the first site is preferred to the reaction of the second site. These results imply that the two heads of myosin are not independent but function cooperatively.

b) Phosphorylation levels in smooth muscle fibers

The conclusion that the two active sites of myosin behave cooperatively was derived from experiments with isolated myosin where complete phosphorylation can be obtained. In contrast the experiments with either intact or skinned smooth muscle fibers show variable levels of phosphorylation but these are never at the theoretical saturation level. Generally at maximum isometric tension the phosphorylation levels are between 50 and 60%. This then raises the question of how could a cooperative model for myosin activation occur in these fibers? A possible solution to this dilemma is found if one considers the available pool of myosin molecules. In the in-vitro studies all of the myosin molecules are available for phosphorylation, but this may not be the case in the fiber experiments. One possibility is that some of the cells in the fiber preparation may not be functional in a particular experiment, either these are not activated or they may be damaged, but their myosin content is included in the calculations of total available sites. Another, and more likely, possibility is that the myosin light chain kinase (MLCK) does not have free access to all of the myosin molecules. This could easily be the situation if MLCK binds to either the myosin or actin filaments, and preliminary experimets suggest the latter. We suggested[5] that MLCK binds to actin and that this binding is not influenced by Ca^{2+} or calmodulin. However, Sobue et al.[22] feel that the MLCK-actin interaction is Ca^{2+}-dependent and occurs only in the absence of Ca^{2+}. Obviously if the MLCK can diffuse freely in the smooth muscle cell then the idea of restricted populations of myosin is not valid. If, on the other hand, the MLCK remains attached to actin at all times then only that myosin within the immediate vicinity can be phosphorylated. This would be particularly critical in isometric contractions where little relative filament sliding is expected. The concentration of MLCK in gizzard smooth muscle[11] is on the order of 4 μM and one would expect for a 1 μm thin filament about 2 molecules MLCK. There are, however, many actin filaments for each thick filament and the observed levels of phosphorylation are within plausible limits.

The idea of limiting the MLCK to the thin filaments is intriguing and raises several points. It would appear to be logical to phosphorylate only those crossbridges that could interact with actin as this would achieve a more efficient use of energy. However, if filament sliding occurs and the phosphorylated myosin move away from the contact area of the MLCK what then is the fate of this myosin? Does it remain phosphorylated and continue crossbridge cycling or is it dephosphorylated? This depends, of course, on the location and activity of the phosphatase and at the moment this is unknown. However, it would seem that some regulation of phosphatase activity is required and an activation of activity in the absence of Ca^{2+} might be predicted. Another interesting point is that if the MLCK remains attached to actin are the 'relaxed' crossbridges close enough to be phosphorylated or is there an additional event involved which modifies either myosin or MLCK to allow the enzyme-substrate interaction?

c) The 6S-10S transition

The pattern of activation of ATPase activity as a consequence of myosin phosphorylation is well established but the molecular changes induced by phosphorylation are unknown. There are, however, some recent results which may help in our understanding of this process. It was shown several years ago[25] that filaments of dephosphorylated myosin in 0.15 MKCl were dissociated by ATP and on ultracentrifugation showed a component of 10S. At higher ionic strengths only a 6S component was formed

which is characteristic of monomeric myosin. Originally it was thought that the 10S component represented a myosin dimer, but it was subsequently shown that monomeric smooth muscle myosin could form both the 10S and 6S species[24] and that the increase in sedimentation coefficient resulted from a drastic decrease in the radius of gyration. The two conformations of smooth muscle myosin were examined by electron microscopy[4,15,27] and it was found that the 10S form represented a folded or looped myosin structure. According to the model proposed by Onishi and Wakabayashi[15] folding of the myosin rod occurs at two regions situated approximately 600 A and 1100 A from the tail end of the molecule and that the looped structure is formed by interaction of the myosin rod with the neck region of the molecule. The 6S myosin conformation is the extended more asymmetric form. It was also shown that the transition of 6S to 10S myosin is favored by dephosphorylation[4,16,27].

We have been studying the 10S-6S transition using viscosity, fluorescence and sedimentation velocity measurements[7,8]. The following conclusions were made concerning the physical properties of myosin: 1) In the presence of ATP the relative viscosity of dephosphorylated myosin decreased markedly at concentrations of KCl of less than 0.3 M and this was due to the formation of the 10S conformer. 2) With phosphorylated myosin the conversion to 10S was more resistant to the effects of decreasing ionic strength and whether or not a monomeric 10S species of phosphorylated myosin could be observed depended on the solvent conditions. At high (10 mM) levels of $MgCl_2$ myosin aggregates were formed before 10S could be identified and 10S could be demonstrated only under conditions where myosin aggregation is reduced, i.e., low $MgCl_2$ and/or high pH. 3) Under appropriate conditions, e.g. 0.2 MKC1, 1 mMATP and 10 mM $MgCl_2$ (pH 7.5) phosphorylation of myosin converts 10S to 6S and for the complete transition phosphorylation of both light chains is required. 4) ATP is not essential for the 6S-10S transition but it does facilitate the conformational change and the latter requires the binding of ATP to each active site, i.e., the transition is aided by low concentrations of ATP consistent with binding of ATP to the high affinity hydrolytic sites. 5) The 6S to 10S transition occurs in the presence of Mg^{2+}-, Ca^{2+}- and K^+-ATP, but is not facilitated by ADP or ATPδS. 6)The ATP-induced tryptophan fluorescence is altered by the 6S-10S transition and is higher for the 10S conformation.

In addition to these effects one other physical property should be mentioned and that is that 6S myosin more easily forms filaments than 10S myosin[23,24] and this feature is thought by some investigators[24,25] to be the most important aspect of the 10S-6S equilibrium.

d) Correlation of myosin shape and enzymatic properties

The inter-conversion of the extended and folded forms of smooth muscle myosin involves a remarkable conformational change but in our opinion a more interesting aspect of the transition is the finding that the 10S and 6S forms exhibit distinct biological properties[7]. Under identical conditions to those used for viscosity experiments the Mg^{2+}- and Ca^{2+}- and K^+EDTA-ATPase activities were measured. It was shown that the 10S and 6S myosins have different ATPase properties and that the enzymatic and conformational transitions were closely correlated. The formation of 10S myosin resulted in a decrease in Mg^{2+}- and Ca^{2+}-ATPase activities and an increase in the K^+ EDTA-ATPase activity. (In an earlier study by Onishi et al.[14] the KCl-dependence of ATPase activities were noted, but at that time the correlation to the monomeric myosin conformation was not realized.) An important point to emphasize is that the enzymatic activity is related directly to the shape of the myosin and not to the phosphorylation level, and it was shown[7] that under conditions where phosphorylated myosin forms the 10S conformation the ATPase activity is altered. A follow up to this point is that under physiological conditions one would expect that phosphorylation drives the 10S-6S transition and, therefore, phosphorylation would be the indirect modifier of enzymatic properties.

Related to the alteration of enzymatic properties for the two myosin conformations is the observed change of ATP-induced tryptophan fluorescence mentioned above[8]. Earlier studies[10,13] have shown that the intrinsic fluorescence of smooth muscle myosin is enhanced by the addition of ATP, as it is with skeletal muscle myosin. The amount of ATP required for the fluorescence enhancement is stoichiometric with the concentration of myosin heads and, therefore, indicates binding of ATP at the hydrolytic sites, since these are the only sites with appropriate binding affinity. In addition, the viscosity, or conformational, transition is facilitated by the binding of ATP to the same sites. Thus the binding of ATP to the high affinity hydrolytic sites can induce both a fluorescence enhancement and a conformational transition. Since the latter is associated with different fluorescence intensities it is reasonable to conclude that the active sites, as portrayed by the tryptophan fluorescence, are altered in the 6S and 10S conformations.

e) Shape-activity hypothesis

In the previous section it was documented that the biological activities to the 10S and 6S forms of myosin are different and it is tempting to speculate that this transition, or some component of it might be involved in the regulation of the contractile apparatus in smooth muscle. The enymatic activity that is subject to regulation in vivo is the actin-activated ATPase activity of myosin and most of the experiments discussed above were for technical reasons carried out in the absence of actin. The major contribution derived from these experiments is the realization that the two conformations of myosin possess different enzymatic properties. Less compelling supportive evidence is the similarity of the phosphorylation-dependence for the conformational change and for the activation of actin-activated myosin ATPase activity; both processes show a non-linear phosphorylation-dependence and a requirement for double phosphorylation. More convincing evidence in support of the 'shape-activity' hypothesis, however, is obtained from experiments done at varying Mg^{2+} concentrations (M. Ikebe, R. Barsotti, S. Hinkins, and D. J. Hartshorne, unpublished observations). With phosphorylated myosin increasing $MgCl_2$ concentrations between 1 and 10 mM (ATP constant at 1 mM) caused a conversion of 10S to 6S myosin and a concommittant increase in actin-activated ATPase activity. In these experiments the extent of actin-activa-

tion was inversely proportional to the percentage of the 10S component. With dephosphorylated myosin relatively high concentrations of $MgCl_2$ (up to 40 mM) caused the conversion of 10S to 6S myosin as judged by viscosity, sedimentation velocity and fluorescence measurements, and this transition again was accompanied by an increase in actin-activated ATPase activity. A tentative conclusion from these results is that the 10S conformation represents an 'inactive' form of myosin and that activation of the contractile system requires the transition to the 'active' 6S species. That a similar process might occur in vivo is suggested by the findings that high levels of $MgCl_2$ (8–20 mM) can induce tension in skinned smooth muscle fibers in the absence of Ca^{2+} and that this is not accompanied by an increase in the level of myosin phosphorylation. Thus under some conditions high Mg^{2+} concentrations can mimic the effects of phosphorylation and can promote the formation of 6S myosin and can also activate the contractile process. The similar effects of phosphorylation and high Mg^{2+} levels is not observed with heavy meromyosin and it may be that Mg^{2+}-dependence is lost on proteolysis.

A point of considerable interest is whether the 6S-10S transition involves the formation of a stable intermediate, or whether the only two states allowed are the folded and extended forms. For examples, what is the conformation of myosin with one light chain phosphorylated? The viscosity results show a slight alteration in hydrodynamic properties, but this could be caused either by a shift in the 6S to 10S equilibrium or by a partial opening, or unfolding, of the 10S state. Another variable that must also be considered with respect to its influence on myosin conformation is the binding of Ca^{2+} by myosin. If a stable intermediate conformation could be identified it would be important to define its actin-binding and enzymatic properties. One objective for these studies would be to attempt a correlation between myosin conformation and the non-cycling attached crossbridges, since the latter cannot be explained with existing biochemical data.

A major reservation of this hypothesis is that, with the exception of the skinned fiber experiments, all of the evidence is derived from in-vitro studies using predominantly monomeric myosin. It is known that in relaxed and contracting smooth muscle myosin exists in the filamentous state[23] and therefore for the theory to have any utility it must be shown to occur in myosin filaments. Measurements of physical parameters in macromolecular aggregates is technically difficult and as yet there is no direct evidence to suggest that the conformation of filamentous myosin is different in the relaxed and contracted states. Furthermore, it is unlikely that the folded 10S state can exist in stable myosin filaments since the length of myosin rod available for packing into the filament structure is drastically reduced and is only about one third of the length of the myosin molecule. However, it is possible that instead of the intramolecular interactions allowed in the monomeric 10S state intermolecular interactions between suitably aligned adjoining molecules could generate an analogous conformation. Under these conditions one might visualize that crossbridge motions are restricted by intermolecular interactions and that the formation of the 6S, or equivalent, allows crossbridge cycling.

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2 Adelstein, R.S, and Eisenberg E., Regulation and kinetics of the actin-myosin-ATP interaction. A. Rev. Biochem. *49* (1980) 921–956.
3 Chacko, S., and Rosenfeld A., Regulation of actin-activated ATP hydrolysis by arterial myosin. Proc. natn. Acad. Sci. USA *79* (1982) 292–296.
4 Craig, R., Smith, R., and Kendrick-Jones, J., Light-chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. Nature *302* (1983) 436–439.
5 Dabrowska, R., Hinkins, S., Walsh, M.P., and Hartshorne, D.J., The binding of smooth muscle myosin light chain kinase to actin. Biochem. Biophys. Res. Commun. *107* (1982) 1524–1531.
6 Dillon, P.F., Aksoy, M.O., Driska, S.P., and Murphy, R.A., Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science *211* (1981) 495–497.
7 Ikebe, M., Hinkins, S., and Hartshorne, D.J., Correlation of enzymatic properties and conformation of smooth muscle myosin. Biochemistry *22* (1983) 4580–4587.
8 Ikebe, M., Hinkins, S., and Hartshorne, D.J., Correlation of intrinsic fluorescence and conformation of smooth muscle myosin. J. biol. Chem. *258* (1983) 14770–14773.
9 Ikebe, M., Ogihara, S., and Tonomura, Y., Nonlinear dependence of actin-activated Mg^{2+}-ATPase activity on the extent on phosphorylation of gizzard myosin and H-meromyosin. J. Biochem., Tokyo *91* (1982) 1809–1812.
10 Ikebe, M., Onishi, H., and Tonomura, Y., Difference between smooth and skeletal muscle myosins in the stoichiometry of their reactions with ATP: Identical and nonidentical two-headed structures of smooth and skeletal muscle myosins. J. Biochem., Tokyo *91* (1982) 1855–1873.
11 Ngai, P.K., Carruthers, C.A., and Walsh, M.P., Isolation of the native form of chicken gizzard myosin light-chain kinase. Biochem. J. *218* (1984) in press.
12 Nonomura, Y., and Ebashi, S., Calcium regulatory mechanism in vertebrate smooth muscle. Biomed. Res. *1* (1980) 1–14.
13 Onishi, H., Possible role of ATP in dimerization of myosin molecules from chicken gizzard muscle. J. Biochem., Tokyo *91* (1982) 157–166.
14 Onishi, H., Suzuki, H., Nakamura, K., Takahashi, K., and Watanabe, S., Adenosine triphosphatase activity and 'thick filament' formation of chicken gizzard myosin in low salt media. J. Biochem., Tokyo *83* (1978) 835–847.
15 Onishi, H., and Wakabayashi, T., Electron microscopic studies of myosin molecules from a chicken gizzard muscle: The formation of the intramolecular loop in the myosin tail. J. Biochem., Tokyo *92* (1982) 871–879.
16 Onishi, H., Wakabayashi, T., Kamata, T., and Watanabe, S., Electron microscopic studies of myosin molecules from chicken gizzard muscle II: The effect of thiophosphorylation of the 20K-Dalton light chain on the ATP-induced change in the conformation of myosin monomers. J. Biochem., Tokyo *94* (1983) 1147–1154.
17 Persechini, A., and Hartshorne, D.J., Phosphorylation of smooth muscle myosins: Evidence for cooperativity between the myosin heads. Science *213* (1981) 1383–1385.
18 Persechini, A., and Hartshorne, D.J., Ordered phosphorylation of the two 20,000 molecular weight light chains of smooth muscle myosin. Biochemistry *22* (1983) 470–476.
19 Scholey, J.M., Taylor, K.A., and Kendrick-Jones, J., Regulation of nonmuscle myosin assembly by calmodulin-dependent light chain kinase. Nature *287* (1980) 233–235.
20 Smith, R.C., Cande, W.Z., Craig, R., Tooth, P.J., Scholey, J.M., and Kendrick-Jones, J., Regulation of myosin filament assembly by light chain phosphorylation. Phil. Trans. R. Soc. Lond. B *302* (1983) 73–82.
21 Sobieszek, A., Vertebrate smooth muscle myosin. Enzymatic and structural properties, in: The Biochemistry of Smooth Muscle, pp. 413–443. Ed. N.L. Stephens. University Park Press, Baltimore 1977.

22 Sobue, K., Morimoto, K., Kanda, K., Fukunaga, K., Miyamoto, E., and Kakiuchi, S., Interaction of 135000-M, calmodulin-binding protein (myosion kinase) and F-actin: another Ca^{2+}- and calmodulin-dependet flip-flop switch. Biochem. Int. *5* (1982) 503–510.
23 Somlyo, A.V., Butler, T.M., Bond, M., and Somlyo, A.P., Myosin filaments have non-phosphorylated light chains in relaxed smooth muscle. Nature *294* (1981) 567–569.
24 Suzuki, H., Kamata, T., Onishi, H., and Watanabe, S., Adenosine triphosphate-induced reversible change in the conformation of chicken gizzard myosin and heavy meromyosin. J. Biochem., Tokyo *91* (1982) 1699–1705.
25 Suzuki, H., Onishi, H., Takahashi, K., and Watanabe, S., Structure and function of chicken gizzard myosin. J. Biochem., Tokyo *84* (1978) 1529–1542.
26 Suzuki, H., Takahaski, K., Onishi, H., and Watanabe, S., Reversible changes in the state of phosphorylation of gizzard myosin, in that of gizzard myosin assembly, in the ATPase activity of gizzard myosin, in that of actomyosin and in the superprecipitation activity. J. Biochem., Tokyo *91* (1982) 1687–1698.
27 Trybus, K.M., Huiatt, T.W., and Lowey, S., A bent monomeric conformation of myosin from smooth muscle. Proc. natn. Acad. Sci. USA *79* (1982) 6151–6155.
28 Walsh, M.P., and Hartshorne, D.J., Actomyosin of smooth muscle, in: Calcium and Cell Function, vol. III, pp. 223–269. Ed. W.Y. Cheung. Academic Press, New York 1982.
29 Walthers, M., and Marston, S.B., Phosphorylation of the calcium ion-regulated thin filaments from vascular smooth muscle. Biochem. J. *197* (1981) 127–139.

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Velocity and myosin phosphorylation transients in arterial smooth muscle: effects of agonist diffusion[1]

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Summary. Transients in myoplasmic [Ca^{2+}] and in phosphorylation of the 20,000 dalton light chain of myosin have been reported following stimulation of vascular smooth muscle by various agonists. Since these transients are rapid compared with the time required to attain a steady-state stress, agonist diffusion rates may be a significant limitation in activation. The purpose of this study was to estimate the effect of agonist diffusion rates on the time course of activation as assessed by mechanical measurements of stress development and isotonic shortening velocities and by determinations of the time course of myosin phosphorylation. The approach was to measure these parameters in K^+-stimulated preparations of the swine carotid media of varying thicknesses and to estimate the theoretical contributions imposed by diffusion rates and the presence of a diffusion boundary layer surrounding the tissue. The results show that the time course of parameters which are tissue averages such as stiffness, active stress, and myosin phosphorylation is dominated by agonist diffusion rates. The sequence of events involved in excitation-contraction coupling including agonist actions on the cell membrane, Ca^{2+} release, activation of myosin light chain kinase, and cross-bridge phosphorylation appear to be very rapid events compared with stress development. Estimates of unloaded or lightly loaded shortening velocities which are not simple tissue averages appear to provide an improved estimate of activation rates.
Key words. Myoplasmic Ca^{2+}; smooth muscle mechanics; active state; potassium depolarization; myosin light chain; swine carotid artery.

Introduction

The kinetics of activation for mammalian smooth muscle tissues in vitro are a function of the rates of several processes: 1) agonist diffusion and action on the cell membrane; 2) an increase in myoplasmic [Ca^{2+}] following release from intracellular stores or influx through the plasma membrane; 3) cross-bridge activation which appears to be a consequence of Ca^{2+}-calmodulin-induced activation of myosin light chain kinase and phosphorylation of the 20,000 dalton myosin light chain[7,18,24]; and 4) cross-bridge cycling resulting in stress development or shortening.

Stimulation of intact smooth muscle strips is associated with increased phosphorylation of myosin, maximal values of which are reached in less than one minute. The initial peak levels of phosphorylation usually decline in time, while active stress rises monotonically to a maintained steady state[6,10,17,33]. It has been proposed that agonist-induced transients in cell calcium concentration[29] can explain a rapid, large phosphorylation transient in the smooth muscle cell[3].

Estimates of myosin light chain phosphorylation are tissue averages. A rapid phosphorylation transient in cells on the edge of the tissue may be virtually complete before the inwardly diffusing agonist reaches excitatory concentrations in the center of the strip. The purpose of the present study was to evaluate the contribution of agonist diffusion times to estimates of rates and magnitudes of activation measured in isolated tissues. The results indicate that 1) the onset of phosphorylation in vascular smooth muscle cells is a very rapid event following stimulation; and 2) that the early time course of parameters such as myosin phophorylation and stress development, which are tissue averages, will be dependent upon agonist diffusion times.

Methods

1. Solutions

The standard physiological salt solution (PSS) used in these studies contained (mM): NaCl, 140.1; KCl, 4.7; Na_2HPO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.6; D-glucose, 5.6;