Vascular Smooth Muscle

Calmodulin and Cyclic AMP-Dependent Protein Kinase Alter Calcium Sensitivity in Porcine Carotid Skinned Fibers

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SUMMARY. Recent work on vascular smooth muscle actomyosin has indicated that the Ca\(^{2+}\) sensitivity of both ATPase and superprecipitation are affected by calmodulin (CaM) and cyclic AMP-dependent protein kinase (cPK). Using a “chemically skinned” arterial preparation, we have extended these observations to the intact structured contractile system. Media from hog carotid artery were skinned with 1% Triton X-100 followed by a 50% glycerol-ATP salt solution, in which the strips were stored at −25°C. Small strips (thickness between 0.1 and 0.2 mm) were mounted isometrically and relaxed in a Mg-ATP salt solution, pH 6.7, Ca\(^{2+}\) 10\(^{-8}\) M, 30°C. Ca\(^{2+}\) elicited a contraction with an ED\(_{50}\) of 10\(^{-7}\) M. Isometric force was between 1 and 4 mM, consistent with the force observed before skinning. With time, the preparation became less sensitive with an increase in ED\(_{50}\) to 10\(^{-5}\) M. CaM (4 \(\mu\)M) reverses this loss, stabilizes the preparation, and sharply accelerates the rate of tension development. The ED\(_{50}\) in the presence of 4 \(\mu\)M CaM shifts to about 10\(^{-7}\) M. This effect is dose-dependent, with the half maximal effect at about 0.4 \(\mu\)M CaM. Submaximal Ca\(^{2+}\) contractions can be reversibly depressed by preincubation of relaxed fibers with cPK catalytic subunit (300 U/ml), even in the presence of 4 \(\mu\)M CaM. An inhibition of about 50% of the contraction at 0.2 \(\mu\)M Ca\(^{2+}\) was obtained, whereas only 20% inhibition was found at 6 \(\mu\)M Ca\(^{2+}\). Our findings suggest that changes in vascular contractility cannot be described solely in terms of changes in cytoplasmic Ca\(^{2+}\) , and that changes in the sensitivity of the contractile protein to a given Ca\(^{2+}\) concentration are also potential mechanisms for vasodilation. (Circ Res 50: 394–399, 1982)

Both vascular smooth muscle actomyosin (Sparrow et al., 1970) and contractility of demembranated (skinned) vascular smooth muscle (Filc et al., 1965) are activated by trace Ca\(^{2+}\) in the micromolar range. Although many aspects of calcium regulation in smooth muscle remain controversial (Ebashi, 1980), it is now clear that calcium binds to calmodulin (Klee et al., 1980) and that this complex activates the myosin light chain kinase (MLCK) phosphorylating the 20,000-dalton myosin light chains (Dabrowska et al., 1978; Blumenthal and Stull, 1980). As observed in chicken gizzard actomyosin (Aksoy et al., 1976; Chacko et al., 1977; Sobieszek, 1977; Sherry et al., 1978), vascular smooth muscle actomyosin ATPase activity often appears to be correlated with the extent of myosin light chain phosphorylation (DiSalvo et al., 1978, Mrwa et al., 1980), and it would appear that myosin phosphorylation is a prerequisite for interaction of actin and myosin (Adelstein and Eisenberg, 1980). Cyclic AMP (cAMP)-dependent protein kinase is also known to play a role in this enzyme cascade by phosphorylating the myosin light chain kinase (Adelstein et al., 1978). The interaction of calcium and calmodulin with myosin light chain kinase is weakened by this phosphorylation, resulting in an inhibition of the myosin light chain kinase activity (Adelstein and Hathaway, 1979). This may result in a reduction of myosin light chain phosphorylation in vascular smooth muscle actomyosin which is associated with an inhibition of actomyosin ATPase (Silver and DiSalvo, 1979; Mrwa et al., 1979). At high free calcium and calmodulin concentrations however, this inhibition can be overcome (Silver et al., 1981).

It has been suggested by Adelstein and Hathaway (1979) and Silver and DiSalvo (1979) that the cAMP-mediated effects just described for isolated actomyosin may play a role in β-adrenergic relaxation of vascular smooth muscle (VSM). In view of this possibility, it seemed desirable to test directly whether cyclic AMP-dependent protein kinase and calmodulin affect the contractility of demembranated VSM. Experiments with skinned VSM complement biochemical studies on the regulation of actomyosin ATPase and provide a link to the mechanism of regulation of actin-myosin interaction in the intact structured muscle. This is of particular interest, as recent experiments on living VSM (Dillon et al., 1981) indicate that isometric force may not necessarily be correlated with the phosphorylation state of the myosin light chains.

Methods

Skinned VSM Preparation

Strips of hog carotid media were dissected as described by Glück and Paul (1977) and skinned by a modification of the procedure of Gordon (1978). They were incubated in 20 mM imidazole, 5 mM EGTA, 50 mM KCl, and 150 mM sucrose (pH 7.4) for 30 minutes at 0°C, then for a further 30 minutes in the same solution with the addition of 1%
Triton X-100, 0.5 mM dithioerythritol (DTE), and 2 μg/ml leupeptin. They were then incubated for 5 minutes at 0°C in a solution of 20 mM imidazole, 4 mM EGTA, 10 mM MgCl₂, 7.5 mM ATP, 1 mM NaN₃, 0.5 mM DTE, and 2 μg/ml leupeptin, at pH 6.7, mixed with 50% glycerol (cf Peterson, 1981) and then stored at −25°C in this solution. Strips used after overnight storage or stored for up to 4 weeks showed similar behavior.

Isometric Force Measurements

Bundles of fibers about 5 mm long and between 100 and 200 μm thick were teased out of the strips and attached horizontally with a cellulose-based glue between an adjustable glass rod mounted on a micrometer drive and a short rod extending from a Statham (UC2) force transducer. Absolute force production by the fiber bundles was 0.5–4 mN. Tissues were bathed in appropriate solutions in 0.4-ml Lucite chambers at 30°C. Compliance of the apparatus amounted to 0.1% shortening at maximum isometric force. The stability of the transducer was on the order of ± 10 μm over the experimental period.

Experimental Protocol

Fibers were bathed in a “relaxing solution” containing (mm) K⁺, 21; Na⁺, 36; Mg (total), 10; EGTA, 4; ATP, 7.5; imidazole, 20; Cl⁻, 35, azide, 1; and an ATP-regenerating system consisting of 10 mM phosphocreatine and 10 U/ml of creatine phosphokinase; pH of the final solution was 6.7. Fibers were stretched (approximately 5%) to a preload between 0.1 to 0.3 mN which was found to be optimal for isometric force. Contraction was induced by partly replacing EGTA with Ca-EGTA, thereby increasing the free Ca²⁺.

The concentration of free Ca²⁺ was calculated from the apparent binding constant for EGTA of 1.2 × 10⁶ M (Portzehl et al., 1964). Catalytic subunit of cAMP-dependent protein kinase from beef heart was obtained from Sigma and was gifts from Dr. J. DiSalvo, Department of Physiology, University of Cincinnati College of Medicine.

Results

Effects of Calmodulin

Skinned carotid arteries incubated in the relaxing solution containing EGTA contract isometrically in response to an increase in the Ca-ion concentration; the half maximum response was found to occur about 10⁻⁶ M calcium. Relaxation occurs when Ca-ion concentration is lowered to less than 10⁻⁸ M by the addition of EGTA. When such concentration/relaxation cycles were repeated several times, the preparations became less calcium sensitive, the ED₅₀ for isometric force was found to change by about 0.3 pCa unit. Figure 1a shows the decreasing response to a given submaximum concentration of Ca-ions. The addition of calmodulin at a concentration of 4 μM, as shown in Figure 1b, was found to reverse the loss of responsiveness to Ca²⁺. Since the maximum isometric force also showed a tendency to decrease after repeated contraction/relaxation cycles (Fig. 1a), myosin light chain kinase was added in an attempt to reverse this loss of force. Figure 2 shows, however, that the decrease cannot be prevented or reversed by the addition of myosin light chain kinase. The addition of calmodulin to a concentration of 4 μM on the other hand was found to quickly reverse the loss of Ca²⁺ sensitivity. It can also be seen in Figures 1 and 2 that the rate of tension development with repeated contraction/relaxation cycles becomes increasingly slower and that addition of calmodulin (4 μM) accelerates the rate of tension development.

Calmodulin was found to have a much larger effect of an isometric force at submaximum Ca-ion concentrations (e.g., 0.34 μM) than at optimal Ca-ion activation (compare a and b, Figure 3). From such cumulative responses to graded Ca-ion concentration, dose-response curves could be constructed as shown in Figure 3c. In the absence of external calmodulin, the threshold for eliciting contraction is about 3 × 10⁻⁷ M Ca²⁺, and half-maximum activation occurs at 10⁻⁶ M. In the presence of 4 μM calmodulin, the calcium dose-response relationship is shifted to the left by about 1 pCa unit; half-maximum activation now occurs at about 10⁻⁷ M Ca²⁺. The effect of calmodulin was found to be dose dependent; as shown in Figure 4,
FIGURE 2. Restoration of calcium responsiveness by calmodulin in the presence of myosin light chain kinase. Repeated contractions elicited at 1, 4, 6, and 9 with $10^{-4}\text{M}\ Ca^{2+}$. Note that the addition of myosin light chain kinase [activity in bath 4 (pmol p./min/ml)] added at 3 had no effect on calcium responsiveness, whereas the addition of 4 μM calmodulin (CaM, at 8) increased responsiveness.

Effects of Catalytic Subunit of cAMP-dependent Protein Kinase

The submaximum response elicited by a given Ca-ion concentration can be reversibly depressed by pre-incubation of relaxed fibers with the catalytic subunit of protein kinase (300 units per ml), even in the presence of calmodulin 4 μM, as shown in Figure 5. The inhibition at $2 \times 10^{-6}\text{M}\ Ca^{2+}$ is about 50% and can be gradually reversed after washing out the catalytic subunit in the presence of calmodulin. At $6 \times 10^{-6}\text{M}\ Ca^{2+}$, the inhibition produced by the catalytic subunit was only 20%. The fact that inhibition is more pronounced at a lower Ca-ion concentration suggests that the Ca sensitivity has been depressed. The changing Ca sensitivity is illustrated in Figure 6, in which the force response is plotted, vs. Ca-ion concentration before and after incubation with catalytic subunit in the presence of calmodulin. In the presence of calmodulin, without the addition of catalytic subunit, the Ca sensitivity of the preparation over repeated contraction/relaxation cycles was found to be constant.

Discussion

The calcium sensitivity, defined as the pCa (negative logarithm of the Ca$^{2+}$ concentration) required for half maximum activation of force production, was found to decrease in skinned carotid smooth muscle when contraction/relaxation cycles were repeated several times. This loss of sensitivity appears to be due to loss of calmodulin, since it can be prevented by the addition of exogenous calmodulin at about 0.1 μM. Larger concentrations (1-4 μM) of calmodulin were found to increase calcium sensitivity from pCa 6 to pCa 7. Since living smooth muscle is reported to contain at least 5 μM calmodulin (Adelstein and Eisenberg, 1980) it seems likely that the intracellular calcium ion concentration required for a threshold contractile response is presumably less than 0.05 μM. This is considerably less than previously found in glycerinated smooth muscle (Filo et al., 1965). As also reported for skinned guinea pig Taenia coli (Sparrow et al., 1981), the inverse relationship between calmodulin concentration and calcium-ion concentration required for threshold contraction suggests that the extent of activation of force in intact smooth muscle depends on the concentration of the calcium-calmodulin complex rather than on that of free calcium alone. Such a relation for the activation of purified myosin light chain kinase has been reported by Blumenthal and Stull (1980) and DiSalvo et al. (1981). However, the calmodulin concentrations required to produce a half maximum effect in our arterial preparations (i.e., for a calcium sensitivity increase of about 0.5 pCa units) was found to be much higher than in skinned Taenia coli and in the experiments of Blumenthal and Stull (1980). This discrepancy may be due in part to differences in the diffusivity of calmodulin between preparations.

In studies using purified proteins the interaction of the calcium-calmodulin complex with myosin light chain kinase (MLCK) can be weakened (Adelstein and Hathaway, 1979) when the MLCK is phosphorylated at site 1 and site 2 (Conti and Adelstein, 1981) by the action of a cAMP-dependent protein kinase. Under these conditions, light chain kinase activity, as well as actomyosin ATPase activity (Silver and DiSalvo, 1979; Mrwa et al., 1979), chicken gizzard (Kerrick and Hoar, 1981), or arterial smooth muscle (this paper), is inhibited. Because of the inhibition of calcium-calmodulin interaction with myosin light chain kinase, the calcium ion concentration required for half maximum activation of actomyosin ATPase (Silver et al., 1981) and skinned fibers is increased even in the presence of a fixed concentration of calmodulin. Conversely, tension production is inhibited by more than 50% at a given submaximal concentration of Ca$^{2+}$ in the presence of calmodulin when the fiber bundle...
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FIGURE 3. a: Cumulative contractile response to Ca²⁺ in the absence of calmodulin. The preparation was relaxed in ATP salt solution at 10⁻⁸ M Ca²⁺; cumulative contractions were elicited with Ca²⁺, at 1: 0.34 μM Ca²⁺; at 2: 1.2 μM Ca²⁺; at 3: 6.6 μM Ca²⁺; at 4: relaxation induced by lowering Ca²⁺ to 10⁻⁸ M. b: Cumulative response to Ca²⁺ in presence of calmodulin (4 μM). The Ca-ion concentrations were 1: 0.09 μM Ca²⁺; 2: 0.2 μM Ca²⁺; 3: 0.34 μM Ca²⁺; 4: 0.53 μM Ca²⁺; at 5: relaxation induced by lowering Ca²⁺ to 10⁻⁸ M. c: Effect of calmodulin on calcium responsiveness of hog carotid skinned smooth muscle fibers. Force (relative values given on ordinate) in relation to Ca-ion concentration (μM) in presence of calmodulin (filled circles) and in the absence of calmodulin (open circles; only experiments on first contraction cycle). The results obtained from several skinned carotid artery strips were normalized with respect to maximum active force.

These results demonstrate that actin-myosin interaction in the structured muscle can be inhibited without reducing the Ca²⁺ concentration. Although the observed inhibition was clearly reduced at high Ca²⁺ concentration, there is some evidence that such a mechanism may be operable in living vascular smooth muscle. Activation of glycogen phosphorylase, a mechanism known to be absolutely dependent on free calcium (Cohen et al., 1980) has been reported to be nearly maximal in the presence of β-adrenergic agonists which fully relaxed porcine coronary vessels (Paul and Doerman, 1980) and rat portal vein (Hellstrand and Paul, 1980). The operation of such a mechanism would, however, appear to be secondary to a reduction of the intracellular free calcium ion concentration to an "intermediate level," perhaps as a consequence of a cAMP-stimulated calcium sequestration (Mueller and van Breemen, 1979). For, as shown by Conti and Adelstein (1981), only at low concentrations of free calcium can the MLCK be phosphorylated at both ‘site 1’ and ‘site 2’ and thus inhibited by the cAMP-dependent protein kinase. However, at high concentrations of calcium-calmodulin, when all the MLCK sites are occupied by calmodulin, the kinase cannot be phosphorylated at site 2, which is required for inhibition. Our results, which indicate a pronounced inhibition at low Ca²⁺ calmodulin concentration and little inhibition of contractility at high calcium-calmodulin concentration, are consistent with the operation of this mechanism in skinned arterial smooth muscle. Our findings suggest that relaxation had been preincubated in the presence of cAMP-dependent protein kinase. Since this enzyme is also present in living arterial muscle (Silver and DiSalvo, 1979) it is not surprising that cyclic AMP added to skinned fibers exerts an effect on calcium sensitivity similar to that of the addition of the catalytic subunit (Ruegg et al., 1981). Extrapolating these findings to the situation in vivo, one might anticipate that cAMP-dependent mechanisms could serve to regulate or modulate the sensitivity of the contractile system to intracellular calcium ion concentration as in the case of heart muscle (Holroyde et al., 1979).

FIGURE 4. Dose-dependent increase in calcium responsiveness by calmodulin in presence of 0.3 μM Ca²⁺. Ordinate: Maximum active force in percent of active force obtained during maximum activation (10⁻⁶ M Ca²⁺). Abscissa: Added calmodulin (μM); note logarithmic scale, with break in scale for plotting the point corresponding to zero exogenous calmodulin, at which the calcium response was just threshold.
of contraction in vascular smooth muscle cannot be described solely in terms of decreasing cytoplasmic free Ca\(^{2+}\). Changes in the sensitivity of the contractile proteins at a given Ca\(^{2+}\) concentration are also potential mechanisms for vasodilation.

**References**


Adelstein RS, Hathaway DR (1979) Role of Ca\(^{2+}\) and cyclic AMP in regulation smooth muscle contraction. Am J Cardiol 44: 783-786


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Figure 5. Contractions elicited by 0.2 μM Ca\(^{2+}\) in the presence of 4 μM calmodulin at 1 before the addition of the catalytic subunit; at 4 immediately after preincubation with the catalytic subunit (c.s., 300 units per ml at 3), and at 6 after washing out the catalytic subunit. The contractions were terminated at (2 and 5) by lowering Ca\(^{2+}\) to 10^{-6} M.

Figure 6. Effect of catalytic subunit of cAMP-dependent protein kinase on calcium response to Ca\(^{2+}\) (m) (shown as a percent of maximum active contractile force) in the presence of 4 μM calmodulin. Filled symbols: Responses before the addition of the catalytic subunit. Open symbols: Responses to Ca\(^{2+}\) immediately after preincubation with catalytic subunit; crosses: after washing out the catalytic subunit.
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Mueller E, van Breemen C (1979) Role of intracellular Ca\textsuperscript{2+}-sequestration in \textbeta-\textalpha-renergic relaxation of a smooth muscle. Nature (Lond) 281: 682-683


Portzehl H, Caldwell PC, Ruegg JC (1964) The dependence of contraction and relaxation of muscle fibres from the crab Maja squinado on the internal concentration of free calcium. Biochim Biophys Acta 79: 581-591


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