Myosin Phosphorylation and Cyclic Adenosine 3',5'-Monophosphate in Relaxation of Arterial Smooth Muscle by Vasodilators

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SUMMARY. Recent evidence indicates that contraction of vascular smooth muscle may be regulated by two calcium-dependent mechanisms: activation of myosin kinase, and calcium binding to a second, unknown regulatory site. This hypothesis implies that vasodilators could modify vascular tone by several mechanisms, including inactivation of myosin kinase. Since relaxation of the carotid artery following agonist removal may occur when myosin phosphorylation is at resting levels, we could determine whether dephosphorylation of myosin is necessarily involved in the molecular mechanisms mediating relaxation in response to vasodilators. The relaxant effects of adenosine, 3-isobutyl-l-methylxanthine, forskolin, sodium nitroprusside, and 8-bromo-cGMP were tested under conditions where myosin phosphorylation was at basal levels (0.08 ± 0.02 mol P_i/mol light chain). All of these agents increased the rate of relaxation in non-steady state experiments where relaxation was induced by stimulus washout. Steady state dose-response curves were obtained for forskolin and 8-bromo-cGMP in the presence of basal myosin phosphorylation. Forskolin caused a dose-dependent increase in cAMP levels at a rate consistent with a cause and effect relationship between relaxation and total tissue cAMP content. Both drugs relaxed the muscles, with no detectable change in myosin phosphorylation. Therefore, dephosphorylation of myosin is not a necessary event in the molecular mechanism of several vasodilators, including some which presumably act via cyclic nucleotides. (Circ Res 54: 83-89, 1984)

BIOCHEMICAL studies of contractile proteins isolated from smooth muscles show that myosin phosphorylation activates actomyosin ATPase (Hartshorne and Siemankowski, 1981; Stull et al., 1980). Experiments using chemically skinned smooth muscle fibers demonstrate a good correlation between Ca^{++}-dependent force development and myosin phosphorylation (Hoar et al., 1979; Chatterjee and Murphy, 1983). Such evidence suggests that myosin phosphorylation is necessary and sufficient for contraction of intact smooth muscle, and implies that dephosphorylation would inevitably lead to relaxation. However, in several intact preparations of smooth muscle, there is substantial evidence that myosin phosphorylation is not always proportional to active stress. Significant dephosphorylation occurs in activated muscles while active stress remains constant (Dillon et al., 1981; Butler and Siegman, 1982; Silver and Stull, 1982; Gerthoffer and Murphy, 1983a). Recent studies of the swine carotid artery provide evidence for a dual regulatory system where active stress is determined by Ca^{++} acting at two sites (Aksoy et al., 1983; Gerthoffer and Murphy, 1983b; Chatterjee and Murphy, 1983): (1) Ca^{++} binds to calmodulin and activates myosin kinase leading to phosphorylation of the 20,000 dalton myosin light chains, and (2) Ca^{++} acts at a second, unknown regulatory site to maintain crossbridge attachment when myosin is dephosphorylated.

If myosin phosphorylation is both necessary and sufficient for smooth muscle contraction, then inhibition of myosin kinase should prevent force development or relax contracting vessels. The net myosin kinase activity in a smooth muscle cell at any given time depends on several factors: the myoplasmic Ca^{++} concentration ([Ca^{++}]), formation of the Ca^{++}-calmodulin-myosin kinase complex, and the degree of phosphorylation of myosin kinase (Adelstein and Hathaway, 1979; Silver and DiSalvo, 1979; Conti and Adelstein, 1981). Each of these may be a direct or indirect site of action of vasodilators. The affinity of myosin kinase for the Ca^{++}-calmodulin complex decreases when this enzyme is phosphorylated by a cAMP-dependent protein kinase, and myosin kinase activity is reduced if the Ca^{++}-calmodulin concentration is limiting (Conti and Adelstein, 1981). Adelstein and Hathaway (1979) suggested that β-adrenergic agonists may relax smooth muscles by increasing cAMP, phosphorylating myosin kinase, and, thus, reducing myosin phosphorylation. There are also cGMP-dependent protein kinases in smooth muscles, and there is some overlap in the substrates for these two kinases (cf. Hardman, 1981). Thus, relaxation mediated by cGMP might also involve covalent modification of myosin kinase and dephosphorylation of myosin.

We tested the hypothesis that dephosphorylation of myosin is necessary in the molecular mech-
anism of action of several vasodilators. The agents tested were adenosine, 3-isobutyl-1-methylxanthine (IBMX), forskolin, sodium nitroprusside (SNP), and 8-bromo-cGMP (Br-cGMP).

Methods

Tissue Preparation

Carotid arteries were obtained from swine after slaughter and stored overnight in cold (0-4°C) physiological salt solution (PSS). The PSS contained (mM): NaCl, 140; KCl, 4.7; MgSO4, 1.2; CaCl2, 1.6; Na2HPO4, 1.2; morpholinepropanesulfonic acid, 2.0; Na2EDTA, 0.02; and d-glucose, 5.6. Thin medial strips were prepared as illustrated by Driska et al. (1981). Before separating the media from the adventitia, we dissected away the intima and 30-50% of the media. This produced a rectangular preparation of medial smooth muscle 0.35 ± 0.02 mm thick (n = 20). Strips were mounted for measurement of isometric contraction and myosin phosphorylation, as described previously (Driska et al., 1981). All strips were initially stretched to a length producing a stress of about 1 x 10^5 N/m² tissue cross-sectional area, and then allowed to equilibrate at 37°C in PSS gassed with 100% O2 for 90 minutes prior to any experimental treatment. We determined the optimum length (L0 = 9.6 ± 0.3 mm, n = 20) for steady state active stress development from partial length-tension curves (Herlihy and Murphy, 1973). K+·PSS (110 mM KCl substituted isosmotically for NaCl) was the stimulus used for the length-tension curves. Tissue cross-sectional area and active stress were calculated as described previously (Dillon et al., 1982).

Experimental Protocols

Two protocols were used to assess the efficacy of the vasodilators:

Non-Steady State Protocol

Muscles were stimulated for 15 minutes with either K+·PSS or 10^-5 M phenylephrine (a maximum dose). The agonist was then washed out with normal PSS to produce relaxation. There was an initial rapid phase of relaxation, lasting about 2 minutes, which was associated with diffusion of agonist from the muscle, and, in the case of K+, dephosphorylation of myosin to basal levels (Gerthoffer and Murphy, 1983b). This was followed by a slow phase of relaxation lasting up to 45 minutes, which reflects detachment of nonphosphorylated crossbridges, which we have termed latchbridges (Dillon et al., 1981, 1982). This slow phase occurs with basal levels of myosin phosphorylation (0.07-0.10 mol Pj/mol 20,000 dalton myosin light chain) and appears to be limited by Ca++ sequestration (Gerthoffer and Murphy, 1983b). We tested the effect of vasodilators on the slow phase by adding ED50 concentrations—or, in the case of forskolin, a maximum dose—2 minutes after washout of the agonist. The experimental protocol is illustrated in Figure 1. ED50 concentrations (concentrations producing 80% of the maximum relaxation) were determined from cumulative dose-response curves for each vasodilator by the protocol described below and illustrated in Figure 3.

Steady State Dose-Response Curves

Cumulative dose-response curves to forskolin and Br-cGMP were obtained by contracting the muscles for 2 minutes with K+·PSS, then partially relaxing the muscles to 71 ± 2% of the maximum steady state stress by changing the solution to one containing 20 mM KCl. The muscles were allowed to equilibrate for 45 minutes in 20 mM K+·PSS. Concentrated doses of the drugs (1% of the bath volume) were added cumulatively. In preliminary experiments, the contraction due to 20 mM K+·PSS was maintained for at least 3 hours. In these experiments, ethanol was the vehicle for forskolin, and it had no relaxant effect at the concentrations used.

Dose-response curves to forskolin were also obtained, using 10^-5 M phenylephrine as the agonist. The muscles were contracted with phenylephrine for 30-40 minutes. Forskolin, dissolved in dimethylsulfoxide, was added cumulatively. The vehicle alone had no relaxant effect. The stress induced by 10^-5 M phenylephrine was 84 ± 2% of the phenylephrine maximum and 55 ± 3% of the stress induced by 110 mM K+·PSS.

Myosin Phosphorylation Assay

Muscle strips were frozen at L0 in an acetone dry ice slurry. All frozen samples were allowed to warm at room temperature in acetone for 60 minutes, and then were homogenized in 1% sodium dodecyl sulfate, 20 mM dithiothreitol, and 10% glycerol (pH 7.2). Isoelectric variants of the 20,000 dalton light chain of myosin corresponding to the nonphosphorylated and phosphorylated forms were separated by two-dimensional gel electrophoresis (Driska et al., 1981). The gels were stained with Coomassie brilliant blue R, and after destaining were scanned to determine the fractional phosphorylation of the light chains.

cAMP Assay

The ability of forskolin to elevate cAMP was tested by adding forskolin to PSS containing free-floating strips of carotid artery. At the appropriate time, the muscles were removed from the PSS, immersed in liquid nitrogen, and homogenized in 95% ethanol, 1 mM HCl (4°C). A sample of the homogenate was removed for the protein assay (see below). The homogenate then was mixed continuously for 30 minutes to extract the cAMP completely. Particulate matter was sedimented by centrifugation at 1100 g for 30 minutes at 4°C. The supernatant was decanted and evaporated to dryness. The residue was suspended in 0.1 M HCl and assayed for cAMP by the method of Brooker et al. (1979). Recovery of a known amount of cAMP was 92 ± 4% (n = 5). The presence of the contractile agonists (20 mM K+ or 10^-5 M phenylephrine) did not alter the ability of forskolin to elevate cAMP in the free-floating strips. Forskolin (10^-4 M) did not cross-react with the radioimmunoassay for protein was assayed by the method of Lowry et al. (1951) after the acid-ethanol was evaporated and the residue was digested with 1 M NaOH. Bovine serum albumin was the protein standard. Results are expressed as pmol cAMP/mg protein.

Drugs

Adenosine, sodium nitroprusside, and Br-cGMP were dissolved in deionized water and diluted with PSS. IBMX was dissolved in 95% ethanol and diluted in PSS. Forskolin (10^-2 M) was dissolved in 95% ethanol or 10% (vol/vol) dimethylsulfoxide and diluted in PSS. Adenosine, sodium nitroprusside, Br-cGMP, phenylephrine, and IBMX were obtained from Sigma Chemical Co. Forskolin was obtained from Calbiochem-Behring.
Statistical Methods

All data are presented as mean ± 1 SEM. Differences between treatment means were tested by one-way analysis of variance. The null hypothesis was rejected when \( P < 0.05 \). Dunnett's test was used to compare the effect of a given treatment on cAMP levels to 'basal' levels. All other comparisons of treatment means were made by the Student-Newman-Keuls method (Steel and Torrie, 1960).

Results

Relaxant Effect of Adenosine

A previous study of relaxation of medial strips of swine carotid artery showed that the slow phase of relaxation after stimulus washout reflected detachment of latchbridges, and that this process was independent of myosin phosphorylation. However, this slow phase of relaxation was sensitive to the extracellular \([Ca^{++}]\) (Gerthoffer and Murphy, 1983b). If the sole mechanism of action of any vasodilator is to modify myosin kinase activity or the activity ratio of myosin kinase/phosphatase, then that vasodilator will not be effective when stress is maintained by latchbridges (i.e., dephosphorylated crossbridges). We tested this hypothesis using the protocol shown in Figure 1. Muscles were stimulated for 15 minutes with \( K^+\)-PSS, and relaxed by washing out the stimulus. The muscles relaxed in several phases: (1) a lag phase of about 20 seconds, presumably reflecting \( K^+\) diffusion from the preparation, (2) a rapid phase which correlated with the time course of myosin dephosphorylation, and (3) an extended period of stress maintenance in the presence of basal levels of myosin phosphorylation \([0.07-0.10 \text{ mol P}/\text{mol light chain} \text{ (Gerthoffer and Murphy, 1983b)}]\). The relaxation rate was markedly enhanced when \( 10^{-5} \text{ M adenosine} \) was added 2 minutes after \( K^+\) washout (Fig. 1).

Effect of Vasodilators which May Act via Cyclic Nucleotides

Several other vasodilator drugs were tested by the same protocol (Figs. 2 and 3) with either \( K^+\)-PSS or \( 10^{-4} \text{ M phenylephrine} \) as the agonist. IBMX, a phosphodiesterase inhibitor (Wells et al., 1975), and forskolin, a direct activator of adenylate cyclase (Seamon and Daly, 1981; Muller and Baer, 1983), both enhanced relaxation rates in the latch state that occurs during \( K^+\) washout (Fig. 2, panel A). We also tested two agents which may relax smooth muscles via cGMP-mediated mechanisms (Murad et al., 1978; Schultz et al., 1979). Sodium nitroprusside and Br-cGMP also increased the relaxation rate during the latch state (Fig. 2, panel B). These agents were all effective at basal levels of myosin phosphorylation. Therefore, relaxation was not the result of reduced myosin kinase activity.

The relaxant effect of \( 10^{-5} \text{ M forskolin} \) was also tested using another agonist (\( 10^{-4} \text{ M phenylephrine} \)) and the same protocol (Fig. 3). Forskolin accelerated the slow phase of relaxation. Phenylephrine was chosen for these studies because it produced the greatest active stress of several agonists tested (94 ± 1% of \( K^+\)-induced stress). The stress induced by norepinephrine, carbachol, and serotonin tended to fade with time, making these agonists unsuitable for analysis of steady state vasodilator dose-response curves.

Forskolin and cAMP Content

Forskolin is known to activate adenylate cyclase in a number of cell types including vascular smooth muscle (Seamon and Daly, 1981; Muller and Baer, 1983). We have conducted further experiments in the carotid artery preparation to determine the relationship between tissue cAMP content and relax-
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Basal myosin phosphorylation ranged from 0.07 to 0.10 mol P$_i$/mol light chain during the non-steady state protocol. Therefore, further studies were carried out with Br-cGMP and forskolin to test the possibility that dephosphorylation to less than the estimated basal values might occur. During a maintained contraction with either 20 mM K$^+$-PSS or phenylephrine, myosin phosphorylation declined to basal levels.

**FIGURE 3.** The effect of forskolin on the relaxation after phenylephrine (PE) washout. The protocol was the same as illustrated in Figure 1, except that 10$^{-4}$ M phenylephrine was the agonist. Control relaxations (●, n = 4) were obtained after 15-minute contraction with phenylephrine. Forskolin (10$^{-5}$ M) was added 2 minutes after phenylephrine removal (●, n = 4). Active stress after 15 minutes in 10$^{-4}$ M phenylephrine was 1.88 ± 0.08 × 10$^5$ N/m$^2$. This value was 94 ± 1.4% of the maximum stress induced by 110 mM K$^+$-PSS in these muscles.

**FIGURE 4.** Time course of changes in cAMP produced by forskolin. Strips of carotid media were incubated in PSS containing 10$^{-4}$ M (●), 10$^{-3}$ M (●), or 10$^{-2}$ M (●) forskolin. One set of muscles was incubated in 20 mM K$^+$-PSS containing 10$^{-5}$ M forskolin (○) to test the possibility that activation by the agonist might alter the response (n = 4 or 5).
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FIGURE 5. Time course of relaxation (O) and elevation of cAMP (D) by 10^{-7} M forskolin. cAMP data are replotted from figure 4. Carotid medial strips were contracted with 10^{-5} M phenylephrine for 15 minutes. Forskolin (10^{-3} M) was added to the bath at time = 0 minute (n = 4).

levels (Fig. 6 and 7). In one set of experiments (Fig. 6) muscles were stimulated with K^-PSS for 2 minutes then partly relaxed to about 70% So in 20 mM K^-PSS. This protocol was used because we knew from a previous study (Gerthoffer and Murphy, 1983b) that myosin phosphorylation would decline to basal levels at 70% So. This observation was confirmed in this experiment. In separate experiments, the muscles were stimulated with 10^{-5} phenylephrine for 30-40 minutes (Fig. 7). Some muscles were frozen after 30-45 minutes in the presence of the agonists to determine control levels of myosin phosphorylation. In separate experiments, cAMP content was measured. Myosin phosphorylation declined to basal levels during this protocol although fairly high levels of active stress were maintained [50-70% of the K^-PSS maximum (Figs. 6 and 7)]. In other tissues, cumulative dose-response curves to forskolin or Br-cGMP were obtained (Fig. 8). There was no decrease in myosin phosphorylation after treatment with the highest dose of forskolin when either K^- or phenylephrine was the agonist; neither did 10^{-4} M Br-cGMP change the phosphorylation state of myosin (0.06 ± 0.02 mol P_i/mol light chain), although both drugs were effective vasodilators. There were significant increases in tissue cAMP levels after exposure to 10^{-5} M forskolin in the presence of either K^- or phenylephrine.

Discussion

The activating effect of myosin phosphorylation on actomyosin ATPase activity led to the hypothesis

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**Figure 5**: Time course of relaxation (O) and elevation of cAMP (D) by 10^{-7} M forskolin. cAMP data are replotted from figure 4. Carotid medial strips were contracted with 10^{-5} M phenylephrine for 15 minutes. Forskolin (10^{-3} M) was added to the bath at time = 0 minute (n = 4).

**Figure 6**: Protocol for vasodilator dose-response curves. Carotid medial strips were stimulated with 110 mM K^-PSS for 2 minutes. The muscles then were partially relaxed in 20 mM K^-PSS to obtain significant stable stress at basal values of myosin phosphorylation. A cumulative dose-response record was obtained for forskolin, as illustrated, or for Br-cGMP using the same protocol. The solid line is the active stress replotted from the record of a typical experiment. The dotted line shows the usual time course of myosin phosphorylation during contraction of the carotid artery with K^-PSS (Dillon et al., 1981; Aksoy et al., 1982). Myosin phosphorylation was measured at the times indicated by the position of the solid bars. Muscles were frozen at rest (basal levels), after 45 minutes in 20 mM K^-PSS, or after the last dose of vasodilator had equilibrated (about 10 minutes). Total tissue cAMP content (open bars) was measured in separate experiments in which free-floating strips of carotid media were exposed to forskolin. The increase in cAMP content was significant at all doses (P < 0.05). The number of experiments is in parenthesis above the bars.

**Figure 7**: Effect of forskolin on myosin phosphorylation, cAMP content, and active stress in carotid medial strips stimulated with 10^{-5} M phenylephrine. Muscles were frozen to assay myosin phosphorylation during contraction or relaxation at the times indicated by the solid bars. A separate set of free floating tissues were frozen in liquid N2 and assayed for cAMP content. Those results are shown by the open bars and are positioned on the time axis to coincide with the appropriate dose of forskolin. The solid line is constructed from the chart record of a typical experiment. The number of experiments are in parentheses above the bars.
that contraction of smooth muscle is mediated by Ca++ activation of myosin kinase which results in phosphorylation of the 20,000 dalton light chains. Relaxation would inevitably follow dephosphorylation of myosin. A corollary hypothesis is that inactivation of myosin kinase by covalent modification (i.e., cAMP-stimulated phosphorylation) or by blocking the interaction of Ca++ with calmodulin would also cause relaxation. Adelstein and Hathaway (1979) suggested that phosphorylation of myosin kinase may underlie cAMP-mediated relaxation of smooth muscle. Studies of isolated proteins and skinned smooth muscles indicate that this mechanism may be sufficient to cause relaxation, at least in those cases in which the contractile element and the regulatory enzymes can be exposed to the catalytic subunit of cAMP-dependent protein kinase (Conti and Adelstein, 1981; Kerrick and Hoar, 1981; Ruegg et al., 1981).

However, our data from preparations with intact cell membranes suggest that Ca++-sequestering mechanisms may be predominant during relaxation, in a variety of circumstances. Myosin dephosphorylation was much faster than relaxation of the carotid artery after stimulus washout, suggesting that myosin dephosphorylation is not the rate-limiting step in relaxation of this muscle (Aksoy et al., 1982; Gerthoffer and Murphy, 1983b). The limiting factor appeared to be the rate of Ca++ sequestration (Gerthoffer and Murphy, 1983b).

Several vasodilators were quite effective in enhancing the relaxation rate after myosin had already been dephosphorylated to basal levels (Figs. 1–3). The results were similar under steady state conditions, when the drug equilibrates at the site or sites of action (Figs. 6 and 7). Both forskolin and Br-cGMP were effective when myosin had been dephosphorylated to basal levels, and there was no further dephosphorylation. Therefore, the effect of vasodilators in our experiments is probably ultimately due to Ca++ sequestration or extrusion. Recent evidence from several laboratories is consistent with this hypothesis (Ito et al., 1982; Miller and Stull, 1983).

Based on our results and the results of previous studies (Gerthoffer and Murphy, 1983b; Chatterjee and Murphy, 1983), it appears that Ca++ sequestration leads to vasodilation by two mechanisms. First, myosin kinase is inactivated due to dissociation of the Ca++-calmodulin complex, and myosin is dephosphorylated by a phosphatase (or phosphatases). This is a fairly rapid process in the carotid artery associated with rapid relaxation due to the inactivation of phosphorylated crossbridges. Second, nonphosphorylated crossbridges (latchbridges) are inactivated by dissociation of Ca++ from a second unknown regulatory site. This regulatory site has a greater sensitivity for Ca++ than myosin kinase, since significant stress is maintained at very low levels of phosphorylation (Figs. 6 and 7; Aksoy et al., 1982; Gerthoffer and Murphy, 1983b; Chatterjee and Murphy, 1983). This hypothesis is supported by the observations that several vasodilators, with different putative mechanisms of action, all have the same qualitative effect on the Ca++-dependent stress maintained by latchbridges. Our results do not exclude phosphorylation of myosin kinase as a possible mechanism for regulating vascular smooth muscle tone. However, it cannot be the primary mechanism for vasodilation by cAMP under the conditions of our experiments.

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