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Effects of Calcium Antagonists and Vasodilators on Arterial Myosin Phosphorylation and Actin-Myosin Interactions

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ABSTRACT

The mechanism of action of many direct-acting vascular smooth muscle relaxant agents is undefined. Moreover, an additional intracellular locus of action for some Ca^{++} entry blockers has been proposed. We have examined the potential for direct action by some of these agents at the level of smooth muscle contractile proteins by quantitating changes in Ca^{++}-dependent superprecipitation of native arterial actomyosin. Moreover, inasmuch as previous research has linked phosphorylation of the 20,000 dalton myosin light chain to Ca^{++}-dependent regulation of contraction, effects on myosin phosphorylation also were quantitated. Whereas the standard calmodulin antagonist W-7 inhibited both parameters by approximately 50% at 10^{-4} M, diazoxide, hydralazine, 3-isobutyl-1-methylxanthine, papaverine, propranolol, nifedipine, nitrendipine, sodium nitroprusside and verapamil did not significantly inhibit either parameter at equimolar concentrations. Cyclandelate significantly delayed the onset of superprecipitation, but did not affect the extent of superprecipitation or myosin phosphorylation. The Ca^{++} antagonists felodipine and diltiazem inhibited superprecipitation by approximately 25%. However, unlike W-7, or felodipine, diltiazem did not concomitantly inhibit myosin phosphorylation. Inhibition of superprecipitation by diltiazem was apparent at a concentration of 10^{-6} M, was manifest by a rightward shift in the pCa relationship and could be attenuated by exogenous calmodulin. These results show that most vasodilators do not have direct effects on smooth muscle contractile protein function. However, diltiazem may inhibit Ca^{++}-dependent arterial actin-myosin interactions by a mechanism which is independent of regulation of myosin light chain phosphorylation.

Although the intracellular concentration of free Ca^{++} regulates the contraction-relaxation cycle in mammalian smooth muscle, the definitive mechanisms responsible for this regulation are undefined. One hypothesis has emerged that has been linked with regulation consistently. This hypothesis invokes a myosin-linked Ca^{++}-calmodulin regulatory system, in which Ca^{++} activates calmodulin-dependent myosin light chain kinase, with subsequent catalysis of phosphorylation of the 20,000 dalton P-light chain of myosin and ultimate stimulation of actin-myosin interactions (see Hartshorne and Gorecka, 1980; Adelstein and Eisenberg, 1980; Silver and Stull, 1982a for reviews). However, alternative hypotheses exist that suggest that Ca^{++}-dependent regulation also may reside on the thin filament (Walters and Marston, 1981; Ebashi *et al.*, 1982).

Evidence supportive of the myosin-linked hypothesis is derived from studies with different sources of smooth muscle as well as from different models of smooth muscle contractile function. In purified contractile protein or actomyosin systems, phosphorylation of the myosin P-light chain is correlated with increases in actin-activated Mg-adenosine triphosphatase activity or superprecipitation of actomyosin, two biochemical correlates of contractile activity (DiSalvo *et al.*, 1978; Silver and DiSalvo, 1979; Silver *et al.*, 1981; Driska *et al.*, 1981; Rees and Frederiksen, 1981; Walsh *et al.*, 1983). The importance of P-light chain phosphorylation also has been demonstrated in more physiological models of contractility. In mechanically or chemically disrupted smooth muscle fibers, phosphorylation of the P-light chain is necessary for the generation of isometric force (Hoar *et al.*, 1979; Walsh *et al.*, 1983). Similarly, the initial development of isometric force and stimulation of cross-bridge cycling during contraction by various physiological/pharmacological agents is dependent on myosin phosphorylation (Driska *et al.*, 1981; Dillon *et al.*, 1981; Silver and Stull, 1982b, 1983).

These studies suggest that pharmacological regulation of smooth muscle contractile activity might occur through modulation of P-light chain phosphorylation (Silver and Stull, 1982a). In addition, modulation might occur conceivably at the contractile proteins distal to regulation of P-light chain phosphorylation. Indirect modulation at either level might involve limitation of the amount of calcium available to the contractile proteins; calcium entry blockers would be an example of this class of agents. Alternatively, direct modulation by alteration of myosin light chain kinase activity might also occur; cyclic

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ABBREVIATIONS: P, phosphorylatable; MOPS, morpholinopropane sulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

AMP-stimulated phosphorylation of myosin light chain kinase with subsequent reduction in affinity for the Ca^{++}-calmodulin complex (Silver and DiSalvo, 1979; Silver *et al.*, 1981) or inhibition of Ca^{++}-calmodulin activation of the kinase by calmodulin-binding agents (Hidaka *et al.*, 1980; Silver and Stull, 1983) are examples of direct modulatory interventions.

Antagonism of the effects of Ca^{++} on the cardiovascular system in certain pathologies has received considerable attention. Although the major mechanism of action of most calcium antagonists (such as the dihydropyridines, verapamil or diltiazem) is through blockade of extracellular calcium channels, an additional intracellular locus of action has been proposed by some investigators (Church and Zsoter, 1980; Boström *et al.*, 1981; Rahwan *et al.*, 1981; Johnson, 1983). Moreover, calmodulin binding by some of these agents has been reported recently (Boström *et al.*, 1981; Johnson, 1983). Similarly, the mechanism of action of other direct-acting vascular smooth muscle antianginal/ antihypertensive agents is undefined. Accordingly, we have examined the possibility of direct modulatory action by various vasodilators at the level of the contractile proteins. Specifically, in this communication, we report on the effects of some of these Ca^{++}-antagonists and smooth muscle vasodilators on Ca^{++}-dependent regulation of arterial actin-myosin interactions and myosin P-light chain phosphorylation in a preparation of purified, mammalian contractile proteins. Our results suggest that most of these agents have little or no effect on Ca^{++}-dependent regulation at this level. However, the Ca^{++} antagonist diltiazem may directly inhibit actin-myosin interactions by a mechanism that is independent of modulation of myosin P-light chain phosphorylation.

Materials and Methods

Experimental procedures were performed according to slight modifications of previously described methods (Silver and DiSalvo, 1979; Silver *et al.*, 1981; Silver and Stull, 1982c).

Preparation of aortic native actomyosin. Bovine aortas from freshly slaughtered steer were obtained from a local abbatoir and packed immediately in ice for transport to the laboratory. Upon arrival at the laboratory, the muscularis was cleaned of connective and endothelial tissue, then subsequently homogenized in 2.5 volumes (wet weight/volume) of a solution containing (millimolar) MOPS, 20 (pH 7.0); KCl, 80; $MgCl_2$, 4; DTT, 1; EGTA, 4; and disodium ATP, 4. This and subsequent procedures were performed at 0–4°C. Crude actomyosin was extracted by stirring (4 hr) and precipitated from a supernatant fraction (30,000 × *g*, 30′) by dialysis against 5 liters of a 4 mM MOPS (pH 7.0), 125 mM KCl, 0.2 mM EGTA and 1 mM DTT solution. After dialysis, the crude actomyosin was collected by centrifugation (10,000 × *g*, 20′) and purified by sequential washing/centrifugation in 2.5 pellet volumes of a solution (pH 7.0) containing 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT in first the presence and then the absence of 1% Triton X-100. The protein concentration of the purified actomyosin was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Routinely, 2 to 3 mg of actomyosin per g of muscularis were obtained. All assays were performed within 24 h after final purification.

Superprecipitation assay. Reaction mixtures (2–4 ml) containing 0.5 to 1.0 mg/ml of actomyosin, 20 mM MOPS (pH 7.0), 50 mM KCl, 0.5 mM DTT and 3 mM EGTA were incubated for 5 min at 25°C before assay. Before transfer to a cuvette, sufficient $CaCl_2$ was added to yield the desired concentration of free Ca^{++} (Blumenthal and Stull, 1980) and the reaction was initiated by the addition of $MgCl_2$-ATP (pH 7.0) to final concentrations of 10 and 2 mM, respectively. Changes in optical density at a wavelength of 660 nm were recorded as described previously (Silver *et al.*, 1981, see fig. 1). In some experiments, purified calmodulin (Caabco, Inc., Houston, TX) was added to a final concentration of 2 μM along with the actomyosin. All drugs were either dissolved in 2 mM MOPS (pH 7.0) and adjusted to pH 7.0 ± 0.5 or dissolved in 95% ethanol just before assay. Appropriate precautions with light-sensitive compounds were followed. Compounds were incubated in the reaction mixtures ($-CaCl_2$) at 10^{-4} M (final concentration) for 5 min at 25°C before assay. Comparisons in the superprecipitation response were made with the appropriate controls (±ethanol) at 1.5, 5 or 12 min after the initiation of the reaction.

Determination of myosin P-light chain phosphorylation. The extent of myosin P-light chain phosphorylation in the reaction mixtures was determined by isoelectric focusing on polyacrylamide gels (DiSalvo *et al.*, 1978; Silver and Stull, 1982c). At the indicated times, aliquots of the reaction mixtures (200–300 μl) were withdrawn and the reactions were halted by denaturation in an equal volume of a solution containing 8 M urea, 15 mM β-mercaptoethanol, 2% Triton X-100 and 2% ampholines (1.6%, pH 4–6, LKB Instruments Inc., Rockville, MD; 0.4%, pH 3–10 Bio-Rad Laboratories, Richmond, CA). Samples (100 μg of protein) were electrofocused on polyacrylamide tube gels (75 × 5 mm) for 4.5 hr at a constant voltage setting of 400 V. Tube gels consisted of 8 M urea, 7.5% polyacrylamide, 1% Triton X-100 and 2% ampholines (same distribution as in the denaturation buffer). The top electrophoresis buffer (cathode solution) was 20 mM NaOH whereas the bottom buffer (anode solution) was 80 mM H_3PO_4. During electrofocusing, the bottom buffer was cooled by a circulating water bath maintained at 12°C. After electrofocusing, the gels were fixed in 15% trichloroacetic acid and washed in 15% methanol-10% acetic acid. Gels were stained with Coomassie Blue R-250 (Sigma Chemical Co., St. Louis, MO) and subsequently destained in methanol-acetic acid. After destaining, the extent of myosin P-light chain phosphorylation was quantitated by densitometric scanning and determination of the peak heights for the nonphosphorylated and phosphorylated forms of the P-light chain.

Statistical comparisons for both superprecipitation and P-light chain phosphorylation were made by either the Student's *t* test or the Dunnett's *t* test for multiple comparisons.

Materials. Compounds were obtained from the following sources: cyclandelate (Cyclospasmol; Ives Laboratories, New York, NY), diazoxide (Schering Corporation, Bloomfield, NJ), diltiazem (Marion Laboratories, Kansas City, MO), hydralazine (Ciba-Geigy Corp., Summit, NJ), 3-isobutyl-1-methylxanthine (Aldrich Chemical Company, Inc., Milwaukee, WI), nitrendipine (Miles Laboratories, Elkhart, IN), papaverine (Sigma), propranolol (Inderal; Ayerst Laboratories, New York, NY), sodium nitroprusside (Sigma) and verapamil (Knoll Pharmaceutical Company, Orange, NJ). Nifedipine and W-7 were synthesized by Wyeth chemists (Philadelphia, PA).

Results

Figure 1 shows the temporal relationship for Ca^{++}-dependent myosin P-light chain phosphorylation and superprecipitation as well as the effect of 1% ethanol (final concentration) on the pCa relationship for both parameters. Experiments were performed with this vehicle as many of the compounds that were tested were soluble in ethanol. Enhanced Ca^{++}-dependent superprecipitation without changes in P-light chain phosphorylation was apparent when ethanol was added to the reaction mixtures. This stimulatory effect of ethanol on arterial actomyosin is in contrast to the previously reported inhibitory effects of ethanol on the troponin C-regulated system of skeletal muscle (Puszkin and Rubin, 1976) and may reflect the differences in the Ca^{++} regulatory systems of the two muscle types. Nevertheless, Ca^{++} sensitivity in arterial actomyosin was still apparent in the presence of ethanol. As a further control, the efficacy of the calmodulin inhibitor W-7 was tested in the presence and absence of 1% ethanol (fig. 2). Inhibition of

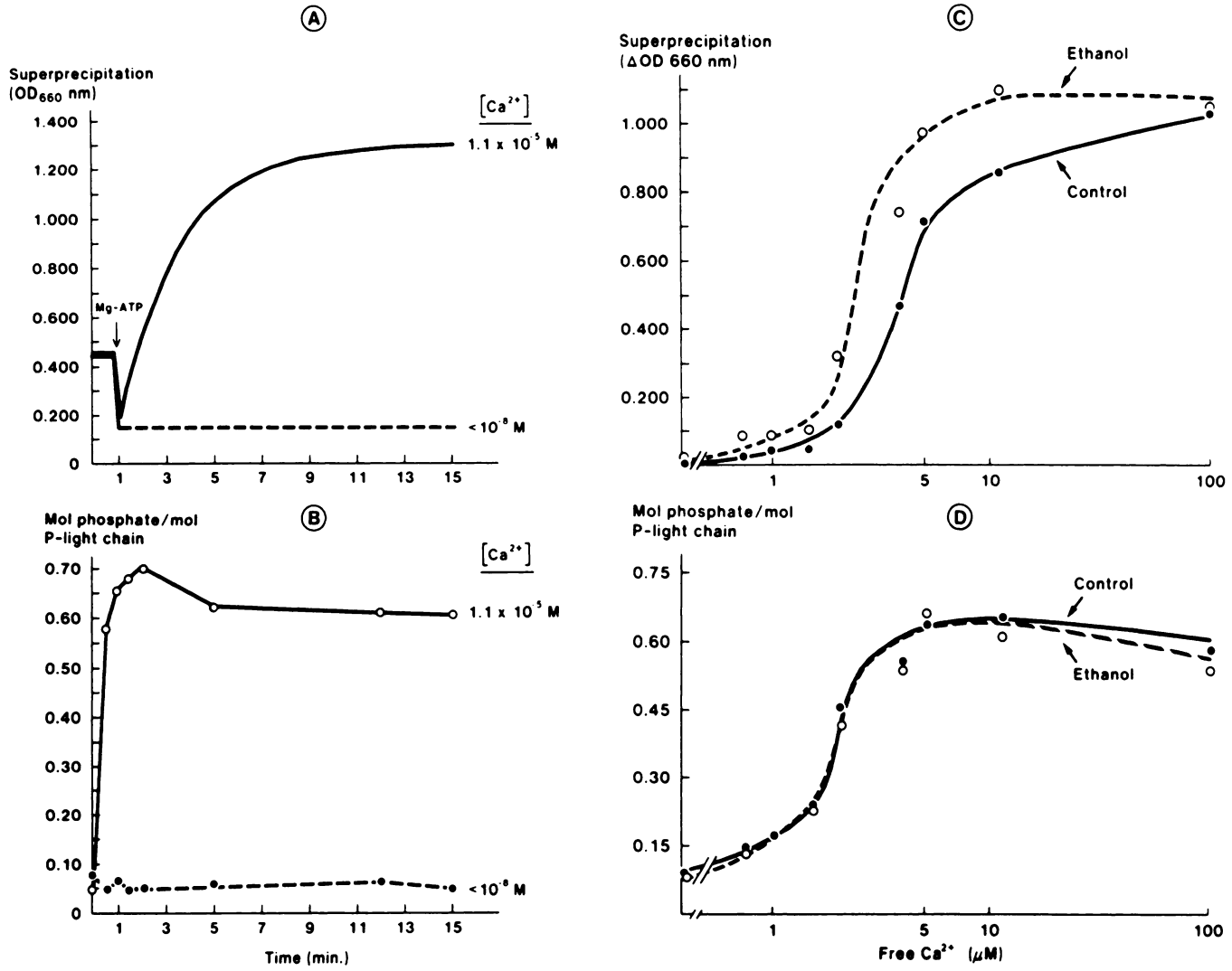

Fig. 1. A and B, time course for arterial superprecipitation and myosin P-light chain phosphorylation. C and D, effect of 1% ethanol on the Ca^{++} sensitivity of superprecipitation and myosin P-light chain phosphorylation. A and B, reaction conditions were as described in the text; changes in superprecipitation (detected at 660 nm) of actomyosin were quantitated after addition of Mg-ATP to the cuvette in either the presence (1.1×10^{-5} M) or absence ($<10^{-8}$ M) of Ca^{++}. At the indicated points, aliquots were removed from the reaction mixture and the extent of myosin phosphorylation (mol phosphate/mol P-light chain) was quantitated as described in the text. Solid line, 1.1×10^{-5} M Ca^{++}; dashed line, $<10^{-8}$ M Ca^{++}. C and D, one percent ethanol (final concentration) was added to the actomyosin reaction mixtures during incubation as described in the text. Superprecipitation was initiated as described in figure 1, A and B. Values (ΔOD660 nm) were obtained by subtracting the initial low reading obtained after the clearing response (base line) from the value obtained 5′ after the initiation of the reaction. The extent of myosin P-light chain phosphorylation was quantitated (mol phosphate/mol P-light chain) after 5′. Free Ca^{++} concentrations in the reaction mixtures were adjusted as described in the text. Values represent the mean of two to three actomyosin preparations. ●——●, control responses (no added ethanol); O- - -O, ethanol-treated actomyosin.

superprecipitation (51%) or myosin P-light chain phosphorylation (47%) in the absence of ethanol was not different from the relative inhibition of either parameter (47% superprecipitation; 49% myosin P-light chain phosphorylation) when assayed in the presence of ethanol. Thus, the potential effects of an ethanol-soluble antagonist could be ascertained in this system.

The effects of Ca^{++} antagonists and smooth muscle vasodilators on smooth muscle actin-myosin interactions were examined at 1.5 (slope phase) and 12 min (plateau phase) of the superprecipitation response (figs. 2 and 3). To adjudge the effects of these agents, the free Ca^{++} concentration in these assays was adjusted to 2 to 5 μM, which produced between 30 and 75% of the maximal response. All compounds were initially tested at a concentration of 10^{-4} M. The calmodulin antagonist W-7 was tested as a standard agent in all preparations. Neither verapamil, nifedipine nor nitrendipine significantly inhibited either phase of superprecipitation or myosin P-light chain phosphorylation (fig. 2). However, felodipine and diltiazem significantly inhibited both phases of superprecipitation, yet in contrast to felodipine, concomitant inhibition of P-light chain phosphorylation was not apparent with diltiazem. Other smooth muscle relaxant/antihypertensive agents which were tested (diazoxide, hydralazine, papaverine, 3-isobutyl-1-methylxanthine, propranolol and sodium nitroprusside) did not significantly affect either phase of superprecipitation or P-light chain phosphorylation (fig. 3). Cyclandelate significantly delayed the onset of superprecipitation (20% inhibition of the slope phase), but did not significantly affect the extent of superprecipitation (plateau phase). Similarly, P-light phosphorylation was not affected by this agent.

Additional studies with diltiazem were performed to charac-

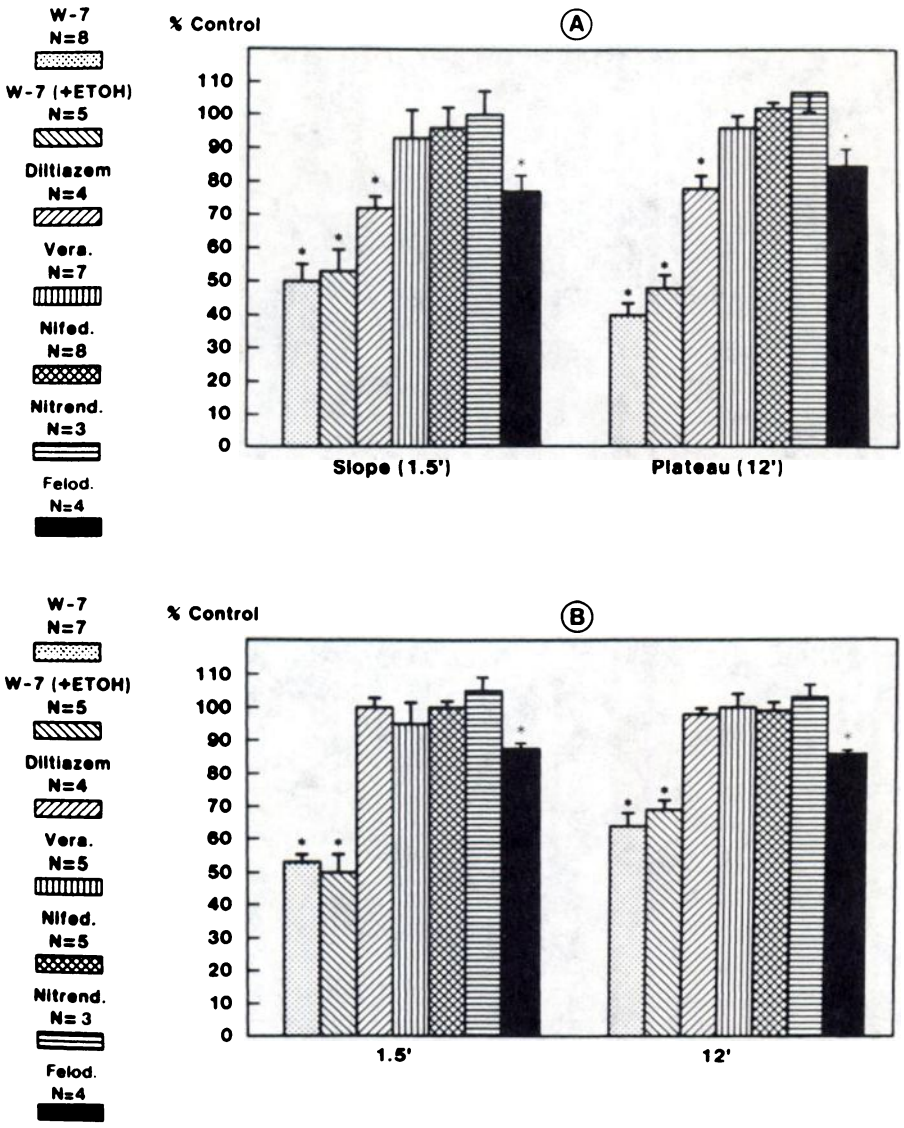

Fig. 2. Effects of Ca^{++} antagonists on A) arterial superprecipitation and B) myosin light chain phosphorylation. The effects of Ca^{++} antagonists (10^{-4} M final concentration) on superprecipitation and myosin light chain phosphorylation of actomyosin at 1.5 and 12 min after initiation of the reaction is shown. The values are the mean ± S.E. for the number (*N*) of preparations tested and are expressed as a percentage of the control responses. The calmodulin antagonist W-7 was tested in both the absence and presence of 1% ethanol (ETOH) as some compounds were only soluble in ethanol. Vera, verapamil; nifed, nifedipine; nitrend, nitrendipine; felod, felodipine. * Denotes statistical significance at the $P < .05$ level using the Dunnett's *t* test for multiple comparisons.

terize further the mechanism of inhibition of actin-myosin interactions. In figure 4, the concentration-response relationship for inhibition by diltiazem at submaximal (1.5–3.8 μM) and maximal (100 μM) concentrations of free Ca^{++} is shown. At submaximal free Ca^{++} concentrations, the threshold concentration of diltiazem for inhibition was 1×10^{-7} M and maximum inhibition (55%) was evident at 5×10^{-4} M. At maximal free Ca^{++} concentrations, inhibition of superprecipitation was not observed at concentrations of diltiazem less than 2.5×10^{-4} M. In further experiments (fig. 5) the effect of diltiazem (5×10^{-4} M) on the pCa relationship for superprecipitation and myosin P-light phosphorylation was compared with the effects of the calmodulin antagonist W-7 (1×10^{-4} M). At lower concentration of free Ca^{++}, inhibition of superprecipitation by W-7 and diltiazem was approximately equal. At higher concentrations of Ca^{++}, the relative inhibition by diltiazem was less when compared with W-7. Both compounds shifted the pCa curve for superprecipitation to the right. However, in contrast to W-7, a similar shift in the pCa relationship for myosin P-light chain phosphorylation did not occur with treatment by diltiazem.

Addition of purified, exogenous calmodulin (2 μM final concentration) attenuated the inhibition of superprecipitation produced by diltiazem (fig. 6). Exogenous calmodulin also stimulated superprecipitation at low (1.5 and 2.0 μM) free Ca^{++}, but not high free Ca^{++} (3.8 and 5.0 μM) in control reaction mixtures (data not shown). Concomitantly, the extent of P-light chain phosphorylation at the lower concentrations of Ca^{++} (1.5 and 2.0 μM) was increased by calmodulin in both the presence and absence of diltiazem. However, calmodulin also attenuated diltiazem-mediated inhibition of superprecipitation at higher free Ca^{++} concentrations (3.8 and 5.0 μM) without significantly increasing P-light chain phosphorylation.

Discussion

The findings presented in this study show that several structurally distinct vasodilator/smooth muscle relaxant agents have little or no effect on regulating either Ca^{++}-calmodulin-dependent myosin light chain phosphorylation or arterial actin-myosin interactions in a contractile protein preparation purified from mammalian vascular smooth muscle. Specifically, the agents that were without effect at either level were hydralazine, diazoxide, papaverine, 3-isobutyl-1-methylxanthine, propranolol and sodium nitroprusside. Because no effects were observed at a relatively high concentration (1×10^{-4} M) of these com-

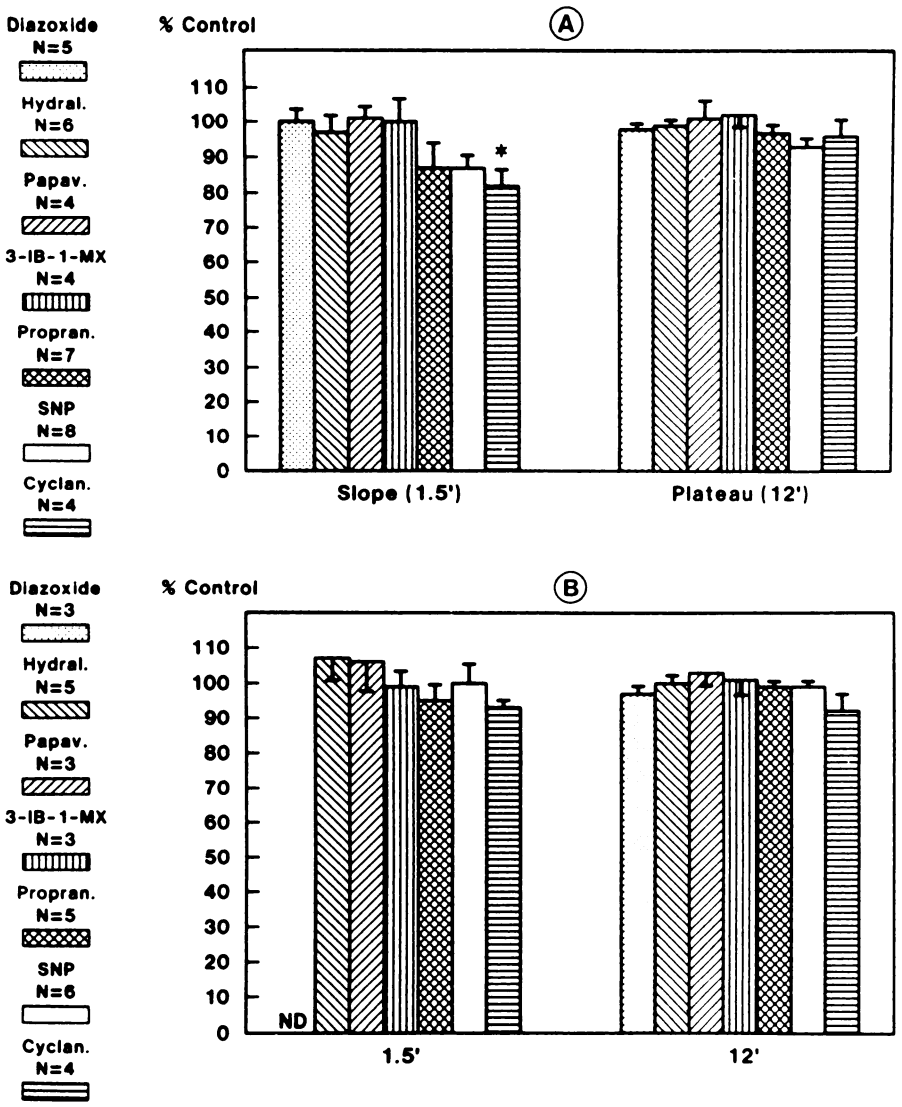

Fig. 3. Effects of other vasodilator agents (10^{-4} M final concentration) on A) arterial superprecipitation and B) myosin P-light chain phosphorylation at 1.5 and 12 min. Assays were performed as described in the text and results are expressed as a percentage of the control responses as described in figure 2. The values are the mean ± S.E. for the number of preparations tested. Hydral, hydralazine; papav, papaverine; 3-IB-1-MX, 3-isobutyl-1-methylxanthine; propran, propranolol, SNP, sodium nitroprusside; cyclan, cyclandelate. * Denotes statistical significance at the $P < .05$ level using the Dunnett's *t* test for multiple comparisons.

pounds, the major site for regulation of Ca^{++}-dependent contraction by these agents is probably proximal to the contractile proteins and most likely involves alteration of membranous handling of Ca^{++} and/or altered cyclic nucleotide metabolism. Cyclandelate, a peripheral vasodilator, significantly delayed a component of the superprecipitation response but the overall extent of superprecipitation and phosphorylation of myosin were unaltered. The reason for this difference and relevance to the mechanism of action of this compound is not apparent.

Although the primary mechanism of action of most Ca^{++} antagonists is *via* blockade of the voltage-dependent Ca^{++}-channel, several investigators have suggested an additional, intracellular site of action for some of these compounds (Church and Zsotér, 1980; Boström *et al.*, 1981; Rahwan *et al.*, 1981; Johnson, 1983). Functional, subcellular studies with most Ca^{++} antagonists have focused on various cardiac membranous fractions (Entman *et al.*, 1972; Watanabe and Besch, 1974; Besch *et al.*, 1981; Colvin *et al.*, 1982) and few reports of effects on contractile proteins are available (Hidaka *et al.*, 1980). In this study with arterial actomyosin, neither nifedipine, nitrendipine nor verapamil inhibited regulation of contractile protein function. Although the concentration of agents tested was several orders of magnitude higher than therapeutic concentrations, they were of the magnitude used in other subcellular studies (Watanabe and Besch, 1974; Besch *et al.*, 1981; Colvin *et al.*, 1982). The demonstration of no inhibition at this relatively high concentration suggests further that these agents probably have no effect at this level which might contribute to the vascular relaxant activity of these drugs. A similar finding was recently reported by Saida and van Breemen (1983) using chemically skinned mesenteric arterial fibers as a model for contractility.

Another subclass of Ca^{++} antagonists, characterized by prenylamine and other lipophilic, weakly basic drugs (such as fendiline, perhexilene and cinnarizine), were not examined in this study. However, prenylamine was shown by Hidaka *et al.*, (1979) in previous studies to directly inhibit chicken gizzard actomyosin adenosine triphosphatase activity and recent studies (Spedding, 1983) with chemically skinned (Triton X-100) guinea-pig tenia coli suggest that fendiline and cinnarizine might have intracellular mechanisms of action which incorporate direct inhibition of contractile protein function. The ability of compounds such as perhexilene and cinnarizine to inhibit directly arterial myosin light chain phosphorylation, the potential mechanism of this inhibition and the relative contribution

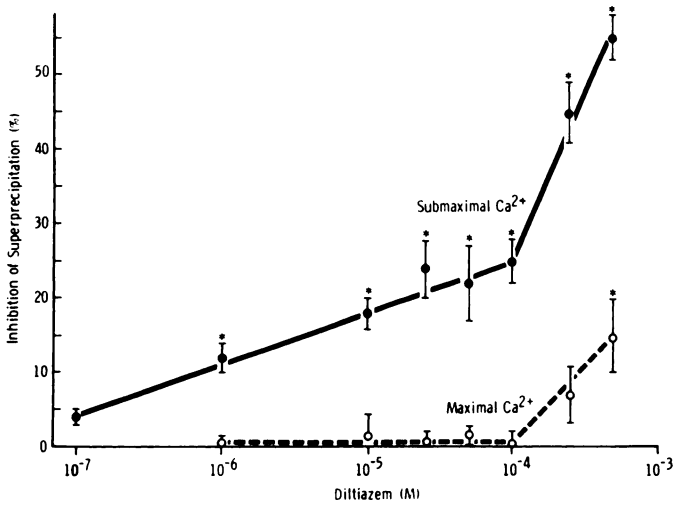

Fig. 4. The concentration-response relationship for inhibition of superprecipitation by diltiazem. Assays were performed with the indicated concentration of diltiazem (molar) at either submaximal [1.5–3.8 μM free Ca^{++} (●)] or maximal [100 μM free Ca^{++} (○)] concentrations of free calcium. Inhibition of superprecipitation (percentage) was quantitated after 5 min as described in figure 1. Values represent the mean ± S.E. for three to four separate actomyosin preparations. * Denotes statistical significance from control values at the $P < .05$ level.

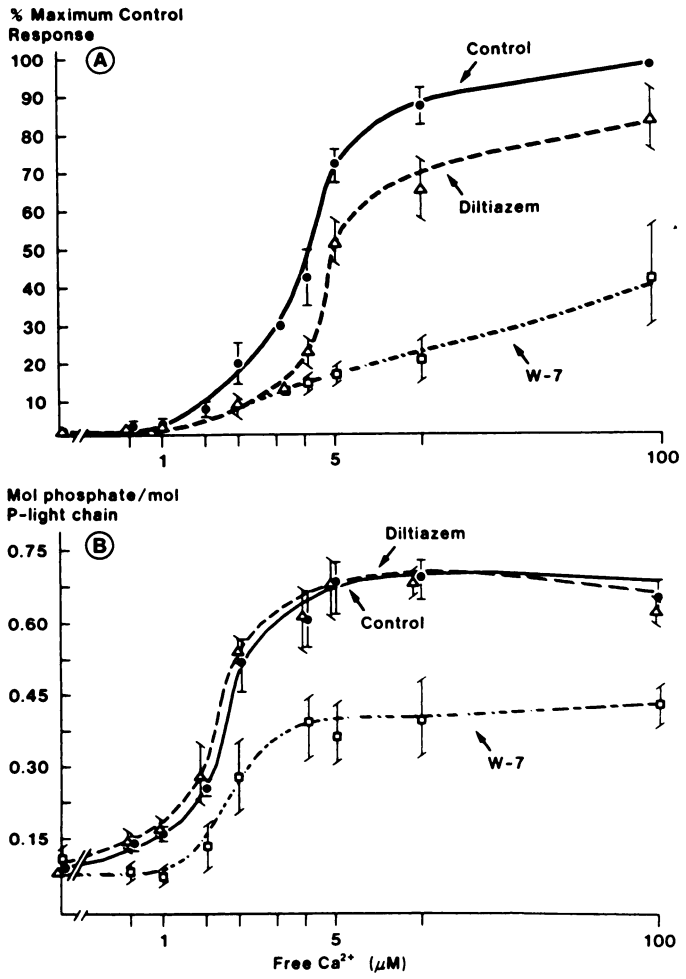

Fig. 5. Effects of diltiazem (5×10^{-4} M) and W-7 (1×10^{-4} M) on the Ca^{++} dependence of A) arterial superprecipitation and B) myosin P-light chain phosphorylation. Results were obtained 5 min after initiation of the reaction as described in figure 1 and are expressed as either A) a percentage of the control, maximal response (100 μM free Ca^{++}) or B) mole phosphate per mole of P-light chain. Values represent the mean ± S.E. for three separate preparations of actomyosin. ●, control responses; △, diltiazem; □, W-7.

of this inhibition to the overall Ca^{++} antagonism displayed by these drugs is currently being examined by our laboratory.

Binding to calmodulin by some Ca^{++} entry blockers has been reported recently (Boström *et al.*, 1981; Johnson, 1983); felodipine and verapamil at micromolar concentrations were shown to bind to calmodulin. However, others have suggested recently that binding of dihydropyridines to calmodulin is unsaturable and occurs with a low affinity, thus questioning the pharmacological relevance of this occurrence (Thayer and Fairhurst, 1983). Our results support the concept that calmodulin binding may *not* be directly related to inhibition of smooth muscle myosin light chain kinase activity, as verapamil (1×10^{-4} M) did not significantly affect myosin P-light chain phosphorylation or superprecipitation of arterial actomyosin (fig. 2) in this study. Felodipine did significantly inhibit superprecipitation and myosin P-light chain phosphorylation, yet inhibition was approximately 23% at this relatively high concentration (1×10^{-4} M). Because this compound completely inhibits smooth muscle contractions in the picomolar-nanomolar range (Boström *et al.*, 1981), inhibition at the level of the contractile proteins is probably not the major site of Ca^{++} antagonism for this agent.

Diltiazem consistently produced significant inhibition of arterial superprecipitation. The threshold concentration for inhibition was 1×10^{-7} M and significant inhibition was evident at concentrations greater than or equal to 1×10^{-6} M. Thus, inhibition occurred over the concentration range which is used for antagonism of receptor-dependent contractions in intact smooth muscle (see Flaim, 1982 for review; Sato *et al.*, 1982). Moreover, a recent report (Saida and van Breemen, 1983) suggests that diltiazem also can inhibit force development in chemically skinned mesenteric arteries. However, significant inhibition was only apparent at concentrations greater than 10^{-5} M. In this study with arterial actomyosin, inhibition of actin-myosin interactions was only apparent at *submaximal* concentrations of free Ca^{++}; higher concentrations of diltiazem ($2.5–5 \times 10^{-4}$ M) were necessary to demonstrate inhibition of maximal Ca^{++} responses. Although these findings are consistent with functional Ca^{++} antagonism by diltiazem at the level of the contractile proteins, it also suggests that the Ca^{++} concentration at the contractile proteins must be submaximal for inhibition to occur. This limitation might occur through the primary mechanism of action of diltiazem, blockade of the Ca^{++} entry channel.

Of further interest is the mechanism by which diltiazem mediates inhibition of arterial actin-myosin interactions. In contrast to inhibition by the calmodulin antagonist W-7 (fig. 5) or cyclic AMP-protein kinase-mediated phosphorylation of myosin light chain kinase (Silver *et al.*, 1981), a rightward shift in the pCa relationship for superprecipitation by diltiazem was *not* associated with a similar shift in Ca^{++} sensitivity of myosin P-light phosphorylation. In fact, there was no significant inhibition of myosin P-light phosphorylation by diltiazem under any experimental conditions. Experiments with purified, exogenous calmodulin (fig. 6) showed that, at low concentrations of free Ca^{++}, addition of calmodulin in the presence of diltiazem increased the extent of P-light chain phosphorylation and attenuated the inhibition of superprecipitation. However, at

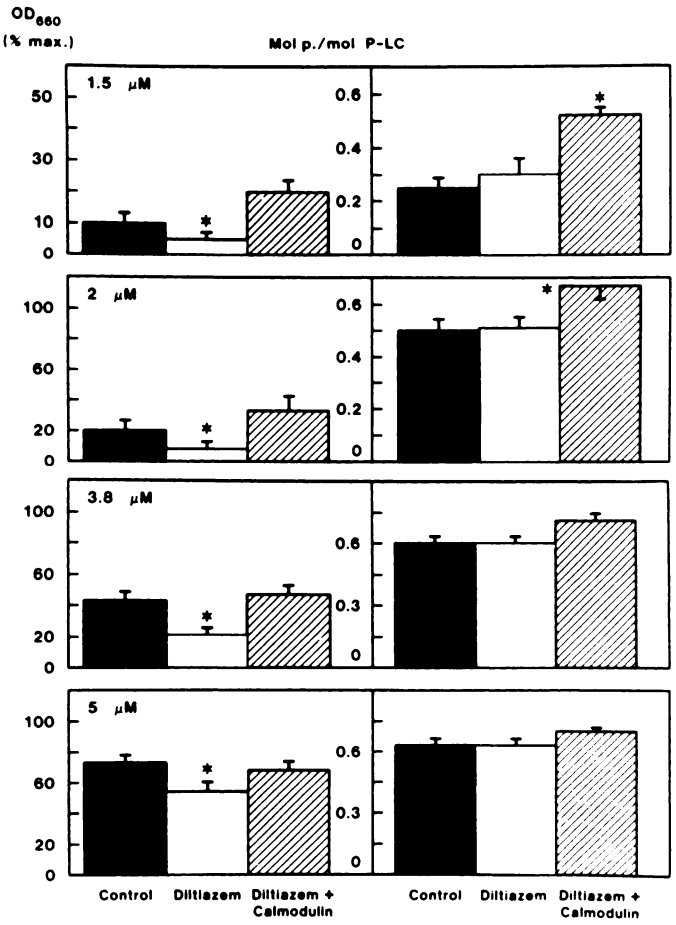

Fig. 6. The effect of exogenous calmodulin on diltiazem-mediated inhibition. Purified calmodulin (2 μM final concentration) was added to reaction mixtures along with diltiazem (5×10^{-4} M) and effects on superprecipitation (left ordinate) and myosin phosphorylation (right ordinate) were quantitated. The values are the mean ± S.E. for three preparations of actomyosin and are expressed as either a percentage of the maximum (100 μM free Ca^{++}) response (superprecipitation) or mole phosphate per mole P-light chain (mol p./mol. P-LC; myosin phosphorylation) as described in figure 5. The concentration of free Ca^{++} (micromolar) for each series is indicated in the upper left hand corner of each panel. Control, solid bars; diltiazem, open bars; diltiazem plus calmodulin, hatched bars. * Denotes statistical significance ($P < .05$) from control values.

higher concentrations of free Ca^{++}, reversal of diltiazem-mediated inhibition by calmodulin was not associated with significant increases in P-light chain phosphorylation. Thus, diltiazem may modulate Ca^{++}-dependent contractions in skinned smooth muscle (Saida and van Breemen, 1983) through inhibition of actin-myosin interactions at a point which is distal to regulation of myosin P-light chain phosphorylation. Future studies might examine the potential for inhibition by diltiazem on the leiotonin system (Ebashi *et al.*, 1982) and/or other proposed Ca^{++} regulatory systems (Walters and Marston, 1981).

Diltiazem, in concentrations similar to those used in this study, can antagonize contractions in smooth muscle produced by a variety of contractile agonists (Flaim, 1982; Cauvin *et al.*, 1982; Sato *et al.*, 1982). Moreover, it exerts the least negative inotropic effect of the most commonly studied Ca^{++} antagonists (Pérez *et al.*, 1982). Both effects are consistent with the data presented in this report. Development of agents which antagonize the effects of Ca^{++}-calmodulin intracellularly at the level of smooth muscle contractile protein function offers the promise for the attainment of smooth muscle-specific Ca^{++} antagonists which may be useful in the treatment of Ca^{++}-related vascular pathologies, such as hypertension or coronary artery vasospasm.

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