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The Role of Myosin Light Chain Kinase Phosphorylation in *Beta*-Adrenergic Relaxation of Tracheal Smooth Muscle

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SUMMARY

Myosin light chain kinase from smooth muscle has been shown to be phosphorylated by cyclic AMP-dependent protein kinase, which leads to a decrease in the affinity of the kinase for Ca^{2+}·calmodulin and, hence, a decrease in enzymatic activity. This event has been proposed as a mechanism for the relaxation of smooth muscle in response to increased intracellular concentrations of cyclic AMP. The ratio of myosin light chain kinase activities measured in the presence of 4 μM or 100 μM Ca^{2+}, at 1 μM calmodulin, permits evaluation of such a change in the calmodulin activation properties of myosin light chain kinase. This activity ratio was decreased by phosphorylation of either purified bovine tracheal smooth muscle myosin light chain kinase, or the endogenous myosin light chain kinase in a homogenate of tracheal smooth muscle, with the addition of the catalytic subunit of cyclic AMP-dependent protein kinase. The ratio was unchanged, however, by activation of the endogenous cyclic AMP-dependent protein kinase in homogenates of tracheal smooth muscle by the addition of cyclic AMP. Incubation of tracheal smooth muscle with isoproterenol, at a concentration sufficient to relax the muscle and to increase phosphorylase *a* formation, had no effect upon the activity ratio. Incubation of tracheal smooth muscle for 2 hr in the presence of carbachol resulted in a transient increase and then a decrease in myosin light chain phosphate content to control values with no decrease in isometric force. The addition of isoproterenol at 2 hr still resulted in relaxation. These findings are inconsistent with a role of myosin light chain kinase phosphorylation in mediating relaxation of tracheal smooth muscle by *beta*-adrenergic agonists. Cyclic AMP-dependent effects on cytoplasmic calcium concentrations may be more important in mediating relaxation.

INTRODUCTION

The contraction of smooth muscle is initiated by an increase in the intracellular concentration of Ca^{2+}, but the nature of the interaction of Ca^{2+} with the contractile apparatus is incompletely understood. According to one widely accepted theory, phosphorylation of the 20,000 M_r myosin light chain subunit is prerequisite to actin activation of myosin ATPase activity and, presumably, to contraction. This process is catalyzed by Ca^{2+}, calmodulin-dependent myosin light chain kinase, and is reversible via one or more Ca^{2+}-independent phosphatases (see refs. 1 and 2 for recent reviews). Other investigators have suggested that smooth muscle contraction need not involve phosphorylation of myosin, but is mediated by the proteins leiotonin A and leiotonin C (3). Still others have proposed a dual regulatory system involving phosphorylation of proteins associated with both the thin and thick filaments (4). Most of the evidence reported to date, however, suggests that phosphorylation of myosin is at least an important event mediating smooth muscle contraction (1, 2).

It is known that *beta*-adrenergic stimulation of many types of smooth muscle results in relaxation. Some previously advanced hypotheses relate *beta*-adrenergic stimulation to a reduction in intracellular Ca^{2+} concentration (5–7). Alternatively, it is possible that the sensitivity of the contractile proteins to activation by Ca^{2+} is decreased. Consistent with the latter hypothesis, myosin light chain kinase purified from turkey gizzard smooth muscle has recently been shown to be phosphorylated by cyclic AMP-dependent protein kinase (8). When phosphorylation occurred in the absence of calmodulin, myosin light chain kinase exhibited a reduced sensitivity to activation by the Ca^{2+}·calmodulin complex; i.e., the calmodulin concentration necessary for half-maximal acti-

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vation increased from 1.2 nM to 25 nM (8). Similar results have been obtained with myosin light chain kinase purified from bovine aortic and stomach smooth muscle (9, 10). Additional evidence for this hypothesis has been provided from studies of smooth muscle native actomyosin (11) and chemically skinned smooth muscle fiber systems (12, 13). In these studies large amounts of cyclic AMP-dependent protein kinase were added. *Beta*-adrenergic stimulation does inhibit myosin light chain phosphorylation concomitant with inhibition of isometric force development in intact tracheal smooth muscle (14). However, these results by themselves do not assess the contributions of multiple mechanisms, such as phosphorylation of myosin light chain kinase and reduction of cytoplasmic Ca^{2+} concentrations, for inhibition of contraction. Both types of mechanisms would decrease P-light chain phosphorylation. To date, evidence that phosphorylation of myosin light chain kinase occurs in intact smooth muscle, and is a component of the regulation of smooth muscle contraction, is lacking. Since purified proteins phosphorylated by protein kinases are not necessarily phosphorylated *in vivo* (2), it is essential that phosphorylation of myosin light chain kinase as well as the anticipated change in the activation properties of the enzyme be demonstrated in living smooth muscle. We have developed an activity ratio assay for smooth muscle myosin light chain kinase that determines whether there is any change in the calmodulin activation properties which might accompany phosphorylation by cyclic AMP-dependent protein kinase. This procedure is analogous to activity ratio measurements used to assess phosphorylation states of phosphorylase, phosphorylase kinase, glycogen synthase, and hormone-sensitive lipase, and has been used to determine whether *beta*-adrenergic stimulation in intact tracheal smooth muscle leads to a decrease in the affinity of myosin light chain kinase for calmodulin.

MATERIALS AND METHODS

Purification of proteins. Myosin light chain kinase was purified 1200-fold from bovine aortic muscularis (15) in collaboration with Dr. Joseph DiSalvo, University of Cincinnati, by methods similar to a previously published procedure (16). The enzyme had a specific activity (V_{max}) of 40 μmoles of phosphate incorporated per minute per milligram of kinase with bovine aortic smooth muscle myosin light chains as substrate. Bovine tracheal smooth muscle myosin light chain kinase was a gift from Dr. Mary Nunnally, Department of Pharmacology, The University of Texas Health Science Center at Dallas. Purified catalytic subunit of cyclic AMP-dependent protein kinase was kindly provided by Drs. Jackie Corbin and Thomas Soderling of Vanderbilt University. Mixed myosin light chains from bovine ventricles were purified as described by Blumenthal and Stull (17). The phosphorylatable light chain content of these preparations was determined by optical densitometry performed on 10% polyacrylamide gels after electrophoresis in the presence of 0.1% sodium dodecyl sulfate or by measurement of maximal radiolabeled phosphate incorporation into the mixed light chains after prolonged incubation with skeletal muscle myosin light chain kinase as previously described (17). Calmodulin was prepared from frozen bovine brain (Pel-Freeze Biologicals, Rogers, Ark.) by the method of Dedman *et al.* (18).

Myosin light chain kinase activity ratio. The activation of myosin light chain kinase involves two sequential reactions:

$$\text{calcium} + \text{calmodulin} \rightleftharpoons \text{calcium}\cdot\text{calmodulin}$$

$$\text{calcium}\cdot\text{calmodulin} + \text{myosin light chain kinase} \rightleftharpoons \text{calcium}\cdot\text{calmodulin}\cdot\text{myosin light chain kinase}$$

The calcium·calmodulin·myosin light chain kinase complex is the active form of the enzyme. It has been proposed that the Ca_i^{2+}·calmodulin complex is the form of calmodulin that activates myosin light chain kinase (17).

The two-step reaction mechanism for activation of myosin light chain kinase indicates that an increase in the K_{CM}[3] value will result in an increase in the calcium concentration required for activation, although there are no changes in the affinity of calmodulin for calcium (Fig. 1). The myosin light chain kinase activity ratio assesses the degree of activation of myosin light chain kinase at a submaximal concentration of the activating complex, Ca_i^{2+}·calmodulin. Myosin light chain kinase activity was determined as previously described (17), except that the reaction mixtures contained 1 μM calmodulin and either 4 or 100 μM free calcium. The lower concentration of calcium was controlled by the use of a calcium/EGTA buffer system (17). The myosin light chain kinase activity ratio is the ratio of kinase activities measured at 4 μM and 100 μM free calcium, respectively. The quantitative model for the activation of myosin light chain kinase (17) predicts that this ratio will decrease in response to a modification of the enzyme, e.g., phosphorylation, which increases K_{CM}, the concentration of Ca_i^{2+}·calmodulin necessary for half-maximal activation (Fig. 1). For example, when $K_{Ca^{2+}}$ = 12 μM [intrinsic activation constant, the product of the K_D values for the four divalent metal binding sites on calmodulin (15, 17)] and K_{CM}, the concentration of Ca_i^{2+}·calmodulin necessary for half-maximal activation, is increased from 1 nM to 20 nM, the activity ratio decreases from 0.80 to 0.16 (Fig. 1). These changes in K_{CM} values are similar to those reported for myosin light chain kinase after phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase (8). Thus, measurement of the myosin light chain kinase activity ratio could be used to evaluate changes in calmodulin activation (i.e., in K_{CM}) expected with phosphorylation of myosin light chain kinase by cyclic AMP-dependent protein kinase. This ratio is easily determined in a dilute tissue extract obtained in the presence of inhibitors of protein kinases and phosphoprotein phosphatases. Free Ca^{2+} concentrations are controlled in the assay system by the use of a Ca^{2+}/EGTA buffer system, and the content of endogenous calmodulin in the diluted extract (1–10 nM) is much less than the amount added to the assay mixture (1 μM).

Phosphorylation of purified myosin light chain kinase. Cyclic AMP-dependent protein kinase catalytic subunit (final activity, 50,000 units/ml, determined using histone IIA as substrate) was added to a reaction mixture containing bovine tracheal smooth muscle myosin light chain kinase (38 μg/ml), 10 mM MOPS, 2 mM EGTA, 15 mM KCl, 10 mM magnesium acetate, 15 mM 2-mercaptoethanol, 15% glycerol, and 1 mM [γ-^{32}P]ATP (380 cpm/mole) (pH 7.0). After a 30-min incubation at 30°, aliquots of the reaction mixture were spotted on Whatman No. 3 MM filter paper squares. The squares were immersed in 10% trichloroacetic acid/4% sodium pyrophosphate and processed by the method of Corbin and Reimann (19). Blanks consisted of the reaction mixture described above, less catalytic subunit. The reaction mixture was diluted 1000-fold in 50 mM potassium phosphate, 4 mM EDTA, and 15 mM 2-mercaptoethanol (pH 6.8) for measurement of the myosin light chain kinase activity ratio. At this dilution the catalytic subunit does not significantly phosphorylate myosin light chain kinase; this eliminates the necessity of separating the catalytic subunit from myosin light chain kinase before activity ratio measurements are performed.

To evaluate the stability of the myosin light chain kinase activity ratio of phosphorylated enzyme in tracheal smooth muscle homogenates, 20-μl aliquots of the phosphorylated kinase were added to 80 μl of a trachealis muscle homogenate (2.5 mg/ml) containing 50 mM potassium phosphate, 6 mM EDTA, 15 mM 2-mercaptoethanol, 0.5% Triton X-100, aprotinin (10 units/ml), and 0.1 mM PMSF (final concentrations), at pH 6.8, and 0°. After a 10-min incubation, aliquots were withdrawn from the supernatant fractions (8000 × *g*, 5 min) of these

[3] The abbreviations used are: CM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; MLCK, myosin light chain kinase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PMSF, phenylmethylsulfonylfluoride.

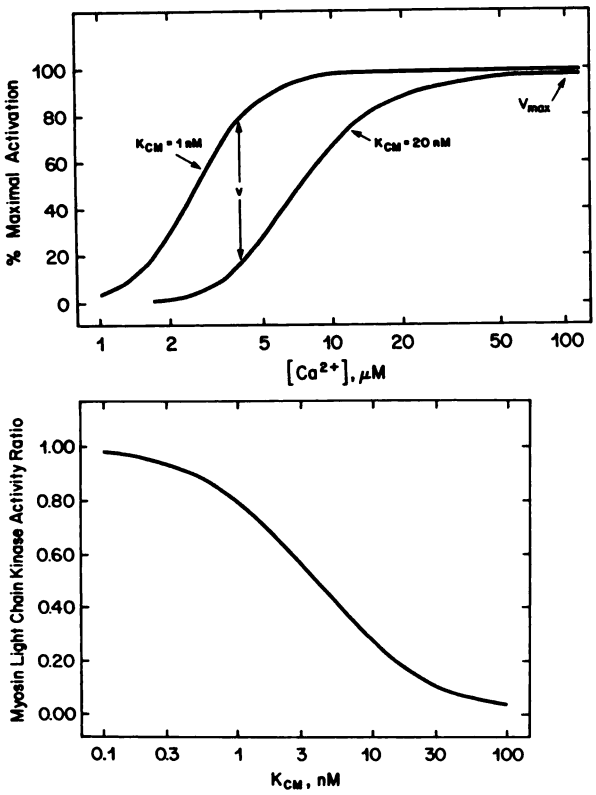

FIG. 1. *Effect of increasing K_{CM} on calcium sensitivity of myosin light chain kinase (top) and relationship between K_{Cm} values and the ratio of myosin light chain kinase activities (bottom)*

Top, effect of increasing K_{CM} on the calcium sensitivity of myosin light chain kinase. At 1 μM calmodulin and 0.2 nM myosin light chain kinase, the calcium concentrations required for activation of enzyme activity with a K_{CM} value of 1 nM are less than those required for activation when K_{CM} equals 20 nM (17). At a high concentration of calmodulin, the increasing calcium concentrations form the active form of calmodulin, i.e., Ca_i^{2+}·calmodulin. Hence, the phosphorylated enzyme, which has a greater K_{CM} value, will require greater concentrations of Ca^{2+} for activation. At 100 μM calcium, the activities of both forms of myosin light chain kinase are similar and close to V_{max} values. At 4 μM calcium, the activity of myosin light chain kinase with a K_{CM} value of 1 nM is greater than the activity of the enzyme with a K_{CM} value of 20 nM.

Bottom, the relationship between K_{CM} values and the ratio of myosin light chain kinase activities at 4 μM Ca^{2+}–100 μM Ca^{2+} in the presence of 1 μM calmodulin. Note that the relationship is not linear and the values for K_{CM} are plotted on a log scale.

homogenates for measurement of protein-bound ^{32}P, and of the myosin light chain kinase activity ratio, at a final enzyme dilution of 1:1000. The enzyme activity determinations at both 4 and 100 μM free Ca^{2+} were corrected by subtraction of the activity measured in the homogenate without the addition of purified enzyme.

Phosphorylation of myosin light chain kinase in tracheal smooth muscle homogenates. Frozen bovine trachealis muscle strips were homogenized in 20 volumes of 20 mM potassium phosphate, 4 mM EGTA, 10 mM NaF, 10 mM magnesium acetate, 1 mM [γ-^{32}P]ATP (200 cpm/pmole), 15 mM 2-mercaptoethanol, 0.5% Triton X-100, aprotinin (10 units/ml), and 0.1 mM PMSF, at pH 6.8 and 30°. For determination of histone phosphorylation rates, some of the homogenates also contained histone IIA (1.5 mg/ml). Control aliquots were withdrawn before addition of 5 μM cyclic AMP and 0.5 mM IBMX or catalytic subunit of cyclic AMP-dependent protein kinase (80 μg/ml) (final concentrations). After incubation intervals of 0.5, 2, and 5 min at 30°, aliquots were withdrawn and diluted in 50 mM potassium phosphate, 4 mM EDTA, and 15 mM 2-mercaptoethanol (pH 6.8) for myosin light chain kinase activity ratio determinations (final tissue dilution, 1:5000). At the same times, 20-μl aliquots were withdrawn and spotted on filter paper squares, which were then immersed in 10% trichloroacetic acid/4% sodium pyrophosphate. Protein-bound ^{32}P was measured as described previously (19). The amount of radioactivity incorporated into protein in the same reaction mixture, less histone IIA, served as a blank for determination of the rate of histone IIA phosphorylation.

Intact tracheal smooth muscle strip studies. Trachealis muscles were removed from fresh bovine trachea, dissected free of the intimal and adventitial layers, and cut into transverse strips measuring approximately 10 mm × 1.5 mm. The strips were mounted in a jacketed muscle bath containing Krebs-Ringer-bicarbonate solution supplemented with 10 mM glucose and 1 mM pyruvate, and aerated with 95% O_2 and 5% CO_2 as described by Katsuki and Murad (20). A passive force of 1.5 g (measured with a Grass FT.03 force displacement transducer) was applied to the strips, and they were allowed to equilibrate for at least 90 min at 36° ± 1°. At the end of the equilibration period, passive force was readjusted to 1.5 g. For rapid freezing of muscle strips for biochemical analyses, the bath was quickly lowered, and the strips were frozen by immersion in dichlorodifluoromethane precooled with liquid nitrogen (14) and stored at −65°. Portions of the frozen muscle strips, weighing 2–3 mg, were homogenized in 1 ml of 50 mM potassium phosphate, 4 mM EDTA, 15 mM 2-mercaptoethanol, 0.5% Triton X-100, aprotinin (10 units/ml), and 0.1 mM PMSF, pH 6.8 and 0°. Myosin light chain kinase activity ratio measurements were performed on the supernatant fractions (8000 × *g*, 5 min) of the homogenates. Phosphorylase activity ratio measurements were performed by the method of Hardman *et al.* (21). The extent of myosin light chain phosphorylation was quantitated by electrophoretic isolation of myosin and subsequent isoelectric focusing to separate the nonphosphorylated and phosphorylated forms of the myosin light chain as previously detailed (22).

Miscellaneous. [γ-^{32}P]ATP was prepared as described by Walseth and Johnson (23). Protein determinations were performed according to the method of Bradford (24), with bovine serum albumin used as a standard. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate essentially as described by Weber *et al.* (25). Statistical comparisons were made by Kruskal-Wallis nonparametric analyses of variance or Newman-Keuls multiple comparisons for parametric analysis (26).

RESULTS

Phosphorylation of purified myosin light chain kinase. Purified myosin light chain kinase from bovine tracheal smooth muscle had a molecular weight of 150,000 on polyacrylamide gels electrophoresed in the presence of sodium dodecyl sulfate. The enzyme was phosphorylated by incubation at 30° with the catalytic subunit of cyclic AMP-dependent protein kinase and 1 mM [γ-^{32}P]ATP. Radiolabeled phosphate was incorporated into myosin light chain kinase to the extent of 2.02 ± 0.2 moles of ^{32}P per mole of enzyme within 30 min (n = 4). Measurements of the myosin light chain kinase activity ratio were performed on the enzyme before and after phosphorylation. As illustrated in Table 1, phosphorylation of the kinase was accompanied by a decrease in the activity ratio from a control value of 0.79 to 0.24, corresponding to a 12-fold increase in the Ca_i^{2+}·calmodulin concentration necessary for half-maximal activity.

To determine whether the decrease in the activity ratio would be maintained during tissue extraction procedures, aliquots of the phosphorylated kinase were diluted 5-fold in 1:400 (w/v) homogenates of bovine tracheal smooth muscle containing EDTA and buffered with potassium phosphate or MOPS, as described under Materials and Methods. After a 10-min incubation, protein-bound ^{32}P in this mixture was 99% of that measured with the purified enzyme before addition to the homogenate. The activity ratio of myosin light chain kinase

TABLE 1

Recovery of phosphorylated myosin light chain kinase added to homogenates of tracheal smooth muscle

Purified myosin light chain kinase from bovine tracheal smooth muscle (38 μg/ml) was incubated with cyclic AMP-dependent protein kinase catalytic subunit as described under Materials and Methods. Aliquots of the reaction mixture were added to the homogenizing buffer containing no trachealis muscle (^{32}P-MLCK + buffer) or 2.5 mg of tracheal muscle per milliliter (^{32}P-MLCK + homogenate).

The extent of myosin light chain kinase phosphorylation catalyzed by catalytic subunit was determined as described under Materials and Methods and is expresed as moles of ^{32}P incorporated per mole of enzyme. Values represent the mean of two experiments with a range not exceeding 5%.

The ratio of myosin light chain kinase activity was measured at 4–100 μM calcium chloride in the presence of 1 μM calmodulin. The activity ratio of the phosphorylated enzymes was determined after incubation for 10 min in buffer or homogenate.

Treatment	Extent of phosphorylation	Activity ratio	cpm[a]
Control	—	0.79	—
^{32}P-MLCK + buffer	2.36	0.24	5950
^{32}P-MCLK + homogenate	2.34	0.27	5904

[a] Protein-bound ^{32}P measured in aliquots of buffer or homogenate removed 20 min after addition of phosphorylated myosin light chain kinase.

following incubation in the homogenate was 0.27. Thus, in the presence of inhibitors of protein kinase and phosphoprotein phosphatase activities, the ratio appears to be stable enough to permit measurement in an extract of intact smooth muscle. Qualitatively similar data were obtained with phosphorylation of purified myosin light chain kinase from aortic smooth muscle. Phosphorylation decreased the activity ratio from 0.79 to 0.21. Addition of the phosphorylated enzyme to homogenates of tracheal smooth muscle did not result in reversal of the activity ratio.

Addition of cyclic AMP or cyclic AMP-dependent protein kinase catalytic subunit of tracheal smooth muscle homogenates. In an attempt to phosphorylate the endogenous myosin light chain kinase in homogenates of tracheal smooth muscle, either 5 μM cyclic AMP and 0.5 mM IBMX were added to activate the endogenous cyclic AMP-dependent protein kinase, or the purified catalytic subunit of cyclic AMP-dependent protein kinase (80 μg/ml) was added. Addition of the catalytic subunit produced a substantial decrease in the myosin light chain kinase activity ratio within 30 sec (Table 2). This decrease coincided with a rate of histone IIA phosphorylation of 500 pmoles of ^{32}P incorporated per minute per milligram of tissue. Addition of 5 μM cyclic AMP increased the activity of the endogenous cyclic AMP-dependent protein kinase, as determined by an increase in the rate of phosphorylation of histone IIA, from 10 to 25 pmoles of ^{32}P incorporated per minute per milligram of tissue. However, cyclic AMP and IBMX had no effect on the myosin light chain kinase activity ratio. Similarly, no change was noted in the activity ratio during the time of incubation when no additions were made. Decreases in the activity ratio with added catalytic subunit were prevented by deletion of MgATP (data not shown). Thus, a sufficiently large amount of cyclic AMP-dependent protein kinase catalytic subunit, but not cyclic AMP alone,

TABLE 2

Myosin light chain kinase activity ratios in tracheal smooth muscle homogenates

Frozen bovine tracheal smooth muscle strips (10 mg) were homogenized in 20 volumes of a buffer which included 50 mM potassium phosphate, 4 mM EGTA, 10 mM NaF, 10 mM magnesium acetate, 1 mM ATP, and protease inhibitors as described under Materials and Methods, before addition of water (control), 5 μM cyclic AMP and 0.5 mM IBMX, or catalytic subunit of cyclic AMP-dependent protein kinase (80 μg/ml) (final concentrations).

Treatment	Time of incubation			
	0 min	0.5 min	2 min	5 min
Control	0.80	0.81	0.83	0.83
Cyclic AMP + IBMX	0.82	0.78	0.79	0.77
Catalytic subunit	0.76	0.50	0.50	0.43

produced a change in the sensitivity of myosin light chain kinase to activation by Ca_i^{2+}·calmodulin, in a manner consistent with phosphorylation of the enzyme.

Extraction of myosin light chain kinase from tracheal smooth muscle strips. In order to study phosphorylation of myosin light chain kinase in intact tissue, it is important to establish conditions under which a large fraction of the total kinase activity can easily be extracted, thus minimizing the possibility that either the phospho- or dephospho-kinase might be preferentially extracted. Therefore, untreated tracheal smooth muscle strips were homogenized under routine conditions (see Materials and Methods). The homogenates were centrifuged at 8000 × *g* for 5 min, and the supernatant fractions were removed for assay. The pellets were re-extracted three times in the same manner, except that the fourth extraction buffer contained 25 mM Mg^{2+} in excess of EDTA (16). Approximately 86% of the total activity was found in the first supernatant fraction, while 9%, 3% and 2% of the total activity were found in the second, third, and fourth supernatant fractions, respectively. We included 0.5% Triton X-100 in the homogenizing buffer, which increased the amount of kinase activity extracted without Triton X-100 in the first supernatant fraction by 30%. This amount of Triton X-100 was diluted 10-fold in the activity ratio assay mixture. Neither 0.5% Triton X-100 added to the homogenizing buffer nor 0.05% added to the assay mixture influenced the activity ratio measurement. The enzyme activity extracted from bovine tracheal smooth muscle in the first supernatant fraction was 870 ± 160 nmoles of ^{32}P incorporated per minute per gram (wet weight).

Intact tissue studies. Initial studies using intact tracheal smooth muscle tested the ability of isoproterenol to relax isolated strips precontracted by exposure to carbachol. At 0.1 μM carbachol, isometric force was approximately 75% of that elicited by maximal concentrations of carbachol, as indicated in Fig. 2. The strips developed an average active force of 11.2 ± 1.4 g with 0.1 μM carbachol after 5 min. Figure 2 also shows the effect of various concentrations of isoproterenol added to a bathing medium containing isolated trachealis strips precontracted by 0.1 μM carbachol. Isoproterenol was added to the bath in increasing cumulative concentrations from 1 nM to 10 μM for 3 min at each concentration. At 10 μM isoproterenol, active force was abolished. The average

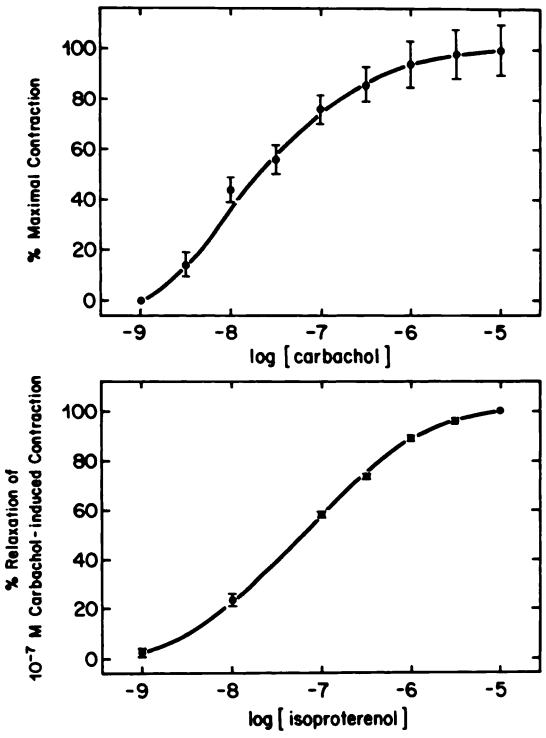

FIG. 2. *Contractile responses of tracheal smooth muscle to carbachol*

Top, percentage of maximal force generated by muscle strips upon addition of increasing cumulative concentrations of carbachol. Bottom, percentage of relaxation, produced by isoproterenol, of strips precontracted with 0.1 μM carbachol. Concentrations of isoproterenol shown are cumulative. Error bars indicate ± 1 SEM for three to five strips.

force decreased to 2.9 ± 0.4 g, or 26% of control values, at 0.3 μM isoproterenol, and this concentration was chosen for further studies. In a separate experiment, five trachealis muscle strips precontracted by 0.1 μM carbachol were exposed to 0.3 μM isoproterenol in a noncumulative fashion. The average active force in these strips was decreased from 13.5 ± 1.3 to 2.2 ± 0.5 g by isoproterenol.

The effect of *beta*-adrenergic stimulation of intact smooth muscle was studied by the addition of 0.3 μM isoproterenol to a muscle bath containing trachealis muscle strips which had not been precontracted. The strips were quick-frozen after incubation periods from 0 to 5 min. Measurements of the myosin light chain kinase and phosphorylase activity ratios were performed on homogenates of the frozen strips. The results (Fig. 3) indicate that phosphorylase *b* to *a* conversion was elevated significantly from control values at times between 1 and 5 min, whereas the myosin light chain kinase activity ratio did not differ from the control value of 0.80 at any time studied. Treatment with isoproterenol also did not affect the total amount of myosin light chain kinase activity extracted from the tissue, i.e., that activity measured at 100 μM calcium.

Upon exposure of trachealis muscle strips to 5 μM isoproterenol for 5 min, the myosin light chain kinase activity ratio decreased slightly, but significantly, from 0.80 ± 0.02 to 0.71 ± 0.02 (Fig. 4). Incubation for up to 30 min did not further decrease the ratio. This concentration of isoproterenol is sufficient to fully relax tracheal smooth muscle precontracted with 0.1 μM carbachol. Similar

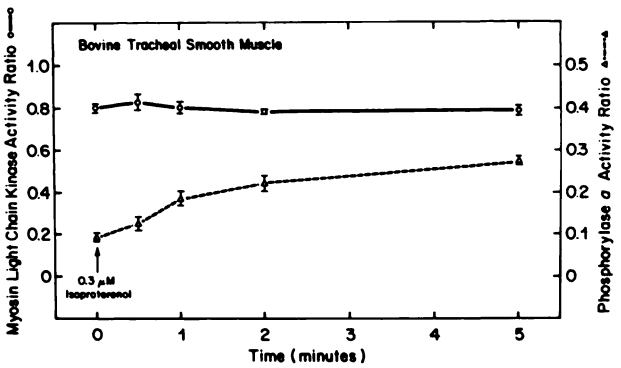

FIG. 3. *Time course of changes in myosin light chain kinase* (o) *and phosphorylase* (△) *activity ratios in bovine tracheal smooth muscle strips following exposure to* 0.3 μM *isoproterenol*

Activity ratio measurements were performed on extracts of strips quick-frozen at the times shown. Error bars indicate ± 1 SEM for four or five muscle samples.

slight decreases in the kinase activity ratio, however, occurred after incubation of muscle strips with 0.1 μM carbachol or with 80 mM KCl, which, like carbachol, contracts tracheal smooth muscle (Fig. 4). The activity ratios were further measured in strips which had been treated with each of these three agents alone, but following removal of the agent from the bath by extensive rinsing. In all three cases, the activity ratios did not differ from control values (Fig. 4), demonstrating that the modest effects produced by isoproterenol, carbachol, and KCl were reversible.

In further experiments, the extent of phosphorylation of the myosin light chain during isoproterenol-mediated relaxation of carbachol-contracted trachealis muscles was determined (Fig. 5). As previously reported by Silver and Stull (14), the phosphate content of the myosin light chain initially increases then decreases, while maximal isometric force is maintained, in trachealis muscles contracted with 1 μM carbachol. From the rate of decline in these previous data, it was estimated that the extent of phosphorylation would return to basal values (approximately 0.10 mole of phosphate per mole of myosin light

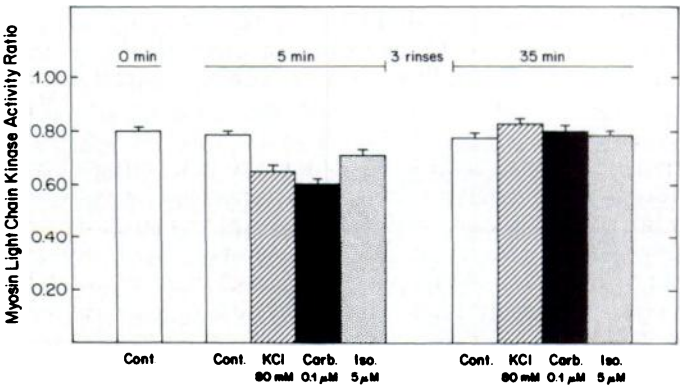

FIG. 4. *Effects of contractile and relaxant agents on myosin light chain kinase activity ratios of tracheal smooth muscle strips*

Left, untreated strips; center, after exposure for 5 min to 80 mM KCl, 0.1 μM carbachol, or 5 μM isoproterenol; right, after exposure to the same agents, followed by removal of the agents from the bath by three rinses over a period of 30 min. Error bars indicate ± 1 SEM for five to eight tracheal smooth muscle strips.

chain) after 2 hr of sustained contraction with carbachol. This estimate was close to actual values (0.11 ± 0.05 mole of phosphate per mole of myosin light chain) determined after 2 hr (Fig. 5). Addition of 5 μM isoproterenol at this time produced immediate, near-maximal relaxation (88%) without decreasing the phosphate content of the light chain (0.13 ± 0.05 mole of phosphate per mole of myosin light chain). Further evidence of activation of the cyclic AMP system was obtained with phosphorylase *a* activity ratio measurements. Ratios measured after 2 hr of carbachol-stimulated contraction (0.28 ± 0.30) more than doubled during this interval of incubation with isoproterenol (0.63 ± 0.02). These data show that relaxation of maximally contracted trachealis smooth muscle by the *beta*-adrenergic agonist may occur with no change (decrease) in the phosphate content of the myosin light chain, and further suggest that *beta*-adrenergic relaxation of intact smooth muscle does not necessarily involve phosphorylation of myosin light chain kinase.

DISCUSSION

Myosin light chain kinase catalyzes the phosphorylation of the 18,500–20,000 M_r myosin light chain subunit in smooth and stirated muscles. Available evidence indicates some differences in the biochemical properties of myosin light chain kinases purified from skeletal, cardiac, and smooth muscles (27) which may, in turn, reflect differences in the physiological significance of myosin phosphorylation among the three muscle types. Myosin light chain kinases purified from skeletal and smooth muscles appear to be similar in their activation properties (15, 17), but the phosphorylation of myosin light chain kinase itself has been shown to alter the activation properties of only the smooth muscle enzyme (8–10, 28, 29).

It has been suggested that the activation of skeletal muscle myosin light chain kinase by Ca^{2+} and calmodulin proceeds by the following reversible sequence (17, 30):

$$4\ Ca^{2+} + CM \overset{K_{Ca^{2+}}}{\rightleftharpoons} Ca_4^{2+}\cdot CM$$

$$Ca_4^{2+}\cdot CM + MLCK_{(inactive)} \overset{K_{CM}}{\rightleftharpoons} Ca_4^{2+}\cdot CM\cdot MLCK_{(active)}$$

where CM and MLCK represent calmodulin and myosin light chain kinase, respectively. In terms of this model, Conti and Adelstein (8) found that phosphorylation of myosin light chain kinase from gizzard smooth muscle reduced its affinity for the Ca_4^{2+}·calmodulin complex. The total intracellular calmodulin concentration is believed to far exceed (31) the concentration of Ca_4^{2+}·calmodulin necessary for half-maximal kinase activity (K_{CM}), hence the concentration of Ca_4^{2+}·calmodulin is limited by the intracellular concentration of free Ca^{2+} (32). Blumenthal and Stull (17) have proposed a quantitative model for the activation of myosin light chain kinase. This relationship predicts the fractional kinase activity (v/V_{max}) for given concentrations of Ca^{2+} and calmodulin when the equilibrium activation constants $K_{Ca^{2+}}$ and K_{CM} are known:

$$\frac{v}{V_{max}} = \frac{[CM]}{K_{CM}(1 + K_{Ca^{2+}}/[Ca^{2+}])^4 + [CM]} \qquad (1)$$

From this relationship, an increase in K_{CM}, accompanying phosphorylation of smooth muscle myosin light chain kinase, would be expected to decrease the fractional kinase activity at limiting free Ca^{2+} concentrations (where calmodulin is present at a high concentration). On the basis of determinations of $K_{Ca^{2+}}$ and K_{CM}, we propose that phosphorylation of smooth muscle myosin light chain kinase in intact tissue may be measured as it affects the ratio of kinase activities measured at 4 μM and 100 μM free Ca^{2+}, in the presence of 1 μM calmodulin.

We have found that, similar to the turkey gizzard smooth muscle kinase, purified bovine tracheal smooth muscle myosin light chain kinase is a substrate for cyclic AMP-dependent protein kinase. The results obtained from activity ratio measurements performed on the purified kinase demonstrate that bovine tracheal smooth muscle myosin light chain kinase is altered in its sensitivity to activation by Ca_4^{2+}·calmodulin, similar to the turkey gizzard kinase. Although the activity ratio measurement itself does not permit determination of the absolute value of K_{CM}, the values of the ratios v/V_{max} and $(v/V_{max})'$ obtained before and after phosphorylation, respectively, may be used to estimate a -fold change from K_{CM} to $K_{CM'}$, based upon a relationship derived from Eq. 1:

$$\frac{(V_{max}/v)' - 1}{(V_{max}/v) - 1} = \frac{K_{CM'}}{K_{CM}} \qquad (2)$$

Thus, a decrease in the activity ratio of purified myosin light chain kinase from 0.79 to 0.24 represents a 12-fold increase in the value of K_{CM} produced by phosphorylation, in close agreement with the findings of Conti and Adelstein (8).

The large, rapid decrease in the myosin light chain kinase activity ratio observed after addition of the catalytic subunit to trachealis muscle homogenates is consistent with the idea that myosin light chain kinase is phosphorylated by the catalytic subunit. However, activation of the endogenous cyclic AMP-dependent protein kinase in tracheal smooth muscle homogenates by the addition of 5 μM cyclic AMP did not lead to a decrease in the myosin light chain kinase activity ratio. The amount of catalytic activity of the added catalytic subunit was 30-fold greater than the catalytic activity of the endogenous cyclic AMP-dependent protein kinase. Hence, at least in the disrupted cell, phosphorylation of myosin light chain kinase by the endogenous cyclic AMP-dependent protein kinase appears to be exceedingly slow or not to occur at all.

To determine the sensitivity of myosin light chain kinase to calmodulin activation in intact tracheal smooth muscle tissue following *beta*-adrenergic stimulation, we studied trachealis muscle strips exposed to isoproterenol in the relaxed state, since Conti and Adelstein (8) suggest that phosphorylation resulting in a change in the activation properties of the kinase would be inhibited when the enzyme was activated, i.e., bound to calmodulin. If *beta*-adrenergic induced relaxation of tracheal smooth muscle were mediated by phosphorylation of myosin light chain kinase, then an effect on the myosin light chain kinase activity ratio should be manifest upon preincubation with 0.3 μM isoproterenol. Previous findings indicate that preincubation with isoproterenol will inhibit

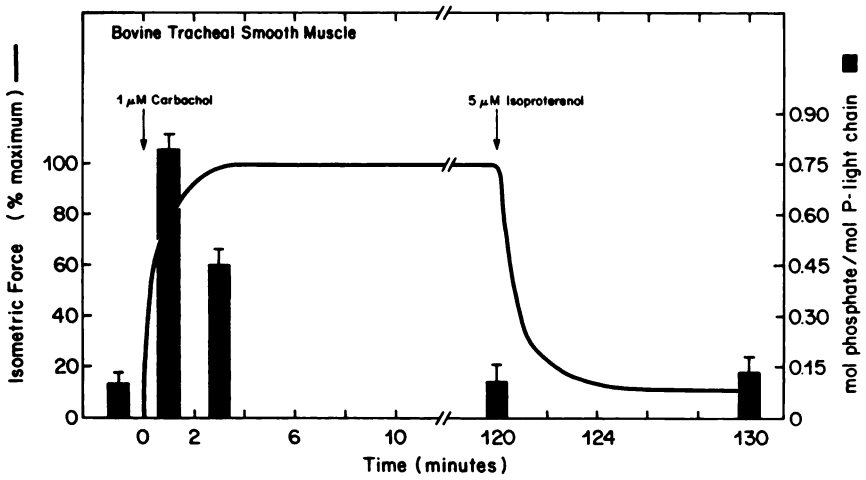

FIG. 5. *Effect of isoproterenol on relaxation and myosin light chain phosphorylation in trachealis smooth muscle*

Muscle strips were contracted for 2 hr by the addition of 1 μM carbachol; after 2 hr, 5 μM isoproterenol was added to the muscle bath for 10 min. The values for isometric force (*% maximum*) are the means for 4–12 muscles per treatment. The extent of myosin light chain phosphorylation (moles of phosphate per mole of P-light chain) was quantitated at the indicated times as described (22). Values represent the mean ± 1 SEM for four to eight muscle strips per time point.

the force-developed and P-light chain phosphorylation in tracheal smooth muscle (14). However, 0.3 μM isoproterenol produced marked relaxation and stimulation of the glycogenolytic cascade, but it did not alter the myosin light chain kinase activity ratio.

Only at the highest concentration tested (5 μM for 5 min) did isoproterenol affect the myosin light chain kinase activity ratio. The decrease from 0.80 to 0.71 (Fig. 4) corresponds to less than a 2-fold increase in K_{CM} (Fig. 1). Similar small changes in the activity ratio resulted from exposure to carbachol or KCl. It is not clear at this time whether these small effects are related to phosphorylation of myosin light chain kinase. It is possible that there are changes in the biochemical properties of myosin light chain kinase, but these modest changes in the activity ratio are found with agents which cause contraction (KCl and carbachol) as well as a high concentration of isoproterenol, which causes relaxation.

The relaxation effects of *beta*-adrenergic receptor stimulation on tracheal smooth muscle are not necessarily dependent upon decreased myosin light chain phosphorylation. Preincubation of tracheal smooth muscle with isoproterenol inhibited myosin light chain phosphorylation and isometric force development in response to carbachol (14). These observations alone do not explain whether a decrease in the availability of activating Ca^{2+} or phosphorylation of myosin light chain kinase, or both, account for the apparent decrease in myosin light chain kinase activity. However, the extent of phosphorylation of myosin light chain in bovine tracheal smooth muscle is not maintained during prolonged isometric tension (14) (Fig. 5). Since *beta*-adrenergic stimulation produces marked relaxation even when the phosphate content of P-light chain has returned to control values, biochemical mechanisms other than phosphorylation of myosin light chain kinase are probably primary determinants of the relaxation response (5–7).

In summary, we have proposed measurement of the myosin light chain kinase activity ratio to measure changes in the calmodulin activation properties of myosin light chain kinase hypothesized to accompany phosphorylation of the enzyme. This technique has yielded evidence to suggest that myosin light chain kinase in purified form or in a smooth muscle homogenate may be phosphorylated in the presence of a sufficiently high concentration of cyclic AMP-dependent protein kinase catalytic subunit. Phosphorylation of smooth muscle kinase produces a decreased sensitivity to activation by Ca_4^{2+}·calmodulin. However, significant phosphorylation of the kinase in intact smooth muscle would depend upon several factors, including the cyclic AMP-dependent protein kinase content in the tissue, the degree of its activation by cyclic AMP following *beta*-adrenergic stimulation, and the catalytic rate of phosphorylation of myosin light chain kinase by cyclic AMP-dependent protein kinase. In fact, no changes in the activation properties of myosin light chain kinase were observed when intact tracheal smooth muscle strips were exposed to concentrations of isoproterenol sufficient to produce marked relaxation. These results suggest that *beta*-adrenergic stimulation did not result in phosphorylation of the enzyme. Among the possible explanations for this finding are (a) that calmodulin, with its calcium-binding sites partially occupied by Ca^{2+}, remains bound to myosin light chain kinase in resting smooth muscle (16), blocking the site of phosphorylation; (b) that the kinase is associated, *in vivo*, with myofibrillar proteins in a way which prevents its phosphorylation; and (c) that phosphorylation of the kinase *in vivo* occurs much too slowly to be an intermediate step in the response to *beta*-adrenergic stimulation. These possibilities may be resolved by additional studies on the properties of phosphorylation of purified smooth muscle myosin light chain kinase by cyclic AMP-dependent protein kinase.

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