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Phosphorylation of Myosin Light Chain and Phosphorylase in Tracheal Smooth Muscle in Response to KCl and Carbachol

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SUMMARY

It has been proposed that Ca^{2+}-dependent myosin light chain (P-light chain) phosphorylation in smooth muscle permits cycling of myosin cross-bridges within myofibrillar elements for muscle shortening, but a second Ca^{2+}-dependent regulatory mechanism is responsible for force generation. Accordingly, we examined P-light chain phosphorylation and another Ca^{2+}-dependent protein phosphorylation reaction, phosphorylase *a* formation, in bovine tracheal smooth muscle during isometric force generation elicited by the cholinergic agonist carbachol or KCl depolarization, two stimuli thought to increase the concentration of sarcoplasmic free Ca^{2+} by mobilizing different pools of Ca^{2+}. Increases in P-light chain phosphorylation reached maximal values of 0.79 and 0.59 mole of phosphate per mole of P-light chain at 1 min and then declined during maintained isometric force developed in response to 1 μM carbachol and 60 mM KCl, respectively. Carbachol elicited approximately twice the amount of force as found in the presence of KCl, and yet a more rapid rate of decline in the phosphate content of P-light chain was apparent. Decreases in maximal levels of phosphorylase *a* also occurred during carbachol-mediated isometric force maintenance, yet did not occur with KCl stimulation. Concentration-dependent responses with carbachol and KCl showed a positive relationship between the extent of P-light chain phosphorylation and extent of developed isometric force after 1 min of contraction with both stimuli. Under no conditions was force generated without P-light chain phosphorylation. The concentration dependence of phosphorylase *a* formation with KCl was similar to isometric force and P-light chain phosphorylation. However, concentrations of carbachol necessary to stimulate phosphorylase *a* formation were much higher than those required for stimulation of isometric force and P-light chain phosphorylation. Furthermore, carbachol attenuated the stimulation of phosphorylase *a* formation by isoproterenol. Thus, carbachol appears to have both an inhibitory and stimulatory effect on phosphorylase *a* formation in bovine tracheal smooth muscle. These results also indicate that maintained isometric force in smooth muscle may be dependent upon the maximal extent of P-light chain phosphorylation obtained during an early temporal transient in phosphorylation.

INTRODUCTION

Contraction of smooth muscle is thought to be dependent upon an increase in the concentration of sarcoplasmic free Ca^{2+} which activates the contractile elements. The source of activator Ca^{2+} may be extracellular or intracellular, and varies with the physiological or pharmacological agent producing the contraction (1–3). For example, K^+ depolarization stimulates influx of extracellular Ca^{2+} through potential-dependent Ca^{2+} channels in the sarcolemma. On the other hand, cholinergic stimulation is thought to stimulate extracellular Ca^{2+} influx through potential-independent as well as potential-dependent sarcolemmal channels, and, in addition, stimulates release of Ca^{2+} from intracellular sites (4, 5).

Calcium regulates several intracellular processes mainly by binding to the ubiquitous Ca^{2+} receptor protein, calmodulin (6). Among these processes is activation of myosin light chain kinase and phosphorylase kinase, which may result in an increase in the extent of phosphorylation of the respective protein substrates, P-light chain[2] and phosphorylase. There is substantial biochemical evidence that implicates myosin P-light chain phosphorylation in the Ca^{2+}-dependent regulation of smooth

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[2] The abbreviation used is: P-light chain, phosphorylatable myosin light chain (20,000 M_r light chain of myosin).

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muscle actin-myosin interactions (7–9). However, alternative hypotheses, suggesting that Ca^{2+}-dependent regulation resides in other contractile elements, also exist (10–12). In intact smooth muscle, the relationship between the extent of isometric force development and extent of P-light chain phosphorylation is not straightforward. As originally reported by Dillon *et al.* (13) for arterial smooth muscle and later by Silver and Stull (14) and Gerthoffer and Murphy (15) for tracheal smooth muscle, the phosphate content of the P-light chain increases during the initial development of isometric force and then declines during the subsequent maintenance of isometric force. Others (16), however, have reported sustained levels of P-light chain phosphorylation during maintained isometric contractions for a short period of time in canine tracheal smooth muscle.

It has been suggested that myosin P-light chain phosphorylation is not required for steady-state force maintenance in response to a contractile stimulus due to the formation of latched myosin cross-bridges (13, 15, 17). Phosphorylation of P-light chain stimulates the rate of myosin cross-bridge cycling and hence has been correlated to shortening velocity in intact arterial smooth muscle. No unique relationship has been found between developed isometric force and the extent of P-light chain phosphorylation. Based on measurements at steady state, it has been proposed that a higher sarcoplasmic Ca^{2+} concentration appears to be necessary for P-light chain phosphorylation than for force maintenance (18). Thus, two Ca^{2+}-dependent mechanisms have been proposed to regulate the mechanical performance of smooth muscles: (a) myosin phosphorylation and shortening velocity and (b) a second unidentified site that allows force maintenance with low cross-bridge cycling rates (latch). Although P-light chain phosphorylation was not measured, low sarcoplasmic Ca^{2+} concentrations were proposed to lead to attached, noncycling cross-bridges in resting taenia coli (19).

In further studies, we have observed qualitative differences in the extent of phosphorylation of the P-light chain and phosphorylase during *beta*-adrenergic stimulation (14) as well as during inhibition of tension development by the phenothiazine derivative, fluphenazine (20). These observations suggest that, in intact smooth muscle, the regulation of these three different Ca^{2+}-dependent processes (myosin P-light chain and phosphorylase phosphorylation, and isometric force development) may be different.

To date, a complete study comparing changes in the regulation of phosphorylation of phosphorylase and the P-light chain in intact smooth muscle under conditions where force development is stimulated by distinct contractile agents is lacking. Accordingly, we have analyzed changes in these parameters in tracheal smooth muscle following contractions elicited by two distinct methods, K^+ depolarization and stimulation of cholinergic muscarinic receptors, which presumably increase the intracellular concentration of calcium. These comparisons have been made on both a *temporal* and *concentration-response* basis and show that different temporal patterns and concentration-response relationships between phosphorylation of these proteins exist. The primary focus of this study, however, was to determine whether isometric force generation was independent of myosin P-light chain phosphorylation; i.e., could isometric force be generated without P-light chain phosphorylation if a second Ca^{2+} regulatory site is more sensitive to activation by Ca^{2+} than myosin light chain kinase (18)? Our results suggest that, regardless of the source(s) of Ca^{2+} responsible for contraction, concomitant increases in phosphate content of the P-light chain parallel increases in the extent of developed isometric force during the early phase of contraction. Furthermore, the maximal extent of P-light chain phosphorylation during the early phase of the contraction is correlated to steady-state isometric force.

MATERIALS AND METHODS

Intact tracheal smooth muscle strip preparation. Trachealis smooth muscle strips were prepared as previously described (14, 20). Briefly stated, muscles were removed from fresh bovine trachea, dissected free of the intimal and adventitial layers, and cut into transverse strips. The strips were mounted vertically in a jacketed muscle bath (36° ± 1°) containing Krebs-Ringer bicarbonate solution supplemented with 10 mM dextrose and 1 mM pyruvate, and aerated with 95% O_2/5% CO_2. A passive force of 1.5 g was applied to the strips, and they were allowed to equilibrate for at least 90 min. Alternatively, muscles were equilibrated for 2 hr without passive force followed by 45 min with passive force. At the end of the equilibration period, passive force was readjusted to 1.5 g. Following prechallenge with 0.1 μM carbachol, the muscle strips were rinsed two or three times with fresh Krebs-Ringer bicarbonate solution until force returned to resting levels, and then three or four additional times for 20 min. After this 20-min equilibration period, the muscle strips were exposed to the pharmacological agents and quick-frozen at the indicated times by rapidly lowering the muscle bath and immersing the strips in dichlorodifluoromethane cooled in liquid nitrogen (14, 21). The pharmacological agents used in this study were atropine (purchased from Burns Biotec), carbachol, isoproterenol HCl, and (±)-propranolol (purchased from Sigma Chemical Company). After freezing, the clipped ends of the muscle strips were chipped away and discarded; the remaining portions were stored in airtight containers at −65° prior to biochemical analysis.

Biochemical analyses. Frozen portions of each muscle strip were divided into approximately equal halves weighing 5–7 mg (frozen wet weight). Each portion was assayed for either P-light chain phosphate content or extent of phosphorylase *a* formation. Thus, both biochemical measurements were made from the same muscle strip, which allowed a direct comparison of the biochemical measurements to the extent of developed isometric force.

The phosphate content of the P-light chain was quantitated by a combination of two electrophoretic procedures as detailed previously (22). Briefly stated, frozen portions of muscle strips were homogenized in 15–20 volumes (w/v) of an extraction buffer containing 100 mM sodium pyrophosphate (pH 8.8), 5 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid, 50 mM sodium fluoride, 10% glycerol, 15 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and aprotinin (Trasylol) (100 units/ml) and centrifuged at 7000 × *g* for 20 min. The supernatant fraction was mixed with 20 μl of a saturated sucrose solution, and an aliquot of this mixture (30–40 μl) was subjected to pyrophosphate-polyacrylamide gel electrophoresis to isolate native myosin from other cellular proteins. After brief staining with Coomassie Blue R-250, the protein band representing myosin and a portion of the top of the pyrophosphate gel were excised, homogenized in an isoelectric focusing denaturant buffer containing 8 M urea, and subjected to isoelectric focusing on polyacrylamide gels to separate the phosphorylated from the nonphosphorylated form of the myosin P-light chain. After staining by an ammonial-silver staining procedure, the phosphate content of the P-light chain was quantitated by measuring the relative amounts of the phosphorylated and nonphosphorylated forms of the P-light chain by densitometry.

To determine phosphorylase *a* content, frozen muscle portions were homogenized in 15 volumes (w/v) of an extraction buffer containing 20 mM β-glycerolphosphate (pH 6.8), 20 mM sodium fluoride, 2 mM EDTA, 0.01% bovine serum albumin, and 15 mM 2-mercaptoethanol. After homogenization, acid-washed Norite (10 mg/ml final concentration) was added, and the extracts were centrifuged at 3000 × *g* for 10 min. Phosphorylase *a* activity in the supernatant fraction was determined using an enzyme-coupled fluorometric procedure (23). Each sample was assayed in duplicate, and the phosphorylase *a* activity ratio was expressed as the ratio of activity measured in the absence and presence of 5′-AMP.

Statistical comparisons were made by either Student's *t*-test or the Newman-Keuls multiple comparisons test.

RESULTS

Temporal comparisons. Differences in the temporal responses in both P-light chain and phosphorylase phosphorylation were apparent during the development and maintenance of isometric force in the presence of 1 μM carbachol or 60 mM KCl (Fig. 1). These concentrations of carbachol (1 μM) and KCl (60 mM) produced respective maximal contractile responses; contractions with KCl were elicited in the presence of 0.1 μM atropine to minimize possible effects due to K^+ depolarization and release of acetylcholine from cholinergic nerve terminals. The maximal extent of isometric force developed in the presence of KCl was approximately 55% of that obtained with carbachol; likewise, the maximal extent of P-light chain phosphorylation was appreciably greater with carbachol (0.79 mole of phosphate per mole of P-light chain) than with KCl (0.59 mole of phosphate per mole of P-light chain). These maximal levels of P-light chain phosphorylation occurred 1 min after the addition of either carbachol or KCl, whereas maximal force development occurred after 3 min for carbachol and 1 min for KCl. The maximal isometric force developed in the presence of either agent was maintained for up to 120 min, yet the phosphate content of the P-light chain declined during this interval. The rate and magnitude of this decline were dependent upon the contractile agent. Muscles stimulated in the presence of KCl showed decreases in phosphate content from 0.59 to 0.40 mole of phosphate per mole of P-light chain after 3 min; this value was unchanged after 120 min. In contrast, muscles contracted in the presence of carbachol showed a continued decrease from the maximal value obtained at 1 min (0.79) and reached basal levels (0.11 mole/mole) after 120 min. When compared with the marked differences in steady-state isometric force at 120 min (12 g with carbachol; 6.3 g with KCl), these findings further emphasize the lack of correlation between the maintained isometric force and steady-state phosphate content of the P-light chain in smooth muscle.

Phosphorylase *a* formation (Fig. 1) also showed a differential response which was dependent upon the contractile agent. Phosphorylase *a* formation reached a maximal value (0.60) by 1 min in the presence of carbachol, whereas approximately 3 min were required to achieve a maximal response in the presence of KCl. After 120 min, the extent of phosphorylase *a* formation was maintained at a maximal value in the presence of KCl. However, in the presence of carbachol, the ratio value at 120 min was decreased to 0.28. This ratio value was significantly greater than the control value (0.13).

Concentration-response relationships. The relationships between concentration of either contractile agent and isometric force development, P-light chain phosphate content, and phosphorylase *a* activity ratio were also examined (Fig. 2). Comparisons were made 1 min after addition of the contractile agent. The abscissae in Fig. 2 were adjusted so that the extent of isometric force obtained with low and medium concentrations of both KCl and carbachol were similar. Under these conditions, concentration-dependent increases in isometric force development were paralleled by concomitant concentration-dependent increases in P-light chain phosphate content during stimulation with either contractile agent. This similarity is reflected in the estimations of concen-

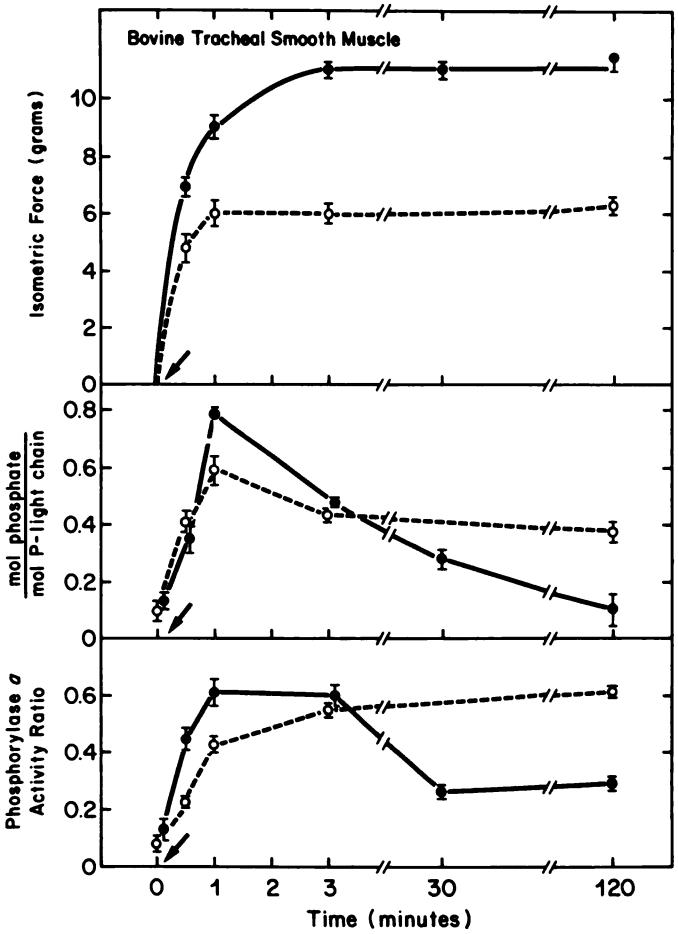

FIG. 1. *Comparison of responses to carbachol and KCl*

Temporal changes in isometric force (top), P-light chain phosphorylation (middle), and phosphorylase *a* formation (bottom) in the presence of 1 μM carbachol (●) or 60 mM KCl and 0.1 μM atropine (○) are shown. Atropine was added 5 min prior to the addition of KCl. Tracheal smooth muscle strips were frozen at the indicated times after the addition (*arrow*) of carbachol or KCl, and all parameters were quantitated as described under Materials and Methods. The values shown represent the mean ± standard error of the mean for three to eight samples per point from a total of twelve different tracheae; experiments with carbachol and KCl were performed on muscle strips obtained from each trachea. All values, with the exception of the P-light chain phosphate content at 120 min with carbachol stimulation, were significantly ($p < 0.05$) different from unstimulated values.

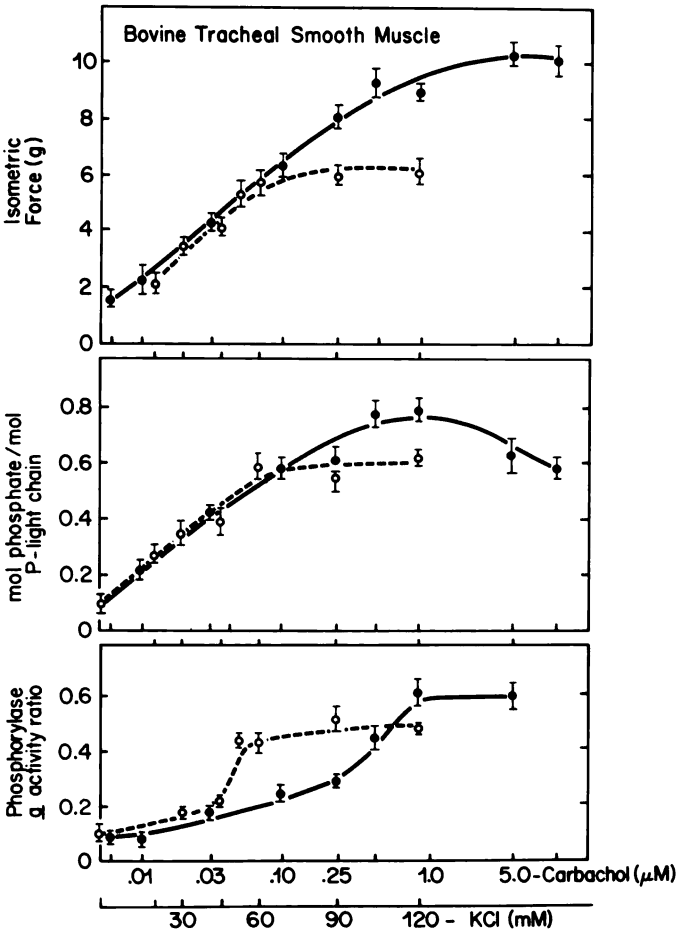

FIG. 2. *Comparison of concentration-response relationships to carbachol and KCl*

Changes in isometric force (top), P-light chain phosphorylation (middle), and phosphorylase *a* formation (bottom) in the presence of carbachol (●) or KCl and 0.1 μM atropine (○) are shown. Muscle strips were frozen 1 min after the addition of either contractile agent. Atropine was added 5 min prior to the addition of KCl. All values represent the mean ± standard error of the mean for three to eight samples per point.

trations required for half-maximal responses for each agent. The concentrations of carbachol required for half-maximal responses for force and P-light chain phosphorylation were 45 nM and 44 nM, respectively. The concentrations of KCl required for half-maximal responses were 28 mM and 23 mM, respectively, for force and P-light chain phosphorylation. However, the concentration relationships between phosphorylase *a* formation and either force or P-light chain phosphorylation were different. The concentration of KCl required for the half-maximal response for phosphorylase *a* formation (47 mM) was approximately 2 times greater than the half-maximal concentrations required for isometric force or P-light chain phosphorylation responses. The value for phosphorylase *a* formation during carbachol stimulation (280 nM) was over 6-fold greater than the value for isometric force or P-light chain phosphorylation. Thus, under conditions in which the extent of isometric force development was similar for KCl and carbachol, phosphorylase *a* formation in the presence of KCl was proportionally greater than phosphorylase *a* formation in the presence of carbachol.

It was possible that this difference in the phosphorylase response may have been due to an increase in cyclic AMP content through stimulation of *beta*-adrenergic receptors by neurotransmitters released during the addition of KCl. To test this possibility, isometric force and phosphorylase responses to KCl were measured in the presence and absence of propranolol. Responses measured 1 min after the addition of 45, 60 or 120 mM KCl showed no differences in either isometric force development or phosphorylase *a* levels in the presence of 0.1 μM propranolol. Thus, it appears that these differences were not due to *beta*-adrenergic involvement.

The possibility of temporal dissociation was also examined in the comparison of isometric force development and phosphorylase *a* formation (Fig. 3). Responses were compared 5 min after the initiation of contraction rather than after 1 min. In this way, steady-state levels of isometric force, rather than initial values obtained during force development, could be compared. A comparison with P-light chain phosphorylation was not made, since the phosphate content of the P-light chain was already declining by this time (Fig. 1; ref. 14). Figure 3 shows that, during KCl stimulation, the difference in the concentration required for half-maximal responses in phosphorylase *a* formation and isometric force obtained at 1 min was eliminated. The half-maximal concentration values for KCl for both parameters were similar (28 mM versus 24 mM). The concentration of carbachol required for a half-maximal response for isometric force was 18

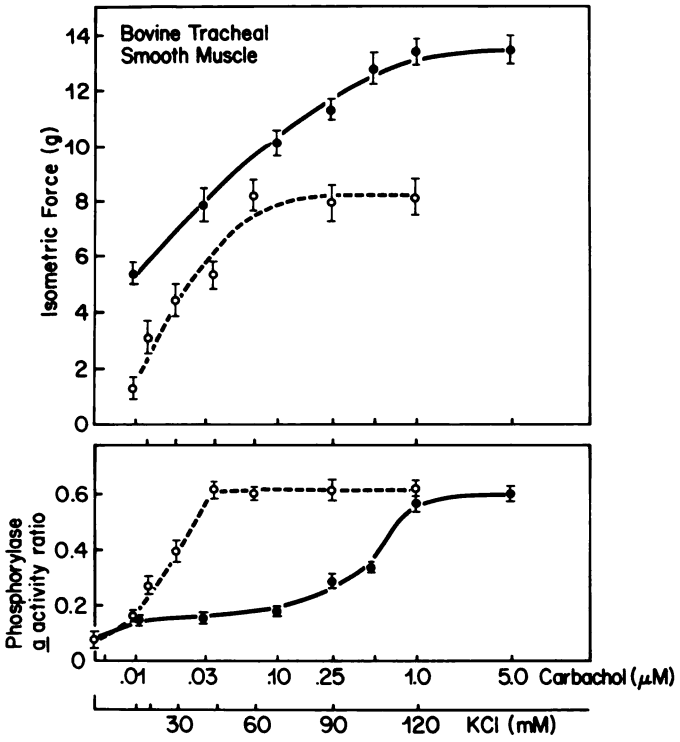

FIG. 3. *Comparison of concentration-response relationships to carbachol and KCl measured 5 min after initiation of contraction*

Changes in isometric force (top) and phosphorylase *a* activity ratio (bottom) during stimulation to contraction with either carbachol (●) or KCl and 0.1 μM atropine (○) are shown. All procedures were as described in Fig. 2; values are the mean ± standard error of the mean for three to six samples per point.

nM, whereas the value for phosphorylase *a* formation was 280 nM. This value for phosphorylase *a* formation at 5 min (280 nM) is not different from that value measured at 1 min.

Inhibition of isoproterenol-mediated phosphorylase a formation by carbachol. The effects of KCl and carbachol on *beta*-adrenergic-mediated phosphorylase *a* formation were also examined (Fig. 4). Incubation with 5 μM isoproterenol for 6 min, in the presence or absence of 0.1 μM atropine, increased the phosphorylase *a* activity ratio to approximately 0.45. Incubation with carbachol (0.1 μM) alone for 1 min also increased the phosphorylase *a* activity ratio (0.25) and isometric force (6.5 g); treatment with isoproterenol completely inhibited the carbachol-dependent increase in force; however, the phosphorylase *a* activity ratio (0.33) was significantly decreased as compared with the ratio obtained with isoproterenol alone. This inhibitory effect of carbachol on phosphorylase *a* formation was blocked by prior treatment with atropine. Stimulation with 30 mM KCl (in the presence of atropine) also increased isometric force and phosphorylase *a* formation. Pretreatment with isoproterenol inhibited the increase in force, yet, in contrast to carbachol, the isoproterenol-mediated increase in the phosphorylase *a* activity ratio was not attenuated by KCl. These results suggest that stimulation of cholinergic receptors can produce both a stimulatory effect and an inhibitory effect on phosphorylase *a* formation in tracheal smooth muscle.

DISCUSSION

This study shows that the development of isometric force in intact bovine tracheal smooth muscle is directly correlated to the extent of phosphorylation of the myosin P-light chain. As shown in Fig. 5A, this correlation occurs over the range of concentrations of both carbachol and KCl for isometric force development, as well as with inhibition of force by relaxant agents such as isoproterenol and fluphenazine. This correlation suggests that myosin P-light chain phosphorylation plays an important role in determining the extent of development of isometric force in tracheal smooth muscle regardless of the conditions producing the contraction. The maximal extent of phosphorylation obtained at 1 min was also related to the final steady-state (10 min) level of isometric force (Fig. 5B). There appeared to be a difference in the slopes between KCl-contracted and carbachol-stimulated muscles. The reason for this apparent difference is not known at this time, but may indicate regulatory mechanisms (10–12) in addition to P-light chain phosphorylation in smooth muscle contraction.

In previous studies on smooth muscle, the dissociation between steady-state force and phosphorylation of P-light chain has been emphasized (13–15, 17, 18, 20). On the basis of responses to changes in Ca^{2+} concentrations in physiological saline solution, it was proposed that isometric force was maintained at lower Ca^{2+} concentrations than P-light chain phosphorylation (18). If force generation and P-light chain phosphorylation are independent events, low Ca^{2+} concentrations should lead to force generation without P-light chain phosphorylation. At low concentrations of KCl and carbachol, the amount of Ca^{2+} released into the sarcoplasm for activation of the contractile elements would be expected to be low. In these experiments on bovine trachealis smooth muscle, development of isometric force was proportional to the extent of phosphorylation of P-light chain measured at 1 min, even with low concentrations of KCl and carbachol. Thus, force generation in intact bovine tracheal smooth muscle was not dissociated from P-light chain phosphorylation. These results also indicate that it is important to establish temporal responses rather than measuring P-light chain phosphorylation at a single time

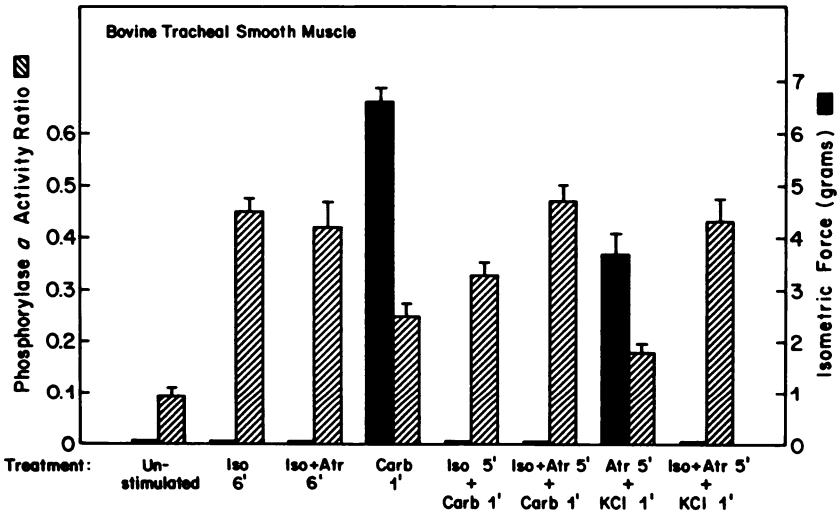

FIG. 4. *Effect of carbachol and KCl on isoproterenol-mediated phosphorylase a formation*

Muscle strips were either unstimulated or exposed to 5 μM isoproterenol (*Iso*), 5 μM isoproterenol plus 0.1 μM atropine (*Iso + Atr*), 0.1 μM carbachol (*Carb*), 5 μM isoproterenol plus 0.1 μM carbachol (*Iso + Carb*), 5 μM isoproterenol and 0.1 μM atropine plus 0.1 μM carbachol (*Iso + Atr + Carb*), 0.1 μM atropine plus 30 mM KCl (*Atr + KCl*), or 5 μM isoproterenol and 0.1 μM atropine plus 30 mM KCl (*Iso + Atr + KCl*) for the indicated time intervals. Isometric force and the phosphorylase *a* activity ratio were determined as described under Materials and Methods. All values are the mean ± standard error of the mean for four to nine samples per group. A comparison of the mean values for the phosphorylase *a* activity ratio using the Neuman-Kuels non-parametric analysis revealed that all groups were significantly different from the unstimulated group; Iso, Iso + Atr, Iso + Atr + Carb, and Iso + Atr + KCl were different from Carb, Iso + Carb, and Atr + KCl.

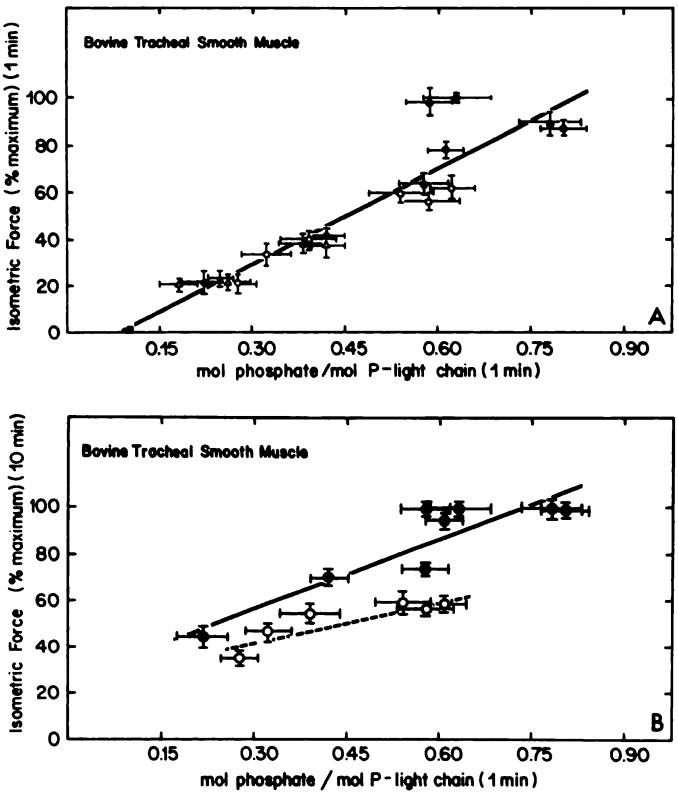

FIG. 5. *Effect of phosphate content on isometric force*

A. Correlation between the extent of isometric force development (% maximum) as a function of the phosphate content of the myosin P-light chain measured 1 min after the initiation of contraction. All values are the mean ± standard error of the mean; values for the different concentrations of carbachol (●) and KCL (○) were obtained from Fig. 2. Values for isoproterenol plus carbachol (▲) and fluphenazine plus KCl (△) were obtained from our previous studies (14, 20). The *solid line* represents the calculated line of regression [$y = 136.2(x) - 11.6$; $r = 0.92$; $p < 0.001$].

B. Correlation between the steady-state level of isometric force (% maximum), measured 10 min after initiation of contraction, and phosphate content of the P-light chain, measured 1 min after initiation of contraction. Values are the mean ± standard error of the mean for varying concentrations of carbachol (●) or KCl (○). The values for phosphate content of the myosin P-light chain were quantitated in muscle strips frozen 1 min after the addition of the respective contractile agent; isometric force was determined in matched muscle strips (same trachea) 10 min after the addition of the contractile agent. The *solid line* represents the calculated line of regression for carbachol-contracted muscles [$y = 27.5 + 99.5(x)$; $r = 0.89$; $p < 0.01$]; the *dashed line* represents the calculated line of regression for KCl-contracted muscles [$y = 26.5 + 56.3(x)$; $r = 0.86$; $p < 0.05$].

after the maximal response. It is apparent that one could obtain significant steady-state force with little or no phosphorylation of P-light chain at extended periods of time.

P-light chain phosphorylation may act as a switch for smooth muscle contraction. Ca^{2+}-dependent P-light chain phosphorylation may stimulate cross-bridge cycling and initiate shortening or development of isometric force. The number of cross-bridges that may be transformed to the attached, noncycling latch state will be proportional to the number of cross-bridges that are initially phosphorylated and hence cycling. If P-light chain is not first phosphorylated, myosin may not be able to form the latch state. This hypothesis is consistent with the recent observation that myosin phosphorylation may be a prerequisite for the latch state in detergent-treated carotid arteries (24). Furthermore, there was no difference in the Ca^{2+} concentrations required for the initial stimulation of P-light chain phosphorylation and stress. However, the subsequent reduction in Ca^{2+} concentrations led to stress maintenance without proportional phosphorylation. Thus, the maintenance of isometric force with the decrease in myosin P-light chain phosphorylation may depend upon Ca^{2+} binding to another myofibrillar site that regulates the latch state.

A recent study on the phosphorylation of purified gizzard myosin indicated that phosphorylation occurs in an ordered fashion, with phosphorylation of the first P-light chain on myosin occurring at a much faster rate than the phosphorylation of the second P-light chain (25). In addition, the relationship between the extent of phosphorylation of P-light chain in myosin and actin-activated myosin ATPase activity was not linear. Phosphorylation of P-light chain in bovine trachealis muscle shows no evidence of sequential phosphorylation or negative cooperativity. Within 1 min there was an apparent linear rate of phosphorylation up to 0.8 mole of phosphate per mole of P-light chain (Fig. 1) (14). In addition, the linear relationship between P-light chain phosphorylation and force generation (Fig. 5A) suggests no positive cooperativity. These results are consistent with the recent observations of Chacko and Rosenfeld (12), who found a linear relationship between the extent of P-light chain phosphorylation and actin-activated myosin ATPase activity for myosin purified from swine pulmonary arteries. Perhaps the nonlinear properties for phosphorylation and ATPase activity are characteristic of gizzard myosin, but not mammalian myosin.

Different temporal responses were apparent in bovine tracheal smooth muscle with the two agents producing contraction. Contractions by both KCl and carbachol were associated with transient increases in P-light chain phosphorylation; however, the rate and magnitude of the decline in extent of P-light chain phosphorylation were much less in KCl-contracted muscles than in carbachol-stimulated muscles. In carbachol-contracted muscles, the maximal extent of isometric force was maintained even though the phosphate content of the P-light chain had declined to basal levels. A similar difference in the temporal response was seen for phosphorylase *a* formation; a decrease from maximal levels was observed with carbachol stimulation, yet no decline was observed with KCl. Since the regulation of the extent of phosphorylation of both the P-light chain and phosphorylase is Ca^{2+}-dependent, it is reasonable to assume that the decrease in the extent of phosphorylation of these two proteins may be due to a decrease in the levels of sarcoplasmic free Ca^{2+} during maintained isometric contractions. This decrease in Ca^{2+} concentration may be greater with carbachol, and thus a greater decrease in extent of phosphorylation of P-light chain and phosphorylase would be expected. This hypothesis is supported by the recent findings of Morgan and Morgan (26). Calcium-dependent aequorin luminescence declined during maintained isometric force in smooth muscle, and the extent of the decrease was greater with a pharmacological agonist than

with K^+ depolarization. This hypothesis is also supported by studies with carotid arterial smooth muscle (18), in which the decrease in P-light chain phosphorylation during K^+-depolarized contractions could be attenuated by increasing the concentration of extracellular Ca^{2+}.

The extent of P-light chain phosphorylation in resting muscles and after a 2-hr exposure to carbachol was similar, with 0.1 mole of phosphate per mole of P-light chain. It could be hypothesized that at rest the phosphorylation is random without contraction, and after carbachol the phosphorylated cross-bridges represent redistribution to latched bridges sufficient to maintain force (i.e., only 10% of the phosphorylated P-light chain is involved in maintaining isometric force). It has been pointed out that the fraction of the total cycle time that an individual cross-bridge is in the attached, force-generating state in smooth muscle is probably much greater than the values (10–50%) estimated for skeletal muscle and may be similar for phosphorylated cross-bridges and nonphosphorylated latched cross-bridges (27). In addition, it has been recently concluded that there are no changes in the kinetics of the myosin light chain kinase-phosphatase system associated with cross-bridge conformations during contraction, and, in particular, P-light chains not involved in cross-bridge formation are not more accessible to dephosphorylation (18).

Not only were temporal differences apparent when phosphorylase *a* formation was compared with P-light chain phosphorylation, the concentration-response relationship measured after 1 min of contraction for both KCl- and carbachol-dependent stimulation was different. The dissociation of phosphorylase *a* formation from force is abolished for KCl stimulation by comparing steady-state levels of isometric force with phosphorylase *a* levels. These data suggest that the *rate* of phosphorylase *a* formation is slower than the *rate* of isometric force development during K^+ depolarization in bovine tracheal smooth muscle. This finding is similar to that previously reported for phosphorylase *a* formation during either spontaneous or serotonin-induced contraction of rat uterine smooth muscle (28). In marked contrast, the lower extent of phosphorylase *a* formation in the presence of carbachol was not due to a temporal difference since the concentration-response curve measured after 5 min was not different than that measured after 1 min. One possible explanation for this observation is a bimodal effect (both stimulatory and inhibitory) of cholinergic stimulation on phosphorylase *a* formation.

Evidence supporting this hypothesis was also obtained in experiments with isoproterenol. We have previously shown (14) that isoproterenol-mediated increases in phosphorylase *a* formation are due to stimulation of *beta*-adrenergic receptors and occur with no change in either P-light chain phosphorylation or isometric force. In this study, the isoproterenol-mediated increase in phosphorylase *a* formation was attenuated by incubation with carbachol. This inhibitory effect was most likely due to stimulation of cholinergic receptors, since attenuation could be blocked by atropine and did not occur with stimulation to contraction by K^+ depolarization. This finding is analogous to regulation of phosphorylase *a* formation in cardiac muscle, where cholinergic stimulation inhibits *beta*-adrenergic-mediated phosphorylase *a* formation (29). However, in contrast to cardiac muscle, cholinergic stimulation of tracheal smooth muscle also increases phosphorylase *a* formation, presumably by increasing sarcoplasmic Ca^{2+} and subsequent activation of phosphorylase kinase. Whether this inhibition is due to modulation of *beta*-adrenergic receptor affinity for isoproterenol (30), inhibition of cyclic AMP formation (31), or inhibition of the cyclic AMP-dependent protein kinase requires further biochemical and pharmacological studies.

In summary, there appears to be a good correlation between the extent of P-light chain phosphorylation and development of isometric force. Although these results are consistent with the hypothesis that P-light chain phosphorylation may play an important role in regulating smooth muscle contraction, it is obvious that there are also other factors that affect regulation. Force maintenance does not require steady-state phosphorylation of P-light chain. The quantitative relationship between P-light chain phosphorylation and isometric force may be different for contractions elicited by membrane depolarization versus receptor occupancy. The role of P-light chain phosphorylation and its relationship to these other potentially important secondary mechanisms in smooth muscle contraction requires additional investigations.

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