MOLECULAR PHARMACOLOGY, 23:665–670



# Effects of the Calmodulin Antagonist, Fluphenazine, on Phosphorylation of Myosin and Phosphorylase in Intact Smooth Muscle

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Received September 7, 1982; Accepted January 11, 1983

SUMMARY

The activities of phosphorylase kinase and myosin light-chain kinase are regulated by $Ca^{2+}$ binding to calmodulin. However, differences in the activation properties of the purified enzymes are apparent, since calmodulin binds to phosphorylase kinase in the absence of $Ca^{2+}$ whereas prior formation of a $Ca^{2+}$·calmodulin complex is necessary for calmodulin to bind to and activate myosin light chain kinase. Since the phenothiazines have been implicated as anticalmodulin drugs and inhibit contractile activity in smooth muscle, we examined the effects of the phenothiazine, fluphenazine, on isometric tension development and phosphorylation of phosphorylase and the phosphorylatable light chain (P-light chain) of myosin in intact bovine tracheal smooth muscle. Preincubation with 50 μM fluphenazine for 5 min inhibited the maximal rate and extent of isometric tension development and P-light chain phosphorylation in the presence of 60 mM KCl. Application of fluphenazine *after* tension and the phosphate content of the P-light chain had reached steady-state levels in response to 60 mM KCl produced little relaxation or dephosphorylation of the P-light chain. KCl-mediated phosphorylase *a* formation was not inhibited by preincubation with fluphenazine for 5 min. However, long periods of preincubation (30–60 min) produced significant inhibition of phosphorylase *a* formation and proportionally greater inhibition of tension and P-light chain phosphorylation. Since phosphorylase *a* formation was not inhibited during short-term preincubation with fluphenazine, KCl-dependent increases in the concentration of free intracellular $Ca^{2+}$ may not have been affected. Moreover, since both isometric tension development and P-light chain phosphorylation were attenuated in a parallel manner, inhibition of contractile activity in intact smooth muscle by anticalmodulin agents may be directly related to inhibition of P-light chain phosphorylation.

## INTRODUCTION

Although changes in the concentration of intracellular free calcium ($Ca^{2+}$) regulate the contraction-relaxation cycle in smooth muscle, the $Ca^{2+}$-dependent mechanism(s) regulating this cycle remain an area of considerable interest and active research. Substantial evidence exists which supports the involvement of $Ca^{2+}$·calmodulin-dependent phosphorylation of the P-light chain[2] as a key regulatory step in initiating actin-myosin interactions in smooth muscle (see refs. 1 and 2 for review). Evidence in favor of this hypothesis comes from studies with purified contractile proteins and mechanically disrupted smooth muscle fibers which show correlative $Ca^{2+}$-dependent increases in both actin-myosin interactions and the extent of P-light chain phosphorylation. In intact smooth muscle, P-light chain phosphorylation is correlated with the development of isometric force (3–7) and appears to stimulate the rate of cross-bridge cycling (6, 7). However, alternative hypotheses exist which suggest that regulation may also be related to thin-filament linked proteins (8, 9).

Phosphorylation of the myosin P-light chain is catalyzed by myosin light-chain kinase, an enzyme which is dependent upon both $Ca^{2+}$ and calmodulin for enzymatic activity. Previous kinetic experiments with skeletal and smooth muscle myosin light-chain kinase suggest that activation of this enzyme by $Ca^{2+}$ requires prior formation of a $Ca_i^{2+}$·calmodulin complex, and thus the active holoenzyme complex may consist of one enzyme catalytic subunit and one calmodulin with all four divalent metal binding sites occupied by $Ca^{2+}$ (10, 11). $Ca^{2+}$-dependent activation of phosphorylase kinase may also involve $Ca^{2+}$

This research was supported, in part, by National Institutes of Health Grant HL26043.

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[2] The abbreviations used are: P-light chain, phosphorylatable light chain (20,000-dalton light chain of myosin); BSS, balanced salt solution.

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binding to calmodulin. However, unlike myosin light-chain kinase, calmodulin is an integral subunit of the enzyme and binds to phosphorylase kinase in the absence of $Ca^{2+}$ (12). Thus, there may be differences in the phosphorylation properties of the P-light chain and phosphorylase in intact smooth muscle during stimulation with agents which increase intracellular levels of $Ca^{2+}$ and activate these calmodulin-dependent kinases.

One approach to take in identifying these possible differences is to use a pharmacological agent which inhibits processes activated by a $Ca^{2+}$·calmodulin complex. The phenothiazine derivatives comprise one type of these purported "anticalmodulin" drugs, as several investigators have shown inhibition of $Ca^{2+}$·calmodulin-mediated processes by these agents in various *in vitro* systems (13–16). In preparations of smooth muscle actomyosin, inhibition of actomyosin Mg-ATPase activity and superprecipitation by phenothiazine and naphthalene-sulfonamide derivatives were related to inhibition of myosin P-light chain phosphorylation (17, 18). In addition, Cassidy *et al.* (19) have shown that the phenothiazine drugs inhibit $Ca^{2+}$-dependent P-light chain phosphorylation and tension development in functionally skinned rabbit ileal and arterial smooth muscle strips.

Relaxation or inhibition of contraction by anticalmodulin drugs has also been cited as proof of involvement of P-light chain phosphorylation in the regulation of the contraction-relaxation cycle in intact smooth muscle (3, 20, 21). However, these studies are complicated by the findings that the high concentrations of the anticalmodulin agents (0.1–1.0 mM) used in these studies often have other pharmacological actions (such as receptor antagonism) in intact tissue (16, 22–25). Moreover, actual measurements of the extent of P-light chain phosphorylation were not made in some of these studies (20, 21), so no direct proof of inhibition of P-light chain phosphorylation was provided. In addition, the temporal component of transient increases in P-light chain phosphorylation which occur in smooth muscle during maintained contractions (5–7) was not examined (3). Thus, the precise relationship between extent of inhibition of myosin P-light chain phosphorylation and inhibition of contractile activity by these anticalmodulin drugs in intact smooth muscle is not known.

In order to elucidate this relationship, we have examined the effects of a relatively low concentration (50 μM) of the phenothiazine derivative, fluphenazine, on isometric tension development and myosin P-light chain phosphorylation in intact bovine tracheal smooth muscle which was contracted by added KCl. Moreover, activation of phosphorylase *a* formation, another $Ca^{2+}$-dependent process, was examined in these muscles in order to gain insight into and to compare the mechanism of intracellular regulation of phosphorylation of phosphorylase and the P-light chain in this tissue.

## MATERIALS AND METHODS

All experimental procedures were as previously described (5, 26).

*Intact tracheal smooth muscle strip preparation.* Smooth muscle was carefully dissected in aerated (95% $O_2$/5% $CO_2$) BSS from bovine trachea. Transverse strips (typically 14 mm × 1.5 mm, 15 mg frozen wet weight) were prepared and fixed vertically in a jacketed (36° ± 1°) muscle bath containing BSS consisting of 118.5 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 $KH_2PO_4$, 24.9 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 10 mM dextrose, and 1 mM pyruvate. The lower end of the strip was attached by a silk thread to a fixed post, and the upper end of the strip was attached to the lever of a Grass FT-.03 force displacement transducer. The muscle strips were then stretched passively to a resting tension of 1.5 g, which resulted in the maximal active isometric tension development for muscle strips of these dimensions (5). After application of passive tension, muscle strips were equilibrated for 90 min in aerated (95% $O_2$/5% $CO_2$) BSS or, alternatively, equilibrated for 2 hr without passive tension followed by 45 min with passive tension. After equilibration, 1.5 g of passive tension was reapplied, and the strips were prechallenged with 0.1 μM carbachol (carbamylcholine chloride; Sigma Chemical Company). The mechanical response of the muscle strip to this test dose of carbachol was used to ascertain the viability of the muscle. Muscle strips which did not attain 5–7 g of active isometric tension (~1%) were eliminated from further study at this point. Following prechallenge with 0.1 μM carbachol, the muscle strips were rinsed two or three times with fresh BSS until tension returned to resting levels, and then three or four additional times for 20 min. As previously determined (5), this equilibration period was judged to be sufficient for the return to basal values of any changes which may have taken place in the phosphate content of the P-light chain or phosphorylase, since pre-carbachol values for both parameters were the same as those obtained 20 min after the prechallenge.

To study the effects of fluphenazine on isometric tension development, contractions were elicited by adding KCl in the presence of 0.1 μM atropine (Atrosed; Burns-Biotec Lab) to the muscle bath. In this way, possible cholinergic antagonistic actions (24) of the phenothiazine on KCl-dependent tension or phosphorylation responses were minimized. The phenothiazines may also antagonize adrenoceptor agonist stimulation (22, 23, 25). Accordingly, the *beta*-adrenergic antagonist, propranolol (0.1 μM), was added to the muscle bath to ascertain the possible effect of KCl-mediated neurotransmitter release and activation of phosphorylase *a* formation through *beta*-adrenergic stimulation. The results of these experiments showed no difference in the magnitude of the KCl-mediated increase in phosphorylase *a* formation in the presence or absence of propranolol (data not shown). Thus, propranolol was not used in experiments with fluphenazine. *Alpha*-adrenergic antagonists were not used since prior experiments demonstrated a lack of tension development during stimulation with *alpha*-adrenergic agonists.

A stock solution (10 mM) of fluphenazine HCl was prepared by dissolving the drug in BSS just prior to use. After exposure to the desired experimental treatment, the muscle strips were frozen by rapidly lowering the muscle bath and immersing the strips in dichlorodifluoromethane (Freon 12) cooled in liquid nitrogen (5). The clamped ends of each strip were chipped away at −30° and the remaining portions of the muscle strips 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 phosphorylase *a*. Thus, both biochemical measurements were made from the same muscle strip, which afforded a direct comparison of the biochemical measurements to the extent of developed isometric tension.

The phosphate content of the P-light chain was quantitated by a combination of two electrophoretic procedures as detailed previously (26). 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, 15 mM 2-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and aprotinin (100 units/ml) (Trasylol), 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. Following 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. Following 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. Following 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 the enzyme-coupled fluorescence procedure described by Hardman *et al.* (27). 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.

## RESULTS

The effect of prior incubation of tracheal smooth muscle with 50 μM fluphenazine on KCl-dependent increases in isometric tension development, myosin P-light chain phosphorylation, and phosphorylase *a* formation is shown in Fig. 1. Previous experiments had determined that addition of 60 mM KCl produced maximal increases in isometric tension which were attainable with this agent. Pretreatment for 5 min with fluphenazine inhibited the initial rate and maximal extent of isometric tension and, concomitantly, the extent of myosin P-light chain phosphorylation during the first 3 min after addition of 60 mM KCl. In marked contrast, KCl-dependent increases in the rate and extent of phosphorylase *a* formation in these same muscles were not inhibited by fluphenazine. Fluphenazine plus atropine in the absence of added KCl had no effect on any of the parameters measured.

Qualitatively similar results were observed during stimulation to contraction with a submaximal contractile concentration (30 mM) of KCl (Table 1). Incubation with 50 μM fluphenazine for 5 min markedly inhibited isometric tension development and myosin P-light chain phosphorylation, yet had no significant effect on phosphorylase *a* formation during stimulation with 30 mM KCl.

As shown in Table 2, increasing the time of incubation with 50 μM fluphenazine increased the extent of inhibition of developed isometric tension. Whereas a 5-min preincubation produced 32% inhibition in maximal active tension development, 30 or 60 min of preincubation produced approximately 60% inhibition of tension. Myosin P-light chain phosphorylation was similarly inhibited to a greater extent with the longer periods of incubation. However, in contrast to the 5-min incubation period with fluphenazine (which had no effect on phosphorylase *a*

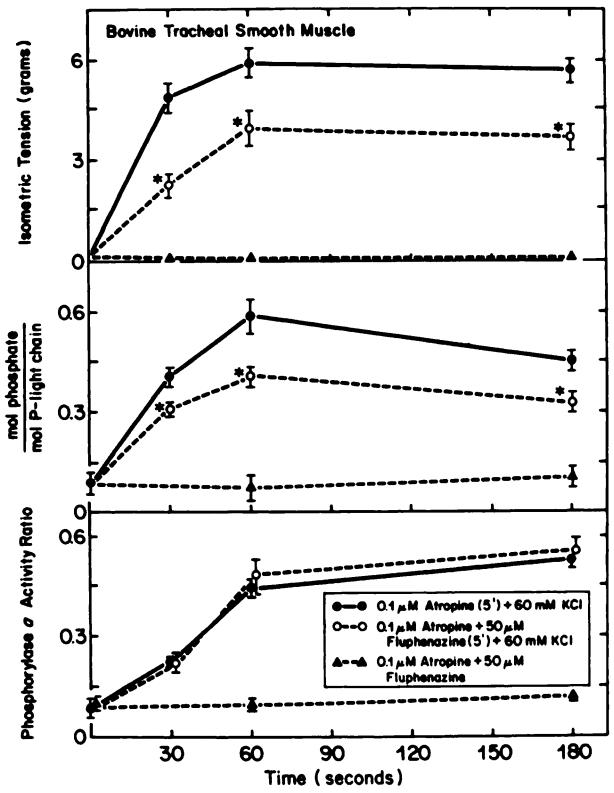


FIG. 1. *Effect of fluphenazine on KCl-mediated isometric tension development, myosin P-light chain phosphorylation, and phosphorylase a formation*

A comparison of responses obtained during stimulation with 60 mM KCl (●——●) with those obtained in response to pretreatment with 50 μM fluphenazine followed by 60 mM KCl (○- - -○) for isometric tension development (top panel), P-light chain phosphorylation (middle panel), and phosphorylase *a* formation (bottom panel) is depicted. All values represent the mean ± standard error of the mean for three to nine muscles, which were frozen at the indicated times and assayed for P-light chain phosphate content and phosphorylase *a* levels as described under Materials and Methods. The values shown for 60 mM KCl were obtained in the presence of 0.1 μM atropine (added 5 min prior to KCl), whereas the fluphenazine-treated values represent muscles which were pretreated for 5 min with 50 μM fluphenazine and 0.1 μM atropine prior to the addition of 60 mM KCl. The *asterisks* denote significant ($p < 0.05$) decreases in KCl-mediated responses in the presence of fluphenazine. ▲- - -▲, No change in any parameter during incubation with 0.1 μM atropine plus 50 μM fluphenazine in the absence of added KCl.

TABLE 1

*Comparison of the effects of fluphenazine on responses to 30 mM and 60 mM KCl*

Values represent the mean ± standard error of the mean for three to eight samples per group. Responses were measured 1 min after the addition of either 30 mM or 60 mM KCl. The presence (+) or absence (−) of pretreatment for 5 min with 50 μM fluphenazine is indicated.

| | Unstimulated | KCl concentration | | | |
|---|---|---|---|---|---|
| | | 60 mM | | 30 mM | |
| | | − | + | − | + |
| Isometric tension (g) | 0 | 5.8 ± 0.4 | 3.9 ± 0.6[a] | 3.7 ± 0.3 | 2.1 ± 0.3[a] |
| Moles phosphate/mole P-light chain | 0.08 ± 0.02 | 0.59 ± 0.05 | 0.42 ± 0.04[a] | 0.35 ± 0.05 | 0.18 ± 0.03[a] |
| Phosphorylase *a* activity ratio | 0.08 ± 0.02 | 0.43 ± 0.03 | 0.49 ± 0.05 | 0.18 ± 0.02 | 0.16 ± 0.02 |

[a] Significantly different ($p < 0.05$) from responses obtained with KCl in the absence of fluphenazine.

TABLE 2

*Effect of incubation time with fluphenazine on KCl-dependent responses*

Values represent the mean ± standard error of the mean for three to seven samples per group. Responses were measured 1 min after the addition of 60 mM KCl.

| | Unstimulated | Time of preincubation with 50 μM fluphenazine | | | |
|---|---|---|---|---|---|
| | | 0 min | 5 min | 30 min | 60 min |
| Isometric tension (g) | 0 | 5.8 ± 0.4 | 3.9 ± 0.6[a] | 2.2 ± 0.3[a] | 2.3 ± 0.4[a] |
| Moles phosphate/mole P-light chain | 0.10 ± 0.03 | 0.59 ± 0.05 | 0.42 ± 0.04[a] | 0.26 ± 0.04[a] | 0.25 ± 0.02[a] |
| Phosphorylase *a* activity ratio | 0.09 ± 0.01 | 0.43 ± 0.03 | 0.49 ± 0.05 | 0.17 ± 0.03[a] | 0.16 ± 0.01[a] |

[a] Significantly different ($p < 0.05$) from responses obtained with KCl in the absence of fluphenazine (0 min).

formation), longer periods of incubation inhibited KCl-dependent increases in phosphorylase *a* formation.

In additional experiments, the effect of adding fluphenazine after contraction and myosin light chain phosphorylation had occurred was examined. As shown in Fig. 2, 60 mM KCl increased isometric tension to maximal levels by 1 min. Phosphorylation of the P-light chain also increased to maximal levels during the 1st min of incubation with KCl, as represented by an increase in the phosphate content of the P-light chain from 0.10 mole of phosphate per mole of P-light chain to a value of 0.60. The phosphate content of the P-light chain subsequently declined to approximately 0.40 mole of phosphate per mole of P-light chain while tension was maintained at maximal levels for 2 hr. In contrast to *pretreatment* with fluphenazine (Fig. 1), addition of 50 μM fluphenazine *after* contraction was initiated produced only a slight (6%) decrease in tension. Moreover, there was no dephosphorylation of the P-light chain after the addition of fluphenazine. One explanation of these data is that after P-light chain phosphorylation has occurred, and presumably $Ca^{2+}$·calmodulin is bound to myosin light-chain kinase, the ability of fluphenazine to inhibit P-light chain phosphorylation and developed isometric tension is attenuated. The muscle strips were not in rigor, nor was the regulatory system controlling the extent of phosphorylation of the P-light chain irreversibly affected by the long-term contractions since rinsing the muscle strips with normal BSS produced immediate, complete relaxation and concomitant dephosphorylation of the P-light chain to basal levels (Fig. 2). Moreover, subsequent readdition of 60 mM KCl in the presence of atropine resulted in restoration of isometric tension and rephosphorylation of the P-light chain to near-maximal values.

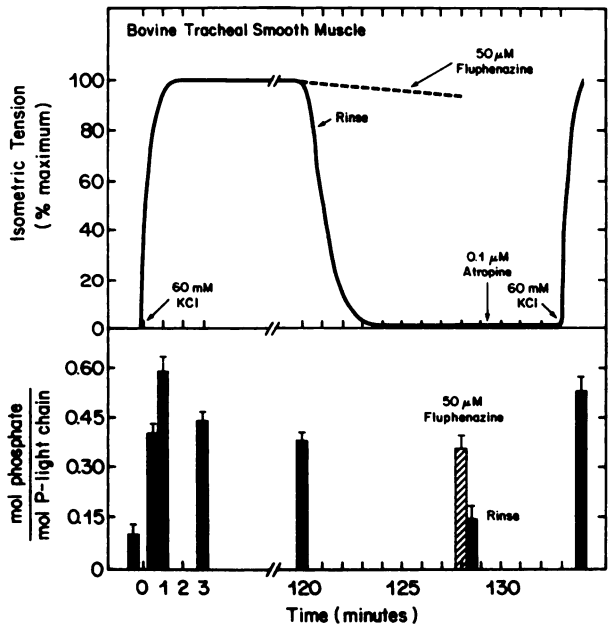


FIG. 2. *Effect of fluphenazine on maintained isometric tension*

The effect of adding fluphenazine after isometric tension had been maintained for 2 hr is shown in the top panel, while the phosphate content of the P-light chain at various points during this interval is depicted in the bottom panel. Muscles were contracted by adding 60 mM KCl five min after incubation with 0.1 μM atropine as described in Fig. 1. After 2 hr of maintained isometric force, the muscles were either exposed to 50 μM fluphenazine (*broken line, hatched bar*) or rinsed with normal BSS (*solid line, stippled bar*). After rinsing with BSS, some msucles were restimulated by addition of 60 mM KCl in the presence of 0.1 μM atropine. The values for isometric tension, expressed as percentage of maximal tension, are the means for 4–12 muscles per treatment. The extent of myosin P-light chain phosphorylation was determined as described under Materials and Methods, and values represent the mean ± standard error of the mean for four to eight muscles.

## DISCUSSION

*Inhibition of isometric tension and P-light chain phosphorylation.* This study shows that inhibition of contraction in intact tracheal smooth muscle by the anticalmodulin agent, fluphenazine, is associated with inhibition of myosin P-light chain phosphorylation. This is the first study linking previous biochemical data from studies with this class of anticalmodulin agent (17–19) to a functional role in mediating tension development and P-light chain phosphorylation in intact smooth muscle under conditions where other, nonspecific actions of the phenothiazine were minimized. These conditions included development of isometric tension by adding KCl in the presence of atropine and the use of a relatively low concentration (50 μM) of fluphenazine. Thus, the possibility of nonspecific actions of fluphenazine through antagonism of receptor stimulation (22–25) were lessened. Barron *et al.* (3) have previously shown that incubation of carotid arterial smooth muscle for 1 hr with either chlorpromazine or trifluoperazine (0.1–1.0 mM) inhibited norepinephrine-stimulated increases in P-light chain phosphorylation and tension development. However, these responses may not have been directly related to anticalmodulin activity, since the concentrations of these phenothiazine derivatives were several orders of magnitude higher than the concentration necessary to displace *alpha*-adrenergic agonists (23). The concentration of flu-

phenazine (50 μM) used in this study was in the range (1–100 μM) used to inhibit calmodulin-dependent processes in other studies *in vitro*, including P-light chain phosphorylation in actomyosin and skinned smooth muscle preparations (13–19).

A correlation was found between fluphenazine-mediated inhibition of myosin P-light chain phosphorylation and isometric tension development (Fig. 3). These data were obtained from responses with submaximal (30 mM) and maximal (60 mM) contractile concentrations of KCl in the presence and absence of fluphenazine (Tables 1 and 2). This positive correlation supports the hypothesis that the extent of P-light chain phosphorylation during the *initial* period of contraction may be important in determining the extent of isometric tension (4–7, 28). However, P-light chain phosphorylation correlates to the velocity of unloaded shortening and not to the *maintenance* of isometric tension (6, 7). Thus, the temporal decrease in P-light chain phosphorylation (Fig. 2) is not associated with a decrease in isometric tension. The transient increase in P-light chain phosphorylation may be due to a transient increase in intracellular $Ca^{2+}$ following KCl treatment. However, other explanations for these observations are possible and should be explored. The close relationship between fluphenazine-mediated inhibition of myosin P-light chain phosphorylation and isometric tension development is consistent with the hypothesis that the mechanism whereby this phenothiazine derivative inhibits contractile activity in smooth muscle is via direct inhibition of myosin P-light chain phosphorylation. This inhibitory effect on tension and P-light chain phosphorylation, when compared on a percentage basis, was greater with 30 mM KCl (when compared with 60 mM KCl). This finding would be consistent with the notion that stimulation with 30 mM KCl produces smaller increases in free intracellular calcium levels than does 60 mM KCl; thus, less $Ca^{2+}$·calmodulin is formed and, in the presence of fluphenazine, a proportionally greater inhibition of myosin P-light chain phosphorylation and isometric tension occurs. These results are similar to those previously found by Hogaboom and Fedan[3] for KCl-depolarized canine tracheal smooth muscle, where it was shown that the extent of trifluoperazine-mediated inhibition of tension was inversely related to the calcium concentration in the muscle bath.

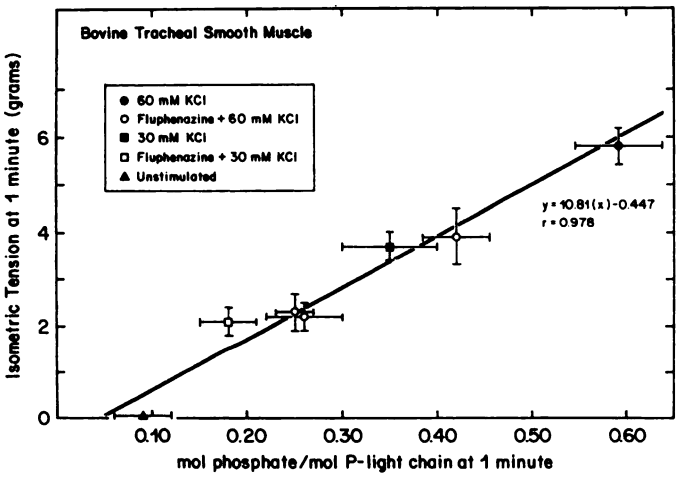


FIG. 3. *Increases in active isometric tension as function of myosin P-light chain phosphorylation after 1 min of stimulation with KCl*

The mean ± standard error of the mean for isometric tension and P-light chain phosphorylation from muscles stimulated by either 30 mM or 60 mM KCl in the presence and absence of fluphenazine is shown. These values were obtained from Tables 1 and 2. The *solid line* represents the calculated line of regression [$y = 10.8(x) - 0.447$; $r = 0.978$; $p < 0.01$].

*Comparison of the effects of fluphenazine on P-light chain and phosphorylase phosphorylation.* Incubation with fluphenazine produced differential effects on phosphorylase *a* formation and myosin P-light chain phosphorylation during KCl-mediated increases in isometric tension (Fig. 1; Table 1). These results suggest that the intracellular regulation of phosphorylation of these substrates by $Ca^{2+}$ is different. Under conditions where maximal increases in the extent and rate of KCl-dependent isometric tension development and P-light chain phosphorylation were inhibited by fluphenazine (Fig. 1), there was no effect on either the extent or rate of phosphorylase *a* formation. Similar effects were also apparent during stimulation with 30 mM KCl (Table 1). As previously discussed, these data would suggest that fluphenazine inhibits tension development directly by inhibiting $Ca^{2+}$·calmodulin-mediated activation of myosin light chain kinase activity and subsequent phosphorylation of the P-light chain. KCl-dependent increases in intracellular levels of $Ca^{2+}$ are presumably not affected by fluphenazine; thus, there is no effect on phosphorylase *a* formation. This finding is also consistent with the notion that calmodulin may be bound to phosphorylase kinase in tracheal smooth muscle in the absence of $Ca^{2+}$, as has been reported for purified skeletal muscle phosphorylase kinase (12). Thus, fluphenazine, which binds to the $Ca^{2+}$·calmodulin complex alone (16), would not inhibit $Ca^{2+}$-dependent activation of phosphorylase kinase via the tightly bound calmodulin subunit.

A comparison of the responses obtained with the submaximal concentration of KCl (30 mM), with those obtained in the presence of fluphenazine plus the maximal concentration (60 mM) of KCl (Table 1) supports this notion. That is, when the increase in active isometric tension development after 1 min was the same (3.7–3.9 g) for these different treatments, the extent of phosphorylation of the P-light chain was also similar (0.35–0.42 mole of phosphate per mole of P-light chain). However, there was a marked difference in the phosphorylase *a* activity ratio measurement (0.18 for 30 mM KCl; 0.49 for fluphenazine plus 60 mM KCl). As previously discussed, the phosphorylase *a* activity ratio obtained with 60 mM KCl alone was not different than the ratio obtained in the presence of fluphenazine, yet isometric tension and P-light chain phosphorylation were significantly inhibited by fluphenazine. These results are consistent with the hypothesis that short-term preincubation with fluphenazine does not inhibit KCl-dependent increases in the concentration of intracellular free $Ca^{2+}$ and hence phosphorylase kinase activity, but may inhibit myosin light-chain kinase activation by $Ca^{2+}$·calmodulin and ultimately depress myosin P-light chain phosphorylation and isometric tension development.

[3] G. K. Hogaboom and J. S. Fedan, personal communication.

In contrast to short periods (5 min) of preincubation with fluphenazine, longer intervals of preincubation (30 or 60 min) markedly inhibited phosphorylase *a* formation (Table 2). The longer preincubation may be associated with other, nonspecific, effects [such as membrane stabilization (25, 29)] by the phenothiazine so that $Ca^{2+}$ entry into the smooth muscle cells was attenuated. Thus, all $Ca^{2+}$-dependent processes would be inhibited. Additional studies with intact smooth muscle and the purified smooth muscle phosphorylase kinase and myosin light-chain kinase are needed to distinguish between this and other possibilities. However, these data do show that a temporal component of incubation is important in distinguishing qualitative differences in effects of the phenothiazines.

In summary, the results reported in this study support the notion that inhibition of contraction in intact smooth muscle by anticalmodulin drugs may be related to inhibition of myosin P-light chain phosphorylation. Myosin light-chain kinase activity may be attenuated by either inhibition of $Ca^{2+}$ influx, and a decrease in the amount of $Ca^{2+}$·calmodulin formed, or by a decrease in the amount of the $Ca^{2+}$·calmodulin complex which can activate the kinase by direct binding of this complex to the phenothiazine. Further studies are needed to gain more insight into the nature of these regulatory mechanisms in intact smooth muscle.

## ACKNOWLEDGMENTS

The authors wish to thank Ms. Nancy Bryant for her excellent assistance in the preparation of the manuscript, and Mrs. Cindy Silver and Mr. Paul Arrambide, III, for their expert technical assistance. We also wish to thank E. R. Squibb & Sons, Inc., for the generous supply of fluphenazine, and Drs. Hogaboom and Fedan (Department of Pharmacology and Toxicology, West Virginia University Medical Center) for a preprint of their work.

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