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GTPγS-Induced phosphorylation of myosin light chain kinase in smooth muscle

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Phosphorylation of myosin light chain kinase by a Ca^{2+}-dependent protein kinase increases the concentration of Ca^{2+}/calmodulin required for half-maximal activation. The Ca^{2+} concentrations required for myosin light chain kinase phosphorylation in permeable smooth muscle are similar to those required for myosin light chain phosphorylation. Both GTPγS and carbachol increase the Ca^{2+} sensitivity of myosin light chain kinase phosphorylation as well as light chain phosphorylation. It is proposed that a similar G-protein mediated mechanism regulates the Ca^{2+}-dependent phosphorylation of these two contractile proteins in smooth muscle.

Smooth muscle contraction; Myosin light chain; Myosin light chain kinase; GTPγS; Protein phosphatase

1. INTRODUCTION

Phosphorylation of the regulatory light chain of smooth muscle myosin by Ca^{2+}/calmodulin-dependent myosin light chain kinase increases actin-activated MgATPase activity and initiates smooth muscle contraction [1,2]. Although the increase in $[Ca^{2+}]_i$ is a primary determinant for myosin light chain phosphorylation, this system may not be the only cellular process regulating smooth muscle contraction [3,4].

Myosin light chain kinase itself is phosphorylated to a high extent at a regulatory site (site A) near the calmodulin-binding domain in smooth muscle stimulated to contract [5,6]. Phosphorylation of this site increases K_{CaM}, the concentration of Ca^{2+}/calmodulin required for half-maximal activity [5,7]. Furthermore, myosin light chain kinase phosphorylation is Ca^{2+}-dependent [5–8] and inhibited by KN-62, an inhibitor of the multifunctional calmodulin-dependent protein kinase II [7]. Thus, it is proposed that phosphorylation of myosin light chain kinase by Ca^{2+}/calmodulin-dependent protein kinase II desensitizes myosin light chain kinase to activation by Ca^{2+}, which provides a cellular mechanism for down regulating the Ca^{2+} signal.

Cell membrane receptors are coupled by G-proteins to multiple signal transduction pathways [9,10]. In permeabilized smooth muscle, GTPγS acts to potentiate Ca^{2+}-dependent force development [11]. The GTPγS-induced potentiation of smooth muscle contraction is secondary to an increase in myosin light chain phosphorylation at serine-19, the site phosphorylated by myosin light chain kinase [12]. It has been proposed that the GTP analogue increases Ca^{2+} sensitivity of myosin light chain phosphorylation through inhibition of myosin light chain phosphatase [11,12]. It is not known whether phosphorylation of myosin light chain kinase is also affected by GTPγS or agonists in permeable smooth muscle. The objective of this study was to determine if a G-protein mediated mechanism might also be involved in regulating the Ca^{2+} sensitivity of myosin light chain kinase phosphorylation in smooth muscle. This possibility was examined in permeable tracheal smooth muscle treated with GTPγS with Ca^{2+} concentrations buffered by EGTA. Agonist stimulation was also examined.

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Abbreviations: $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; KN-62, 1-[*N*-*O*-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; G-protein, guanine nucleotide-binding protein; PSS, physiological salt solution; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-[*N*-morpholino]propane-sulfonic acid; EGTA, ethyleneglycol-bis (β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; K_{CaM}, concentration of Ca^{2+}/calmodulin required for half-maximal activation; PIPES, piperazine-*N*,*N*′-bis[2-ethane-sulfonic acid].

2. MATERIALS AND METHODS

2.1. *Isometric force*

Bovine tracheas were obtained from a local abattoir (Dallas City Packing), and smooth muscle tissue was dissected into small strips (0.1 × 2 × 0.02 mm). Organ transport and tissue dissection conditions were performed in a salt solution (PSS) as described previously [5,6]. The strip was mounted on a force transducer in a small quartz chamber (10 μl capacity) filled with PSS which was gassed with 95% O_2/5% CO_2. The chamber solution was perfused at 2–3 s intervals. After eliciting a maximal contraction with 10 μM carbachol, the tissue was

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permeabilized with β-escin for 25 min at room temperature in a relaxing solution [12,13]. The lightly skinned preparation demonstrated 3–4 highly reproducible contractions without calmodulin being added to the perfusion solution. Furthermore, the Ca^{2+} dependence of light chain phosphorylation (Fig. 1) is similar to results reported with intact fibers or cells [1–4,6,7]. Thus, it is unlikely that there was a significant loss of essential contractile proteins including calmodulin from the skinned fiber with this experimental protocol. This solution also contained 1 μM leupeptin to minimize proteolysis and 10 μM ionomycin to deplete Ca^{2+} from the sarcoplasmic reticulum. After skinning, the tissue was perfused in relaxing solution for 5 min.

2.2. *Myosin light chain phosphorylation*

Phosphorylation of myosin light chain was measured in skinned tracheal tissue prepared as described for the force measurements. The strips were fixed in acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol that was precooled to −60°C. The strips were warmed to room temperature in the same solution and washed three times with diethyl ether to remove residual trichloroacetic acid. The protein was dissolved in 8 M urea-sample buffer [6]. The extent of myosin light chain phosphorylation was measured by separation of nonphosphorylated and phosphorylated forms of the light chain by glycerol/PAGE followed by electrophoretic transfer of the protein to nitrocellulose paper. Quantitation of the relative amounts of each form by an immunoblot procedure was performed as previously described [6,14].

2.3. *Myosin light chain kinase activity ratio*

Changes in the calmodulin activation properties of myosin light chain kinase associated with phosphorylation of site A were measured in skinned tracheal tissue prepared as described for the force measurements [5,6,15]. Quick-frozen tissues were homogenized at 0°C for 15 s in 20 vols. (w/v) of buffer containing 50 mM MOPS, 0.1% Nonidet P-40, 100 mM sodium pyrophosphate, 100 mM NaF, 250 mM NaCl, 1 mM dithiothreitol, 50 μM PMSF, 1 μM leupeptin at pH 8.6 and stored in liquid nitrogen until assay. After thawing to 0°C the homogenates were diluted 8–10-fold with a buffer containing 50 mM K_2PO_4, 4 mM EDTA and 1 mM dithiothreitol at pH 7.0; 5 μl were added to 20 μl of a reaction solution containing (final concentration): 50 mM MOPS at pH 7.0, 10 mM magnesium acetate, 1 mM dithiothreitol, 50 μM cardiac light chain, 400 nM calmodulin, [γ-^{32}P]ATP (200–300 cpm/pmol) and 4 mM EGTA with $CaCl_2$ added to give a free Ca^{2+} concentration of 1 or 100 μM. The reaction was incubated at 30°C for 8 min, and the ^{32}P incorporated into light chain was measured.

When there is an increase in the concentration of calmodulin required for half-maximal activity (K_{CaM}), the ratio of activity measured at 1 (v) to that 100 (V_{max}) μM free Ca^{2+} decreases quantitatively. An increase in the K_{CaM} value associated with kinase phosphorylation at the regulatory site decreases the fractional kinase activity at a limiting Ca^{2+} concentration (1 μM) under standardized assay conditions. The magnitude of the change in K_{CaM} after myosin light chain kinase phosphorylation can be derived from the following relationship: $\{(V_{max}/v)' - 1\}/\{(V_{max}/v) - 1\} = K_{CaM}'/K_{CaM}$, where the values of the ratios (v/V_{max}) and (v/V_{max})′ are obtained before and after phosphorylation, respectively, and K_{CaM}' is the value of K_{CaM} after phosphorylation. It should be noted that the relationship between the decrease in the activity ratio and the increase in K_{CaM} is not linear [5,6,15].

2.4. *Miscellaneous*

The composition of PSS was (in mM): 120.5 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.6 $CaCl_2$, 1.2 NaH_2PO_4, 20.4 $NaHCO_3$, 10.0 glucose and 1.0 pyruvate. The solution was continuously bubbled with 95% O_2/5% CO_2 resulting in pH 7.6 at room temperature.

The relaxing solution included (in mM): 90 potassium methanesulfonate; 5 magnesium methanesulfonate; 20 PIPES; 4 ATP; 4 EGTA (pH 6.8, ionic strength 0.17).

β-escin and GTPγS were obtained from Sigma, ionomycin from Calbiochem, and leupeptin from Vega Biotechnologies Inc. All chemicals used for experiments were analytical grade.

2.5. *Statistical analyses*

Statistical analyses were performed by independent t-test for force development, myosin light chain phosphorylation and myosin light chain kinase activity ratio with experimental responses compared to control values. Two-tailed values were used with P values < 0.05 considered significant. Data are expressed as mean ± S.E.M.

3. RESULTS

Fig. 1 shows that the Ca^{2+} dependence of force development in tracheal smooth muscle made permeable with β-escin is similar to results reported for saponin-skinned tracheal smooth muscle [12]. Myosin light chain phosphorylation was also Ca^{2+}-dependent with a maximal extent of phosphorylation (0.56 mol phosphate/mol light chain) observed at 3 and 10 μM Ca^{2+} (Fig. 1). Increases in the Ca^{2+} concentration also decreased the activity ratio for myosin light chain kinase from 0.81 (resting value) to 0.40 at 10 μM Ca^{2+} (Fig. 1). These data show that the Ca^{2+} dependence of the change in K_{CaM}, presumably due to phosphorylation at site A [5–7], is similar to the Ca^{2+}-dependence of myosin light chain phosphorylation. These results are consistent with results obtained with smooth muscle cells in culture [7].

Previously, we observed that GTPγS did not evoke a contraction in relaxing solution containing 4 mM EGTA [12]. Therefore, we selected a low Ca^{2+} concentration to optimize the effect of GTPγS on smooth muscle contraction. At 0.3 μM Ca^{2+} myosin light chain phosphorylation increased from 0.02 to 0.16 mol phosphate/mol light chain. This effect of Ca^{2+} was potentiated by 10 μM GTPγS where the extent of phosphorylation increased to 0.29 mol phosphate/mol light chain (Fig. 2). We also examined the effect of GTPγS on the myosin light chain kinase activity ratio. Increasing Ca^{2+} to 0.3 μM resulted in a decrease in myosin light chain kinase activity ratio from a control value of 0.81 to 0.68

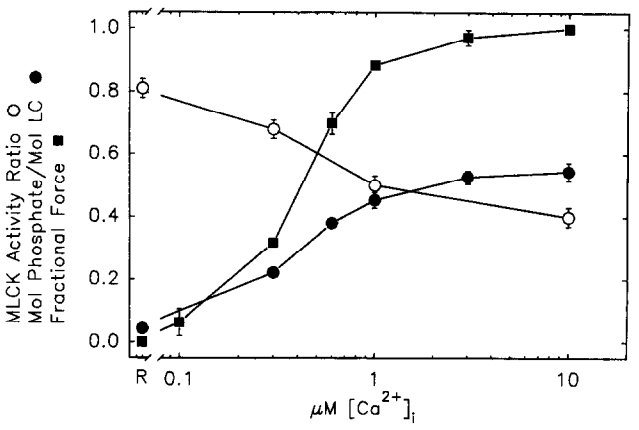

Fig. 1. Ca^{2+}-dependent properties of β-escin permeable bovine tracheal smooth muscle. Smooth muscle strips were permeabilized by β-escin in relaxing solution and incubated in different concentrations of Ca^{2+}. Force responses were normalized to the amplitude of contraction evoked by 10 μM Ca^{2+}. Mean values represent 3–4 observations. Standard error bars smaller than symbol size are not shown.

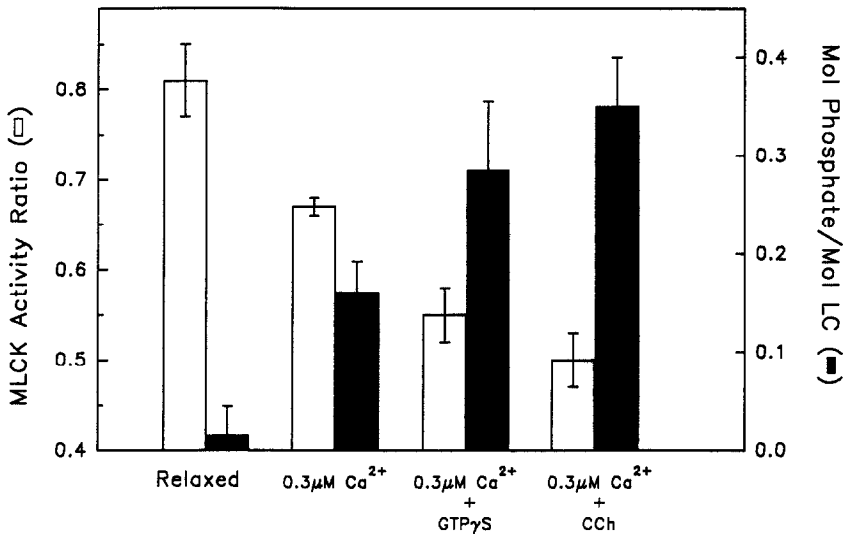

Fig. 2. Effect of GTPγS and carbachol on myosin light chain kinase activity ratio and myosin light chain phosphorylation. Smooth muscle strips in relaxing solution were treated with 0.3 μM Ca^{2+}, 0.3 μM Ca^{2+} plus 10 μM GTPγS or 0.3 μM Ca^{2+} plus 10 μM carbachol (CCh). Myosin light chain kinase activity ratio and myosin light chain phosphorylation were determined as described in Section 2.

(Fig. 2). Addition of 10 μM GTPγS led to a further decrease in the activity ratio to 0.55 (Fig. 2). Addition of 10 μM carbachol to permeable smooth muscle cells in 0.3 μM Ca^{2+} also potentiated the myosin light chain and myosin light chain kinase phosphorylation responses (Fig. 2). Myosin light chain phosphorylation increased from 0.16 to 0.35 mol phosphate/mol light chain. Similarly, myosin light chain kinase activity ratio decreased from 0.67 to 0.50.

4. DISCUSSION

The decrease in myosin light chain kinase activity ratio with increasing Ca^{2+} concentrations in permeable smooth muscle is consistent with the Ca^{2+}-dependent phosphorylation of the enzyme [5–7,15]. Most importantly, the experiments with the permeable fibers show that Ca^{2+} alone is sufficient to affect the activity ratio supporting the hypothesis that Ca^{2+}/calmodulin-dependent protein kinase II phosphorylates myosin light chain kinase [7]. This phosphorylation occurs at concentrations of Ca^{2+} that also effect myosin light chain phosphorylation, indicating a similar responsiveness to physiological stimuli that increase cytosolic Ca^{2+} concentrations.

We also show that both GTPγS and carbachol enhance the Ca^{2+}-dependent decrease in myosin light chain kinase activity ratio, as well as increasing myosin light chain phosphorylation in permeable smooth muscle. This result is consistent with the effect of carbachol in intact tracheal smooth muscle [6]. It has been shown previously that the decrease in the myosin light chain kinase activity ratio is related to the extent of site A phosphorylation [5,7]. Furthermore, it has been proposed that the GTPγS-induced Ca^{2+} sensitization of myosin light chain phosphorylation is mediated by inhibition of a myosin light chain phosphatase, probably protein phosphatase-1 [16–18]. Since smooth muscle myosin light chain kinase is also dephosphorylated in vitro by protein phosphatase-1 [19], the possibility is raised that GTPγS acts to potentiate myosin light chain kinase and myosin light chain phosphorylation through inhibition of protein phosphatase-1 that dephosphorylates both proteins.

Potentiation of the phosphorylation of myosin light chain and myosin light chain kinase would have opposing physiological effects. The Ca^{2+} sensitivity of myosin light chain phosphorylation is dependent upon the relative activities of myosin light chain kinase and myosin light chain phosphatase. Because distinct G-protein-mediated signal transduction pathways may exist for inhibition of light chain phosphatase via arachidonic acid [17] and phosphorylation of myosin light chain kinase by Ca^{2+}/calmodulin-dependent protein kinase II [6,7], the Ca^{2+} sensitivity of myosin light chain phosphorylation may be subject to combined regulatory processes. Different pharmacological and physiological effectors could differentially modulate Ca^{2+} sensitivity due to specific actions on the two pathways [3,4].

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