Myosin Light Chain Kinase Phosphorylation in Tracheal Smooth Muscle*

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Purified myosin light chain kinase from smooth muscle is phosphorylated by cyclic AMP-dependent protein kinase, protein kinase C, and the multifunctional calmodulin-dependent protein kinase II. Because phosphorylation in a specific site (site A) by any one of these kinases desensitizes myosin light chain kinase to activation by Ca++/calmodulin, kinase phosphorylation could play an important role in regulating smooth muscle contractility. This possibility was investigated in 32P-labeled bovine tracheal smooth muscle. Treatment of tissues with carbachol, KCl, isoproterenol, or phorbol 12,13-dibutyrate increased the extent of kinase phosphorylation. Six primary phosphopeptides (A-F) of myosin light chain kinase were identified. Site A was phosphorylated to an appreciable extent only with carbachol or KCl, agents which contract tracheal smooth muscle. The extent of site A phosphorylation correlated to increases in the concentration of Ca++/calmodulin required for activation. These results show that cyclic AMP-dependent protein kinase and protein kinase C do not affect smooth muscle contractility by phosphorylating site A in myosin light chain kinase. It is proposed that phosphorylation of myosin light chain kinase in site A in contracting tracheal smooth muscle may play a role in the reported desensitization of contractile elements to activation by Ca++.

Cyclic AMP-dependent protein kinase phosphorylates myosin light chain kinase purified from gizzard smooth muscle (Adelstein et al., 1978). In the absence of Ca++/calmodulin, two sites (sites A and B) are phosphorylated, and the concentration of Ca++/calmodulin required for half-maximal activation (K_CaM) increases 10-fold (Conti and Adelstein, 1981). In the presence of Ca++/calmodulin, phosphate is incorporated into site B with no effect on myosin light chain kinase activity. Similar observations have been made with myosin light chain kinases purified from mammalian smooth muscles including trachea (Miller et al., 1983), stomach (Walsh et al., 1982), myometrium (Higashi et al., 1983), aorta (Vallet et al., 1981), and carotid artery (Bhalla et al., 1982). It has been suggested that phosphorylation of site A by cyclic AMP-dependent protein kinase could decrease the extent of myosin light chain kinase activation with a resultant decrease in myosin light chain phosphorylation and inhibition of contraction (Conti and Adelstein, 1981).

It was shown subsequently that a 17-fold stimulation of cyclic AMP formation with forskolin resulted in myosin light chain kinase phosphorylation in 32P-labeled tracheal smooth muscle (de Lanerolle et al., 1984). The sites of phosphorylation were not identified. However, treatment of tracheal smooth muscles with isoproterenol at a concentration sufficient for relaxation had no effect on the Ca++/calmodulin activation properties of myosin light chain kinase from these muscles (Miller et al., 1983). At a high concentration (5 μM) of isoproterenol, however, K_CaM increased slightly (less than 2-fold). Interestingly, K_CaM increased more with carbachol or KCl treatments alone, both of which resulted in contraction (Miller et al., 1983). Although sites of phosphorylation in myosin light chain kinase were not identified, the conclusion was reached that β-adrenergic relaxation does not require an increase in K_CaM for myosin light chain kinase.

Purified myosin light chain kinase from smooth muscle is phosphorylated by other protein kinases. Protein kinase C incorporates phosphate into two sites in gizzard myosin light chain kinase in the absence of Ca++/calmodulin (Ikebe et al., 1985; Nishikawa et al., 1985). There is disagreement as to whether one of these sites is identical to site A. In any case, phosphorylation by protein kinase C leads to a reduced affinity of the kinase for Ca++/calmodulin. This result is similar to the effect produced by phosphorylation with cyclic AMP-dependent protein kinase. Calmodulin-dependent protein kinase II also phosphorylates myosin light chain kinase to a molar stoichiometry of 2.77 with an associated increase in K_CaM (Hashimoto and Soderling, 1990). Peptide mapping and sequence analysis showed that both calmodulin-dependent protein kinase II and cyclic AMP-dependent protein kinase phosphorylated the second of the two adjacent serines in phosphopeptide A which is located near the calmodulin-binding domain. These biochemical observations with protein kinase C and calmodulin-dependent protein kinase II are interesting in light of the previous report (Miller et al., 1983) that contraction with carbachol or KCl was associated with an increase in K_CaM for myosin light chain kinase in tracheal smooth muscle. Carbachol stimulation of muscarinic receptors could result in activation of both protein kinase C and calmodulin-dependent protein kinase, whereas KCl depolarization would lead to activation of calmodulin-dependent protein kinase II.

The goals of this study were to determine if myosin light chain kinase was phosphorylated in tracheal smooth muscle under conditions that activate protein kinases in addition to cyclic AMP-dependent protein kinase, to identify the number of sites phosphorylated in myosin light chain kinase by phosphopeptide mapping, and to evaluate the functional signifi-

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Myosin Light Chain Kinase Phosphorylation

**EXPERIMENTAL PROCEDURES**

*Tissue Preparation*—Strips of smooth muscle (10–20 mg, wet weight) were dissected from bovine tracheae as described previously (Kamm and Stull, 1985). Organ transport, tissue dissection, and experimental protocols were performed in a physiological salt solution of the following composition (millimolar): NaCl, 120.5; KCl, 4.8; (Kamm and Stull, 1985). Organ transport, tissue dissection, and experimental protocols were performed in a physiological salt solution containing 60 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 100 mM Tris, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM TPCK, 1 mM TAME, 100 mM MgCl₂, and 50 mM NaCl by the addition of solid NaCl, and concentrated in an Amicon Concentrator (PM-30 filter). The concentrate was brought to 10% sucrose and deassed before it was applied to a Bio-Gel A-5 m column (5 x 90 cm) that was equilibrated with 20 mM MOPS, 1 mM EDTA, and 1 mM TPCCK, 0.1 mM Tris[32P]ATP (Kamm et al., 1989), C;ch et al., 1989). The pelleted protein was resuspended in urea/glycerol-PAGE and immunoblotting with a modification (Kamm et al., 1989) of the procedure described by Persechini et al. (1986). Relative

1. **The abbreviations used are:** SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PDBu, phosphorol 12,13-dibutyrate; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene diaminetetra acetic acid; TPCCK, 1,1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; DTT, dithiorbitol.

2. **Fig. 1. Immunoprecipitation of myosin light chain kinase (MLCK)** from 52P-labeled bovine tracheal smooth muscle tissues. A representative Coomassie Blue-stained polyacrylamide gel is shown in the left panel with purified myosin light chain kinase on the left and immunoprecipitated kinase on the right. The right panel shows representative autoradiograms of tissue samples treated with no addition (C), 3 μM isoproterenol for 20 min (I), 0.1 μM carbachol for 25 min (CCh), or a combination of isoproterenol and carbachol (I + CCh).

3. **The supernatant fraction was diluted with 20 mM MOPS, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT at pH 7.0 to 7.5 millisiemens.** Diethylaminoethyl cellulose (DE-52) equilibrated in the same buffer (150 g dry) was added and stirred 1 h. After washing the resin on a scintillated glass funnel, the kinase was eluted by bringing the conductivity of the resin in 100 ml of buffer to 15 millisiemens with solid NaCl. The solution was collected, diluted to a conductivity of less than 2.5 millisiemens, and added to DE-52 (50 g dry) equilibrated in 20 mM MOPS, 1 mM EGTA, 1 mM EDTA, and 1 mM DTT at pH 7.0, and containing protease inhibitors as above. After stirring 1 h, the resin was drained and washed two times on a scintillated glass funnel and poured into a column. The kinase was eluted with a 1.5-liter gradient containing 0–300 mM NaCl in the equilibration buffer. Fractions containing kinase activity were pooled, brought to 500 mM NaCl by the addition of solid NaCl, and concentrated in an Amicon Concentrator (PM-30 filter). The concentrate was brought to 10% sucrose and deassed before it was applied to a Bio-Gel A-5 m column (5 x 90 cm) that was equilibrated with 20 mM MOPS, 0.5 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM TPCK, 0.1 mM TLCK, and 10 μM leupeptin at pH 7.0. Active kinase fractions were pooled and dialyzed against 20 mM MOPS, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 50 mM NaCl at pH 7.0. The dialyzed pool was applied to a phycohecellulose column (5 x 4 cm) equilibrated in the same buffer. Myosin light chain kinase was eluted with 400 ml of equilibration buffer plus 0.4 mM NaCl.

4. **The kinase fraction was brought to 2 mM CaCl₂, 2 mM magnesium acetate, and 1 mM DTT at pH 7.0. This fraction was applied to a calmodulin-Sepharose column (2.5 x 10 cm).** The column was washed extensively with 10 mM MOPS, 1 mM CaCl₂, 2 mM magnesium acetate, 0.2 mM NaCl, 1 mM DTT, 10 μM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride at pH 7.0. Myosin light chain kinase was eluted with wash buffer containing 10 mM EGTA and dialyzed against 10 mM potassium phosphate and 1 mM DTT at pH 7.0. The dialyzed sample was applied to a hydroxylapatite column (1 x 4 cm). After washing the column in the same buffer, the kinase was eluted with a 10 to 300 mM potassium phosphate gradient containing 1 mM DTT at pH 6.8 in a total volume of 120 ml. The fractions containing myosin light chain kinase activity were brought to 10% glycerol and stored at -60°C.

5. **Miscellaneous Procedures**—The portion of each 52P-labeled tissue (approximately 5 mg) not used for immunoprecipitation of myosin light chain kinase was homogenized in 200 μl of 10% trichloroacetic acid at 0°C. After centrifugation at 7000 x g for 1 min, the supernatant fraction was removed for analysis of radioactivity of specific activity of tissue [gamma-32P]ATP (Kamm et al., 1989). The pellet protein was reprecipitated in urea sample buffer and processed for urea/glycerol-PAGE and immunoblotting with a modification (Kamm et al., 1989) of the procedure described by Persechini et al. (1986). Relative activity of phosphorylation in terms of changes in calmodulin activation properties.
amounts of nonphosphorylated and monophosphorylated light chain were quantitated from densitometric scans of immunostained nitrocellulose blots. Under the experimental conditions described herein, diphosphorylated light chain was not observed. Purified myosin light chain kinase was phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase in the presence and absence of Ca++/calmodulin as described by Conti and Adelstein (1981). To assess changes in \( K_{\text{CaM}} \), myosin light chain kinase activity ratios were determined as described previously by Miller et al. (1983) with minor modifications. Activities were measured at 1 and 100 \( \mu \text{m} \) free Ca++ in the presence of 400 nM calmodulin. When there is an increase in the \( K_{\text{CaM}} \) value, the ratio of activity measured at 1 to that at 100 \( \mu \text{m} \) free Ca++ decreases.

Data are expressed as mean \( \pm \) S.E.

RESULTS

Myosin Light Chain Kinase Phosphorylation—Treatment of bovine tracheal tissue with 3 \( \mu \text{m} \) isoproterenol resulted in no increase in myosin light chain phosphorylation or in force (Fig. 2). However, the extent of myosin light chain kinase phosphorylation increased from a control value of 0.86 mol of phosphate/mol of kinase to 1.28 and 2.56 mol by 1 and 20 min, respectively (Fig. 2). Carbachol treatment (0.1 \( \mu \text{m} \)) resulted in a transient increase in the extent of myosin light chain phosphorylation to a maximal value of 0.70 mol of phosphate/mol of light chain by 5 min while force increased to a sustained level. The extent of myosin light chain kinase phosphorylation also increased in the presence of carbachol to 1.94 mol of phosphate/mol of kinase by 25 min. The addition of isoproterenol to tissues that had been treated with carbachol for 5 min resulted in a prompt relaxation and decrease in myosin light chain phosphorylation. However, the extent of myosin light chain kinase phosphorylation was similar to values obtained with carbachol alone. Thus, the effects of isoproterenol and carbachol on myosin light chain kinase phosphorylation were not additive. Furthermore, there was no simple correlation between contractile force and myosin light chain kinase phosphorylation.

PDBu, an activator of protein kinase C, also stimulated myosin light chain kinase phosphorylation (Fig. 3). Treatment of tracheal tissues with 1 \( \mu \text{M} \) PDBu for 25 min resulted in an increase in myosin light chain kinase phosphorylation to 2.73 mol of phosphate/mol of kinase. Fractional force and myosin light chain phosphorylation increased slightly. These conditions lead to a predominant phosphorylation of protein kinase C phosphorylation sites in myosin light chain (Kamm et al., 1989) which has little apparent effect on smooth muscle contractile properties (Sutton and Haeberle, 1990). Treatment of tissues with a combination of carbachol and PDBu did not result in any greater increase in myosin light chain kinase phosphorylation compared to PDBu alone. Thus, the effects of PDBu and carbachol on kinase phosphorylation were not additive.

KCl stimulated myosin light chain phosphorylation and force development in tracheal smooth muscle (Fig. 3). KCl treatment also increased myosin light chain kinase phosphorylation to 1.60 mol of phosphate/mol of kinase.

These results show that treatment of tracheal smooth muscle with agents that activate different protein kinases stimulated myosin light chain kinase phosphorylation. However, there was no simple correlation between the extent of kinase phosphorylation and the contractile state. Since myosin light chain kinase can be phosphorylated at multiple sites by different protein kinases (Conti and Adelstein, 1981; Nishikawa et al., 1985; Ikebe et al., 1985; Hashimoto and Soderling, 1990), phosphopeptide mapping was used to identify specific sites of phosphorylation.

Phosphorylation Sites in Myosin Light Chain—Myosin light chain kinase purified from bovine tracheal smooth muscle was phosphorylated by the catalytic subunit of the cyclic AMP-dependent protein kinase. In the presence of Ca++ and calmodulin, the extent of phosphorylation was 1.6 mol of \(^{32}\text{P} \) incorporated/mol of myosin light chain kinase. Phosphopeptide mapping revealed a single phosphopeptide B (Fig. 4). In the presence of EGTA, \(^{32}\text{P} \) incorporation increased to 2.0 mol of \(^{32}\text{P} \) incorporated/mol of myosin light chain kinase and peptide mapping showed two phosphopeptides, A and B. Identical phosphopeptide maps were obtained with diphosphorylated myosin light chain kinase from gizzard smooth muscle (data not shown). Based on previous reports with the avian enzyme, phosphopeptide A was identified as the serine site phosphorylated near the calmodulin-binding domain (Conti and Adelstein, 1981; Lukas et al., 1986; Hashimoto and Soderling, 1989). Phosphorylation of both sites when calmodulin is not bound to myosin light chain kinase increases \( K_{\text{CaM}} \). Site B, phosphorylated whether or not calmodulin is bound, has been identified on the C-terminal side of site A; phosphorylation of site B itself has no effect on \( K_{\text{CaM}} \) (Payne et al., 1989).
FIG. 4. Two-dimensional trypic phosphopeptide maps of purified tracheal myosin light chain kinase phosphorylated by cyclic AMP-dependent protein kinase. A, myosin light chain kinase (MLCK) was phosphorylated to 1.0 mol of $^{32}$P/mol of kinase. B, myosin light chain kinase was phosphorylated to 2.0 mol of $^{32}$P/mol of kinase. C, diphosphorylated myosin light chain kinase was added to a tracheal tissue homogenate. Phosphorylation, immunoprecipitation, SDS-PAGE, trypsin digestion, and peptide-mapping methods are described under "Experimental Procedures." The circle in the lower left-hand corner of each panel identifies the origin.

FIG. 5. Two-dimensional trypic phosphopeptide maps of $^{32}$P-labeled myosin light chain kinase from stimulated trachealis. Myosin light chain kinase was isolated by immunoprecipitation from homogenates of $^{32}$P-labeled tracheal tissues followed by SDS-PAGE. Myosin light chain kinase was excised, incubated with trypsin, and two-dimensional phosphopeptide mapping was performed as described under "Experimental Procedures." The circle in the lower left-hand corner of each panel identifies the origin. The treatment conditions include: A, control; B, 3 μM isoproterenol for 20 min; C, 1 μM PDBu for 25 min; D, 0.1 μM carbachol for 25 min; E, 0.1 μM carbachol (25 min) plus 3 μM isoproterenol (20 min); F, 65 mM KCl (5 min) and 0.1 μM atropine (15 min).

al., 1986). Purified protein kinase C and Ca$^{2+}$/calmodulin-dependent protein kinase II also phosphorylated site A, but not site B (data not shown).

Purified $^{32}$P-labeled tracheal smooth muscle myosin light chain kinase diphosphorylated by cyclic AMP-dependent protein kinase was added to a bovine tracheal tissue extract in a buffer system that inhibited protein kinase and protein phosphatase activities. There was no dephosphorylation of the kinase during the time required for immunoprecipitation and processing. Furthermore, there was no alteration of the mobility of phosphopeptides A and B (Fig. 4).

Myosin light chain kinase was immunoprecipitated from $^{32}$P-labeled tracheal tissues and subjected to phosphopeptide mapping (Fig. 5). Six phosphopeptides were identified consistently after treatment with various agents. Phosphopeptides A and B coincide to the phosphopeptides A and B identified in myosin light chain kinase diphosphorylated by cyclic AMP-dependent protein kinase; co-electrophoresis of digests of the purified myosin light chain kinase and kinase obtained from $^{32}$P-labeled tissues resulted in coincident migrations of A and B phosphopeptides, respectively (data not shown). Under control conditions, $^{32}$P was found in peptides B, C, D, and F. Thus, there are multiple phosphorylation sites when the extent of myosin light chain kinase phosphorylation is 0.86 mol of phosphate/mol of kinase. Treatment of tracheal tissues with isoproterenol for 20 min results in four primary phosphopeptides from myosin light chain kinase, A, B, C, and D. The relative amounts of $^{32}$P in phosphopeptides A and B are less than C and D. PDBu treatment resulted in no significant $^{32}$P incorporation into phosphopeptide A or B and the appearance of another phosphopeptide E. The appearance of phosphopeptides B, C, D, and E on a diagonal to the origin may indicate that they are the same peptide with different extents of phosphorylation, i.e., the incorporation of each phosphate decreases the mobility in both the electrophoretic and chromatographic dimensions. This type of relationship was observed with a monophosphorylated and diphosphorylated peptide from smooth muscle myosin light chain (Colburn et al., 1988).

Stimulation of muscarinic receptors for 25 min with carbachol resulted in significant $^{32}$P incorporation into all six phosphopeptides. The combination of carbachol and isoproterenol gave a phosphopeptide pattern intermediate to the treatments with the individual agonists. Interestingly, phosphopeptide A was the most prominent $^{32}$P-labeled peptide after treatment of tracheal smooth muscle with 65 mM KCl (Fig. 5).

The relative amounts of $^{32}$P incorporated into $^{32}$P-labeled peptides from myosin light chain kinase were analyzed (Table I). Under control conditions there was no significant phosphorylation of peptide A, and the radioactivity was evenly distributed to peptides B, C, D, and F. With the addition of
phosphorylation, but interestingly no significant phosphorylation of peptide B could be due to its phosphorylation and subsequent phosphate incorporation, then the relative decline of phosphopeptide B. If phosphopeptides B, C, and D also increased the extent of myosin light chain kinase phosphorylation (Fig. 2). Prolonged treatment of tracheal smooth muscle with KCl treatments, respectively.

Isoproterenol alone increased myosin light chain kinase phosphorylation, but only a small fraction of the radioactivity was incorporated into peptide A (Table I). The maximal extent of 32P incorporation was 0.20 mol of phosphate/mol of peptide A for carbachol and KCl treatments, respectively. Isoproterenol alone increased myosin light chain kinase phosphorylation, but only a small fraction of the radioactivity was incorporated into peptide A (Table I). The maximal extent of 32P incorporation was 0.20 mol of phosphate/mol of peptide A for carbachol and KCl treatments, respectively.

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Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Myosin light chain kinase</th>
<th>32P-labeled peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>0.86 ± 0.08</td>
<td>0 ± 0 (0)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>1.28 ± 0.07</td>
<td>35 ± 6 (0.42)</td>
</tr>
<tr>
<td>1 min</td>
<td>1.66 ± 0.26</td>
<td>43 ± 9 (0.71)</td>
</tr>
<tr>
<td>5 min</td>
<td>1.94 ± 0.26</td>
<td>20 ± 2 (0.39)</td>
</tr>
<tr>
<td>KCl, 5 min</td>
<td>1.60 ± 0.26</td>
<td>50 ± 2 (0.80)</td>
</tr>
<tr>
<td>Isoproterenol, 1 min</td>
<td>1.28 ± 0.15</td>
<td>12 ± 2 (0.15)</td>
</tr>
<tr>
<td>20 min</td>
<td>2.56 ± 0.35</td>
<td>8 ± 1 (0.20)</td>
</tr>
<tr>
<td>PDBu, 25 min</td>
<td>2.73 ± 0.33</td>
<td>3 ± 3 (0.08)</td>
</tr>
<tr>
<td>Carbachol (5 min) + isoproterenol (1 min)</td>
<td>1.43 ± 0.24</td>
<td>23 ± 5 (0.33)</td>
</tr>
<tr>
<td>Carbachol (25 min) + isoproterenol (20 min)</td>
<td>1.66 ± 0.13</td>
<td>14 ± 5 (0.23)</td>
</tr>
<tr>
<td>Carbachol (25 min) + PDBu (20 min)</td>
<td>3.09 ± 0.36</td>
<td>7 ± 2 (0.22)</td>
</tr>
</tbody>
</table>

* Moles of phosphate per mol of myosin light chain kinase. Data from Figs. 2 and 3.
* Numbers in parentheses are calculated moles of phosphate per mol of peptide A.
* Carbachol, 0.1 μM; isoproterenol, 3 μM; PDBu, 1 μM; KCl, 65 mM + 0.1 μM atropine.

It is predicted that phosphorylation of site A in myosin light chain kinase increases KcαM irrespective of the kinase that may phosphorylate this site (Kamm and Stull, 1985, 1989). Changes in the calmodulin activation properties were assessed by measurements of the kinase activity ratio in tissue homogenates. The ratio of enzymatic activity at 1 μM Ca2+ to the activity at 100 μM Ca2+ in the presence of 400 nM calmodulin is dependent quantitatively upon the KcαM value (Miller et al., 1983). As shown in Fig. 6, there was a direct correlation between the myosin light chain kinase activity ratio and the extent of phosphorylation of peptide A in myosin light chain kinase. This relationship was observed with tissues treated with carbachol, KCl, isoproterenol, and PDBu. Thus, changes in KcαM are probably determined by the extent of phosphorylation of site A.

The extent of phosphorylation at site A in myosin light chain kinase was originally proposed to be related inversely to myosin light chain phosphorylation (Conti and Adeleson, 1981). Thus, a high extent of site A phosphorylation in smooth muscle would lead to an increase in KcαM, inhibition of myosin light chain kinase activity, and diminished myosin light chain phosphorylation. However, as shown in Fig. 7, there is a positive relationship with a high level of myosin light chain phosphorylation associated with a high extent of site A phosphorylation. In tracheal smooth muscle depleted of Ca2+ by incubation in the presence of 5 mM EGTA (Ratz and Murphy, 1987), 0.1 μM carbachol did not result in the development of...
Myosin light chain kinase is phosphorylated in tracheal smooth muscle, six primary phosphorylation sites were identified. Phosphorylation of purified tracheal smooth muscle myosin light chain kinase in the presence of Ca$^{2+}$/calmodulin by cyclic AMP-dependent protein kinase results in a single phosphorylation peptide B that is identical in terms of migration to the single site phosphorylated under similar conditions in the avian smooth muscle myosin light chain kinase. The partial amino acid sequence of the avian kinase has been deduced from a cDNA clone and includes the catalytic domain, pseudosubstrate region, calmodulin-binding domain, and the extended C-terminal portion (Guerrero et al., 1986; Pearson et al., 1988). From this sequence the predicted structure of a small phosphopeptide obtained after extensive trypsin digestion would be KASGSPTPSPINADK. Although the sequence around site B is not known for tracheal myosin light chain kinase, the structure of the peptide for the mammalian kinase is probably very similar, if not identical, to the avian enzyme. It has been demonstrated previously that the site phosphorylated in the presence of Ca$^{2+}$/calmodulin by the cyclic AMP-dependent protein kinase is . . . RKAS(P)GSSTPSPINADK . . . where the phosphorylatable serine is preceded by a neutral amino acid and 2 basic residues (Pavne et al., 1986). This limited sequence is representative of a consensus phosphorylation sequence for cyclic AMP-dependent protein kinase (Edelman et al., 1987).

It is interesting that peptide B contains 3 additional serine residues and 1 additional threonine residue with a total potential of five phosphorylation sites. The incorporation of additional phosphate may result in a proportional decrease in the migration of the peptide in both the electrophoretic and chromatographic dimensions (B, C, D, and E phosphopeptide) (Colburn et al., 1988). Kinases that could phosphorylate these additional residues have not been identified. Protein kinase C (Iikebe et al., 1985; Nishikawa et al., 1985) and Ca$^{2+}$/calmodulin-dependent protein kinase II (Hashimoto and Soderling, 1990) do not phosphorylate this peptide. A recently described proline-directed serine/threonine protein kinase is a candidate (Vulliet et al., 1989) since there are 2 potential phosphorylation residues in the peptide within the consensus sequence -X-S-P-X-. Another kinase that could phosphorylate this peptide includes glycogen synthase kinase 3 which recognizes -S-X-X-X-S(P) (Fiol et al., 1987). This possibility would involve a synergistic action of protein kinases since the phosphorylation sites would be recognized after post-translational phosphorylation. However, other kinases should also be considered. It is not clear what functions, if any, phosphorylation of peptides B, C, D, and E may play in the regulation of myosin light chain kinase, but it is tempting to speculate that they may affect binding to contractile elements or other noncatalytic properties.

The sequence of phosphopeptide A has been determined for avian smooth muscle myosin light chain kinase (Lukas et al., 1986; Hashimoto and Soderling, 1990). It includes -L-S-S(P)-M-A-M-I-S-G-M-S-G-R- where S(P) identifies the specific serine residue phosphorylated by cyclic AMP-dependent protein kinase and Ca$^{2+}$/calmodulin-dependent protein kinase II (Hashimoto and Soderling, 1990). This serine is probably also phosphorylated by protein kinase C, because we and Nishikawa et al. (1985) found $^{32}$P incorporation into this peptide. There are no reports that the other serine residues in this peptide are phosphorylated, and no known consensus phosphorylation sequences for protein kinases are identified. In addition, the phosphopeptide mapping of myosin light chain kinase phosphorylated under a variety of conditions in tracheal smooth muscle gave no evidence that phosphopeptide $\Delta$ could be phosphorylated at additional serines, i.e. there were no phosphopeptides on a diagonal from phosphopeptide A relative to the origin.

Physiologically, site A is not readily phosphorylated under conditions in which cyclic AMP formation is stimulated. At
a high concentration of isoproterenol, the maximal extent of phosphorylation was 0.2 mol of phosphate/mole of peptide A, and the myosin light chain kinase activity ratio decreased slightly. The small decrease in the activity ratio with a high concentration of isoproterenol is consistent with previous observations (Miller et al., 1983). Furthermore, lower concentrations of isoproterenol that result in appreciable relaxation of contracted tracheal smooth muscle did not change the myosin light chain kinase activity ratio (Miller et al., 1983). These results indicate that phosphorylation of myosin light chain kinase in site A is not necessary for smooth muscle relaxation. Decreases in cytosolic Ca²⁺ concentrations probably account for the relaxation response to increases in cyclic AMP formation (Gunst and Bandyopadhyay, 1989; Felbel et al., 1988; Taylor et al., 1989).

When is cyclic AMP formation such a poor stimulus for site A phosphorylation? The sequence around the phosphorylation site (-R-X-S-X-) is a consensus sequence for Ca²⁺/calmodulin-dependent protein kinase II, not for cyclic AMP-dependent protein kinase (-R-X-S-X). Myosin light chain kinase is phosphorylated at a rate that is 1% or less than the rates of phosphorylation of physiological substrates (Stull et al., 1986). Therefore, the rate of phosphorylation at site A by cyclic AMP-dependent protein kinase may not be sufficiently greater than the rate of dephosphorylation by a protein phosphatase; thus only minor extents of phosphorylation can be achieved.

Purified smooth muscle myosin light chain kinase is phosphorylated by protein kinase C (Nishikawa et al., 1985; Ikebe et al., 1985). Although there is disagreement about the sites of phosphorylation, both groups of investigators found that phosphorylation reduced the affinity of myosin light chain kinase for Ca²⁺/calmodulin. The addition of PDBu, an activator of protein kinase C, to tracheal smooth muscle resulted in substantial phosphorylation of myosin light chain kinase. However, there was no phosphorylation of phosphopeptide A, and there was no decrease in the myosin light chain activity ratio. The possibility exists that protein kinase C may activate another kinase that phosphorylates myosin light chain kinase. However, the function of these phosphorylations is not clear because there is not a significant decrease in the myosin light chain kinase activity ratio.

Myosin light chain kinase is phosphorylated to a high extent in site A in tracheal smooth muscle contracted with carbachol or KCl. Furthermore, there is a positive correlation between the extents of phosphorylation of myosin light chain and myosin light chain kinase. These data plus the inhibition of myosin light chain kinase phosphorylation at site A by EGTA treatment suggest that it is catalyzed by a Ca²⁺/calmodulin-dependent protein kinase would be a likely candidate (Hashimoto and Soderling, 1990), because it also phosphorylates myosin light chain kinase at site A, and this site is highly phosphorylated in contracting tissue. However, as noted by Hashimoto and Soderling (1990), when calmodulin is bound to myosin light chain kinase, phosphorylation of this regulatory site is blocked. The autophosphorylated Ca²⁺/calmodulin-dependent protein kinase II was used by these investigators for rapid phosphorylation without Ca²⁺/calmodulin binding to myosin light chain kinase. Myosin light chain kinase could be phosphorylated by the Ca²⁺/calmodulin dependent protein kinase II during contraction if a large fraction of the myosin light chain kinase did not have calmodulin bound. Alternatively, a small population of nonbound kinase may be phosphorylated rapidly. During the time required for development of force, Ca²⁺/calmodulin will associate and dissociate from myosin light chain kinase. While dissociated, the kinase may be phosphorylated, so that over some period of time substantial phosphorylation will be obtained.

The possibility must also be considered, however, that another Ca²⁺-dependent protein kinase may phosphorylate myosin light chain kinase when Ca²⁺/calmodulin is bound. It is known that a protein phosphatase dephosphorylates site A when Ca²⁺/calmodulin is bound to myosin light chain kinase (Pato and Adelstein, 1983). Thus the phosphate moiety is not covered by calmodulin or otherwise totally inaccessible. Evidence has also been presented that site A is not necessary for the high affinity binding of Ca²⁺/calmodulin to a 17-residue synthetic peptide representing the calmodulin-binding domain of the avian myosin light chain kinase (Lukas et al., 1986). Thus the phosphorylatable serine in site A may not bind directly to Ca²⁺/calmodulin and could be accessible for phosphorylation by an unidentified, Ca²⁺-dependent protein kinase.

The predicted effect of site A phosphorylation in myosin light chain kinase is to decrease the sensitivity of kinase activation to Ca²⁺. Cytosolic Ca²⁺ concentrations were not measured in this study, and therefore direct assessment of the consequences of myosin light chain phosphorylation is not possible. However, there are a number of recent physiological studies that have demonstrated a desensitization of contractile elements to cytotoxic Ca²⁺ concentrations in contracting smooth muscles: i.e. there are time-dependent decreases in the ratio of cytosolic Ca²⁺ concentrations to the force generated (Morgan and Morgan, 1984; Rembold and Murphy, 1988; Yagi et al., 1988; Karaki, 1989; Himpens et al., 1989). We propose that phosphorylation of myosin light chain kinase with a resultant increase in Kₘₐₜ could play an important role in this desensitization process. Direct measurements of cytosolic Ca²⁺ concentrations, myosin light chain kinase phosphorylation in site A, and myosin light chain phosphorylation will be needed to substantiate this hypothesis.

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