Phosphorylation of the 20,000-Dalton Light Chain of Smooth Muscle Myosin by the Calcium-activated, Phospholipid-dependent Protein Kinase

PHOSPHORYLATION SITES AND EFFECTS OF PHOSPHORYLATION*

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Smooth muscle heavy meromyosin (HMM) is phosphorylated by the Ca\(^{2+}\)-activated phospholipid-dependent protein kinase, i.e. protein kinase C, at three sites on each 20,000-dalton light chain. Phosphorylation of three sites also is observed with isolated 20,000-dalton light chain and HMM subfragment 1. The phosphorylation sites are serine 1, serine 2, and threonine 9. Threonine is phosphorylated most rapidly followed by either serine 1 or 2. Phosphorylation of the third site occurs only on prolonged incubation. Phosphorylation is a random process. HMM phosphorylated at two sites per light chain by protein kinase C can be dephosphorylated, as shown using two phosphatase preparations. Increasing levels of phosphorylation of HMM by protein kinase C causes a progressive inhibition of the subsequent rate of phosphorylation of serine 19 by myosin light chain kinase and causes a progressive inhibition of actin-activated ATPase activity of HMM, prephosphorylated by myosin light chain kinase. Inhibition of ATPase activity is due to a decreased affinity of HMM for actin rather than a change in V\(_{max}\). Previous results with HMM and protein kinase C (Nishikawa, M., Sellers, J. R., Adelstein, R. S., and Hidaka, H. (1984) J. Biol. Chem. 259, 8808–8814) examined effects induced by phosphorylation of the threonine residues. Our results confirm these and consider also the influence of higher levels of phosphorylation by protein kinase C.

Contraction of smooth muscle requires phosphorylation of the 20,000-dalton light chains of myosin (see review in Ref. 1) by the calmodulin-dependent myosin light chain kinase (MLC kinase). The site of phosphorylation usually is serine 19, although under some circumstances, for example at high concentrations of MLC kinase, a second site is phosphorylated and this has been identified as threonine 18 (2). Phosphorylation of either serine or serine plus threonine increases the actin-activated ATPase activity of myosin (3, 4), and it has been suggested that the change in enzymatic activity reflects a conformational change of myosin (5). Recently, it has also been shown that the Ca\(^{2+}\)-activated phospholipid-dependent protein kinase, i.e. protein kinase C, phosphorylates the smooth muscle myosin 20,000-dalton light chain, either isolated or incorporated into HMM or myosin (6, 7). For HMM the phosphorylation of one site per light chain was observed and threonine was the major phosphoamino acid formed (7). With isolated light chain, up to 3 mol of phosphate could be incorporated per mol of light chain (7). Phosphorylation by protein kinase C alone does not affect the enzymatic properties of myosin (6, 7), although phosphorylation of HMM by both protein kinase C and MLC kinase causes a reduction of actin-activated ATPase activity of myosin compared to the activity obtained following phosphorylation only by MLC kinase (8). The inhibition is due to an increase in K\(_{m}\) for actin (7). It has also been shown that the phosphorylation of smooth muscle myosin by protein kinase C does not induce a change in conformation (8).

In our laboratory, the stoichiometry of phosphorylation differed from that observed by Nishikawa et al. (7) in that levels approaching 6 mol of P/mol of HMM were obtained, and both threonine and serine residues were phosphorylated. In this paper, the effects of phosphorylation of HMM by protein kinase C on actin-activated ATPase activity and subsequent phosphorylation by MLC kinase are reported. A primary objective of this study was to identify the sites of phosphorylation by protein kinase C, since this is a prerequisite for the understanding of the molecular effects induced by light chain phosphorylation via both protein kinase C and MLC kinase. It was found that the three potential sites are serine 1, serine 2, and threonine 9. If phosphorylation of myosin by protein kinase C has physiological relevance, then dephosphorylation must also occur. Using two preparations of phosphatase, dephosphorylation of HMM is documented. An interesting distinction was observed in that one preparation dephosphorylated both serine and threonine residues, whereas the other preparation was active only toward phosphothreonine.

MATERIALS AND METHODS

The following materials and procedures were used for the isolation of proteins: myosin (9), MLC kinase (10), and myosin light chain phosphatase (11) from frozen turkey gizzards; HMM and S1 by digestion of gizzard myosin with Staphylococcus aureus protease (12) and by digestion with a-chymotrypsin (13); the 20,000-dalton light chain from gizzard myosin (14); protein kinase C from bovine brain (15); actin from rabbit skeletal muscle (16); and calmodulin from bull.
Phosphorylation of Smooth Muscle Myosin by Protein Kinase C

**RESULTS**

The phosphorylation of HMM by protein kinase C under different assay conditions is shown in Fig. 1. Phosphatidylserine is essential for kinase activity and, in addition, either PMA or Ca²⁺ is required. It is interesting that, in the presence of PMA and phosphatidylserine and the absence of Ca²⁺, a higher rate of phosphorylation is observed. Negligible phosphorylation is found with phosphatidylserine plus EGTA, EGTA alone, and Ca²⁺ alone. Under the conditions shown in Fig. 1, it is clear that 1 residue is phosphorylated relatively rapidly, followed by a more slowly reacting residue. On more prolonged incubation, phosphorylation levels higher than 2 mol of P/mol of light chain were observed; for example, after 120 min, 2.5 mol of P/mol of light chain were incorporated. These results indicate that there are at least three potential phosphorylation sites on the 20,000-dalton light chain and that successive sites are phosphorylated at reduced rates. Analysis of the phosphorylated amino acids (Fig. 1) showed that phosphothreonine is formed first followed by phosphoserine (see samples taken at 2 and 10 min in Fig. 1). From the inset of Fig. 1 it can be estimated that the rate of phosphorylation of threonine is approximately 6.5 times faster than the rate for the second site. At a higher level of phosphorylation (2.5 mol of P/mol of light chain) no other phosphoamino acids were formed and only phosphoserine and phosphothreonine were detected.

The phosphorylation of threonine and serine residues by protein kinase C was observed for HMM prepared by proteolysis with a- chymotrypsin, for S1 prepared by proteolysis with S. aureus protease, and for isolated 20,000-dalton light chain. The rates of phosphorylation for the a-chymotryptic HMM and S1 are similar to those shown in Fig. 1, and the phosphorylation of the isolated light chain is slightly slower (about 30% slower for the first two sites). In Fig. 2 are shown urea gels sampled during time courses of phosphorylation by protein kinase C of isolated 20,000-dalton light chain and HMM. The autoradiograms of these gels are also shown. The phosphorylation of two sites is confirmed by these results, as bands corresponding to singly and doubly labeled light chain are detected. With longer reaction times than those used for Fig. 2, the third site can be detected by the appearance of a band corresponding to the triply labeled light chain (data not shown). An additional point illustrated in Fig. 2 is that phosphorylation of the first two sites is not sequential, as the dephosphorylated, singly, and doubly phosphorylated light chains can coexist (see Fig. 2A, lane 3, or Fig. 2B, lane 4). The dephosphorylation of HMM, phosphorylated at two light chain sites by protein kinase C, was investigated using two preparations of phosphatase and the results are shown in Fig. 3. Using the spontaneously active phosphatase from bovine aorta only one band is dephosphorylated, and by phosphoamino acid analysis this site was shown to be phosphothreonine. The rate of dephosphorylation (as determined by the inset of Fig. 3A) is 11.3 times slower than the dephosphorylation of serine 19 by the same preparation of phosphatase.
Phosphorylation by protein kinase C of isolated 20,000-dalton light chain and HMM. The time points, from left to right, are: 0, 0.33, 1.5, 5, 15, 30, 45, and 70 min. On the left are the Coomassie Brilliant Blue R-250-stained gels (upper left, isolated light chain, and lower left, HMM) and on the right are the corresponding autoradiograms (using Kodak Min-R 100 x-ray film). The phosphorylation of light chains (0.1 mg/ml) and HMM (0.68 mg/ml) was as described in the legend to Fig. 1 with PMA, EGTA, and phosphatidylserine. LC20, 20,000-dalton light chain; LC17, 17,000-dalton light chain.

Fig. 2. Urea gel electrophoresis for time courses of phosphorylation by protein kinase C of isolated 20,000-dalton light chains and HMM. The time points, from left to right, are: 0, 0.33, 1.5, 5, 15, 30, 45, and 70 min. On the left are the Coomassie Brilliant Blue R-250-stained gels (upper left, isolated light chain, and lower left, HMM) and on the right are the corresponding autoradiograms (using Kodak Min-R 100 x-ray film). The phosphorylation of light chains (0.1 mg/ml) and HMM (0.68 mg/ml) was as described in the legend to Fig. 1 with PMA, EGTA, and phosphatidylserine. LC20, 20,000-dalton light chain; LC17, 17,000-dalton light chain.

Fig. 3. Dephosphorylation of phosphorylated HMM by two preparations of phosphatase. HMM was phosphorylated by protein kinase C as described in the legend to Fig. 1 with PMA, EGTA, and phosphatidylserine and applied to a TSK DEAE-5PW (HPLC) column equilibrated with 30 mM Tris-HCl (pH 7.5) and 0.5 mM dithiothreitol. The phosphorylated HMM (approximately 4 mol of P/mol of HMM) was eluted by a linear KC1 gradient and assayed with the spontaneously active bovine aorta phosphatase (A) and the turkey gizzard phosphatase (B). Neither phosphatase preparation was homogeneous.

In contrast, the phosphatase prepared from turkey gizzard dephosphorylates both sites (Fig. 3B), and dephosphorylation is a random process, as judged by the fit to a single exponential, shown in the inset of Fig. 3B. The rate of dephosphorylation is 2.4 times slower than the dephosphorylation of serine 19.

Amino acid analyses of the phosphorylated peptides P-2, P-3, and P-4 (see "Materials and Methods") are shown in Table I. P-2 contained 0.8 mol of P/mol of peptide and there are two potential sites represented by the 2 threonine residues.

Amino acid compositions and sequences of the phosphorylated tryptic peptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>P-2 (Observed)</th>
<th>P-3 (Observed)</th>
<th>P-4 (Observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>2.9</td>
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<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>1.0</td>
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</tr>
<tr>
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<td>0.2</td>
<td>0.2</td>
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<tr>
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</tr>
<tr>
<td>Gly</td>
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<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala</td>
<td>2.2</td>
<td>2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Part of the N-terminal sequence of the 20,000-dalton light chain (19) is shown in the top line and the deduced sequences for the tryptic peptides (P-2 to P-4) are shown below. Phosphorylation sites are threonine 9, serine 1, and serine 2 (shown by *).

Phosphorylation sequence of P-2 on the gas-phase sequenator gave the sequence Ala-Lys-Ala-Lys-__Thr-Lys; at step 5 no phenylthiohydantoin amino acid was observed. This would be expected for phosphothreonine because the phosphate is bound to the filter disc in the sequenator, and the breakdown products are not observed in high yield. Thus it is concluded that threonine 9 is the initial site of phosphorylation by protein kinase C. It was suggested previously (2), based on analysis of the products of sequential α-chymotryptic hydrolysis, that the threonine phosphorylated by protein kinase C is located close to the N terminus of the 20,000-dalton light chain and could be either threonine 9 or 10.

For peptides P-3 and P-4 the phosphate contents were 2.05 and 1.14 mol/mol of peptide, respectively. Direct analysis of these peptides in the sequenator was not possible since the N terminus is blocked. However, the sequences can be deduced from the amino acid analyses (Table I), and it is apparent that site 1 and 2 are potential phosphorylation sites. Digestion of P-4 with carboxypeptidase B releases only arginine, and incubation with carboxypeptidase Y, under conditions that result in extensive digestion of the insulin B chain (23), releases nothing. Presumably, the presence of the phosphate group(s) adjacent to lysine 3 inhibit the release of lysine by carboxypeptidase. (Arginine also is removed quantitatively from the tetrapeptide by trypsin hydrolysis (1:10 weight ratio), and the phosphorylation site(s) is retained in the residual peptide.)

From these and previous data (shown in Fig. 1) it is clear that the initial, or preferred, site of phosphorylation is threonine 9 and that the second site is either serine 1 or serine 2. Since the hexapeptide P-3 contained 2 phosphorylated residues it is reasonable to assume that both serines are phosphorylated. The presence of two phosphates was confirmed on analysis of P-3 by tandem mass spectrometry. A peptide of mass 878.5 was detected. The calculated mass for this hexapeptide including the N-terminal acetyl group and the phosphorylation site(s) is 878.4. Thus, it is concluded that both serine residues can be phosphorylated. The three potential phosphorylation sites, therefore, are serine 1, serine 2, and threonine 9.

Analysis of peptide P-3 by triple quadrupole mass spectrometry gave two ions of mass 780 and 682, reflecting the loss of one...
and two phosphate groups, respectively. Analysis of the tetrapeptide (P-4) by tandem mass spectrometry did not reveal a component of appropriate mass corresponding to a phosphorylated peptide, despite the presence of a $^{32}$P-labeled residue(s).

The effect of phosphorylation by protein kinase C on some of the biological properties of HMM was investigated next. In Fig. 4, the influence of phosphorylation by protein kinase C on the actin-activated ATPase activity of HMM was monitored. HMM was prephosphorylated by MLC kinase (to approximately 2 mol of P/mol of HMM) and then phosphorylated to varying extents by protein kinase C. At the indicated levels of phosphorylation by protein kinase C, actin-activated ATPase activity was measured. As shown (Fig. 4), increasing levels of phosphorylation decrease the actin-activated ATPase. At stoichiometries of 1 and 2 mol of P/mol of light chain (incorporated by protein kinase C) the actin-activated ATPase activities are approximately 50 and 25% of the control activity, respectively. At a level of 1 mol of P/mol of light chain similar results were obtained by Nishikawa et al. (6, 7). The inhibition of ATPase activity is due to an increase in $K_a$ (the apparent dissociation constant for actin as determined by ATPase measurements), as shown in Fig. 5. For HMM phosphorylated only by MLC kinase (approximately 2 mol of P/mol of HMM), $K_a$ and $V_{max}$ are approximately 60 $\mu$M and 0.77 $\mu$mol/min mg (4.5 $s^{-1}$), respectively. Phosphorylation of this HMM by protein kinase C (to an additional 2.3 mol of P/mol of light chain) did not alter $V_{max}$ but changed $K_a$ to 340 $\mu$M. Phosphorylation of HMM by protein kinase C alone (up to 2.3 mol of P/mol of light chain) did not increase the actin-activated ATPase activity.

**Fig. 4.** Dependence of actin-activated ATPase activity of HMM (prephosphorylated by MLC kinase) on the extent of phosphorylation by protein kinase C. HMM (1.0 mg/ml) was phosphorylated by MLC kinase (2 $\mu$g/ml) in 30 mM Tris-HCl (pH 7.5), 300 mM KCl, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 0.5 mM ATP, and 5 $\mu$g/ml calmodulin for 15 min at 25 $^\circ$C. Reaction was stopped by addition of EGTA to 1 mM, and the solution was diluted with 2 volumes of 30 mM Tris-HCl (pH 7.5) and applied to a DEAE-SPW column attached to a Perkin-Elmer HPLC system. HMM was eluted at 0.25 mM NaCl by a linear NaCl gradient. The phosphorylated HMM was dialyzed against 30 mM Tris-HCl (pH 7.6) and the level of phosphorylation checked by urea gel electrophoresis. The level of phosphorylation was approximately 2 mol of P/mol of HMM. The phosphorylated HMM was incubated with protein kinase C for varying times, with conditions as in Fig. 1 with PMA, EGTA, and phosphatidyserine. Samples were filtered through Millex-GV (Millipore Corp.) to remove phosphatidyserine. Actin-activated ATPase activities were assayed with 1 mg/ml F-actin, 0.15 mg/ml HMM, 30 mM Tris-HCl (pH 7.5), 1 mM MgCl$_2$, 1 mM EGTA, and 0.5 mM [gamma-$^{32}$P]ATP at 25 $^\circ$C. The extent of phosphorylation by protein kinase C was also measured (see "Materials and Methods"). LC, light chain.

The effect of phosphorylation by protein kinase C on the subsequent phosphorylation rate by MLC kinase on HMM prephosphorylated to varying levels by protein kinase C. HMM (1.0 mg/ml) was phosphorylated by protein kinase C in EGTA, PMA, and phosphatidyserine for varying times as described in the legend to Fig. 1. Samples were filtered through Millex-GV (Millipore Corp.) and applied to a DEAE-SPW (as in Fig. 4), eluted by a linear NaCl gradient and dialyzed versus 30 mM KCl, 30 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol. For each sample the extent of phosphorylation was determined from incorporated $^{32}$P and by urea gel electrophoresis. Phosphorylation of HMM (0.3 mg/ml) by MLC kinase was assayed at 25 $^\circ$C in 30 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 0.5 mM [gamma-$^{32}$P]ATP, 0.1 $\mu$g/ml MLC kinase, and 5 $\mu$g/ml calmodulin. The MLC kinase rate was calculated from the initial linear phase of phosphorylation.

**Fig. 5.** Actin dependence of ATPase activity of HMM phosphorylated by MLC kinase and protein kinase C. Conditions of phosphorylation were as in Fig. 4. HMM phosphorylated only by MLC kinase (C) and HMM phosphorylated by MLC kinase and protein kinase C to a level 2.3 mol of P/mol of light chain (●) are shown.

**Fig. 6.** Dependence of the rate of phosphorylation by MLC kinase on HMM prephosphorylated to varying levels by protein kinase C. HMM (1.0 mg/ml) was phosphorylated by protein kinase C in EGTA, PMA, and phosphatidyserine for varying times as described in the legend to Fig. 1. Samples were filtered through Millex-GV (Millipore Corp.) and applied to a DEAE-SPW (as in Fig. 4), eluted by a linear NaCl gradient and dialyzed versus 30 mM KCl, 30 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol. For each sample the extent of phosphorylation was determined from incorporated $^{32}$P and by urea gel electrophoresis. Phosphorylation of HMM (0.3 mg/ml) by MLC kinase was assayed at 25 $^\circ$C in 30 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 0.5 mM [gamma-$^{32}$P]ATP, 0.1 $\mu$g/ml MLC kinase, and 5 $\mu$g/ml calmodulin. The MLC kinase rate was calculated from the initial linear phase of phosphorylation.

The effect of phosphorylation by HMM of protein kinase C on the subsequent phosphorylation rate by MLC kinase is shown in Fig. 6. HMM was initially phosphorylated by protein kinase C to the levels indicated in Fig. 6 and then subject to phosphorylation by MLC kinase. (The rate of phosphorylation for MLC kinase was determined from the initial linear phase of phosphorylation.) As can be seen, prior phosphorylation by protein kinase C markedly reduces the rate of phosphorylation by MLC kinase. For a level of 2 mol of P/mol of
light chain, incorporated by protein kinase C, the rate of phosphorylation by MLC kinase is reduced over 90% compared to the control rate.

**DISCUSSION**

It is shown that the 20,000-dalton light chain incorporated into HMM can be phosphorylated at three sites by protein kinase C. The most rapid, or the preferred, site is threonine 9 and subsequently serine 1 and serine 2 are also phosphorylated. Despite differences in the rates of phosphorylation for each site, it is thought that the pattern of phosphorylation is random rather than sequential. Whether one of the two serines is a better substrate than the other is not known, but it is possible that there is little initial preference for the two serines and phosphorylation of either serine induces a slower phosphorylation of the second serine. In terms of stoichiometry of phosphorylation, our results with HMM are similar to those of Nishikawa et al. (7) using isolated light chains. However, with HMM as substrate, Nishikawa et al. (7) detected only the threonine sites.

If phosphorylation of myosin by protein kinase C has any physiological relevance, then dephosphorylation is an obligatory component. The above results document that dephosphorylation can occur, and this is shown using two preparations of phosphatase. It is interesting that the spontaneously active bovine phosphatase removes only the phosphate attached to threonine 9 and apparently is ineffective with the serine phosphates. Whether or not this reflects an in vitro distinction is not known. However, since conditions are not established under which phosphorylation of myosin by protein kinase C occurs in intact, or skinned, smooth muscle fibers, it is inappropriate to speculate on possible mechanisms of dephosphorylation.

It was shown previously (7) that phosphorylation of HMM by protein kinase C to a level of 1 mol of P/mol of light chain decreased the subsequent rate of phosphorylation by MLC kinase and also decreased actin-activated ATPase of HMM prephosphorylated by MLC kinase. Our results confirm these data and in addition show that higher levels of phosphorylation by protein kinase C enhance these effects and increase inhibition of both the MLC kinase rate and actin-activated ATPase. In agreement with Nishikawa et al. (7), the inhibition of ATPase activity results from an alteration of the actin-binding affinity of HMM rather than a change in Vmax.

It is intriguing that, within the first 19 N-terminal residues of the 20,000-dalton light chain, there are five potential phosphorylation sites (serine 19 and threonine 18 for MLC kinase (2), and threonine 9, serine 1, and serine 2 for protein kinase C). Despite their proximity, phosphorylation of these sites have markedly different effects on the enzymatic activity of myosin. Phosphorylation of serine 19 and threonine 18 increases the actin-activated ATPase activity (2-4) and phosphorylation of threonine 9 (6, 7), serine 1, and serine 2 suppresses the level of activity achieved by phosphorylation with MLC kinase. Although phosphorylation of HMM by protein kinase C alone does not increase actin-activated ATPase activity, it is not known whether phosphorylation of any, or all, of the three protein kinase C sites influences other properties of myosin, enzymatic or physical. Since it is possible that interactions between the N-terminal region of the 20,000-dalton light chain and the myosin heavy chain play an important role in determining the rates of phosphorylation for specific myosin, it would be interesting to examine in more detail the effects of phosphorylation by protein kinase C.

The substrate determinants of protein kinase C have not been unequivocally identified, although it is clear that the sequence requirements are distinct from the cAMP-dependent protein kinase and MLC kinase (for references, see Ref. 24). It has been suggested (24) that basic residues, particularly arginine, N-terminal to the phosphorylation site are important substrate determinants for protein kinase C, and it was proposed also that basic residues C-terminal to the phosphorylation site can promote phosphorylation (25). The preferred light chain site for phosphorylation by protein kinase C, i.e. threonine 9, satisfies each criteria since there are several basic residues both on its C- and N-terminal sides (see Table I). Phosphorylation of the second and third light chain sites, i.e. serine 1 and serine 2, is probably facilitated by the lysine and arginine residues at positions 3 and 4, respectively. Recently it was shown that the acetylated N-terminal serine of chick muscle lactate dehydrogenase was phosphorylated in vitro by protein kinase C (26) and the presence of an adjacent basic residue C-terminal to the site facilitated phosphorylation.

**REFERENCES**