Activation of Actin-activated ATPase in Smooth Muscle by Phosphorylation of Myosin Light Chain with Protease-activated Kinase I*

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The 20,000-dalton light chain of myosin from chicken gizzard has been shown to be phosphorylated in a Ca2+ and calmodulin-independent manner by the activated form of a protease-activated kinase from rabbit reticulocytes. Protease-activated kinase I incorporates phosphate stoichiometrically into the phosphorylatable light chain (P-light chain) in isolated myosin light chains and in actomyosin. The same serine residue appears to be phosphorylated by the protease-activated kinase and the Ca2+-dependent myosin light chain kinase. This conclusion is based on results from two-dimensional peptide maps of chymotryptic and tryptic digests of the phosphorylated P-light chain and from phosphoamino acid analysis of acid hydrolysates. Phosphorylation of the P-light chain by the proteolytically activated protein kinase stimulates the actin-activated Mg-ATPase activity of myosin in the absence of Ca2+. The extent of stimulation of the ATPase activity is similar to that observed upon phosphorylation of actomyosin by the Ca2+-dependent myosin light chain kinase. A proteolytically activated protein kinase with chromatographic properties and substrate specificity similar to protease-activated kinase I from reticulocytes has also been identified in gizzard. Protease-activated kinase I has been shown to be distinct from the Ca2+-dependent myosin light chain kinase by the mode of activation and specificity with other substrates, including phosphorylation of a unique site on myosin P-light chain from skeletal muscle (Tuazon, P. T., Stull, J. T., and Traugh, J. A. [1982] Biochem. Biophys. Res. Commun. 108, 910-917).

The Ca2+, calmodulin-dependent phosphorylation of the 20,000-dalton light chain of myosin (P-light chain) has been implicated in the regulation of contraction in smooth muscle and nonmuscle systems (for review, see Refs. 1 and 2). Considerable support for this comes from in vitro studies with crude or reconstituted actomyosin which show the actin-activated Mg-ATPase activity of myosin is stimulated by phosphorylation of the P-light chain (3-11). In addition, changes in contractile activity or tension have been correlated with the extent of phosphorylation of the P-light chain in intact muscles (12-17) and chemically skinned fibers (18-20).

Recently we reported that the P-light chain in isolated myosin light chains from skeletal muscle is phosphorylated by a protease-activated protein kinase in a Ca2+, calmodulin-independent manner (21, 22). Incorporation of phosphate into the P-light chain from skeletal muscle is stoichiometric and the site phosphorylated by the protease-activated kinase is different from that phosphorylated by the Ca2+, calmodulin-dependent myosin light chain kinase (21, 22). Antibody prepared to myosin light chain kinase from rabbit skeletal muscle inhibits the activity of the myosin light chain kinase, but does not affect the activity of the protease-activated kinase (21).

Protease-activated kinase I has been isolated from a number of tissues including rabbit reticulocytes and skeletal muscle (22, 23), and chicken liver and brain.1 The enzyme is activated by limited proteolysis with trypsin, but is not affected by Ca2+ and calmodulin or by Ca2+, phospholipids, and diacylglycerol (21, 23). In addition to myosin light chain, protease-activated kinase I phosphorylates histones 2B and 4 (23), eukaryotic initiation factors (24), and ribosomal protein S10 (24, 25). On the basis of the differences in mode of activation and substrate specificity with myosin light chain from skeletal muscle and other substrates, protease-activated kinase I appears to be distinct from the Ca2+-dependent myosin light chain kinase. Thus, phosphorylation of isolated myosin light chains and actomyosin from chicken gizzard by protease-activated kinase I has been examined and quantified, and a correlation of this phosphorylation with activation of the Mg-ATPase activity of myosin has been undertaken.

EXPERIMENTAL PROCEDURES

Materials—Trypsin (diphenylcarbamyl chloride-treated), soybean trypsin inhibitor, and mixed histone IIAs were obtained from Boehringer Mannheim; trifluoperazine dihydrochloride was from Smith Kline and French Laboratories. Precoated plastic-helicated thin layer cellulose sheets (20 x 20 cm, 0.16 mm thick, without fluorescent indicator) were purchased from Eastman. [γ-32P]ATP was prepared as previously described (26).

Protease-activated kinase I was purified from rabbit reticulocytes by chromatography on DEAE-cellulose, phosphocellulose, and hydroxylapatite as described by Tahara and Traugh (23) and stored in buffer A (15 mM potassium phosphate, pH 6.8, 20 mM 2-mercaptoethanol, 1 mM EDTA; 2 mM EGTA; 0.02% NaN3) at 4 °C. The enzyme obtained in this manner was free of other protein kinase activities and was purified 44-fold based on the activity recovered following DEAE-cellulose chromatography. Highly purified preparations of myosin light chain kinase from bovine aorta muscle, calmodulin from bovine brain, and myosin light chains from rabbit skeletal muscle were generously provided by Dr. James T. Stull, University of Texas Health Science Center, Dallas, TX. Mixed myosin light chains were prepared from chicken gizzard according to Perrie and Perry (27). Concentrations of the P-light chain were determined by densitometric scanning of polyacrylamide gels stained with Coomassie blue using bovine serum albumin as a standard. The endogenous

1 K. S. Morley and J. A. Traugh, unpublished results.
2 The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′′,N′′'-tetraacetic acid.

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Myosin Light Chain Phosphorylation by Protease-activated Kinase

Phosphate content of isolated myosin light chains was determined as previously described by Pearson and Perry (28).

Actomyosin was prepared from chicken gizzard according to the procedure of Sobieszek and Small (29) with the following modifications. In all buffers, 15 mM 2-mercaptoethanol replaced cysteine; streptomyacin was omitted. Homogenization of minced muscle was carried out in 10 volumes of buffer in a Waring blender, and the myocardins were washed two times with 1% Triton X-100. Actomyosin isolated this way contained 27% myosin as determined from densitometric scanning of the P-light chain. In some cases "washed" actomyosin was prepared as described by Sobieszek and Bremel (30) by precipitating the crude actomyosin two times with 25% ammonium sulfate to remove endogenous myosin light chain kinase and tropomyosin.

Protein Kinase Assays—Assays to identify the protease-activated kinase were carried out with histone (1 mg/ml) as substrate (23) with the indicated concentrations of trypsin-activated or nonactivated enzyme. Assays with myosin P-light chain (10.7 pM) were carried out under the same reaction conditions as with histone except that reaction mixtures were 0.003 ml, and the concentration of MgCl2 was 10 mM (21). Incorporation of 32P into P-light chain was monitored by counting gel slices excised from 15% polyacrylamide gels containing sodium dodecyl sulfate (31). Myosin light chain kinase activity was measured as previously described (21) with the indicated concentration of myosin light chains.

Assays for Actin-activated Myosin ATPase Activity—The actin-activated Mg-ATPase activity of crude actomyosin was measured in 0.20-ml reaction mixtures containing 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 60 mM KCl, 2.5 mM ATP, 0.01 mg/ml of either 6 MgEGTA or 0.4 mM CaCl2, and protein kinase as indicated. The reactions were initiated by addition of ATP and were incubated at 30 °C for 10 min. Liberated inorganic phosphate was determined as described by Jarrett and Penniston (32) and was shown to be linear with time. The protease-activated kinase used for these assays was dialyzed in 20 mM Tris-HCl, pH 7.4, and 15 mM 2-mercaptoethanol before use.

Other Methods—Protein concentrations were determined by the Coomassie blue dye-binding method of Bradford (33) with γ-globulin as standard. Two-dimensional peptide mapping involving thin layer chromatography in butanol:acetic acidwater (5:1:1) was carried out as previously described (31). The phosphoamino acid determination of phosphorylated myosin light chain has been described elsewhere (31).

RESULTS

Phosphorylation of P-Light Chain in Isolated Myosin Light Chains and Actomyosin—Isolated myosin light chains from chicken gizzard were analyzed as substrate for protease-activated kinase I from rabbit reticulocytes. The protein kinase phosphorylated the 20,000-dalton light chain as identified by glycerol-urea polyacrylamide slab gel electrophoresis (27). The isolated myosin light chains were shown to be phosphorylated approximately six times faster than actomyosin (Table I).

In order to quantify the total amount of phosphate which could be incorporated into the P-light chain, an 8- to 10-fold increase in protease-activated kinase was used. Under these conditions, optimal phosphate incorporation was observed at 15 min; 0.6 mol of phosphate was incorporated per mol of P-light chain by protease-activated kinase I. A similar value was obtained with myosin light chain kinase using isolated myosin light chains. When actomyosin was examined, 0.5 mol of phosphate was incorporated per mol of P-light chain by either enzyme. Phosphorylation of myosin light chain by the two enzymes was not additive; when mixed light chains or actomyosin were maximally phosphorylated by protease-activated kinase I in the presence of EGTA, no further incorporation was observed upon addition of Ca2+ and myosin light chain kinase.

Comparison of Sites Phosphorylated by Protease-activated Kinase I and Myosin Light Chain Kinase—In order to determine whether protease-activated kinase I and the Ca2+-, calmodulin-dependent myosin light chain kinase phosphorylated the same site, chymotryptic digests of phosphorylated myosin light chains in actomyosin were analyzed by two-dimensional peptide mapping (Fig. 2). A single chymotryptic phosphopeptide was obtained when phosphorylation was carried out with either the protease-activated kinase or myosin light chain kinase. When a mixture of the chymotryptic digests was analyzed, only one phosphopeptide was obtained. The same results were obtained when isolated light chains were phosphorylated with either the protease-activated kinase from reticulocytes or the purified Ca2+-dependent myosin light chain kinase from aorta or skeletal muscle (data not shown).

Peptide mapping of tryptic digests of the P-light chain from actomyosin or isolated light chain also showed a single phosphopeptide when the light chain was phosphorylated with protease-activated kinase I (Fig. 2). An identical phosphopeptide was obtained when phosphorylation was carried out with the Ca2+-dependent myosin light chain kinase and was confirmed by analyzing a mixture of the tryptic digests.
Experimental Procedures. Values are moles of phosphoacceptor amino acid. Both protease-activated kinase I and the Ca²⁺-dependent incorporated into the light chain was quantitated as described under polyacrylamide gels containing sodium dodecyl sulfate and the "Pi

\[ \text{pmol} \] were phosphorylated with protease-activated kinase I (3.0 µg) for 5 and 30 min, respectively. Reactions were analyzed on 15% polyacrylamide gels containing sodium dodecyl sulfate and the \( ^{32}\text{P} \). incorporated into the light chain was quantitated as described under "Experimental Procedures." Values are moles of \( ^{32}\text{P} \) incorporated per mol of P-light chain.

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol/min</th>
<th>mol/mol</th>
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<tbody>
<tr>
<td>Isolated light chains</td>
<td>9.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>1.4</td>
<td>0.5</td>
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</table>

*Phosphorylation of mixed light chains (350 pmol) or actomyosin (340 pmol) by protease-activated kinase (0.65 µg) was carried out in a 0.060-ml reaction mixture.

Mixed light chains (186 pmol) and "washed" actomyosin (24 pmol) were phosphorylated with protease-activated kinase I (3.0 µg) for 5 and 30 min, respectively. Reactions were analyzed on 15% polyacrylamide gels containing sodium dodecyl sulfate and the \( ^{32}\text{P} \). incorporated into the light chain was quantitated as described under "Experimental Procedures." Values are moles of \( ^{32}\text{P} \) incorporated per mol of P-light chain.

Partial acid hydrolysates of maximally phosphorylated myosin P-light chains were analyzed by high voltage electrophoresis in order to determine the phosphoacceptor amino acid. Both protease-activated kinase I and the Ca²⁺-dependent myosin light chain kinase phosphorylated serine residues (data not shown).

**Effect of Phosphorylation of Actomyosin on the Actin-activated Mg-ATPase Activity**—The effect of phosphorylation on the actin-activated Mg-ATPase activity of crude actomyosin was measured at low ionic strength (60 mM KCl) in the presence of Ca²⁺ at pH 7.4 (Fig. 3). In these experiments, the actomyosin contained approximately 0.5 mol of endogenous phosphate/mol of P-light chain. When the reaction was carried out in EGTA, without added protein kinase, the Mg-ATPase activity was 4.2 ± 0.2 nmol/min/mg of myosin (Table II). The Mg-ATPase activity was shown to increase as the amount of added protease-activated kinase I was increased in the reaction mixture. The maximum activity attained with protease-activated kinase was 36 ± 4 nmol/min/mg (Table II). A similar value was obtained with the endogenous myosin light chain kinase in the presence of Ca²⁺.

No further increase was observed when exogenous myosin light chain kinase was added. Similarly, addition of protease-activated kinase to assays containing Ca²⁺ did not result in further stimulation of the ATPase activity.

**Activation of the Mg-ATPase activity by protease-activated kinase I in the absence of Ca²⁺** was shown to be accompanied by phosphorylation of the 20,000-dalton light chain. Phosphorylation of the P-light chain of actomyosin in the presence of EGTA was observed only upon addition of the activated form of the protease-activated kinase. No significant phosphorylation was observed in the absence of added enzyme or upon addition of the inactive holoenzyme. Addition of increasing amounts of protease-activated kinase resulted in increasing phosphorylation of the P-light chain (Fig. 4). The extent of phosphorylation was the same as that observed with endogenous or added myosin light chain kinase in the presence of Ca²⁺. No further increase was observed when protease-activated kinase I was added to reactions in the presence of Ca²⁺.

The time course of ATP hydrolysis in the presence of protease-activated kinase I and EGTA was examined and compared to the time course of phosphorylation of the myosin P-light chain. ATP hydrolysis and phosphorylation of the P-light chain were shown to increase with increasing incubation times; maximum ATP hydrolysis occurred with maximum phosphate incorporation into the P-light chain (data not shown).

**Effect of Trifluoperazine on the Mg-ATPase Activity and Phosphorylation of Actomyosin**—The effect of trifluoperazine on the Mg-ATPase activity and phosphorylation of actomyosin was examined (Table II). Phosphorylation of P-light chain by the endogenous myosin light chain kinase in the
presence of Ca\textsuperscript{2+} was inhibited 50% with 35 \textmu M trifluoperazin.
No inhibition of phosphorylation by the protease-activated kinase was observed in the presence or absence of Ca\textsuperscript{2+} at this concentration of trifluoperazin. Addition of 150 \textmu M trifluoperazin inhibited the Ca\textsuperscript{2+}-activated Mg-ATPase activity by 92%, whereas it had no effect on the Mg-ATPase activity stimulated by protease-activated kinase I in the presence of EGTA or Ca\textsuperscript{2+} and endogenous myosin light chain kinase.

Identification of a Protease-activated Kinase in Chicken Gizzard—Extracts of chicken gizzard were chromatographed on DEAE-cellulose at pH 8.0 to determine if a proteolytically activatable protein kinase was present in gizzard. Protease-activated kinase activity was assayed before and after limited proteolytic digestion with trypsin. A major peak of protease-activated kinase eluted at 0.05 M KCl (Fig. 5), a position similar to that observed for protease-activated kinase I from reticulocytes and skeletal muscle (22, 23). The protease-activated kinase was identified by phosphorylation of mixed histone and myosin light chains. The Ca\textsuperscript{2+}-dependent myosin light chain kinase was observed to elute at 0.15 M KCl as shown by phosphorylation of myosin light chains in the presence of Ca\textsuperscript{2+} and calmodulin. Integration of the peaks of activity for protease-activated kinase I and myosin light chain kinase with myosin light chain as substrate showed the amount of protease-activated kinase in gizzard was 7–10% of that of the Ca\textsuperscript{2+}-dependent myosin light chain kinase.

![Fig. 4. Effect of protease-activated kinase I on the phosphorylation of actomyosin from chicken gizzard. Phosphorylation was carried out in 0.030-ml reaction volumes under the conditions of the Mg-ATPase assay except that 0.4 mM [\gamma\textsuperscript{32}P]ATP replaced 2.5 \textmu M ATP. Reactions were analyzed on 15% polyacrylamide gels containing sodium dodecyl sulfate. \textsuperscript{32}P incorporation was quantitated as described under "Experimental Procedures." \textsuperscript{32}P incorporation was quantitated with protease-activated kinase (O); without protease-activated kinase in the presence of Ca\textsuperscript{2+} (\bullet).

![Fig. 5. Identification of a protease-activated kinase from chicken gizzard by chromatography on DEAE-cellulose. Extract from chicken gizzard (30 g) was chromatographed on DEAE-cellulose (1.8 × 2.3 cm). Aliquots (0.01 ml) of 1-ml fractions were assayed before and after activation by trypsin with histone (1 mg/ml) and with myosin light chains as substrates (10.7 \textmu M) in the presence of 1 mM EGTA. The fractions were also assayed with myosin light chains in the presence of Ca\textsuperscript{2+} (0.8 mM) and calmodulin (CM; 267 \textmu M).

![Table 1](https://example.com/table1.png)

<table>
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<tr>
<th>Addition</th>
<th>Mg-ATPase activity</th>
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<tbody>
<tr>
<td></td>
<td>EGTA</td>
</tr>
<tr>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>Activated PAK</td>
<td>314</td>
</tr>
<tr>
<td>Trifluoperazin</td>
<td>42</td>
</tr>
<tr>
<td>Activated PAK, trifluoperazin</td>
<td>307</td>
</tr>
</tbody>
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\* PAK, protease-activated kinase.

**DISCUSSION**

Phosphorylation of the 20,000-dalton light chain by the Ca\textsuperscript{2+}, calmodulin-dependent myosin light chain kinase appears to regulate muscle contraction in smooth muscle systems via stimulation of the actin-activated Mg-ATPase activity (3–11). We have shown that the P-light chain from gizzard is phosphorylated stoichiometrically by protease-activated kinase I in a Ca\textsuperscript{2+}, calmodulin-independent manner and that phosphorylation of actomyosin results in a stimulation of Mg-ATPase activity equivalent to that observed with the Ca\textsuperscript{2+}-activated myosin light chain kinase. The extent of phosphorylation of the P-light chain by protease-activated kinase I using purified light chains and crude actomyosin from chicken gizzard is the same with both substrates; approximately 0.5 mol of phosphate is incorporated per mol of light chain using either the Ca\textsuperscript{2+}-dependent myosin light chain kinase or protease-activated kinase I. Since the isolated light chains have been shown to contain endogenous phosphate by electrophoresis in urea gels, the total phosphate content of the P-light chain following phosphorylation is approximately 1 mol/mol. The rate of phosphorylation of the isolated light chains by protease-activated kinase I is six times faster than that of actomyosin. Similar differences in rates between the isolated
myosin light chains and actomyosin have been observed with the Ca"+-dependent myosin light chain kinase (34, 35).

Protease-activated kinase I and the Ca"+-dependent myosin light chain kinase appear to phosphorylate the same site on the P-light chain of myosin. Phosphorylation by the two protein kinases is not additive, suggesting that the same site is being phosphorylated or phosphorylation of one site inhibits phosphorylation of a second closely associated site. Two-dimensional peptide mapping of the tryptic and chymotryptic digests of the phosphorylated P-light chain indicates that the phosphopeptide obtained with the protease-activated kinase is the same as that obtained with the Ca"+-dependent myosin light chain kinase. With both enzymes, the major phosphorylated residue has been determined to be serine. The site phosphorylated by the Ca"+-dependent myosin light chain kinase has been identified as Ser-19 (2, 36). This same residue appears to be phosphorylated by the protease-activated kinase. Examination of the amino acid sequence of the P-light chain (36, 37) reveals three serine residues, Ser-1, -2, and -19, in the chymotryptic peptide. However, following tryptic digestion, the first 3 amino acids are removed and Ser-19 is the only seryl group in the peptide. It has been reported that CaM-dependent protein kinase also phosphorylates isolated myosin light chains from turkey gizzard in the site phosphorylated by the Ca"+, calmodulin-dependent myosin light chain kinase (38). However, in contrast to the data obtained with the protease-activated kinase I, actomyosin is not phosphorylated by the CaM-dependent protein kinase (39). It is not unusual that more than one enzyme phosphorylates the same serine residue. For example, it has been shown that CaM-dependent protein kinase, phosphorylase kinase, and glycogen synthase kinase-4 phosphorylate the same serine residue in glycogen synthase (40, 41). In recent studies, we have observed that the isolated P-light chain from gizzard is also phosphorylated by casein kinase II, but at a different site. However, casein kinase II does not phosphorylate the P-light chain in actomyosin suggesting this phosphorylation event is of little interest.

In myosin P-light chain from skeletal muscle, protease-activated kinase I phosphorylates a site different from that phosphorylated by the Ca"+-dependent myosin light chain kinase, as indicated by the different chymotryptic phosphopeptides obtained (21, 22). The observed difference in phosphorylation of gizzard and skeletal P-light chain by protease-activated kinase I is probably due to the amino acid sequence around the phosphorylation sites. Protease-activated kinase I appears to recognize a basic amino acid two or three residues toward the NH2-terminal side of serine (e.g. lysine in the sequence Lys-Ala-Thr-Ser-Asn-Val in gizzard P-light chain). In the P-light chain from skeletal muscle, the amino acid sequence around the site phosphorylated by the Ca"+-dependent myosin light chain kinase is Gly-Gly-Ser-Val-Phe (2, 42). Since the basic amino acid is not present, Ser-15 is not recognized by the protease-activated kinase. A likely phosphorylation site for the protease-activated kinase in P-light chain from skeletal muscle is Ser-131. This is the only serine with a basic amino acid 2 residues toward the NH2 terminus.

The protease-activated kinase appears to be a unique protein kinase when compared with the Ca"+, calmodulin-dependent myosin light chain kinase. It does not have a calmodulin-binding site as evidenced by a lack of stimulation by calmodulin and a lack of inhibition by trifluoperazine on both phosphorylation and Mg-ATPase activity. The protease-activated kinase does not appear to be a partially proteolyzed form of myosin light chain kinase that has lost the calmodulin-binding site as has been recently reported by Walsh et al. (43) for the following reasons. The protease-activated kinase has an extended substrate specificity and phosphorylates ribosomal protein S10, translational initiation factors, and histones (21-25). It phosphorylates a unique site on the P-light chain of skeletal myosin (21, 22). When the P-light chain from skeletal muscle is phosphorylated in the presence or absence of Ca"+ with myosin light chain kinase pretreated with trypsin, the chymotryptic phosphopeptides obtained are identical with those obtained with the Ca"+-activated myosin light chain kinase (22) indicating activation by proteolysis has not altered the substrate specificity of the enzyme.

Protease-activated kinase I has been identified and partially purified from rabbit skeletal muscle, chicken liver, and brain (22). A proteolytically activated enzyme having similar chromatographic properties on DEAE-cellulose and similar substrate specificity with histone and mixed myosin light chains has been identified in gizzard. Further studies are in progress to identify the physiological activator of the enzyme and the possible function of the protein kinase in muscle contraction.

Acknowledgments—We wish to thank Dr. James T. Stull for generously providing myosin light chain kinase from bovine aorta, calmodulin from bovine brain, and myosin light chains from skeletal muscle. We also wish to thank Drs. James T. Stull and Robert Adelstein for critical reading of the manuscript.

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Myosin Light Chain Phosphorylation by Protease-activated Kinase

Activation of actin-activated ATPase in smooth muscle by phosphorylation of myosin light chain with protease-activated kinase I.

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