Smooth Muscle Myosin Kinase Requires Residues on the COOH-terminal Side of the Phosphorylation Site

PEPTIDE INHIBITORS*

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The COOH-terminal residue in peptide analogs of the phosphorylation sequence site in smooth muscle myosin light chains, Lys11-Lys12-Arg13-Ala-Ala-Arg16-Ala-Thr-Ser19-(P)Asn20-Val21-Phe22-Ala23, were shown to have a strong influence on the kinetics of peptide phosphorylation. The peptides 11–19, 11–20, 11–21, 11–22, and 11–23 were all phosphorylated by the myosin light chain kinase with similar apparent K_m values in the range 11–17 μM. The V_max varied 40-fold, with the peptides 11–19, 11–20, 11–21, 11–22, and 11–23 having V_max values of 0.035, 0.045, 0.32, 1.74, and 1.43 amol⋅mg\(^{-1}\)⋅min\(^{-1}\) respectively. These results indicated that Ala23 was not essential whereas Phe22 and Val21 had a strong influence on the V_max of peptide phosphorylation.

The series of peptides competitively inhibited myosin light chain phosphorylation with K_i values similar to their respective K_m values. Peptide 11–19 had a K_i value of approximately 10 μM and a V_max less than 0.1% of the value with myosin light chains and is therefore an effective inhibitor of the smooth muscle myosin kinase.

The myosin light chain kinase is thought to act as the principal regulatory enzyme in the control of smooth muscle contraction (1). This enzyme is responsible for phosphorylating Ser19 in smooth muscle myosin light chains (2, 3). We have recently reported that smooth muscle myosin kinase phosphorylates synthetic peptides corresponding to the region around Ser19, the phosphorylation site in the myosin light chain (4, 5). From structure-function studies it was found that the four basic residues, 11, 12, 13, and 16 in the sequence Lys11-Lys12-Arg13-Ala-Ala-Arg16-Ala-Thr-Ser19-Asn20-Val21-Phe22-Ala23 had a strong influence on the kinetics of peptide phosphorylation. Moreover, the spatial relationships between Ser19, Arg16, and Arg13 were found to be important. Relocation of Arg16 to position 15 caused a complete switch in specificity from the natural site of phosphorylation Ser19 to Thr19. In addition to the basic residues, one of the other striking structural features of the known myosin light chain phosphorylation site sequences (6) is that they all contain an invariant region, Ser(P)-Asn-Val-Phe, on the COOH side of the phosphorylated serine. In this paper we report the contribution of these residues to the kinetics of peptide phosphorylation and the development of peptide inhibitors of the myosin kinase with K_i values in the micromolar range.

EXPERIMENTAL PROCEDURES

All materials were reagent-grade unless otherwise indicated. [γ-32P]ATP was obtained from New England Nuclear.

Peptide Synthesis and Purification—The synthetic peptides were synthesized as the COOH-terminal amide form by the Merrified solid-phase synthesis procedure (7). Amino acid derivatives protected with the t-butyloxycarbonyl group in the α-amino position and benzyl-phenol were obtained from the Protein Research Foundation (Osaka, Japan). The peptides were assembled manually with ninhydrin testing at each step (8). The completed peptides were simultaneously deprotected and cleaved from the resin in anhydrous HF (9) and purified by ion-exchange chromatography (10). Peptide purity was assayed by quantitative amino acid analysis, high-voltage electrophoresis at pH 1.9 and 6.4, and reverse-phase HPLC (Brownlee RP-300 column, 0.1% (v/v) trifluoroacetic acid with a gradient 0 to 60% (v/v) acetonitrile, 30 min, 1.0 ml/min) (data not shown).

Protein Purification—The purification of myosin light chains (11), calmodulin (12), and chicken gizzard myosin kinase (13) was by published procedures modified as described previously (4).

Protein Kinase Assay—Myosin kinase was assayed in a volume of 0.08 ml of 40 mM Hepes buffer, pH 7.0, 5 mM magnesium acetate, 0.50 mM [γ-32P]ATP (100–200 cpm/pmol), 0.55 mM CaCl_2, 5 μg of calmodulin, 1 mg/ml bovine serum albumin, 0.1% (w/v) Tween 80, and myosin light chains or peptide as indicated. The enzyme was diluted in 25 mM Tris-HCl, 1 mM dithiothreitol buffer, pH 7.5, containing 0.1% (w/v) Tween 80 to prevent loss of enzyme through binding to plastic and to ensure an accurate measure of the enzyme-specific activity (14). Incubations were carried out at 30 °C and aliquots (0.03 ml) were taken at 3 and 6 min. The aliquots were applied to phosphocellulose ion-exchange paper and were washed and counted as described (5). In experiments to determine the apparent K_m and V_max of the peptide analogs, the parent peptide MLC 11–23 was used as an internal control for the V_max. Inhibition of myosin light chain phosphorylation by synthetic peptide was assessed by trichloroacetic acid precipitation, 10% (w/v) on filter papers, as described by Reimann et al. (15). Under these conditions the synthetic peptide is not retained on the filter paper and does not contribute to the measured [32P] phosphate transferred to the myosin light chains.

RESULTS AND DISCUSSION

Since all of the known regulatory light chain sequences (6) have an invariant sequence, Ser(P)-Asn-Val-Phe, on the COOH-terminal side of the phosphorylated serine, it was of interest to assess whether this region influenced the kinetics of peptide phosphorylation by the myosin kinase. In the study, all peptides were prepared as their carboxyl-terminal amides so that the results would not be influenced by the presence of a free carboxyl. The parent peptide Lys11-Lys12-Arg13-Ala-Ala-Thr-Ser19-Asn20-Val21-Phe22-Ala23 contained Ala at positions 14 and 15 in place of Pro-Gln found in the chicken gizzard myosin light chain (M, 20,000) sequence (2). This peptide is phosphorylated with kinetic parameters comparable to those of the corresponding peptide containing Pro-Gln (15). While these peptides are phosphorylated with K_m values

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; MLC, myosin light chain.
approximately 25-fold lower (Table 1). Whether Ser or Thr was phosphorylated basic residues, particularly Arg1', had a powerful influence on the production in the \( v_{\text{max}} \) over that obtained with the 11-20 peptide.

Deletion of the hydrophobic residues Phe2' and Val2' had a marked effect on the effect on the apparent \( K_m \). In contrast deletion of the hydrophobic residues on the sequence by Ala-Ala-Ala resulted in a peptide with a low \( K_m \) and very poor \( v_{\text{max}} \) comparable to the peptide 11-19 (Table 1). These results indicate that the peptide chain length is not significantly effected. Replacement of the Asn-Val-Phe light chains (5). In contrast to the strong effects of the removal of the hydrophobic residues in the pyruvate kinase peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly was accompanied by a dramatic increase in the \( v_{\text{max}} \) obtained with the myosin light chains (5). In contrast to the strong effects of the removal of the hydrophobic residues on the \( v_{\text{max}} \), the apparent \( K_m \) was not significantly effected. Replacement of the Asn-Val-Phe sequence by Ala-Ala-Ala resulted in a peptide with a low \( K_m \) and very poor \( v_{\text{max}} \) comparable to the peptide 11-19 (Table 1). These results indicate that the peptide chain length is not a dominant factor but rather that the hydrophobic residues Val-Phe have a strong influence on the \( v_{\text{max}} \).

Previously we have reported that the spatial location of the basic residues, particularly Arg5', had a powerful influence on whether Ser19 or Thr19 was phosphorylated (5). The peptides containing deletions of the carboxyl-terminal residues were phosphorylated predominantly on Ser1' as follows: MLC 11-19 (97%), MLC 11-20 (91%), MLC 11-21 (90%), MLC 11-22 (96%), and MLC 11-22, A30, A21, A22 (86%). The site of phosphorylation was assessed by partial acid hydrolysis (5.7 M HCl, 110 °C, 2 h), high voltage paper electrophoresis at pH 1.9, autoradiography, and liquid scintillation counting of the O-phosphothreonine and O-phosphoserine spots. The results were not corrected for the differences in the acid stability of the phosphoaminoacids (16) and therefore tend to underesti-

\[ \frac{V}{V_{\text{Peptide conc. (\( \mu M \times 10^{-2} \))}} \]
amidine analogs Leu-Arg-Arg-Ala-Ser-Leu-NH₂ and Leu-Arg-Arg-Ala-Ser-NH₂ also show the same trend with increasing the apparent phosphorylation of 90 of the peptide 11-19 was 10

The very poor V_max and relatively low apparent K_m of the myosin light chain peptide 11-19 raised the possibility that this peptide may be a useful inhibitor of the myosin kinase. The capacity of this peptide to inhibit myosin light chain phosphorylation was studied (Fig. 2). The K_m obtained with the peptide 11-19 was 10 μM. This value was comparable to the apparent K_m (17 μM) obtained with peptide as substrate. Previously we had found that the peptide Ser-Ser-Lys-Thr-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser competitively inhibited myosin light chain phosphorylation with a K_i of 590 μM compared with an apparent K_m for phosphorylation of 90 μM (18). This peptide differs from the ones described in the present study in not having the tribasic recognition site Lys-Lys-Arg. Several studies with peptide substrates of the cGMP-dependent protein kinase (19, 20) have found that the K_i values are higher than the corresponding apparent K_m values. Direct binding studies with the cAMP-dependent protein kinase and synthetic peptides have shown that the K_i for peptide binding is also higher than the apparent K_m for phosphorylation, by almost two orders of magnitude. The results obtained with myosin light chain peptide 11-19 were therefore unexpected. For this reason we examined the capacity of each of the truncated peptides to inhibit myosin light chain phosphorylation. In all cases the K_i values obtained were comparable to the apparent K_m values for peptide phosphorylation (Tables I and II). Inspection of the double reciprocal plots for peptide inhibition (see Fig. 2) indicated that the lines did not intersect precisely on the ordinate. This did not reflect experimental variation, as the double reciprocal plots for the other four peptides tested all had intersection points slightly to the right of the ordinate. Thus, increasing concentrations of the inhibitory peptide give the effect of increasing the apparent V_max. One possible explanation for this observation is suggested by recent studies of Bhatnagar and his colleagues (21) in which it was found that peptide substrates of the cGMP-dependent protein kinase cause an unexpected 9-fold increase in the K_i for ADP. In the event of ADP release being rate-limiting for the myosin light chain kinase, a peptide inhibitor that increased the K_i for ADP would have the effect of increasing the apparent V_max.

The results of this study demonstrate that it is possible to construct relatively potent synthetic peptide inhibitors of the smooth muscle myosin light chain kinase simply by removing the residues on the COOH-terminal side of the phosphorylated serine. Furthermore, the Asn-Val-Phe peptide that is homologous on all myosin light chains has an important influence on the kinetics of peptide phosphorylation by the myosin kinase.

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