Minimum requirements for inhibition of smooth-muscle myosin light-chain kinase by synthetic peptides

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Although the amino acid residues that are important for peptide substrates of myosin light-chain kinase have been reported, those that are important for peptide inhibitors of this enzyme have not previously been investigated. Synthetic peptides based on the sequence Lys¹¹-Lys¹²-Arg¹³-Ala-Ala-Arg¹⁶-Ala-Thr-Ser¹⁹-Asn-Val²¹-Phe²²-Ala of the chicken gizzard myosin light chain were tested as inhibitors of pig carotid-artery myosin light-chain kinase. The basic amino acid residues of the known myosin light-chain kinase inhibitor Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-NH₂ (IC₅₀ = 14 μM) [Pearson, Misconi & Kemp (1986) J. Biol. Chem. 261, 25–27] were shown to be the important residues that contribute to inhibitor potency, as evidenced by the finding that the hexapeptide Lys-Lys-Arg-Ala-Ala-Arg-NH₂ had an IC₅₀ value of 22 μM. This indicates that binding of the phosphorylatable serine residue to myosin light-chain kinase, which is of obvious importance for a substrate, does not enhance the potency of an inhibitor. With the aim of preparing more potent inhibitors, peptides Lys-Lys-Arg-Ala-Ala-Arg-Ala-Ala-Xaa-NH₂ were prepared with a variety of amino acids substituted for the phosphorylatable serine residue. None of these peptides was a more potent inhibitor than the serine peptide.

INTRODUCTION

Phosphorylation of the 20000-*M_r* myosin light chains (MLCs) by the Ca²⁺-calmodulin-dependent MLC kinase is important in the regulation of force in smooth muscle (for a review see ref. [1]). However, there is currently a controversy as to whether this phosphorylation is a necessary and sufficient prerequisite to force generation. Some laboratories have found that an increase in MLC phosphorylation always precedes force development [2,3], whereas others have found evidence for force development in the absence of significant increases in the phosphorylation state of the MLCs [4–6]. The resolution of this controversy has proved difficult because it has not been possible to demonstrate force development in the complete absence of MLC phosphorylation.

A specific inhibitor of MLC kinase would be an invaluable tool in elucidating the precise role of MLC phosphorylation in smooth-muscle contraction. Optimally, the inhibitor should not act by calmodulin antagonism because of the numerous other biochemical processes controlled by this Ca²⁺-binding protein. Owing to the ubiquity of pharmacologically important ATP-binding sites, inhibitors competitive with ATP might be expected to show only marginal specificity. For these reasons, and because MLC kinase exhibits a very narrow substrate specificity, we sought an inhibitor of MLC kinase that was competitive with substrate. One of the most potent inhibitors of MLC kinase known that is competitive with substrate is the nonapeptide Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-NH₂, having a reported

K_i value of 10 μM [7]. This inhibitor was discovered through a series of studies addressing the substrate-specificity determinants for MLC kinase [8,9].

Investigations concerning the important features of MLC kinase inhibitor peptides have not previously been reported. The present study was designed to identify the residues of the nonapeptide that contribute to the inhibition of MLC kinase and to determine whether smaller peptides are equipotent to this nonapeptide as competitive inhibitors of MLC kinase. Smaller inhibitor peptides might show increased transport into smooth-muscle cells, thus facilitating their use as tools in smooth-muscle preparations. In the present study, a series of synthetic peptides was examined that defines the residues responsible for the inhibitory activity of the nonapeptide. Hexapeptides have been identified that are potent inhibitors of MLC kinase. Synthetic peptides that contain, in place of the phosphorylatable serine residue, amino acids that may be capable of interacting with catalytic residues of the active site of the enzyme were also tested as inhibitors of MLC kinase.

MATERIALS AND METHODS

Preparation of protected unnatural amino acids

L-[N²-Boc-N³-(2-chloro-Cbz)-2,3-diaminopropionic acid] (for the preparation of peptide 20) was prepared by the reaction of L-(N²-Boc-2,3-diaminopropionic acid) [10] with di-*t*-butyl dicarbonate in aq. NaOH/dioxan [11]. L-(N²-Boc-N⁴-(2-chloro-Cbz)-2,4-diaminobutyric acid) (for the preparation of peptide 21) and L-(N²-Boc-N⁵-(2-chloro-Cbz)-2,5-diaminopentanoic acid) (for the

Abbreviations used: Boc, *t*-butoxycarbonyl; Cbz, benzyloxycarbonyl; MLC, myosin light chain.

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preparation of peptide **22**) were prepared from L-2,4-diaminobutyric acid and L-ornithine respectively by two-step procedures. The first step involved formation of a copper(II) complex, reaction of the side-chain amine with 2-chloro-Cbz chloride and liberation of the complex with disodium EDTA [12]. Reaction of the α -amine with di-*t*-butyl dicarbonate in aq. NaOH/dioxan afforded the fully protected amino acids [11]. ^1H - and ^{13}C -n.m.r. spectra were in accord with the assigned structures.

L-[*N*²-Boc-*N*³-(2-chloro-Cbz)-2,3-diaminopropionic acid] had m.p. 63–65 °C (uncorr.), $[\alpha]_{\text{D}} -10.1^\circ$ (*c* 1.0 in methanol) (Found: C, 51.3; H, 5.9; Cl, 9.2; N, 7.1; $\text{C}_{16}\text{H}_{21}\text{ClN}_2\text{O}_6$ requires C, 51.6; H, 5.7; Cl, 9.5; N, 7.5%).

L-[*N*²-Boc-*N*⁴-(2-chloro-Cbz)-2,4-diaminobutyric acid] had m.p. 73–76 °C, $[\alpha]_{\text{D}} -12.85^\circ$ (*c* 2.28 in methanol) (Found: C, 52.3; H, 6.2; Cl, 9.3; N, 7.1; $\text{C}_{17}\text{H}_{23}\text{ClN}_2\text{O}_6$ requires C, 52.2; H, 6.0; Cl, 9.1; N, 7.2%).

L-[*N*²-Boc-*N*⁵-(2-chloro-Cbz)-2,5-diaminopentanoic acid] had m.p. 114–115 °C, $[\alpha]_{\text{D}} -2.37^\circ$ (*c* 1.5 in acetic acid) (Found: C, 53.5; H, 6.1; Cl, 9.1; N, 6.9; $\text{C}_{18}\text{H}_{25}\text{ClN}_2\text{O}_6$ requires C, 53.9; H, 6.3; Cl, 8.8; N, 7.0%).

Peptide synthesis and purification

Boc-amino acids and esters and resins were obtained from Bachem. Peptides **1–4** and **9–12** were prepared by using solution techniques with ϵ -Cbz-lysine and δ -nitro-arginine protecting groups. The preparation of Boc-Arg(NO_2)-pyrrolidinamide and all amide couplings were performed in methylene chloride or *NN*-dimethylformamide by using 1-(3-dimethylamino)propyl-3-ethylcarbodi-imide hydrochloride and 1-hydroxybenzotriazole. The Boc group was removed with 98% formic acid/37% HCl (10:1, v/v), and the amine hydrochloride was neutralized with *N*-methylmorpholine for subsequent couplings. Acetylations were performed on the amine hydrochloride salts by using 10 equiv. each of sodium acetate and acetic anhydride in tetrahydrofuran. The free acid of peptide **11** was prepared by the hydrolysis of the fully protected tripeptide methyl ester with 1.75 equiv. of NaOH in water/tetrahydrofuran (2:1, v/v). Protecting groups were removed by hydrogenation over $\text{Pd}(\text{OH})_2$ in methanol/water/acetic acid (2:1:1, by vol.).

Peptides **5–8** and **13–25** were manually prepared as the C-terminal amide forms by the Merrifield solid-phase synthesis procedure with the use of benzhydrylamine resin [13]. Peptide **24** was prepared with the use of Merrifield resin. Couplings were performed by using the 1-hydroxybenzotriazole ester in methylene dichloride/*NN*-dimethylformamide (1:1, v/v). The Boc group was removed with methylene dichloride/trifluoroacetic acid (1:1, v/v) and the free amine was generated with 5% (v/v) ethyldi-isopropylamine in methylene dichloride. Acetylations were performed with acetic anhydride/pyridine (1:1, v/v). Peptides were simultaneously deprotected and cleaved from the resin in anhydrous HF at 0 °C for 1 h in the presence of anisole [14].

All peptides, isolated as acetate salts, were purified to homogeneity (> 98%) by ion-exchange chromatography on a CM-cellulose column eluted with a 0–0.5 M gradient of ammonium acetate, pH 5.75. Peptide purity was assessed by electrophoresis and reverse-phase h.p.l.c. [YMC 100A ODS column, with 5–50% (v/v) of acetonitrile in 0.1% (v/v) trifluoroacetic acid, 20 min, 1.5 ml/min]. All peptides were characterized by using ^1H - and ^{13}C -n.m.r. spectroscopy (all resonances and integrations

were consistent with the assigned structures), fast-atom-bombardment m.s. (all peptides exhibited protonated and deprotonated molecular ions as well as supporting fragments) and elemental analysis (all peptides exhibited the correct C/N ratio and the hydration values allowed the determination of peptide content).

Protein purification

MLCs were isolated from fresh bovine ventricles. All procedures were carried out at 4 °C unless otherwise specified. Standard buffers contained 15 mM-2-mercaptoethanol and 20 mM-Tris, pH 8. The ventricles were cleaned of fat, homogenized in 6 vol. of standard buffer containing 50 mM-KCl and 0.2 mM-magnesium acetate and centrifuged at 4000 *g* for 15 min. The pellet was resuspended in standard buffer including 1% (v/v) Triton X-100, centrifuged as above, and the supernatant was discarded. This centrifugation step was repeated four times until the pellet was nearly white and the supernatant nearly clear. The pellets were then homogenized in standard buffer containing 8 M-urea and 1 mM-EDTA and stirred at room temperature for 2 h. An equal volume of 50% (v/v) ethanol was added and the mixture was stirred at 4 °C for 1 h. The mixture was then centrifuged at 14000 *g* for 20 min, the pellets were discarded and the supernatant was filtered through glass-wool. DEAE-cellulose (Whatman) was added to the filtrate and the mixture was stirred overnight. The resin was allowed to settle and the supernatant was discarded. The resin was washed with standard buffer, centrifuged at 170 *g* for 10 min and the supernatant was discarded. The resin was gently mixed with standard buffer and re-centrifuged four more times. The resin was then packed into a column (24 cm $\times$ 5 cm) and the mixed MLCs were eluted with standard buffer containing 150 mM-KCl. The protein was pooled, freeze-dried and dialysed against several changes of buffer containing 4 mM-Mops, pH 7, and 15 mM-2-mercaptoethanol. The mixed MLCs were stored in small portions at –60 °C.

MLC kinase was isolated from pig carotid arteries, which were obtained at slaughter and transported to the laboratory in ice-cold physiological salt solution of the following composition: 140 mM-NaCl/4.7 mM-KCl/1.2 mM-MgSO₄/1.6 mM-CaCl₂/0.02 mM-EDTA/1.2 mM-NaH₂PO₄/2.0 mM-Mops, pH 7.4. The adventitia was stripped off the arteries [15] and discarded. MLC kinase was isolated by a minor modification of the method of Hathaway *et al.* [16]. Rather than using a column of Blue Sepharose in the last step of the purification procedure, a calmodulin affinity column was used and protein was eluted with 5 mM-EGTA. The fractions containing protein were pooled, and enough glycerol was added to bring its final concentration to 10% (v/v). The pooled protein fractions were stored in small portions at –60 °C.

SDS/polyacrylamide-gel-electrophoresis studies indicated that the pooled mixture was composed primarily (> 80%) of a protein with an *M_r* of approx. 140000. Another band of protein running at a slightly lower *M_r* could be seen with a heavier loading of the gels.

MLC kinase assay

MLC kinase was assayed at 30 °C in a volume of 75 μl of 50 mM-Mops buffer, pH 7, containing 15 mM-2-mercaptoethanol, 10 mM-magnesium acetate, 0.1 mM-CaCl₂, 0.1 μM -calmodulin, 0.04 mg of human serum

albumin, 1 mM- $[\gamma^{32}\text{P}]\text{ATP}$ (100–2000 c.p.m./pmol) and MLCs and/or synthetic peptides as indicated. For IC_{50} determinations, the concentration of MLCs used was $32\text{ }\mu\text{M}$ ($\frac{1}{2} K_m$). The assay components were mixed and the reaction was started by addition of $[\gamma^{32}\text{P}]\text{ATP}$. Samples (20 μl) were taken at two or three time points, applied to phosphocellulose ion-exchange paper and washed as described by Glass *et al.* [17]. For inhibition studies with the synthetic peptides that contained a phosphorylatable serine residue, samples of the assay mixture were applied to Whatman no. 3MM filter paper and washed as described by Corbin & Reimann [18].

IC_{50} values were determined graphically by plotting the percentage MLC kinase activity against the concentration of inhibitor peptide. Apparent K_m and V_{max} values were determined by the method of Cleland [19]. The K_i values were determined from replots of the Lineweaver–Burk plots in which the data were fitted to a line by using the method of least squares.

RESULTS AND DISCUSSION

Assay of synthetic peptide inhibitors

Initial experiments indicated that the concentrations of calmodulin, CaCl_2 , magnesium acetate and ATP in the assay were 10–100 times greater than the concentrations necessary to produce maximal activity (results not shown). The apparent K_m and V_{max} values for cardiac mixed MLCs were $60\text{ }\mu\text{M}$ and $13\text{ }\mu\text{mol/min per mg}$ respectively (see ref. [20] for values for the turkey gizzard enzyme).

Synthetic peptides based on fragments of the sequence of the MLC were prepared and assayed for inhibition of pig carotid-artery MLC kinase. Enzyme activity was determined at several concentrations of MLCs in the presence and in the absence of the inhibitors. The data in Fig. 1 show the results of a typical experiment with Lys-Lys-Arg-Ala-Ala-Arg-OMe (peptide 4) as the inhibitor. The double-reciprocal plot for peptide 4 as well as the plots for the other peptide inhibitors are similar to that reported for the inhibition of the chicken gizzard enzyme by peptide 6, with the lines intersecting to the right of the ordinate [7]. This phenomenon has been discussed previously [7]. To calculate the K_i , the apparent K_m and V_{max} values were determined at each concentration of inhibitor, and a secondary plot from the mean data of several experiments (Fig. 1 inset) was made. The K_i was taken as the intersection of the abscissa and the line through the data points. For eight of the peptides both the IC_{50} and the K_i values were determined. These values were closely correlated ($r = 0.98$), demonstrating that valid comparisons of potency can be made by using the IC_{50} values.

Effect of peptide length on inhibitor potency

MLC kinase exhibits a very narrow substrate specificity, the only known physiological substrate being the regulatory light chain of myosin. The amino acid sequence around the phosphorylatable serine residue of MLCs from chicken gizzard is -Lys¹¹-Lys¹²-Arg¹³-Pro-Gln-Arg¹⁶-Ala-Thr-Ser¹⁹-Asn-Val²¹-Phe²²-Ala- [21]. With the use of the model substrate Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ala-NH₂, studies of the substrate specificity of chicken gizzard MLC kinase have implicated four regions of the chain in substrate recognition and phosphorylation [7–9]. To facilitate

Fig 1. Inhibition of MLC phosphorylation by peptide 4

Lineweaver–Burk plot of pig carotid-artery MLC kinase activity versus the concentration of MLC in the absence (●) and in the presence of $15\text{ }\mu\text{M}$ (○), $30\text{ }\mu\text{M}$ (■) or $60\text{ }\mu\text{M}$ (□) concentrations of peptide 4. Each assay was run in duplicate. Inset. A replot of mean data from several experiments like the one described above. The ratio of the apparent K_m to the V_{max} was plotted at each concentration of peptide 4 studied. Points are means $\pm$ S.E.M. ($n = 3$).

Fig 2. Model of the subsite regions of the MLC and corresponding regions of MLC kinase

Regions of the smooth-muscle MLC, designated as PR (peptide region), that are important for substrate recognition and phosphorylation by smooth-muscle MLC kinase. A full description of the model is given in the text.

discussion, the shorthand 'PR' is used to refer to these 'peptide regions' (Fig. 2). The phosphorylation occurs at Ser¹⁹, defined as PR₀. The corresponding subsite on the enzyme, which is probably contiguous to the ATP-binding site, may contain residues directly involved in catalysis. Towards the N-terminal, a spacer that appears to require a critical length of exactly two residues intervenes between PR₀ and the PR₁ subsite, Arg¹⁶. Replacement of Arg with Ala in the PR₁ subsite results in a substantial increase in apparent K_m and a large decrease in V_{max} . [9]. The enzyme region that interacts with PR₁ is thus involved both in binding of substrate and in increasing the rate of catalysis. Another spacer, optimally two or three residues, intervenes between PR₁ and the PR₂ subsite, which is the basic triad Lys¹¹-Lys¹²-Arg¹³. Replacement of any of these three basic residues with Ala substantially increases the apparent

Table 1. Effect of peptide length and *N*-terminal acetylation on potency

Peptide	Sequence	IC ₅₀ (μ M)	K _i (μ M)	Ratio of K _i values acetylated/ free	K _m (μ M)	V _{max} (μ mol/min per mg)
1	Ac-KKR-OMe	2300	2000			
2	KKR-OMe	730	850	2.4		
3	Ac-KKRAAR-OMe	205	210			
4	KKRAAR-OMe	27	23	9.1		
5	Ac-KKRAARATS-NH ₂	360	360		> 1000	0.034
6	KKRAARATS-NH ₂	14	12	30.0	24	0.024
7	Ac-KKRAARATSNVFA-NH ₂	900	450		46	0.30
8	KKRAARATSNVFA-NH ₂	18	24	18.8	12	0.30

K_m but does not affect V_{max}, indicating that PR₂ is involved in substrate recognition and not in catalysis [8]. On the C-terminal side of PR₀ is a one-residue spacer followed by subsite PR₁, consisting of the Val-Phe sequence. Deletion of these two residues or replacement of the Asn²⁰-Val²¹-Phe²² sequence with Ala-Ala-Ala has no effect on apparent K_m but produces peptides with a very low V_{max} for phosphorylation [7]. The PR₁ residues provide binding energy that is utilized to drive the catalytic process and that does not appear in the apparent K_m [22]. Therefore deletion of the PR₁ region of the model substrate produces the nonapeptide inhibitor Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-NH₂, which inhibits chicken gizzard MLC kinase with a K_i value of 10 μ M [7]. The importance of the residues comprising the PR₂, PR₁ and PR₀ subsites to the potency of inhibitors of MLC kinase has not been determined.

Peptide 6, based on the 11–19 residue sequence of the MLC, inhibited pig carotid-artery MLC kinase with a similar potency (K_i = 12 μ M; Table 1) to that previously shown with chicken gizzard MLC kinase [7]. Peptide 2, consisting only of the PR₂ tripeptide sequence (Lys-Lys-Arg-OMe), was found to be a weak inhibitor of pig carotid-artery MLC kinase (K_i = 850 μ M). The hexapeptide 4 (Lys-Lys-Arg-Ala-Ala-Arg-OMe), containing the basic regions PR₁ and PR₂ separated by a two residue spacer, was a fairly potent inhibitor (K_i = 23 μ M). It is nearly equipotent to the longer peptide that includes the PR₀ subsite, the MLC 11–19 nonapeptide 6. The inhibitory potency of the hexapeptide indicates that the Ala-Thr-Ser fragment of the nonapeptide does not provide a significant binding interaction for peptide 6 as an inhibitor, even though this peptide has a residue capable of interacting with catalytic-site amino acid residues of MLC kinase. Lengthening the peptide to include the hydrophobic residues on the C-terminal side of the phosphorylatable serine residue, giving peptide 8, did not further improve inhibitor potency, as was previously reported with the chicken gizzard enzyme [7].

Effect of *N*- and *C*-terminal modifications on inhibitor potency

These small peptide inhibitors of MLC kinase are based on internal sequences of the MLC, where neutral polarized groups occupy the sites analogous to the termini of the synthetic peptides. It was expected that peptide inhibitors would better mimic the natural substrate and thus be more potent inhibitors if their termini were uncharged and therefore more like amide bonds. Peptide

Table 2. Effects of modifications and substitutions on inhibitor potency

Peptide	Sequence	IC ₅₀ (μ M)
Terminal modifications		
9	H ₂ N-[CH ₂] ₅ CO-KR-OMe	3800
1	Ac-KKR-OMe	2300
2	K(L)KR-OMe	730
10	K(D)KR-OMe	1100
11	KKR-OH	8000
12	KKR-N	260
Varying the amino acids between Arg ¹³ and Arg ¹⁶		
4	KKRAAR-OMe	27
13	K(L)KRAAR-NH ₂	22
14	K(D)KRAAR-NH ₂	180
15	KKRPAR-NH ₂	44
16	KKRPQR-NH ₂	77

11, Lys-Lys-Arg-OH (IC₅₀ = 8000 μ M), with a C-terminal carboxylate, was a much poorer inhibitor than peptide 2, indicating that the MLC 11–13 tripeptide was indeed a more potent inhibitor of MLC kinase when the C-terminal was uncharged. Peptide 12 (IC₅₀ = 260 μ M), with a C-terminal pyrrolidinamide, which mimicks Pro¹⁴ of the MLC sequence, was slightly more potent than peptide 2. However, acetylation of the *N*-terminus of the peptides consistently resulted in poorer inhibition. The acetylated peptides were 2–30 times less potent inhibitors compared with the desacetyl peptides (Table 1). The effect of acetylation on increasing the K_i value was more marked for the larger peptides. Despite their decreased potency, kinetic plots for the acetylated peptides were similar to those for the unacetylated peptides in that the lines through the data points intersected to the right of the ordinate (results not shown).

By using the tripeptide 2 based on MLC residues 11–13, the effect of *N*-terminal modifications was further explored. A peptide without an *N*-terminal amine, peptide 9, was a 5-fold poorer inhibitor than peptide 2 (Table 2), indicating that the acetyl group was not simply sterically interfering with binding. The *N*-terminal ammonium group therefore appeared to provide a favourable electrostatic binding interaction. The binding interaction was also sensitive to stereochemistry, as shown by the poorer potency of the 11–13 tripeptide 10 (IC₅₀ = 1100 μ M), which incorporates a D-lysine residue

into the *N*-terminal position of the MLC 11–13 basic triad. The deleterious effect of unnatural D-lysine at the *N*-terminal was confirmed by comparing the potency of the hexapeptides Lys(D)-Lys-Arg-Ala-Ala-Arg-NH₂, peptide 14 (IC₅₀ = 180 μM), and Lys(L)-Lys-Arg-Ala-Ala-Arg-NH₂, peptide 13 (IC₅₀ = 22 μM).

Effect of changes at positions 14 and 15 on inhibitor potency

In studies of peptide substrates of MLC kinase, replacement of Pro¹⁴ and Glu¹⁵ of the chicken gizzard MLC with alanine residues produced a peptide substrate with a 3-fold lower *K_m* value [9]. In order to probe the effect of these alanine replacements on peptide inhibitors, hexapeptide inhibitors 15 and 16 were prepared in which the spacer between the basic subsites contains the dipeptide sequences Pro-Ala and Pro-Gln respectively. These peptides were nearly equipotent with the Ala¹⁴-Ala¹⁵-containing hexapeptide (Table 2). Because of the bend that proline enforces in a peptide chain, this suggests that even the peptides containing the Ala¹⁴-Ala¹⁵ sequence may bind to the enzyme with a bend near residue 14.

Effect of *N*-terminal acetylation on substrate peptides

In view of the large effect of acetylation on the inhibitor potency of these peptides, it was of interest to determine the effect of this group on the ability of a peptide to act as a substrate. Peptide 8 was an effective substrate for pig carotid-artery MLC kinase with kinetic constants of 0.30 μmol/min per mg for the *V_{max}*, and 12 μM for the apparent *K_m* (Table 1). Peptide 8 exhibited nearly identical values of apparent *K_m* and of *K_i* for inhibition of MLC phosphorylation. The behaviour of the acetylated peptide 7 contrasts strikingly with that of peptide 8 in that peptide 7 was a much poorer inhibitor of MLC kinase-catalysed phosphorylation of MLC compared with peptide 8 (*K_i* = 450 μM and 24 μM respectively) while being nearly equally effective as a substrate of MLC kinase. Peptide 7 exhibited an apparent *K_m* only 3-fold higher than that of peptide 8 and had an identical *V_{max}* value. Studies with cyclic AMP-dependent protein kinase have also reported peptide substrates that exhibit *K_i* values greater than apparent *K_m* values [23]. In that case, studies were performed that showed that the *K_d* for substrate binding, like its *K_i* value, was also higher than the apparent *K_m*. To explain these findings, a mechanism was proposed whereby the substrate binds with high affinity to or enhances the formation of a specific enzyme-ATP conformation and catalysis ensues. The apparent *K_m* is lowered by the enhanced affinity of the substrate for a particular enzyme conformation along the reaction pathway. The relevance of this mechanism to MLC kinase is unclear, considering that for some substrates the apparent *K_m* is equal to the *K_i* whereas in several other cases the *K_i* is greater than the apparent *K_m*.

Peptides 5 and 6 were significantly poorer substrates of pig carotid-artery MLC kinase compared with peptides 7 and 8. Peptide 6 was phosphorylated by pig carotid-artery MLC kinase with a similar *K_m* but a lower *V_{max}*, compared with the *C*-terminal extended peptide 8. Like peptide 8, it exhibited nearly identical values of apparent *K_m* and of *K_i* for inhibition of MLC phosphorylation. Peptide 5 was a poorer substrate for pig carotid-artery MLC kinase with a *V_{max}* value similar to that for the unacetylated peptide 6 but with an apparent *K_m* value

Table 3. Effect of phosphorylation-site substitutions on inhibitor potency

Peptide	Sequence	IC ₅₀ (μM)
Neutral		
6	KKRAARATS-NH ₂	14
17	KKRAARAAS-NH ₂	16
18	KKRAARAAG-NH ₂	52
Cationic		
19	KKRAARAAH-NH ₂	40
20	KKRAARAA-NH- -CONH ₂	30
21	KKRAARAA-NH- -CONH ₂	17
22	KKRAARAA-NH- -CONH ₂	17
23	KKRAARAAG-NH ₂	22
Anionic		
24	KKRAARAAG-OH	195
25	KKRAARAAD-NH ₂	130

much higher than that for peptide 6. The apparent *K_m* value for peptide 5 was significantly higher than its *K_i* for inhibition of MLC phosphorylation. The *K_m* value for peptide 5 was difficult to determine precisely because very high concentrations of [γ-³²P]ATP were required to incorporate measurable radioactive phosphate into the peptide. This led to high background levels of radioactivity on the assay paper and difficulty in determining radioactivity incorporated into substrate.

Effect of phosphorylation-site substitutions on inhibitor potency

The chemical mechanism of cyclic AMP-dependent protein kinase has been postulated to involve general base catalysis [24]. A group with a p*K* of 6.2, which must be deprotonated for catalysis to occur, is hypothesized to facilitate attack of the serine oxygen atom on the γ-phosphate of ATP by accepting the hydroxy-group proton. The chemical mechanism of MLC kinase is not known, but general base catalysis is suggested by the sharp decline in kinase activity that occurs as the pH is lowered from 6.5 to 5.5 [25]. The fact that the hexapeptide 13 (Lys-Lys-Arg-Ala-Ala-Arg-NH₂) and the nonapeptide 6 (Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-NH₂) inhibit MLC kinase with nearly identical IC₅₀ values implies that the phosphorylatable serine residue provides little or no binding energy for an inhibitor. With the aim of improving inhibitor potency, we prepared nonapeptides containing residues at the phosphorylation site that would be capable of hydrogen-bond or ion-pair interactions with the putative catalytic base.

The equipotent inhibition shown by peptides 6 and 17 (Table 3) indicated that the threonine residue does not contribute to inhibitor binding, and so the remaining

analogues were prepared with alanine in place of threonine. The glycine amide-containing peptide **18** was a slightly poorer inhibitor ($IC_{50} = 52 \mu M$). Five peptides were prepared with side chains containing a group that would be cationic at the pH of the assay, and these were all fairly potent inhibitors. The peptide **20**, containing aminoalanine, a residue isosteric with serine but with an ammonium ion in place of the hydroxy group, was nearly equipotent ($IC_{50} = 30 \mu M$) with the serine-containing nonapeptide. Indeed, inhibitors with other cationic side chains, including aminoethyl (peptide **21**, $IC_{50} = 17 \mu M$), aminopropyl (peptide **22**, ornithine, $IC_{50} = 17 \mu M$) and aminobutyl (peptide **23**, lysine, $IC_{50} = 22 \mu M$), were equipotent to the serine peptide and to each other. Only peptides **24** ($IC_{50} = 195 \mu M$) and **25** ($IC_{50} = 130 \mu M$), in which the phosphorylation site contained a carboxy-group substitution, a group that is anionic at the pH of the assay and that will experience a repulsive interaction with a general base, were significantly poorer inhibitors.

The lack of improved binding by a peptide containing a phosphorylation-site cation may be explained in several ways. First, MLC kinase may not use general base catalysis in its chemical mechanism. Secondly, these analogues may not have the correct spacing or geometry to take advantage of an interaction with a general base at the active site of MLC kinase. A third possibility is that the binding energy of the Asn-Val-Phe sequence, which is necessary to drive the phosphorylation reaction, is needed to allow the interaction of the phosphorylation-site residue of an inhibitor with the catalytic machinery of MLC kinase.

With MLC kinase, we have now shown that a minor change in a peptide substrate can drastically alter its ability to act as an inhibitor of MLC phosphorylation while causing only minor changes in the kinetics of its own phosphorylation. Further studies will be required to clarify the mechanism underlying this phenomenon. We have shown that a hexapeptide containing only the residues comprising the basic recognition sites of MLC kinase is an inhibitor nearly equipotent to a larger peptide that also contains a residue occupying the phosphorylation site. This and other studies have shown that the PR_2 subsite, the basic Lys¹¹-Lys¹²-Arg¹³, and the PR_1 subsite, Arg¹⁶, provide binding energy both for substrate and inhibitor peptides [8,9]. The hydrophobic region PR_1 , comprising Val²¹ and Phe²², provides binding energy that drives the catalytic reaction but that is not available to an inhibitor [7]. This study has shown that any binding energy available to the active-site serine residue PR_0 in the phosphorylation reaction is not utilized by an inhibitor peptide. In addition, replacement of the phosphorylatable serine residue of MLC peptides with a number of analogues designed to interact with the putative general base at the active site of MLC kinase failed to provide more potent inhibitors of the enzyme.

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