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SUBSTRATE BASED INHIBITORS OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE*

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Summary: Activation of myosin light chain kinase is a prerequisite for smooth muscle activation. In this study, short peptide analogs of the phosphorylation site of the myosin light chain were studied for their effects on several contractile protein systems. The peptides inhibited phosphorylation of isolated ventricular and smooth muscle myosin light chains by smooth muscle myosin light chain kinase, but they were only weak inhibitors of phosphorylation of intact myosin and actomyosin. The peptides were also unable to block force development or myosin light chain phosphorylation in glycerol permeabilized fibers of swine carotid media. Apparently, the association of the myosin light chain with myosin changes its conformation such that substrate analogs which are potent inhibitors of the phosphorylation of isolated myosin light chains by myosin light chain kinase are ineffective at blocking phosphorylation of the intact molecule.

The primary stimulus for contraction of vascular smooth muscle is an increase in the intracellular free Ca^{2+} concentration. Ca^{2+} activates the calmodulin-dependent MLC kinase which phosphorylates a specific Ser residue (Ser^{19}) on the 20 kDa MLC. MLC phosphorylation has been shown to initiate actin-activated myosin ATPase activity and superprecipitation in isolated

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Abbreviations: ATP, adenosine triphosphate; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid; MLC, 20 kDa myosin light chain; MOPS, 2-(N-morpholino)-propanesulfonic acid; PSS, physiological salt solution.

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contractile protein systems and to allow crossbridge cycling and force development in permeabilized and intact smooth muscle tissue (for review, see 1). However, several studies have demonstrated that force development in permeabilized fibers can occur in the absence of increases in MLC phosphorylation (2,3,4). In addition, Gerthoffer (5) has shown that withdrawal of Ca^{2+} can relax intact smooth muscle without concomitantly decreasing MLC phosphorylation. Therefore, it is likely that MLC phosphorylation-dephosphorylation does not act as a simple Ca^{2+} dependent switch as previously suggested (6).

One of the more interesting approaches to address the role of MLC phosphorylation in smooth muscle regulation has been the use of inhibitors of MLC kinase, some of which are peptide analogs of the MLC. Peptide analogs of the region around the phosphorylatable Ser^{19} of the MLC have been shown to be potent inhibitors of the isolated MLC kinase (7,8,9).

In this study, three peptide analogs of the MLC were studied which have been previously shown to inhibit the phosphorylation of isolated bovine ventricular MLC by swine carotid artery MLC kinase (7). The goal of this study was to determine if these peptides would inhibit: i) phosphorylation of isolated gizzard smooth muscle MLC or intact myosin by gizzard MLC kinase, ii) actin-activated myosin ATPase activity and superprecipitation of bovine aortic actomyosin, or iii) force development and maintenance or MLC phosphorylation in permeabilized vascular smooth muscle fibers.

Materials and Methods

All assays were conducted in the absence and presence of several concentrations of synthetic peptide inhibitor. The inhibitors used in these studies were: SQ 31,511 (KKRAARATSNVFA) an analog of amino acids 11 through 23 of the MLC, SQ 31,430 (KKRAARATS) an analog of amino acids 11 through 19 of the MLC, and SQ 32,055 (KKRAAR) an analog of amino acids 11 through 16 of the MLC. The actual MLC contains Pro at position 14 and Gln at position 15 in place of the Ala-Ala sequence used in these synthetic analogs. Concentrated stock solutions of the peptides were prepared in H_2O and stored refrigerated.

Calmodulin independent chicken gizzard MLC kinase activity was assayed at pH 7.5 and 25° C in the presence of (in mM): 100 KCl, 0.1 [γ-^{32}P]-ATP, 1 $MgCl_2$, 1 EGTA, and 30 Tris-HCl (10). Either 0.5 or 1 μg/ml calmodulin independent MLC kinase was assayed against 0.1 mg/ml isolated gizzard MLC or 0.48 mg/ml gizzard myosin, respectively. MLC kinase activity in the presence of inhibitor is reported as a percent of control (absence of inhibitor). Calmodulin independent MLC kinase, calmodulin, MLC, and myosin were isolated from chicken gizzard as previously described (10).

Bovine aortae were obtained shortly after slaughter and transported to the laboratory in a standard MOPS-buffered PSS. Ca^{2+}-sensitive actomyosin was prepared as described by Litton et al. (11). Actomyosin ATPase activity and superprecipitation were determined at pH 7.0 and 37° C in the presence of (in mM): 0.03 Ca^{2+}, 5 Mg^{2+}, 2 MgATP, 20 imidazole, 1 DTT, and appropriate KCl to maintain ionic strength at 0.12 M. ATPase activity was determined by the measurement of inorganic phosphate using the method of Penney (12). Ca^{2+} sensitive ATPase activity (total activity minus activity in the presence of 1 mM EGTA) is reported as μmol P_i produced/g actomyosin, min. Superprecipitation was measured as a change in absorbance following the addition of ATP to the reaction mixture in a DU-9 Beckman spectrophotometer as described by Moreland and Ford (13). Superprecipitation response is reported as the maximal Ca^{2+} sensitive absorbance (total response minus response in 1 mM EGTA).

Swine carotid arteries were obtained soon after slaughter and transported to the laboratory in ice-cold MOPS-buffered PSS. On the day before experimentation, adventitial-medial strips were dissected from the arteries and exposed to calcium-free PSS containing 1 mM EGTA for one

hour followed by overnight refrigerated storage in a "glycerol skinning solution" composed of 50% glycerol and the following (in mM): 5 EGTA, 20 imidazole (pH 7.0), 50 K-acetate, 1 DTT, and 150 sucrose. On the day of the experiment, the adventitia was removed from the tissue and circumferential strips (150 - 200 μm x 2 mm x 8 mm) were cut and mounted for isometric force recording at 22° C. The glycerol skinned strips were subsequently treated as described by Moreland and Murphy (14). All solutions contained three protease inhibitors: 10 μM leupeptin, 10 μM bestatin, and 1 μM amastatin. Preliminary studies demonstrated that these protease inhibitors did not affect force development or MLC phosphorylation levels. The composition of the relaxing and contracting solutions and the computer program for solving the multi-equilibrium association equations are described in detail by Moreland and Murphy (14).

The levels of MLC phosphorylation were determined on all strips by quantitative 2-dimensional electrophoresis as described in detail by Aksoy et al. (15).

Results

The effects of the three peptide inhibitors, SQ 31,511, SQ 31,430, and SQ 32,055, on phosphorylation of gizzard smooth muscle MLC by the Ca^{2+} and calmodulin independent gizzard MLC kinase are shown in Fig. 1A. In agreement with our previous results using isolated cardiac MLC, all three peptide analogs of the MLC inhibited phosphorylation of isolated gizzard MLC. The potency of the peptides was, however, less using gizzard MLC as the substrate as compared to cardiac MLC. The IC_{50} values fell between 62 - 100 μM with gizzard smooth muscle MLC as compared to 14 - 22 μM using cardiac MLC. In contrast to the potent inhibition of phosphorylation of isolated cardiac and gizzard smooth muscle MLC, less than 20% inhibition of gizzard smooth muscle myosin could be demonstrated (Fig. 1B) even at peptide concentrations of 1 mM.

The three smooth muscle MLC analogs were examined for inhibitory effects on actin-activated myosin ATPase activity and the superprecipitation response of bovine actomyosin. All three peptide inhibitors decreased the Ca^{2+} dependent superprecipitation response of actomyosin (Fig. 2), however, the estimated IC_{50} values were greater than 1 mM. High concentrations of SQ

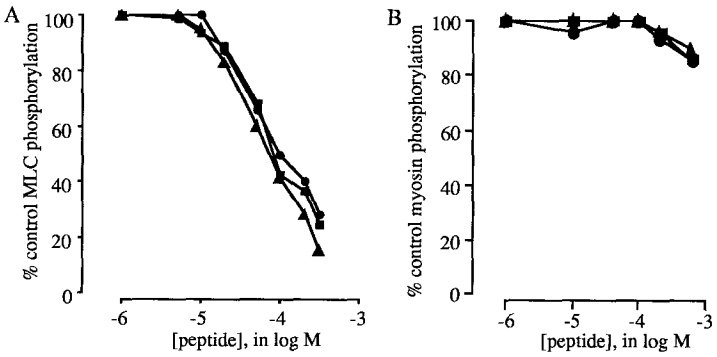

Figure 1. A. Inhibition of phosphorylation of gizzard smooth muscle MLC by Ca^{2+} and calmodulin independent gizzard MLC kinase in the presence of increasing concentrations of SQ 31,511 (▲), SQ 31,430 (●), and SQ 32,055 (■). B. Inhibition of phosphorylation of isolated chicken gizzard smooth muscle myosin by Ca^{2+} and calmodulin independent gizzard MLC kinase in the presence of increasing concentrations of SQ 31,511 (▲), SQ 31,430 (●), and SQ 32,055 (■).

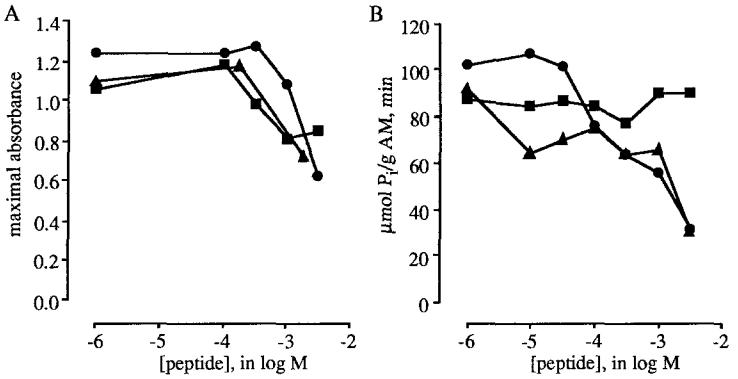

Figure 2. Superprecipitation of bovine aortic actomyosin (A) and actin-activated myosin ATPase activity (B) in the presence of increasing concentrations of SQ 31,511 (▲), SQ 31,430 (●), and SQ 32,055 (■).

31,511 and SQ 31,430 also depressed actin-activated myosin ATPase activity, but SQ 32,055 had no effect.

The smallest inhibitor, the hexapeptide SQ 32,055, was further tested in swine carotid medial fibers permeabilized with glycerol. Pretreatment of the fibers with 100 μM SQ 32,055 had no significant effect on the initiation of stress development or MLC phosphorylation levels in response to stimulation with 5 μM Ca^{2+} (Fig. 3). Additionally, no effect of 100 μM SQ 32,055 was seen if it was added during the steady state maintenance of stress and MLC phosphorylation in response to 5 μM Ca^{2+} (data not shown). A similar lack of effect was noted in fibers permeabilized with saponin or Triton X-100 (data not shown).

Discussion

Ca^{2+} and calmodulin dependent MLC kinase activity is believed to be a prerequisite for stress development in smooth muscle. Although several lines of evidence provide strong support

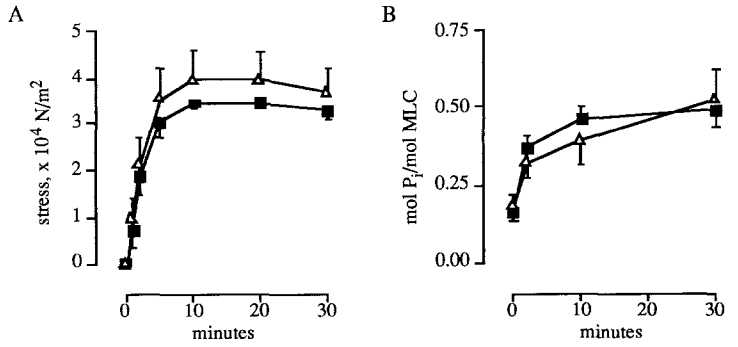

Figure 3. Increase in stress (A) and MLC phosphorylation (B) in response to 5 μM Ca^{2+} in glycerol skinned fibers of swine carotid media in the absence (■) and presence (Δ) of 100 μM SQ 32,055. Data are plotted as mean ± SEM, n = 4 strips from different arteries.

for this hypothesis (for review, see 1), there are also reports suggesting that other Ca^{2+} dependent processes are important in smooth muscle regulation (for review, see 16). Specific inhibitors of MLC kinase would be useful in the resolution of the controversy. The micro-injection of peptides patterned after either the calmodulin binding site (RS-20) or the pseudosubstrate inhibitory site (SM1) of the MLC kinase inhibit calcium-induced shortening of single smooth muscle cells (17,18). We have described three small peptides that are potent inhibitors of the phosphorylation of cardiac ventricular MLC by swine carotid MLC kinase (7). In the present study, we have further characterized the effect of these peptide inhibitors by examining their actions on isolated chicken gizzard and bovine aortic contractile proteins and on permeabilized swine carotid arteries.

The peptides were somewhat more potent inhibitors of phosphorylation of cardiac MLC than of gizzard MLC. A possible explanation for this finding lies in the amino acid sequence of the peptides. Arg^{16} of the gizzard smooth muscle MLC has been shown to be important for substrate recognition by the MLC kinase (19). In cardiac muscle MLC, however, this position contains Gln (20). The peptides may have appeared to be more potent inhibitors when assayed against the poorer cardiac substrate than they did against the smooth muscle MLC. In addition, the inhibitor peptides contained Ala substitutions at positions 14 and 15, whereas smooth muscle MLC contains Pro^{14} and Gln^{15}. These substitutions may have favored inhibition of cardiac MLC phosphorylation over smooth muscle MLC phosphorylation.

Bovine aortic actin-activated myosin ATPase activity and the actomyosin superprecipitation response were inhibited by these peptides, but only at significantly higher concentrations than were required for inhibition of cardiac MLC phosphorylation. Significantly higher concentrations of SQ 32,055 were also necessary to inhibit gizzard MLC phosphorylation as compared to cardiac MLC phosphorylation. Both of these results may be due to the difference in affinities of smooth muscle (gizzard or aortic) MLC and cardiac MLC for smooth muscle MLC kinase discussed above.

The threshold concentrations for inhibition of actomyosin activity by the peptides were in the mM range. Because actin-activated myosin ATPase activity and possibly the actomyosin superprecipitation response are believed to be tightly coupled to MLC phosphorylation (21), the decrease in activity shown can be assumed to be due to inhibition of MLC kinase activity. It is probable that the relatively weak inhibitory effect on the actomyosin preparation as compared to isolated MLC are due to differences in structure and conformation between the isolated MLC and intact myosin. Consistent with this hypothesis was the finding of weak inhibitory potency of the peptides with intact gizzard myosin as the substrate as compared with the complete and potent inhibition when isolated gizzard MLC was used as the substrate. Another explanation might be a difference in the affinity of MLC kinase for isolated MLC as compared to intact myosin. This is not the case however, as we have shown that the K_m for isolated MLC is similar to that for intact myosin (data not shown). It is also possible that the inhibitory peptides bind directly to the isolated MLC to inhibit phosphorylation, a process that may be sterically inhibited with intact myosin. Lastly, binding of MLC kinase to the isolated MLC may be different from that with intact myosin. Intact myosin, as compared to the isolated MLC, may have multiple contact sites with the MLC kinase, one of which is not involved in the catalytic reaction and therefore does not contribute to the K_m, but may prevent inhibitor binding. Although our results do not specifically address these

possibilities, they do suggest that association of the MLC with myosin significantly alters its conformation such that the peptides are no longer effective inhibitors of the MLC kinase.

Given the decreasing potency of the peptides for inhibition of intact myosin phosphorylation and actomyosin ATPase activity, the lack of effect of SQ 32,055 in the permeabilized fiber was not surprising. Although not directly assessed, the lack of effect is most likely not due to endogenous protease activity because three general protease inhibitors were included in all solutions containing the peptide. In addition, there was no difference whether the peptide was added prior to or after the initiation of contraction, conditions that would alter the time available for proteolytic activity. Because sufficient time was allowed for diffusion of the peptide into the permeabilized fibers (15 min), time-dependent diffusional barriers may also not be responsible for the lack of effect. However, this does not address charged-membrane fragments acting as diffusional barriers. It is possible that the peptide simply did not gain access to the intracellular space of the permeabilized fibers. In preliminary experiments, various permeabilization methods, 0.5% Triton X-100, 0.5 mg/ml saponin, as well as 50% glycerol, were used to examine peptide effects. No peptide inhibitor tested had any effect on these permeabilized fibers.

Inhibition of MLC kinase by the peptide analogs of MLC used in this study was somewhat less than that of peptide analogs of similar sequence from the MLC kinase autoinhibitory domain; compare SQ 31,511 **KKR**AA**R**ATSNVFA, K_i = 24 μM (7) with **RRK**WQ**K**TGHA**V**, K_{iapp} = 3 μM (22). However, inhibition by these MLC analogs was markedly weaker than that of N-terminal extended analogs of the autoinhibitory domain (e.g., LSKDRMKKYMA**RRK**WQ, K_{iapp} = 30 nM) (22,23). The increase in binding affinity supplied by the residues N-terminal to the pseudosubstrate sequence is reminiscent of the heat stable inhibitor of cAMP-dependent protein kinase, where for example an aromatic amino acid 8 residues N-terminal to the consensus recognition site RRX(A/S) is critical to tight binding (24). The similar increase in MLC kinase inhibition by residues N-terminal to the consensus recognition sequence **KKR**XX**R** suggests that more than a simple analog of the substrate is required for potent inhibition of MLC kinase.

Large peptide inhibitors of the MLC kinase have been shown to inhibit force in intact single cells (17) and force and MLC phosphorylation in permeabilized fibers (25). The inhibitors used in those studies were based on the calmodulin binding domain of the smooth muscle MLC kinase rather than on the phosphorylation site of the MLC. In both studies, increasing concentrations of calmodulin could depress or reverse the effect of the inhibitor. Calmodulin antagonists also inhibit stress and MLC phosphorylation in detergent skinned swine carotid media (26). The peptides used in the present study did not affect the fluorescence of dansyl-calmodulin at concentrations as high as 1 mM (data not shown) and, therefore, are not likely to be calmodulin antagonists.

In summary, peptides capable of inhibiting the phosphorylation of isolated cardiac MLC by swine carotid artery MLC kinase and of isolated gizzard MLC by gizzard MLC kinase were ineffective inhibitors of phosphorylation of intact gizzard myosin by gizzard MLC kinase. The peptides weakly inhibited bovine aortic actomyosin activity and were completely ineffective in inhibiting force and MLC phosphorylation in permeabilized swine carotid strips. These findings suggest an intimate relationship between MLC kinase and myosin which may prevent inhibitory

peptides from gaining access to the enzyme active site. The conformation and structure of the myosin-MLC kinase system is important to consider in the development of biochemical tools; the complex interactions between enzyme and substrate in various models of contraction must be taken into account. These results suggest that the autoinhibitory domain of the MLC kinase contains more information than a simple analog of the substrate. These results also demonstrate that caution must be used in the interpretation of studies using peptide inhibitors of MLC kinase function in structured systems as compared to single isolated proteins.

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