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*Biochemistry*, 1997, 36 (8), 2063-2067 • DOI: 10.1021/bi9624651 • Publication Date (Web): 25 February 1997

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## A Subset of Protein Kinase C Phosphorylation Sites on the Myosin II Regulatory Light Chain Inhibits Phosphorylation by Myosin Light Chain Kinase<sup>†</sup>

Kirsi Turbedsky,<sup>‡,||</sup> Thomas D. Pollard,<sup>§,||</sup> and Anne R. Bresnick<sup>\*,§</sup>

Department of Biophysics and Biophysical Chemistry and Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received October 1, 1996; Revised Manuscript Received December 20, 1996<sup>⊗</sup>

**ABSTRACT:** Protein kinase C (PKC) phosphorylates the regulatory light chains of smooth muscle and cytoplasmic myosin II at three known sites: S1, S2, and T9 [Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1987) *J. Biol. Chem.* 262, 9569–9573]. Phosphorylation at these sites inhibits the actomyosin ATPase and inhibits phosphorylation of S19 on the regulatory light chain by myosin light chain kinase (MLCK) [Nishikawa, M., Sellers, J. R., Adelstein, R. S., & Hidaka, H. (1984) *J. Biol. Chem.* 259, 8808–8814]. To compare the effects of phosphorylation at a subset of PKC sites on the rate of MLCK phosphorylation, we substituted alanines for the known PKC phosphorylation sites in the *Xenopus* regulatory light chain (XRLC). PKC phosphorylation of S1A/S2A/T9A revealed secondary phosphorylation sites at T7 and T10, which are accessible both on isolated S1A/S2A/T9A and S1A/S2A/T9A-myosin hybrids. Apparent kinetic constants were determined for MLCK phosphorylation of WT XRLC and XRLC mutants: T9A, S1A/S2A, S1A/S2A/T9A, and T7A/T9A/T10A. PKC prephosphorylation of S1/2 had no effect on the rate of MLCK phosphorylation, while PKC prephosphorylation of T7/9/10 inhibited MLCK phosphorylation due to a 6-fold increase in  $K_m$ . Our results suggest that phosphorylation of RLC S1/2 as observed *in vivo* may not be responsible for an inhibition of MLCK phosphorylation.

Differential phosphorylation of the 20 kD regulatory light chain (RLC) regulates the activity of smooth muscle and nonmuscle myosin II. The calcium-calmodulin dependent myosin light chain kinase (MLCK) phosphorylates the RLC primarily on serine 19 (S19) while protein kinase C (PKC) phosphorylates serine 1 and/or serine 2 (S1/2) and threonine 9 (T9) [Ikebe et al., 1987; Bengur et al., 1987]. Phosphorylation at S19 enhances the actin-activated ATPase activity of myosin II [Ikebe & Hartshorne, 1985] and is essential for the *in vitro* motility of actin filaments [Sellers et al., 1985; Okagaki et al., 1991]. MLCK phosphorylation also promotes filament assembly *in vitro* [Suzuki et al., 1978]. PKC phosphorylation inhibits myosin II activity directly by decreasing the actin-activated ATPase activity of myosin II phosphorylated at S19. In addition, phosphorylation by PKC decreases the affinity of the RLC for MLCK such that the rate of MLCK phosphorylation at S19 is 2-fold slower [Nishikawa et al., 1984]. Thus, PKC phosphorylation not only inhibits the rate of MLCK phosphorylation but it also decreases the ATPase activity of the myosin II once it is phosphorylated by MLCK. The opposing effects of MLCK and PKC phosphorylation on the activity of myosin II *in vitro* have been proposed to regulate the localized reorganization of myosin II during secretion [Choi et al., 1994] and the timing of contractile events such as cytokinesis [Satterwhite et al., 1992].

Phosphorylation at both the MLCK and PKC sites on myosin II occurs *in vivo*. Stimulation of smooth muscle cells with muscarinic agonists such as histamine causes muscle contraction and phosphorylation at S19 of the RLC [Kamm et al., 1989]. Activation of platelets by thrombin [Daniel et al., 1981] and antigenic stimulation of basophilic leukemia cells [Choi et al., 1994] also result in phosphorylation of S19. Unlike MLCK phosphorylation, which clearly initiates smooth muscle contraction and platelet activation, the physiological role of PKC phosphorylation is unclear. Phorbol esters which activate PKC stimulate RLC phosphorylation of S1/2 in platelets [Naka et al., 1983; Kawamoto et al., 1989], intact smooth muscle [Kamm et al., 1989; Singer et al., 1989; Singer, 1990], and basophilic leukemia cells [Choi et al., 1994]. S1/2 is phosphorylated in mitotically arrested REF-4A cells [Yamakita et al., 1994], possibly due to cyclin-p34<sup>cdc2</sup> kinase, which phosphorylates the PKC sites of RLC *in vitro* [Satterwhite et al., 1992]. Many investigators have observed phosphorylation of the PKC sites S1/2 *in vivo*, but no one has reported phosphorylation of T9. A smooth muscle phosphatase, which preferentially dephosphorylates T9 on isolated regulatory light chains, may account for these observations [Erdodi et al., 1989].

It is not known if PKC phosphorylation inhibits myosin II *in vivo*. PKC phosphorylates isolated RLC more rapidly on T9 than on S1/2 [Ikebe et al., 1987, 1990]. Therefore, *in vitro* experiments measure the effect of PKC phosphorylation primarily at T9 and do not distinguish between phosphorylation at T9 and S1/2. PKC phosphorylation of heavy meromyosin (HMM) at RLC T9 [Nishikawa et al., 1984] or at RLC T9 and S1/2 [Bengur et al., 1987] inhibits the actin-activated ATPase similarly. However, it is unclear

<sup>†</sup> This work was supported by grants from the Human Frontiers Science Program (A.R.B.) and by NIH grant GM26132.

<sup>\*</sup> Present address: Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

<sup>‡</sup> Department of Biophysics and Biophysical Chemistry.

<sup>§</sup> Department of Cell Biology and Anatomy.

<sup>||</sup> Present address: Structural Biology Lab, The Salk Institute, 10010 N. Torrey Pines Rd., La Jolla, CA 92037.

<sup>⊗</sup> Abstract published in *Advance ACS Abstracts*, February 1, 1997.

if the inhibition of MLCK phosphorylation and ATPase activity observed *in vitro* results from PKC phosphorylation at T9 alone, S1/2 alone, or both T9 and S1/2. Since phosphorylation has been observed only at S1/2 *in vivo*, the effect of phosphorylation at S1/2 alone needs to be determined *in vitro*.

To compare the effects of phosphorylation at individual PKC sites, we made alanine substitutions in the *Xenopus* RLC (XRLC) at T9 (T9A), S1/2 (S1A/S2A), and at both T9 and S1/2 (S1A/S2A/T9A). Substitution of T9 with alanine revealed additional PKC phosphorylation sites at T7 and T10. Phosphorylation of T7/9/10 inhibited MLCK phosphorylation to a similar extent as phosphorylation at both S1/2 and T9 in WT XRLC, while phosphorylation only at S1/2 did not affect MLCK phosphorylation. Therefore, PKC phosphorylation of T7/9/10 is sufficient to fully inhibit MLCK phosphorylation of isolated light chains.

## MATERIALS AND METHODS

**Cloning, Expression, and Purification of XRLC Mutants.** Site directed mutagenesis of the *Xenopus* RLC cDNA was performed to mutate T9 to alanine using the Biorad Muta-Gene Phagemid *in vitro* mutagenesis kit. The S1A/S2A, S1A/S2A/T9A, T7A/T9A/T10A, and S1A/S2A/T7A/T9A/T10A mutants were created by PCR. All mutants were sequenced throughout their entire length prior to expression in BL21(DE3). Recombinant XRLCs were purified as described previously (Bresnick et al., 1995).

**Protein Purification.** Unphosphorylated smooth muscle myosin was prepared from chicken gizzards according to Sellers et al. (1981). Chicken gizzard MLCK was purified as described by Conti and Adelstein (1991). Bovine calmodulin and rat brain PKC were purchased from Calbiochem. Recombinant gizzard RLC as characterized by Trybus and Chatman (1993) was kindly provided by Lee Sweeney.

**PKC Phosphorylation of XRLCs.** Recombinant XRLCs (1.6 mg/mL) were phosphorylated by PKC in 34 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 9 mM KCl, 10 mM NaCl, 50 μM EDTA, 50 μM EGTA, 1% glycerol, 0.2 mM CaCl<sub>2</sub>, 1.5 mM DTT, 0.24 mM ATP, 50 μg/mL phosphatidylserine, 0.8 μg/mL 1,2-dioleoyl-sn-glycerol (Avanti Polar Lipids), and 3.5 μg/mL PKC at 25 °C for 2 h. The extent of phosphorylation was monitored by glycerol polyacrylamide gel electrophoresis according to Perrie and Perry (1970).

**Preparation of XRLC-Myosin Hybrids.** Myosin hybrids containing PKC-mutant XRLCs were prepared as described previously (Bresnick et al., 1995).

**Amino Acid Sequencing of Radiolabeled XRLCs.** S1A/S2A/T9A XRLC was PKC phosphorylated using [ $\gamma$ -<sup>32</sup>P]ATP and applied to a Sephadex G25 (coarse) spin column to remove free ATP. The XRLC was incubated with cyanogen bromide (5 mg/mg XRLC) in 70% formic acid for 20 h in the dark. The mixture was diluted 7-fold with water, frozen, and lyophilized. The sample was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a reversed phase C18 column (220 × 4.6 mm, Vydac 218TP54). The peptides were eluted with a series of gradients using solution A (0.1% aqueous TFA) and solution B (0.1% TFA in 90% acetonitrile) at a flow rate of 1 mL/min as follows: 0–10% B over 5 min, 10–40% B over 55 min, and 40–100% B over 20 min. Peptides were detected by monitoring the absorbance of the eluant at 215 and 280 nm. Peaks containing radioactively phosphorylated peptides were identified by

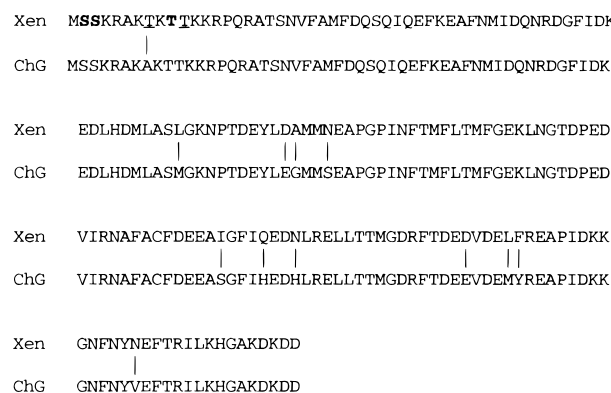


FIGURE 1: Alignment of the gizzard RLC with *Xenopus* RLC amino acid sequences with substitutions marked by vertical lines. Bold indicates known PKC phosphorylation sites. Underline indicates secondary PKC phosphorylation sites observed in XRLC mutants.

Cerenkov counting. The peaks with the highest counts were dried and sequenced as described by Leszyk et al. (1989) on an Applied Biosystems model 477A sequencer equipped with an online model 120A phenylthiohydantoin (PTH) analyzer (John Collins; University of Maryland, Baltimore, MD). In addition, S1A/S2A/T9A XRLC phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP was covalently attached to a polyvinylidene difluoride membrane, derivatized with aryl amine groups using a Millipore Sequelon AA Reagent Kit (Bedford, MA) and sequenced from the N-terminus (Protein and Nucleic Acid Chemistry Laboratories; Washington University School of Medicine, St. Louis, MO). The radioactive phosphate was extracted from the cartridge using reagents and cycles as described by Russo et al. (1992). Cycle programming required minor modifications to adapt it to the Model 477A from the originally used Model 473A. Fractions were Cerenkov counted on a Beckman Model 3801 Scintillation Counter (Fullerton, CA). This permitted the identification of specifically phosphorylated residues.

**MLCK Assays.** MLCK (3 nM) was added to a reaction mix containing XRLCs (0.5–30 μM) in 35 mM Tris-HCl, pH 7.5, 10 mM KCl, 4.2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1.7 mM DTT, 0.4 μM calmodulin, and 0.2 mM ATP at 25 °C. Aliquots were removed at 20 s intervals and spotted on 1 cm × 1 cm P81 phosphocellulose squares. The squares were washed repeatedly in 75 mM phosphoric acid (8 × 120 mL) and measured for incorporation of <sup>32</sup>P using a Beckman LS 7000 scintillation counter. The dependence of initial rate on the substrate concentration was fit to an equation for a hyperbola using the computer program Regression.

## RESULTS

**XRLC Alanine Mutants at Known PKC Phosphorylation Sites.** The *Xenopus* RLC is 94% identical to the chicken gizzard RLC with a single substitution (T for Ala7) in the first 60 residues (Figure 1) (Bresnick et al., 1995). Alanine substitution mutants of the *Xenopus* RLC were made at the known PKC phosphorylation sites S1, S2, and T9. They consisted of a single mutant (T9A) with a substitution at T9, a double mutant (S1A/S2A) with substitutions at S1 and S2, and a triple mutant (S1A/S2A/T9A) with alanine substitutions at all known PKC phosphorylation sites.

**PKC Phosphorylation of Mutant XRLCs.** PKC phosphorylated wild type and mutant XRLC to varying extents depending on the available sites (Figure 2). As anticipated, WT XRLC and T9A were each doubly phosphorylated;

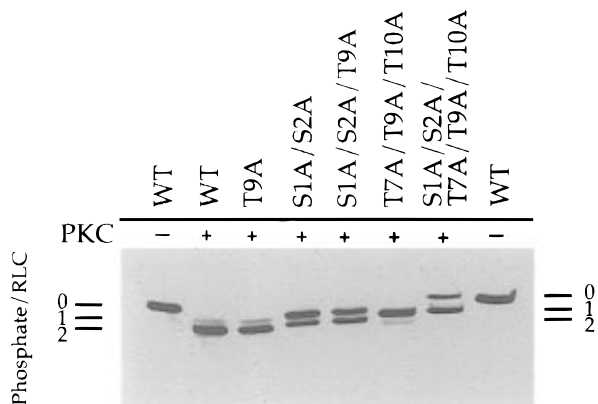


FIGURE 2: Recombinant XRLCs phosphorylated with PKC. Phosphorylation was analyzed by glycerol PAGE stained with Coomassie Blue. Marks indicate the mobility of light chains with zero, one, or two phosphates.

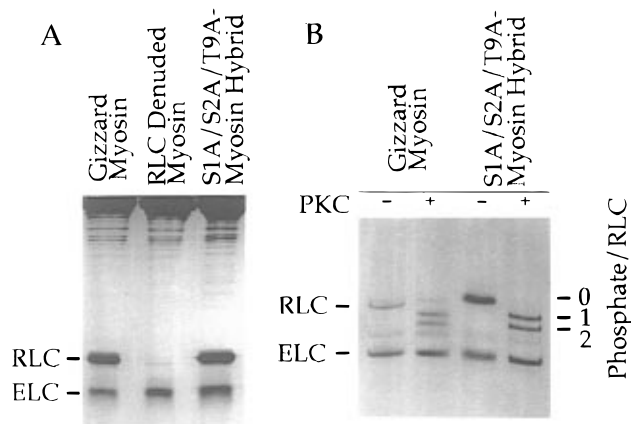


FIGURE 3: (A) Reconstitution of denuded gizzard myosin II with S1A/S2A/T9A XRLC. Myosin analyzed by SDS-PAGE and stained with Coomassie Blue. (B) Myosin and S1A/S2A/T9A-myosin hybrid phosphorylated with PKC. Phosphorylation was analyzed using glycerol PAGE stained with Coomassie Blue.

however, S1A/S2A and S1A/S2A/T9A were mixtures of singly and doubly phosphorylated species. On the basis of the known PKC phosphorylation sites, the S1A/S2A double mutant was expected to be singly phosphorylated on T9 and the S1A/S2A/T9A triple mutant was not expected to be phosphorylated.

**PKC Phosphorylation of S1A/S2A/T9A-Myosin hybrids.** To determine if the S1A/S2A/T9A XRLC was phosphorylated by PKC when bound to the myosin heavy chain, we prepared S1A/S2A/T9A-myosin hybrids (Figure 3A). PKC phosphorylated native gizzard myosin and the S1A/S2A/T9A-myosin hybrid to a similar extent, each incorporating a mixture of one and two phosphates per light chain (Figure 3B). The phosphorylation patterns of isolated S1A/S2A/T9A and the S1A/S2A/T9A-myosin hybrid are identical (Figures 2 and 3B), indicating that the additional PKC sites are accessible to MLCK when the RLC is bound to the myosin heavy chain.

**Identification of Secondary PKC Phosphorylation Sites on S1A/S2A/T9A.** To identify the secondary PKC phosphorylation sites, we cleaved <sup>32</sup>P-phosphorylated S1A/S2A/T9A XRLC with CNBr and separated the seven resulting peptides by HPLC. Sequencing of the radioactive peptide (N-terminal fragment, residues 1-24) indicated that the phosphorylation sites were located in the first 24 residues. To further identify these sites, the radioactivity at each cycle of Edman degra-

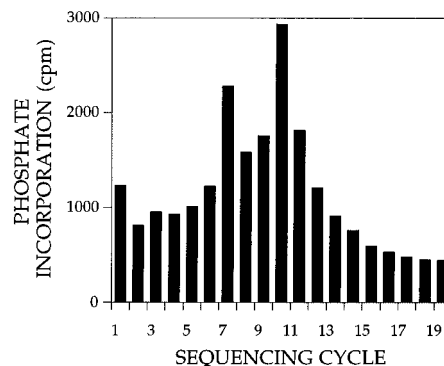


FIGURE 4: Radioactive content in cycles of Edman degradation of PKC phosphorylated S1A/S2A/T9A. Approximately 500 pmol at 200 cpm/pmol was sequenced. Cycle number corresponds to residue number in S1A/S2A/T9A XRLC. T7 and T10 are phosphorylated.

dation starting at the N-terminus was measured. The cycles corresponding to T7 and T10 stood out over a background of radioactivity produced from <sup>32</sup>P-phosphorylated S1A/S2A/T9A XRLC (Figure 4). The highest recovery of <sup>32</sup>P was at T10; therefore, the secondary site T10 appears to be phosphorylated first. PKC phosphorylated neither S19, the primary MLCK phosphorylation site, nor T18 the secondary MLCK site.

**PKC Phosphorylation of XRLC Alanine Mutants at Secondary PKC Sites.** We made alanine substitutions at T7 and T10 to remove the effect of PKC phosphorylation at these secondary sites. Mutant T7A/T9A/T10A has PKC phosphorylation sites only at S1/2, while mutant S1A/S2A/T7A/T9A/T10A was designed to lack PKC phosphorylation sites. T7A/T9A/T10A incorporated one phosphate per XRLC (Figure 2). However, the mutant with all identified PKC phosphorylation sites removed was partially singly phosphorylated (Figure 2). Since the only serines or threonines remaining in the N-terminal region of the XRLC are T18 and S19 (MLCK sites), we measured the extent of MLCK phosphorylation of this mutant. While MLCK incorporated one phosphate per XRLC in control samples, S1A/S2A/T7A/T9A/T10A was MLCK phosphorylated to a level of 0.3 mol of phosphate per mol of XRLC (Figure 5). Therefore, in the absence of PKC and PKC secondary sites, PKC phosphorylated the MLCK sites S19 and/or T18.

**Inhibition of MLCK Phosphorylation.** MLCK phosphorylated wild type and mutant XRLCs to a final level of 1 mol of phosphate per mol of XRLC independent of PKC phosphorylation (except for S1A/S2A/T7A/T9A/T10A as discussed above). However, the time course for MLCK phosphorylation differed among the mutants (Figure 5). To compare the various levels of MLCK inhibition observed, we determined apparent steady state kinetic constants of MLCK activity using each mutant as substrate (Figure 6, Table 1). The *K<sub>m</sub>* for MLCK phosphorylation of isolated recombinant gizzard RLC (14.6 μM) agreed with literature values of both recombinant and tissue purified gizzard RLC, which range between 5 and 12 μM (Ikebe et al., 1994; Zhi et al., 1994; Nishikawa et al., 1984). The *K<sub>m</sub>* for all *Xenopus* RLCs (1.6 μM), both wild type and mutant, was 9-fold less. Alanine substitutions at the PKC sites did not affect the binding of MLCK to the XRLC in the absence of PKC phosphorylation. The *V<sub>max</sub>* (3.0 μmol/min mg) was the same for wild type and each mutant independent of PKC phosphorylation. PKC phosphorylation of wild type and all mutants except T7A/T9A/T10A increased the *K<sub>m</sub>* for MLCK phosphorylation. The largest increases (6-fold) were ob-

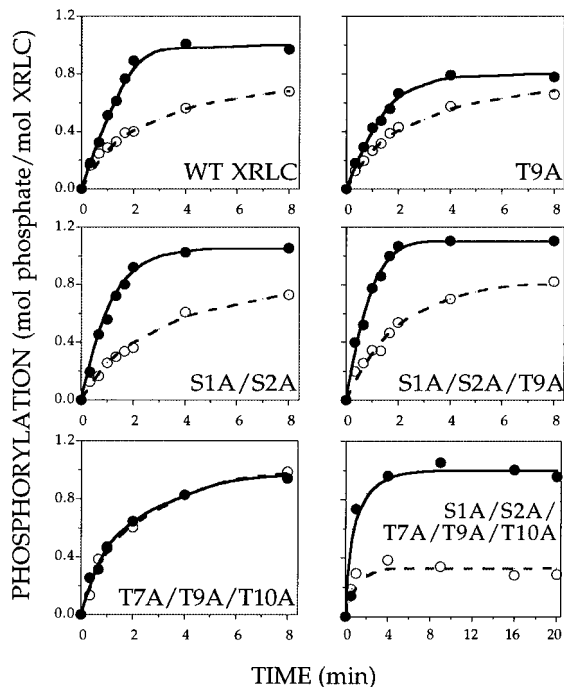


FIGURE 5: Time course of MLCK phosphorylation of XRLC mutants. MLCK phosphorylation of WT XRLC and mutants T9A, S1A/S2A, S1A/S2A/T9A, T7A/T9A/T10A, and S1A/S2A/T7A/T9A/T10A was analyzed by measuring protein bound  $^{32}\text{P}$ . (Solid circles, solid lines) WT or mutant XRLCs untreated; and (open circles, broken lines) WT or mutant XRLCs prephosphorylated by PKC. MLCK phosphorylation plateaued at 1 mol phosphate per mol XRLC for WT and mutants except for S1A/S2A/T7A/T9A/T10A which plateaued at 0.3 mol phosphate per mol XRLC.

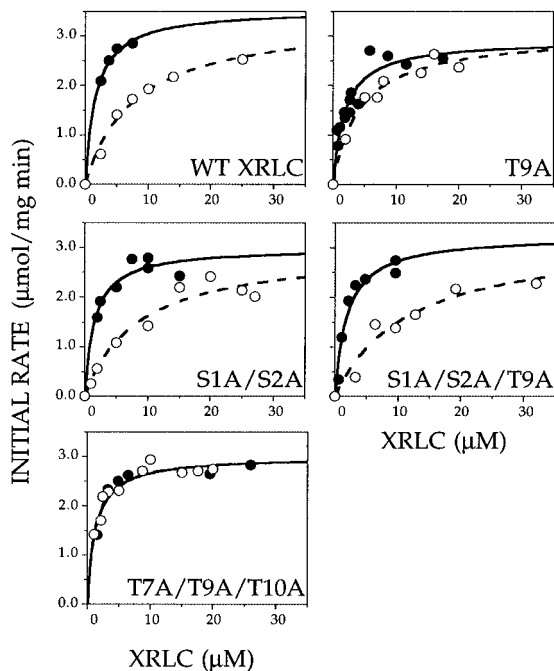


FIGURE 6: Dependence of the initial rate of MLCK phosphorylation on XRLC concentration. (Solid circles, solid lines) WT or mutant XRLCs untreated; and (open circles, broken lines) WT or mutant XRLCs prephosphorylated by PKC. Points were fit to a curve according to the equation for a hyperbola using the computer program Regression.

served for PKC prephosphorylated WT, S1A/S2A, and S1A/S2A/T9A. Each of these mutants are phosphorylated at T7/9/10 (Table 1). T9A which is phosphorylated at S1/2 and T7/10 had a smaller increase in  $K_m$  (2-fold) while T7A/T9A/T10A phosphorylated solely at S1/2 had no effect on  $K_m$ .

Table 1:  $K_m$  Values for MLCK Phosphorylation of Mutant XRLCs and PKC Phosphorylated Mutant XRLCs

substrate	phosphorylation sites <sup>a</sup>					$K_m$ ( $\mu\text{M}$ )	
	phosphate per RLC	S1/2	T7	T9	T10	XRLC	XRLC + PKC
WT XRLC	2	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>	1.6	7.8
T9A	2	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>	1.8	4.2
S1A/S2A	1, 2		<u>P</u>	<u>P</u>	<u>P</u>	1.5	8.5
S1A/S2A/T9A	1, 2		<u>P</u>	<u>P</u>	<u>P</u>	1.9	11.6
T7A/T9A/T10A	1	<u>P</u>				1.3	1.3

<sup>a</sup> P, available; P, preferred.

Therefore, PKC phosphorylation at T7/9/10 decreases the affinity of the RLC for MLCK while PKC phosphorylation at S1/2 has no effect on subsequent MLCK phosphorylation.

## DISCUSSION

Experiments with the triple mutant S1A/S2A/T9A lacking all three known PKC phosphorylation sites revealed two new PKC phosphorylation sites on the *Xenopus* regulatory light chain. Sequencing of radioactively phosphorylated S1A/S2A/T9A identified T7 and T10 as the new sites, which we will refer to as secondary PKC sites. On the basis of the incorporation of radioactive phosphate, T10 appears to be the preferred secondary PKC site. PKC did not phosphorylate the MLCK sites, S19 or T18 on the S1A/S2A/T9A mutant. However, in the absence of T7 and T10, PKC partially phosphorylated the XRLC mutant at the MLCK sites. Many substrates have a loose consensus site for PKC phosphorylation consisting of an arginine separated from the acceptor serine or threonine by one or two neutral residues. Multiple basic residues surround T7 and T10 at the N-terminus of the XRLC (Figure 1), so it is not surprising that PKC phosphorylates these sites in addition to T9.

In contrast to several other kinases (cAMP dependent kinase, casein kinase-1, casein kinase-2) that phosphorylate isolated RLCs but not native myosin (Singh et al., 1983), PKC can phosphorylate all three primary sites on wild type RLCs and both secondary sites on mutant RLCs when the light chains are reconstituted on myosin heavy chains. Thus, the secondary sites are accessible to PKC when RLC is bound to myosin heavy chain.

The higher affinity of MLCK for XRLC as compared to gizzard RLC facilitated our analysis of mutations in the PKC phosphorylation sites. In spite of the 94% sequence identity between *Xenopus* and gizzard RLCs, the  $K_m$  for MLCK phosphorylation of *Xenopus* is 9-fold less. The only substitution located within the first 60 residues occurs at position 7 (*Xenopus* has a Thr in place of gizzard's Ala). However, the  $K_m$  for XRLC mutant T7A/T9A/T10A in which T7 reverts back to the alanine found in gizzard is identical to WT XRLC. Therefore, other unidentified residues more distant in the sequence appear to play a role in binding recognition. Similarly, MLCK phosphorylation of chimeras consisting of smooth and skeletal RLC indicates that residues between 29 and 102 are involved in MLCK binding (Zhi et al., 1994).

Ikebe et al. (1987) has shown that PKC phosphorylates T9 before S1/2 where the choice of site S1 or S2 is random. Since phosphorylation of T7/10 is not observed in the presence of T9, phosphorylation of T9 is preferred over phosphorylation of T7/10 (Table 1). Our experiments have shown that in the absence of primary PKC sites, PKC

phosphorylates T10 before T7 (Figure 4); however, it is unclear whether PKC prefers T10 over S1/2 in the absence of T9. The lower  $K_m$  obtained for the T9A mutant suggests that some of the sample which is doubly phosphorylated (Figure 2) may not be phosphorylated at T7/10 since phosphorylation at these sites greatly increases the  $K_m$  (Table 1). On the basis of these results, it appears that the order of preferential PKC phosphorylation sites is T9 > S1/2 > T10 > T7. Although S1A/S2A/T7A/T9A/T10A was designed to remove all PKC phosphorylation sites, PKC phosphorylated approximately one-third of the XRLC sample at the MLCK sites (S19, T18). PKC phosphorylation did not occur at the MLCK sites in S1A/S2A/T9A or T7A/T9A/T10A based on sequencing of the radioactively phosphorylated protein (Figure 4) and the incorporation of 1 mol of phosphate per mol of XRLC by MLCK (Figure 5).

XRLC mutants with alanine substitutions at known PKC phosphorylation sites S1, S2, and T9, as well as secondary sites T7 and T10, allowed us to assess the effects of phosphorylation at individual sites. We determined the rate of MLCK phosphorylation of the XRLC mutants with and without PKC prephosphorylation to compare MLCK inhibition by PKC phosphorylation at S1/2 versus T9. We expected the T9A mutant to provide information about the effects of PKC phosphorylation at S1/2, the sites observed *in vivo*, but secondary threonine sites were phosphorylated whenever S1/2 was phosphorylated. Instead, the mutant T7A/T9A/T10A allowed us to analyze the effects of PKC phosphorylation at S1/2 alone.

The  $V_{max}$  for MLCK phosphorylation was identical for all light chains, independent of phosphorylation, whereas the  $K_m$  increased 6-fold upon PKC phosphorylation of T7/9/10. PKC phosphorylation of the secondary sites T7 and T10 in S1A/S2A/T9A inhibited MLCK phosphorylation just as effectively as phosphorylation of the primary sites in WT XRLC. The  $K_m$  for T9A increased 2-fold upon PKC phosphorylation. Phosphorylation at S1 and S2 instead of T7 and T10 may account for this decreased MLCK inhibition. PKC phosphorylation of S1/2 in T7A/T9A/T10A did not affect MLCK phosphorylation. Therefore, phosphorylation of sites in the vicinity of T9 (T7/9/10) is sufficient to inhibit MLCK phosphorylation of isolated XRLCs.

Phosphorylation has been observed *in vivo* only at S1/2 (not T9) in smooth muscle and nonmuscle cells. Our results suggest that phosphorylation at this site has no effect on MLCK phosphorylation. A phosphatase has been identified which preferentially dephosphorylates T9 over S1/2, T18, and S19 (Erdodi et al., 1989). Therefore, phosphorylation may occur transiently at T9 and consequently inhibit MLCK phosphorylation. On the basis of our results, T9 appears to be the physiologically relevant site; however, its rapid dephosphorylation may have prevented its identification *in vivo*. We have focused on MLCK phosphorylation of isolated XRLCs rather than reconstituted XRLC-myosin hybrids; however, the increase in  $K_m$  for PKC phosphorylation of HMM (9-fold) (Nishikawa et al., 1984) is consistent with the change seen in isolated XRLCs (6-fold). PKC phosphorylation has also been shown to decrease the actomyosin ATPase activity of myosin with MLCK phosphorylated RLC (Nishikawa et al., 1984), but the differential effect of phosphorylation at S1/2 versus T7/9/10 has not been studied. Thus, PKC phosphorylation may still play a prominent role in down-regulating the effects of MLCK phosphorylation.

Our results indicate that purified PKC readily phosphorylates a number of different sites on the isolated XRLC *in vitro*. It is unclear if PKC or other enzymes which phosphorylate the PKC sites (cyclin-p34<sup>cdc2</sup> kinase) will also phosphorylate the secondary PKC sites *in vivo*. Our biochemical studies show that to assess the role of PKC phosphorylation *in vivo* at specific PKC sites, one must consider the presence of secondary PKC sites at T7 and T10 which also inhibit MLCK phosphorylation.

## ACKNOWLEDGMENT

We thank Dr. Lee Sweeney for providing gizzard light chains and Dr. John Collins for assistance with HPLC and protein sequencing.

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