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Phosphorylation and partial sequence of pregnant sheep myometrium myosin light chain kinase

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Abstract

The function of the uterine smooth muscle in gestation and parturition is affected by a variety of hormones and biomolecules, some of which alter the intracellular levels of cAMP and Ca^{2+}. Since the activity of smooth muscle MLCK has been shown to be modulated by phosphorylation, the effect of this modification of pregnant sheep myometrium (psm) MLCK by the catalytic subunit of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) was studied. In contrast to other smooth muscle MLCK reported, PKA incorporates 2.0–2.2 moles phosphate into a mole of psm MLCK both in the presence and absence of Ca^{2+}-calmodulin. Modification of serine residues inhibited the activity of the enzyme. PKC also incorporated 2.0–2.1 moles of phosphate per mole psmMLCK under both conditions but had no effect on the MLCK activity. Sequential phosphorylation by PKC and PKA incorporated 3.8–4.1 moles phosphate suggesting that the amino acid residues modified by the two kinases are different. Phosphoamino acid analysis of the MLCK revealed that PKC phosphorylated serine and threonine residues. The double reciprocal plots of the enzyme activity and calmodulin concentrations showed that the V_{max} of the reaction is not altered by phosphorylation by PKA but the calmodulin concentration require for half-maximal activation is increased about 4-fold. Only 10 out of 17 monoclonal antibodies to various regions of the turkey gizzard MLCK cross-reacted with psmMLCK suggesting structural differences between these enzymes. Comparison of the deduced amino acid sequence of the cDNA encoding the C-terminal half of the psmMLCK molecule showed that while cgMLCK and psmMLCK are highly homologous, a number of nonconservative substitutions are present, particularly near the PKA phosphorylation site B (S^{828}). (Mol Cell Biochem **149/150:** 59–69, 1995)

Key words: myometrium, myosin light chain kinase, calmodulin, phosphorylation

Abbreviations: MLCK – myosin light chain kinase; psm – pregnant sheep myometrium; tg – turkey gizzard; cg – chicken gizzard; ru – rabbit uterine; bs – bovine stomach; PKA – catalytic subunit of cAMP-dependent protein kinase; PKC – Ca^{2+} and phospholipid dependent protein kinase; CaM Kinase II – multifunctional Ca^{2+} and calmodulin dependent protein kinase

Introduction

Phosphorylation of the 20,000 Da light chains of myosin by a calcium-calmodulin dependent enzyme, Myosin Light Chain Kinase (MLCK) is essential for the initiation and regulation of contractile activity in smooth muscles [1]. MLCK has been purified from skeletal, cardiac, and smooth muscles, and non-muscle cells [2]. A common property of these enzymes is their requirement for Ca^{2+} and calmodulin for activation. They have a narrow substrate specificity for the 20 kD light chain of myosin, the only protein observed to be phosphorylated at a rapid rate. The structural properties of MLCK from skeletal muscle and avian gizzards have been extensively characterized. The complete amino acid sequence of the rabbit skeletal muscle MLCK has been determined [3, 4] and its functional domains delineated [5]. The cDNAs encoding MLCK from rat skeletal muscle [6], chicken gizzard [7], chicken embryo fibroblast (8), rabbit uterine smooth muscle [9] and bovine stomach [10] have been isolated, sequenced and characterized.

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Purification and characterization of MLCK from pregnant sheep myometrium revealed that like other MLCK reported, it requires calmodulin for activation but its molecular weight (160,000) is higher than most MLCK [11]. Structural difference between the avian and pregnant sheep myometrium (psm) MLCK is suggested by the observation that some polyclonal antibodies prepared against psmMLCK did not cross-react with turkey gizzard (tg) and chicken gizzard (cg) MLCK. The epitopes of these antibodies are mainly localized on a 28 kD peptide at the N-terminal region as suggested by immunoblot analysis of the tryptic digests of psmMLCK. To identify other differences between psmMLCK and the avian enzymes, the phosphorylation of these enzymes by the catalytic subunit of the cyclic AMP dependent protein kinase (PKA) and the Ca^{2+} and phospholipid dependent protein kinase, Protein Kinase C (PKC) were compared.

This paper reports that phosphorylation of psmMLCK by PKA, both in the presence and absence of Ca^{2+}-calmodulin, incorporated two moles of phosphate and decreased the activity of the enzyme. On the other hand, phosphorylation of psmMLCK by PKC in the presence and absence of Ca^{2+}-calmodulin did not alter the activity of the enzyme. To understand the biochemical basis for these observations, the cDNA encoding the C-terminal region of psmMLCK which contains the catalytic domain, calmodulin binding site, pseudosubstrate region and PKA phosphorylation sites was cloned, sequenced and analyzed.

Materials and methods

Preparation of proteins

Myosin light chain kinase was prepared from fresh turkey gizzard according to the procedure described by Adelstein and Klee [12]. The 40–60% $(NH_4)_2SO_4$ fraction of the extract was subjected to gel filtration on Sephacryl S-300, ion-exchange on DEAE-Sephacel and affinity chromatography on Calmodulin-Sepharose. A modified procedure was used for the purification of MLCK from myometrium of pregnant sheep killed in the late gestation period (135–145 days) [12]. Frozen tissues was used in the latter procedure and proteolytic inhibitors were included in all buffers used in the preparation. Smooth Muscle Phosphatase-I was purified from turkey gizzard [13] while calmodulin was prepared from bovine brain [14]. The 20 kD light chains of myosin were isolated from turkey gizzards [15] and separated from contaminating calmodulin by affinity chromatography on tg MLCK-Sepharose in the presence of Ca^{2+}. PKA was prepared from rat skeletal muscle as described in [16]. The resins used for the purification of the proteins were purchased from Pharmacia Fine Chemicals. The concentration of the proteins used in this study were determined by the Bradford method [17].

Phosphorylation of MLCK

Phosphorylation of MLCK by PKA in the presence of Ca^{2+}-calmodulin was carried out in a reaction mixture (200–300 µl) containing 0.6–1.2 µM MLCK in 50 mM Tris. HCl, 10 mM Mg^{2+}, 0.1 mM [^{32}P]ATP, 0.2 mM Ca^{2+}, 3 µM calmodulin incubated at room temperature. Following addition of PKA, aliquots of the reaction mixture were taken at 2, 10, 30, 60 min for analysis on SDS-polyacrylamide gel and determination of phosphate incorporation. At 30 min, half of the reaction mixture was made to 1 mM EGTA and aliquots of the resulting reaction mixture were taken for analysis at 32, 40 and 60 min. The unphosphorylated enzyme (control) was also subjected to the same treatment except for the omission of PKA in the reaction mixture. Phosphorylation of MLCK in the absence of Ca^{2+}-calmodulin was carried out, under 3 different conditions, in the same reaction mixture described above but (a) containing 1 mM EGTA, (b) without the addition of Ca^{2+}, and (c) without the addition of Ca^{2+} and calmodulin. Samples for SDS-polyacrylamide gel electrophoresis were mixed with equal volume of 0.1 M Tris.HCl (pH 6.8), 2% SDS, 20% glycerol, bromophenol blue, boiled immediately, and subsequently, analyzed on a 12.5 % SDS-polyacrylamide gel according to the procedure of Laemmli [18]. The samples for the determination of phosphate incorporation were spotted on a strip (2 × 2 cm) of phosphocellulose paper (Whatman P81 ion exchange chromatography paper) [19]. The paper strips were immersed immediately in 75 mM H_3PO_4, washed with the same solution three times and counted in a scintillation counter. Phosphorylation of MLCK by PKC (gift of Dr. James R. Sellers, NIH) was carried out in reaction mixture containing 25 mM Tris.HCl (pH 7.5), 0.1 mM [^{32}p]ATP, 0.2 mM Ca^{2+}, 10 µg/ml phosphatidylserine and 1 µg/ml diolein, as described by Nishikawa *et al.* [20]. Aliquots of reaction mixture were taken for determination of kinase activity, phosphate incorporation, SDS polyacrylamide gel electrophoresis and phosphoamino acid analysis.

Peptide mapping

An aliquot of the phosphorylation reaction mixture was made to 6 M guanidine hydrochloride to terminate the phosphorylation reaction and denature the proteins. It was incubated for 60 min at 37°C and dialyzed against 0.5 M KCl, 0.2 M NH_4HCO_3 (pH 8.0), 1 mM EGTA, 5 mM dithiothreitol overnight with several changes [21]. Tryptic digestion of the enzyme was performed by the addition of L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at a

trypsin to substrate ratio of 1: 50 and incubated at 37°C for 16 h. The digest was lyophilyzed, dissolved in 2% NH_4OH and electrophoresced on a thin layer cellulose sheet (Eastman, 20 × 20 cm) at 4°C, 1000 V for 105 min in acetic acid: formic acid: H_2O (15:5:80) pH 1.9 [20]. The cellulose sheet was dried and autoradiographed.

Identification of the phosphoamino acid

The identification of the phosphoamino acid was carried out according to the procedure of de Lanerolle and Nishikawa [22]. Aliquots of the phosphorylated psmMLCK were lyophilized and hydrolyzed with 6 M HCl at 110°C for 4 h. The HCl hydrolysates were lyophilized, dissolved in water and analyzed by high voltage thin layer chromatography on cellulose sheet at pH 3.5 in pyridine:acetic acid: H_2O (1:10:189) for 45 min at 1000 volts. Following chromatography, the cellulose sheet was dried and autoradiographed. The phosphoamino acids were identified by comparison with standards, phosphoserine and phosphothreonine, which were visualized with ninhydrin.

MLCK activity assay

The effect of phosphorylation on the activity of the MLCK was determined by assaying aliquots taken after phosphorylation by PKA or PKC in the presence and absence of Ca^{2+}-calmodulin as described above but using nonradioactive ATP. The MLCK activity was determined in a reaction mixture (50 µl) containing 50 mM Tris.HCl (pH 7.4), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM [^{32}p] ATP, 50–100 µM 20 kD myosin light chains, 1 mM dithiothreitol and varying concentrations of calmodulin at room temperature. The reaction was initiated by the addition of MLCK. The reaction time was determined from the linear portion of the activity curve of the unphosphorylated MLCK with respect to time in the presence of 100 nM calmodulin. Aliquots of the reaction mixture were applied to strips of phosphocellulose paper, washed with H_3PO_4 and counted in a scintillation counter.

Immunoassay

Immunodot blot analysis was performed by spotting equal volumes of purified psmMLCK to pieces of nitrocellulose paper. The nitrocelulose blots were blocked with 3% gelatin for 1 h, incubated separately with 17 different monoclonal anti tgMLCK antibodies (gift of Dr. M. Ikebe, Case Western University) for 4 h and then incubated with immunoglobulin conjugated with alkaline phosphatase for 1 h. After washing the nitrocellulose blots with 20 mM Tris.HCl (pH 7.5), 500 mM NaCl, 0.05% Tween, the secondary antibodies were detected by addition of the substrates of alkaline phosphatase, 5-bromo-4-chloro-3-indoyl phosphate toluidine salt and p-nitro blue tetrazolium chloride.

Construction of sheep myometrial cDNA library

Total RNA was obtained from myometrial tissue collected from a pregnant sheep at day 127 of gestation. The myometrial tissue was snap frozen in liquid nitrogen and total RNA was extracted from the tissue according to a modification of the method of Chirgwin *et al.* [23] using a cesium trifluoroacetate (Pharmacia) density gradient.

PolyA$^+$ RNA was isolated from the sheep myometrial total RNA using Stratagene Poly A Quik Kit. The quality of this messenger RNA was assessed by Northern analysis using PCR product (see below) as probe. The mRNA was used to generate a cDNA library using the UniZap cDNA Library kit (Stratagene). The cDNA library was packaged using the Lamda Zap packaging kit (Stratagene).

Preparation of oligonucleotide probes by PCR

Degenerate oligonucleotides were synthesized based on sequences of the catalytic domain (amino acid residues 639–645) and calmodulin binding domain (amino acid residues 800–805) of cgMLCK [7] which show high degree of sequence similarity between chicken gizzard and rat skeletal MLCK. These sequences are about 500 bp apart in the cgMLCK. A Sal 1 site was added to the 5'end of the catalytic domain oligonucleotide, 5'GGGTCGACc/tTc/gAAGCCAGAa/gAA3', and a Cla 1 site was added to the 5'end of the calmodulin binding domain oligonucleotide, 5'GGATCGATGCAt/at/gGc/ac/ag/tg/tTTTTCTt/gCC3'.

Total myometrial RNA was primed with an oligo dT primer (Pharmacia) and transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Boehringer Mannheim) at 37°C for 30 min. Polymerase Chain Reaction was performed using the above synthetic oligonucleotide primers and Taq polymerase (Promega). The Perkin Elmer Cetus thermal cycler was programed to cycle 30 times: denaturing at 95°C for 60 sec, annealing at 59°C for 30 sec and elongating at 72°C for 2 min. The PCR products were cut using Cla 1 and Sal 1 (Boehringer Mannheim) and the digests were electrophoresced on a TAE low melting point agarose minigel. A DNA band around the expected size of 500 bp was cut from the gel and extracted using Geneclean reagents (BioRad). The PCR fragments were ligated into pSP72 and the recombinant DNA were transformed in DH5α cells. Ampicillin resistant colonies were amplified for plasmid isolation and purification using standard protocol [24]. Clones were verified by

hybridizing a random primed cgMLCK cDNA (gift of Dr. A. R. Means, Baylor College of Medicine) to a blot of a minigel containing the Cla 1/Sal 1 digested plasmids. Clones that hybridized to the cgMLCK cDNA probe were sequenced using the Sequenase kit (United States Biochemical) and were found to have a very high degree of similarity to that of the cgMLCK sequence.

Screening of cDNA library and sequencing of smMLCK clone

The original cDNA library was amplified once. Then 2×10^5 phage were plated, and lifted and fixed onto nylon membranes (Amersham). Primary screening of the membranes with the PCR products revealed 4 positive plaques. Two of these plaques were rescreened and isolated as Lambda DNA in pBluescript (Stratagene). The pBluescript clones were characterized by sequencing their ends, mapping their restriction sites and determining their ability to hybridize to the PCR products by Southern Blot analysis. The complete sequence of the longer clone 2B (1.4 kb) was determined using the M13 sequencing vectors mp18 and mp19, and the extension reagents provided with the Sequenase kit (United States Biochemicals).

Results

Figure 1 shows a comparison of the time course of phosphorylation of tgMLCK which was used as control, and psmMLCK by PKA in the presence and absence of Ca^{2+}-calmodulin. As previously described by Conti and Adelstein [21], one mole of phosphate is incorporated into tgMLCK in the presence of Ca^{2+}-calmodulin and 2 moles phosphate in the absence of Ca^{2+}-calmodulin (Fig. 1A). Similarly, 2.2 moles phosphate were incorporated into a mole of psmMLCK in the absence of Ca^{2+}-calmodulin but in contrast to tgMLCK, 2.0 moles of phosphate were also incorporated in the presence of Ca^{2+}-calmodulin (Fig. 1B) suggesting that the two sites of phosphorylation in psmMLCK are accessible to PKA under both conditions. Addition of EGTA to the reaction mixture containing Ca^{2+}-calmodulin after 30 min of incubation to dissociate the complex, resulted only in a slight increase of phosphorylation of psmMLCK but incorporation of another mole of phosphate into tgMLCK. The phosphorylation in the absence of Ca^{2+}-calmodulin was carried out in three different conditions: in a reaction mixture containing Ca^{2+}-calmodulin and 1 mM free EGTA; in the presence of calmodulin but no Ca^{2+}; and in the absence of both Ca2+ and calmodulin. In all cases, the time course of phosphorylation observed were similar to that shown in Fig. 1B.

The aliquots of the phosphorylation reaction mixtures in

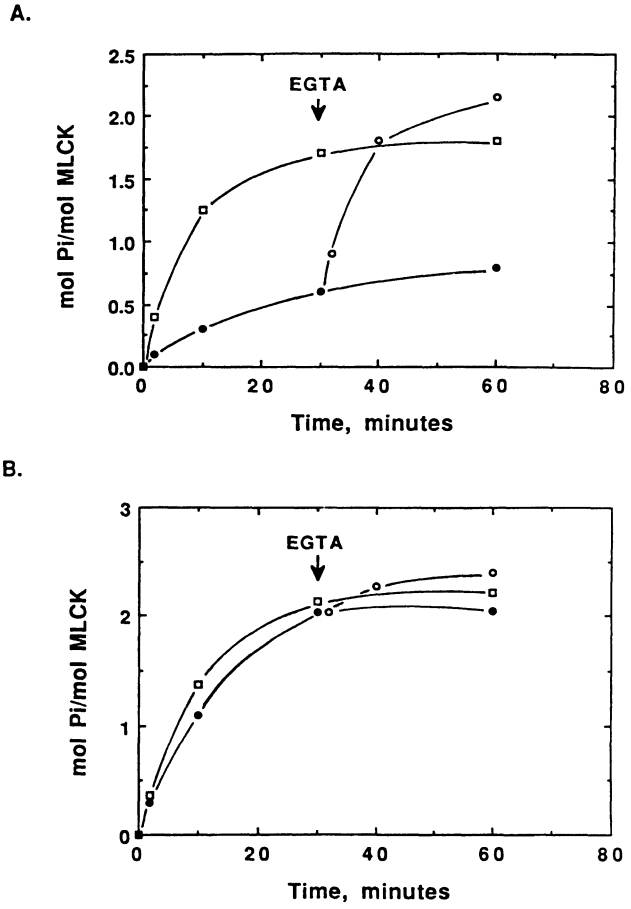

Fig. 1. Time course of phosphorylation of MLCK from turkey gizzards (A) and pregnant sheep myometrium (B) by PKA in the absence (□) and presence (●) of bound Ca^{2+}-calmodulin as described in the 'Materials and methods'. After 30 min of phosphorylation, half of the latter reaction mixture containing Ca^{2+}-calmodulin was made to 1 mM EGTA (○). Aliquots of the reaction mixtures were taken at the times indicated for determination of phosphate incorporation.

Figs 1A and B taken at 0, 30 and 60 min were analyzed on SDS-polyacrylamide gels. The autoradiographs of these gels show that all the radioactivity were confined to the MLCK band and confirmed the lack of significant difference in the extent of phosphorylation of psmMLCK in the presence and absence of Ca^{2+}-calmodulin. As expected, the phosphate incorporation into tgMLCK in the absence of Ca^{2+}-calmodulin is about twice that in the presence of Ca^{2+}-calmodulin.

To eliminate the possibility that the purified psmMLCK is already partially phosphorylated, psmMLCK was incubated with Smooth Muscle Phosphatase-I [13] from turkey gizzards for 60 min at 30°C. This enzyme has been shown previously to dephoshorylate MLCK which has been phosphorylated by PKA. The phosphatase was then inhibited by the addition of okadaic acid, a potent phosphatase inhibitor [25] which did not affect the activities of the MLCK and PKA. Phosphorylation of the phosphatase-treated-psmMLCK by PKA in the presence and absence of Ca^{2+}-calmodulin gave the same re-

sults as that described in Fig. 1B, proving that the discrepancy observed between the psmMLCK and tgMLCK is not due to a partially phosphorylated psmMLCK (data not shown).

Comparison of the tryptic peptide maps of the psmMLCK phosphorylated in the presence and absence of Ca^{2+}-calmodulin with that of the diphosphorylated tg-MLCK revealed that the phosphopeptides of psmMLCK have the same mobility as the tryptic phosphopeptides of tgMLCK phosphorylated in the absence of Ca^{2+}-calmodulin (Fig. 2) suggesting that the sites of phosphorylation of tg- and psmMLCK by PKA are the same. Analysis of the HCl hydrolysate of the phosphorylated psmMLCK by high voltage thin layer electrophoresis showed that the amino acids modified by PKA in the presence and absence of Ca^{2+}-

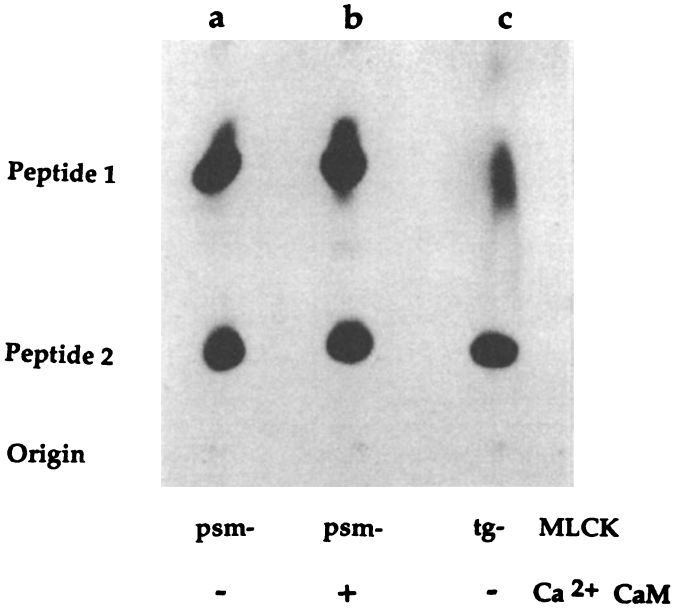

Fig. 2. Autoradiograph of ^{32}P-labeled tryptic peptides of psmMLCK after thin layer cellulose electrophoresis. Aliquots of the reaction mixtures in Fig. 1 were taken after 60 min incubation, lyophilized and digested with trypsin as described in the 'Materials and methods'. The tryptic peptides of psmMLCK (lanes a and b) and tgMLCK (lane c) phosphorylated by PKA in the absence (lanes a and c) and presence (lane b) of Ca^{2+}-calmodulin were subjected to thin layer electrophoresis.

calmodulin are serine residues (data not shown).

Nishikawa *et al.* [20] reported that phosphorylation of tgMLCK by PKC altered the activity of the enzyme while Ikebe *et al.* [26] did not observe any effect of phosphorylation. Phosphorylation of psmMLCK with PKC in the presence and absence of Ca^{2+}-calmodulin resulted in the incorporation of 2.0–2.1 moles phosphate into one mole of enzyme under both conditions (Fig. 3). Addition of PKA to psmMLCK phosphorylated with PKC resulted in further phosphate incorporation to a total of about 4 mol phosphate/mol

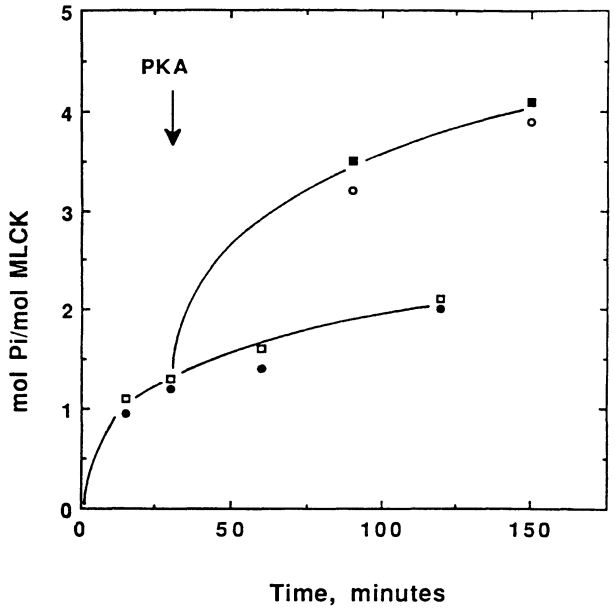

Fig. 3. Time course of phosphorylation of psmMLCK by PKC and PKA. Phosphorylation of psmMLCK by PKC was carried out in the presence (□) and absence (●) of Ca^{2+}-calmodulin. After 30 min of incubation, PKA was added to half of the reaction mixtures (■, ○). Aliquots were taken for determination of phosphate incorporation.

enzyme suggesting that the sites of phosphorylation by the PKA and PKC are different. This conclusion is supported by the results of the phosphoamino acid analysis showing that one of the amino acids modified by PKC is a threonine residue (Fig. 4).

To determine the effect of phosphorylation on the kinetic properties of psmMLCK, the activities of the phosphorylated and unphosphorylated psmMLCK were assayed at varying calmodulin concentrations. Figure 5A shows that the activity curves of the enzyme phosphorylated by PKA in the presence and absence of Ca^{2+}-calmodulin are the same, and that the phosphorylated enzyme requires higher calmodulin concentration for activation than the unphosphorylated species. The double reciprocal plots of the activity of MLCK and concentration of calmodulin showed that phosphorylation of psmMLCK by PKA does not have an effect on the V_{max} of the reaction but increases the K_{CAM} 3-fold from 3–9.2 nM (data not shown). In contrast, phosphorylation by PKC did not affect the calmodulin activation dose response curve (Fig. 5B), and the K_{CAM} and V_{max} of the reaction (Fig. 6). Figure 6 also shows that the kinetic properties of psmMLCK phosphorylated by PKA is not affected by prephosphorylation by PKC.

The disimilarity of the effect of phosphorylation of psmMLCK and tgMLCK by PKA and PKC may be due to differences in the structural properties of these enzymes. To verify this possibility, the cross-reactivity of psmMLCK with 17 monoclonal antibodies which have been demonstrated to

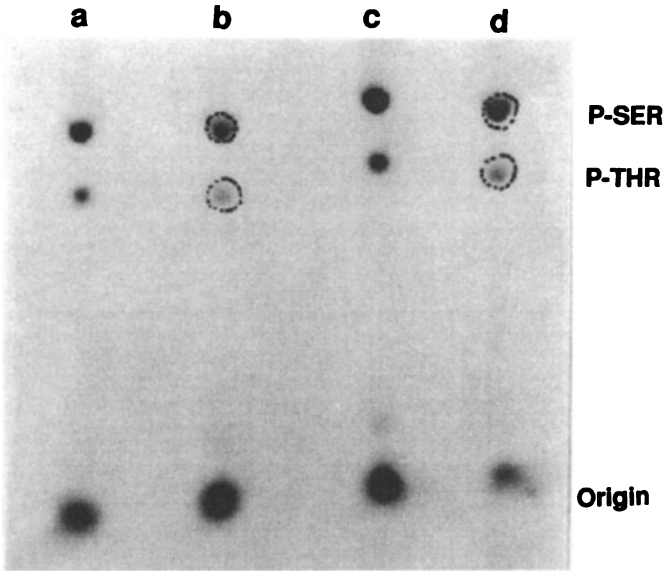

Fig. 4. Identification of the amino acids phosphorylated by PKC. psmMLCK samples which were phosphorylated by PKC (Fig. 3) in the presence (lanes a and b) and absence (lanes c and d) of Ca^{2+}-calmodulin were digested with HCl. The hyrolysates were electrophoresed on a thin layer cellulose sheet at pH 3.5 at 1000 V for 45 min. Standard amino acids, phosphoserine and phosphothreonine were added to the samples applied to lanes b and c and were visualized by ninhydrin (broken circle). The ^{32}p labelled amino acids were detected by autoradiography.

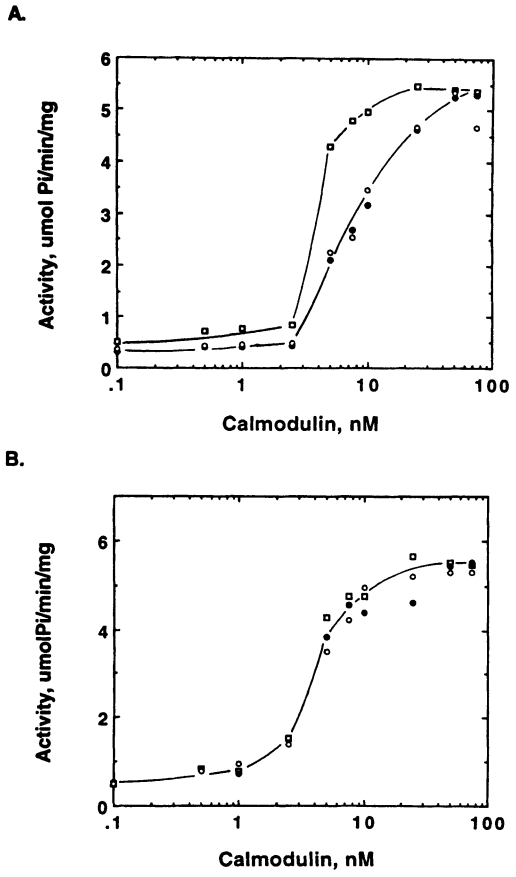

Fig. 5. Activation by calmodulin of unphosphorylated psmMLCK (□), psmMLCK phosphorylated in the presence (●) and absence (○) of Ca^{2+}-calmodulin by PKA (A) and PKC (B). Following incubation of psmMLCK with PKA for 60 min as described in Fig. 1B and with PKC for 2 h as described in Fig. 3, the MLCK activity was determined at various calmodulin concentration.

cross-react with different regions of tgMLCK molecule by Araki and Ikebe [27] was studied. Immunodot blot analysis showed that only 10 of these antibodies cross-reacted strongly with psmMLCK (data not shown). The epitopes of 5 of these antibodies, mAb LKH 2, 4, 8, 19, and 20, may be at the N-terminal region or at the calmodulin binding site while those of LKH 7, 10 and 14 are on the 64 kD tryptic peptide of tgMLCK. LKH 18 mAb which cross-reacts with the calmodulin binding region and LKH 16 which cross-reacts with all the tryptic peptides of tgMLCK tested also bound to psmMLCK. Very weak interaction, if any, was observed with the other antibodies with epitopes at the N-terminal region or calmodulin binding site (LKH 6, 17, 21 and 22) and on the 64 kD tryptic peptide (LKH 15). Interestingly, 2 mAb with epitopes on the 23 kD tryptic phosphopeptide (LKH 1 and 3) did not cross-react with psmMLCK. These results provide direct evidence for the presence of structural differences between these enzymes.

To compare further the structural properties of the avian and psmMLCK, the cDNA encoding the C-terminal region of psmMLCK was cloned and sequenced. The nucleotide and amino acid sequences of this clone are shown in Figs 7A and B, respectively, and are compared to equivalent regions of cgMLCK [7] and ruMLCK [9]. The psmMLCK cDNA encodes the region from the amino acid residue 553 of cgMLCK or 711 of ruMLCK to the C-terminus. This region encompasses part of the catalytic domain (cgMLCK 526–762), psuedosubstrate domain (cgMLCK 787–807) as well as the PKA phosphorylation site A (Ser^{815}) and site B (Ser^{828}).

The homology of the nucleotide sequence of the psmMLCK to that of ruMLCK is 91.2%, and 78.9% to that of cgMLCK. Of the 438 amino acids encoded by the psmMLCK cDNA, only 12 residues (2.7%) differ from those of ruMLCK and 33 residues (7.5%) from those of cgMLCK. Interestingly, 5 of the 12 amino acids differences between ru- and psmMLCK are unchanged in the cgMLCK. Of the 33 amino acids differences between the ru- and cgMLCK, 8 amino acids are unchanged in psmMLCK. Many of these amino acid substitutions are nonconservative. More recently, the sequence of the cDNA of the 155 kDa bovine stomach (bs) MLCK was reported [10]. Comparison of the sequence of the psmMLCK with the same region of the bsMLCK (F740 to the C-terminus) revealed only 4 amino acid differences. A glycine residue at position 1142 of psmMLCK and 1170 of bsMLCK is not present in either ru- and cgMLCK. Furthermore, there are two sets of 3 amino acids in the cgMLCK sequence (at 1134

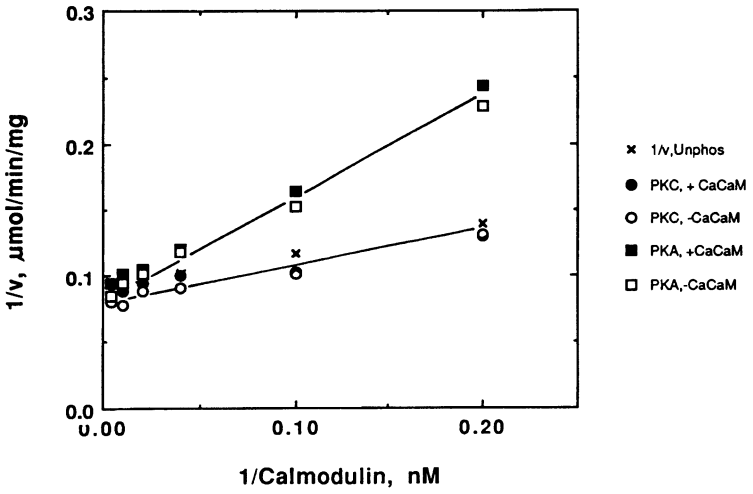

Fig. 6. Double reciprocal plots of the activities of unphosphorylated (x) and phosphorylated psmMLCK at varying calmodulin concentrations. The activities of psmMLCK phosphorylated by PKC (circles), and both PKC and PKA (squares) in the absence (open symbols) and presence (filled symbols) of Ca^{2+}-calmodulin were determined.

and 1143 of the ruMLCK sequence) that are deleted in psm-, bs- and ruMLCK. While there are notable differences between psm- and cgMLCK near phosphorylation site B (2 polar amino acids substituted with hydrophobic residues and one vice versa), there are no differences in the immediate vicinity of site A.

Discussion

Myometrial contractility is influenced by fluctuating concentrations of a variety of hormones and agents which alter the intracellular concentrations of cAMP and Ca^{2+}, particularly during gestation and parturition [28]. A mechanism that has been proposed for the modulation of contractile activity of smooth muscles is through the alteration of the activity of MLCK by phosphorylation by protein kinases. *In vitro* studies have shown that cAMP dependent protein kinase, PKA, and Ca^{2+} dependent protein kinases, PKC and a multifunctional Ca^{2+}-calmodulin dependent protein kinase, Cam Kinase II, phosphorylate MLCK. PKA modifies two serine residues, sites A and B in the absence of Ca^{2+}-calmodulin, but only site B is phosphorylated when Ca^{2+}-calmodulin is bound to the enzyme [21]. Site A has been localized to the C-terminus of the calmodulin binding site and identified as S^{815} of cgMLCK [29] while site B is S^{828} [30]. The lack of phosphorylation of site A in the presence of Ca^{2+}-calmodulin has been attributed to its inaccessibility to the protein kinase when calmodulin is bound to the enzyme. Phosphorylation of site B alone has no effect on its activity while diphosphorylation of MLCK inhibited its activity due to a 10–20-fold decrease of its affinity for Ca^{2+} and calmodulin [21]. This observation led to the hypothesis that agents which increase the intracellular concentration of cAMP modulate contractile activity by altering the MLCK activity through phosphorylation of the kinase. However, there are evidence to suggest that modification of MLCK activity *in vivo* involves CaM kinase II rather than PKA. Site A is phosphorylated *in vitro* by Cam Kinase II in the absence of Ca^{2+}-calmodulin with concomitant increase in K_{CAM} [31]. The association of site A phosphorylation with the alteration of activity of MLCK is supported by the direct correlation observed by Stull *et al.* [32] between the MLCK activity and extent of phosphorylation of site A of tracheal MLCK in tissues treated with carbachol, KCl, isoproterenol and PDBu. Furthermore, a decrease in the K_{CAM} of MLCK in KCl stimulated tracheal smooth muscle cells has been attributed to phosphorylation of MLCK by Cam Kinase II [33]. However, the higher activation constant for calmodulin for CaM kinase II (20–100 nM) than for MLCK (1-2 nM) [33] poses a problem with the suggestion that CaM kinase II regulates MLCK activity *in vivo* because calmodulin will bind to MLCK before it activates CaM Kinase II.

Contrary to the stoichiometry of phosphorylation of smooth muscle MLCK by PKA, the extent of phosphorylation of psmMLCK by PKA both in the presence and absence of Ca^{2+}-calmodulin are similar (2.0–2.2 mol phosphate/mol MLCK). The sites of phosphorylation of psmMLCK by PKA are most likely to be sites A and B because comparison of the primary sequences of the C-terminal regions of psmMLCK and other MLCK shows high homology and that S^{815} and S^{828} are conserved. Moreover, the tryptic maps of the diphosphorylated psm- and tgMLCK are the same. Thus, the phosphorylation stoichiometry of psmMLCK suggests that binding of Ca^{2+}-calmodulin to psmMLCK does not interfere with the phosphorylation of site A as it does in tgMLCK. The reason for this property is not evident from the primary struc-

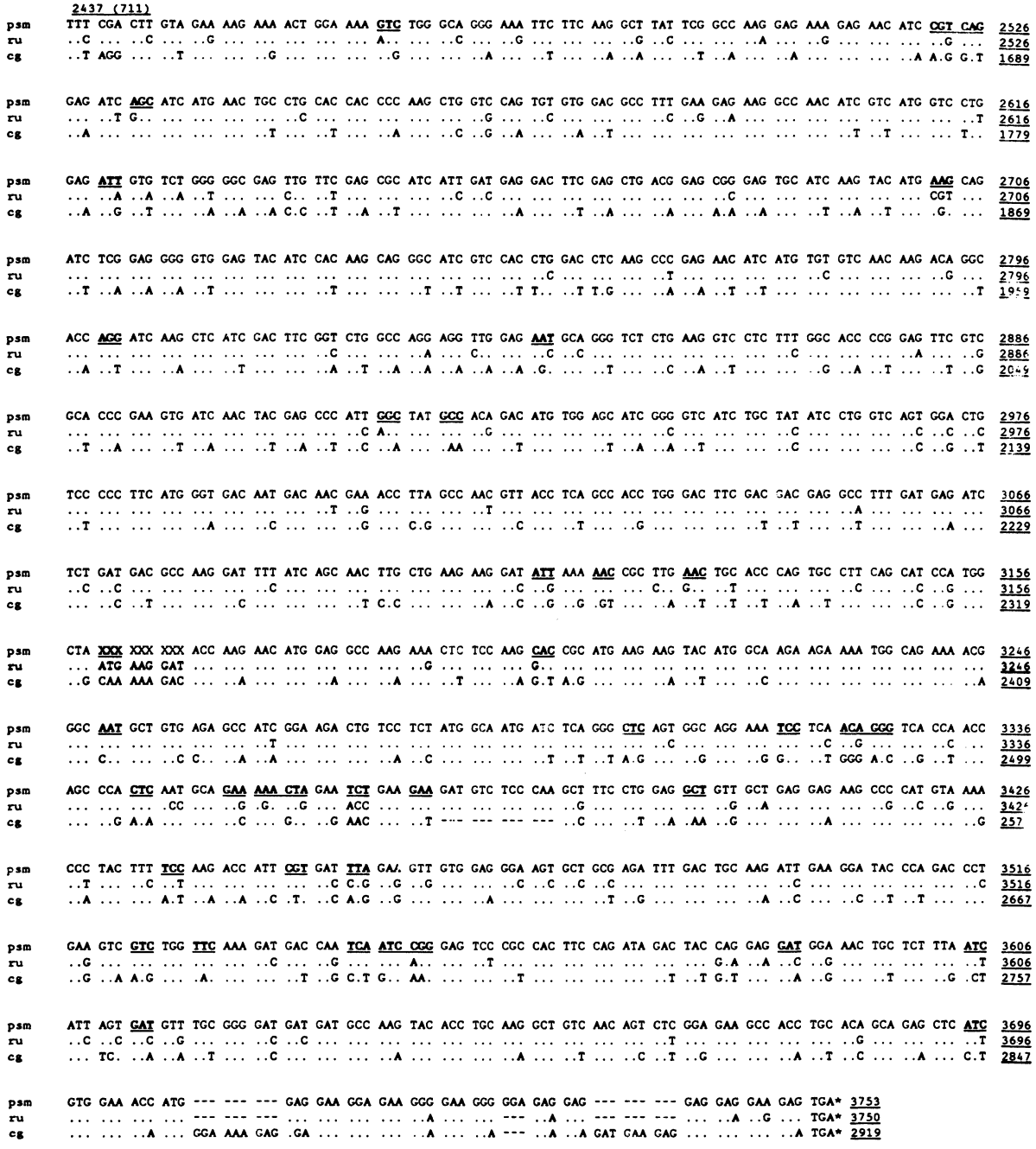

Fig. 7A. Nucleotide sequence of the cDNA encoding the C-terminal region of psmMLCK is aligned with the nucleotide sequences of rabbit uterine smooth muscle (ru) and chicken gizzard (cg) MLCK. All nucleotides are identical (denoted by ·) unless otherwise stated. Letters in bold type and underlined represent triplets in the sheep sequence which encode different amino acids from either the rabbit or chicken sequences. Numbering for rabbit uterine muscle [9] and chicken gizzard [7] sequences are taken from published sources. The numbering of psmMLCK is aligned at bp 2437 of the ruMLCK sequence for comparison purposes. Dashes represent positions where nucleotides need to be added to maintain alignment.

ture of psmMLCK since the sequence of the chicken and sheep MLCK around serine residue (S^{815}) are the same. Changes in primary structure near site B include substitution of the hydrophobic amino acids before and after S^{828} with polar amino acids in psmMLCK, a non-conservative substitution of G^{830}, and a 4 amino acid insertion between D^{845} and A^{846}. It is possible that these differences may cause conformational change to make site A accessible to PKA for phosphorylation in the presence of bound calmodulin. It is interesting that two monoclonal antibodies to tgMLCK with epitopes on the tryptic peptide containing the phosphorylatable serine residues did not cross react with psmMLCK.

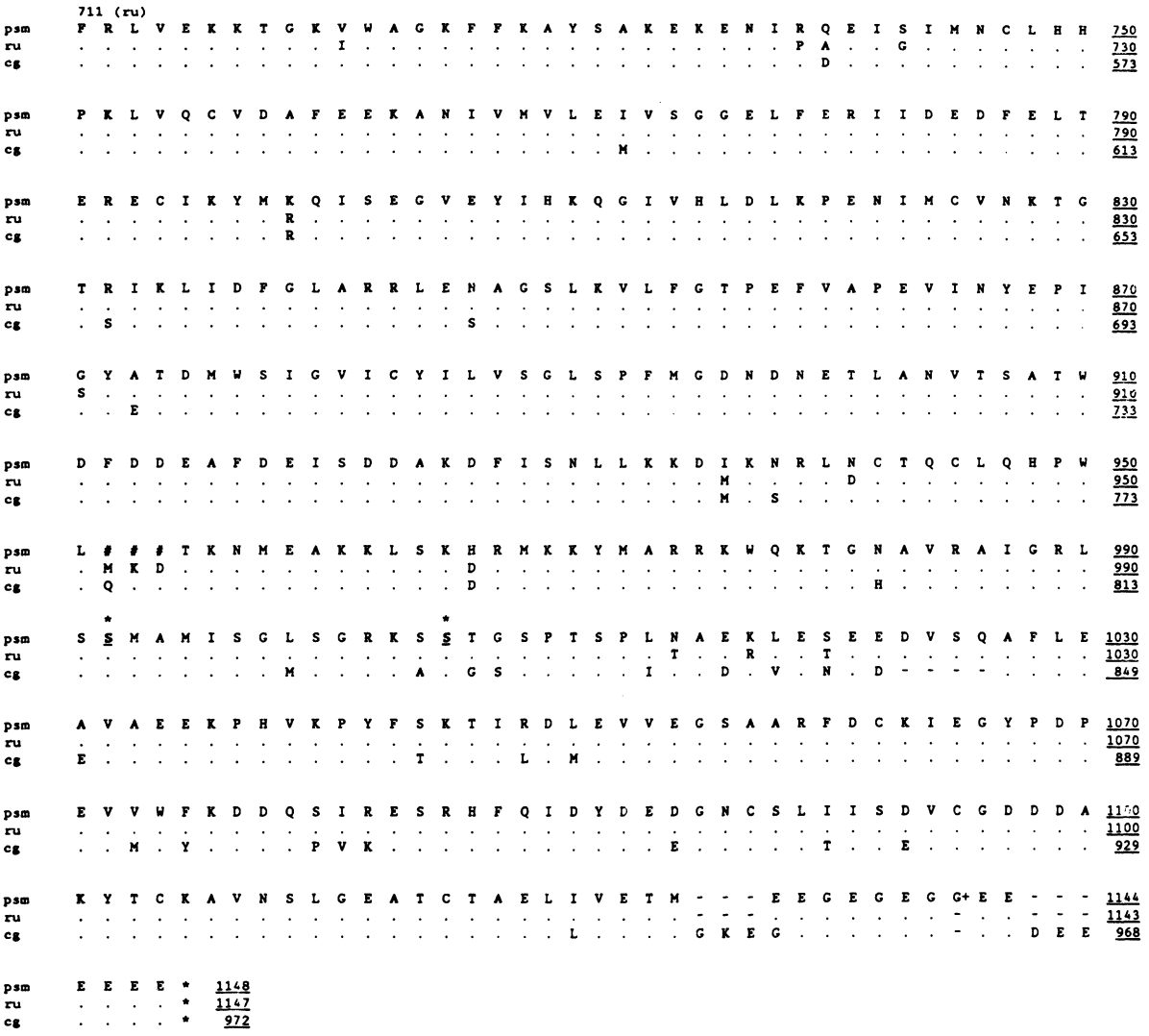

Fig. 7B. Deduced amino acid sequence of the C-terminal region of psmMLCK from the nucleotide sequence of a cDNA encoding this region. The psmMLCK amino acid sequence is aligned to published sequences of rabbit uterine smooth muscle (ru) and chicken gizzard (cg) MLCK. All amino acids are identical unless otherwise stated. Numbering for the ru- and cg MLCK is taken from published sources. The numbering of psmMLCK is aligned at amino acid 711 of the ruMLCK sequence for comparison purposes. Dashes represent positions where the amino acid residues need to be added to maintain alignment. + denotes an additional glycine in psmMLCK not present in chicken or rabbit sequences. The serine residues phosphorylatable by PKA are denoted by asterisks. #denotes undetermined amino acids.

Although the residues at the calmodulin binding that are essential for binding L^{813}, I^{810}, W^{800}, G^{804} and R^{812} [35], are conserved in the psmMLCK, there are two substitutions in this domain which might affect the binding of calmodulin to the enzyme allowing phosphorylation of site A in the presence of Ca^{2+}-calmodulin. These amino acids are D^{789} and H^{805} which are histidine and asparagine, respectively, in psmMLCK.

Nishikawa *et al.* [20] have demonstrated that phosphorylation of human platelet MLCK by PKC in the presence of Ca^{2+}-calmodulin incorporated 0.8–1.1 mole phosphate into a site different from those phosphorylated by PKA and did not affect the activity of the enzyme. On the other hand, 2–2.4 moles phosphate were incorporated (sites A and B) in the absence of Ca^{2+}-calmodulin resulting in the inhibition of enzyme activity due to 3.3-fold increase in the concentration of calmodulin required for half maximal activation. Ikebe *et al.* [26] also observed incorporation of 2 moles phosphate into a mole of tgMLCK in the absence of Ca^{2+}-calmodulin and about 10-fold decrease in the affinity for calmodulin of the diphosphorylated enzyme. But the residues modified by PKC were different from those phosphorylated by PKA. In the presence of Ca^{2+}-calmodulin, no phosphorylation of MLCK was observed. In this study the phosphorylation of psmMLCK by PKC incorporated 2.0–2.1 mole phosphate both in the presence and absence of Ca^{2+}-calmodulin with no effect on the activity of the MLCK suggesting that site A is

not phosphorylated under these conditions. Indeed, subsequent phosphorylation by PKA incorporated additional phosphate into sites A and B and inhibited the enzyme activity. These results differ from that of Nishikawa *et al.* [20] and Ikebe *et al.* [26] but are consistent with the finding of Stull *et al.* [32] that treatment of tracheal muscle with PDBu resulted in phosphorylation of MLCK but no significant phosphorylation was found at site A.

The discrepancy in the extent of phosphorylation of psmMLCK and other smooth muscle MLCK by PKA and PKC may be due to difference in their structures. This is suggested, firstly, by the difference in the molecular weights of avian and psmMLCK. We have previously shown that a 28 kD peptide is present in the N-terminal region of psmMLCK but not in tgMLCK. It is not obvious how this structure could affect the phosphorylation of residues near the C-terminus of the molecule without knowledge of the three dimensional structure of MLCK. Secondly, some monoclonal antibodies to tgMLCK did not cross-react with psmMLCK. Thirdly, there are notable differences in the primary structures of the C-terminal regions of cg- and psmMLCK.

Although increase in cAMP levels have been shown to inhibit contraction by decreasing the intracellular Ca^{2+} concentration [36, 37], the possibility that psmMLCK is regulated by PKA phosphorylation cannot be discounted. Our data showing that even in the presence of Ca^{2+}-calmodulin, psmMLCK can be phosphorylated by PKA with subsequent decrease in enzyme activity is consistent with the well known physiologic response of the myometrium to β-adrenergic agonists. In this smooth muscle, β-adrenergic agonists produce profound inhibition of both spontaneous contractions and those induced by agents that act by increasing intracellular Ca^{2+} concentration such as oxytocin, prostaglandin $F_{2\alpha}$, and carbachol [38, 39, 40]. Indeed, this ability to relax the myometrium even in the presence of stimulants is used clinically when hypertonus develops during oxytocin administration for labor induction [41].

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