Site-Specific Dephosphorylation of Smooth Muscle Myosin Light Chain Kinase by Protein Phosphatases 1 and 2A†

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ABSTRACT: Smooth muscle myosin light chain kinase is phosphorylated at two sites (A and B) by different protein kinases. Phosphorylation at site A increases the concentration of Ca^{2+}/calmodulin required for kinase activation. Diphosphorylated myosin light chain kinase was used to determine the site-specificity of several forms of protein serine/threonine phosphatase. These phosphatases readily dephosphorylated myosin light chain kinase in vitro and displayed differing specificities for the two phosphorylation sites. Type 2A protein phosphatase specifically dephosphorylated site A, and binding of Ca^{2+}/calmodulin to the kinase had no effect on dephosphorylation. The purified catalytic subunit of type 1 protein phosphatase dephosphorylated both sites in the absence of Ca^{2+}/calmodulin but only dephosphorylated site A in the presence of Ca^{2+}/calmodulin. A protein phosphatase fraction was prepared from smooth muscle actomyosin by extraction with 80 mM $MgCl_2$. On the basis of sensitivity to okadaic acid and inhibitor 2, this activity was composed of multiple protein phosphatases including type 1 activity. This phosphatase fraction dephosphorylated both sites in the absence of Ca^{2+}/calmodulin. However, dephosphorylation of both sites A and B was completely blocked in the presence of Ca^{2+}/calmodulin. These results indicate that two phosphorylation sites of myosin light chain kinase are dephosphorylated by multiple protein serine/threonine phosphatases with unique catalytic specificities.

Smooth muscle myosin light chain kinase has been shown to be phosphorylated in vitro at two sites by cyclic AMP-dependent protein kinase in the absence of Ca^{2+}/calmodulin (Conti & Adelstein, 1981). These sites have been designated A and B on the basis of peptide mapping studies and correspond to serines-815 and -828 in the gizzard smooth muscle kinase (Olson et al., 1990; Lukas et al., 1986; Payne et al., 1986). The doubly-phosphorylated enzyme has a 10-fold higher dependence on Ca^{2+}/calmodulin concentration for half-maximal activation (Conti & Adelstein, 1981). In the presence of Ca^{2+}/calmodulin, only site B is phosphorylated, and there is no effect on kinase activation properties.

Myosin light chain kinase is also phosphorylated in vitro by protein kinase C (Ikebe et al., 1985; Nishikawa et al., 1985), calmodulin-dependent protein kinase II (Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990), and cyclic GMP-dependent protein kinase (Nishikawa et al., 1984). Protein kinase C phosphorylates purified myosin light chain kinase at two sites, leading to a reduced affinity for Ca^{2+}/calmodulin (Ikebe et al., 1985; Nishikawa et al., 1985). Calmodulin-dependent protein kinase II also phosphorylates myosin light chain kinase at two sites which causes an increase in the concentration of Ca^{2+}/calmodulin required for half-maximal activation (Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990). One of the sites phosphorylated by calmodulin-dependent protein kinase II is the same site A serine residue (serine-815) that is phosphorylated by cAMP-dependent protein kinase. There is disagreement as to whether it also phosphorylates site B. Cyclic GMP-dependent protein kinase only phosphorylates site B with no significant changes in the calmodulin activation properties (Nishikawa et al., 1984). In all cases, site A phosphorylation is blocked when calmodulin is bound to myosin light chain kinase whereas other sites of phosphorylation are not affected.

The protein serine/threonine phosphatases in eukaryotic organisms have been grouped into four major families (types 1, 2A, 2B, and 2C) on the basis of biochemical properties (Cohen, 1989; Shenolikar & Nairn, 1991). Protein phosphatases 1 and 2A (PP1 and PP2A)[1] account for the bulk of the serine/threonine phosphatase activity in most tissues. The catalytic subunits of PP1 and PP2A have broad and overlapping substrate specificies in vitro although microinjection studies have shown they have more limited specificities in intact cells (Fernandez et al., 1990). Comparisons of the sequences surrounding the phosphorylation sites of substrate proteins have not revealed any clear-cut requirements for particular amino acid residues adjacent to the phosphorylated serine or threonine. It has been shown that peptides corresponding to the phosphorylation sites in normally good substrates are poorly dephosphorylated by PP1, indicating that the primary sequence surrounding the phosphorylation site is not sufficient for substrate recognition (McNall & Fischer, 1988; Agostinis et al., 1987). In contrast, PP2A readily dephosphorylates short peptides with kinetic constants comparable to the intact proteins (Agostinis et al., 1987). It is also clear that the activity and substrate specificity of both types of catalytic subunits can be altered significantly by association with regulatory subunits (Cohen, 1989; Shenolikar & Nairn, 1991).

Relatively little is known about the phosphatases that dephosphorylate myosin light chain kinase. A smooth muscle

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[1] Abbreviations: PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

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protein phosphatase (SMP-I) with a subunit structure most closely related to PP2A (Cohen, 1989) was purified from turkey gizzard smooth muscle (Pato & Adelstein, 1983). SMP-I could readily dephosphorylate both the A and B sites of myosin light chain kinase. If calmodulin was bound to the diphosphorylated kinase, only the A site was readily dephosphorylated. Biochemical analyses and inhibitor sensitivity studies have shown that both smooth (Ishihara et al., 1989; Erdodi et al., 1989) and striated (Chisholm & Cohen, 1988) muscle contain significant amounts of type 1 protein phosphatase activity associated with actomyosin. An additional form of turkey gizzard phosphatase (SMP-IV) has also been shown to associate with smooth muscle mysoin (Sellers & Pato, 1984). Since myosin light chain kinase associates tightly with both actin and myosin (Sellers & Pato, 1984), actomyosin-associated phosphatase activity could play a significant role in dephosphorylation of myosin light chain kinase in intact muscle. The two phosphorylation sites within the regulatory region of this kinase are surrounded by different local sequences that may provide test substrates to identify factors controlling protein phosphatase specificity. In this study, we show that both type 1 and type 2A protein phosphatases, as well as protein phosphatase activity associated with smooth muscle actomyosin, can dephosphorylate myosin light chain kinase in vitro. These phosphatases have differential specificities for site A and site B, and dephosphorylation is altered by the presence of bound calmodulin.

EXPERIMENTAL PROCEDURES

Purification of Proteins. Myosin light chain kinase was purified from chicken gizzard (Adelstein & Klee, 1981), calmodulin was purified from bovine testes (Blumenthal & Stull, 1982), and the catalytic subunit of cAMP-dependent protein kinase was purified from bovine cardiac muscle (Beavo et al., 1974). The heterotrimeric ABC (PT-1) and heterodimeric AC (PT-2) forms of PP2A were purified from bovine ventricular tissue (Mumby et al., 1987), and the PP2A catalytic subunit (PT-C) was purified from the same source by ethanol precipitation (Mumby et al., 1985). The catalytic subunit of PP1 from rabbit skeletal muscle, purified by ethanol precipitation (Cohen et al., 1988a), was provided by Dr. Shirish Shenolikar, Duke University School of Medicine.

Actomyosin was purified from chicken gizzard by a modification of a previously described procedure (Litten et al., 1977). Chicken gizzard tissue was minced with scissors and homogenized with a Polytron homogenizer in 2.5 volumes of buffer containing 50 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 4 mM ATP, 2 mM dithiothreitol, and 20 mM MOPS, pH 7.0. This homogenate was centrifuged at 1600*g* for 1 h. The supernatant fraction was filtered through cheesecloth and dialyzed overnight against three changes of 500 volumes of dialysis buffer containing 25 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 1 mM dithiothreitol, and 5 mM MOPS, pH 7.0. The dialyzed preparation was centrifuged at 10000*g* for 15 min to collect the precipitate which was then washed 4 times in 8 volumes of dialysis buffer containing 1% Triton X-100 and 4 times in 8 volumes of detergent-free dialysis buffer.

Protein phosphatase activity was extracted from actomyosin as follows: washed actomyosin was resuspended in 80 mM $MgCl_2$/10 mM Tris-HCl, pH 7.5, and incubated on ice for 1 h. This extract was centrifuged at 200000*g* for 30 min. The supernatant fraction was removed, dialyzed overnight at 4 °C against 2 L of 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 5 mM Tris-HCl, pH 7.5, and clarified by centrifugation at 200000*g* for 30 min.

Phosphatase Assays. Myosin light chain kinase was phosphorylated to 2 mol of P_i/mol of protein using the catalytic subunit of cAMP-dependent protein kinase as described previously (Pato & Adelstein, 1983). Dephosphorylation of myosin light chain kinase was carried out by incubation with various protein phosphatases in a reaction mixture containing 0.5 μM myosin light chain kinase, 1 mM EGTA or 0.5 mM $CaCl_2$ plus 1 μM calmodulin, 20 mM Tris-HCl, pH 7.5, and the amounts of phosphatase indicated in the figure legends. The protein phosphatase activity associated with actomyosin was activated by pretreatment with 5 mM Mn^{2+} for 5 min at 30 °C to obtain maximal activity. Preincubation with Mn^{2+} did not alter the site-specificity of this phosphatase activity (data not shown).

Dephosphorylation reactions were initiated by the addition of phosphatase, and aliquots of the reaction mixture were added to trichloroacetic acid at various times. The radioactivity released was determined as described previously (Mumby et al., 1985). The moles of P_i released from myosin light chain kinase was calculated from the specific activity of the ^{32}P and the amount of protein added to the assays. The activities of the different protein phosphatase preparations were normalized using myosin light chains as substrate as described below.

In some experiments, phosphatase activity was determined with 2 μM ^{32}P-labeled myosin light chains as described previously (Mumby et al., 1987). The activities of the different protein phosphatase preparations are expressed in units, where one unit is defined as the amount of phosphatase activity that releases 1.0 nmol of phosphate per minute from myosin light chains. The contribution by type 1 protein phosphatase was determined in some experiments using inhibitor 2, a specific inhibitor of type 1 protein phosphatases (Cohen, 1989; Shenolikar & Nairn, 1991). One unit of inhibitor 2 is defined as the amount that inhibits 0.01 milliunit of protein phosphatase 1 catalytic subunit by 50% in a standard phosphatase assay (Cohen et al. 1988b). Purified inhibitor 2 (Cohen et al., 1988b) from rabbit skeletal muscle was a gift of Dr. Shirish Shenolikar, Duke University Medical Center. For assays involving okadaic acid (Moana Bioproducts, Inc., Honolulu, HI), the dried material was dissolved in dimethyl sulfoxide, and all reactions were adjusted to contain the same final concentration (0.4%) of the solvent.

Phosphopeptide Mapping. ^{32}P-Labeled myosin light chain kinase was incubated with phosphatases as described above for 1 h and the dephosphorylation of specific sites monitored by phosphopeptide mapping (Stull et al., 1990; Yamaguchi et al., 1980). The reactions were stopped with SDS sample buffer and subjected to 7.5% SDS–PAGE. After the gels were stained and destained, they were washed extensively with distilled water, and the bands of myosin light chain kinase were excised. Gel slices were incubated in 0.5 mL of 25 mM NH_4HCO_3 (pH 8.4) and 10 μg of TPCK-treated trypsin (sequence grade from Boehringer) at 30 °C overnight. Another 10 μg of trypsin was added to each tube and incubated an additional 5 h at 30 °C. After removal of the gel slice, the solution was lyophilized, resuspended in electrophoresis buffer (acetic acid/formic acid/water 15:5:80), and subjected to high-voltage thin-layer electrophoresis at 1000 V for 70 min. The ^{32}P was detected by autoradiography using Kodak X-Omat AR film.

RESULTS

Dephosphorylation of Myosin Light Chain Kinase by Purified Phosphatases. Myosin light chain kinase was

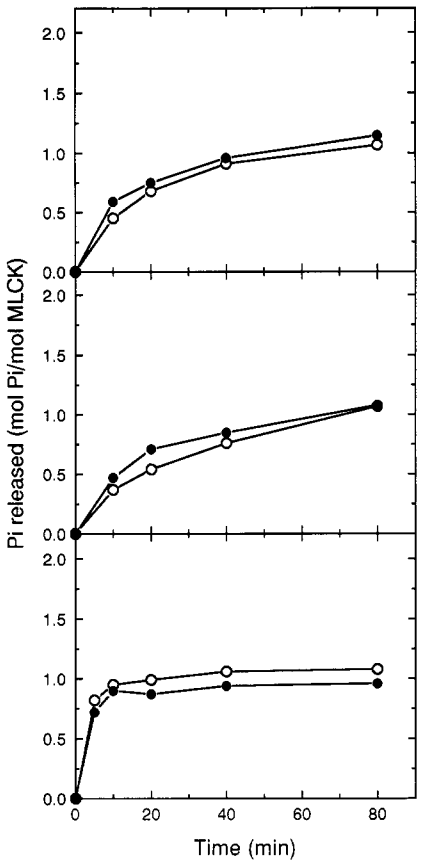

FIGURE 1: Time course of the dephosphorylation of myosin light chain kinase by various forms of type 2A protein phosphatase. Diphosphorylated myosin light chain kinase was incubated with 0.8 μg/mL ABC (0.008 unit/mL), 0.56 μg/mL AC (0.01 unit/mL), and 0.3 μg/mL free C (0.05 unit/mL) subunit forms of PP2A. Incubations were carried out for the times indicated in the absence (○) or presence (●) of Ca^{2+}/calmodulin.

phosphorylated in the absence of Ca^{2+}/calmodulin with [γ-^{32}P]-ATP using the catalytic subunit of cAMP-dependent protein kinase. Two moles of phosphate was incorporated per mole of myosin light chain kinase. Diphosphorylated myosin light chain kinase was then dephosphorylated by various protein phosphatases in the presence or absence of Ca^{2+}/calmodulin. In dephosphorylation experiments using PP2A, three different forms of the purified bovine cardiac enzyme were used. These forms included the heterotrimeric ABC form, the heterodimeric AC form, and the free catalytic subunit. Previous data have shown that the presence of the A and B subunits in the multimeric complexes results in alterations in the activity and specificity of the C subunit (Mumby et al., 1987; Kamibayashi et al., 1991). The time course of dephosphorylation of myosin light chain kinase by these three forms of PP2A is shown in Figure 1. All three forms of the enzyme dephosphorylated myosin light chain kinase in a time-dependent fashion. In each case, a maximum of 1 mol of phosphate could be released. The presence of Ca^{2+}/calmodulin had no effect on the extent of dephosphorylation of diphosphorylated myosin light chain kinase by any of the forms of PP2A. The initial rates, calculated from the linear portions of the curves, were 37.5, 48.2, and 250 nmol·min^{-1}·mg^{-1} for the ABC, AC, and free C forms, respectively, in the absence of Ca^{2+}/calmodulin. These rates corresponded to turnover rates of 5.9, 4.9, and 9.5 min^{-1}, indicating each form had similar activity. The apparent rates of dephosphorylation by the ABC and AC forms were slightly increased in the presence of Ca^{2+}/calmodulin.

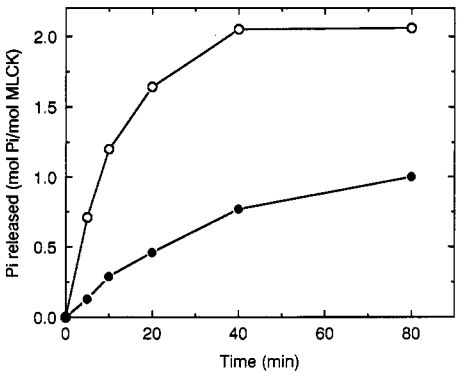

FIGURE 2: Time course of dephosphorylation of myosin light chain kinase by the catalytic subunit of type 1 protein phosphatase purified from rabbit skeletal muscle. Diphosphorylated myosin light chain kinase was incubated with 5.2 μg/mL (0.008 unit/mL) purified PP1 catalytic subunit. Dephosphorylation reactions were carried out in the absence (○) or presence (●) of Ca^{2+}/calmodulin.

The ability of protein phosphatase 1 to dephosphorylate myosin light chain kinase was also determined. Myosin light chain kinase was dephosphorylated by the purified catalytic subunit of PP1 purified from rabbit skeletal muscle. In the presence of Ca^{2+}/calmodulin, 1 mol of phosphate was released while nearly 2 mol was released in the absence of Ca^{2+}/calmodulin (Figure 2). Addition of Ca^{2+}/calmodulin had no effect on the dephosphorylation of another substrate, myosin light chain, indicating that inhibition of site A dephosphorylation was not due to interaction of calmodulin with the phosphatase (data not shown).

Dephosphorylation of Myosin Light Chain Kinase by Actomyosin-Associated Protein Phosphatase. There are substantial amounts of protein serine/threonine phosphatase activity associated with smooth muscle actomyosin preparations. Since the actomyosin-associated phosphatase may be involved in dephosphorylation of myosin light chain kinase in vivo, we characterized the phosphatase activity associated with gizzard smooth muscle actomyosin by sensitivity to okadaic acid and inhibitor 2. Phosphatase activity was extracted from chicken gizzard actomyosin with 80 mM $MgCl_2$ as described under Experimental Procedures. A significant fraction of the actomyosin-associated phosphatase (>80%) was extracted by this procedure. This actomyosin-associated fraction appeared to contain multiple protein phosphatase activities. The activity was inhibited by okadaic acid with an IC_{50} of 63 nM and was nearly completely inhibited (96%) by 3 μM okadaic acid (Figure 3). Forty percent of the extracted activity was inhibited by inhibitor 2 (Figure 4). The protein phosphatase activity extracted from actomyosin by $MgCl_2$ dephosphorylated both sites of myosin light chain kinase in the absence of Ca^{2+}/calmodulin (Figure 5). In the presence of Ca^{2+}/calmodulin, very little phosphate was released from diphosphorylated myosin light chain kinase, indicating that dephosphorylation of both sites was blocked. The presence of Ca^{2+}/calmodulin had no effect on the dephosphorylation of myosin light chain by this preparation.

Phosphopeptide Mapping. The sites that were dephosphorylated by the different protein phosphatase activities were examined by phosphopeptide mapping. To identify the two phosphorylated sites, myosin light chain kinase diphosphorylated by cAMP-dependent protein kinase was digested with trypsin, and the phosphopeptides were analyzed by thin-layer electrophoresis as described under Experimental Procedures. Diphosphorylated myosin light chain kinase yielded two distinct phosphopeptides (A and B) when subjected to this analysis (Figure 6, lane A). Incubation with the free catalytic

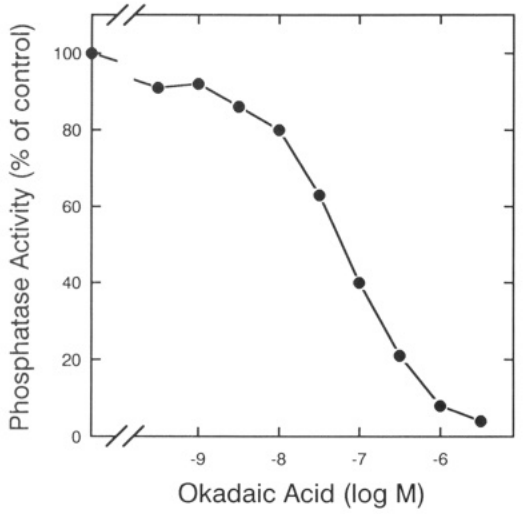

FIGURE 3: Inhibition of actomyosin-associated phosphatase activity by okadaic acid. The activity of the protein phosphatase extracted from gizzard actomyosin was assayed for myosin light chain phosphatase activity in the presence of the indicated final concentrations of okadaic acid. The final concentration of the phosphatase preparation used in the assay was 14 μg/mL (0.01 unit/mL). Phosphatase activity is expressed relative to the control activity in the absence of okadaic acid.

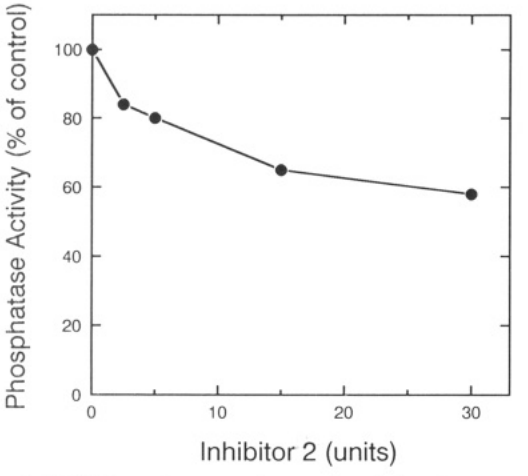

FIGURE 4: Inhibition of actomyosin-associated phosphatase activity by inhibitor 2. The myosin light chain phosphatase activity of the actomyosin-associated phosphatase was assayed in the presence of the indicated amounts of inhibitor 2 as described under Experimental Procedures.

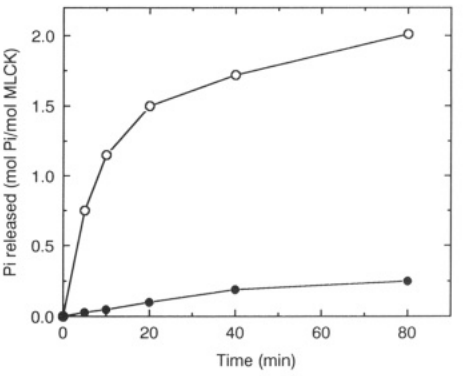

FIGURE 5: Time course of dephosphorylation of myosin light chain kinase by protein phosphatase activity extracted from chicken gizzard actomyosin. Diphosphorylated myosin light chain kinase was incubated with 14 μg/mL (0.01 unit/mL) $MgCl_2$ extract of actomyosin. Dephosphorylation reactions were carried out in the absence (○) or presence (●) of Ca^{2+}/calmodulin.

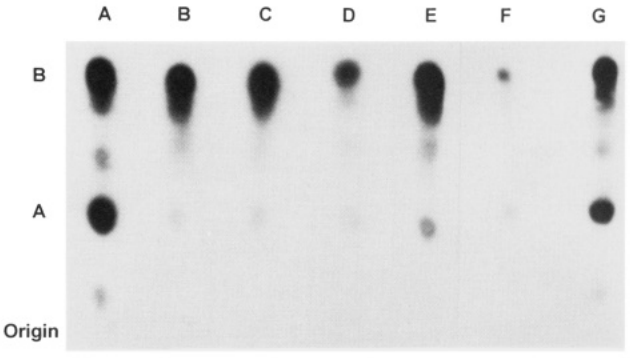

FIGURE 6: Site specificity for dephosphorylation of diphosphorylated myosin light chain kinase by different protein phosphatases. Phosphopeptide analysis of ^{32}P-labeled myosin light chain kinase incubated with various protein phosphatases was carried out as described under Experimental Procedures. The dephosphorylation reactions were carried out for 1 h as described for Figures 1, 2, 3, and 6 in the absence (B, D, F) or presence (C, E, G) of Ca^{2+}/calmodulin. The protein phosphatases used included the free C subunit of bovine cardiac PP2A (B, C), the purified catalytic subunit of rabbit skeletal muscle PP1 (D, E), and the chicken gizzard actomyosin-associated phosphatase preactivated by incubation with Mn^{2+} (F, G). Lane A contains a sample of diphosphorylated myosin light chain kinase that had not been incubated with phosphatase. Digested peptides were subjected to high-voltage thin-layer electrophoresis to resolve the two phosphopeptides (A and B) which were identified by autoradiography.

subunit of cardiac PP2A for 1 h resulted in the complete dephosphorylation of site A (Figure 6, lanes B and C) but had no effect on site B. Consistent with Figure 1, dephosphorylation of site A was not affected by the presence of Ca^{2+}/calmodulin. Similar results were observed using the multisubunit forms of PP2A (data not shown). The purified catalytic subunit of skeletal muscle PP1 dephosphorylated both sites (Figure 6, lane D). A small amount of site B phosphopeptide remained after 60 min, indicating a reduced rate of dephosphorylation of this site relative to site A. Dephosphorylation of site B, but not site A, was inhibited in the presence of Ca^{2+}/calmodulin (Figure 6, lane E). The protein phosphatase activity extracted from chicken gizzard actomyosin also dephosphorylated both sites in the absence of Ca^{2+}/calmodulin (Figure 6, lane F). Consistent with the stoichiometry of dephosphorylation (Figure 5), dephosphorylation of both sites was inhibited in the presence of Ca^{2+}/calmodulin (Figure 6, lane G).

DISCUSSION

We have examined the dephosphorylation of smooth muscle myosin light chain kinase by different protein phosphatases including phosphatase activity associated with smooth muscle actomyosin. Each of the different oligomeric forms of protein phosphatase 2A, including the free catalytic subunit, specifically dephosphorylated site A (Table I). The rates of dephosphorylation of site A were similar for each form of PP2A and were not altered by the presence of Ca^{2+}/calmodulin. These observations are distinct from those observed with smooth muscle phosphatase I (SMP-I) which has been classified as a heterotrimeric form of protein phosphatase 2A on the basis of enzymatic properties and subunit composition (Pato & Kerc, 1990). Both sites A and B were dephosphorylated by SMP-I in the absence of Ca^{2+}/calmodulin. In the presence of Ca^{2+}/calmodulin, SMP-I dephosphorylated a single site that corresponded to site A (Pato & Adelstein, 1983). This result suggested that dephosphorylation of site B by SMP-I was blocked by the presence of bound calmodulin. It is unlikely these differences

Table I: Summary of the Dephosphorylation of Smooth Muscle Myosin Light Chain Kinase by Protein Phosphatases[a]

		phosphorylation sites			
		-CaM		+CaM	
phosphatase	forms	A	B	A	B
protein phosphatase 2A	catalytic subunit	+	-	+	-
	heterodimer	+	-	+	-
	heterotrimer	+	-	+	-
protein phosphatase 1	catalytic subunit	+	+	+	-
actomyosin associated		+	+	-	-

[a] The information presented summarizes the data presented in the figures. A (+) symbol indicates dephosphorylation whereas a (-) symbol indicates little or no dephosphorylation.

are due to the catalytic subunits since the primary sequence is highly conserved between species (Cohen, 1989; Shenolikar & Nairn, 1991; Orgad et al., 1990). The A and B sunits of PP2A play important roles in controlling the activity and specificity of the catalytic subunit (Mumby et al., 1987; Chen et al., 1989; Imaoka et al., 1983; Pato & Kerc, 1986; Umi et al., 1988; Kamibayashi et al., 1991). It is possible that the B subunit present in SMP-I provides a gain of function that allows dephosphorylation of site B in the absence of Ca^{2+}/calmodulin.

The specificity of cardiac muscle PP2A for site A of myosin light chain kinase was remarkable since PP2A, and its catalytic subunit in particular, has a broad substrate specificity in vitro and is capable of dephosphorylating serine and threonine residues phosphorylated by many protein kinases (Cohen, 1989; Shenolikar & Nairn, 1991). Sites A (serine-815) and B (serine-828) are close to each other and are adjacent to the overlapping calmodulin binding (residues 796–813) and autoinhibitory (residues 787–807) domains (Olson et al., 1990). It has been postulated that in the absence of Ca^{2+}/calmodulin the autoinhibitory domain binds to the active site of gizzard myosin light chain kinase and that activation involves binding of calmodulin to the same (or at least partially overlapping) domain (Pearson et al., 1988). Our data show that the dephosphorylation of serine-815 by PP2A, even though it is very close to the C-terminal borders of the autoinhibitory and calmodulin binding domains, is not affected by interaction of the adjacent domains with the active site or with calmodulin. Our data also suggest that the inability of PP2A to dephosphorylate site B is an intrinsic property of this site since it is not dephosphorylated in either the inactive or the calmodulin-activated kinase. The inhibition of dephosphorylation does not appear to be due to simple stearic hindrance since site B was dephosphorylated by PP1 which is approximately the same size as the catalytic subunit of PP2A. Studies with synthetic peptides have shown that the local sequence surrounding the phosphorylation site plays a role in the substrate specificity of PP2A (Agostinis et al., 1987; Kamibayashi et al., 1991). The sequence surrounding site A in chicken gizzard myosin light chain kinase is -R-L-S-S(P)-M-A- (Lukas et al., 1986) which conforms to the consensus phosphorylation sites of both cyclic AMP-dependent protein kinase and calmodulin-dependent protein kinase II (Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990). The sequence of the region surrounding site B is -G-R-K-A-S(P)-G-S-S-P-T-S-P- (Payne et al., 1986). The presence of a proline residue on the C-terminal side of a phosphothreonine in peptides corresponding to the phosphorylation sites of pyruvate kinase or inhibitor 1 [e.g., -R-R-A-T(P)-P-A-] is a strong negative determinant for dephosphorylation by several forms of PP2A (Agostinis et al., 1987). The presence of prolines on the C-terminal side of serine-828 may act as a negative determinant for dephosphorylation of this site by PP2A.

The pattern of dephosphorylation of myosin light chain kinase by type 1 protein phosphatase was distinct from that of PP2A (Table I). In the absence of calmodulin, the catalytic subunit of PP1 from rabbit skeletal muscle released nearly 2 mol of phosphate from diphosphorylated myosin light chain kinase. The time course was biphasic, indicating that the two sites were dephosphorylated at different rates. Addition of Ca^{2+}/calmodulin completely blocked dephosphorylation of site B. This effect is distinct from effects seen with protein kinases where site A phosphorylation, but not site B phosphorylation, is blocked when Ca^{2+}/calmodulin binds to myosin light chain kinase (Conti & Adelstein, 1981; Olson et al., 1990; Lukas et al., 1986; Payne et al., 1986; Ikebe et al., 1985; Nishikawa et al., 1985; Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990).

Protein phosphatase activity extracted from gizzard smooth muscle actomyosin was composed primarily of type 1 and/or type 2A protein phosphatases since activity was completely inhibited by 1–3 μM okadaic acid (Haystead et al., 1989). The IC_{50} for okadaic acid was 63 nM, but only 40% of the activity was inhibited by inhibitor 2. The inhibitor 2-insensitive activity could be due to the presence of PP2A, an SMP-IV-like activity, or a distinct protein phosphatase. In the absence of Ca^{2+}/calmodulin, the actomyosin-associated protein phosphatase activity completely dephosphorylated myosin light chain kinase. Unlike the PP1 catalytic subunit, the dephosphorylation of both sites was blocked in the presence of Ca^{2+}/calmodulin, suggesting that regulatory subunits in this preparation inhibit dephosphorylation when calmodulin is bound to myosin light chain kinase. Ca^{2+}/calmodulin does not appear to interact with some component of the phosphatase preparation since it did not inhibit myosin light chain dephosphorylation. The notion that unique forms of protein phosphatases exist in avian smooth muscle is supported by the recent report on SMP-III (Tulloch & Pato, 1991). The presence of type 1 protein phosphatase activity associated with chicken gizzard smooth muscle actomyosin has been reported previously (Ishihara et al., 1989). A myosin phosphatase (SMP-IV) purified from the soluble fraction of turkey gizzard smooth muscle (Pato & Kerc, 1985) has relatively high affinity for phosphorylated myosin in vitro (Sellers & Pato, 1984). Although this enzyme preferentially dephosphorylates the β subunit of phosphorylase kinase, a hallmark of type 1 protein phosphatases, it is insensitive to inhibitor 2. It has been suggested that SMP-IV may be the smooth muscle equivalent of $PP\text{-}1_M$, a myosin-associated phosphatase in skeletal and cardiac muscles (Cohen, 1989). Association of the M_r 40 000 catalytic subunit with the M_r 58 000 subunit of SMP-IV may prevent inhibition by inhibitor 2.

Although the phosphorylation of site A by all known protein kinases is inhibited when calmodulin binds to myosin light chain kinase (Conti & Adelstein, 1981; Ikebe et al., 1985; Nishikawa et al., 1985; Hashimoto & Soderling, 1990; Ikebe & Reardon, 1990), site A dephosphorylation was not necessarily inhibited when calmodulin was bound to the kinase (Table I). These data indicate that calmodulin does not simply cover the phosphorylatable serine which is only two residues removed from the calmodulin binding domain. Furthermore, calmodulin inhibited the dephosphorylation of site B with some of the protein phosphatases (Table I). These results indicate that the effects of calmodulin extend well beyond the calmodulin binding domain since the phosphorylatable serine

in site B is 15 residues from the carboxyl end of the calmodulin binding domain (Lukas et al., 1986; Payne et al., 1986; Guerriero et al., 1986). It should also be noted that the catalytic core and inhibitory domain are on the amino-terminal side of the calmodulin binding domain where effects of calmodulin binding are manifested by activation of the kinase.

As summarized in Table I, the dephosphorylation of the two sites of myosin light chain kinase by protein serine/theronine phosphatases displayed distinct patterns of specificity. PP2A only dephosphorylated site A and was not affected by calmodulin. All of the PP1 preparations used, including actomyosin-associated activity, dephosphorylated both sites, and calmodulin had differential effects that depended on the form used. This result demonstrates that although the substrate specificities of the catalytic subunits of PP1 and PP2A overlap with many substrates (Cohen, 1989; Shenolikar & Nairn, 1991), the determinants required for substrate recognition are not identical. Another recent example of this level of substrate differentiation by these enzymes is histone H1 phosphorylated by $p34^{cdc2}$ (Sola et al., 1991). These types of in vitro biochemical data support data obtained by microinjection of the PP1 and PP2A catalytic subunits (Fernandez et al., 1990) and by yeast genetics (Kinoshita et al., 1990; Healy et al., 1991) that indicate that these enzymes have distinct physiological roles. Identification of the characteristics of sites A and B of myosin light chain kinase that define the basis of selectivity by PP1 and PP2A should provide important information on the molecular basis of substrate recognition by these related protein serine/threonine phosphatases.

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Registry No. PP, 9025-75-6; Ca, 7440-70-2; myosin light chain kinase, 51845-53-5.