Dephosphorylation of distinct sites on the 20 kDa myosin light chain by smooth muscle myosin phosphatase

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Abstract The dephosphorylation of the myosin light chain kinase and protein kinase C sites on the 20 kDa myosin light chain by myosin phosphatase was investigated. The myosin phosphatase holoenzyme and catalytic subunit, dephosphorylated Ser-19, Thr-18 and Thr-9, but not Ser-1/Ser-2. The role of non-catalytic subunits in myosin phosphatase was to activate the phosphatase activity. For Ser-19 and Thr-18, this was due to a decrease in K_m and an increase in k_{cat} and for Thr-9 to a decrease in K_m. Thus, the distinction between the various sites is a property of the catalytic subunit.

Key words: Smooth muscle myosin; Myosin light chain; Myosin phosphatase; Myosin light chain kinase; Protein kinase C

1. Introduction

It is established that the contractile activity in smooth muscle and non-muscle cells is regulated by a mechanism that involves phosphorylation and dephosphorylation of the 20 kDa light chain of myosin (MLC20) [1] [1]. Under most experimental conditions, MLC20 can be phosphorylated to 1 mol P/mol MLC20 by the myosin light chain kinase (MLCK) and the site of phosphorylation has been identified as Ser-19 [2]. At high concentrations of MLCK, a second site is phosphorylated and this site is Thr-18 [3]. MLC20 can also be phosphorylated by Ca^{2+}-activated, phospholipid-dependent protein kinase (PKC) on distinct serine and threonine residues [4–6]. Three potential sites of in vitro phosphorylation have been identified as Ser-1, Ser-2 and Thr-9 [6]. Other kinases such as calmodulin-dependent protein kinase II [7], Rho-associated kinase [8] and the p21-activated protein kinase [9] also phosphorylate MLC20 at Ser-19. In general, phosphorylation at Ser-19 and Thr-18 activates the actin-activated ATPase activity of myosin, while the PKC sites have an inhibitory effect [3–6].

On the other hand, the phosphatases that dephosphorylate myosin are not as well understood. A number of protein phosphatases have been reported that can dephosphorylate MLC20 at Ser-19 at least in vitro [10]. Some are also involved in dephosphorylation of other sites, i.e. Thr-18 and Ser-19 by MLCK [4,11], Ser-1, Ser-2 and Thr-9 by PKC [6,11]. Recently, a myosin phosphatase (MP) effective with intact phosphorylated myosin as a substrate was isolated from smooth muscle (reviewed in [12]). This MP was composed of three subunits: 110, 38 and 20 kDa. The 38 kDa subunit is the δ isoform of the catalytic subunit of type 1 protein phosphatase (PP1cδ) (also referred to as PP1cβ) and the 110 kDa subunit is a putative regulatory or target molecule for myosin and PP1cδ. The function of the 20 kDa regulatory subunit (M20) is not known. MP was found to account for nearly all of the MP activity in myofibrils [13] and thus it is generally accepted that the trimeric MP is the major phosphatase for myosin in smooth muscle [12]. Recently, MP has attracted the attention because its regulatory mechanisms may be linked to the Ca^{2+} sensitization of smooth muscle contraction [14].

All previous studies on MP have focused on the dephosphorylation of Ser-19 and dephosphorylation of other sites has not been considered. In this article, it is shown that MP dephosphorylates both of the MLCK sites, Ser-19 and Thr-18, but only dephosphorylates one of the PKC sites, i.e. Thr-9. The ability of MP to discern between sites is a property of the catalytic subunit PP1cδ and does not require the non-catalytic subunits. Kinetic analyses show that the effect of the non-catalytic subunits is to increase the rate of dephosphorylation for the different sites.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Du Pont-NEN (Boston, MA, USA), phorbol 12-myristate 13-acetate, L-α-phosphatidyl-L-serine, TPCK-treated trypsin, L-phosphoserine, L-phosphothreonine and L-phosphotyrosine from Sigma Chemical (St. Louis, MO, USA), microcystin-LR from Wako Pure Chemical (Osaka, Japan). All other chemicals were of reagent grade.

2.2. Protein preparations

MP was purified from chicken gizzard as described previously [15]. Smooth muscle myosin, MLC20, PP1cδ and MLCK from chicken gizzard and calmodulin from bovine brain were purified as listed previously [16]. PKC was purified from human platelets as described previously [17].

2.3. Preparation of ^{32}P-labelled substrate

Phosphorylation of myosin at Ser-19 was carried out as follows: myosin (5 mg/ml) was incubated at 30°C for 12 min with 5 μg/ml calmodulin and 10 μg/ml MLCK in solvent A (20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 300 mM KCl, 0.1 mM $CaCl_2$, 1 μM microcystin-LR and 0.5 mM [γ-^{32}P]ATP. Phosphorylation of myosin at Ser-19 plus Thr-18 was carried out at as follows: myosin (5 mg/ml) was

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Abbreviations: MP, myosin phosphatase; PP1, type 1 protein phosphatase; PP1cδ, the δ isoform of the catalytic subunit of PP1; PP2A, type 2A protein phosphatase; PP2Ac, the catalytic subunit of PP2A; MLCK, myosin light chain kinase; MLC20, 20 kDa myosin light chain; PKC, Ca^{2+}-activated, phospholipid-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel eletrophoresis

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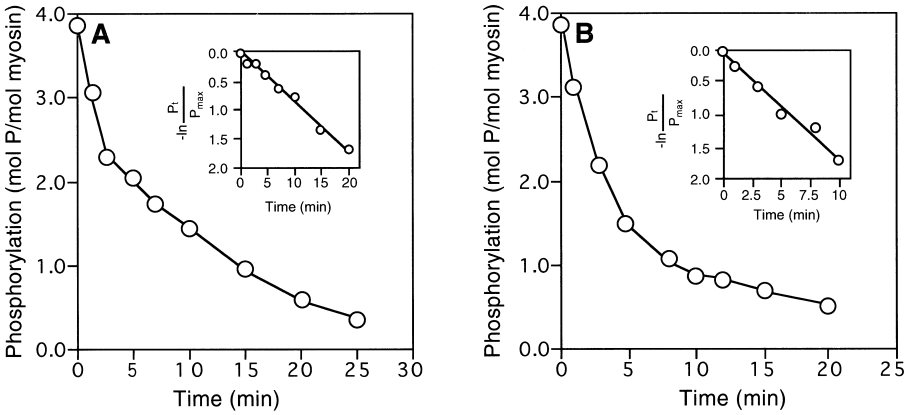

Fig. 1. Dephosphorylation of doubly phosphorylated myosin. Myosin was phosphorylated to 3.9 mol P/mol myosin by MLCK and the double ^{32}P-labelled myosin was assayed with 1 μg/ml of MP holoenzyme (A) and 10 μg/ml of PP1cδ (B) as described in Section 2. Insets show semilogarithmic plots of time course data, where P_{max} is the experimental maximum level of phosphorylation and P_t is the phosphorylation at time t.

incubated at 30°C for 20 min with 20 μg/ml calmodulin and 100 μg/ml MLCK in solvent A. Phosphorylation of myosin at Thr-9, Ser-1 and/or Ser-2 by PKC was carried out as follows: myosin (5 mg/ml) was incubated at 30°C for 2 h with 10 μg/ml PKC in 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 300 mM KCl, 1 mM EGTA, 100 ng/ml phorbol 12-myristate 13-acetate, 100 μg/ml L-α-phosphatidyl-L-serine, 1 μM microcystin-LR and 0.5 mM [γ-^{32}P]ATP [6]. The levels of phosphorylation obtained were 1.9, 3.9 and 3.4 mol P/mol myosin, respectively. The phosphorylated samples were dialyzed against 30 mM Tris-HCl, pH 7.5, 300 mM KCl and 0.2 mM dithiothreitol. ^{32}P-labelled MLC20 at Ser-19 plus Thr-18 by MLCK and at Thr-9, Ser-1 and/or Ser-2 by PKC were prepared as described previously [18].

2.4. Phosphatase assays

Phosphatase assays were carried out at 30°C using ^{32}P-labelled myosin (final concentration 2 μM) or ^{32}P-labelled MLC20 as the substrate (final concentration 5 μM). The assay conditions were 30 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mg/ml bovine serum albumin and enzyme as indicated in the figure legends. The reactions were started by the addition of substrate and terminated by the addition of trichloroacetic acid to 5%. After sedimentation at 5000×g for 5 min, the radioactivity of the supernatant was determined by Cerenkov counting.

2.5. Determination of the sites of dephosphorylation on the myosin light chain

Aliquots of phosphorylated myosin were resolved by 10% SDS-PAGE and stained with Coomassie blue. The bands corresponding to MLC20 were excized and processed for the digestion (in the gel) by TPCK-treated trypsin [19]. A two dimensional phosphoamino acid analysis was then performed [20].

2.6. Other procedures

SDS-PAGE was carried out with the discontinuous buffer system of Laemmli [21]. Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) procedures, using bovine serum albumin as a standard.

3. Results

To determine if MP of smooth muscle could dephosphorylate both of the MLCK sites, the phosphatase rate was assayed using double-labelled myosin by MLCK (3.9 mol P/mol myosin). The time courses of dephosphorylation by the MP holoenzyme and PP1cδ are shown in Fig. 1A and B, respectively. Both the holoenzyme and PP1cδ dephosphorylated double-labelled myosin (Thr-18/Ser-19) almost completely, suggesting that MP and PP1cδ are effective with both of the MLCK phosphorylation sites. The insets of Fig. 1 show semilogarithmic plots of the time course data and single first order rate processes are indicated. These results suggest that both the MP holoenzyme and PP1cδ have no apparent preference for Thr-18 or Ser-19. The MP holoenzyme and PP1cδ also completely dephosphorylated double-labelled MLC20 (data not shown).

Dephosphorylation of the PKC sites was studied next using myosin phosphorylated by PKC (3.4 mol P/mol myosin). As

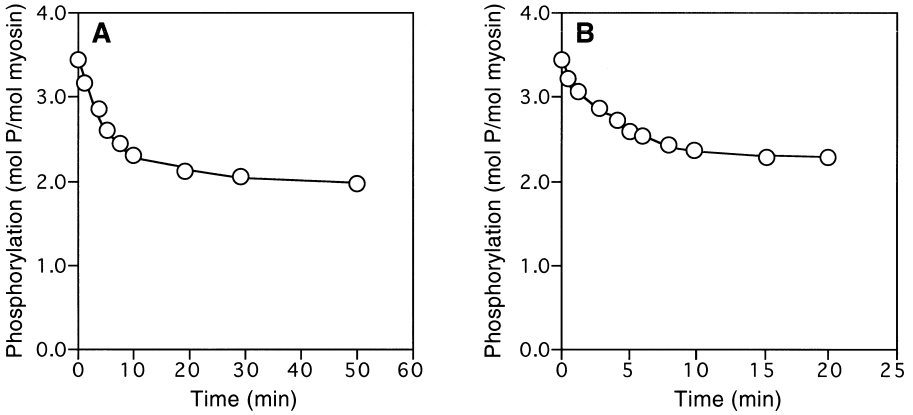

Fig. 2. Dephosphorylation of multiple phosphorylated myosin. Myosin was phosphorylated to 3.4 mol P/mol myosin by PKC and the multiple ^{32}P-labelled myosin was assayed with 1 μg/ml of MP (A) and 10 μg/ml of PP1cδ (B) as described in Section 2.

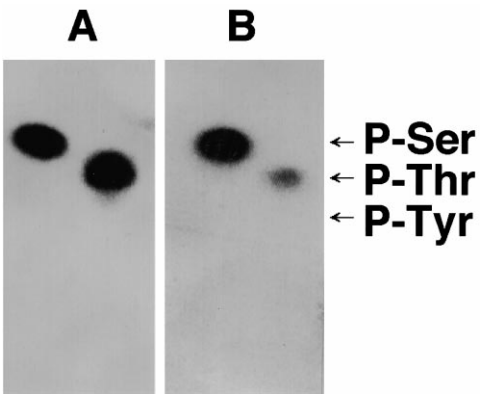

Fig. 3. Determination of the dephosphorylation site. Aliquots of [^{32}P]myosin phosphorylated by PKC were incubated without (A) or with 1 μg/ml of MP (B) for 60 min and then the samples were subjected to the phosphoamino acid analysis as described in Section 2. The radioactive spots were scraped from the thin layer plates and the radioactivities corresponding to phosphoserine and phosphothreonine were determined by Cerenkov counting. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

shown in Fig. 2, the dephosphorylation achieved by MP holoenzyme and PP1cδ was only partial. After 10 min incubation, only about 30–40% of the total phosphate was released and the remaining sites were resistant to further dephosphorylation. With MLC20 phosphorylated by PKC as substrate, similar results were obtained (data not shown). These results suggest that between the three potential PKC sites, there is some preference as substrates for the holoenzyme and PP1cδ. When myosin is phosphorylated by PKC to 3.4 mol P/mol myosin, both phosphoserine and phosphothreonine were detected (Fig. 3A). After dephosphorylation by MP (Fig. 3B), phosphothreonine disappeared and about 90% of the radioactivity was present as phosphoserine. In the control sample (no phosphatase treatment), both phosphoserine and phosphothreonine were detected. These results indicated that of the three PKC sites only Thr-9 can be effectively dephosphorylated by the MP holoenzyme. Similar results were obtained with PP1cδ (data not shown).

Phosphatase assays were carried out to determine kinetic parameters of MP and PP1cδ using mono-phosphorylated myosin (Ser-19), di-phosphorylated myosin (Thr-18/Ser-19) and the multi-phosphorylated myosin (Thr-9, Ser-1 and/or Ser-2). The respective values are compared in Table 1. The following points should be made: (1) with mono-phosphorylated and di-phosphorylated myosin as substrates, the effects of the holoenzyme were about a 10-fold decrease in K_m and a 9-fold increase in k_{cat}, compared to PP1cδ. (2) With PKC-phosphorylated myosin as substrate, the holoenzyme showed a 5-fold decrease in K_m but had no effect on k_{cat}.

4. Discussion

The above results document that the dephosphorylation of both Ser-19 and Thr-18 can be catalyzed by the MP holoenzyme. A single rate of dephosphorylation was indicated thus implying a lack of preference between the two sites. Diphosphorylation of MLC20 at Thr-18 and Ser-19 has been observed not only in vitro [3,4] but also in intact smooth muscles following agonist stimulation [22,23]. In vitro experiments show that diphosphorylation of Thr-18 and Ser-19 increases the actin-activated ATPase activity of myosin [3,4] and stabilizes myosin filaments [24]. However, some effects of diphosphorylation of MLC20 are controversial. In permeabilized smooth muscle fiber preparations, the additional phosphorylation at Thr-18 did not affect the tension and shortening velocity [25], nor did it increase the velocity of myosin-coated beads in the in vitro mobility assays [26]. On the other hand, diphosphorylation of MLC20 has been reported to correlate in situ with thrombin-induced platelet aggregation [27] and with the rate and extent of degranulation in RBL-2H3 cells [28]. It is interesting that treatment with 10–100 nM calyculin A (a protein phosphatase inhibitor) in SM-3 cells [29] or guanosine 5′-*O*-(3-thiotriphosphate), which is thought to inhibit MP, in permeabilized pig aortic smooth muscle cells [30], strongly induced diphosphorylation of MLC20. In addition, diphosphorylation of MLC20 was observed in various pathological models, including intimal hyperplasia in rabbit carotid artery [31] and coronary artery spasm in a swine model [32]. Whether the generation of di-phosphorylated MLC20 is caused in part by the inhibition of MP in spasm smooth muscle is an interesting point but is beyond the scope of the present study. Thus, these data suggest that if diphosphorylation (by MLCK) occurs under physiological conditions, both sites would be subject to dephosphorylation by MP.

Phosphorylation of MLC20 by PKC has also been demonstrated in vitro for smooth muscle [4–6] and non-muscle myosin [33–38]. Although the effects of PKC phosphorylation of myosin in the smooth muscle system remain obscure, possible effects of phosphorylation at the PKC sites in human platelets [33,35] and the mast cell line RBL-2H3 [36] have been proposed. Yamakita et al. [37] demonstrated the importance of phosphorylation of myosin on Ser-1 and Ser-2 catalyzed by cdc2 kinase in mitotically arrested cells. Our results show that MP effectively dephosphorylated Thr-9, whereas the other two sites (Ser-1 and/or Ser-2) were resistant to the phosphatase. Recently, evidence has accumulated to indicate that phosphorylation of Ser-1 and/or Ser-2 was detectable in intact smooth muscle and non-muscle cells, but phosphorylation of Thr-9 was not detectable in vivo ([28,29,35,36,39]). On the other hand, Turbedsky et al. [40] found that phosphorylation of Ser-1/Ser-2 of the *Xenopus* myosin regulatory light chain did not inhibit the subsequent phosphorylation of Ser-19 by

Table 1
Kinetics of MP and PP1cδ for distinct phosphorylated myosin[a]

Substrates	MP		PP1cδ	
	K_m (μM)	k_{cat} (μM)	K_m (per min)[b]	k_{cat} (per min)[b]
Myosin (Ser-19)	4.3 ± 0.9	791 ± 27	39.3 ± 3.3	115 ± 14
Myosin (Thr-18/Ser-19	7.4 ± 2.1	949 ± 52	44.7 ± 4.1	106 ± 17
Myosin (Ser-1/Ser-2/Thr-9	12.2 ± 1.5	82.6 ± 17.3	59.2 ± 3.8	80.6 ± 11.5

[a]Values are means ± S.D. ($n = 3$). [b]The molecular weights used were 168 kDa for MP and 37 kDa for PP1cδ.

MLCK and suggested that the inhibition of MLCK was due to phosphorylation of Thr-9 and secondary sites Thr-7 and Thr-10. Thus, it is suggested that if phosphorylation of Thr-9 is involved in a given process, the relevant phosphatase is a type 1 enzyme, as in MP. If Ser-1/Ser-2 are phosphorylated then it is likely that a different phosphatase is required.

Several protein phosphatases isolated from smooth muscle tissues have been reported to dephosphorylate multiple sites on MLC20. Ikebe et al. showed that a spontaneously active phosphatase from bovine aorta could dephosphorylate Ser-19, Thr-18 and Thr-9 but not Ser-1 and Ser-2 [4,6]. Erdödi et al. also reported that the catalytic subunit of a heparin-agarose bound phosphatase effectively dephosphorylated Thr-18, Ser-19 and Thr-9, whereas it was less effective in dephosphorylation of Ser-1 and Ser-2 in actomyosin [11]. The site preference for these phosphatases is similar to MP, suggesting that they may be related, possibly via the PP1 catalytic subunit.

The catalytic subunit, PP1cδ, showed the same site selection as the holoenzyme. In this respect it differs from PP2A, since Ser-19 of MLC20 in intact myosin was de-phosphorylated by PP2Ac but not by PP2A holoenzyme [41]. The role of the non-catalytic subunit in MP, therefore, is to activate PP1cδ, as suggested in earlier studies [12,16]. Activation was observed for each of the three sites and for Ser-19 and Thr-18 was achieved by a decrease in K_m and an increase in k_{cat}. For Thr-9, the activation was primarily due to a decrease in K_m. Thus, it is suggested that an important factor in the activation of the phosphatase activity as seen for the holoenzyme, reflects an increased affinity of binding to the phosphorylated substrate. Probably, this is due to targeting via the large non-catalytic subunit.

In summary, we showed that MP is effective with Ser-19, Thr-18 and Thr-9, but ineffective with Ser-1 and/or Ser-2 of MLC20 in intact myosin. The ability to discern among these sites is a property of the catalytic subunit, PP1cδ, and does not require the non-catalytic subunits.

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