Biochimica et Biophysica Acta, 971 (1988) 163–169
Elsevier

BBA 12309

The myosin-bound form of protein phosphatase 1 ($PP\text{-}1_M$) is the enzyme that dephosphorylates native myosin in skeletal and cardiac muscles

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(Received 12 April 1988)

Key words: Protein phosphorylation; Protein phosphatase; Myosin; Muscle contraction; Striated muscle

The myosin-bound form of protein phosphatase 1 ($PP\text{-}1_M$) and the glycogen-bound form ($PP\text{-}1_G$) together account for virtually all the phosphatase activity in rabbit skeletal muscle extracts towards native myosin. $PP\text{-}1_M$ has a 3-fold higher activity towards native myosin than does $PP\text{-}1_G$ and accounts for at least 60% of the myosin phosphatase activity in rabbit skeletal muscle. $PP\text{-}1_M$ accounts for 90% of the myosin phosphatase activity in bovine cardiac muscle, where $PP\text{-}1_G$ is essentially absent. The high activity of $PP\text{-}1_M$ towards native myosin appears to arise from interaction of the catalytic subunit with the putative myosin-binding subunit, since chymotryptic digestion liberates a catalytic subunit having the same characteristics as that released by limited proteolysis of $PP\text{-}1_G$. Protein phosphatase 2A in skeletal and cardiac muscles is very active towards the isolated myosin P-light chain, but ineffective in dephosphorylating native myosin. The results suggest that $PP\text{-}1_M$ is the enzyme that dephosphorylates myosin in skeletal and cardiac muscle.

Introduction

Four serine/threonine-specific protein phosphatase catalytic subunits have been identified in eukaryotic cells and divided into two groups (type 1 and type 2) on the basis of their sensitivity to the heat-stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2) (type-) and specificity for the α (type 2) or β (type 1) subunits of phosphorylase kinase [1,2]. These enzymes have broad and overlapping substrate specificities in vitro and identification of their in vivo substrates has become a major current problem in protein phosphatase research.

The active forms of the type-1 phosphatases are largely particulate, and the type-2 phosphatases cytosolic, providing an important clue to the likely functions of type-1 enzymes [3]. Evidence is accumulating that type-1 phosphatases are directed to particular subcellular locations through interaction of the catalytic subunit ($PP\text{-}1_C$) with specific 'targetting' subunits. In low ionic strength EDTA extracts of skeletal muscle, most of the directly measurable type-1 phosphatase activity is associated with glycogen, because $PP\text{-}1_C$ is complexed to a glycogen-binding G subunit [4,5]. This species, $PP\text{-}1_G$, is also present in the myofibrillar fraction from which it can be extracted with Triton [6]. This suggests that $PP\text{-}1_G$ may also be associated with membrane, or with glycogen-particles that are associated with membranes.

Abbreviations: PP-1(2A), protein phosphatase 1(2A); TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; I-1(2), heat-stable protein, inhibitor-1(2)

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A further form of PP-1 is present in the myofibrillar fraction which is tightly bound to myosin [6]. This species, PP-1_M, is equally sensitive to I-1 and I-2, but in contrast to PP-1_G does not bind to glycogen and is not immunoprecipitated by antibody directed against the G subunit. PP-1_C (37 kDa) derived from either PP-1_M (110 kDa) or PP-1_G does not bind to either myosin or glycogen [6]. These observations suggest that PP-1_M is composed of PP-1_C complexed to a myosin-binding 'M subunit'. Here we show that PP-1_M is the principal enzyme that dephosphorylates the P-light chain of native myosin in striated muscles. This conclusion is in disagreement with the work of Mumby and co-workers [7].

Materials and Methods

Preparation of native myosin and isolated myosin light chains. Native myosin was purified from rabbit skeletal muscle and bovine cardiac muscle and freed from its accessory proteins (C, F, H and X) as described in Ref. 8, except that DEAE-Sepharose was used instead of DEAE-cellulose. Isolated mixed myosin light chains were purified from rabbit skeletal muscle and bovine cardiac muscle as described in Ref. [9], and myosin light chain kinase from rabbit skeletal muscle as described in Ref. 10.

Preparation of ^{32}P-labelled myosin and myosin P-light chains (10^6 cpm / nmol). Myosin (30 mg/ml) or myosin light chains (2 mg/ml) were dephosphorylated by incubation for 30 min at 30 °C with PP-1_M (10 mU/ml) in 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol (solution A) containing 0.1 M NaCl in a total volume of 0.5 ml. Both incubations were terminated by addition of 0.05 ml of 0.5 M NaF.

100% (w/v) trichloroacetic acid (TCA, 0.05 ml) was added to the solution containing isolated myosin light chains and, after 15 min at 0 °C, the suspension was centrifuged for 2 min at 13000 × *g* and the supernatant was discarded. The pellet was washed three times with 10% (w/v) TCA, dissolved in 0.1 M $NaHCO_3$ and dialysed against solution A.

The solution containing native myosin was diluted 5-fold with 2 ml of solution A and, after 60 min at 0 °C, the myosin was collected by centrifugation for 30 min at 80000 × *g*. The supernatant was discarded, the pellet was redissolved in 0.5 ml of solution A/0.15 M NaCl and reprecipitated by dilution with 2.0 ml of solution A. After centrifugation, the myosin was redissolved in solution A/0.1 M NaCl.

Phosphatase-treated native myosin (7.5 mg/ml) or isolated myosin light chains (0.5 mg/ml) were incubated in 50 mM Hepes (pH 7.4), 0.1% 2-mercaptoethanol, 0.1 mM $CaCl_2$, 5 mM magnesium acetate, 0.1 μM calmodulin, 2.5 μg/ml myosin light chain kinase and 0.1 mM [γ-^{32}P]ATP. After 30 min at 30 °C, phosphorylation of the isolated light chains was stopped by addition of 0.1 vol. of 100% TCA and the protein was collected by centrifugation as described above. The pellet was washed several times with 10% TCA, redissolved in 0.1 ml of $NaHCO_3$, dialysed against solution A and stored at −20 °C.

Phosphorylation of native myosin was stopped by addition of 0.1 vol. of 0.1 M EDTA, 0.5 M NaF, 0.1 M sodium pyrophosphate and the protein was precipitated by addition of 5 vol. of solution A containing unlabelled ATP (1 mM). The protein was collected by centrifugation as described above, redissolved in 0.5 ml of solution A/0.15 M NaCl/1 mM ATP and reprecipitated by addition of 2.0 ml of solution A. This cycle was repeated twice more, but omitting unlabelled ATP in the final cycle. The ^{32}P-labelled native myosin was redissolved in solution A/150 mM NaCl dialysed against solution A/100 mM NaCl and stored at 0–4 °C.

SDS-polyacrylamide gel electrophoresis followed by autoradiography showed that the P-light chain was the only protein phosphorylated, using either isolated light chains or native myosin (data not shown). Incorporation of phosphate into the isolated 17 kDa P-light chain was 0.57 mol/mol (skeletal muscle) and 0.51 mol/mol (cardiac muscle), assuming that the P-light chain comprised 50% of the protein by weight of the mixed light chain preparations. The amounts of phosphate incorporated into native skeletal and cardiac myosin (250 kDa) were 0.49 mol/mol and 0.42 mol/mol, respectively. Protein was measured according to Bradford [11] using bovine serum albumin as standard.

Preparation of muscle extracts. These were prepared essentially as described in Ref. 6. Briefly, muscle was homogenised in 2.5 vol. of 20 mM Tris-HCl (pH 7.0), 0.1% mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine (solution B) containing 4 mM EDTA, centrifuged at $5000 \times g$ and the supernatant, termed 'EDTA extract', was decanted. The myofibrillar pellet was extracted twice by rehomogenisation with solution B containing 2 mM EGTA and 0.5% Triton X-100 and, following centrifugation, the combined supernatants, termed 'Triton extract', were collected. The Triton-extracted myofibrils were resuspended in solution B containing 2 mM EGTA and 0.6 M NaCl and are termed the 'myofibrillar suspension'. The myofibrillar suspension was freed from actomyosin by fractionation with 3–12% poly(ethylene glycol) [6]. The 'EDTA extract' was centrifuged for 90 min at $100\,000 \times g$ to pellet the glycogen-protein particles and this fraction was resuspended in solution B at a concentration of 0.5 U/ml and used as a source of $PP\text{-}1_G$ where specified.

Protein phosphatase assays. The dephosphorylation of phosphorylase *a* was carried out at 30 °C in 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.01% (w/v) Brij 35 in a total volume of 0.03 ml as described in Ref. 12. Dephosphorylation of native myosin or isolated myosin P-light chains was performed in an identical manner, except that 50 mM NaCl was included to solubilise native myosin. This concentration of NaCl inhibited the dephosphorylation of isolated P-light chains by less than 10%. Assays were performed with skeletal muscle myosin, and not cardiac myosin, unless stated otherwise. All substrates were used at a final concentration of 10 μM. 1 unit of phosphatase activity was that amount which catalysed the release of 1.0 μmol of phosphate from each substrate in 1 min. The release of phosphate from each substrate was restricted to less than 20%, and no phosphate was released in the absence of added phosphatase. When I-1 or I-2 were present, diluted phosphatase was preincubated with the inhibitors for 15 min before initiating the reaction with substrate [13].

Other materials and methods. Chymotrypsin was purchased from Worthington, PMSF was from Sigma, heparin-Sepharose and Sephadex G-100 were from Pharmacia. I-1, I-2 and $PP\text{-}1_G$ were purified from rabbit skeletal muscle by Dr. Michael Hubbard and Dr. Carol MacKintosh in this laboratory [13]. Affinity-purified antibody to the G subunit was prepared and used as in Ref. 6. Other procedures are also described in Ref. 6.

Results

Myosin phosphatase activity in different fractions prepared from rabbit skeletal muscle

We have previously shown that large amounts of phosphorylase phosphatase activity are present in the EDTA extract, Triton extract and myofibrillar suspension [6]. Activity can be inhibited by I-2 by 85% (EDTA extract) or by more than 90% (Triton extract and myofibrillar fraction) indicating that dephosphorylation is predominantly catalysed by type-1 protein phosphatases. About 75% of the protein phosphatase 1 activity in the EDTA and Triton extracts can be immunoprecipitated by antibody to the G subunit, since $PP\text{-}1_G$ is the major active type-1 phosphatase in these fractions. Less than 10% of the phosphorylase phosphatase activity in the myofibrillar suspension is immunoprecipitated by this antibody, as $PP\text{-}1_M$ is the predominant type-1 phosphatase [6].

In the present work, very similar observations were made using native myosin as substrate. Most of the myosin phosphatase activity in the EDTA and Triton extracts could be inhibited by I-2 and immunoprecipitated by antibody to the G subunit (Fig. 1), whereas myosin phosphatase activity in the myofibrillar suspension was blocked by I-2, but not immunoprecipitated by antibody to the G subunit (Fig. 1). It should also be noted that the activity ratio myosin phosphatase/phosphorylase phosphatase was nearly 3-fold higher in the myofibrillar fraction than in either the EDTA or Triton extracts. As a consequence, about 60% of the myosin phosphatase activity, but only 40% of the phosphorylase phosphatase activity was present in the myofibrillar fraction (Fig. 1).

The myosin phosphatase activity in the myofibrillar suspension had exactly the same sensitivity to I-1 and I-2 as the phosphorylase phosphatase activity (Fig. 2) and copurified with phosphorylase phosphatase through chromatog-

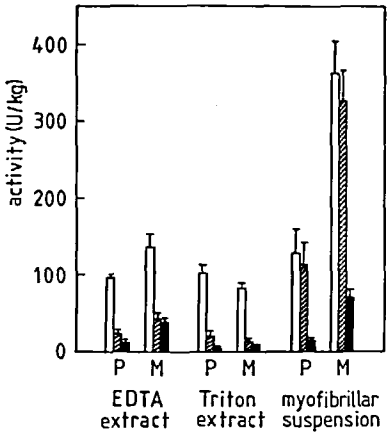

Fig. 1. Phosphorylase phosphatase (P) and native myosin phosphatase (M) activities in different fractions from rabbit skeletal muscle. Values are given ± S.E. for three different preparations. The hatched bars show activity remaining after immunoprecipitation with antibody to the G subunit of PP-1_G, after correction for the observation that only 88% of purified PP-1_G was immunoprecipitated in parallel experiments. The filled bars show activity measured in the presence of 0.25 μM I−2 and the open bars show activity measured in the absence of antibody or I-2.

raphy on heparin-Sepharose (Fig. 3) and gel-filtration on Sephadex G-100 (Fig. 4). These results indicate that the myosin phosphatase activity is catalysed by PP-1_M.

The other enzyme with significant phosphorylase phosphatase activity in mammalian tissues is protein phosphatase 2A (PP-2A). PP-2A in skeletal muscle is largely present in the EDTA extract [6] and can be resolved from PP-1 by chromatography on heparin-Sepharose [14–16]. PP-1, but not PP-2A, is retained by the column and can be eluted with 0.5 M NaCl. Chromatography of the EDTA extract on heparin-Sepharose revealed that PP-2A was 30-fold more effective in dephosphorylating isolated myosin P-light chains than native myosin and 10-fold more active towards the isolated P-light chains than phosphorylase *a* (Fig. 3). By contrast, PP-1_G dephosphorylated all three substrates at comparable rates in the EDTA and Triton extracts (Fig. 3), while PP-1_M was more active towards native myosin than isolated P-light chains or phosphorylase *a* (Fig. 3) as expected.

Myosin phosphatase activity in bovine cardiac muscle

Bovine cardiac muscle was fractionated in an identical manner to that used for rabbit skeletal muscle, and the EDTA extract, Triton extract and myofibrillar suspension were assayed for phosphatase activities in the presence and absence of I-2. The EDTA extract had a relatively high activity towards isolated skeletal muscle myosin P-light chains, but its activity towards native skeletal muscle myosin and phosphorylase *a* was 30-fold and 10-fold lower, respectively (Fig. 5). This result, and the minimal effect of I-2 on the dephosphorylation of any substrate (Fig. 5), showed that the cardiac EDTA extract contained almost no active PP-1, and that PP-2A accounted for virtually all the phosphatase activity in this fraction.

The Triton extract contained very little activity compared to the corresponding fraction in rabbit skeletal muscle, but that which was present was mostly I-2 sensitive (Fig. 5). By contrast, the myofibrillar suspension was very active towards all three substrates, native skeletal muscle myosin

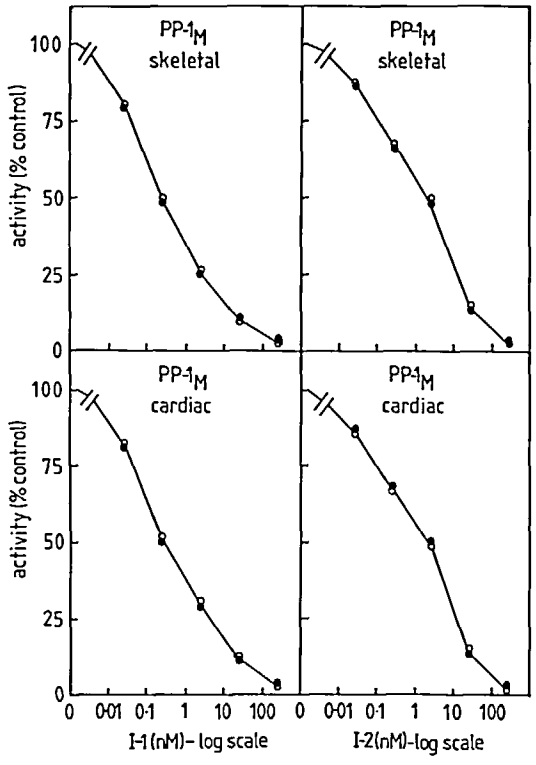

Fig. 2. Effect of inhibitors 1 and 2 on phosphatase activities in the myofibrillar suspensions of skeletal and cardiac muscle. The myofibrillar fraction was fractionated by poly(ethylene glycol) precipitation and assayed with phosphorylase (○) or native myosin (●) as substrate.

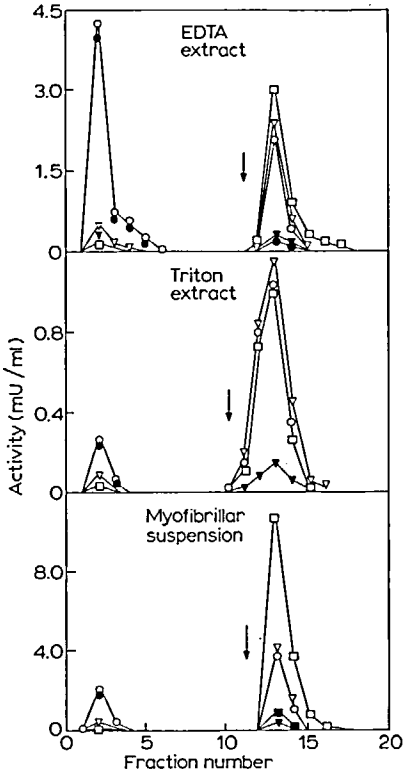

Fig. 3. Separation of protein phosphatases 1 and 2A on heparin-Sepharose. Aliquots (2 ml) of the skeletal muscle EDTA extract, Triton extract and myofibrillar suspension that had been fractionated with poly(ethylene glycol) were diluted to 10 ml in solution B containing 1.0 mM EGTA, 5% (v/v) glycerol and 0.1 M NaCl and applied to the column (5 ml) equilibrated in the same buffer. Arrows indicate the points at which the columns were eluted with 0.5 M NaCl. Fractions (7 ml) were assayed in the presence (closed symbols) and absence (open symbols) of 0.25 μM I-2 using native myosin (■, □), isolated myosin P-light chains (●, ○) and phosphorylase *a* (▼ ▽). The yield of activity from each column was at least 90:

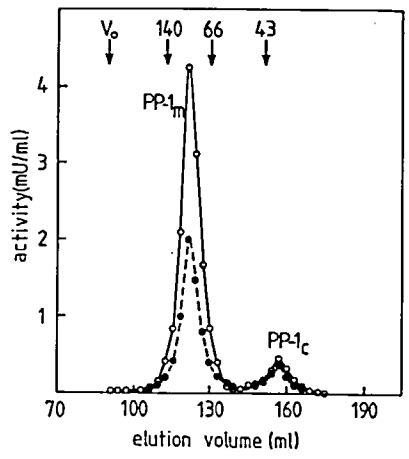

Fig. 4. Gel-filtration of skeletal muscle protein phosphatase 1_M on Sephadex G-100 Superfine. The myofibrillar suspension that had been fractionated by poly(ethylene glycol) precipitation (42 mU, 2 ml) was applied to the column (55×2.5 cm) equilibrated in 0.05 M Tris-HCl (pH 7.0, 25°C), 1.0 mM EGTA, 5% (v/v) glycerol, 0.1% 2-mercaptoethanol, 0.05 M NaCl, 0.1 mM PMSF and 1.0 mM benzamidine. The flow rate was 4 ml/h and fractions of 3 ml were collected. The arrows show the elution positions of the void volume, V_0, and the marker proteins liver alcohol dehydrogenase (140 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa). Symbols: phosphorylase phosphatase, ●------●; native myosin phosphatase, ○———○. About 10% of the PP-1_M in this preparation had converted to PP-1_C.

being dephosphorylated at a rate 3-fold higher than that of the isolated P-light chain from skeletal muscle or phosphorylase *a*. The dephosphorylation of each substrate was inhibited almost completely by I-2 (Figs. 2 and 5). These results are very similar to those observed in the myofibrillar fraction from rabbit skeletal muscle (Fig. 1). It should be noted that the cardiac myofibrillar suspension contained about 90% of the activity towards native skeletal muscle myosin, whereas the EDTA extract contained about 70% of the activity towards the isolated myosin P-light chains (Fig. 5).

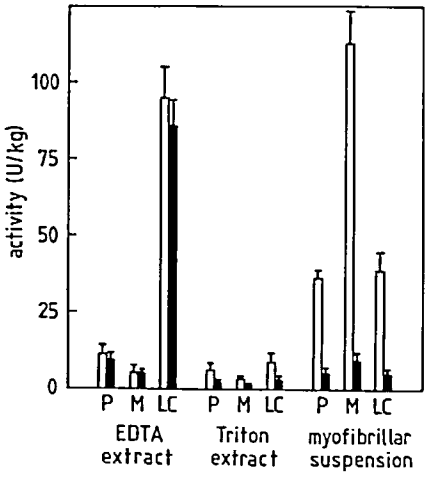

Fig. 5. Phosphatase activity in different fractions from bovine cardiac muscle towards phosphorylase (P), native myosin (M) and the isolated P-light chain (LC). Values are given as ±S.E. for five different preparations. The filled bars show activity measured in the presence of 0.25 μM I−2 and the open bars show activity in the absence of I-2.

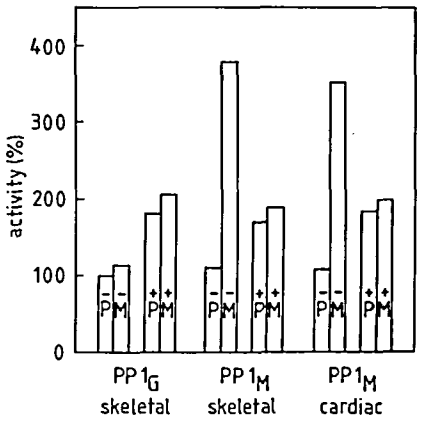

Fig. 6. Effect of chymotrypsin on the activities of protein phosphatases 1_G and 1_M towards phosphorylase (P) and native myosin (M). Glycogen-protein particles were used as the source of PP-1_G, and PP-1_M was partially purified from the myofibrillar suspensions of skeletal and cardiac muscle by fractionation with poly(ethylene glycol). The preparations were incubated for 10 min at 30 °C in the presence (+) and absence (−) of 0.3 mg/ml chymotrypsin as described in Ref. 17. Very similar results were obtained with four different preparations of PP-1_G and four of PP-1_M.

Identical results to those shown in Fig. 5 were obtained when native cardiac myosin and isolated cardiac P-light chains isolated from bovine cardiac muscle were substituted for the rabbit skeletal muscle proteins (data not shown). The rates of dephosphorylation of native myosin or isolated P-light chain by either PP-1 or PP-2A were very similar whether the substrates were isolated from skeletal muscle or cardiac muscle.

Effect of chymotrypsin on the glycogen and myosin-bound forms of protein phosphatase 1

Incubation of PP-1_G from skeletal muscle with either trypsin or chymotrypsin degrades the G subunit, releasing the catalytic subunit (PP-1_C) and enhancing phosphorylase phosphatase activity by 80% [17]. In the present work, chymotryptic digestion of PP-1_G increased activity towards native myosin in a similar manner to that of the phosphorylase phosphatase activity (Fig. 6). By contrast, chymotryptic digestion of PP-1_M decreased activity towards native myosin by 50%, whereas phosphorylase phosphatase activity increased by 50% (Fig. 6). The myosin phosphatase/phosphorylase phosphatase activities of PP-1_G and PP-1_M became very similar after chymotryptic digestion (Fig. 6), as would be expected if they contained the same catalytic subunit. Proteolysis of PP-1_M, like PP-1_G, liberated PP-1_C, as judged by a decrease in molecular mass from 110 kDa (Fig. 4) to 35 kDa (data not shown).

Discussion

The finding that PP-1_M is the only phosphatase that is bound to myosin [6] and that it accounts for about 60% and 90% of the phosphatase activity towards native myosin in rabbit skeletal muscle and bovine cardiac muscle, respectively, strongly suggests that this enzyme dephosphorylates the myosin P-light chain in vivo. However, Mumby and co-workers [7] reported that PP-2A accounted for virtually all the measurable phosphatase activity in bovine cardiac muscle towards either the isolated myosin P-light chain or native myosin. Their finding that PP-2A is the dominant activity in bovine cardiac cytosol and that this enzyme dephosphorylates isolated myosin P-light chains much more rapidly than native myosin has been confirmed in the present work (Fig. 5). The reason why these investigators did not detect the large amount of PP-1 activity associated with the myofibrillar fraction is unclear.

The present work is more consistent with observations made in avian smooth muscle. Pato and co-workers [18,19] reported that two enzymes termed smooth muscle phosphatases 1 and II (classified in Ref. 20 as protein phosphatases 2A and 2C, respectively) were very active towards isolated myosin P-light chains, but dephosphorylated native myosin very poorly. Two further enzymes termed smooth muscle phosphatases III and IV were effective in dephosphorylating native myosin as well as the isolated P-light chains [18]. Smooth muscle phosphatase IV could bind to myosin in vitro and dephosphorylated the β-subunit of phosphorylase kinase specifically, suggestive of a type-1 phosphatase. However, its activity was reported to be unaffected by I-2 [21]. Furthermore, smooth muscle phosphatases III and IV were isolated from turkey gizzard cytosol, whereas PP-1_M is tightly associated with the myofibrils in skeletal and cardiac muscle and not present in cytosol. It is possible that smooth muscle phosphatase IV is related to PP-1_M, while smooth

muscle phosphatase III may represent another type-1 phosphatase, since even PP-1_G and PP-1_C (Figs. 1, 3, 4 and 6) are capable of dephosphorylating native myosin at a comparable rate to that of the isolated P-light chain.

The much higher activity of PP-2A towards the isolated P-light chain relative to native myosin (Figs. 3 and 6) emphasizes the importance of using the native substrate when trying to identify the relevant phosphatase in vivo. A similar problem has been encountered in the liver, where PP-1 and PP-2A are both effective in dephosphorylating muscle glycogen synthase that has been phosphorylated by A-kinase, while only PP-1 is able to reactivate at a significant rate the highly phosphorylated preparations of liver glycogen synthase that are extracted from this tissue (reviewed in Ref. 22).

Chymotryptic digestion of glycogen-protein particles enhances PP-1_G activity towards phosphorylase *a* and native myosin, while proteolysis of PP-1_M enhances activity towards phosphorylase *a*, yet decreases activity towards native myosin (Fig. 6). Since chymotryptic attack releases PP-1_C, and the catalytic subunits liberated from PP-1_G and PP-1_M have identical phosphorylase phosphatase/myosin phosphatase activity ratios (Fig. 6), it would appear that interaction with the myosin-binding subunit enhances PP-1_C activity towards native myosin several-fold. This is also supported by gel-filtration experiments shown in Fig. 4. Analogous observations have been made with hepatic PP-1_G, where proteolysis of the putative glycogen-binding subunit stimulates phosphorylase phosphatase activity, while suppressing glycogen synthase phosphatase activity [17]. Thus, the subunits which target PP-1 to particular subcellular locations may also stimulate activity towards certain substrates present in these same locations.

PP-1_M dephosphorylates native 10 μM myosin several-fold more rapidly than 10 μM phosphorylase *a*, previously recognised to be the best substrate for PP-1. Furthermore, the K_m for myosin of PP-1_M and PP-1_G is much higher than 10 μM, whereas the K_m for phosphorylase *a* is approx. 5 μM (unpublished experiments). These observations indicate that the V_{max} for myosin is higher than for phosphorylase *a*. The very high activity of PP-1 for myosin and phosphorylase *a* may be related to the intracellular concentrations of these enzymes, which are two of the most abundant phosphoproteins in mammalian cells, their intracellular concentrations in skeletal muscle being 200 μM and 80 μM, respectively.

Acknowledgements

This work was supported by a Programme Grant and Group Support from the Medical Research Council, London and by The Royal Society.

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