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A New Heat-Stable Regulatory Factor is Associated with Aortic Polycation-Modulated (PCM)-Phosphatase (42207)

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Abstract. An aortic phosphatase which dephosphorylates several proteins including phosphorylase a and the 20-kDa myosin light chains is subject to modulation *in vitro* by polycationic effectors such as lysine-rich histone-H_1 and polylysine. This study was based on the hypothesis that polycationic modulation of expressed enzymic activity involves interactions between the effectors and a regulatory site associated with the polycation-modulated (PCM)-phosphatase. Basal PCM-phosphatase activity expressed against myocardial myosin light chains (MLC, 1258 nmole/min/mg) was about eightfold greater than activity expressed against phosphorylase a (149 nmole/min/mg). However, dephosphorylation of phosphorylase a was stimulated four- to sevenfold by low concentrations of polylysine (M_r = 13,000; 0.01–0.1 μM), whereas MLC phosphatase activity was virtually abolished. Higher concentrations of polylysine inhibited dephosphorylation of either substrate. Interestingly, a heat-stable fraction prepared from the PCM-phosphatase reversed the stimulatory effect of polylysine on phosphorylase phosphatase activity and the inhibitory effect on dephosphorylation of MLC. No reversal of the modulatory effects of polylysine occurred when protein phosphatase inhibitor 1 or inhibitor 2 was substituted for the heat-stable factor derived from the PCM-phosphatase. Sucrose density centrifugation of the enzyme yielded a single peak (M_r = 63,000) exhibiting polycation-modulated activity against phosphorylase a and MLC. Moreover, heating each of the gradient fractions showed the presence of a heat-stable factor which reversed the modulatory effects of polylysine on dephosphorylation of either phosphorylase a or MLC. These results show that a specific heat-stable factor, which differs from both inhibitor 1 and 2, is associated with the PCM-phosphatase. The results suggest that polycationic modulation of expressed PCM-phosphatase activity may involve interactions between the polycationic effector and the enzyme-associated regulatory factor.

A large body of evidence suggests that Ca^{2+}-dependent phosphorylation of the 20-kDa myosin light chains is obligatory for contraction of smooth muscle (see 1, 2, for reviews). In addition, glycogen phosphorylase is converted to its more active phosphorylated form (phosphorylase a) when smooth muscle contracts (3–5). Accordingly, modulation of protein phosphatase activity may participate in regulating contraction and metabolism of smooth muscle.

In this context, we recently identified a phosphatase in aortic vascular smooth muscle which dephosphorylates aortic native myosin, myocardial myosin light chains, protein phosphatase inhibitor 1, and phosphorylase a (6–9). A unique feature of the enzyme, which we call polycation-modulated (PCM)-phosphatase is that expressed phosphorylase and light chain phosphatase activities are differentially susceptible to modulation *in vitro* by polycationic effectors such as lysine-rich histone-H_1 or polylysine (8, 9). Low concentrations of either effector (<25 μg/ml) stimulate phosphorylase phosphatase activity six- to eightfold, while higher concentrations suppress stimulation. In contrast, only inhibition of light chain phosphatase activity occurs in the presence of either histone-H_1 or polylysine. Whether or not such modulation occurs *in vivo* is unknown. Nevertheless the reported studies strongly suggest that the expression of protein phosphatase substrate specificity may be subject to regulation by specific effectors.

Available evidence indicates that polycation-mediated modulation of PCM-phosphatase activity involves direct interactions between effector and enzyme (8, 9, 10). Since limited proteolysis of aortic PCM-phosphatase reduces its susceptibility to polycationic modulation, we hypothesized that a protease-sensitive regulatory site, responsible for interaction with the effector, is associated with the enzyme (8).

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We now report that a heat-stable regulatory factor copurifies with aortic PCM-phosphatase. Moreover, this factor is apparently different from protein phosphatase inhibitors 1 and 2 (11, 12) which are thought to regulate a number of phosphatases such as the ubiquitious ATP·Mg-dependent phosphatase (13–15). The heat-stable regulatory factor associated with the PCM-phosphatase, unlike either inhibitor 1 or 2, reverses both the stimulatory effect of polylysine on expressed phosphorylase phosphatase activity and the inhibitory effect on light chain phosphatase activity. To our knowledge, this is the first reported evidence indicating that differential modulation of phosphatase activity and expression of substrate specificity may be ascribable to direct interactions between a given effector and a specific regulatory component associated with the enzyme.

Methods. PCM-phosphatase, as described previously (8–9), was prepared from bovine aortic muscularis by sequential steps involving ion exchange chromatography on DEAE–Sephacel, polylysine–sepharose and two cycles of chromatography on a Mono Q HR5/5 anion exchange column using the Pharmacia system for fast protein liquid chromatography. The purified enzyme was stored at −60°C in 20 m*M* Tris, pH 7.4, and 0.5 m*M* dithiothreitol (Buffer A) containing 50% glycerol.

Phosphorylatable myosin light chains were prepared from bovine myocardium according to Blumenthal and Stull (16), and phosphorylase was prepared from rabbit skeletal muscle by the method of Krebs *et al.* (17). As described previously (8, 9), assays for PCM-phosphatase activity were performed at 30°C in a reaction mixture (30 μl) containing either [^{32}P]phosphorylase a (10 μM) or ^{32}P-myosin light chains (4 μM). Because basal light chain phosphatase activity was about eightfold greater than phosphorylase phosphatase activity (see under Results), assays with light chains were performed for 3 min while assays with phosphorylase a were performed for 10 min using the same dilution of enzyme. All assays were started by addition of substrate to the reaction mixture and stopped by the addition of ice-cold 20% trichloroacetic acid (8, 9). Activity was expressed as units and 1 unit (U) was defined as that amount of enzyme which releases 1 nmole ^{32}P per minute under the assay conditions tested.

To test for the presence of heat-stable factor(s) associated with the PCM-phosphatase, aliquots of the enzyme (150–300 μl) were incubated in a 90°C water bath for 10 min, cooled on ice, and centrifuged to remove precipitated protein. This treatment destroyed all enzymatic activity. In several experiments, samples (150–300 μl) of the unheated PCM-phosphatase, or the supernatant obtained from heated enzyme, were dialyzed against Buffer A to remove glycerol and subjected to sucrose density centrifugation according to the method of Martin and Ames (18). Briefly, 100 μl of the dialyzed sample was layered on 4 ml 5–20% sucrose–Buffer A and centrifuged at 40,000 rpm for 15 hr in a Beckman SW 50 rotor at 4°C (6, 9). [^{14}C]Ovalbumin (M_r = 40,000; NEN) was used as an internal marker.

Modulatory effects of polylysine (M_r = 13,000; Sigma) on expressed PCM-phosphatase activity were assessed after preincubating the enzyme with the desired concentration of polylysine for 10 min at 30°C. Similarly, in tests of the effects of heated preparations on both basal or polycation-modulatable activity, the heated fraction was also preincubated (10 min) with the enzyme in either the presence or absence of polylysine.

Protein phosphatase inhibitor 1 was purified from rabbit skeletal muscle as described by Nimmo and Cohen (19), and inhibitor 2 was purified from the same tissue using the method of Yang *et al.* (20). Bovine aortic ATP·Mg-dependent phosphatase, also called F_c (see 14, 15, for reviews) was purified as detailed earlier (21). Since myosin light chains are a poor substrate for this enzyme (22), assays were performed using only phosphorylase a as substrate. It is important to recall that the ATP·Mg-dependent phosphatase exists in an inactive form consisting of a catalytic subunit and inhibitor 2 (14, 15, 23, 24). Phosphatase activity is expressed following incubation with ATP (0.5 m*M*), Mg^{2+} (2.5 m*M*), and an activating protein, F_A, which is identical to glycogen synthase kinase 3 (25). In order to avoid confusion, we have adopted the designation F_A/GSK 3 as suggested by Roach *et al.* (26). Maximal phosphorylase phosphatase activity

of the aortic ATP • Mg-dependent phosphatase used in this study was 1300 U/mg protein. In addition, an inhibitor 2-deficient form of the enzyme, called *free* F_c (14, 27), was prepared by anion exchange chromatography on HR 5/5 (Pharmacia). Since *free* F_c cannot be activated by F_A/GSK-3, ATP, and Mg^{2+} unless exogenous inhibitor 2 is added to the reaction mixture, it may be used as a sensitive assay for inhibitor 2 (14, 27). Where appropriate, protein was determined by the method of Lowry *et al.* (28) using bovine serum albumin as standard.

Results. *Modulation of PCM-phosphatase activity by polylysine.* Basal activity of the PCM-phosphatase expressed against myocardial myosin light chains (1258 ± 48 U/mg, n = 30) was about eightfold greater than basal activity expressed against phosphorylase a (149 ± 19 U/mg, n = 27). However, in accordance with our earlier studies (8, 9), low concentrations of polylysine (0.002–0.064 μM) virtually abolished light chain phosphatase activity, whereas dephosphorylation of phosphorylase a was stimulated 6.5-fold (Fig. 1).

The differential modulatory effects of polylysine on light chain and phosphorylase phosphatase activities were also demonstrable following sucrose density centrifugation of the PCM-phosphatase (Fig. 2). Thus, the enzyme migrated (M_r = 63,000) as a single peak of activity expressable against both phosphorylase a and myosin light chains. Moreover, dephosphorylation of phosphorylase a by each of the active gradient fractions was stimulated four- to sixfold in the presence of a suboptimal concentration of polylysine (0.04 μM). However, in keeping with the data shown in Fig. 1, this concentration of polylysine virtually abolished light chain phosphatase activity (Fig. 2A).

Association of heat-stable regulatory factor with PCM-phosphatase. A heat-stable factor which reversed the modulatory effects of polylysine on PCM-phosphatase activities was present in the supernatant obtained from heated enzyme (Fig. 3). That is, polylysine-mediated stimulation of phosphorylase phosphatase activity was progressively reversed as the concentration of the heat-stable factor was increased in the reaction mixtures (Fig. 3A). Similarly, polylysine-mediated inhibition of

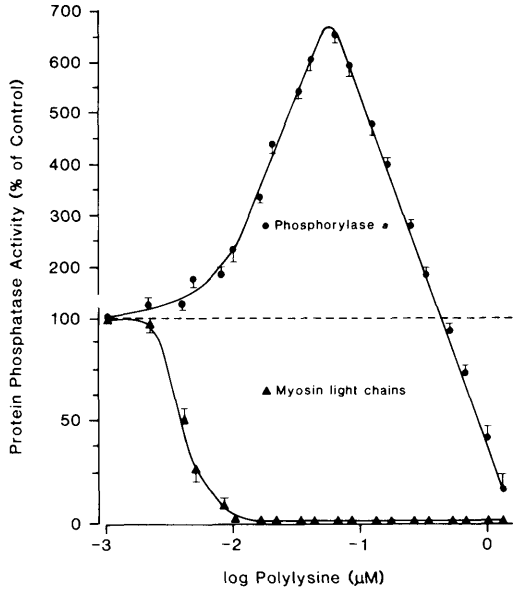

FIG. 1. Influence of polylysine (M_r = 13,000; 0.001–8.0 μM) on dephosphorylation of phosphorylase a (●, 10 μM) and phosphorylated cardiac myosin light chains (▲, 4 μM) by aortic PCM-phosphatase. Each point is the mean of three experiments. Small vertical bars show 1 SEM. Control, or basal, phosphorylase phosphatase activity was 2.4 ± 0.4 mU/ml assay, whereas basal light chain phosphatase activity was 18.1 ± 0.3 mU/ml assay.

light chain phosphatase activity was also reversed in the presence of the heat-stable factor (Fig. 3B). The data in Fig. 3 also show that the efficacy of the factor in reversing polycationic modulation of enzymic activity decreased when the concentration of polylysine tested was increased. Thus, although comparable stimulation of phosphorylase phosphatase activity occurred with 0.03 and 0.13 μM polylysine, the concentration of heat-stable factor required to reverse the stimulatory effect of polylysine by 50% was almost 10-fold higher (3.2 Ui/ml; see Fig. 3 and below for definition of inhibitory unit, Ui) in the presence of 0.13 μM polylysine than it was in the presence of 0.03 μM polylysine (0.35 Ui/ml). In similar fashion, 50% reversal of the inhibitory effects on light chain phosphatase activity required about a 15-fold (3.8 Ui/ml) greater concentration of the factor at the higher concentration of polylysine tested as compared to the lower concentration of the polycationic effector (0.25

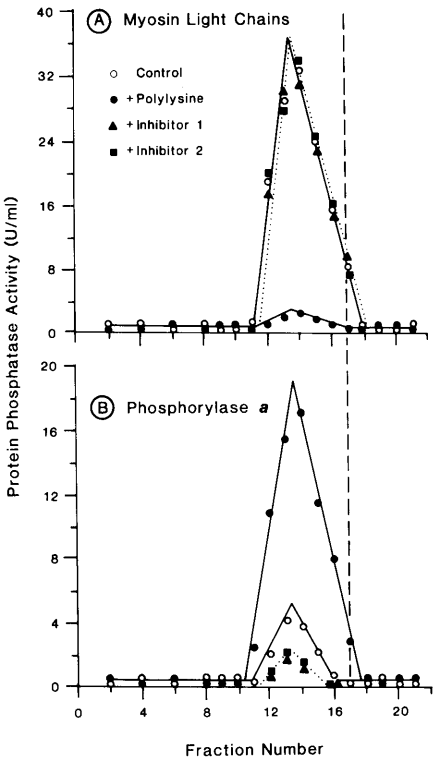

FIG. 2. Sucrose density centrifugation of PCM-phosphatase on a 4 ml 5–20% sucrose gradient. Twenty-three fractions of 170 μl each were collected. The dashed vertical line shows the peak migration of [^{14}C]ovalbumin (M_r = 40,000) which was used as an internal marker. (A) shows the migration of light chain phosphatase activity, while the migration of phosphorylase phosphatase activity is shown in (B). Fractions were assayed in the presence of either 0.04 μM polylysine (●), protein phosphatase inhibitor 1 (▲, 230 ng/ml), or inhibitor 2 (■, 132 ng/ml) and in the absence of any additive (○).

Ui/ml). In contrast, only a 2-fold increase in the concentration of the factor was needed for complete recovery of light chain phosphatase activity (i.e., 4 Ui/ml at 0.03 μM polylysine, and 8 Ui/ml at 0.15 μM polylysine).

Sucrose-density centrifugation of the PCM-phosphatase showed that the heat-stable factor was associated with the peak of enzymic activity (Fig. 4). Heat treatment of each of the gradient fractions exhibiting phosphatase activity revealed the presence of a factor which reversed polylysine-mediated inhibition of light chain phosphatase activity (Fig. 4A) and polylysine-mediated stimulation of phosphorylase phosphatase activity (Fig. 4B). However, centrifugation of the heated supernatant derived from the enzyme revealed that the migration of the *free* heat-stable factor (M_r = 30,000; Fig. 5) was much lower than that of the unheated enzyme (M_r = 63,000; Figs. 3 and 4) from which it was derived.

Comparative studies between heat-stable factor and protein phosphatase inhibitors 1 and 2. Inhibitor 1, inhibitor 2, and the heat-stable factor derived from the PCM-phosphatase

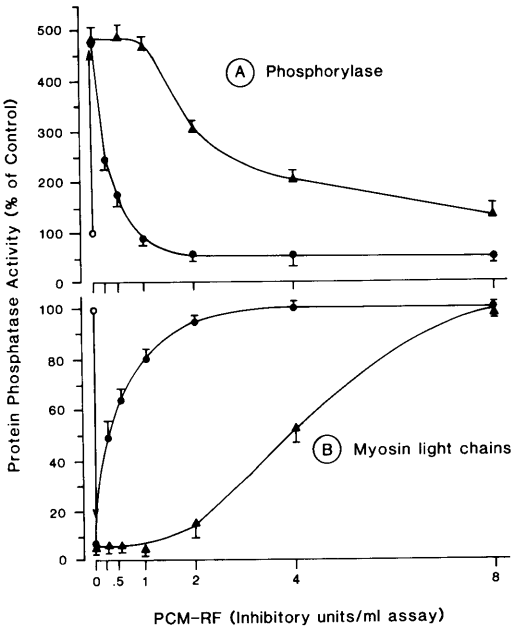

FIG. 3. The influence of heat-stable factor (PCM-RF, see under Methods) on polylysine-mediated modulation of phosphorylase (A) and light chain phosphatase activities (B) at a low (●, 0.03 μM) and high (▲, 0.13 μM) concentration of polylysine. Each point is the mean of three experiments and small vertical bars represent 1 SEM. Basal phosphorylase phosphatase activity (○, 1.6 ± 0.4 mU/ml assay) was stimulated (upward arrow) to about the same extent in the presence of the low (228 ± 12 mU/ml assay) and high (237 ± 14 mU/ml assay) concentrations of polylysine tested. Basal light chain phosphatase activity (○, 590 ± 7 mU/ml assay) was inhibited (downward arrow) to the same extent (28–30 mU/ml assay) in the presence of the two concentrations of polylysine tested. One unit of inhibitory activity is that amount of PCM-RF protein which inhibits 70 mU/ml of ATP·Mg-dependent phosphatase activity by 50% (see text).

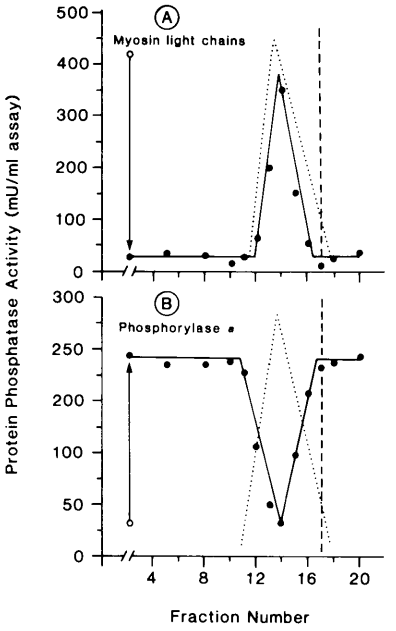

FIG. 4. Comigration of heat-stable factor (PCM-RF) with PCM-phosphatase. Results are from the same gradient shown in Fig. 2: the dotted peak in each panel shows the migration of PCM-phosphatase. Aliquots (50 μl) of gradient fractions were heated at 90°C for 10 min, cooled on ice, and tested for effects on polylysine-mediated modulation of PCM-phosphatase activity using an unheated sample of the enzyme. (A) Shows that basal light chain phosphatase activity (○; 240 mU/ml assay) was markedly reduced (downward arrow, ●; 32 mU/ml assay) in the presence of 0.03 μ*M* polylysine, and that this inhibition was reversed by heated fractions coincident with the initial peak of enzymic activity. (B) Shows that basal phosphorylase phosphatase activity (○; 24 mU/ml assay) was greatly stimulated (upward arrow, ●; 412 mU/ml assay) by 0.03 μ*M* polylysine and that such stimulation was also reversed by heated fractions coincident with the initial peak of enzymic activity. The dashed vertical line through each panel shows the migration of [^{14}C]ovalbumin (M_r = 40,000).

(PCM-RF) were similar to each other in that they all inhibited dephosphorylation of phosphorylase a by activated F_c (i.e., ATP • Mg-dependent phosphatase) in a concentration-dependent manner (Fig. 6A). In contrast, the three preparations differed markedly from each other with respect to their effects on *free* F_c (Fig. 6B). This form of the ATP • Mg-dependent phosphatase apparently is deficient in functionally intact inhibitor 2 (14, 27) so that activation of the enzyme by F_A/GSK-3 occurs only in the presence of added inhibitor 2 (see under Methods). In keeping with this requirement, inclusion of low concentrations of inhibitor 2 in the assay mixtures resulted in up to a fivefold increase in ATP • Mg-dependent phosphatase activity. However, reflecting the known inhibitory effect of inhibitor

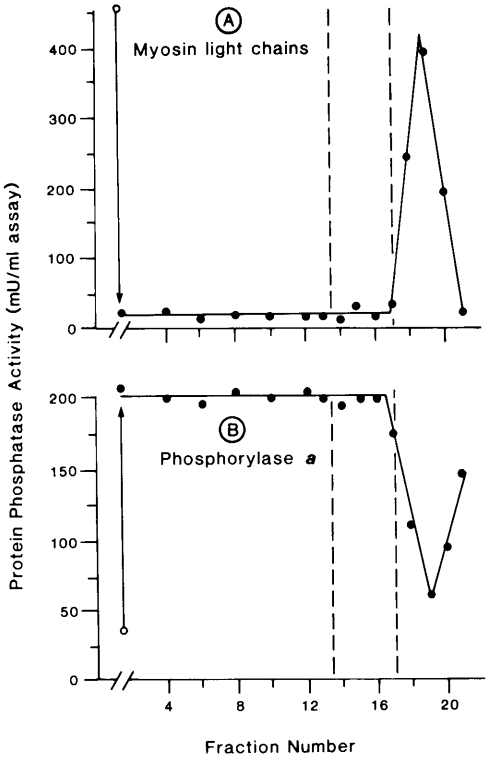

FIG. 5. Sucrose density centrifugation of heat-stable factor (PCM-RF) obtained from PCM phosphatase. Following centrifugation, the influence of an aliquot (15 μl) of each fraction on polylysine-mediated modulation of light chain (A) and phosphorylase phosphatase (B) was assessed. Basal light chain phosphatase activity (○; 452 mU/ml assay) was expectedly inhibited (downward arrow, ●; 27 mU/ml assay) in the presence of 0.03 μ*M* polylysine; however, this inhibition was reversed in the presence of appropriate fractions from the gradient. As shown in (B), basal phosphorylase phosphatase activity (○; 37 mU/ml assay) was stimulated almost sixfold (upward arrow, ●; 212 mU/ml assay) by the same concentration of polylysine (●). Such stimulation was reversed by same gradient fractions which reversed the inhibitory effect of polylysine on light chain phosphatase activity. The vertical dashed lines through each panel show the peak migration of unheated PCM-phosphatase (left, M_r = 65,000) and [^{14}C]ovalbumin (right, M_r = 40,000).

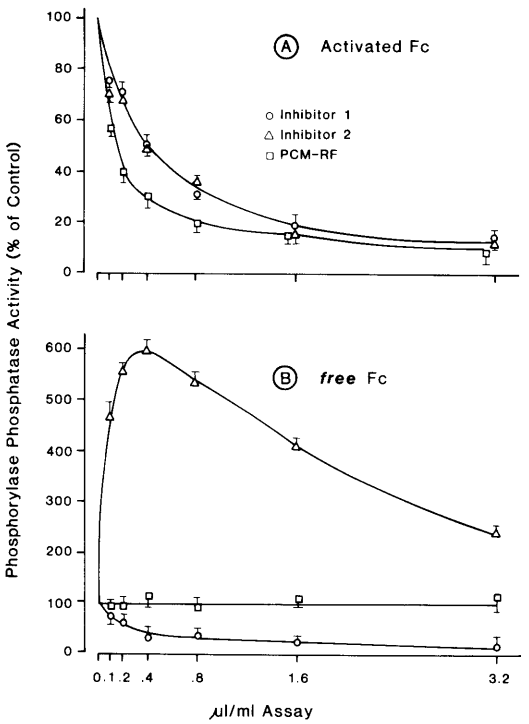

FIG. 6. (A) Shows the effects of protein phosphatase inhibitor 1 (○), inhibitor 2 (△), and PCM-RF (□) on phosphorylase phosphatase activity of aortic ATP·Mg-dependent phosphatase (activated F_c; 70 ± 6 mU/ml assay). Phosphorylase phosphatase activity prior to activation with F_A/GSK-3, ATP, and Mg^{2+} was 1.6 ± 0.8 mU/ml assay (see under Methods). (B) Shows the effects of the same preparations on phosphorylase phosphatase activity of *free* F_c, the inhibitor 2-deficient form of the ATP·Mg-dependent phosphatase. Control activity expressed in the presence of F_A/GSK-3, ATP and Mg^{2+} was only 0.8 ± 0.3 mU/ml assay. In both panels each point is the mean of three experiments and small vertical bars represent 1 SEM. The concentration of protein in the stock solutions were respectively 1.4 mg/ml (inhibitor 1), 0.8 mg/ml (inhibitor 2), and 1.5 mg/ml (PCM-RF).

2 on activated ATP·Mg-dependent phosphatase [Fig. 6A, Refs. (13–15)], activation of *free* F_c was much less pronounced as the concentration of added inhibitor 2 was increased further. Contrasting sharply with inhibitor 2, neither inhibitor 1 or PCM-RF promoted activation of *free* F_c (Fig. 6B). Instead PCM-RF exerted no influence on phosphorylase phosphatase activity measured in the presence of ATP, Mg^{2+} and F_A/GSK-3, whereas inhibitor 1 expectedly reduced activity.

To facilitate further comparisons between PCM-RF, inhibitor 1, and inhibitor 2 at normalized or effectively equivalent concentrations, we defined 1 unit of inhibitory activity (Ui) as that amount of protein in each preparation which inhibits 70 mU of ATP·Mg-dependent phosphorylase phosphatase activity/ml of assay (2 mU/assay). On this basis, basal light chain phosphatase activity expressed by the PCM-phosphatase was essentially unaffected by 0.1–8 Ui/ml of either PCM-RF, inhibitor 1, or inhibitor 2 (Fig. 7), whereas basal phosphorylase phosphatase was inhibited 40–60% by each of the preparations (Fig. 8). Thus, within the framework of the nomenclature suggested by Cohen *et al.* (15), the PCM-phosphatase behaved as an apparent inhibitor-insensitive type 2 phosphatase when light chains were used as substrate. In contrast, the enzyme behaved as an inhibitor-sensitive type 1 phosphatase when phosphorylase a was used as substrate. Nevertheless, this concomitant inhibitor-insensitive dephosphorylation of the light chains and inhibitor-sensitive dephosphorylation of phosphorylase was also evident following sucrose density centrifugation of the enzyme (Fig. 2).

PCM-RF (0.25–4 Ui) progressively and completely reversed the modulatory effects of 0.064 μM polylysine on PCM-phosphatase activity when either light chains (Fig. 7A) or phosphorylase a was used as substrate (Fig. 8A). However, underscoring the specificity of PCM-RF and a fundamental difference between it and the other two inhibitory proteins tested, neither inhibitor 1 (Figs. 7B and 8B) or inhibitors 2 (Figs. 7C and 8C) reversed the modulatory effects of polylysine.

Discussion. This study was guided by the hypothesis that polycationic modulation of PCM-phosphatase activity involves interactions between a regulatory component of the enzyme and polycationic effector such that phosphatase activity expressed against a given substrate is either enhanced (e.g., phosphorylase a) or suppressed (e.g., myosin light chains). We suspected that the putative regulatory component might be heat-stable because several purported regulators of certain phosphatases, such as protein phosphatase inhibitors 1 and 2, have been characterized as heat-stable proteins (11–15).

Two major points were developed from this

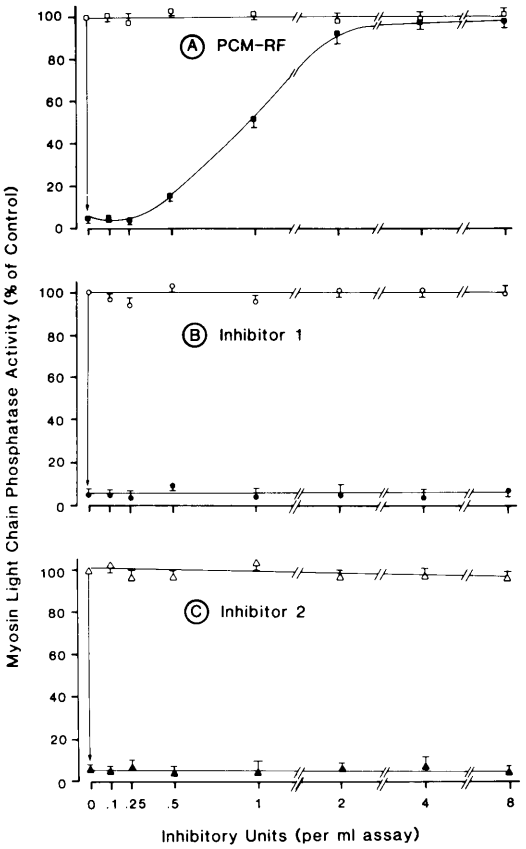

FIG. 7. Influence of PCM-RF (A), inhibitor 1 (B), and inhibitor 2 (C) on light chain phosphatase activity expressed by PCM-phosphatase in the presence (filled symbols) and absence (open symbols) of 0.064 μM polylysine. Basal activity (579 ± 9 mU/ml assay) was inhibited 93% (downward arrow, 40 ± 6 mU/ml assay in the presence of polylysine. Each point is the mean of three experiments and small vertical bars represent 1 SEM.

study. First, a heat-stable factor, PCM-RF, which is associated with the PCM-phosphatase, reverses the modulatory effects of polylysine on expression of phosphatase activity with respect to both phosphorylase a and myosin light chains (Figs. 3–5, 7, and 8). Second, no reversal of polylysine-mediated modulation occurs when either inhibitor 1 or inhibitor 2 is substituted for PCM-RF (Figs. 7 and 8). These results suggest that PCM-RF may be a regulatory component of the PCM-phosphatase which is different from inhibitor 1 or 2.

The comigration of PCM-RF and phosphatase activity during sucrose density centrifugation of the unheated enzyme (Fig. 3) indicates that PCM-RF is either an integral component of the PCM-phosphatase, or that the phosphatase and PCM-RF are tightly bound to each other. Although not distinguishing between these possibilities, sucrose density centrifugation of the supernatant from heated PCM-phosphatase showed that the apparent M_r of PCM-RF (Fig. 5, M_r = 30,000) is about half that of the unheated enzyme (Fig. 2, M_r = 63,000). Accordingly, it is tempting to speculate that the PCM-phosphatase may consist of a catalytic domain or subunit of about 30,000–35,000 Da, similar to that described for several other phosphatases (13–15, 24, 29) and a heat-stable regulatory domain or subunit of about 30,000 Da corresponding to PCM-

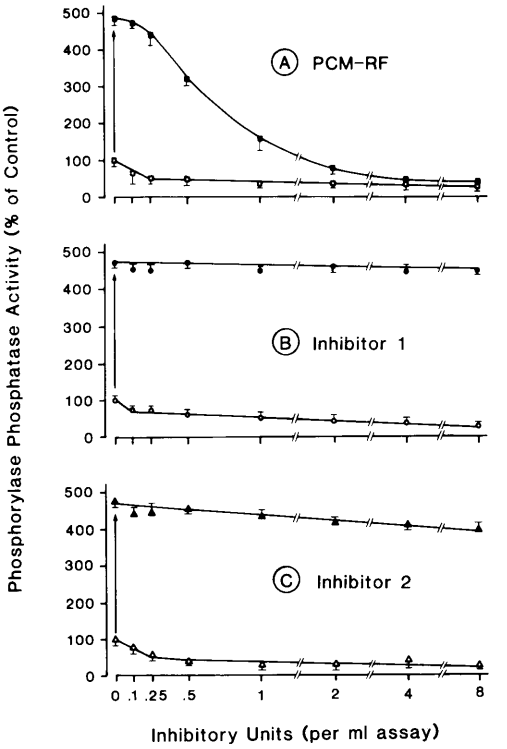

FIG. 8. Influence of PCM-RF (A), inhibitor 1 (B), and inhibitor 2 (Panel C) on phosphorylase phosphatase activity expressed by PCM-phosphatase in the presence (filled symbols) and absence (open symbols) of 0.064 μM polylysine. Basal phosphorylase phosphatase activity (73 ± 8 mU/ml assay) was stimulated almost sixfold (upward arrow, 426 ± 7 mU/ml assay) in the presence of polylysine. Each point is the mean of three experiments and small vertical bars represent 1 SEM.

RF. However, in the absence of additional data, such as definition of the subunit structure of the enzyme, this hypothesis must be viewed with caution.

It is likely that reversal of polylysine-mediated modulation of PCM-phosphatase activities by PCM-RF involves interactions between PCM-RF and polylysine. This inference is suggested by the observations that such reversal is independent of the substrate studied (Figs. 7 and 8), but dependent upon the concentration of polylysine tested (Fig. 4). Conceivably, interactions between PCM-RF and polylysine could effectively lower the free, or available, concentration of polylysine in the assay mixtures. Since the modulatory effects of polylysine on expressed PCM-phosphatase activity are concentration dependent (Fig. 1), an apparent decrease in the concentration of available polylysine should be associated with decreased inhibition of light chain phosphatase activity and decreased stimulation of phosphorylase phosphatase activity. However, the suggested interaction between polylysine and PCM-RF is probably complex. For example, the relationship between concentration of PCM-RF and reversal of polylysine-mediated inhibition of light chain phosphatase activity appears to be hyperbolic when a low concentration of polylysine is included in the assays (Fig. 4B). In contrast, the relationship appears to be sigmoidal when a high concentration of polylysine is tested, perhaps indicating that the suggested interaction between polylysine and PCM-RF involves cooperative mechanisms.

Studies reported from other laboratories have established that protein phosphatase inhibitor 1 and inhibitor 2 are different proteins (11–15). In this context, susceptibility to inhibition by inhibitors 1 and 2 has been advocated as a criterion for classification of protein phosphatases (15). Inhibitor sensitive enzymes such as the ATP · Mg-dependent phosphatase were categorized as *type 1* enzymes, whereas inhibitor insensitive enzymes were called *type 2* phosphatases. It appears that the PCM-phosphatase used in this and other studies in this laboratory (8, 9, 30, 31) can be fitted into each of the two groups because dephosphorylation of the light chains is inhibitor-insensitive (Fig. 7, i.e., type 2 behavior), while phosphorylase phosphatase activity in inhibitor-sensitive (Fig. 8, i.e., type 1 behavior). This apparent disparity may suggest that the PCM-phosphatase is contaminated with a type 1 phosphatase. Alternatively, the results may simply reflect a distinct property of the PCM-phosphatase used in this study. Several authors have called attention to the need for guarded caution in attempting to classify phosphatases on the basis of their sensitivity to inhibitors 1 and 2 (13, 29, 32, 34). Nevertheless, the present study shows that PCM-RF is different from both inhibitor 1 and inhibitor 2. Thus, only PCM-RF reversed polylysine-mediated modulation of PCM-phosphatase activity (Figs. 7 and 8), and only inhibitor 2 promoted activation of *free* F_c (Fig. 6B), the inhibitor 2-deficient form of the ATP · Mg-dependent phosphatase.

To our knowledge, only histone-H_1 has been identified as a naturally occurring polycationic modulator of the PCM-phosphatase. Further studies are required to establish whether other cellular modulators also exist. Nevertheless, since the PCM-phosphatase dephosphorylates aortic native myosin and thereby decreases actin–myosin interaction (30, 31, 35), the enzyme is likely to be involved in contraction–relaxation of smooth muscle. The present study shows that a new heat-stable factor, PCM-RF, which is associated with the PCM-phosphatase, may regulate its susceptibility to polycationic modulation. The results are also consistent with the hypothesis that expression of PCM-phosphatase substrate specificity could be ascribable to interactions between PCM-RF and polycationic effector.

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