PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184**, 64–73 (1987)

Glycosaminoglycans and a Newly Purified Aortic Chondroitin Proteoglycan Block Polycationic Modulation of Protein Phosphatase Activity (42447)

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Abstract. Recently, we described a bovine aortic phosphatase which we called PCM-phosphatase (**p**olycation **m**odulable) because its activity *in vitro* can be modulated by polycations such as polylysine and histone-H_1 (Di Salvo J, Gifford D, Kokkinakis A. Modulation of aortic protein phoshatase activity by polylysine. Proc Soc Exp Biol Med **177**:24–32, 1984). We suspected that polycationic modulation might be inhibited by polyanionic glycosaminoglycans. Accordingly, an aortic anionic substance was purified by sequential steps including (a) heating aortic extracts at 90°C, (b) precipitation of protein with $(NH_4)_2$ SO_4, and (c) anionic-exchange chromatography on a Mono Q HR 5/5 column using the Pharmacia fast protein liquid chromatography system. Electrophoresis (polyacrylamide–agarose) of the purified substance revealed one band which stained metachromatically with toluidine blue; however, no staining occurred with Coomassie blue. Electrophoretic mobility increased following proteolytic digestion of the substance with papain. The substance produced concentration-dependent reversal of polylysine-mediated inhibition of myosin light chain dephosphorylation, and it also reversed polysine-mediated stimulation of phosphorylase phosphatase activity expressed by PCM-phosphatase. Its ability to inhibit or reverse polycationic modulation was abolished after incubation with either chondroitinase AC or chondroitinase ABC. Based on these properties the substance was identified as a chondroitin proteoglycan. Commercially available glycosaminoglycans (heparin and chondroitin sulfates) also reversed polycationic modulation. The results show that modulation of phosphatase activity may be significantly modified by naturally occurring glycosaminoglycans. These studies may also have an important bearing on the purported roles of phosphatase(s) and glycosaminoglycans in calcification of soft tissues.

Diverse physiological mechanisms are regulated by phosphorylation and dephosphorylation of specific enzymes and regulatory proteins (see (1–4) for reviews). In principle, the extent to which a given protein is phosphorylated can be influenced by modulating activities expressed by kinases responsible for phosphorylation and/or phosphatases responsible for dephosphorylation.

Recent studies *in vitro* show that protein phosphatase activity can be altered markedly by polyions. For example, Wilson *et al.* reported that histone-H_1 stimulated dephosphorylation of phosphorylase *a* by a renal phosphatase preparation (5). Shortly afterward, we described an aortic phosphatase which was stimulated 6- to 15-fold by low concentrations of cationic histone-H_1 or polylysine when phosphorylase *a* was used as substrate (6, 7). However, phosphatase activity was virtually abolished when myocardial myosin light chains were used as substrate. We suggested that enzymes of this kind be referred to as polycation-modulable (PCM-) phosphatases. Further studies in this (8–10) and other laboratories (11) established that apparently different forms of PCM-phosphatase exist. The aortic PCM-phosphatase used in this and other studies also dephosphorylates structurally and functionally intact smooth muscle myosin (12–14). Dephosphorylation of myosin is associated with decreases in actin–myosin interaction as reflected by decreased actomyosin ATPase activity and enhanced relaxation of detergent-skinned smooth muscle fibers. In a subsequent report, Tung and Cohen showed that dephosphorylation of phosphorylase *a* by several skeletal muscle phosphatases belonging to the group which they call *type 2A* was stimulated with low concentrations of polycations (15). They also confirmed our earlier observation showing that dephosphorylation of phosphorylase by the catalytic subunit of *type 1* phosphatases was inhibited by low concen-

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trations of polycation (5). Thus, polycationic modulation of expressed enzyme activity may be a general property of type 2 phosphatases.

Differential modulation of phosphatases also occurs in response to polyanions such as the glycosaminoglycan heparin. Gergely *et al.* (16) reported that heparin inhibits phosphorylase phosphatase activity expressed by type 1 phosphatases and that such inhibition can be blocked by polybrene, a cationic heparin antagonist (17). However, whether polycation-mediated modulation of PCM-phosphatase activities is blocked by heparin or other glycosaminoglycans is unknown. Such information is of interest because of the purported role of phosphatases in promoting calcification of soft tissues (18) and also because of suspected functional relationships among glycosaminoglycans, proteoglycans, and calcification (19–22).

In this communication we report that several glycosaminoglycans block cationic modulation of aortic PCM-phosphatase activities. We also describe the purification and identification of an aortic chondroitin proteoglycan which effectively reverses polylysine modulation of PCM-phosphatase.

Materials and Methods. PCM-phosphatase (mol wt = 90,000; sucrose density centrifugation) was purified from bovine aortic muscularis as described previously (7–10). Basal phosphorylase phosphatase activity was 164 ± 21 U/mg and myosin light chain phosphatase activity was 1350 ± 33 U/mg(n = 4). In accordance with our earlier reports, low concentrations of L-polylysine (0.01–0.1 μM, mol wt = 13,000; Sigma) stimulated phosphorylase phosphatase activity 6- to 12-fold, whereas dephosphorylation of the light chains was virtually abolished.

Phosphorylase was prepared from rabbit skeletal muscle (23), while phosphorylatable myosin light chains were prepared from bovine myocardium (24). Substrates were phosphorylated as detailed earlier (7–10). Assays for phosphorylase phosphatase activity were performed at 30°C in a reaction mixture (30 μl) containing 20 mM Tris, pH 7.4, 5 mM caffeine, 0.5 mM dithiothreitol, 1 mg/ml bovine serum albumin (Sigma), and 10 μM [^{32}P]phosphorylase a (7). Assays for light chain phosphatase activity were performed under similar conditions except that caffeine was omitted from the reaction mixtures and the concentration of substrate was 4 μM (7). Since basal light chain phosphatase activity was six- to ninefold greater than phosphorylase phosphatase activity, light chain assays were performed for 3 min whereas phosphorylase assays were performed for 10 min using the same dilution of enzyme. Under these conditions one unit (U) of phosphatase activity is that amount of enzyme which releases 1 nmole ^{32}P/min from the substrate tested. Specific activities were expressed as U/mg protein. Protein was determined by the method of Lowry *et al.* (25) using bovine serum albumin as standard.

The influence of different glycosaminoglycans (μg) and aortic proteoglycan (μg protein) on expressed PCM-phosphatase activities were assessed in the presence and absence of 32 nM polylysine. This concentration of polylysine stimulated phosphorylase phosphatase activity 600–800% and reduced light chain phosphatase activity by about 90% (7–10). All assays were started by addition of substrate to the reaction mixture after a 10-min preincubation of the PCM-phosphatase in the presence and the absence of polylysine and/or glycosaminoglycan. Where appropriate, additional procedural details are given in figure and table legends. Heparin, heparinase, chondroitin sulfates A, B, and C, chondroitinase AC, and chondroitinase ABC were purchased from Sigma; papain was obtained from Boehringer–Mannheim. The concentrations of heparinase or chondroitinases used were expressed in units of activity as defined by the supplier.

To purify aortic proteoglycan, 400 g of aortic muscularis was homogenized in 4 vol of 50 mM Tris, pH 8.0, 0.5 mM dithiothreitol (DTT), 0.5 mM benzamidine, 0.1 mM Na-p-tosyl-L-lysine chloromethyl ketone, 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine tetraacetic acid, and 2 mM ethylene-bis-(β-aminoethyl ether), N,N'-tetraacetic acid at 4°C. Following centrifugation (6000g, 45 min), the supernatant was filtered through glass wool, rapidly heated to 90°C, cooled on ice, and centrifuged to remove coagulated protein. Protein in the resulting supernatant was precipitated with 25–75% $(NH_4)_2SO_4$, redissolved in 25–50 ml of 0.5 mM DTT and 20 mM Tris, pH 7.4 (Buffer A), and extensively dialyzed against the same

buffer. Following dialysis, the solution was centrifuged to remove undissolved protein and the supernatant was interacted with 100 ml of DEAE-Sephacel equilibrated in Buffer A. The Sephacel was washed with 0.1 *M* NaCl–buffer A until the absorbance of the flow-through at 280 n*M* was less than 0.05, and packed into a column (2.5 × 19 cm). Adsorbed protein was eluted with a linear gradient of 0.1–0.8 *M* NaCl in Buffer A (total vol = 600 ml). As described under Results, the ability of the proteoglycan to reverse polylysine-mediated stimulation of phosphorylase phosphatase activity was exploited in order to monitor its presence in eluted fractions. Selected fractions were pooled, dialyzed against Buffer A, and subjected to one cycle of anion-exchange chromatography on a Mono Q HR 5/5 column using the Pharmacia fast protein liquid chromatography system. Bound protein was eluted with a 48-ml linear gradient of 0.6 to 1.3 *M* NaCl–Buffer A. Appropriate fractions were pooled, dialyzed against Buffer A, concentrated by dialysis against Buffer A containing 50% glycerol, and stored in the same solution at −60°C. Electrophoresis on polyacrylamide–agarose gels, according to the method of McDevitt and Muir (26), was used to assess the purity of the preparations.

Results. *Influence of glycosaminoglycans on polycationic modulation of PCM-phosphatase activity.* Heparin, and each of three isomers of chondroitin sulfate, produced concentration-dependent blockade of the stimulatory effect of polylysine on dephosphorylation of phosphorylase *a* and its inhibitory effect on light chain phosphatase activity expressed by the PCM-phosphatase (Fig. 1). Similar concentrations (40–80 ng/ml) of chondroitin sulfate B, chondroitin sulfate C, or heparin were required to reduce the modulatory effects of polylysine by 50% with respect to either of the two substrates tested. Moreover, when the concentration of polylysine was increased, the concentration of glycosaminoglycan required for 50% blockade or reversal of modulation increased in parallel fashion (Table I). For example, as shown in previous studies (7–10) and in Table I, comparable stimulation of phosphorylase phosphatase activity occurred in the presence of 0.03 and 0.13 μM polylysine. However, about five times as much chondroitin sulfate B was needed for 50% reversal

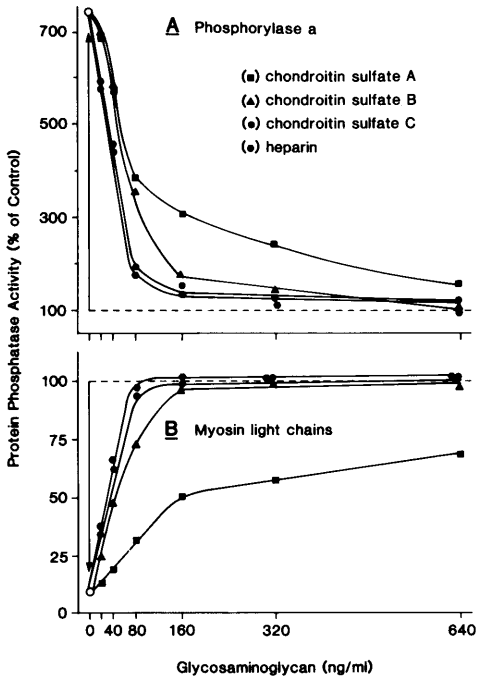

FIG. 1. Influence of glycosaminoglycans on polylysine-mediated modulation of phosphorylase (A) and myosin light chain (B) phosphatase activities expressed by PCM-phosphatase. In each panel, basal activities are shown as 100% of control (dashed horizontal lines). Basal phosphorylase phosphatase activity (62 mU/ml) was stimulated about 7.5-fold in the presence of 32 n*M* polylysine (A, upward arrow), whereas basal light chain phosphatase activity (525 mU/ml) was inhibited by about 90% in the presence of the same concentration of polylysine (B, downward arrow). As indicated, the glycosaminoglycans tested were chondroitin sulfate A (■), chondroitin sulfate B (▲), chondroitin sulfate C (●), and heparin (●).

at 0.13 μM polylysine than at 0.03 μM polylysine.

Complete reversal of both polylysine-mediated stimulation of phosphorylase phosphatase activity and inhibition of light chain phosphatase activity occurred with either heparin or chondroitin sulfates B and C (Fig. 1). In contrast, reversal of modulation with chondroitin sulfate A was incomplete over the range of concentrations tested.

Purification and influence of aortic chondroitin proteoglycan on polycationic modulation of PCM-phosphatase activity. Ion-exchange chromatography of a heated aortic extract on DEAE-Sephacel yielded a broad polydisperse peak of phosphorylase phospha-

TABLE I. THE INFLUENCE OF THE CONCENTRATION OF POLYLYSINE (13 kDa) TESTED ON THE CONCENTRATION OF CHONDROITIN SULFATE B (CSB) OR AORTIC PROTEOGLYCAN (PG) REQUIRED FOR 50% REVERSAL ($R_{0.5}$) OF CATIONIC MODULATION OF PCM-PHOSPHATASE ACTIVITY

Assay conditions		Phosphatase activity (mU/ml assay)	$R_{0.5}$	
Substrate	Polylysine (μM)		CSB (ng/ml)	PG (ng/protein/ml)
Phosphorylase a	0	53 ± 9		
	0.03	328 ± 13	68 ± 14	70 ± 12
	0.13	352 ± 15	335 ± 17	356 ± 17
Myosin light chains	0	402 ± 15		
	0.03	12 ± 4	72 ± 15	71 ± 14
	0.13	9 ± 3	352 ± 22	348 ± 19

Note. The values given are the means ± SE for four experiments.

tase activity expressed by aortic PCM-phosphatase (Fig. 2A). This polydispersity persisted during chromatography on a Pharmacia Mono Q HR 5/5 anion-exchange column (Fig. 2B). Nevertheless, only a single band, which stained metachromatically with toluidine blue, was revealed when the purified material was subjected to electrophoresis on polyacrylamide–agarose gels (Fig. 2C). However, no bands were revealed when Coomassie blue was used in place of toluidine blue (not shown).

The purified material reversed the modulatory effects of polylysine on expressed PCM-phosphatase activity in a concentration-dependent manner when either phosphorylase *a* (Fig. 3A) or phosphorylated myosin light chains were used as substrate (Fig. 3B). The concentration of the material needed for 50% reversal of the stimulatory effect of 0.03 μM polylysine on phosphorylase phosphatase activity (84 ng protein/ml) was virtually identical to the concentration needed for 50% reversal of the inhibitory effect of polylysine on expressed light chain phosphatase activity (80 ng protein/ml). In accordance with results obtained in the presence of glycosaminoglycans, about a fivefold increase in the concentration of the material was required for 50% reversal of modulation when the concentration of polylysine was increased to 0.13 μM (Table I).

We suspected that the purified material might be a proteoglycan because of its apparent polydispersity during ion-exchange chromatography and its unusual staining characteristics (Fig. 2). To gain insight into this possibility we studied the influence of proteolytic digestion with papain and incubation with enzymes which degrade glycosaminoglycans (e.g., chondroitinases and heparinase) on several properties of the purified material. In this context, the behavior of the material during electrophoresis was altered markedly following incubation with either papain or chondroitinase (Fig. 4). Additionally, however, the purified material also protected each of the two hydrolyases tested against heat-induced denaturation. Thus, reflecting its susceptibility to proteolytic degradation, the mobility of the material was increased significantly after 2 hr of incubation with unheated papain (Fig. 4, gel A′). Surprisingly, however, the same change in mobility occurred when papain and purified material were heated immediately after mixing (Fig. 4, gel D′). In contrast, no change in mobility was evident after 2 hr of incubation with papain which had been heated before mixing it with the purified material (Fig. 4, gel B′).

No change in either electrophoretic mobility or intensity of staining with toluidine blue occurred after 2 hr of incubation with heat-denatured chondroitinase (Fig. 4, gel B). However, the intensity of staining was reduced drastically after incubation with unheated chondroitinase (Fig. 4, gel A). This suggests that the chondroitinase had extensively hydrolyzed the purified material. Interestingly, two metachromatic bands were revealed in mixtures of the purified material and chondroitinase which were heated immediately after mixing (Fig. 4, gel D). One of these bands, probably corresponding to unhydrolyzed material, exhibited no change in electrophoretic

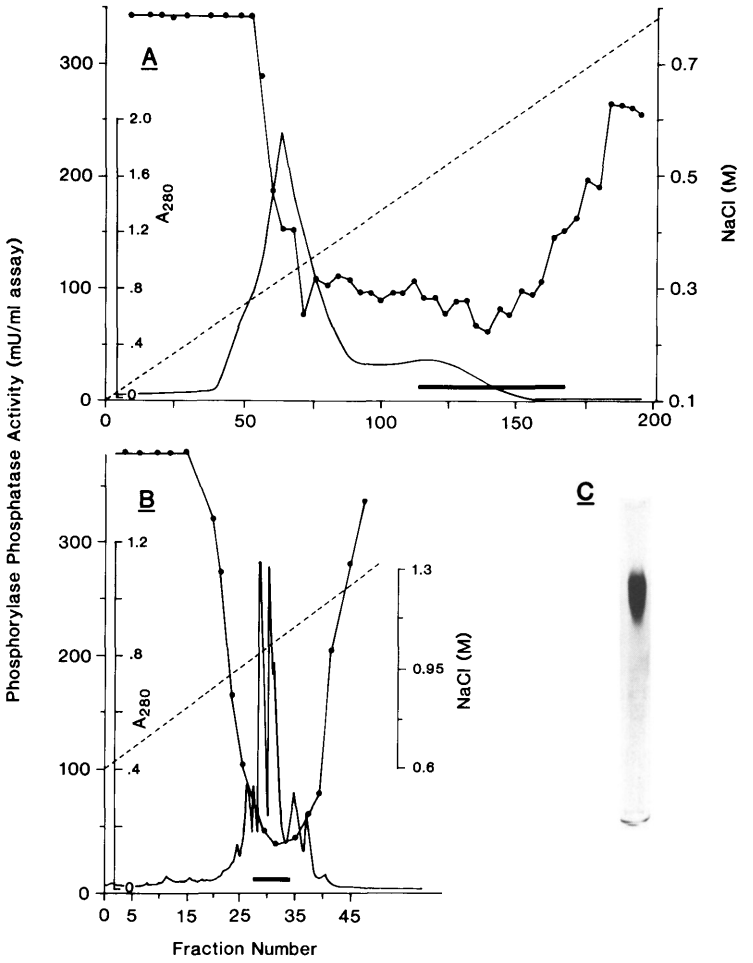

FIG. 2. Purification of a substance from bovine aortic muscularis which reverses polylysine-mediated stimulation of dephosphorylation of phosphorylase *a* by PCM-phosphatase. (A) Elution profile of a heated aortic extract subject to ion-exchange chromatography on DEAE-Sephacel. Fractions included by the horizontal bar were pooled, dialyzed to remove salt, and chromatographed on an HR 5/5 anion-exchange column (B). Active material was identified by its ability to reverse polylysine-mediated stimulation of phosphorylase phosphatase activity expressed by PCM-phosphatase. Fractions were diluted and assayed in a mixture (30 μl) containing 42 mU/ml PCM-phosphatase, 32 n*M* polylysine, and 10 μM phosphorylase *a*. Expressed stimulated phosphorylase phosphatase activity was 348 mU/ml. Further details are given under Materials and Methods. (C) A polyacrylamide–agarose electrophoretic gel of the purified product (20 μg protein) stained with toluidine blue.

mobility. The other band, probably corresponding to a hydrolytic product of the purified material, exhibited a marked increase in mobility. No change in electrophoretic mobility, intensity of staining with toluidine blue, or efficacy in reversing polylysine modulation of PCM-phosphatase activity occurred when heparinase (20–300 mU/ml) was used in place of chondrotinase AC or chondroitinase ABC. Collectively, these observations allow for identification of the active material as an aortic chondroitin proteoglycan.

Digestion of the proteoglycan with chondroitinase AC or chondroitinase ABC produced concentration and time-dependent decreases in its ability to reverse polylysine modulation of PCM-phosphatase activity (Figs. 5 and 6). Furthermore, these decreases in effi-

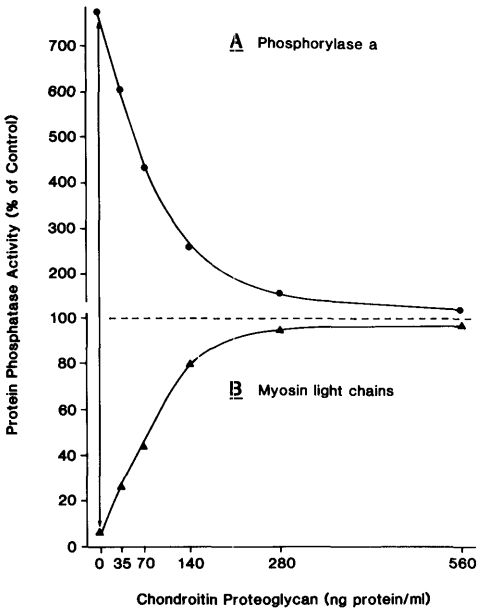

FIG. 3. Reversal of polylysine-mediated stimulation of phosphorylase phosphatase activity (A) and polylysine-mediated inhibition of myosin light chain phosphatase activity (B) by the active material purified in Fig. 2. Basal activities are expressed as 100% of control (dashed horizontal line): basal phosphorylase phosphatase activity was 32 mU/ml, whereas basal light chain phosphatase activity was 460 mU/ml. Phosphorylase phosphatase activity was stimulated almost eightfold by 32 n*M* polylysine (A, upward arrow), while light chain phosphatase activity was inhibited by about 95% (B, downward arrow). Though not shown, no change in basal phosphorylase or light chain phosphatase activity occurred with any concentration of the substance tested.

cacy were associated with marked changes in electrophoretic behavior. For example, prior to digestion about 75 ng of proteoglycan/ml was required for 50% reversal of the stimulatory effect of polylysine on expressed phosphorylase phosphatase activity (Fig. 5A), and only a single intensely stained band was visualized following electrophoresis (Fig. 5B). Following digestion of the proteoglycan with a low concentration of chondroitinase AC (20 mU/ml) about a fivefold increase in the concentration of proteoglycan (350 ng/ml) was required for 50% reversal of polylysine modulation. This change was associated with an apparent decrease in the intensity of the proteoglycan band and the appearance of a second metachromatic band of greater mobility. Digestion with a high concentration of chondroitinase AC (320 mU/ml) completely abolished its ability to reverse polylysine stimulation of phosphorylase phosphatase activity. This effect was associated with the disappearance of metachromatically staining proteoglycan. Qualitatively similar results were obtained when the proteoglycan was digested for different periods of time with an intermediate concentration of chondroitinase AC (40 mU/ml; Fig. 6).

Discussion. A major point of this study is that glycosaminoglycans and a newly purified aortic proteoglycan reverse the modulatory effects of polylysine on PCM-phosphatase activity. Several lines of evidence suggest that such reversal involves interactions between polycationic polylysine and polyanionic glycosaminoglycans or proteoglycan. First, reversal or blockade of enzymic modulation was

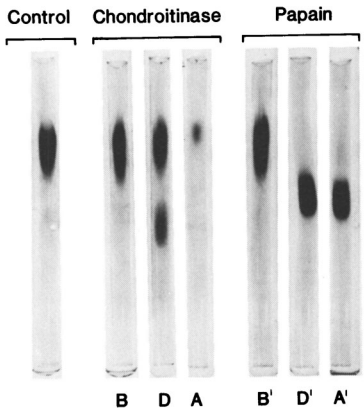

FIG. 4. Changes in electrophoretic behavior of substance purified in Fig. 2 (20 μg protein) after incubation with either chondroitinase AC (80 mU/ml) or papain (25 μg/ml). A control sample of the purified material (no added chondroitinase AC or papain) subjected to electrophoresis on a polyacrylamide–agarose gel is shown at left. Test gels are identified as follows: (B) sample incubated for 2 hr at 30°C with chondroitinase AC which had been denatured by heating at 90°C at 10 min; (D) mixture of sample and chondroitinase heated immediately after mixing (90°C/10 min) and then incubated at 30°C for 2 hr; (A) mixture of sample and chondroitinase AC incubated for 2 hr at 30°C and then heated for 10 min at 90°C; (B') sample incubated with heat-denatured papain; (D') mixture of sample and papain heated immediately after mixing and then incubated; (A') mixture of sample and papain heated after 2 hr of incubation. All gels were stained with toluidine blue.

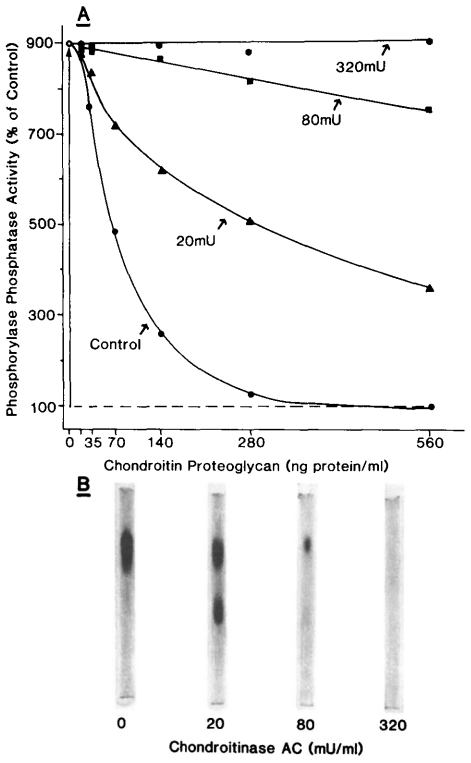

FIG. 5. Influence of preincubation of the purified material (chondroitin proteoglycan, see text) with different concentrations of chondroitinase AC for 2 hr at 30°C on efficacy in reversing polylysine stimulation of phosphorylase phosphatase activity (A) and electrophoretic behavior (B). Basal phosphorylase phosphatase activity (48 mU/ml, 100% of control) of the PCM-phosphatase was stimulated ninefold in the presence of 32 n*M* polylysine (A, upward arrow). Following incubation with the concentration of chondroitinase AC indicated, mixtures were heated at 90°C for 10 min and stored on ice: an aliquot of the mixture was diluted serially and assayed for ability to reverse polylysine stimulation of phosphorylase phosphatase activity (A), and a different aliquot corresponding to 20 μg of initial proteoglycan protein was subjected to electrophoresis on polyacrylamide–agarose gels. Virtually identical results were obtained when chondroitinase ABC was used in place of chondroitinase AC.

independent of the substrate tested. That is, each of the polyanions effectively reversed polylysine-mediated stimulation of phosphorylase phosphatase activity and polylysine-mediated inhibition of light chain phosphatase activity (Figs. 1 and 3). Second, although the concentration of phosphorylase *a* (10 μM) in the assay mixtures was 2.5fold greater than that of the light chains (4 μM), the concentration of a given polyanion required for 50% reversal of polylysine-modulation was virtually the same for the two phosphorylated substrates (Table I, Fig. 3). Collectively, these observations argue against the possibility that glycosaminoglycan- or proteoglycan-induced reversal of polylysine modulation is due to a substrate-directed mechanism. Third, an enzyme-directed mechanism also seems unlikely because no change in basal PCM-phosphatase activities was evident at concentrations of polyanion which blocked polylysine modulation. Fourth, direct interaction between po-

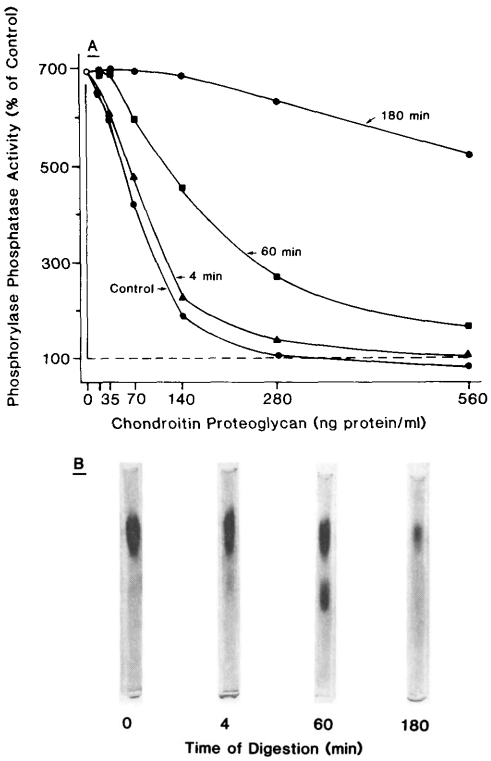

FIG. 6. Influence of preincubation of the purified material (chondroitin proteoglycan; see text) with 40 mU/ml of chondroitinase AC for different times on efficacy in reversing polylysine stimulation of phosphorylase phosphatase activity (A) and electrophoretic behavior (B). Basal phosphorylase phosphatase activity (54 mU/ml, 100% of control) of the PCM-phosphatase was stimulated sevenfold in the presence of 32 n*M* polylysine (A, upward arrow). Reaction mixtures were processed as described in Fig. 5. Essentially the same results were obtained when chondroitinase ABC was substituted for chondroitinase AC.

lyanion and cationic polylysine is also indicated by the observation that the concentration of polyanion required for 50% reversal of either polylysine-mediated stimulation of phosphorylase phosphatase activity or polylysine-mediated inhibition of light chain phosphatase activity increased about fivefold when the concentration of polylysine was increased fivefold (Table I).

Moreover, studies in other laboratories have established that direct interactions between polylysine and glycosaminoglycans do occur. For example, Suzuki and Koide recently reported that binding of heparin to polylysine–Sepharose can be used to advantage in separating heparins of different chain length (27). Similarly, earlier spectropolarimetric studies reported by Gelman *et al.* (28) showed that ionic bonds form between the sulfate groups of chondroitin sulfate C and polylysine. Accordingly, it is reasonable to conclude that chondroitin sulfate A, chondroitin sulfate B, and the aortic proteoglycan studied can also interact directly with polylysine. Such ionic interactions would effectively reduce the concentration of free polylysine available for modulation of PCM-phosphatase activity in the reaction mixtures. Within this framework, polylysine-mediated stimulation of phosphorylase phosphatase activity and inhibition of light chain phosphatase activity would be progressively reversed as the concentration of polyanion is increased (Figs. 1 and 3). In this context, it is noteworthy that previous studies have shown that the modulatory effects of polylysine are concentration dependent and related to the number of cationic sites present in polymers of different chain length (7–10).

Another point of this study was the purification and identification of a material in aortic extracts which reversed the modulatory effects of polylysine on PCM-phosphatase activity as a chondroitin proteoglycan. We suspected that the material was a proteglycan because of its polydisperse elution pattern during anion-exchange chromatography, the relatively high salt concentration required for elution and its metachromatic staining with toluidine blue (Fig. 2). These properties are characteristic of a variety of proteoglycans, including those reportedly present in aortic tissue (26, 29–34). Several reports have shown that different types of proteoglycans, including chondroitin 4- and chondroitin-6-sulfate, dermatan sulfate, heparan, and hyaluronate, are present in aortic tissue from different species (29–31). Susceptibility to degradation with chondroitinase AC of the material which we purified strongly suggests that it is a chondroitin proteoglycan free of dermatan sulfate (i.e., chondroitin sulfate B; Figs. 4–6). However, further studies are required to characterize the chondroitin proteoglycan purified in this study with respect to structure and composition of glycosaminoglycan chains, core protein, and interactions with other macromolecules.

The present data show that this aortic proteoglycan blocks the modulatory effects of polylysine on enzymic activities expressed by PCM-phosphatase (Fig. 3) and that such blockade probably involves interactions between the proteoglycan and polylysine (Table I). The persistence of this activity following proteolysis with papain probably is attributable to preserving the glycosaminoglycan side chains. That is, incubation with papain probably produces degradation of the core protein without altering the structural integrity of the anionic glycosaminoglycan side chains. This inference is supported by the observation that digestion with papain was associated with an increase in electrophoretic mobility of the proteoglycan without altering its metachromatic staining with cationic toluidine blue (Fig. 3). In contrast, time- and concentration-dependent digestion with chondroitinase AC was associated with progressive loss of metachromasia and ability to reverse polylysine modulation of PCM-phosphatase activity (Figs. 4–6). Thus, maintenance of the structural integrity of the anionic glycosaminoglycan side chains, reflected by persistent metachromasia (26, 33), probably is required for reversal of cationic modulation of expressed phosphatase activity.

Glycosaminoglycans and proteoglycans are generally considered to be located extracellularly or bound to the external surface of the plasma membrane (32, 34), whereas the PCM-phosphatase appears to be a soluble cytosolic enzyme (5–12). Accordingly, the physiological significance of the effects of these polyanions on modulated phosphatase activity is obscure. However, as recently reviewed by Anderson (18), progressive development of calcific diseases, including arterial calcification, is

thought to involve extrusion of cellular matrix vesicles containing cytosolic phosphatase activity. The phosphatases, apparently acting on phosphoproteins in close proximity to the vesicles, are believed to increase the local concentration of inorganic phosphates, thereby promoting deposition of insoluble phosphates such as hydroxylapitite. Moreover, chondroitin proteoglycans and other polyanions retard growth and sedimentation of calcium phosphates precipitating *in vitro* (19–22), and *in vivo* calcification of cartilage is associated with proteoglycan degradation (33, 35). Conceivably, the actions of chondroitin proteoglycans and related glycosaminoglycans on modulated phosphatase activity described in this study, as well as the reported effects of these polyanions on formation and deposition of hydroxylapetite, may contribute to progressive calcific vascular disease. Though speculation of this kind must certainly be viewed with guarded caution, the idea is an interesting working hypothesis for designing future studies. In addition, the efficacy of polyanions in reversing cationic modulation of PCM-phosphatase activity should prove useful as a tool for purifying cellular modulators of the enzyme(s).

This study was supported by NIH Grants HLB 20196 and HLB 22619. We are grateful to Ms. A. Kokkinakis for excellent technical assistance and to Ms. A. Tolle for typing the manuscript.

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Received July 21, 1986. P.S.E.B.M. 1987, Vol. 184.
Accepted September 26, 1986.