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Modulation of Aortic Protein Phosphatase Activity by Polylysine (41907)

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The Ca^{2+}-regulatory mechanism for contraction of smooth muscle involves phosphorylation of the 20,000-Da myosin light chains. In the fully relaxed muscle the intracellular concentration of Ca^{2+} is low ($<10^{-9}$ M) and no interaction between the contractile proteins, actin and myosin, is evident. Following excitation, however, release of Ca^{2+} from intracellular storage sites and influx of Ca^{2+} from the extracellular space through voltage-dependent and receptor-operated channels in the sarcolemma results in a marked increase in the intracellular concentration of Ca^{2+} ($>10^{-6}$ M). Subsequently, formation of the Ca^{2+}–calmodulin complex results in activation of myosin light-chain kinase and phosphorylation of the regulatory light chains. In this form, actin–myosin interaction is activated resulting in hydrolysis of ATP and cross-bridge cycling. The physiological correlate of these biochemical events is contraction of the smooth muscle cell (see (1–4) for reviews). Although recent studies suggest that cross bridges may remain attached after dephosphorylation of the light chains (5), cyclic phosphorylation and dephosphorylation appear to be requisite for recurring contraction and relaxation of smooth muscle.

Phosphorylase, the rate-limiting enzyme in glycogenolysis, is also phosphorylated during contraction of a variety of smooth muscles (6–9). Moreover, recent studies suggest that glycogenolysis may participate in generating ATP required for contraction of vascular smooth muscle (10). Accordingly, phosphatases which are effective in dephosphorylating phosphorylase *a* and the myosin light chains may function in coordinating metabolism and contractility in smooth muscle (11–13).

Smooth Muscle Phosphatases. *Gizzard.* At least four phosphatases, designated SMPI–IV, have been identified in the smooth muscle of turkey gizzard (14–17). Two of these have been purified to apparent homogeneity: SMPI consists of three subunits (M_r = 65,000; 50,000; 38,000) and SMPII is a Mg^{2+}-dependent monomeric enzyme (M_r = 43,000) which appears to be closely related to a phosphatase isolated from rat liver (18) and bovine myocardium (19). Both SMPI and SMPII exhibited high activity against isolated myosin light chains and relatively low activity against phosphorylase *a*. However, neither SMPI nor SMPII were effective in dephosphorylating native myosin so that their functional significance in terms of modulating actin–myosin interaction is obscure. In contrast, both SMPIII, an enzyme of unknown structure, and SMPIV (M_r = 58,000; 40,000) were reportedly active against native myosin.

Onishi *et al.* (20) also purified a trimeric phosphatase (M_r = 67,000; 54,000; 34,000) from turkey gizzard which resembled Pato's SMP-I. However, the enzyme described by Onishi was active against native myosin and inhibited actin–myosin interaction.

Bovine aorta. Multiple forms of protein phosphatase(s) have also been identified in mammalian vascular smooth muscle (11–13, 21–24). Werth *et al.* (21) described an aortic phosphatase consisting of two subunits (M_r = 67,000; 38,000) which was active against native myosin, but exhibited relatively low activity against phosphorylase *a*. This enzyme, however, reportedly required unphysiological concentrations of Mn^{2+} or Co^{2+} for activity.

We identified several protein phosphatases in aortic smooth muscle which are pertinent to this discussion (11–13, 22–25). One of these was the multisubstrate ATPMg-dependent protein phosphatase known to be present in a variety of tissues (12). This phosphatase, effective in dephosphorylating phosphorylase *a*, glycogen synthase, phosphorylase kinase, and other proteins, is apparently the inactive form of the major physiologically relevant phosphatase involved in coordinating glycogen synthesis and breakdown, and it appears to be subject to hormonal regulation (see (26, 27) for reviews). However, ATPMg-dependent phosphatase had relatively low activity against isolated light chains (13).

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Whether or not this enzyme can dephosphorylate native myosin, and therefore participate in modulating actin–myosin interaction, is unknown.

The second aortic phosphatase which we identified was about five times more active against isolated light chains than it was when phosphorylase *a* was used as substrate (13, 22). Enzymatic activity was unaffected by either inhibitor 1 or inhibitor 2 (modulator protein) suggesting that it was a type 2 phosphatase (28). The enzyme was effective in dephosphorylating myosin in aortic native actomyosin and it inhibited actin–myosin interaction (22). More recent studies suggested that this enzyme was identical to an aortic phosphatase whose activity against phosphorylase *a* was markedly stimulated by polylysine. That is, subsequent to a report by Wilson *et al.* (29) showing that histone H1 could stimulate the activity of a phosphorylase phosphatase isolated from porcine kidney, we identified an H1-stimulable phosphorylase phosphatase in bovine aorta (23). We also reported that synthetic polylysine could effectively substitute for lysine-rich histone H1 and that stimulation with either of these effectors was limited to phosphatases which were insensitive to both modulator protein (inhibitor 2) and inhibitor 1 (i.e., type 2 phosphatases). However, when myosin light chains were used as substrate, dephosphorylation was markedly inhibited (see below). Since this phosphatase is active against both phosphorylase *a* and native myosin we believe that it may be functionally involved in coordinating arterial metabolism and contractility. We also believe that modulation of the enzyme by cationic effectors such as polylysine or histone H1 provides meaningful insight into potential regulatory mechanisms operative *in vivo*.

Studies with Polylysine-Modulated Phosphatase. *Dephosphorylation of phosphorylase a and cardiac myosin light chains.* In four different aortic enzyme preparations studied to date we found that myosin light-chain phosphatase activities (800–1200 U/mg protein) were three- to sixfold greater than activities measured against phosphorylase *a* (200–260 U/mg). However, when assays were performed in the presence of either lysine-rich histone H1 (5–10 μg/ml) or synthetic polylysine (13,000 Da, 2–5 μg/ml) phosphorylase phosphatase activity was stimulated 6- to 10-fold. In contrast, myosin light-chain phosphatase activity was virtually abolished by either histone H1 or polylysine.

Electrophoresis of the phosphatase under nondenaturing conditions revealed the presence of a major and minor peak of enzymatic activity (Fig. 1). In each instance, enzyme extracted from the electrophoretic gel was effective in dephosphorylating both phosphorylase *a* (Fig. 1A) and myosin light chains (Fig. 1B). Basal activity expressed against the light chains was about threefold greater than activity expressed against phosphorylase *a*. Polylysine inhibited myosin light-chain phosphatase activity, but markedly stimulated phosphorylase phosphatase activity. Though not shown, similar results were obtained when histone H1 was substituted for polylysine. The pH optimum for basal phosphorylase phosphatase activity (pH 6.8–7.4) was the same for both the major and minor peaks of activity. Moreover, the time course for thermal inactivation at either 45 or 50°C was also the same for each of the two peaks. However, further studies are required to establish whether or not the two peaks represent different forms of the same enzyme. Nevertheless, the findings are consistent with the view that a single catalytic species can dephosphorylate both phosphorylase *a* and the myosin light chains, and that phosphatase activities against these substrates are differentially affected by polylysine.

We would like to suggest that histone H1 or a polylysine-like effector of phosphatase activity functions in the cell. Modulation of phosphatase activity by such an effector, with apparently bifunctional attributes, may be important in regulating the substrate specificity of a multisubstrate enzyme according to the existing needs of the cell. In addition, by concomitantly enhancing dephosphorylation of one substrate (e.g., phosphorylase *a*) and suppressing dephosphorylation of a different substrate (e.g., myosin light chains) the effector can facilitate functional integration of different cellular mechanisms.

Influence of polylysine on phosphorylase phosphatase activity. We exploited the availability of different linear polymeric chain lengths of polylysine to study relationships

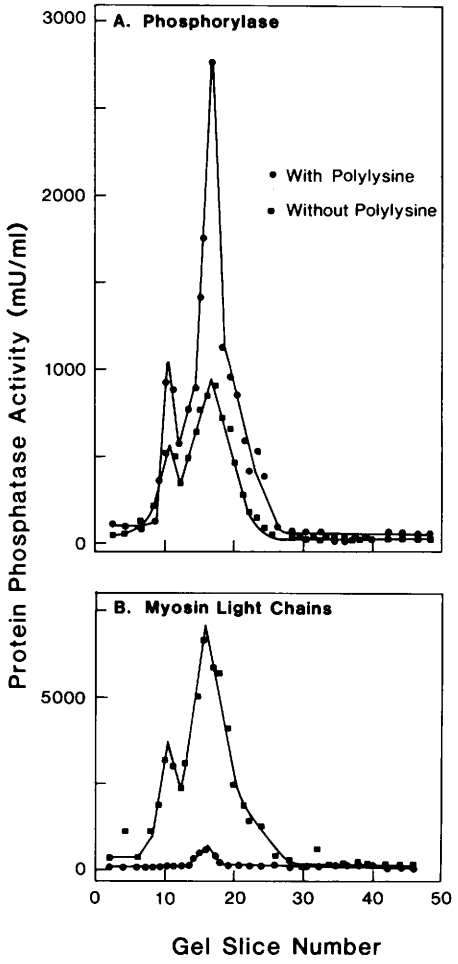

FIG. 1. Phosphorylase (Panel A) and myosin light-chain phosphatase (Panel B) activities of polylysine-modulated phosphatase from bovine aorta are shown following electrophoresis under nondenaturing conditions. Gel slices (2 mm) were extracted for 24 hr at 4°C in 20 mM Tris, pH 7.4, 15 m*M* 2-mercaptoethanol, and bovine serum albumin (1 mg/ml). Assays for phosphatase activity, shown in this and subsequent figures, were performed at 30°C as described previously (11–13) using either 10 μM [^{32}P]phosphorylase *a* or 4 μM ^{32}P-labeled cardiac myosin light chains. One unit (U) of phosphatase activity is that amount of enzyme which releases 1 nmole of phosphate per minute under the assay conditions. Assays were performed in the presence and absence of polylysine (Sigma, M_r = 13,000; 13 n*M*). Although extracts from each gel slice which exhibited phosphorylase phosphatase activity also exhibited light-chain phosphatase activity, the activity expressed against light chains was about threefold greater than the activity expressed against phosphorylase *a* (note different scales in A and B). Polylysine stimulated phosphorylase phosphatase activity but it inhibited light-chain phosphatase activity.

between polymer concentration and phosphorylase phosphatase activity (Fig. 2). Several observations merit comment. First, modulation of phosphorylase phosphatase activity by each of the polylysine polymers tested (4000–200,000 Da) was biphasic (Fig. 2A). That is, enzymatic activity was markedly and progressively stimulated by each of the polymers over well-defined ranges of increasing concentrations. However, further increases in polymer concentration were associated with lower levels of stimulation so that activity returned to control level and was subsequently inhibited. Second, the range of concentrations resulting in biphasic modulation of enzymatic activity was shifted progressively to the left (e.g., lower concentra-

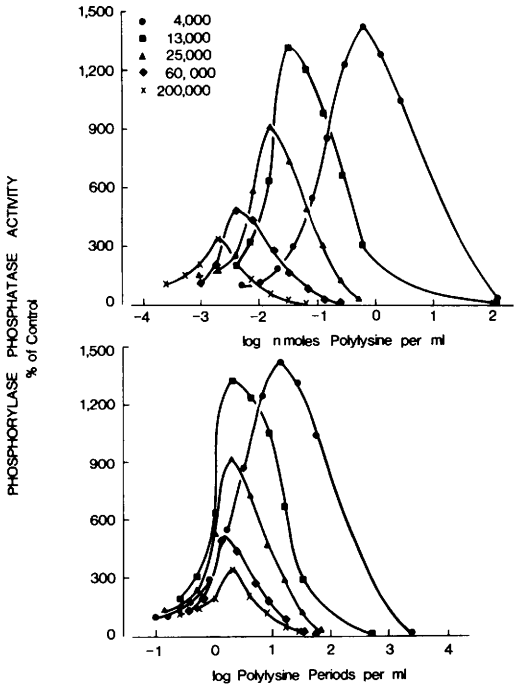

FIG. 2. Effects of different concentrations of polylysine polymers (M_r = 4000–200,000) on phosphorylase phosphatase activity are shown in the top panel. In the lower panel concentration is expressed as lysine periods (monomer residues). Control phosphorylase phosphatase activity was 70 mU/ml of assay mixture. The concentration of polylysine polymer required for maximal stimulation and the extent of stimulation were inversely proportional to the molecular weight of the polymer. However, the concentration of lysine periods eliciting maximal stimulation was the same for polymers of M_r = 13,000–200,000.

tions) with increasing molecular weight of the polylysine polymers. Third, the magnitude of the stimulatory effect decreased as the molecular weight of the polylysine polymer increased. Thus, the phosphorylase phosphatase activity expressed by the preparation could be widely regulated since maximal stimulation ranged from 3.5-fold (200,000 Da) to almost 15-fold (4000 Da) depending upon the polymer length of the polylysine studied. Fourth, the concentration of polylysine required for maximal expression of enzymatic activity decreased as the molecular weight or chain length of the polymer increased and ranged from 2 n*M* for the largest polymer (200,000 Da) to 65 n*M* for the smallest (4000 Da). In sharp contrast, however, replotting the same data as expressed phosphorylase phosphatase activity against the concentration of lysine residues or periods, rather than the concentration of polylysine polymer, showed that maximal stimulable activity was attained at essentially the same concentration of lysine residues (1 to 2 μM) for polymers of 13,000 to 200,000 Da (Fig. 2B). This finding suggests that the number of lysine residues, perhaps reflecting the number of cationic sites, interacting with either the enzyme or substrate is the primary event responsible for stimulation of activity. Since the number of lysine residues available for interaction is directly proportional to the molecular weight of the polymer, the concentration of polymer required for maximal stimulation should be inversely proportional to its molecular weight. Conceivably, the extent of stimulation attained with each polymer may be limited by structural parameters imposed by the polylysine chain length. Although the nature of such structural restraints is unknown they may also contribute to the inhibitory effects seen at high concentrations of polylysine. In the absence of additional data, this hypothesis should be viewed with caution.

It is noteworthy that the concentration of phosphorylase *a* (10 μM) in the assay mixtures was 5- to 10-fold greater than the concentration of lysine residues (1–2 μM) eliciting maximal stimulation of basal activity. However, the smallest polymer tested (4000 Da) stood apart from the four larger polymers (13,000–200,000 Da) in that the concentration of lysine residues (12 μM) producing maximal stimulation of activity was comparable to the concentration of substrate. The mechanism underlying this behavior is unknown. Nevertheless, the large molar excess of phosphorylase *a* relative to polylysine residues (M_r = 13,000–200,000) suggests that stimulation of phosphorylase phosphatase may be ascribable to interactions between polylysine and the enzyme. Interestingly, Schlender and Mellgren (30) concluded that the stimulatory effect of histone H1 on phosphatase activity was also enzyme directed, perhaps suggesting that both effectors share a common mechanism.

To gain further insight into whether or not polylysine interacts with the phosphatase or phosphorylase *a* we studied the influence of different concentrations of polylysine (13,000 Da) on expressed enzymatic activity using either different concentrations of enzyme and a fixed concentration of substrate (Fig. 3A), or different concentrations of substrate and a fixed concentration of enzyme (Fig. 3B). The results showed that higher concentrations of polylysine were required to maximally stimulate activity as the initial concentration of enzyme in the assay mixtures was increased. Thus, maximal stimulation was attained with only 0.03 μM polylysine when the initial concentration of enzyme was 0.05 U/ml. A fivefold increase in enzyme concentration (0.25 U/ml) was associated with a comparable increase in the concentration of polylysine (0.14 μM) required for maximal stimulation of enzymatic activity. It is also noteworthy that the percentage increase in phosphatase activity was virtually identical (1000–1100%) for each concentration of enzyme initially included in the reaction mixtures. In contrast to results obtained with different concentrations of enzyme, the concentration of polylysine needed for maximal stimulation of activity was unaltered over an eightfold range of substrate concentration (Fig. 3B). These data provide additional evidence suggesting that the stimulatory effect of polylysine on phosphorylase phosphatase activity is largely ascribable to interactions between polylysine and the enzyme. In this context, it is also worth recalling that, since the initial studies of Gratecos *et al.* (31), binding of a variety of phosphatase

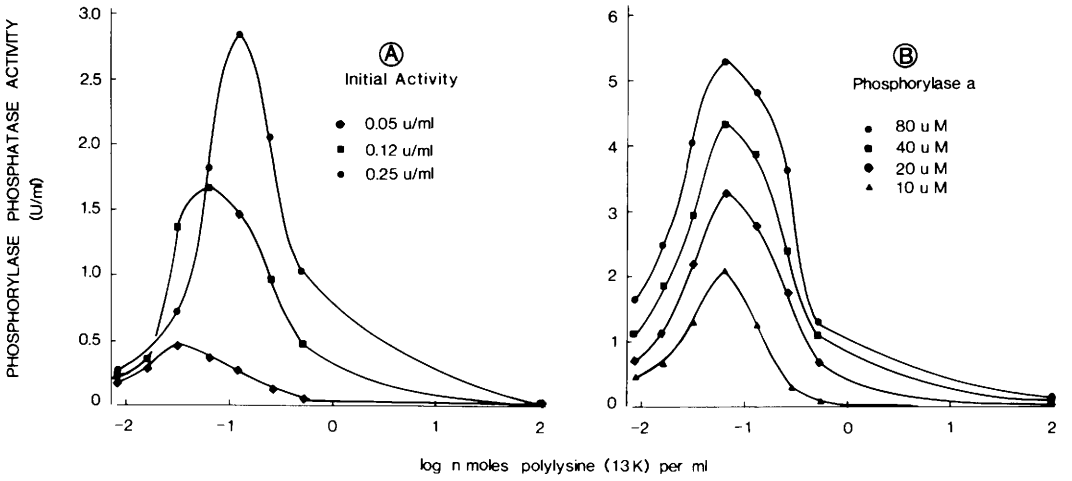

FIG. 3. Panel A shows the relationship between expressed phosphorylase phosphatase activity (mU/ml assay mixture) and concentation of polylysine (M_r = 13,000) for three different initial concentrations of enzyme (0.05–0.25 U/ml) in the reaction mixtures. Panel B shows the relationship between activity and concentration of polylysine at four different concentrations of substrate (10–80 μM). The concentration of polylysine required for maximal stimulation of activity increased as the initial concentration of enzyme added to the reaction mixtures was increased (Panel A), but it was essentially unchanged in the presence of different concentrations of phosphorylase *a* (Panel B).

preparations to polylysine–Sepharose has been routinely used as a key preparative step in the purification of the enzymes (11–13, 22–26).

Whether or not the inhibitory effect on phosphorylase phosphatase activity observed with high concentrations of each of the polymers studied (Figs. 2 and 3) is also ascribable to enzyme-directed effects is less certain. Conceivably, substrate-directed effects may become operative when the concentration of polymer is markedly increased. The present data do not permit firm conclusions to be drawn regarding this point. Nevertheless, it is interesting to note that inhibition, or depressed stimulation of activity, does not become apparent until the concentration of polymer exceeds the concentration of phosphorylase *a*. Ample evidence for both enzyme-directed and substrate-directed effects of a given compound on protein phosphatase has been elegantly described by Li (32, 33).

Effects of limited tryptic digestion on phosphorylase phosphatase activity. Limited proteolysis of the enzyme preparation with trypsin increased basal phosphorylase phosphatase activity and concomitantly altered the response to polylysine (Fig. 4). Enhancement of expressed activity following limited proteolysis was dependent upon the concentration of trypsin used for proteolytic digestion (Fig. 4A). Thus, phosphorylase phosphatase activity increased progressively as the concentration of trypsin was increased from 0.75 to 6 μg/ml. However, the level of expressed activity attained after digestion with higher concentrations of trypsin, though greater than the control level of activity, decreased with further increases in the concentration of trypsin (>6 mg/ml). Enhancement of phosphatase activity following limited tryptic digestion has been well documented for several preparations (see (33, 34) for reviews). Probably, Lee (34) should be credited with introducing the concept that limited proteolysis may cause selective degradation of an inhibitory subunit of the enzyme resulting in a consequent increase in activity. More extensive proteolysis may involve progressive degradation of the catalytic subunit so that enzymatic activity subsequently decreases.

However, we also found that the stimulatory effect of polylysine (13,000 Da) decreased precipitously following limited proteolysis of the preparation (Fig. 4B). For example, in the experiment shown in Fig. 4, polylysine stimulated activity about 5 1/2-fold prior to proteolytic digestion. After digestion with a

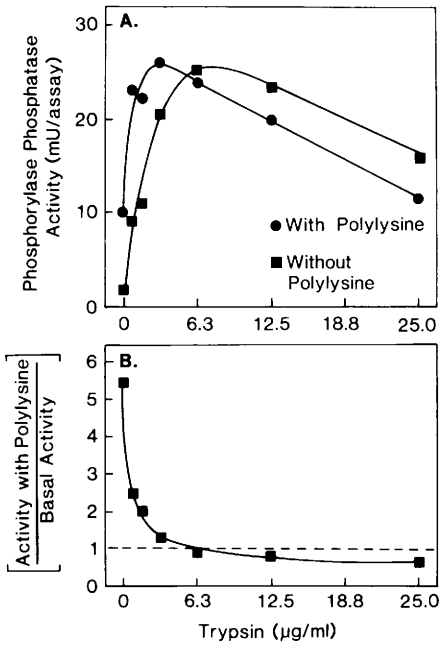

FIG. 4. Panel A shows the influence of limited tryptic digestion on spontaneously expressed phosphorylase phosphatase activity (■) and the activity expressed after the digested enzyme was assayed in the presence of polylysine (●). Enzyme was incubated in the presence of different concentrations of trypsin (abscissa) for 10 min at 30°C. Proteolysis was stopped by adding a 50-fold excess of soybean trypsin inhibitor (controls in which protease inhibitor was added prior to addition of trypsin showed that proteolysis was prevented). The mixtures were diluted 300-fold and assayed in the presence and absence of polylysine (M_r = 13,000; 30 nM with respect to polymer or 2 μM with respect to monomer). Panel B shows the ratio of phosphorylase phosphatase activity obtained in the presence to that obtained in the absence of polylysine (i.e., stimulability). All points above the interrupted horizontal line (ratio = 1) reflect stimulation by polylysine, whereas points below reflect inhibition (see text).

low concentration of trypsin (1.5 μg/ml) phosphatase activity increased by roughly 6 fold. However, when assays of the digested enzyme were repeated in the presence of polylysine, stimulation of activity was markedly less pronounced (2 fold) than it was before proteolysis (5.5-fold). Moreover, the stimulatory effect of polylysine was virtually abolished and appeared to be reversed to a slight inhibitory effect following digestion with higher concentrations of trypsin (>6 mg/ml). Collectively, these findings may be interpreted to suggest that the native enzyme contains an inhibitory or modulatory subunit which suppresses activity against phosphorylase *a*. Within this framework, it is possible that lysine residues in polylysine interact with the modulatory subunit thereby relieving suppression of activity and enhancing dephosphorylation of phosphorylase *a*. In similar fashion, limited tryptic digestion may selectively degrade the same modulatory subunit so that spontaneously expressed phosphorylase phosphatase activity is enhanced. In this setting, however, interaction with lysine residues is compromised resulting in progressively lower levels of polylysine-stimulated activity. Although this hypothesis is probably oversimplified it provides a useful model for further study.

Influence of polylysine on myosin light-chain phosphatase activity. Dephosphorylation of cardiac myosin light chains was inhibited in a concentration-dependent manner by each of the polylysine polymers studied (Fig. 5). The concentration of polymer required for 50% inhibition of phosphatase activity was inversely related to the molecular weight of the polymer and ranged from about 1 nM for the largest polymer tested (200,000 Da) to 80 nM for the smallest polymer tested (4000 Da). However, in terms of the concentration of lysine residues, 50% inhibition of activity was manifest over a very narrow range (1–1.5 μM). Complete inhibition was apparent at 5 to 10 μM with respect to lysine residues. These concentrations are comparable to the concentration of phosphorylated light chains (4 μM) which were used in the assays for phosphatase activity. Accordingly, the possibility of direct salt-like interactions between the acidic light chains and basic lysine residues in the polylysine polymers merits consideration. Such interactions could render the light chains structurally less suitable to serve as a substrate for the phosphatase. However, the possibility that enzyme-directed effects of polylysine also contribute to the inhibitory efect cannot be discounted on the basis of currently available data.

Physiological Role of Modulated Phosphatase. A phosphatase subject to modulation by either polylysine to histone H1 has been identified in skeletal muscle, kidney, and aortic smooth muscle (23, 29, 30). The aortic

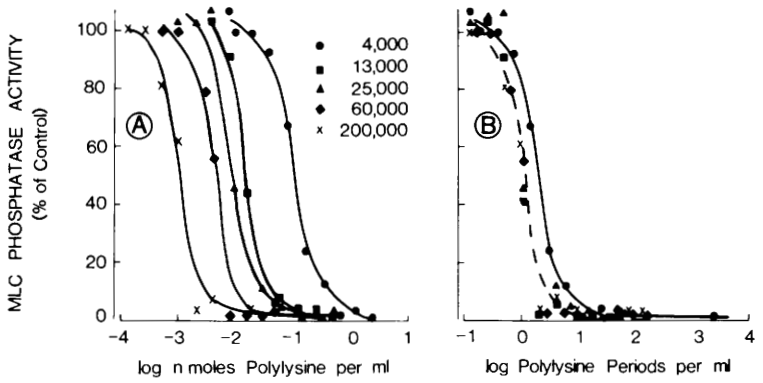

FIG. 5. Effects of different concentrations of polylysine polymers (M_r = 4000–200,000) on myosin light-chain phosphatase activity are shown in Panel A. The concentration of polymer required for 50% inhibition of activity increased progressively (1–80 nM) as the molecular weight of the polymer tested was decreased. In contrast, the concentration of lysine residues (Panel B) required for 50% inhibition was essentially the same (1–1.5 μM) for each of the polymers. Control myosin light-chain phosphatase (cardiac light chains) was 260 mU/ml assay mixture.

enzyme is active against phosphorylase *a*, native myosin, isolated light chains, and inhibitor 1 (22–25). This suggests that the enzyme may participate in coordinating arterial metabolism and contractility. Modulation of its activity by histone H1, or a polylysine-like substance existing *in vivo*, may serve to modulate its substrate specificity, and thereby regulate its participation in different cellular mechanisms. This interesting possibility is underscored by our finding that polylysine and histone H1 exerted differential effects on the dephosphorylation of phosphorylase *a* and the myosin light chains (Fig. 1).

Interestingly, polylysine modulation of phosphorylase phosphatase activity was biphasic exhibiting marked stimulation at low concentrations and depressed stimulation (inhibition) at high concentrations (Fig. 2). The data suggests that the stimulatory effect may be ascribable to enzyme-directed modulation, and that it is probably dependent upon the number of cationic lysine residues interacting with the enzyme. The suppression of stimulation evinced at high concentrations, or by polylysine polymers of increasing molecular weight, may involve either or both enzyme-directed and substrate-directed mechanisms. Although further studies are required to test these possibilities, our finding that modulation of activity is also dependent on polymer length could be physiologically relevant. Thus, Fullilov *et al.* (35) recently reported that the enzymatic activity of a phosphatase isolated from rabbit reticulocytes can be stimulated by a series of peptides of different molecular weights which are derived from a single larger peptide of 230,000 Da. Accordingly, the possibility that a series of polylysine-like effectors derived from a larger precursor can contribute to modulation of phosphatase activity *in vivo* appears to merit careful consideration. The biphasic pattern of phosphorylation phosphatase modulation observed in this study may provide a means for fine tuning of expressed enzymatic activity. Moreover, the apparently wide-spread occurrence of the modulated phosphatase among functionally diverse tissues suggests it could be involved in diverse cellular mechanisms regulated by phosphorylation–dephosphorylation of specific proteins.

We recognize that many questions regarding the structure of this phosphatase, the molecular mechanisms underlying modulation of its enzymatic activity, and identification of polylysine-like effectors *in vivo* remain to be elucidated. However, we believe that answers to these questions promise to provide valuable insight into the physiological role(s) of the enzyme and cellular mechanisms of its regulation.

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