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MODULATION OF LATENT PROTEIN PHOSPHATASE ACTIVITY FROM VASCULAR SMOOTH MUSCLE BY HISTONE-H_1 AND POLYLYSINE

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An apparently latent phosphatase which migrated as a protein of M_r 130,000 during sucrose density centrifugation, and a spontaneously active phosphatase (M_r 68,000) were isolated from bovine aortic smooth muscle. Basal phosphorylase phosphatase activity of the latent preparations was stimulated 12 fold by low concentrations of lysine-rich histone-H_1 (30 μg/ml) and 6 fold by polylysine (M_r 17,000; 12 μg/ml), whereas the spontaneously active enzyme was only slightly affected. The enzymatic activity of the spontaneously active preparation was completely destroyed by β-mercaptoethanol. In contrast, the apparently latent enzyme was converted to a more active form of lower molecular weight (M_r 86,000) following treatment with β-mercaptoethanol and this form of the enzyme was still stimulateable by histone-H_1. These findings show that the aortic spontaneous and apparently latent phosphatase actives are ascribable to separate enzymes and they suggest that the activity of latent phosphatase in living cells may be modulated by cationic proteins such as histones or similar effector molecules.

Contraction of arterial smooth muscle is associated with phosphorylation of phosphorylase (1,2) and the regulatory 20,000 dalton myosin light chain (3-6). However, phosphorylase is subsequently dephosphorylated while contractile force is maintained, suggesting that modulation of phosphoprotein phosphatase(s) may participate in coordinating arterial metabolism and contractility (7-9). Adequate testing of this hypothesis requires further understanding of the types of phosphoprotein phosphatase present in vascular smooth muscle and the mechanisms whereby their enzymatic activities are regulated. One potential regulatory mechanism involves stimulation of apparently latent phosphatase(s).

Although latent or normally unexpressed phosphoprotein phosphatase activity has been revealed in preparations from liver and skeletal muscle such activity has not been described in vascular smooth muscle. Expression or stimulation of latent phosphatase

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activity in the tissues studied has required treatment of crude extracts or partially purified preparations with harsh procedures such as precipitation with 80% ethanol, freezing and thawing in 0.2 M β-mercaptoethanol, incubation with 6M urea or limited proteolysis with either trypsin or endogenous Ca^{2+} dependent proteases (for reviews see 10,11).

In this context, Wilson, Mellgren and Schlender (12) reported that an acid and heat-stable protein isolated from porcine kidney markedly stimulated dephosphorylation of phosphorylase by either a high or low molecular weight form of phosphoprotein phosphatase. Subsequently, the same group identified the stimulatory protein as lysine-rich histone-H_1 (13). We reasoned that the stimulatory effect of H_1 might be partly ascribable to activation of a copurifying latent phosphoprotein phosphatase and that H_1 could be a useful investigative tool for unmasking and purifying apparently latent phosphatase(s).

This is the first communication showing that an apparently latent phosphatase which can be stimulated by H_1 exists in vascular smooth muscle and that the same enzyme is also stimulable by polylysine. Moreover, the latent enzyme is converted to a spontaneously active form of lower molecular weight by freezing and thawing with β-mercaptoethanol: this form of the enzyme is also markedly stimulated by H_1. These findings suggest the exciting possibility that expression of latent phosphatase activity in living cells may be modulated by cationic proteins such as histone-H_1 or related effector molecules.

METHODS

Methods used for the extraction and purification of aortic latent and spontaneously active phosphatases were adapted from procedures described previously (7-9). Briefly, 1 Kg of aortic muscularis was homogenized at 4°C in 5 vols of 50 mM Tris pH 8.0, 0.15 mM dithiothreitol (DTT), 1 mM ethylenediamine tetraacetic acid, 0.5 mM benzamidine, 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone, 0.1 mM phenlymethylsulfonyl fluoride and 2 mM ethylene-bis-(β-aminoethyl ether), NN'-tetraacetic acid (EGTA). The homogenate was centrifuged (6,000 g, 45 min) and the resulting supernatant was filtered through glass wool and interacted with 500 ml of DEAE Sephacel (Pharmacia) equilibrated in 20 mM Tris pH 7.0, 2 mM EGTA and 0.5 mM DTT (Buffer A). The Sephacel was washed batch-wise with 3 to 5 L of 0.1 M NaCl in Buffer A and bound protein was eluted from a column (5 x 25 cm) with a linear gradient of 0.1-0.4 M NaCl in Buffer A (1500 ml, total vol). Collected fractions (12 ml) were assayed for protein (A_{280}) and phosphorylase a phosphatase activity (see below) in the presence and absence of 30 μg/ml of H_1 (Sigma, type VS). Appropriate fractions (12 ml) containing H_1-stimulated phosphatase activity were pooled and precipitated with 30-50% $(NH_4)_2SO_4$. The precipitate was dissolved in 2-3 ml of Buffer A, dialyzed against 1000 vols of the same buffer, centrifuged to remove insoluble protein and subjected to gel filtration through a column (2.5 x 80 cm) of Ultrogel AcA-34 (LKB) equilibrated in Buffer A. Following elution with Buffer A, appropriate fractions (2.5 ml) showing H_1-stimulated activity were again pooled and applied to a

column of (1 x 10 cm) of polylysine-Sepharose. After thoroughly washing the column with 0.1 M NaCl-Buffer A, bound protein was eluted with a gradient of 0.1-0.6 M NaCl-Buffer A (150 ml, total vol). Selected fractions (2.5 ml) showing largely H_1-stimulated (latent) phosphatase activity, and those showing largely H_1-independent (spontaneous) phosphatase activity were pooled separately, dialyzed against Buffer A, concentrated by dialysis against 20% polyethylene glycol in the same buffer and stored at -20°C. Apparent molecular weights were estimated by sucrose density gradient centrifugation (100µl of appropriately diluted enzyme in 4.5 ml 5-20% sucrose, 40,000 rpm/15h, Beckman SW50 rotor at 4°C) according to the method of Martin and Ames (14) using ^{14}C-ovalbumin (M_r 45,000) as internal marker.

Assays for phosphatase activity, as described earlier (7-9), were performed at 30°C in a reaction mixture (30 µl) containing 20 mM Tris pH 7.0, 5 mM caffeine, 0.5 mM DTT, 1 mg/ml bovine serum albumin (Sigma), and 2 mg/ml of ^{32}P-phosphorylase a prepared from skeletal muscle according to Krebs et al. (15). One unit (U) of phosphatase activity is that amount of enzyme which releases 1 nmol of ^{32}P/min. Assays were also performed in the presence and absence of polylysine (17,000 daltons, Sigma), active protein phosphatase inhibitor-1 (0.1-6.7µg/ml) prepared from rabbit skeletal muscle as described by Nimmo and Cohen (16), and modulator protein (inhibitor-2, 0.8-53µg/ml) prepared from the same tissue according to Yang et al. (19).

RESULTS AND DISCUSSION

This study was based on the hypothesis that apparently latent phosphatase activity exists in vascular smooth muscle and that such activity might be revealed in the presence of histone-H_1. Moreover, since H_1 is a naturally occurring lysine-rich cationic protein, we suspected that synthetic cationic polylysine might also be effective in promoting expression of aortic latent phosphatase activity.

The chromatographic elution profiles for spontaneously active and H_1-stimulated phosphorylase phosphatase activity are shown in Fig. 1 for each stage of purification. The activity of the major peak of spontaneously active phosphatase which eluted from DEAE-Sephacel at 0.28-0.34 M NaCl was stimulated 3 to 4 fold when assays were performed in the presence of H_1 (Fig. 1A). Precipitation of the indicated fractions with 30-50% $(NH_4)_2SO_4$ followed by gel-filtration on AcA-34 (Fig. 1B) revealed a polydisperse elution pattern of spontaneously activity phosphatase(s). However 2 peaks of H_1-stimulated activity were also apparent. H_1-induced stimulation of phosphatase activity was considerably more pronounced in the first peak than in the second peak. Chromatography of the pooled AcA-34 fractions on polylysine-Sepharose permitted a clear separation of 2 peaks of activity: the first, designated L, was apparently latent since expression of its enzymatic activity was almost entirely dependent on H_1, whereas the second peak of activity, designated S, was largely spontaneously active and independent of H_1.

These results provide strong evidence for the existence of an apparently latent phosphatase in vascular smooth muscle (pool L) and show that its activity can be markedly

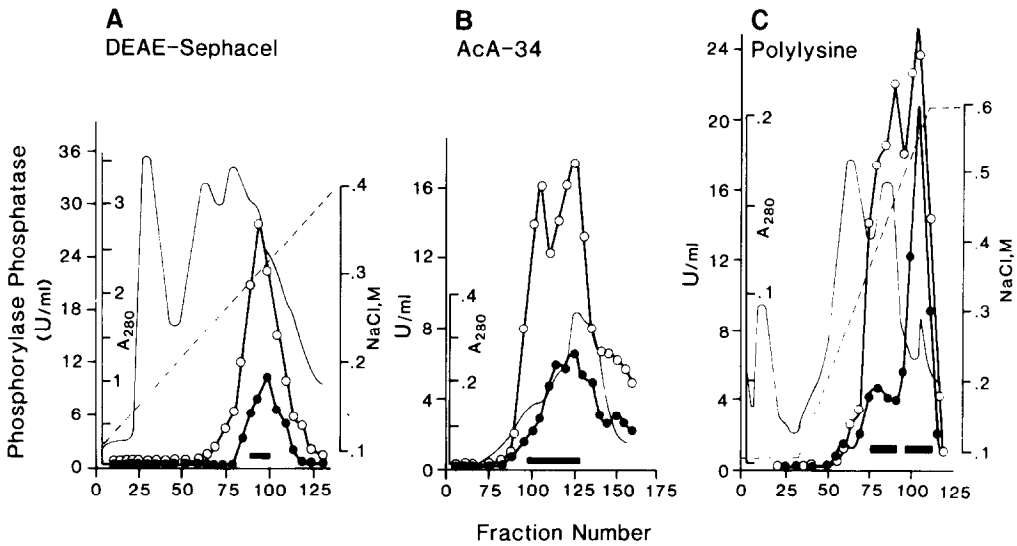

Figure 1. Chromatographic elution profiles of aortic phosphorylase phosphatase activity are shown for fractions assayed in the presence (o, 30μg/assay) and absence (●) of histone H_1. Protein (A_{280}) is shown by the continuous curve, and where appropriate, the concentration of NaCl in the elution gradient is given by the dashed line. Horizontal bars indicate fractions which were pooled following ion exchange chromatography on DEAE-Sephacel (Panel A), gel filtration on Ultrogel AcA-34 (Panel B), and chromatography on polylysine-Sepharose (Panel C). The predominantly latent or H_1-dependent activity (pool L) and spontaneous or H_1 independent activity (pool S) were pooled separately, concentrated by dialysis against polyethylene glycol and stored at -20°C (see METHODS). Protein concentrations were 2.2 mg/ml for pool L and 0.8 mg/ml for pool S.

enhanced in the presence of H_1. Thus, the basal or H_1-independent phosphatase activity in the concentrated L-pool (20U/mg) was only 1/12 of the activity measured in the presence of H_1 (240 U/mg). Part of the small degree of basal activity in the preparation probably reflects low level contamination with the spontaneously active phosphatase present in pool S. In sharp contrast, however, the enzymatic activity of the S-pool (175 U/mg) was only about 2½ fold greater (460 U/mg) in the presence of H_1. This small degree of stimulation is very likely attributable to contaminating latent activity.

The spontaneously active enzyme migrated as a single symmetrical peak corresponding to a molecular weight of 65,000 during sucrose density gradient centrifugation (Fig. 2A). A very minor shoulder of higher molecular weight activity, was also present. It is especially noteworthy that little or no stimulation of activity was seen with H_1 and that all enzymatic activity was destroyed after freezing and thawing the fractions in 0.2 M β-mercaptoethanol.

Sucrose density gradient centrifugation of the apparently latent enzyme showed a single peak of low level phosphatase activity with a molecular weight of 130,000 (Fig. 2B).

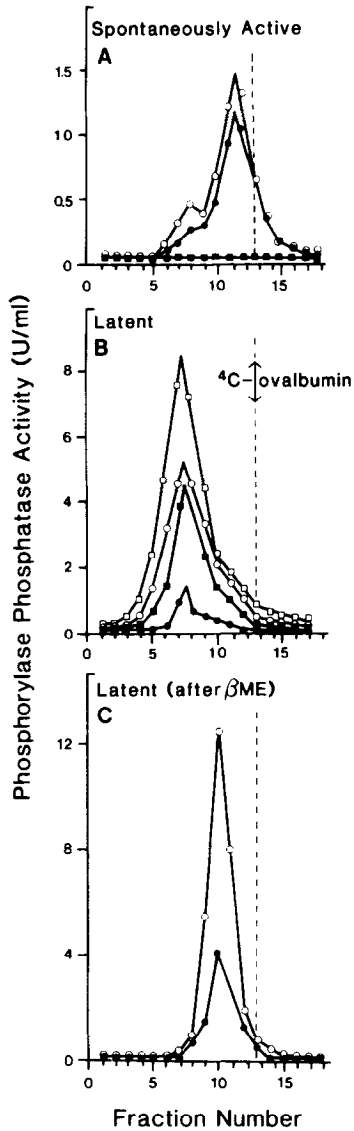

Figure 2. Phospnatase activity profiles are shown following sucrose density gradient centrifugation of aortic spontaneously active phosphatase (pool S), and the apparently latent phosphatase (pool L) before and after treatment with β-mercaptoethanol. The dashed vertical line extending through each panel gives the position of ^{14}C-ovalbumine (Mr 45,000). Panel A shows that the spontaneously active enzyme (●) migrated as a protein of M_r 65,000 and that its activity was slightly stimulated by H_1 (○) but completely destroyed after freezing and thawing in 0.2 M β-mercaptoethanol (■). Based on the areas under the peaks, Panel B shows that the apparently latent enzyme (●) which migrated as a protein of M_r 130,000 was stimulated 7 fold by H_1 (○), 5 fold after freezing and thawing in β-mercaptoethanol (■) and 11 fold after the β-mercaptoethanol treated fractions were assayed with H_1 (□). Sucrose density gradient centrifugation of the latent enzyme after treatment with β-mercaptoethanol (Panel C) decreased its Mr to 80,000 (●) but stimulation in response to H_1 was retained (○).

Unlike the spontaneously active preparation, the activity of the latent enzyme was enhanced 7 fold in the presence of H_1. Moreover, comparable stimulation was manifest after each of the fractions was frozen and thawed in β-mercaptoethanol and assayed

without H_1. β-mercaptoethanol effectively converted the latent enzyme into a spontaneously active form which retained the ability to be stimulated by H_1 thereby clearly distinguishing it from the spontaneously active enzyme depicted in Fig. 2A. It is also evident that the expressable activity of the latent enzyme after treatment with β-mercaptoethanol and H_1 was more than 10 fold greater than its basal activity, a value approximately the sum of the activities manifest with either H_1 or β-mercaptoethanol alone. Conversion of the apparently latent enzyme to a spontaneously active form after treatment with β-mercaptoethanol was associated with a decrease of its molecular weight from its initial value of 130,000 (Fig. 2B) to 86,000 (Fig. 2C). However, this smaller enzymatically active species was larger than the spontaneously active phosphatase in the S-pool (Fig. 2A), and in accord with the results shown in Fig. 2B, it was still stimulable by H_1. In contrast to results obtained with crude extracts from liver (10,11), no evidence for production of a catalytically active 35,000 dalton protein was obtained following treatment with β-mercaptoethanol.

The effects of H_1 on phosphatase activity were concentration dependent and qualitatively different for different types of phosphatases studied (Fig. 3). These enzymes included the aortic latent (pool L) and spontaneously active preparations (pool S) and two purified 35,000 dalton catalytic subunits of phosphoprotein phosphatase from rabbit skeletal muscle. One of the purified catalytic subunits was inhibited by the well characterized phosphatase inhibitor-1 and inhibitor-2 (modulator protein) initially described by Huang and Glinsmann (18), while the second catalytic subunit was unaffected by these proteins. As shown in Fig. 3, the activity of each of the four phosphatases studied was markedly inhibited by high concentrations of H_1. In contrast, at low concentrations of H_1 the activity of the latent enzyme was stimulated by 200-500%, whereas the spontaneously active preparation was only slightly stimulated (30%). A significant stimulatory effect (50-90%) was apparent with phosphatase-inhibitor insensitive catalytic subunit, but only suppression of activity occurred when the inhibitor-sensitive catalytic subunit was tested. These findings suggest that the stimulatory effect of H_1 may be specific for phosphatases which are insensitive to inhibitor-1, and modulator protein. This hypothesis is supported by the observation that the latent enzyme is insensitive to either inhibitor-1 or modulator protein (inhibitor-2). Thus, the activity of the latent enzyme was

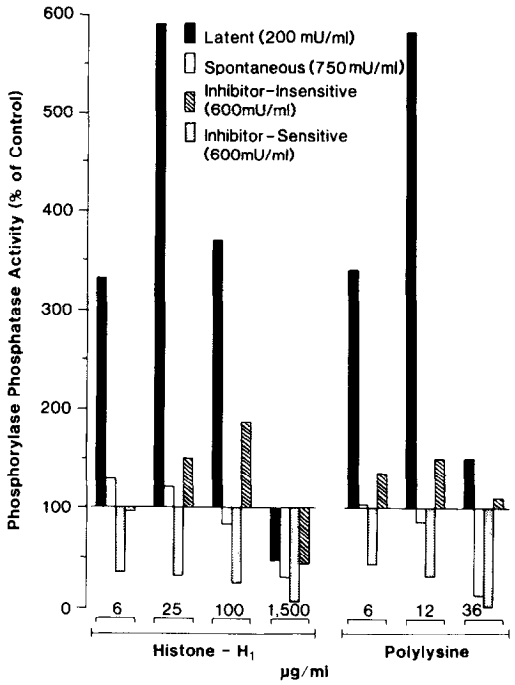

Figure 3. Concentration dependent effects of H_1 and polylysine on phosphatase activity are shown for the aortic latent phosphatase, aortic spontaneously active enzyme, and the purified 35,000 dalton catalytic subunits of inhibitor-sensitive and inhibitor-insensitive protein phosphatases purified from rabbit skeletal muscle. Control activities (mU/ml) which are listed for reference, were normalized to 100% for each of the four phosphatases studied (see text for details).

unaffected by inhibitor-1 (0.1-6.7 µg/ml) or modulator protein (0.8-53 µg/ml) either before (100 mU latent enzyme/ml) or after activation with β-mercaptoethanol (500 mU/ml) or H_1 (600 mU/ml).

Low concentrations of polylysine (M_r 17,000) also profoundly stimulated (50-400%) the apparently latent aortic phosphatase (Fig. 3). This suggests that the stimulatory effect of H_1 is probably related to its high content of lysine (19). In accord with the results obtained with H_1, moderate degrees of polylysine-induced stimulation (10-50%) also occurred with the inhibitor-insensitive catalytic subunit, but only inhibition occurred with the purified catalytic subunit sensitive to phosphatase inhibitor-1 and modulator protein. Contrasting sharply with the aortic latent enzyme, the predominant effect of polylysine on the aortic spontaneously active preparation was inhibition of activity. Although, the inhibitory effect of polylysine on phosphatase activity has been well documented (20), the stimulatory effect described in this study is a new observation.

Stimulation of the aortic latent phosphatase in vitro with either H_1 or polylysine suggests that modulation of latent phosphatases in vivo may be mediated by cationic proteins or similar effector molecules. However, it is important to emphasize that a particular phosphatase may appear latent with respect to one substrate but express significant enzymatic activity with respect to a different substrate. Accordingly, the ability to influence such activity in vivo with histone-like compounds may also contribute to regulating the substrate specificity of the enzyme.

In conclusion our results show that an apparently latent phosphatase exists in vascular smooth muscle and that its enzymatic activity can be enhanced by either lysine-rich histone-H_1, polylysine, or treatment with β-mercaptoethanol. This enzyme may participate in modulating the state of phosphorylation of contractile and regulatory proteins and thereby contribute to the coordination of arterial metabolism and contractility.

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