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INHIBITOR-1 PHOSPHATASE ACTIVITY IN VASCULAR SMOOTH MUSCLE

Etienne Waelkens[X], Jozef Goris[X], Joseph Di Salvo[+] and Wilfried Merlevede[X]

[X]*Afdeling Biochemie, Faculteit Geneeskunde, KatholiekeUniversiteit Leuven, Leuven B-3000, Belgium*

[+]*Department of Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267*

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The histone-H_1 and polylysine stimulated "latent" phosphorylase phosphatase, characterized by a molecular weight of 260,000 in gel filtration and 130,000 in sucrose density gradient centrifugation has been identified as a major inhibitor-1 phosphatase in vascular smooth muscle. Its substrate: protein phosphatase inhibitor-1, was also shown to be present in the same tissue. Following treatment with β-mercaptoethanol the enzyme dissociates into a lower molecular weight (38,000) form with a higher specific activity.

Investigation of the different types of phosphoprotein phosphatases present in vascular smooth muscle led to the discovery of a latent phosphorylase phosphatase (1) which migrated as a protein of M_r 130,000 in sucrose density gradient centrifugation. The enzyme activity could be stimulated several fold by low concentrations of lysine-rich histone-H_1 or polylysine. Treatment of this enzyme form with β-mercaptoethanol led to a decrease in molecular weight and an increase in activity; further stimulation by histone-H_1 was still possible. The present study provides evidence that this latent protein phosphatase is spontaneously active towards inhibitor-1, and may represent a major inhibitor-1 phosphatase in vascular smooth muscle. Inhibitor-1 phosphorylated by cyclic AMP-dependent protein kinase (2) is a powerfull inhibitor of the multisubstrate protein phosphatase (3) and has been identified in a variety of tissues (4,5). Relaxation of coronary arterial smooth muscle produced in response to β-adrenergic stimulation is correlated to activation of the cyclic AMP-dependent protein kinase (6), whereas contraction is associated with activation of

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phosphorylase (7). Accordingly, regulation of the state of phosphorylation by either cyclic AMP-dependent protein kinase or inhibitor-1 phosphatase may participate in coordinating arterial metabolism and contractility.

EXPERIMENTAL PROCEDURES

Materials and methods used for the extraction and purification of aortic "latent" protein phosphatase and sucrose density gradient centrifugations were essentially as described previously (1) with slight modifications (see Results and Discussions). The ATP,Mg-dependent protein phosphatase, kinase F_A, ^{32}P-labeled phosphorylase *a*, the catalytic subunit of cyclic AMP-dependent protein kinase, modulator protein, and ^{32}P-labeled inhibitor-1 were prepared as described in (8-10). Spontaneously active protein phosphatase (50,000 U/mg) was purified from dog liver glycogen pellets as in (11). Protein phosphatase inhibitor-1 was prepared according to (12). Brain calmodulin was kindly provided by Dr. F.Wuytack (Division of Physiology, Louvain).

Assays for phosphorylase phosphatase activity were performed in the presence or absence of histone-H_1 as in (1). Inhibitor-1 phosphatase activity was measured by incubation of the appropriate sample with an equal volume (10 µl) of ^{32}P-labeled inhibitor-1 (40 µg per ml) during 20 min at 30°C. ^{32}P-release was measured as in (8) using 25% trichloroacetic acid for protein precipitation. Routine assays of inhibitor-1 phosphatase activity were carried out in the absence of histone-H_1, since the enzyme activity was found to be unaffected by this protein (see "Results and Discussion"). One unit of protein phosphatase activity is defined as the amount of enzyme which releases 1 nMol of ^{32}P phosphate per min. Assays of phosphorylase *a* as well as inhibitor-1 phosphatase activity of the enzyme fraction investigated are linear only up to 10% conversion of the substrate. Therefore appropriate dilutions of the phosphatase were made in 20 mM Tris-HCl, pH 7.4, 1 mg per ml bovine serum albumin and 0.5 mM dithiothreitol.

In the assay for inhibitor-1 aliquots were preincubated 20 min at 30°C in a solution consisting of 20 mM Tris-HCl buffer (pH 7.4), 0.3 mM ATP, 3 mM $MgCl_2$, 1 mg/ml bovine serum albumin, in the presence or absence of the catalytic subunit of cyclic AMP-dependent protein kinase (3 U/ml). After this preincubation, the mixture was boiled for 5 min, and assayed with phosphorylase phosphatase as in (5) using the ATP,Mg-dependent or the spontaneously active protein phosphatase.

RESULTS AND DISCUSSION

1. Evidence for the presence of protein phosphatase inhibitor-1 in bovine aortic smooth muscle

Bovine aortic muscularis (100 g) was homogenized in two volumes of 4 mM EDTA, pH 7. All steps were carried out at 4°C unless indicated otherwise. The homogenate was centrifuged

(10,000 g, 10 min) and the resulting supernatant filtered through glasswool. The next steps -differential trichloroacetic acid precipitation (2-15%), dialysis and heat treatment- were performed as in the procedure for skeletal muscle (12). The solution thus obtained was diluted with water to adjust the ionic strength and applied to a column of DEAE-cellulose (1.5x10 cm) equilibrated in 10 mM Tris-HCl, pH 7.8 containing 0.5 mM dithiothreitol (Buffer A). The column was washed thoroughly with the same buffer and inhibitor-1 was then eluted with 200 ml of a linear salt gradient (0-0.4 mM NaCl) in buffer A. The active fractions were pooled and concentrated by lyophilisation, redissolved in a minimum volume of buffer A and dialyzed against the same buffer.

Preincubation with the catalytic subunit of cyclic AMP-dependent protein kinase resulted in an increase of the inhibitory capacity of the preparation measured with the spontaneously active protein phosphatase isolated from dog liver glycogen pellet (Fig. 1) as well as with the kinase F_A activated ATP,Mg-dependent phosphatase isolated from rabbit skeletal muscle (not shown). As could be expected (see further) the

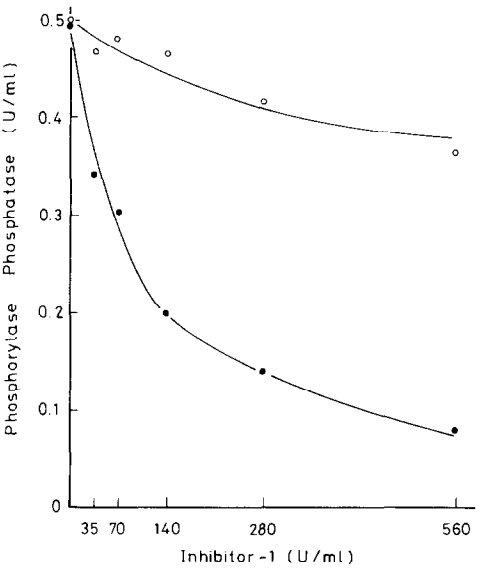

Fig. 1. *Protein kinase-dependent activation of the inhibitor-1 like material isolated from aortic smooth muscle.* Aliquots of inhibitor-1 were incubated for 10 min at 30°C in the presence (•) or absence (o) of the catalytic subunit of cyclic AMP-dependent protein kinase and then assayed for inhibitory activity on the spontaneously active protein phosphatase (0.5 U/ml) isolated from dog liver glycogen pellet.

presumed inhibitor-1 like material could be dephosphorylated and inactivated by the aortic latent protein phosphatase.

2. Isolation of the inhibitor-1 phosphatase from bovine aortic muscularis

All operations were performed at 4°C according to a previously described procedure (1) except for some slight adaptations made in the use of inhibitor-1 as a substrate for the isolation of the enzyme. The low speed supernatant (3,250 ml) was filtered through glasswool and interacted with 500 ml of DEAE-Sephacel equilibrated in 20 mM Tris-HCl, pH 7.0, 1 mM EDTA and 0.5 mM dithiothreitol (Buffer B). The DEAE-Sephacel was washed batchwise with 4x500 ml 0.1 M NaCl in Buffer B and the bound protein was then eluted from the column (5x36 cm) with 1,500 ml of a linear gradient of 0.1-0.45 M NaCl in Buffer B. The collected fractions (12 ml) were assayed for protein (A_{280}), phosphorylase *a* phosphatase activity in the presence and absence of histone H_1 (Sigma, type VS) and for inhibitor-1 phosphatase activity. The fractions were pooled according to their inhibitor-1 phosphatase activity (Fig. 2A). In addition an inconsistant amount of inhibitor-1 phosphatase activity eluted from the column at a higher salt concentration (0.36 M NaCl). Further purification of the latter enzyme fraction using the same procedure produces a phosphatase characterized by an apparent M_r of 91,000 in sucrose density gradient centrifugation. After β-mercaptoethanol treatment a slight shift of M_r to 83,000 was observed. No separation of inhibitor-1 phosphatase and phosphorylase *a* phosphatase could be observed. These fractions, when present, were not further investigated.

The 30 to 50% $(NH_4)_2SO_4$ cut of the pooled fractions was redissolved in a minimal volume of Buffer B and dialyzed against the same buffer. The dialyzed preparation was then chromatographed on an Ultrogel ACA-34 column (2.5x90 cm) equilibrated in Buffer B. Two peaks of histone-H_1 stimulated phosphorylase *a* phosphatase activity were apparent (Fig. 2B). Only the higher M_r-peak displaying the most pronounced stimulation by histone-H_1 coincided with inhibitor-1 phosphatase activity. The molecular weight of this phosphatase was thus estimated at 260,000 in gel filtration, assuming the enzyme to be a globular protein. The pooled fractions of the inhibitor-1 phosphatase were then ap-

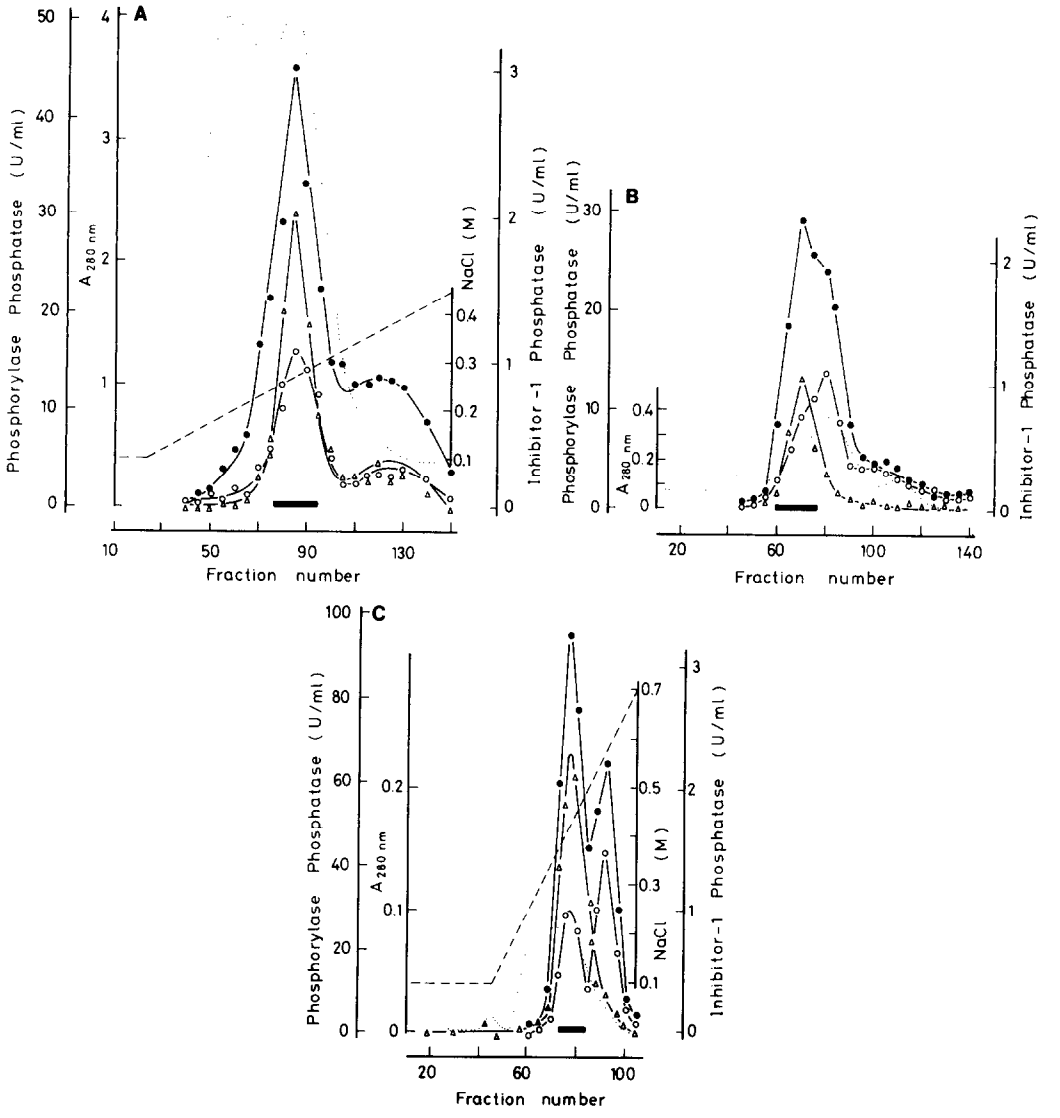

Fig. 2. *Purification of vascular smooth muscle inhibitor-1 phosphatase.*
(A) DEAE-Sephacel gradient elution profile; (B) Ultrogel ACA-34 elution; (C) Polylysine-Sepharose 4B gradient elution profile. Assays for inhibitor-1 phosphatase activity (Δ) and phosphorylase phosphatase activity in the presence (●) or absence (o) of histone-H_1 were performed as outlined in the text. The dotted line indicates the absorbance at 280 nm. Fractions were pooled as indicated by the horizontal bars.

plied to a polylysine-Sepharose 4B column (0.9x10 cm). The column was washed with 0.1 M NaCl in Buffer B and eluted with 160 ml of a linear salt gradient (0.1 to 0.7 M NaCl in Buffer B). The first of the two peaks of phosphorylase *a* phosphatase activity in the elution profile (Fig. 2C) shows a considerable

Table I. *Purification of inhibitor-1 phosphatase ("latent" enzyme with phosphorylase a as a substrate) from vascular smooth muscle*

Purification step	Total protein mg	Phosphorylase phosphatase - Histone Total units	Phosphorylase phosphatase - Histone Units/mg	Phosphorylase phosphatase + Histone Total units	Phosphorylase phosphatase + Histone Units/mg	Inhibitor-1 phosphatase Total units	Inhibitor-1 phosphatase Units/mg	Inhibitor-1 phosphatase Yield %
1. DEAE-Sephacel	860	1720	2	4730	5.5	161	0.2	100
2. 30-50% ammonium sulfate precipitation	100	1600	16	3900	39	154	1.5	96
3. Ultrogel ACA-34	22	720	33	1570	71	63	2.9	39
4. Polylysine-Sepharose 4B	1.6	270	169	1053	660	23	14.4	14

Starting material was 1 kg of aortic smooth muscle. Total activity of inhibitor-1 phosphatase activity in the crude extract was estimated to be 500 U. The total activity of the DEAE-Sephacel was taken as 100%.

stimulation by histone-H_1 overlapping perfectly with the inhibitor-1 phosphatase activity. These peak fractions were pooled as indicated and concentrated about 10 fold by dialysis against 20% polyethyleneglycol in Buffer B and an additional 5 fold by dialysis against 60% glycerol in the same buffer. The preparation could be stored as such at -20°C. The purification results are summarized in Table I.

The molecular weight of the inhibitor-1 phosphatase in sucrose density gradient centrifugation was estimated at 130,000 (Fig. 3A). After treatment with 0.2 M β-mercaptoethanol the molecular weight was shown to be 38,000 (Fig. 3B). Neither one of these two forms of inhibitor-1 phosphatase was stimulated by histone-H_1 in contrast to the results obtained using phosphorylase *a* as a substrate.

From these results it appears that at each stage of the isolation procedure the histone-H_1 stimulated phosphorylase *a* phosphatase activity, described recently as "latent" (1), co-purified with inhibitor-1 phosphatase activity. Even freezing and thawing in the presence of 0.2 M β-mercaptoethanol resulted in the conversion of the "latent" as well as the inhibitor-1 phosphatase activity to the same lower molecular weight form.

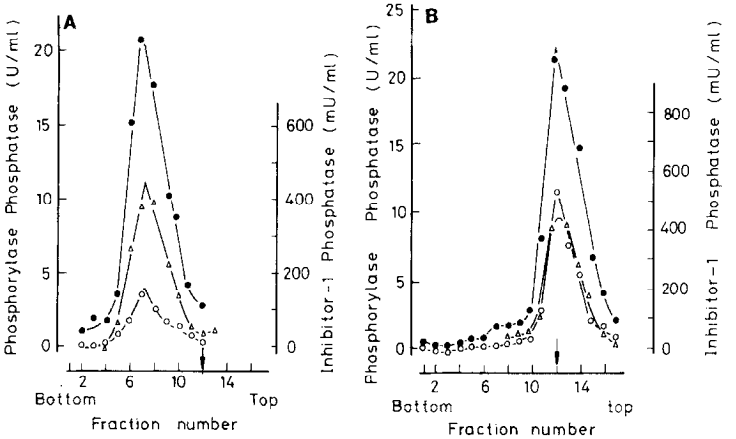

Fig. 3. *Sucrose density gradient centrifugation profiles of the protein phosphatase before (A) and after (B) treatment with β-mercaptoethanol.*
Treatment with β-mercaptoethanol and assays for inhibitor-1 phosphatase activity (Δ) and phosphorylase phosphatase activity in the presence (●) or absence (o) of histone-H_1, were performed as outlined in the text. The arrow gives the position of ^{14}C-ovalbumin (M_r=45,000) used as an internal marker.

These observations strongly suggest a single phosphatase responsible for both enzyme activities.

No relationship between this inhibitor-1 phosphatase and calcineurin (13,14) could be found since no effects of 0.1-10 mM Ca^{2+}, 2 μM calmodulin, 1-2 mM ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid, 1-2 mM EDTA or 150 μM trifluoperazine could be detected. The activity of the enzyme measured as inhibitor-1 phosphatase or as phosphorylase *a* phosphatase in the presence or absence of histone-H_1, was unaffected by up to 3000 U per ml of the phospho-form of inhibitor-1 as well as by up to 100,000 U/ml modulator protein, in contrast with the ATP,Mg-dependent phosphatase or its spontaneously active form (3,5).

Several protein phosphatases displaying inhibitor-1 phosphatase activity under likely physiological conditions have been described: the inhibitor-1 phosphatase found in vascular smooth muscle and described in this paper, calcineurin (13,14) and the spontaneously active multisubstrate protein phosphatase isolated from the liver glycogen pellet which expresses its activity only in the presence of the deinhibitor protein or in the presence of unphysiological concentrations of Mn^{2+} (10). The specific activity of the inhibitor-1 phosphatase isolated

from vascular smooth muscle (14 U/mg) is comparable to the specific activity of calcineurin from bovine brain: 20 U/mg as calculated from the data presented in (14). Furthermore the highly purified latent protein phosphatase from skeletal muscle recently isolated in this laboratory (15) shows a similar specific activity towards inhibitor-1: 13 U/mg (not shown). Which phosphatase eventually dephosphorylates inhibitor-1 *in vivo* is unknown. Conceivably different physiological stimuli, leading to the dephosphorylation of inhibitor-1, may act through a different protein phosphatase.

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