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PHOSPHOPROTEIN PHOSPHATASE IN BOVINE TRACHEAL SMOOTH MUSCLE

MULTIPLE FRACTIONS AND MULTIPLE SUBSTRATES

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Summary

Phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) from bovine tracheal smooth muscle extracts was isolated and its activity determined using two [^{32}P]phosphorylated proteins as substrates, i.e. phosphorylated histone (H-P) and a phosphorylated muscle specific substrate protein (MS-P) for the tracheal smooth muscle protein kinase. The enzyme was purified by the use of DEAE-cellulose followed by a two stage chromatography on a histone-Sepharose affinity column. Elution from the affinity column resolved the phosphoprotein phosphatase into four activity fractions. While fractions expressed phosphatase activity against both tested substrates the relative amounts of either activity varied. The ratio of activity towards H-P to activity towards MS-P changed from 11.5 to 0.12. The characterization of four phosphoprotein phosphatase fractions was based on the differences found in the following parameters: substrate specificity; sensitivity to NaF; influences of nucleotides (ATP, 5′-AMP, cyclic AMP, cyclic GMP) and the requirement of Mn^{2+} for maximal activity. Mg^{2+}, Ba^{2+} or Ca^{2+} could not substitute for Mn^{2+}.

Introduction

β-Adrenergic drugs are used clinically to produce relaxation of bronchotracheal smooth muscle. The role of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in mediating the effects of β-receptor stimulation has been questioned in vascular [1] as well as uterine smooth muscle [2—4], however, recent data by Katsuki and Murad [5] support the hypothesis that cyclic AMP is involved in the β-adrenergic drug-induced relaxation of tracheal smooth muscle. At present the only known intracellular mechanism of action of cyclic AMP in mammalian systems is the activation of the enzyme cyclic AMP-dependent pro-

tein kinase. This was proposed by Kuo and Greengard [6] based on the widespread distribution of this enzyme. The occurrence of a cyclic AMP-dependent protein kinase has been also reported in tracheal smooth muscle [7]. If the intracellular mechanism of action of cyclic AMP involved the activation of the protein kinase, then one would expect to find enzymes which reversed this reaction, i.e. removed phosphate from the serine and threonine residues of the substrate proteins. Therefore phosphoprotein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) are expected to follow protein kinases in their far-reaching distribution. But, although isolation of these enzymes has been described from various mammalian tissues [8—18], there are only a few reports on non-mammalian species [19—23].

Investigation of the role of phosphoprotein phosphatase has been hampered by our lack of knowledge concerning the physiological substrates for the enzyme. It is difficult to compare various reports on phosphoprotein phosphatases, because of vastly differing purification procedures employed and the determination of phosphatase activity towards a variety of substrates. Similar characteristics of the enzymes regarding stability, requirement for divalent cations, sensitivity to inhibitors and influence by cyclic nucleotides could not be established from the literature. In spite of this lack of common features several observations suggested very little substrate specificity of phosphatase activity [10—13].

This paper represents the first study of the isolation of phosphoprotein phosphatase activity from bovine tracheal smooth muscle. Phosphatase activity was characterized either towards phosphorylated histone (H-P) or towards phosphorylated muscle specific substrate protein (MS-P) [24] for the cyclic AMP-dependent tracheal smooth muscle protein kinase [7]. We report on the existence of four different phosphoprotein phosphatase fractions with activity towards both substrates.

Materials

Fresh bovine tracheae were obtained from local slaughterhouses and transported to the laboratory within 30 min in ice-cold isotonic sucrose solution. Cyclic AMP-dependent protein kinase was prepared from bovine tracheal smooth muscle according to the procedure of Sands et al. [7]. The muscle specific substrate for this enzyme was isolated from the tissue as previously reported by Sands et al. [24]. [γ-^{32}P]ATP was either purchased from ICN Chemical and Radioisotope Division, or it was synthesized from carrier-free ^{32}P using the method of Maxam and Gilbert [25].

Methods

Phosphorylation of phosphatase substrates. Histone (5 mg/ml) and the muscle specific protein kinase substrate (approx. 1.24 mg/ml) were phosphorylated with [γ-^{32}P]ATP (2 μCi/mol) using partially purified bovine tracheal smooth muscle cyclic AMP-dependent protein kinase in the presence of 10^{-6} M cyclic AMP according to Sands et al. [26].

Phosphatase assay. Phosphoprotein phosphatase activity was measured on the basis of the release of radioactive orthophosphate from ^{32}P-labeled sub-

strates. Phosphatases were routinely assayed by the modified method of Maeno and Greengard [8] in either 20 mM histidine (pH 7.2) or 20 mM Tris · HCl (pH 7.0) as indicated. The buffers contained 5 mM $MgCl_2$, 55 mM KCl, 10 mM NaCl and 708 mM sucrose. The final reaction volume of 0.3 ml contained 2—200 μg of enzyme protein and 66 or 134 μg of labeled muscle specific substrate or histone, respectively. The reaction was started with the addition of the labeled substrate (containing $2 \cdot 10^5$—$2.5 \cdot 10^5$ cpm) and was terminated after 10 min of incubation at 37°C by the addition of trichloroacetic acid to a final concentration of 12%. The trichloroacetic acid extract was assayed for radioactive inorganic phosphate using the molybdate extraction procedure described by Plaut [27] as modified by Maeno and Greengard [8]. Zero reaction time values were subtracted from those determined in incubated samples. The calculation of the amount of inorganic phosphate released from substrate protein was based on the specific activity of the radioactive ATP used as a precursor in the protein phosphorylation reaction (neglecting unlabeled seryl phosphate or threonyl phosphate present in the non-phosphorylated proteins). The specific phosphatase activity was defined as pmol "P_i" released per mg of protein per 10 min.

Other methods. Protein was estimated according to Heil and Zillig [28] using bovine serum albumin as standard. The histone affinity column was prepared according to the method described by Cuatrecasas [29]. Linear sucrose gradients (5—22%) in 25 mM Tris · HCl (pH 7.5), 1 mM EDTA, 5 mM MgCl, 30 mM β-mercaptoethanol and 5 mM $MnCl_2$ (Buffer A) were employed for molecular weight determinations via sucrose density centrifugation [30]. The phosphatase fractions were concentrated 10-fold by vacuum-dialysis before application to the gradients. Mean values (± S.E.) were averages from at least three experiments. Comparison of experimental results was done by the Student's t-test; $P < 0.05$ significant.

Results

Purification scheme for phosphoprotein phosphatase from bovine tracheal smooth muscle

Table I summarizes the procedures that yielded four partially purified phosphoprotein phosphatase fractions from tracheal tissue.

Preparation of the crude homogenate. Tracheal smooth muscle (approx. 250 g wet weight) was homogenized using a Servall Omni-Mixer in one volume Buffer A at 16 000 rev./min, four times each for 30 s. Stability of the enzyme was achieved only when 5 mM $MnCl_2$ and 30 mM β-mercaptoethanol were included in the buffer system. The homogenization and all following procedures were carried out a 4°C. The homogenate was centrifuged at 8000 $\times g$ for 30 min and the supernatant filtered through four layers of gauze with one layer of glass-wool in between. At every stage of purification the phosphatase activities towards H-P and MS-P as substrates were determined and specific activities as well as their ratio were calculated.

$$\text{Ratio } (R) = \frac{\text{Activity towards H-P}}{\text{Activity towards MS-P}}$$

TABLE I

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASES FROM BOVINE TRACHEAL SMOOTH MUSCLE

Phosphoprotein phosphatases were purified as described in the text. Specific activity and total activity were determined at each stage of purification using H-P or MS-P as substrate in histidine buffer, pH 7.2.

Purification step	Protein (mg)	Specific activity (units/mg)		Total activity (10^3)		Yield (%)		Purification (fold)		Ratio H-P/MS-P
		H-P	MS-P	H-P	MS-P	H-P	MS-P	H-P	MS-P	
8000 X *g* supernatant	2463.12	83.73	53.40	206	132	100	100			1.60
DEAE-Sephadex chromatography	337.60	207.96	163.18	70	55	34	42	2.5	3.1	1.30
Histone-Sepharose affinity chromatography										
1. Column (gradient)										
A	147.00	159.97	62.38	24	9	11	7	1.9	1.2	2.60
B	35.29	656.20	471.16	23	17	11	13	7.8	8.8	1.40
C	23.11	498.64	834.30	12	19	6	15	6.0	15.6	0.60
D	9.20	331.44	2825.57	3	26	1.5	20	4.0	53.0	0.12
2. Column (batches)										
A_1	29.16	1648.58	143.71	48	4	23	3	19.7	2.7	11.50

DEAE-cellulose chromatography. The 8000 ×*g* supernatant fraction, containing approx. 2.5 g of protein, was applied to a DE-52 column equilibrated with Buffer A. Elution of this column (5 ×18 cm) with 1 l of a 0.05 M linear NaCl gradient (pH 7.5) resulted in a broad phosphatase peak with activity towards both substrates (the several peaks of phosphatase activity seen were difficult to reproduce). The elution pattern is shown in Fig. 1. Active fractions eluted between 0.1 and 0.3 M NaCl, were pooled. The sample of 300 ml was dialyzed three times against 1.5 l Buffer A for a total of 9 h. A 2.5- and 3.1-fold purification of enzyme activity towards H-P and MS-P, respectively, was obtained.

First histone affinity chromatography. The dialyzed DEAE preparation (approx. 340 mg of protein) was put on a histone-Sepharose 4B affinity column, which had been equilibrated with Buffer A. Elution from this column (4 ×12 cm) with 1 l of a 0—2 M linear NaCl gradient (pH 7.5) resolved the phosphatase activity into four distinct peaks, which were eluted at the following NaCl concentrations: A, between 0.2 and 0.4 M; B, between 0.6 and 0.8 M; C, between 0.8 and 1.0 M; and D, between 1.3 and 1.6 M (Fig. 2). Samples between 90 and 150 ml were dialyzed twice against 3.5 l Buffer A for a total of 9 h. While B, C and D contained very low protein concentrations (63—346 μg/ml), peak A coincided with the main protein peak (1960 μg/ml). No further purification of either activity was seen in fraction A. Fraction B showed equal increases in the specific activities against both phosphorylated substrates. In fraction C 6- and 16-fold purifications were obtained for activity towards H-P and MS-P, respectively. In fraction D the major phosphatase activity found was

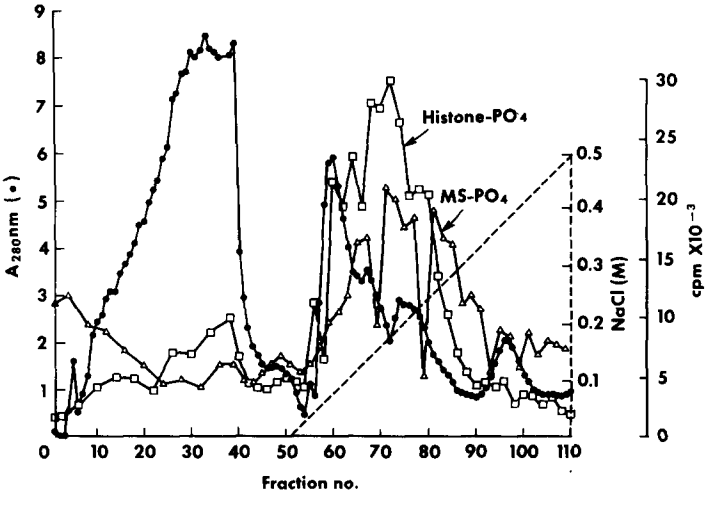

Fig. 1. DEAE-cellulose chromatography of smooth muscle phosphoprotein phosphatase. The supernatant of 8000 ×*g* centrifugation (2.5 g protein) was applied to a DEAE-cellulose column (5 × 18 cm) equilibrated with 25 mM Tris · HCl, 1 mM EDTA, 5 mM $MgCl_2$, 30 mM β-mercaptoethanol, 5 mM $MnCl_2$, pH 7.5. Enzyme activity was eluted with a linear gradient of 1000 ml NaCl (0—0.5 M) in the equilibration buffer. Absorbance at 280 nm (•———•) was measured against a buffer blank eluted from the column. Phosphatase activity was determined as phosphate released from phosphorylated histone (□———□) and phosphorylated muscle specific substrate (△———△). Assays were conducted at pH 7.2; other conditions as described in Methods. Fractions 64—85 were pooled.

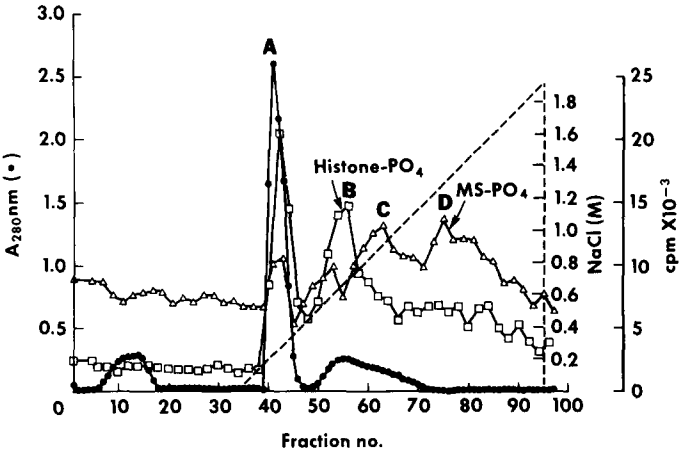

Fig. 2. Affinity chromatography of smooth muscle phosphoprotein phosphatase. Approx. 340 mg protein, obtained from the DEAE-cellulose column, were applied to a histone-Sepharose 4B column (4 X 12 cm), prepared as described in Methods, which was equilibrated with 25 mM Tris · HCl, 1 mM EDTA, 5 mM $MgCl_2$, 30 mM β-mercaptoethanol, 5 mM MnCl, pH 7.5. Enzyme activity was eluted with a linear gradient of 1000 ml NaCl (0—2 M) in the equilibration buffer. Absorbance at 280 nm (•————•) was measured against a buffer blank eluted from the column. Phosphatase activity was determined as phosphate released from phosphorylated histone (□————□) and phosphorylated muscle specific substrate (△————△). Assays were conducted at pH 7.2; other conditions as described in Methods. Fractions 40—45 (peak A), 51—58 (peak B). 59—65 (peak C) and 73—82 (peak D) were pooled.

directed towards MS-P and was 53-fold purified compared to a 4-fold purification of activity towards H-P. The activity ratios of the enzymes are shown in Table I.

Second histone affinity chromatography. After the histone affinity column had been washed extensively with 3 M NaCl in Buffer A to remove residual bound protein, the gel was re-equilibrated with Buffer A and fraction A was recycled and refractionated with batches of 0.3, 0.6, 0.9, 1.2 and 1.5 M NaCl (Table II). The activity peak eluted at 0.6 M NaCl (A_I) was identified as the

TABLE II

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASE ON A SECOND HISTONE AFFINITY COLUMN

Elution profile of phosphoprotein phosphatase activity in peak A (150 mg protein), obtained from the preceding affinity column, when refractionated on the histone-Sepharose 4B column and eluted with batches (150 ml) of NaCl (0.3—1.5 M) in the equilibration buffer (25 mM Tris · HCl, 1 mM EDTA, 5 mM $MgCl_2$, 30 mM β-mercaptoethanol, 5 mM MnCl, pH 7.5). Absorbance at 280 nm was measured against the buffer blank eluted from the column. Phosphatase activity was determined as phosphate released from phosphorylated histone and phosphorylated muscle specific substrate. Assays were conducted at pH 7.2; other conditions as described in Methods.

	Wash	Fraction NaCl (M)				
		0.3	0.6	0.9	1.2	1.5
Absorbance	0.092	0.438	0.393	0.123	0.072	0.096
^{32}P released from H-P (cpm)	0	0	2152	0	0	0
^{32}P released from MS-P (cpm)	0	0	0	0	0	0

main phosphohistone phosphatase with 20- and 3-fold increased activity towards H-P and MS-P, respectively (R = 11.5), when compared to the activities in the 8000 $\times g$ supernatant fraction. The final yields of the partially purified phosphoprotein phosphatases in relation to the 8000 $\times g$ supernatant were, for H-P as substrate: 23% (A_I), 11% (B), 6% (C) and 1.5% (D), for MS-P as substrate: 3% (A_I), 13% (B), 15%(C) and 20% (D).

Characterization of the phosphoprotein phosphatase fractions

Divalent cations. The stabilizing effect of manganese on phosphatase activity from smooth muscle has been discussed earlier. To evaluate the degree of Mn^{2+} dependence the purified enzymes were extensively dialyzed against Mn^{2+}-free buffer. This procedure did not change the phosphatase activity towards H-P of A, B and C and slightly inactivated D; the activity towards MS-P, however, was strongly diminished after manganese had been removed (Table III). The gradual readdition of Mn^{2+} to the assay medium did not affect the dephosphorylation of H-P caused by A_I, B and C (with an inhibitory effect of higher concentrations on A_I), but restored the activity of D almost completely. The MS-P phosphatase activity was increased upon stepwise readdition of Mn^{2+}. The activity of A_I and C was comparable to that observed in the undialyzed samples when 0.5 mM Mn^{2+} was present, whereas in B and D, the addition of 10 mM Mn^{2+} restored 75% of the original activity (Table III). In order to investigate if Mn^{2+} could be replaced by another divalent cation, 5 mM Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+} or spermidine were added to the dialyzed samples and their effects were compared to that of Mn^{2+}. The results can be seen in Table III. The dephosphorylation of H-P, unaffected by Mn^{2+} removal, was not altered by Mn^{2+}, Mg^{2+} or Ba^{2+}, but was markedly inhibited by the other ions tested in the order $Ca^{2+} < Zn^{2+} <$ spermidine. The phosphatase activity towards MS-P, almost abolished by Mn^{2+} removal, could not be restored by any other divalent cation but Mn^{2+}. It should

TABLE III

EFFECTS OF DIVALENT CATIONS ON PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

Effects of various divalent cations on phosphoprotein phophatase activities against histone and phosphorylated muscle specific substrate compared with the effect of Mn^{2+}. The enzymes stored in the presence of 5 mM Mn^{2+} were extensively dialysed against Mn^{2+}-free buffer and different divalent cations (5 mM) were added to the Mn^{2+}-depleted sample. Assays were conducted at pH 7.2; other conditions as described in Methods. Results are expressed in percent of control activity.

	Enzyme fraction							
	A_I		B		C		D	
Substrate:	H-P	MS-P	H-P	MS-P	H-P	MS-P	H-P	MS-P
Control	100	100	100	100	100	100	100	100
Control after dialysis	107	2	91	15	78	40	68	9
Mn^{2+}	76	84	96	88	93	88	95	75
Mg^{2+}	97	10	87	22	78	19	69	31
Ca^{2+}	32	7	49	15	49	46	39	18
Ba^{2+}	105	10	89	17	79	34	81	14
Spermidine	9	4	36	19	31	21	35	2
Zn^{2+}	4	1	4	2	2	3	3	17

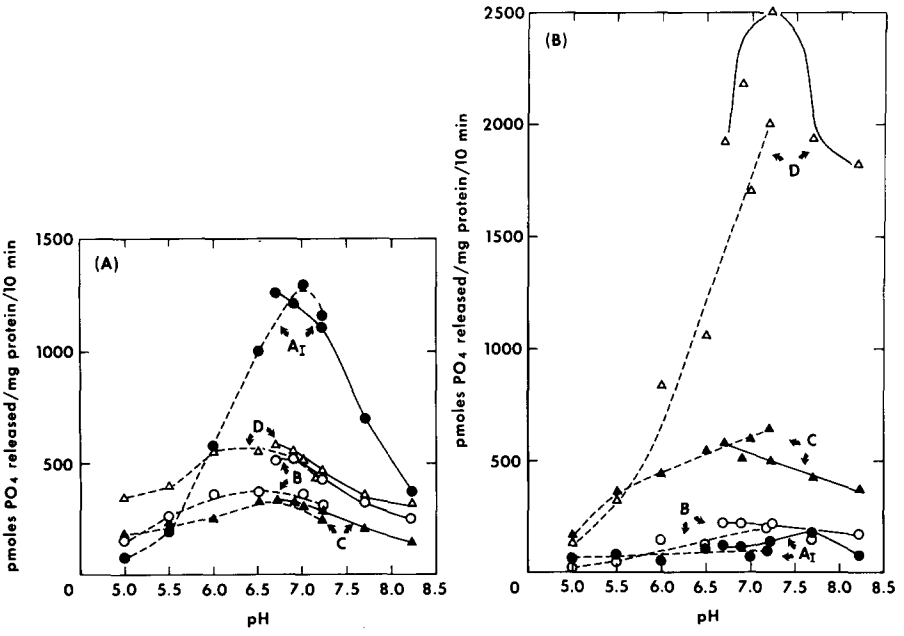

Fig. 3. Phosphoprotein phosphatase activity as a function of pH with H-P (A) or MS-P (B) as substrate. Conditions of assay as described in Methods except for the variation in pH. - - - - - -, histidine buffer; ———, Tris buffer.

be pointed out that the use of spermidine phosphate (instead of the free base) in this concentration cannot account for the observed 100% inhibition of phosphatase activity towards either substrate (see Inhibitor Section).

pH optimum. Maximal phosphatase activity was in the range of pH 6.5—7.2 for all four phosphoprotein phosphatase fractions and towards both enzyme substrates (Figs. 3a and 3b).

Molecular weights. The sedimentation behavior of the phosphatase fractions on sucrose density gradients revealed broad bands of activity in the range of 50 000—80 000 daltons for both substrates. A second peak of activity against MS-P was obtained at approx. 165 000 daltons. Precise molecular weights were not obtainable because of the lack of resolution with sucrose gradient centrifugation, possibly due to dilution of enzymatic activity.

Inhibitors of phosphatase activity. The inhibitory effects of sodium molybdate (1, 5, 10 mM), NaF (10, 50 mM) and potassium phosphate (1, 10 mM) on the isolated phosphatases are presented in Table IV. In the case of A_I these studies were only conducted with H-P as substrate because the activity towards MS-P was too low to allow definitive evaluations. Sodium molybdate was identified as the most potent inhibitor. 10 mM Na_2MoO_4 produced 100% inhibition of phosphatase activity in all four enzymes. At low concentrations of Na_2MoO_4 (1 and 5 mM) the phosphatase activity towards MS-P was inhibited significantly more than the activity towards H-P. When NaF or potassium phosphate was used, A_I was found to be significantly more sensitive to these inhibitors than the other enzymes.

Nucleotides. The influence of ATP, 5'-AMP, cyclic AMP and cyclic GMP on

TABLE IV

EFFECTS OF NUCLEOTIDES AND INHIBITORS ON PHOSPHATASE ACTIVITY

Phosphoprotein phosphatase activities using the two substrates were measured under standardized conditions in Tris · HCl buffer, pH 7.0. The data are the means ± S.E. of 3—5 observations. * indicates P values <0.05 when compared to untreated enzymes. Results are expressed in percent of control activity.

	Enzyme fraction							
	A_I		B		C		D	
Substrate:	H-P	MS-P	H-P	MS-P	H-P	MS-P	H-P	MS-P
ATP (1 mM)	43 ± 3*	—	53 ± 5*	37 ± 9*	69 ± 6	70 ± 7	69 ± 10	54 ± 14
5′-AMP (1 mM)	117 ± 16	—	76 ± 3*	32 ± 2*	100 ± 2	73 ± 7	102 ± 13	60 ± 11
Cyclic AMP (0.1 mM)	67 ± 6*	—	94 ± 2	115 ± 28	91 ± 4	77 ± 2*	82 ± 7	83 ± 9
Cyclic GMP (0.1 mM)	76 ± 11	—	94 ± 2	93 ± 6	82 ± 3*	66 ± 6*	108 ± 15	97 ± 18
Na_2MoO_4								
1 mM	92 ± 3	—	96 ± 5	69 ± 12	96 ± 7	59 ± 6	81 ± 9	50 ± 5
5 mM	58 ± 4*	—	61 ± 3*	11 ± 6*	58 ± 3*	16 ± 7*	49 ± 3*	20 ± 2*
10 mM	2 ± 1*	—	5 ± 5*	2 ± 2*	3 ± 2*	0 ± 0*	0.3 ± 0.3 *	1 ± 1*
NaF								
10 mM	19 ± 2*	—	58 ± 1*	71 ± 11	70 ± 10	57 ± 6*	69 ± 4	73 ± 10
50 mM	2 ± 1*	—	63 ± 2*	41 ± 6*	63 ± 9*	34 ± 2*	49 ± 6*	48 ± 9*
KH_2PO_4								
1 mM	21 ± 2*	—	43 ± 3*	34 ± 7*	51 ± 6*	61 ± 10*	62 ± 5*	44 ± 14*
10 mM	10 ± 1*	—	25 ± 8*	21 ± 8*	32 ± 6*	40 ± 8*	41 ± 4*	27 ± 4*

phosphatase activities, shown in Table IV can be summarized as follows: A_I: Significant inhibition of H-P activity by ATP and cyclic AMP. No significant effect of 5′-AMP or cyclic GMP. B: Significant inhibition of H-P and MS-P activities by ATP and 5′-AMP. No significant effect of cyclic AMP or cyclic GMP. C: Significant inhibition of H-P activity by cyclic GMP, of MS-P by cyclic AMP and cyclic GMP. No significant effect of ATP or 5′-AMP. D: No significant effect of ATP, 5′-AMP, cyclic AMP or cyclic GMP.

Discussion

The results presented here indicate the presence of multiple fractions of phosphoprotein phosphatase activity within tracheal smooth muscle. These different fractions have been resolved by the use of histone affinity chromatography and their activities were measured towards both phosphorylated histone (H-P) and phosphorylated muscle specific protein kinase substrate (MS-P). The fractions differed in their relative activities towards each substrate ranging from fraction A_I with activity mainly towards H-P to fraction D with activity mainly towards MS-P. There was a 100-fold difference in the activity ratios of these two fractions. The various fractions also differed with regard to inhibition by NaF and sodium molybdate and influence of nucleotides. These data suggest that tracheal smooth muscle might contain four distinct phosphoprotein phosphatases. However, the possibility that the characterized phosphatase fractions are different forms of the same enzyme produced by proteolytic activity or polymerization-depolymerization cannot be totally discounted. The fact that fractions with similar characteristics have been repeatedly obtained, diminishes the probability of the former occurrence. With regard to the latter, multiple forms of phosphoprotein phosphatase activity of polymeric origin have been reported to differ in their elution behavior on DEAE-cellulose, substrate specificity, degree of Mn^{2+} stimulation and molecular weight [31—33]. There appears to be a gradient of activities from fraction A_I (high H-P, low MS-P) to fraction D (low H-P, high MS-P) and most of the characteristics also change gradually from A to D. It could be that fraction B and C are mixtures of A_I and D.

The tracheal smooth muscle is relatively hetergeneous. It is, therefore, possible that these different fractions of phosphatase activity are located within different cell types in vivo. Abe and Tsuiki [34] described the presence of six phosphatase forms in rat liver, not derived from enzyme degradation or aggregation, and distinguishable on the basis of molecular weight and tissue distribution. The different effects of cyclic nucleotides on the activity of the fractions A_I, B, C and D support the proposal of the presence of four different phosphoprotein phosphatases in tracheal smooth muscle. However, since significant cyclic nucleotide effects on the phosphatase activity required concentrations of 10^{-4} M, these results may be physiologically unimportant. Most studies on phosphoprotein phosphatases did not find effects of cyclic AMP [22,23] or cyclic GMP [23,35]. However, there are two reports on soluble phosphorylase phosphatase demonstrating cyclic AMP-induced inactivation of the enzyme at nucleotide concentrations of $5 \cdot 10^{-7}$—$1 \cdot 10^{-5}$ M [36] or $1 \cdot 10^{-4}$ M [37], and the membrane-bound phosphoprotein phosphatase from toad bladder has been shown to be stimulated by cyclic AMP [19].

The data presented here using two substrates for tracheal smooth muscle phosphoprotein phosphatase demonstrated different requirements for Mn^{2+}. After dialyzing the four phosphatase fractions against Mn^{2+}-free buffer the activity towards H-P was unchanged or slightly reduced, while the activity towards MS-P was lost almost completely. The readdition of Mn^{2+} to the assay medium did not drastically alter the activity towards H-P. However, the readdition of Mn^{2+} did restore the phosphatase activity towards MS-P of all four fractions. There are at least two possible explanations for these observations: The first is that the actual substrate in the case of MS-P is the MS-P · Mn^{2+} complex. The second explanation is that there are different catalytic binding sites for the two substrates. The MS-P binding site binds Mn^{2+} loosely, while the H-P binding site binds Mn^{2+} too tightly to be removed by dialysis. The data do not differentiate between these two possibilities. Other cases of unusual Mn^{2+} dependence of phosphoprotein phosphatase activity have been reported [15,38—41]. For example, Nakai and Thomas [12] showed that Mn^{2+} could either stimulate or inhibit phosphatase activity depending on the substrate used.

The information provided in this report expands our knowledge of the biochemistry of phosphoprotein phosphatases. This knowledge is essential for the full elucidation of the mechanism by which protein phosphorylation regulates cellular function.

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References

1 Diamond, J. and Blisard, K.S. (1976) Mol. Pharmacol. 12, 688—692
2 Verma, S.C. and McNeill, H.J. (1976) J. Pharmacol. Exp. Ther. 198, 539—543
3 Nesheim, B.-I., Osnes, J.-B. and Øye, I. (1975) Br. J. Pharmacol. 53, 403—407
4 Harbon, S., DoKhac, L. and Vesin, M.-F. (1976) Mol. Cell. Endocrinol. 6, 17—34
5 Katsuki, S. and Murad, F. (1977) Mol. Pharmacol. 13, 330—341
6 Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349—1355
7 Sands, H., Meyer, T.A. and Rickenberg, H.V. (1973) Biochim. Biophys. Acta 302, 267—281
8 Maeno, H. and Greengard, P. (1972) J. Biol. Chem. 247, 3269—3277
9 Murray, A.W., Froscio, M. and Kemp, B.E. (1972) Biochem. J. 129, 995—1002
10 England, P.J., Stull, J.T. and Krebs, E.G. (1972) J. Biol. Chem. 247, 5275—5277
11 Zieve, F.J. and Glinsmann, W.H. (1973) Biochem. Biophys. Res. Commun. 50, 872—878
12 Nakai, C. and Thomas, J.A. (1974) J. Biol. Chem. 249, 6459—6467
13 Khandelwal, R.L., Vandenheede, J.R. and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850—4858
14 Titanji, V.P.K., Zetterqvist, O. and Engström, L. (1976) Biochim. Biophys. Acta 422, 98—108
15 Li, H.-C. and Hsiao, K.-J. (1977) Arch. Biochem. Biophys. 179, 147—156
16 Kirchberger, M.A. and Raffo, A. (1977) J. Cyclic Nucl. Res. 3, 45—53
17 Chou, C.-K., Alfano, J. and Rosen, O.M. (1977) J. Biol. Chem. 252, 2855—2859
18 Titanji, V.P.K. (1977) Biochim. Biophys. Acta 481, 140—151
19 DeLorenzo, R.J. and Greengard, P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1831—1835
20 Liu, A.Y.-C and Greengard, P. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3869—3873
21 Severson, D.L., Khoo, J.C. and Steinberg, D. (1977) J. Biol. Chem. 252, 1484—1489
22 Tellez De Inon, M.T. and Torres, H.N. (1973) Biochim. Biophys. Acta 297, 399—412
23 Albin, E.E. and Newburgh, R.W. (1975) Biochim. Biophys. Acta 377, 381—388
24 Sands, H., Penberthy, W., Meyer, T.A. and Jorgensen, R. (1976) Biochim. Biophys. Acta 445, 791—801

25 Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U.S. 74, 560—564
26 Sands, H., Mascali, J. and Paietta, E. (1977) Biochim. Biophys. Acta, in the press
27 Plaut, G.W.E. (1963) in Methods in Enzymology (Colowick and Kaplan, eds.), Vol. VI, pp. 319—324, Academic Press, New York
28 Heil, A. and Zillig. W. (1970) FEBS Lett. 11, 165—168
29 Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059—3065
30 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
31 Kato, K., Kobayashi, M. and Sato, S. (1974) Biochim. Biophys. Acta 371, 89—101
32 Kobayashi, M., Kato, K. and Sato, S. (1975) Biochim. Biophys. Acta 377, 343—355
33 Kobayashi, M. and Kato, K. (1977) J. Biochem. 81, 93—97
34 Abe, N. and Tsuiki, S. (1974) Biochim. Biophys. Acta 350, 383—391
35 Nuttall, F.Q., Gannon, M.C., Corbett, V.A. and Wheeler, M.P. (1976) J. Biol. Chem. 251, 6724—6729
36 Chelala, C.A. and Torres, H.N. (1970) Biochim. Biophys. Acta 198, 504—513
37 Merlevede, W. and Riley, G.A. (1966) J. Biol. Chem. 241, 3517—3524
38 Kato, K. and Sato, S. (1974) Biochim. Biophys. Acta 358, 299—307
39 Li, H.-C. (1975) FEBS Lett. 55, 134—137
40 Kato, K., Kobayashi, M. and Sato, S. (1975) J. Biochem. 77, 811—815
41 Yang, H.-Y., T., Costa, E., Majane, E.A. and Hong, J. (1977) J. Neurochem. 28, 1075—1080