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CYCLIC AMP-BINDING PROTEINS : ENHANCED RETENTION ON MEMBRANE FILTERS BY HISTONE AND PROTAMINE SULFATE

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SUMMARY : Polycations such as histone and protamine sulfate increase the efficiency of retention of cAMP-binding protein complexes on cellulose ester membrane filters. A 40,000 dalton cAMP-binding protein from calf ovaries was not retained on the membrane filters when assayed above pH 5.5. The addition of histone or protamine sulfate to the cAMP-binding assay completely abolished the pH dependency and resulted in retention of the complex. The retention of larger cAMP-binding proteins as well as the ovarian cAMP-dependent protein kinases showed little effect of histone at low pH values while a significant enhancement was observed above pH 7.0.

INTRODUCTION : Adenosine 3',5'-monophosphate (cAMP) is one of the important intracellular mediators of hormonal action in many tissues (1). Much interest has been devoted to the binding of cAMP to the regulatory subunit of the cAMP-dependent protein kinase (ATP : protein phosphotransferase EC2.7.1.37) (2, 3). Cyclic AMP acts on this enzyme by dissociating the inactive holoenzyme (RC) to yield free catalytic subunit (C) and cAMP-regulatory subunit complex (R-cAMP).

The membrane filter assay (4,5) is widely used today to assay for cAMP-binding proteins. This technique relies on the ability of cellulose ester filters to quantitatively adsorb the cyclic [^{3}H] AMP : receptor complex. Therefore any factors which affect the binding of cAMP will be of importance. The pH of the binding assay has been described to affect both the ability to collect the bound cAMP and the amount bound (6). It has been reported that certain polycations such as protamine sulfate and histone enhance the binding of cAMP to protein kinases using this membrane filter assay (7,8,9). This has been interpreted by some (8) as a stimulation of cAMP-binding activity. In this communication we have examined the basis for this enhancement of cAMP-binding by polycations. Our data provide experimental evidence to the theoretical speculations of Tao and Hacket (7) that the effect of polycations is merely an enhanced retention of the cAMP-binding protein on the membrane filter.

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MATERIALS AND METHODS : Materials for Sephadex gel filtrations were obtained from Pharmacia. Bovine serum albumin, aminophylline, cAMP, phosvitin, ovalbumin and histone (total) were obtained from Sigma. Protamine sulfate and theophylline were obtained from Merck [γ-^{32}P] ATP (4-8 Ci/mmol) and cyclic [^{3}H] -AMP (37 Ci/mmol) were obtained from New England Nuclear.

The cytosol fraction was prepared from calf ovaries and chromatographed on DEAE-cellulose as previously described (10). Protein kinase activity was measured using protamine sulfate as substrate in the presence of 3.3 μMcAMP (10). Binding of cyclic [^{3}H] -AMP to protein was routinely determined by modifications of the membrane filter assay (4,5) in a total incubation volume of 0.3 ml. The reaction mixture contained 33mM acetate buffer, pH 4.5 containing 4mM EDTA, 5mM aminophylline and 60nM cyclic [^{3}H] -AMP. Incubation was carried out for 90 min at 4^{o} followed by the addition of 1ml of ice-cold 20mM acetate buffer, pH 4.5 containing 1.5mM EDTA and 2mM aminophylline. The samples were filtered immediately through Millipore filters (PHWP 0.25μm) previously immersed in the same buffer. The reaction tubes and filters were washed with an additional 8 ml of the same buffer, the filters containing the protein-bound cyclic [^{3}H] -AMP were dissolved in 8 ml of Instagel (Packard Corp.), and counted for radiocativity. Values presented represent specific cAMP binding as determined by reduction in binding activity with unlabelled cAMP and after correcting for unspecific binding in the absence of protein.

RESULTS : Two cAMP-binding proteins were partially purified from a calf ovary cytosol (Fig. 1). DEAE-cellulose chromatography separated a free cAMP- binding protein (D-1) from the major cAMP-dependent protein kinase activity which was partially resolved into two peaks. Sephadex G-200 chromatography of D-1 revealed a cAMP-binding protein BP-1 with an estimated molecular weight of 145,000 daltons. This component may represent the free dimer of the regulatory subunit (R_2) of the cytosol cAMP-dependent protein kinase (10). In addition to BP-1 a smaller peak of cAMP-binding activity eluted at 44,000 daltons (Fig. 1B). Gel filtration of the first cAMP-dependent protein kinase peak (D-2) was able to resolve two peaks of cAMP-binding activity (Fig. 1C). The first coincided with the protein kinase activity and represents the R_2-subunit of the 230,000 dalton cAMP-dependent protein kinase. The second binding peak (BP-2) was not associated with protein kinase activity and represents a free cAMP-binding protein of 44,000 dalton. This small cAMP-binding protein is probably a proteolytic fragment of the larger subunit (11). Controlled protease digestion of the 230,000 dalton holoenzyme resulted in completed conversion to an 88,000 dalton cAMP-dependent protein kinase. There was specific cleavage of the regulatory subunit, which yields a small cAMP-binding protein of approximately 44,000 dalton (manuscript in preparation). A small cAMP-binding protein of similar size has also been observed in the nuclear fraction of calf ovaries (11).

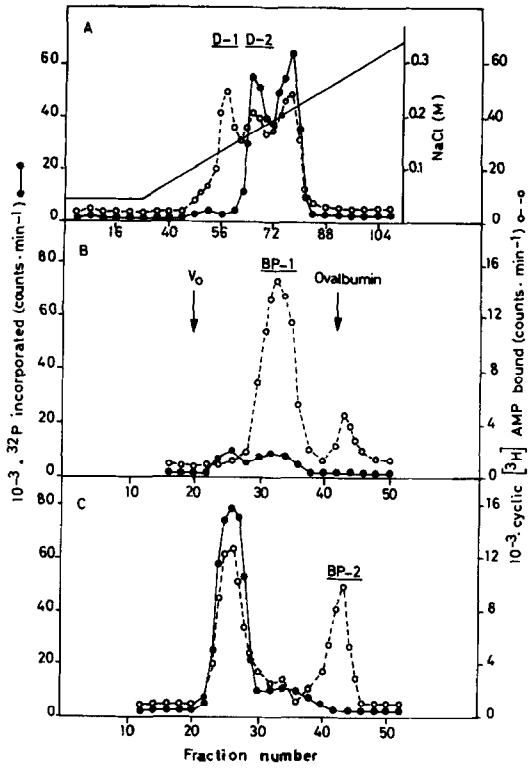

Fig. 1 :

Chromatography of calf ovarian protein kinases and cAMP-binding proteins (A) DEAE-cellulose. An ammonium sulfate fraction (11) was applied to a DEAE-cellulose column (2.5 x 35 cm). Elution was carried out with a 75 - 350 mM NaCl gradient in 10 mM Tris buffer, pH 7.4, containing 6 mM 2-mercaptoethanol. Fractions of 15 ml were collected (B) Sephadex G-200. The fractions in peak D-1 from the DEAE column were pooled, concentrated and a 2 ml aliquot was applied to a Sephadex G-200 column (2.0 x 80 cm). Elution was carried out with 20 mM Tris buffer, pH 7.4, containing 200 mM NaCl and 6 mM 2-mercaptoethanol. Fractions of 3.0 ml were collected. (C) Sephadex G-200. Peak D-2 from the DEAE column was concentrated and a 2 ml aliquot was applied to the Sephadex G-200 column and chromatographed as described above. Aliquots of 5 μl from the DEAE column and 25 μl from the Sephadex columns were assayed for protein kinase activity as previously described (11). Aliqutos of 25 μl were assayed for cAMP-binding as described in Methods.

During the characterization of the ovarian cytosol cAMP-binding proteins it was evident that using the membrane filter assay significant differences existed in the cAMP-binding activities depending upon the incubation conditions. This is demonstrated in Fig. 2 where BP-1 and BP-2 were assayed at different pH values. The binding activity of BP-1 declined rapidly above pH 5.0 and was similar to background beyond pH 6.5. However, if the assay was carried out in the presence of histone the activity was significantly elevated and was relatively

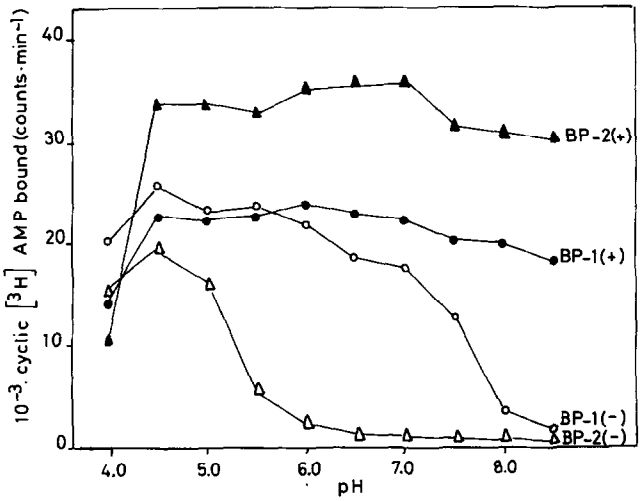

Fig. 2 : Effect of pH and histone on the cAMP-binding activities of BP-1 and BP-2. The two cAMP-binding proteins obtained from the Sephadex G-200 chromatography were assayed in the absence (o—o, Δ—Δ) and presence (●—●, ▲—▲) of 100 μg of total histone at the indicated pH calues : pH 4.0 - 5.5, sodium acetate buffer ; pH 6.0 - 7.0, potassium phosphate buffer ; pH 7.5 - 8.5, Tris/HCl buffer. The respective buffers at the indicated pH values were also utilized in the washing of the filters. The concentrations of buffer, EDTA and aminophylline were as described in Methods.

TABLE 1

EFFECT OF VARIOUS PROTEINS ON THE cAMP-BINDING ACTIVITIES OF BP-1 AND BP-2

	BP-1		BP-2	
Protein	pH 5.0	pH 8.0	pH 5.0	pH 8.0
	cAMP-binding activity(cpm x 10^3)			
none	21.7	6.8	13.1	0.4
BSA	25.1	10.7	21.8	0.6
phosvitin	21.4	9.9	15.4	0.7
ovalbumin	23.0	10.2	19.8	0.5
protamine	24.5	22.5	19.2	21.5
histone	24.8	23.4	31.2	37.4

The two cAMP-binding proteins were assayed at pH 5.0 and pH 8.0 in the presence of 100 μg of the indicated proteins.

constant between pH 4.5 and pH 8.5. The enhancement of the binding activity by histone was about 1.5 fold at pH 4.5 and over 7-fold at pH 5.5. In contrast the cAMP-binding activity of PB-1 was only slightly affected by histone between pH 4.5 and pH 7.0. Above pH 7.0 a pronounced enhancement by histone was also observed with this protein but to a lesser extent than BP-2.

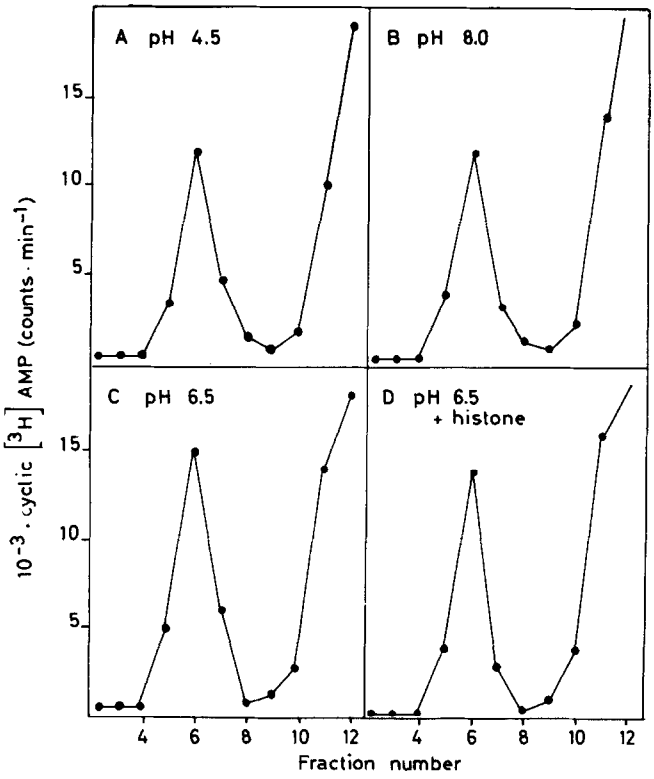

Fig. 3 :
Sephadex G-25 chromatography. Binding protein BP-2 was assayed at pH 4.5 (Fig. 3A), pH 8.0 (Fig. 3B) and pH 6.5 in the absence (Fig. 3C) and presence of 100 µg of histone (Fig. 3D). For the separation of bound and free cAMP the incubation mixtures were passed over a Sephadex G-25 column (0.7 x 12 cm) and the column was eluted with the respective buffers. Fractions of 0.5 ml were collected and counted for radioactivity. In the absence of binding protein no radioactivity was detected in fractions 5 - 8.

To ascertain the protein specificity of this phenomena, several different proteins were examined at two pH values where high and low cAMP-binding activities were observed. Table 1 illustrates that polycations (histone and protamine sulfate) were able to enhance the cAMP-binding activity whereas bovine serum albumin, phosvitin and ovalbumin had no effect.

This effect of polycations could represent an actual stimulation of cAMP-binding activity or it may be an artifact of the membrane filter assay. This question was resolved by determining the cAMP-binding activity of PB-2 using Sephadex G-25 chromatography to separate the protein-bound and free ligand. BP-2 was assayed at pH 4.5 (Fig. 3A), pH 8.0 (Fig. 3B), pH 6.5 in the absence (Fig. 3C) and presence (Fig. 3D) of histone. The cAMP-binding activity using this assay procedure showed only a slight pH dependency and there was no observable effect of histone on the binding of cAMP. Similar results were also obtained for BP-1.

Comparable cAMP-binding activities were found using the Sephadex G-25 procedure and the membrane filter assay when histone was included in the incubation buffer. Thus the apparent stimulatory effect of histone is a result of higher retention of the cAMP-binding protein complex on the filters. Support for this explanation was obtained by assaying BP-2 at pH 6.5 in the absence of histone and collecting the buffer passing through the filter. Histone was added to this filtrate and the mixture filtered again. The majority of the original ligand-receptor complex had passed through the filter and was only recovered after histone had been added.

DISCUSSION : The retention of the cAMP-receptor complex in the absence of histone is a function of both pH and molecular size. BP-2 was only retained at pH values below 5.5, while BP-1 showed a significant drop in the binding activity only above pH 7.5. The addition of histone to the incubation buffer or directly to the filter resulted in a significant increase in the retention of BP-2. Histone also aided in the retention of BP-1 above pH 7.0. The 230,000 dalton cAMP-dependent protein kinase exhibited similar cAMP-binding properties (pH and histone dependency) as BP-1.

Both cAMP-binding proteins (BP-1 and PB-2) are acidic proteins with isoelectric points between pH 4.7 and pH 5.3 (manuscript in preparation). Above pH 5.5 they are negatively charged and not be completely retained on the filters due to this charge. It is possible that polycations, being positively charged, are able to neutralize this charge and thus increase the efficiency of adsorption. It is likewise conceivable that the increased retention at low pH is due to aggregation of these proteins at or near their isoelectric points. Polycations may form aggregates with these proteins and in this way increase retention.

The results described here make it clear that caution must be exercised in analyzing for cAMP-binding activity. The results both quantitative and qualitative can be strongly influenced by the pH and the method of assay. In the membrane filter assay there can be preferential loss of some classes of binding proteins due to lack of retention on the filters. This latter difficulty as well as the pH effect of the retention can be overcome by the inclusion of histone in the cAMP-binding assay.

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