PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 698–701 (1975)

Specific Adenosine Binding Proteins from Rat Liver (38882)

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The roles of adenosine in the regulation of cellular processes have been increasingly recognized. In cell-free systems adenosine was found, depending on its concentration, to activate (1) or inhibit (2, 3) adenyl cyclase from various tissues. In addition, adenosine inhibits phosphodiesterase (4–6) and protein kinase (7) from a variety of sources. Most interestingly, adenosine acts synergistically with biogenic amines to elicit accumulations of cAMP in brain slices (8). It reacts synergistically also with parathyroid hormone to accumulate cAMP[1] in cultured bone cells (9). In order to understand the specific role of adenosine, it is important to study in detail the interactions between intracellular compartments and adenosine. This report describes the chromatographic fractionation of adenosine-binding proteins from rat liver in relation to activities of cAMP binding and histone phosphokinase.

Methods. Adenosine-binding proteins were prepared essentially according to the procedure of Walsh *et al.* (10) for the preparation of protein kinase from bovine muscle. Fresh livers (10 g) of Holtzman rats were homogenized in 3 vol of 4 m*M* EDTA. The homogenates were adjusted to pH 5.5 with 1 *N* acetic acid. The resulting precipitate was removed by centrifugation (13,000*g*, 10 min) and discarded and the pH of the supernate readjusted to 6.8 with 1 *M* potassium phosphate buffer (pH 7.5). Solid ammonium sulfate (0.325 g/ml) was added slowly and the solution stirred for 60 min at 4°. The precipitate which then resulted was collected by centrifugation (13,000*g*, 10 min) then dissolved in 1.0 tissue volume of 5 m*M* potassium phosphate buffer containing 2 m*M* EDTA (pH 7.0). The protein solution was dialyzed overnight against 250 vol in the same buffer and diluted to final 1.5 tissue volumes then centrifuged at 75,000*g* for 30 min. The supernate was then applied to a column of DEAE–cellulose (1.2 × 12 cm) equilibrated with the same buffer. The collection of 1-ml fractions of eluate was started immediately after the enzyme was applied. Development and elution continued by application of a linear gradient of 5 m*M* to 0.4 *M* potassium buffer (in a total volume of 200 ml). The flow rate was 0.3 ml/min.

For assay of cAMP and adenosine binding, duplicates or triplicates were prepared by adding 20 μl of each fraction to 0.2 ml of 0.05 *M* potassium phosphate buffer (pH 7.0) containing (unless otherwise indicated) 10,000–15,000 cpm of [^{3}H]adenosine or cAMP (30 Ci/mmole, New England Nuclear). The mixtures were incubated for 60 min at 4°. A modified method based on charcoal adsorption was used to separate the free, from the protein-bound, adenosine or cAMP (11). After the incubation period, 1 ml of the well-stirred suspension of cold charcoal mixture (2.5% charcoal, 0.3% Dextran T-70 and 1% human serum albumin) was added to each mixture. After mixing the tubes then stood for 10 min at 4°. After centrifugation at 2500 rpm at 4° for 10 min the supernates were decanted into scintillation vials which contained 10 ml of toluene–Triton X-100–fluors mixture as described by Boyce and Farley (12). The blanks contained no eluate, i.e., no binding proteins.

The protein kinase was assayed according to the method of Kuo and Greengard (13). The reaction mixture contained 0.05 *M* sodium acetate buffer, pH 6.0, 0.01 *M* magnesium acetate, 1 μ*M* [γ-^{32}P]ATP (10 Ci/mmole, New England Nuclear), 40 μg calf thymus histones (unfractionated, Sigma Chemical), 0.2 m*M* EGTA, and 2.5 × 10^{-6}*M* cAMP (when required), in a total volume of 0.2 ml. Unless otherwise indicated, the

[1] Abbreviations: cAMP, adenosine 3′,5′-monophosphate; EDTA, disodium ethylene diaminetetraacetate; EGTA, ethylene glycol bis (β-amino-ethyl ether)-*N*,*N*′-tetraacetic acid.

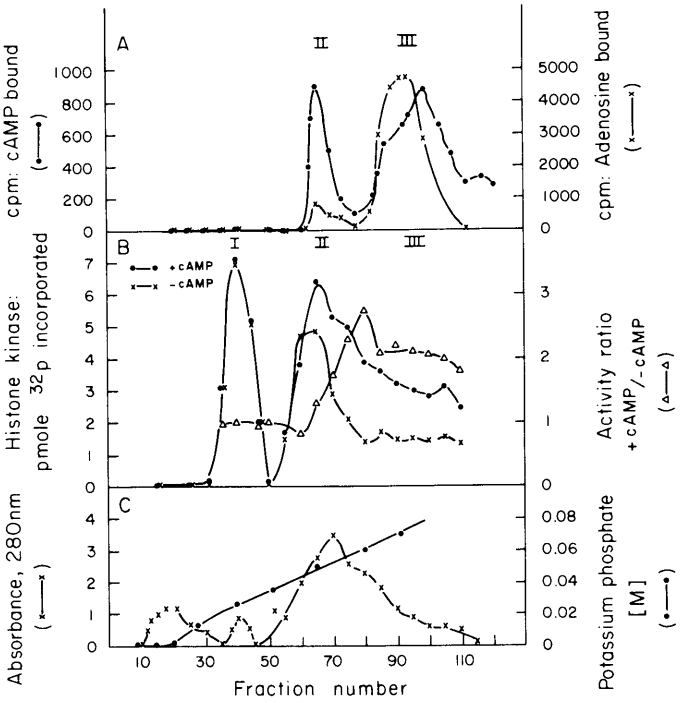

FIG. 1. Chromatography of cAMP, adenosine-binding proteins (A), and histone phosphokinase (B) on a DEAE-cellulose column. Experimental details are as described under Methods. A 20-μl aliquot from each fraction was assayed for histone phosphokinase and binding activities.

reaction was initiated by the addition of 20-μl aliquots of fractions. Incubations were carried out for 5 min at 30° with shaking (100 rpm). In each experiment enzyme and histone blanks were included.

Results. The fractionation of liver extract on a DEAE-cellulose column yielded an elution pattern which showed three major peaks (Fig. 1A and B) associated with varying degrees of cAMP or adenosine binding and histone phosphokinase activity. Peak I represents histone phosphokinase activity not stimulated by cAMP. Neither cAMP nor adenosine-binding activity was found in the fraction represented by Peak I. Peak II represents cAMP, adenosine-binding activities, and histone phosphokinase which was slightly (30%) stimulated by 2.5 μ*M* cAMP. As cAMP and adenosine-binding activities associated with Peak II were progressively decreased, the cAMP dependency of histone phosphokinase rapidly increased. It is of interest to note that the ability of cAMP to stimulate protein kinase peaks in the trough between Peak II and III (Fig. 1A and B)

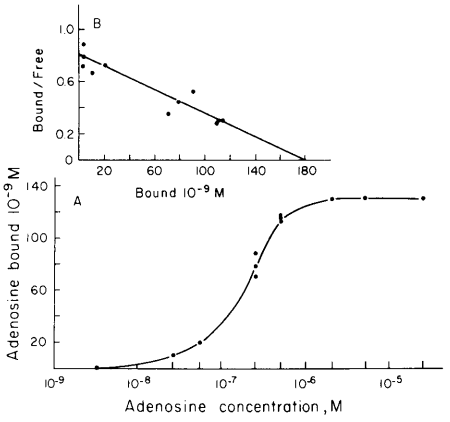

FIG. 2. The dose-response curve (A) and Scatchard plot (B) of adenosine binding. Aliquots of 20-μl fractions represented by Peak III and varying concentrations of [^{3}H]adenosine were used for binding studies. The details of binding assay are as described under Methods.

and plateaus throughout Peak III. Peak III represents major adenosine-binding activity (Fig. 1A). The major cAMP binding activity was found in Peak II. Further increases in

cAMP concentration (not plotted) increase its binding in Peak II but not in Peak III. Adenosine at $2.5 \times 10^{-5}M$ and $2.5 \times 10^{-4}M$ inhibited histone phosphokinase activities in all three peaks by 45 and 87%, respectively (unpublished observations).

Since the major part of adenosine binding (and cAMP stimulation) were found in Peak III, the dose-response curve and Scatchard plot (14) of adenosine binding as depicted in Fig. 2A and B were studied with fractions represented by Peak III. The apparent dissociation constant was $2.4 \times 10^{-7}M$ and the total binding was 18 nmole/mg protein (14, 15).

TABLE I. EFFECT OF ADENOSINE ANALOGS ON ADENOSINE BINDING[a]

Compounds	Concentrations	% Inhibition[b]
cAMP	$5.0 \times 10^{-5}M$	0
	$2.5 \times 10^{-4}M$	68
5'-AMP	$5.0 \times 10^{-5}M$	0
Adenine	$5.0 \times 10^{-6}M$	0
	$2.5 \times 10^{-5}M$	46
ATP	$5.0 \times 10^{-6}M$	0
	$2.5 \times 10^{-5}M$	42
Theophylline	$5.0 \times 10^{-4}M$	0

[a] [^{3}H]-adenosine at 2.5 nM (15,000 cpm) with or without an analog was incubated with 20-μl aliquots of fractions represented by Peak III for 1 hr at 4°. The details of the binding assay are as described under Methods.

[b] % Inhibitor = $100 - \frac{[^3\text{H}]\text{adenosine bound (cpm) in the presence of analog}}{[^3\text{H}]\text{adenosine bound (cpm) in the absence of analog}}$

The specificity of adenosine binding to constituents represented by Peak III is indicated in Table I. Five analogs (adenine, cAMP, ATP, AMP, theophylline) were tested for their ability to inhibit [^{3}H]adenosine binding to this fraction. These analogs exerted inhibition of binding only at 1000-fold or higher concentrations. The order of effectiveness of the inhibitors was adenine > ATP > cAMP > theophylline.

To test the possibility that cAMP independency of histone phosphokinases in fractions represented by Peaks I and II was due to dissociation of catalytic subunits from regulatory subunits (16, 17), the effect of mixing two fractions on protein kinase activity is shown in Table II. The nonadditive effect was seen especially when an aliquot represented by Peak III was present in the mixture. The inhibitory effect was not appreciably different in the presence and absence of cAMP. The effect of mixing did not seem to inhibit the stimulation by 2.5 μM cAMP when fractions from Peak III were present.

Discussion. The fractionation of rat liver extracts on DEAE-cellulose column indicate that only one of the two adenosine-binding fractions is associated with cAMP-stimulated histone phosphokinase (Fig. 1A and B). Adenosine inhibits the kinase activities to the same extent in those fractions where the adenosine binding is either absent or present (Peak I and II). Therefore, the

TABLE II. EFFECT OF COMBINATION OF PEAKS FROM DEAE-CELLULOSE COLUMN ON HISTONE PHOSPHOKINASE[a]

Fractions	cAMP and adenosine binding	Ratio of the combined activity[a] to the sum of activity of each peak		$\frac{+\text{cAMP}}{-\text{cAMP}}$[b]
		+cAMP	−cAMP	
Peak I	−	—	—	1.0
Peak II	+	—	—	1.3
Peak III	+	—	—	2.3
Peaks I + II	+	0.89	0.90	1.1
Peaks I + III	+	0.64	0.65	1.6
Peaks II + III	+	0.70	0.60	2.1

[a] An aliquot from each of the three peaks was selected so that the activity was equalized to 7 pmole of ^{32}P incorporated into histone per 0.2 ml of reaction mixture.

[b] The ratio of histone phosphokinase activity in the presence of cAMP to the activity in its absence.

present studies do not indicate whether the function of adenosine binding is to minimize the inhibitory effect of adenosine on protein kinases.

Fractions represented by Peak III associated with the major adenosine contains factors which inhibit cAMP-independent kinase activities in fractions from Peaks I and II (Table II). The possibility that the inhibitory factors are regulatory subunits is unlikely since the addition of cAMP did not reduce the inhibition resulting from mixing material from various peak fractions (Table II).

As in the case of cAMP receptor (18, 19) it is conceivable that adenosine receptors potentiate the action of cAMP or adenosine in various systems by arresting excessive amounts of free adenosine in the cell. It is of interest to note that similar types of adenosine receptors have been obtained recently from rabbit erythrocytes (20). The function of these adenosine receptors remains to be established.

Summary. Specific adenosine-binding proteins from homogenates of rat liver have been fractionated on a DEAE–cellulose column. Three major peaks have been identified with respect to histone phosphokinase and cAMP and adenosine-binding activities. Peak I contains only histone phosphokinase activity not stimulated by cAMP. Peak II contains histone phosphokinase slightly stimulated by cAMP. Both cAMP- and adenosine-binding activities are found in this fraction. The major adenosine-binding protein is associated with Peak III. Histone phosphokinase in Peak III which also binds cAMP is stimulated 2-fold by 2.5 μM cAMP whereas adenosine at $2.5 \times 10^{-4} M$ inhibits these enzymes equally well in each of three peaks. The specificity of adenosine binding is discussed.

The author is indebted to Dr. R. M. Archibald for his encouragement and invaluable suggestions in the preparation of this manuscript. The generous support, suggestions, and assistance of Drs. R. Eger, D. Rifkin, J. Sy, G. Vidali, and C. Stone and Mrs. P. Macklin are gratefully acknowledged. The work was supported in part by Grant 2R01-AM-11431 from NIH.

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Received Feb. 25, 1975. P.S.E.B.M., 1975, Vol. 149.