A cAMP RECEPTOR FROM MOUSE LIVER CYTOSOL WHOSE BINDING CAPACITY IS ENHANCED BY Mg^{++}-ATP

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SUMMARY: A receptor with a dissociation constant of $2 \cdot 10^{-6}$M for cyclic 3',5'-AMP (cAMP) has been found in mouse liver cytosol. This cAMP binding activity can be differentiated from the cAMP-dependent protein kinase holoenzymes and the free regulatory subunits also found in the cytosol. Mg^{++}-ATP increases the number of binding sites for cAMP several fold. This increased capacity for cAMP binding persists after Sephadex G-25 filtration, and incubation for 14 hours in the presence of 5 mM EDTA. Among several adenosine- and guanosine-derivatives tested, only AMP, ADP and ATP compete efficiently with [^{3}H] cAMP for the cAMP binding site.

The role of cAMP-dependent protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37.) as mediator for several intracellular cAMP-effects seems established (1,2).

The diversity of effects ascribed to cAMP makes it tempting to speculate that other cAMP binding proteins can mediate the cAMP-effect either directly (3), or by regulating the amount of cAMP available to activate the protein kinase.

A cAMP receptor with an established function exists in E.coli (4). cAMP receptors without protein kinase activity have also been found in eukaryotes: yeast (5), embryonic Drosophila melanogaster (6), rabbit reticulocytes (7), and rat liver (8). Until now, no physiological role has been ascribed to these eukaryotic receptors.

We report here the existence in mouse liver cytosol of a cAMP receptor not related to the cAMP-dependent protein kinase, whose cAMP binding capacity is influenced by Mg^{++}-ATP.

MATERIALS AND METHODS: Chemicals were of the highest purity commercially available. All nucleotides were from Sigma. [^{3}H] cyclic 3', 5'-AMP (27 Ci/mmole) was from The Radiochemical Centre. [γ-^{32}P] ATP (approx. 16 Ci/mmole) was prepared according to the method of Glynn & Chappel (9). -Binding of [^{3}H] cAMP was assayed at 30°C in 15 mM Hepes-buffer pH 7.0 with 150 mM KCl and 5 mM mercaptoethanol. When added, Mg^{++} was present as 10 mM $Mg(CH_3COO)_2$. The concentrations of the other ingredients varied from experiment to experiment, and are given together with the other experimental details. The binding reaction was terminated by pipetting an aliquot of the incubation-mixture into 8 times its own volume of ice-cold 80% $(NH_4)_2SO_4$ and vortexing. The precipitate was collected by suction

through Millipore filters (HAWP-0.45μ). The filters were put in scintillation-vials, the precipitate dissolved in 1.5 ml 1% SDS, and 7 ml Unisolve (Koch-Light) added.

-Protein kinase activity was assayed essentially as described (10). The incubation-mixture contained: 15 mM Hepes pH 7.0, 10 mM $Mg(CH_3COO)_2$, 3.10^{-5} M ATP, [γ-^{32}P]-ATP (1μ Ci/ml), Calf Thymus Histone (Sigma type II, 0.67 mg/ml), and enzyme preparation.

-Preparation of cytosol. Finely minced livers from adult N.M.R.I. mice were homogenized (1/5 v/v) with 2 strokes at 465 rev./min in a teflon-glass homogenizer. Homogenization buffer was 15 mM Tris-HCl pH 7.6 with 6 mM EDTA, 5 mM mercaptoethanol, and 0.25 M sucrose. After a high-speed pelletation (20,000 x g for 10 min), the cytosol fraction was obtained by spinning 100,000 x g for 1 h. The cytosol was passed through a Sephadex G-25 column equilibrated with the starting buffer for the DEAE-cellulose column: 10 mM Tris-HCl pH 7.5 containing 4 mM EDTA and 5 mM mercaptoethanol, hereafter referred to as Tris-buffer. All steps were carried out at 2°C.

RESULTS AND DISCUSSION: The distribution of protein kinase activity and high-affinity cAMP binding activity in the DEAE-cellulose eluate of a normal liver cytosol is shown in Fig. 1A. The two major protein kinase isoenzymes in this tissue (PKI and PKII, termed after their order of elution) are both associated with high-affinity cAMP binding activities. The peak of cAMP binding material eluting between PKI and PKII has the high Kd ($2 \cdot 10^{-10}$ M) for cAMP characteristic of the free regulatory subunit (12), and a peak appears in this same position after cAMP induced dissociation of the holoenzyme (Fig. 1B).

We have found a close correlation between cAMP-activation of and cAMP binding to PKII (unpublished observations). A discrepancy existed however for the PKI-preparation (Fig. 2). Saturation of cAMP binding sites required doses of cAMP several fold higher than those necessary to fully activate the enzyme. It was also noted that more cAMP could be bound when the ATP-concentration was raised from $3 \cdot 10^{-5}$M to $3 \cdot 10^{-3}$M (Fig. 2).

Assay of DEAE-cellulose fractions for cAMP binding in the presence of high concentrations of cAMP and ATP, revealed a major peak of high-capacity cAMP binding activity, which co-eluted with PKI (Fig. 1A). On rechromatography of the peak fractions incubated with cAMP, the high-capacity cAMP binding activity eluted in the same position as before, clearly separated from the protein kinase subunits. The site responsible for the high capacity cAMP binding is thus not located on the PKI regulatory subunit.

A clear separation of the high capacity cAMP binding substance and PKI was accomplished by QAE-Sephadex chromatography (Fig. 1C).

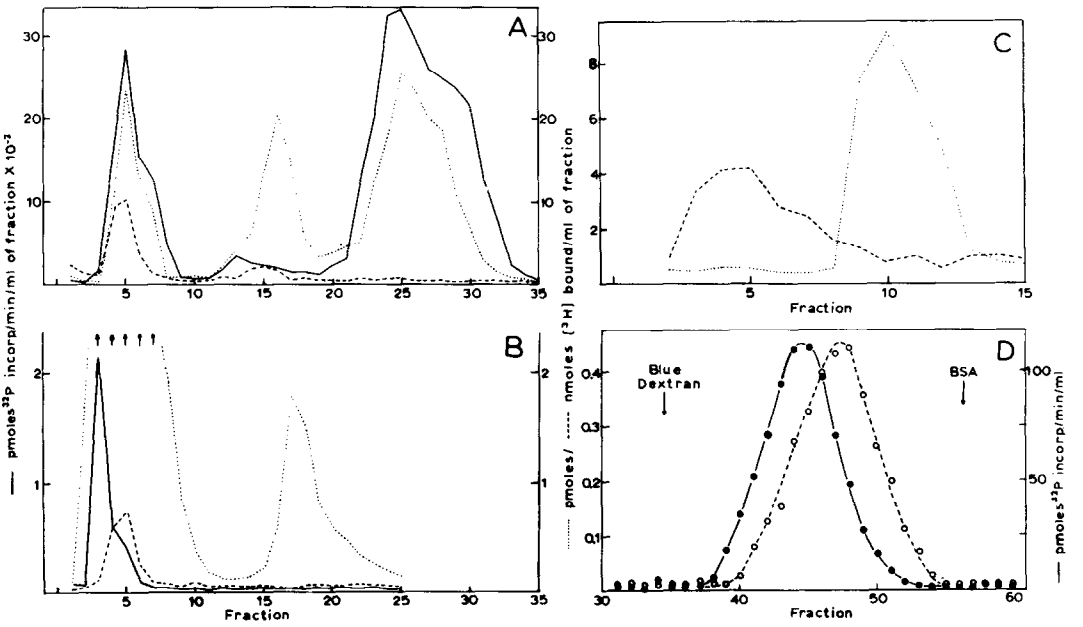

Fig. 1A. DEAE-cellulose chromatography of mouse liver cytosol. Cytosol prepared from 20 g liver was applied to a DE-52 column (2.6 x 45 cm) equilibrated with Tris-buffer. A linear KCl-gradient (0-0.3 M) was started at fraction 4. Fraction volume was 20 ml. Solid line: Protein kinase activity assayed in the presence of $2 \cdot 10^{-6}$M cAMP.
Dotted line: cAMP binding assayed with 10^{-8}M[^{3}H] cAMP.
Dashed line: cAMP binding assayed in the presence of 10 mM Mg^{++}, 3 mM ATP, and $6 \cdot 10^{-5}$M [^{3}H] cAMP.

Fig. 1B. Dissociation of PKI into subunits. Fractions 3-6 (Fig. 1A) were pooled, precipitated with 65% $(NH_4)_2SO_4$, redissolved, and desalted by passing through a Sephadex G-25 column equilibrated with Tris-buffer. This desalted preparation was divided into three portions. One portion was incubated at 0°C for 30 min in the presence of $2 \cdot 10^{-6}$M [^{3}H] cAMP, applied to a DE-52 column (0.9 x 15 cm) and eluted with a linear 0-0.3 M KCl gradient starting at fraction 5. Fractions of 5 ml were collected.
Solid line: Protein kinase activity (independent of cAMP).
Dotted line: Amount of [^{3}H] cAMP remaining bound from the pre-incubation. 1 ml aliquots from the fractions were mixed with 7 ml Unisolve and counted.
Dashed line: cAMP binding assayed in the presence of 10 mM Mg^{++}, 3 mM ATP and $6 \cdot 10^{-5}$ M [^{3}H] cAMP.

Fig. 1C. Separation of cAMP-binding activities by QAE-Sephadex chromatography. One portion of the preparation described above was applied to a QAE-Sephadex (A-25) column (0.9 x 30 cm). The activity was eluted with 30 ml Tris-buffer followed by a linear 0-0.6 M KCl-gradient, starting at fraction 7. Fractions of 5 ml were collected.
Dotted line: cAMP binding assayed in the presence of $5 \cdot 10^{-10}$ M [^{3}H] cAMP.
Dashed line: cAMP binding assayed in the presence of 10 mM Mg^{++}, 3 mM ATP, $6 \cdot 10^{-5}$ M [^{3}H] cAMP.

Fig. 1D. Sephadex G-200 chromatography of PKI and cAMP receptor. A 1.5 ml portion of the desalted preparation described in legend to fig. 1B was run by reverse flow on a Sephadex G-200 column (1.6 x 62 cm). Fraction volume was 1.5 ml.
(●-●): Protein kinase activity assayed in the presence of $2 \cdot 10^{-6}$M cAMP.

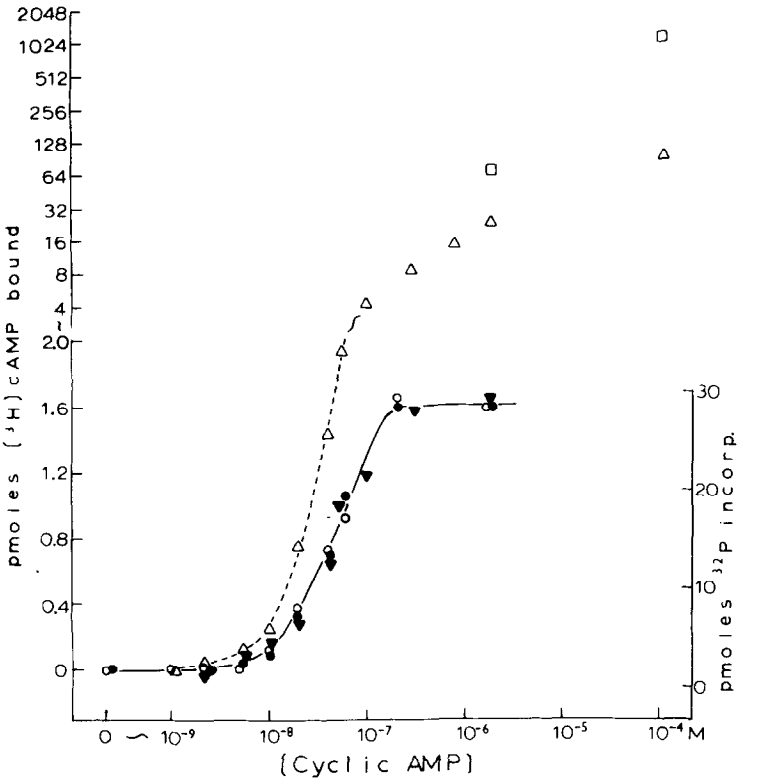

Fig. 2. Binding of [^{3}H] cAMP and activation of protein kinase as a function of cAMP-concentration. Kinase and binding activities were measured in the medium described for the protein kinase assay (150 mM KCl was included in all assays, and unlabelled ATP and [^{3}H]-labelled cAMP used in the binding reaction). Incubation time was 15 min.
Crude PKI (DEAE-cellulose fractions 3-6, Fig. 1A, B) was tested for kinase activity (O), and cAMP binding activity (Δ) under the conditions described. This preparation was also tested for cAMP binding in the presence of 6 mM ATP (□). The desalted 35-65% $(NH_4)_2SO_4$ fraction from QAE-Sephadex fractions 10-12 (Fig. 1C) was tested for kinase activity (●) and cAMP binding (▲). Left ordinate: pmoles of cAMP bound /ml of incubate. Note the transition from linear to logarithmic scale.
Right ordinate: protein kinase activity per ml of incubate expressed as pmoles of [^{32}P] incorporated into histone per min.

When the holoenzyme, now devoid of the high capacity cAMP receptor, was reassayed (Fig. 2), a close correlation was found between cAMP binding and cAMP activation of the kinase. The activation of PKI as a function of cAMP concentration was

(0-0): cAMP binding assayed in the presence of 10 mM Mg^{++}, 3 mM ATP and $6 \cdot 10^{-5}$M [^{3}H] cAMP.
The peak fractions for Blue Dextran and bovine serum albumin are indicated by vertical arrows.

not affected by the removal of high capacity cAMP-receptor from the PKI preparation.

On Sephadex G-200 chromatography, the high capacity cAMP binding activity eluted after the PKI holoenzyme (Fig. 1D). The symmetrical sharp peak obtained for the high capacity cAMP receptor, indicates that the molecules carrying the cAMP binding site(s) are of homogenous molecular size.

The data presented in the first row of Table I show that the enhanced capacity for cAMP binding brought about by ATP is present after removal of free ATP by Sephadex G-25 filtration, and even after 14 hours of incubation in the presence of 5 mM EDTA.

The possibility existed that the cAMP receptor was preferentially inactivated in the absence of ATP during the incubation prior to Sephadex G-25 filtration. When cAMP binding was assayed in the presence of Mg^{++}-ATP (second row of Table I), there was no evidence indicating preferential loss of activity for the receptor preparation pre-incubated without Mg^{++}-ATP.

When the receptor was assayed for cAMP binding in the presence of 10 mM EDTA, half maximal binding was observed at $2 \cdot 10^{-6}$ M cAMP and maximal binding at $2 \cdot 10^{-5}$ M cAMP. The data for binding of cAMP presented in Fig. 3A were obtained in the presence of $2 \cdot 10^{-5}$ M cAMP, which is a saturating concentration under the conditions of the assay. Several purine derivatives were tested (in the presence of 10 mM Mg^{++}) for their ability to increase the cAMP binding capacity of the receptor. After pre-incubation with the particular nucleotide, the samples were passed through Sephadex G-25 columns in order to remove the excess nucleotide, which could interfere with the subsequent cAMP binding assay. ATP enhances the binding of cAMP far more than the other purine derivatives tested. ATP was more potent in this respect in the presence of Mg^{++}, but had a significant effect also in the absence of this ion. As the enhanced binding brought about by Mg^{++}-ATP was observed at saturating concentrations of cAMP, it seems reasonable to assume that pre-incubation of the receptor preparation in the presence of Mg^{++}-ATP had made more sites available for cAMP.

The specificity of the cAMP binding site was examined (Fig. 3B) by testing the ability of a number of purine derivatives to inhibit the binding of cAMP. Only AMP, ADP and ATP were

TABLE I

Persistence of Mg^{++}-ATP effect on cAMP receptor after Sephadex G-25 filtration and exposure to EDTA.

	Preincubated in the presence of EDTA		Preincubated in the presence of Mg^{++}-ATP	
	assayed immediately	assayed after 14h-5mM EDTA	assayed immediately	assayed after 14h-5mM EDTA
A	505 c.p.m.	530 c.p.m.	5400 c.p.m.	4400 c.p.m.
B[1]	1800 c.p.m.	1500 c.p.m.	1200 c.p.m.	1050 c.p.m.

One portion of the desalted 35-65% $(NH_4)_2SO_4$-fraction from QAE-Sephadex fractions 10-12 (Fig. 1C) was incubated for 1 h at 30°C in 15 mM Hepes-buffer pH 7.0 in the presence of 15 mM KCl, 5 mM mercaptoethanol and 10 mM EDTA. Another portion was incubated under the same conditions, except that 10 mM Mg^{++} and 6 mM ATP replaced the EDTA. The incubate was filtrated through Sephadex G-25 columns equilibrated with 15 mM Hepes-buffer pH 7.0 in order to remove free Mg^{++}-ATP and EDTA. A 100 µl portion from each of the two incubates (supplemented with human Hb to visualize the fraction excluded from the gel) was carefully layered on the top of 0.8 x 3.5 cm Sephadex G-25 columns. The high-molecular-weight material was quantitatively recovered in 400 µl of eluate, well separated from the low-molecular weight components. One portion of this eluate was assayed directly for cAMP binding. The remaining 200 µl of the eluate was mixed with 5 µl of 200 mM neutralized EDTA and left for 14 hours in the cold before assay for cAMP binding.

A: cAMP binding assay in the presence of $6 \cdot 10^{-5}$ M [^{3}H] cAMP (120 mCi/mmole) and 10 mM EDTA.

B: cAMP binding assay in the presence of $6 \cdot 10^{-5}$ M [^{3}H] cAMP (120 mCi/mmole) and 10 mM Mg^{++}, 4.5 mM ATP.

1/ Conditions are suboptimal for [^{3}H] cAMP binding in the presence of Mg^{++} -4.5 mM ATP, probably related to inhibition of binding of [^{3}H] cAMP by the ATP present.

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effective among the inhibitors tested. Notably, cGMP, 2',3' cAMP, and adenosine added in 100 fold excess of the [^{3}H] cAMP present, failed to inhibit significantly the binding of [^{3}H] cAMP. The lack of inhibition of cAMP binding by adenosine serves to differentiate the receptor described by us, from the adenosine-cAMP receptor purified from rabbit reticulocytes, the binding of cAMP to which can be completely inhibited by adenosine (7).

The inhibition by adenine nucleotides of the binding of cAMP to the receptor described by us, resembles the data given for rabbit muscle phosphofructokinase (12). In that system ADP was more efficient inhibitor of cAMP binding than was AMP, whereas we have found the order of inhibitory power to be AMP>ADP>ATP.

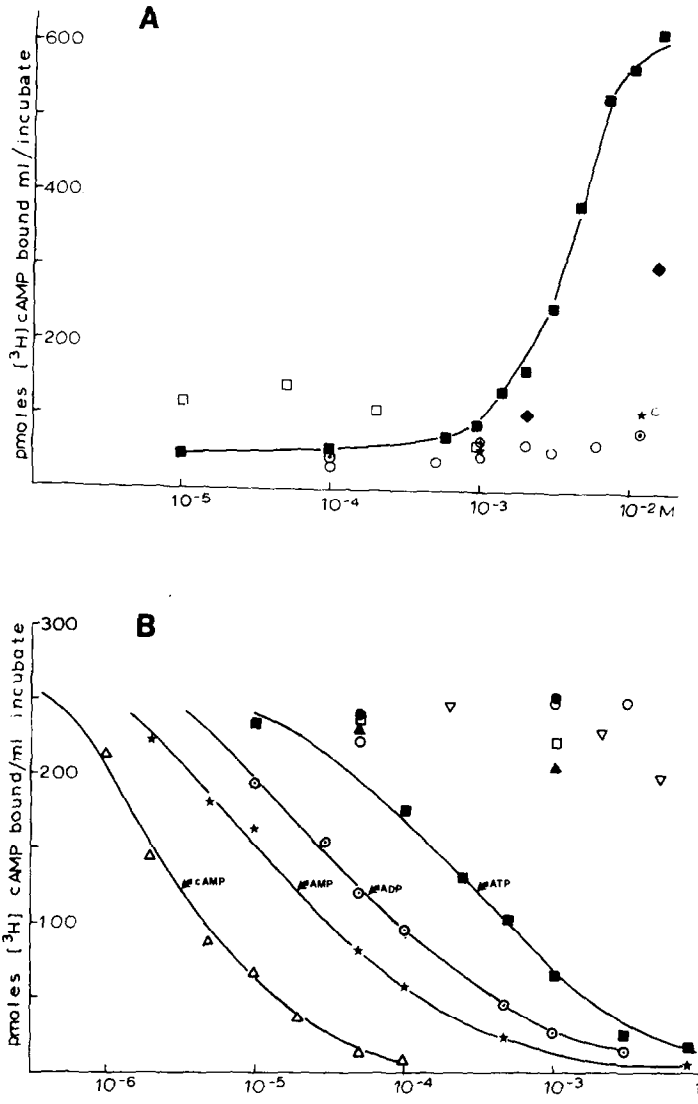

Fig. 3A. Capacity for cAMP binding of receptor-preparation after preincubation with various purine derivatives. Pre-incubation and Sephadex G-25 filtration were performed as described in legend to Table I. Pre-incubations were in the presence of 10 mM Mg^{++} (except where indicated) and the concentrations of the purine derivatives given on the abscissa. ■: ATP, ⊙: ADP, ★: AMP, □: Adenosine, ◆: GTP, ⊙: ATP preincubated in the presence of 10 mM EDTA and no Mg^{++}. Pre-incubation was for 30 min, assay for cAMP binding was for 15 min in the presence of $2 \cdot 10^{-5}$M [^{3}H] cAMP and 10 mM EDTA.

Fig. 3B. Inhibition of [^{3}H] cAMP binding by purine derivatives. Desalted cAMP receptor pretreated with 10 mM Mg^{++}-ATP for 30 min, was incubated for 15 min, in the presence of 10 mM EDTA, $2 \cdot 10^{-6}$M [^{3}H] cAMP, and the concentration of inhibitors indicated on the abscissa. ■: ATP, ⊙: ADP, ★: AMP, □: Adenosine, ●: cyclic 2',3'-AMP, ▲: cyclic 3',5'-GMP, ○: GTP, ▽: Theophylline.

This order of inhibitory potency, taken together with the low efficiency of AMP and ADP as compared to ATP, to make binding sites available for cAMP (Fig. 3A), suggests that the relative concentrations of AMP, ADP and ATP can regulate the amount of cAMP bound to the receptor. High concentrations of ATP should then tend to increase the amount of cAMP which can be bound; whereas high concentrations of AMP and ADP should tend to release cAMP from the receptor.

To find if the cAMP binding factor described functions as regulator of available cAMP in the cell or not, and if it has other functions, requires further purification and characterization.

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