Purification and Characterization of Adenosine–Adenosine Cyclic 3′,5′-Monophosphate Binding Protein Factors from Rabbit Erythrocytes†

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ABSTRACT: Two adenosine cyclic 3′,5′-monophosphate (cAMP) binding protein factors (receptors I and II) were purified to homogeneity from rabbit erythrocytes. The molecular weight of each of these receptors is approximately 240,000, as estimated by gel filtration and polyacrylamide gel electrophoresis. Electrophoresis of both receptors in the presence of sodium dodecyl sulfate revealed a single protein component of approximately 48,000 daltons. Likewise, a single protein band was also obtained when electrophoresis was performed in the presence of 8 M urea. The data suggest that these receptors are pentamers composed of monomeric units of identical mass and charge. The sedimentation of these receptors in sucrose density gradient was not affected by cAMP; and a sedimentation coefficient of 9.3 S was obtained for both proteins. An apparent K_m of 3×10^{-7} M was obtained for the interaction of the receptors with cAMP. The binding of cAMP as measured by the Millipore filtration technique was enhanced by protamine. Of several nucleosides and nucleotides tested, only adenosine significantly inhibited the binding of cAMP by these proteins. Adenine was also found to be inhibitory but required a higher concentration. Since receptor I constitutes the major component in all preparations, the binding properties of this protein has been investigated in greater detail than receptor II. The binding of adenosine to receptor I had a K_m of about 1×10^{-7} M. Whereas adenosine could completely inhibit the binding of the cyclic nucleotide, cAMP had no effect on the binding of adenosine. There is evidence to suggest that receptor I may contain two types of binding sites: one specific for adenosine and the other for cAMP. Neither receptor I nor II had any regulatory function in modulating the activity of the catalytic subunit of cAMP dependent protein kinases of rabbit erythrocytes. Thus, they were apparently different from the regulatory subunit of these kinases. These receptor preparations were also devoid of any phosphofructokinase and protein kinase activities.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] exerts a multitude of regulatory functions (Robison *et al.*, 1971). These functions may be tissue or cell specific. Clearly, if cAMP is to perform its function, it must interact with some receptor molecules in the cell. To date, several protein factors have been isolated from different sources, all of which strongly bind the cyclic nucleotide. A cAMP receptor protein has been found in *Escherichia coli* (Zubay *et al.*, 1970; de Crombrugghe *et al.*, 1971) whose function is to promote the transcription of mRNA of inducible enzymes. On the other hand, a receptor isolated from rat liver appears to effect the release of tyrosine aminotransferase from the ribosome at the translational level (Donovan and Oliver, 1972). cAMP binding proteins, which are characterized as regulatory subunits of widely occurring cAMP dependent protein kinases, have also been described (Walsh *et al.*, 1968; Tao *et al.*, 1970; Gill and Garren, 1970; Reimann *et al.*, 1971; Rubin *et al.*, 1972). Yet another cAMP receptor protein of unknown function has been isolated from yeast (Sy and Richter, 1972).

During the course of our investigation into the role of cAMP in red blood cells, we have observed the presence of at least two non-kinase associated cAMP binding protein factors. These factors are not derived from the cAMP dependent protein kinases since they have no inhibitory effect on the catalytic moiety of these enzymes. Interestingly, these protein factors also bind adenosine. The binding of adenosine may exclude the binding of cAMP but not *vice versa*. This report deals with the purification and characterization of these binding proteins.

Experimental Section

Materials. Young (8–12 weeks) rabbit red blood cells were purchased from Pel-Freez Biologicals, Inc. Lysine-rich histone, histone mixture, protamine, ovalbumin, bovine serum albumin, and, unless indicated otherwise, all the enzymes were supplied by Sigma Chemical Co. Rabbit liver phosphofructokinase was a generous gift of Dr. Robert Kemp. All radioisotopes were the products of New England Nuclear.

cAMP Binding Assay. The assay of the binding proteins was based on the ability of these molecules to bind cAMP and the retention of the resulting complex on Millipore filters. The standard assay was performed in a total volume of either 0.1 or 0.2 ml, containing 1 mM Tris-HCl (pH 7.5), 1.0 μM [^{3}H]cAMP or [^{32}P]cAMP, 30 μg/ml of protamine, 4.0 mM $MgCl_2$, and binding proteins. The blanks received no binding proteins. The mixture was incubated at 37° for 3 min and transferred to an ice-water bath. Under these conditions, the binding had attained equilibrium, *i.e.*, further incubation did not cause an increase in binding. Each sample was then diluted with about 2.0 ml of ice-cold 1.0 mM Tris-HCl (pH 7.5) buffer containing 4.0 mM $MgCl_2$ (buffer A), and filtered through a Millipore filter (HA 0.45-μm

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[1] Abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; SDS, sodium dodecyl sulfate; mol wt, molecular weight.

pore size), which had been presoaked in the same buffer. The filter was washed ten times with 2-ml portions of cold buffer A and dissolved in 5.0 ml of Bray's (1960) solution, and the radioactivity was determined. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Assay of Protein Kinase Activity. Protein kinase activity was assayed as described previously by measuring the amount of ^{32}P incorporated into calf thymus lysine-rich histone or histone mixture (Tao and Hackett, 1973).

Preparation of Catalytic Subunits of Rabbit Erythrocyte cAMP Dependent Protein Kinases. cAMP dependent protein kinases I, IIa, and IIb were prepared from rabbit erythrocytes as described previously (Tao and Hackett, 1973). The catalytic moieties of these enzymes were isolated by dissociation of the enzyme complex with protamine and separation of the two dissimilar functional subunits in a sucrose density gradient (Tao, 1972; Tao and Hackett, 1973). Protamine formed a heavy complex with the regulatory subunit causing it to sediment to the bottom of the tube while leaving the catalytic moiety behind.

Sucrose Density Gradient Centrifugation. Studies employing sucrose density gradient centrifugation were conducted by the method of Martin and Ames (1961), using a Beckman SW65K rotor at 50,000 rpm, 0°, for 16.5 hr (unless otherwise stated). Linear gradients of 5–20% sucrose in 0.02 M Tris-HCl (pH 7.5) and 1.0 mM dithiothreitol were employed. Three-drop fractions were collected from each gradient and an aliquot was withdrawn from each fraction and assayed for binding of cAMP. The protein markers used were phosphorylase *b* (8.4 S) and beef liver catalase (11.3 S).

Electrophoretic Analyses. Polyacrylamide disc gel electrophoresis was performed by the procedure of Gabriel (1971). The buffer system used was Tris (3.0 g/l.)-glycine (14.4 g/l.) (pH. 8.3). A separating gel of 7.5% and a stacking gel were polymerized in a 5 mm × 6 cm glass column. About 0.1 ml of the enzyme solutions containing 15–50 μg of protein in 20% sucrose were applied to each of the gels. Electrophoresis was performed in a cold room at 2 mA/gel for about 2 hr or until the dye (Bromophenol Blue) front approached the bottom of the tube. The gels were stained with Coomassie Brilliant Blue (0.2% in 10% acetic acid and 45% methanol) for 1 hr and destained electrophoretically in 5% methanol and 7% acetic acid. In experiments where an incubation mixture of [^{3}H]cAMP and receptor was subjected to electrophoresis, the radioactivity in each 1-mm gel slice (Hoefer Scientific Instruments gel slicer) was determined after dissolving it in 0.5 ml of 30% H_2O_2 at 54° for 2 hr, followed by 10 ml of Aquasol (New England Nuclear).

Disc gel electrophoresis in the presence of 8 M urea was performed at pH 10 by a modified procedure of Duesberg and Rueckert (1965). Both the stacking and the separating gels were polymerized in the presence of 8 M urea and 10 mM methylamine hydrochloride. Electrophoresis was conducted at 2 mA/gel until the tracking dye had migrated to the bottom of the tube. Staining and destaining of the gels were performed as described above.

The determination of molecular weight by polyacrylamide gel electrophoresis was carried out as described by Hedrick and Smith (1968) in gel concentrations of 5–12%, at 1% increment. Catalase (mol wt 244,000), creatine kinase (mol wt 80,000), bovine serum albumin (mol wt 68,000), D-amino acid oxidase (mol wt 90,000), lactic dehydrogenase (mol wt 140,000), and xanthine oxidase (mol wt 275,000) were employed as protein standards. Measurements and calculations were performed as described by Hedrick and Smith (1968).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (1970) on slab gel made up of 4.5% stacking gel and 10% separating gel. The proteins were dissolved in 63 mM Tris-HCl (pH 6.8) buffer containing 2% SDS, 10% sucrose, 1 mM dithiothreitol, and 0.001% Bromophenol Blue; and incubated at 100° for 5 min. Electrophoresis was carried out with a constant current of 25 mA for a 10 × 14 cm slab of 1.5 mm thick (Hoefer Scientific Instruments). At the end of about 6 hr the dye front had migrated to the bottom of the gel. The proteins were stained and destained according to the procedures of O'Farrell *et al.* (1973). Measurements and calculations of molecular weights were performed as described by Weber and Osborn (1969) using phosphorylase *b* (mol wt 93,000), bovine serum albumin (mol wt 68,000), catalase (mol wt 60,000), creatine kinase (mol wt 40,000), ovalbumin (mol wt 43,000), and pepsin (mol wt 35,000) as standards.

Estimation of Molecular Weights by Gel Filtration. A Sephadex G-200 column (1.6 × 84 cm) was equilibrated with 0.02 M Tris-HCl (pH 7.5) at 4°. About 0.5 ml of each of the protein solutions was applied to the column and the elution was carried out with the same buffer at an upward flow rate of 12 ml/hr. Blue Dextran T-2000 was used to determine the void volume of the column. The protein standards used were phosphorylase *a* (mol wt 370,000), catalase (mol wt 244,000), phosphorylase *b* (mol wt 185,000), lipoxidase (mol wt 97,000), creatine kinase (mol wt 80,000), peroxidase (mol wt 49,000), and ovalbumin (mol wt 43,000).

Results

Purification. Unless stated otherwise, all steps were performed at 0–4°; and centrifugations were carried out in a Sorvall RC2-B centrifuge. The enzyme solutions obtained at various stages of purification may be stored in liquid nitrogen without loss of binding capacity.

The initial purification steps were essentially similar to those described for the preparation of cAMP dependent protein kinases (Tao and Hackett, 1973); 2 l. of young rabbit red blood cells was lyzed with 4 volumes of 2.5 mM $MgCl_2$. The crude supernatant fraction, freed of cell debris by centrifugation, was brought to 50% saturation with respect to ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 0.02 M Tris-HCl (pH 7.5) containing 1 mM dithiothreitol (buffer B), and dialyzed overnight against this buffer.

After dialysis, the solution was clarified by centrifugation and applied to a 3.8 × 34 cm DEAE-cellulose column which had previously been equilibrated with buffer B. Following adsorption of the protein, the column was washed with 1 l. of buffer B or until all the reddish-colored substance (presumably hemoglobin) had been removed. The column was eluted with a 2-l. linear gradient of 0–0.35 M KCl in buffer B. Fractions of about 20 ml were collected and assayed for kinase activity and cAMP binding capacity. Two cAMP binding peaks with their corresponding kinase activities were eluted from the column. The non-kinase associated cAMP binding proteins appeared to cochromatograph with cAMP dependent protein kinase I.

The cAMP binding fractions which coeluted with cAMP dependent protein kinase I were pooled. Ammonium sulfate

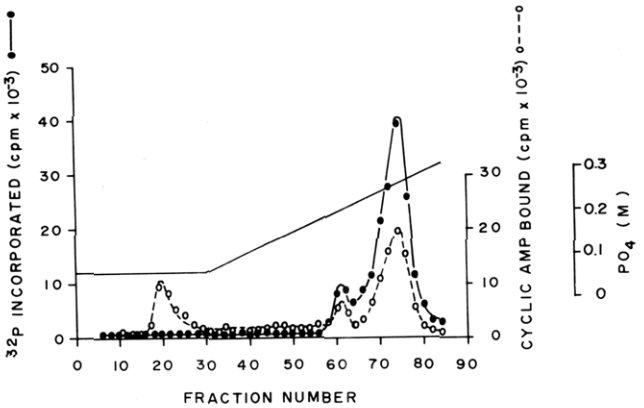

FIGURE 1: Separation of receptors from cAMP dependent protein kinases by hydroxylapatite column chromatography. The experimental details are given in the text. The kinases were assayed in the presence of 1.0 μM cAMP. The specific activities of [γ-^{32}P]ATP and [^{3}H]cAMP were 23 and 2600 cpm/pmol, respectively.

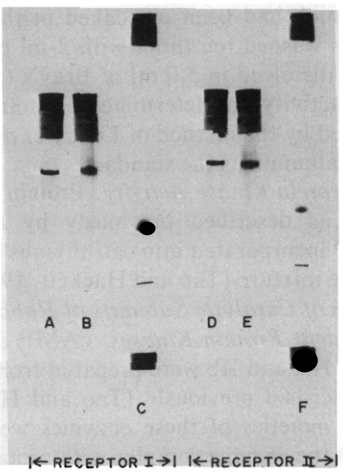

FIGURE 3: Polyacrylamide gel electrophoresis of receptors I and II under various conditions. Receptor I: (A) 18 μg, control; (B) 18 μg, in 8 M urea; (C) 44 μg, in 0.1% SDS. Receptor II: (D) 40 μg, control; (E) 40 μg, in 8 M urea; (F) 56 μg, in 0.1% SDS.

was added to yield 50% saturation, and the resulting precipitate was centrifuged and dissolved in and dialyzed against 0.05 M potassium phosphate buffer (pH 6.8) containing 1 mM dithiothreitol (buffer C). The enzyme solution was subsequently applied to a hydroxylapatite (2.8 × 14 cm; Bio-Gel HTP, Bio-Rad Laboratories) column which had been previously equilibrated with buffer C. This step separated the binding protein factors from the kinases and their corresponding cAMP binding proteins. These binding protein factors will be tentatively identified as receptors while the binding factors associated with the cAMP dependent protein kinases as regulatory subunits. This is an arbitrary distinction. The receptors were not adsorbed to the gel and emerged at the breakthrough, while a higher salt concentration was required to elute the cAMP dependent protein kinases. No kinase activity was eluted with the receptors (Figure 1).

The receptor fractions were pooled, concentrated by ammonium sulfate precipitation, and dissolved in and dialyzed against 0.1 M Tris-HCl (pH 6.5) containing 1.0 mM dithiothreitol (buffer D). The protein solution was applied to a QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc.) column (2.8 × 23 cm). The column was washed with two bed volumes of buffer D and eluted with a 600-ml linear gradient of 0–0.3 M KCl in buffer D. Fractions of 9.0 ml were collected and assayed for cAMP binding capacity. As shown in Figure 2, two receptor peaks were eluted from the column, receptor I emerged at a salt concentration of about 0.1 M and receptor II at about 0.2 M. The cAMP binding

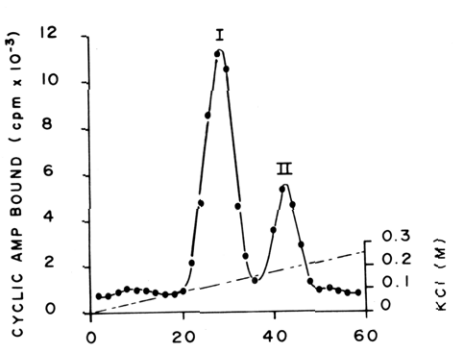

FIGURE 2: Resolution of receptors I and II by QAE-Sephadex ion exchange chromatography. See text for details of the chromatographic procedure. The specific activity of [^{3}H]cAMP was 2100 cpm/pmol.

fractions under each peak were pooled, concentrated to about 0.5–1.0 ml by Diaflo ultrafiltration using a PM-10 membrane (Amicon Corp.), and dialyzed overnight against buffer B. Interestingly, receptor II was absent in some preparations.

Receptors I and II were chromatographed separately on a Sephadex G-200 column (1.6 × 84 cm), previously equilibrated with buffer B; and elution was performed with the same buffer at an upward flow rate of about 12 ml/hr. Fractions (6.4 ml) were collected, and those containing significant cAMP binding capacity were pooled and concentrated by Diaflo ultrafiltration to about 1.0 ml.

Although both receptors I and II had reached a high state of purity after the Sephadex G-200 filtration step, one or two minor contaminants were still present when analyzed by polyacrylamide disc gel electrophoresis. However, a relatively homogeneous preparation of these receptors could be obtained by sedimentation through 5–20% linear sucrose density gradients. Sucrose density gradient centrifugations were carried out as described in the Experimental Section. Those containing cAMP binding capacity were pooled and frozen in liquid nitrogen. No effort was made to remove the sucrose in the enzyme solution since it did not seem to interfere with the binding assay. Because of interfering activities in the initial steps of purification attributed to cAMP dependent protein kinases, meaningful data regarding the number of fold of purification and the yield of these receptors could not be obtained.

Polyacrylamide Gel Electrophoresis. The purity of the receptor preparations was established by polyacrylamide gel electrophoresis under various conditions. As shown in Figure 3, each receptor migrated as a single component on polyacrylamide gel electrophoresis in the presence and in the absence of either SDS or 8 M urea.

The electrophoretic profile of receptor I was not altered by the binding of cAMP to the protein as shown in Figure 4. The A and B gels in Figure 4 represent receptor I incubated in the absence and in the presence of [^{3}H]cAMP at 37° for 3 min, respectively. The results presented in Figure 4 indicate that the radioactivity in the gel comigrates with the visible protein staining component. The coincidence of the migration of the radioactivity with the protein band further suggests that our receptor I preparation is homogeneous and that the protein band represents that of the re-

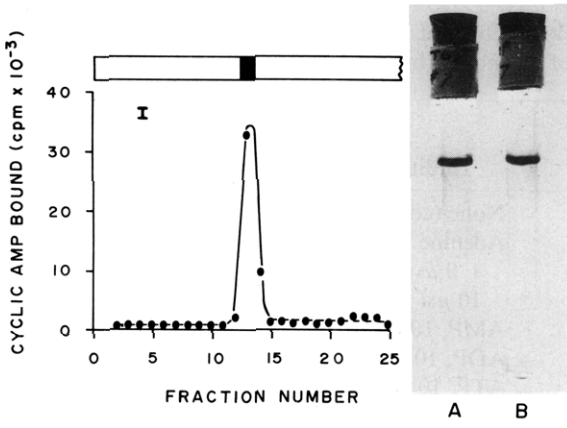

FIGURE 4: Electrophoretogram of receptor I incubated with and without cAMP. Receptor I (90 μg) was incubated for 3 min at 37° in 0.02 M Tris-HCl (pH 7.5) containing 0.5 μM [^{3}H]cAMP (2900 cpm/pmol) and 20% sucrose, in a final volume of 0.2 ml. The entire incubation mixture was then applied to the gel and subjected to electrophoresis, and the radioactivity was determined as described in the Experimental Section. Each of the stained gels, A (control) and B (+ cAMP), contained about 45 μg of protein.

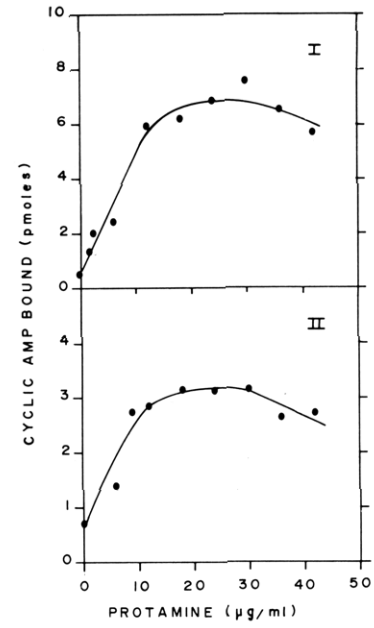

FIGURE 5: Effect of varying amounts of protamine on the binding of cAMP by receptors I and II. cAMP binding assay was carried out as described in the Experimental Section with either 70 μg/ml of receptor I or 90 μg/ml of receptor II, in the presence of varying amounts of protamine. Specific activity of [^{3}H]cAMP was 2900 cpm/pmol.

ceptor. A similar result was obtained with receptor II.

Molecular Weight and Subunit Structure. Both receptors I and II sedimented as a 9.3S component in sucrose density gradient. An apparent mol wt of 240,000 was obtained for both protein factors by Sephadex G-200 gel filtration and by polyacrylamide gel electrophoresis. Molecular weight determinations of these protein factors under denatured conditions yielded a value of 48,000 for both factors. As shown previously, each receptor electrophoresed as a homogeneous band in a polyacrylamide gel containing 8 M urea. The results indicate that these receptors are pentamers composed of subunits with identical charge at pH 10 and of mol wt 48,000.

Stability. The cAMP binding capacities of these receptors were completely destroyed after heat treatment for 5 min at 100°. They were insensitive to RNase, DNase, phospholipase *c*, and lysozyme. Following a 30-min incubation with trypsin (200 μg/ml), about 10% of the receptor I and 40% of the receptor II cAMP binding capacities were abolished. Both receptors were equally sensitive to chymotrypsin and Pronase. About 70–80% of the binding capacities of these receptors were destroyed when incubated for 30 min at 37° with 200 μg/ml of each of the proteolytic enzymes. The results suggest that these receptors are proteinaceous in nature.

Effect of Protamine on cAMP Binding. It was previously reported that protamine enhanced the binding of cAMP to the regulatory subunit of cAMP dependent protein kinases of rabbit red blood cells as measured by the Millipore filtration assay (Tao and Hackett, 1973). Protamine caused a similar enhancement of the binding of cAMP to receptors I and II. As shown in Figure 5, a 5- to 10-fold increase in binding was observed in the presence of protamine. For each experimental point shown in Figure 5, a correction was made for the nonspecific retention of radioactivity due to variation in protamine concentration.

Test for Inhibitory Action on Protein Kinases. Reports from this laboratory (Tao *et al.*, 1970; Tao and Hackett, 1973) as well as others (Gill and Garren, 1970; Reimann *et al.*, 1971; Rubin *et al.*, 1972) have demonstrated that cAMP dependent protein kinases derived from several sources are constructed of two dissimilar functional subunits: a regulatory subunit which binds cAMP and a catalytic subunit. It was therefore of interest to determine whether receptor I or II could also function as regulator of rabbit red cell protein kinase activity. Table I shows that neither of the two receptors had significant effect on the activity of the catalytic moiety of rabbit erythrocyte cAMP dependent protein kinase I in the presence or absence of cAMP. Essentially similar results were obtained with the catalytic moiety of cAMP dependent protein kinases IIa and IIb. The conditions employed in these studies were similar to those described earlier for the reversible subunit interaction of the cAMP dependent protein kinases (Tao and Hackett, 1973).

Binding Studies. In addition to the Millipore assay and gel electrophoresis (Figure 4), an alternate method employ-

TABLE I: Lack of Inhibitory Activity of Receptors I and II on the Catalytic Moiety of cAMP Dependent Protein Kinase I.[a]

Receptor Added	(μg)	^{32}P Incorporated (pmol) −cAMP	+cAMP
I	0	300	330
	12	330	300
	24	350	320
	60	340	250
II	0	600	590
	26	500	510
	52	510	570
	130	470	610

[a] Details regarding subunit isolation and the reaction mixture are given in the Experimental Section. Receptor I was tested in the presence of 2.5 μg of the catalytic subunit of cAMP dependent protein kinase I, whereas receptor II, 4.0 μg of the subunit.

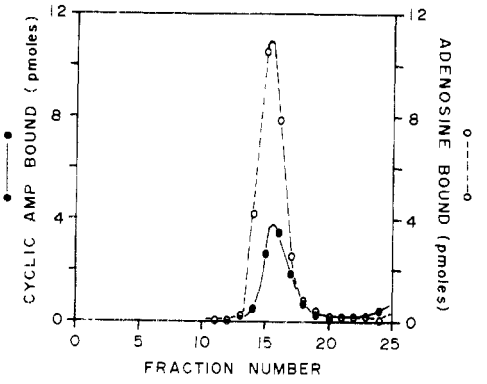

FIGURE 6: Binding of cAMP and adenosine to receptor I as determined by Sephadex G-50 gel filtration. Receptor I (60 μg/ml) was incubated for 1 hr at 37° in the presence of 1.0 mM Tris-HCl (pH 7.5) containing 2.5 μM of either [^{32}P]cAMP or [^{3}H]adenosine. The mixture (0.1 ml) was then applied to a Sephadex G-50 column (0.7 × 12 cm) and eluted with 1.0 mM Tris-HCl (pH 7.5) buffer. Three-drop fractions were collected for radioactivity determinations. The specific activities of [^{32}P]cAMP and [^{3}H]adenosine were 8100 and 3700 cpm/pmol, respectively.

ing Sephadex G-50 gel filtration was used to confirm the binding of cAMP to the receptors. Figure 6 shows the elution profile of a mixture of receptor I and [^{32}P]cAMP from the column. A portion of the radioactivity coeluted with the protein at the void volume. This excluded material was identified as cAMP by thin-layer chromatography on impregnated cellulose sheet and developed in 95% ethanol–1 M ammonium acetate (75:30; v/v) as described by Tao and Lipmann (1969). Similar results were obtained with receptor II.

Receptor I appeared to have some interesting binding properties and was investigated in greater detail. Due to a limitation in material, a similar study employing receptor II had not been carried out. As shown in Figure 6, adenosine could also bind to receptor I and was excluded from the gel. Inhibition studies further indicate that at equimolar concentration, adenosine inhibited the binding of cAMP by about 60% (Table II). Adenine at high concentration was also inhibitory. Other nucleosides such as uridine, cytidine, guanosine, thymidine, and their phosphorylated derivatives did not interfere with the binding of cAMP by receptor I, even at concentrations ten times that of the cyclic nucleotide. The interactions of receptor I with adenosine and cAMP had apparent K_m values of aobut 1×10^{-7} and 3×10^{-7} M, respectively. These values were calculated from double reciprocal plots of bound *vs.* free ligand concentrations in accordance with the method suggested by Rodbard (1973). All these experiments were carried out employing the Sephadex G-50 binding assay since the adenosine–receptor I complex appeared to bind poorly to Millipore filter.

TABLE II: Effect of Adenine and Its Derivatives on the Binding of cAMP by Receptor I.[a]

Addition	cAMP Bound, % of Control
None (control)	100
Adenine	
1.0 μM	86
10 μM	66
Adenosine	
1.0 μM	39
10 μM	6
AMP, 10 μM	90
ADP, 10 μM	98
ATP, 10 μM	92

[a] Receptor I (12 μg) was incubated for 3 min at 37° in the presence of 1.0 mM Tris-HCl (pH 7.5), 10% sucrose, 1.0 μM [^{32}P]cAMP, and adenine or its derivatives at concentrations indicated in the table. The binding of cAMP to receptor I was determined by Sephadex G-50 gel filtration as described in the legend to Figure 7.

TABLE III: Effect of Adenine and Its Derivatives on the Binding of Adenosine by Receptor I.[a]

Addition	Adenosine Bound, % of Control
None (control)	100
Adenine	
1.0 μM	52
10 μM	32
AMP, 10 μM	108
ADP, 10 μM	116
ATP, 10 μM	111
cAMP	
10 μM	98
0.1 mM	92

[a] The experimental details were as in Table II except [^{32}P]cAMP was replaced by [^{3}H]adenosine.

The results presented in Figure 6 further indicate that under saturating condition, receptor I bound more adenosine than cAMP. The difference in the binding capacities of receptor I for adenosine and cAMP may be due in part to a greater affinity of the receptor for adenosine and/or to the contamination of the [^{32}P]cAMP preparation with adenosine. The latter possibility could lead to a lower estimate of the binding of the cyclic nucleotide due to interference by contaminating adenosine. Furthermore, a difference in binding could also be explained by the presence of additional binding sites for adenosine. Inhibition studies have provided initial evidence for the existence of two types of binding sites in receptor I. As shown earlier in Table II, high concentrations of adenosine could almost completely abolish the binding of cAMP by receptor I. On the other hand, cAMP has no significant effect on the binding of adenosine (Table III). A number of compounds had been tested for their ability to interfere with the binding of adenosine. As shown in Table III, only adenine displays some inhibition of the binding of adenosine by receptor I. The inability of cAMP to block the binding of adenosine provides evidence for an adenosine-binding site(s) distinct from the site(s) for cAMP.

Additional data suggesting the presence of at least two types of binding sites in receptor I are presented in Table IV. The purpose of these experiments was to determine whether the binding of adenosine could be detected under conditions where the receptor had maximally bound cAMP. In this case, varying amounts of [^{3}H]adenosine were incubated with receptor I in the presence of saturating amount

TABLE IV: Binding of Adenosine to Receptor I in the Presence of Saturating Amount of cAMP.[a]

[^{3}H]Adenosine (μM)	cAMP Bound (pmol)	Adenosine Bound (pmol)
0	11.0	0
0.10	10.1	5.8
0.25	8.4	13.1
0.50	5.7	17.7
2.50	3.5	30.8

[a] Varying amounts of [^{3}H]adenosine (3600 cpm/pmol) were incubated with receptor I (6 μg) in the presence of 2.5 μM [^{32}P]cAMP (3300 cpm/pmol). Other experimental details were as described in the legend to Figure 6.

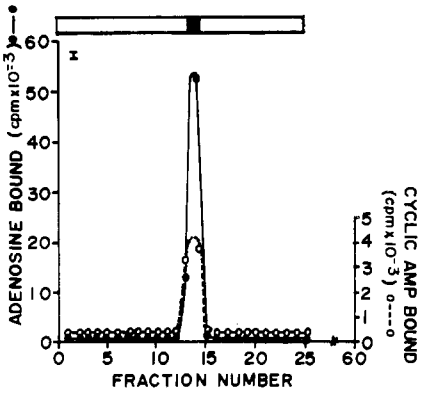

FIGURE 7: Electrophoretogram of receptor I incubated with cAMP and adenosine. Receptor I (40 μg) was incubated for 3 min at 37° in the presence of 0.02 M Tris-HCl (pH 7.5), 1.0 μM [^{32}P]cAMP (9900 cpm/pmol), 1.0 μM [^{3}H]adenosine (1900 cpm/pmol), and 10% sucrose. Other experimental details are described in the Experimental Section.

of [^{32}P]cAMP. As shown in Table IV, a considerable amount of [^{3}H]adenosine was bound to receptor I at concentrations of [^{3}H]adenosine insufficient to cause a significant decrease in [^{32}P]cAMP binding. The binding of adenosine was not due to contaminating enzyme(s) present in the receptor preparation. Figure 7 shows the electrophoretic profile of an incubation mixture of receptor I, [^{3}H]adenosine, and [^{32}P]cAMP on polyacrylamide gel. Both the [^{3}H]adenosine and [^{32}P]cAMP radioactive peaks comigrated with the protein band suggesting that both labels were bound to the same protein. The above data clearly demonstrate the presence of specific binding site(s) for adenosine.

Discussion

In an attempt to elucidate the functions of cAMP in red blood cells, we have initiated studies toward the isolation and characterization of protein factors with high affinity for the cyclic nucleotide. At least three cAMP dependent protein kinases are present in rabbit red blood cells (Tao and Hackett, 1973). The interrelationships and the functions of these multiple kinases are presently under investigation. The experiments reported here clearly establish the existence of at least two additional protein factors in the red cell capable of binding cAMP. Since the function of these cAMP binding protein factors is not known, they will be tentatively identified as receptors. These receptors have been purified to homogeneity based on criteria of polyacrylamide gel electrophoresis under various conditions.

The two receptors appear to have very similar molecular properties but they are clearly separable by QAE-Sephadex chromatography. As shown in Figure 2, receptor II appears to be present as a minor component. In some preparations, we have failed to detect the presence of this receptor. In view of the similarity in the molecular properties of the two proteins, it is possible that they may be interrelated.

Both factors have a molecular weight of approximately 240,000, as determined by gel filtration and polyacrylamide gel electrophoresis. Electrophoresis on SDS-polyacrylamide gel reveals that both proteins are constructed of five subunits, each of approximately 48,000. The assignment of molecular weights and number of subunits are tentative and are subject to the limitations of the methods by which they are derived. Further physicochemical analyses are needed to firmly establish these parameters. As determined by sucrose density gradient centrifugation, the sedimentation coefficient of both protein factors is 9.3 S. In contrast to the cAMP dependent protein kinases (Tao and Hackett, 1973), these receptors present no evidence of dissociation when sucrose density gradient centrifugation is performed in the presence of cAMP.

The nature of the effect of protamine on the binding of cAMP by the receptor proteins is not clear. It is not unreasonable to assume that these effects may be confined to the Millipore binding assay. For example, if protamine forms a large molecular aggregate with the receptor-cAMP complex, this could very well increase the efficiency of retention of the complex on the filter. Based on the data presented in Figures 4 and 6, it is evident that protamine is not essential for the binding of cAMP to the receptors. The Millipore binding assay, used mainly for monitoring the receptors during purification, gives a poor measurement of the binding capacity of the receptor for adenosine as compared to determination by Sephadex G-50 gel filtration. Furthermore, the interaction of receptor I with cAMP appears to reach equilibrium within 3 min of incubation under the Millipore binding assay. This is certainly not the case when assayed by Sephadex G-50 gel filtration which requires 30–40 min of incubation (unpublished observation). The reason for the anomalous behavior between the two methods of assay of binding capacity is not immediately apparent to us but may reflect the complex nature of the Millipore binding assay.

Both receptors bind cAMP with an apparent K_m of approximately 3×10^{-7} M. The binding of cAMP by both receptors is inhibited about 60% by equimolar concentration of adenosine. The interaction of adenosine with receptor I has been investigated. A similar study with receptor II is hampered by the limited amount of this protein available to us at this time. Evidence at hand indicates that receptor I may contain two types of binding sites: one for cAMP and the other for adenosine. Under the same conditions, considerably more adenosine is bound than cAMP, with a ratio of about 3:1. The data suggest that there are more binding sites for adenosine than for cAMP. Interestingly, cAMP has no effect on the binding of adenosine while, as mentioned earlier, adenosine inhibits the binding of cAMP. This clearly indicates that the two binding sites are different. Additional evidence supporting the presence of two types of binding sites in receptor I is based on the observation that adenosine could be bound to the receptor saturated with cAMP. Although we have conclusively shown that cAMP does not ineract with the adenosine-binding site(s), as yet

we do not know whether adenosine could occupy the cAMP binding site. At least two interpretations are available for the inhibition of cAMP binding by adenosine. It is possible that in addition to the adenosine-binding site(s), the nucleoside could also interact with the cAMP binding site. Alternatively, the saturation of the adenosine-binding site(s) by adenosine alters the conformation of the receptor to a state of low or no affinity for cAMP. Whichever is the correct interpretation must await further kinetic analyses.

Preliminary estimate indicates that about 0.44 molecule of cAMP is bound to each molecule of receptor I. The reason for the relatively low amount of cAMP bound is not immediately apparent to us and may be attributed to several contributing factors. One of the possibilities may be that only a fraction of the protein molecules of the receptor preparation are active and participate in the binding reaction. Whether a large population of the proteins are denatured (inactive) or whether cofactors are required for optimum binding remains to be established.

The function of these receptor proteins in red blood cells is not known. The binding specificity of these receptors remotely resembles that of rabbit muscle phosphofructokinase (Kemp and Krebs, 1967). However, no phosphofructokinase activity is found to be associated with these receptor preparations. In addition, both rabbit liver and muscle phosphofructokinase do not cause the retention of cAMP on Millipore filters when assayed under the same conditions employed for the receptors (unpublished observation). These results suggest that the binding protein factors are not related to phosphofructokinase. Our efforts to demonstrate any protein phosphokinase activity associated with these receptors have not been successful. The reason for this may be trivial, *i.e.*, the experiments may not have been conducted with the ideal substrate and/or under optimum conditions. On the other hand, it is conceivable that these receptors do not have protein phosphokinase activity and participate in some as yet undefined metabolic processes. The possibility that they may function only as regulators without a direct involvement in catalysis cannot be overlooked. In relation to a potential regulatory role, however, these receptors do not seem to be affiliated with any of the cAMP dependent protein kinases of the red blood cell since they are unable to inhibit the catalytic moiety of these enzymes.

Reports from several laboratories have indicated a relationship between adenosine and cAMP metabolism. For example, adenosine inhibits the norepinephrine-stimulated accumulation of cAMP in fat cells (Fain, 1973; Schwabe *et al.*, 1973) by preventing the activation of adenylate cyclase (Fain *et al.*, 1972). In other systems such as slices of rat (Perkins and Moore, 1973; Schultz and Daly, 1973) and guinea pig cerebral cortex (Sattin and Rall, 1970; Daly *et al.*, 1972), the nucleoside is reported to potentiate the increase in cAMP levels caused by biogenic amines. Other evidence implicating adenosine as a metabolic regulator have been documented. In human red blood cells, adenosine can inhibit the synthesis of nucleotides (Manohar *et al.*, 1968). Furthermore, adenosine could also act as a vasodilator and cause an increase in blood flow to cardiac tissue (Rubio and Berne, 1969). Whether these receptors have a role in the aforementioned processes remains to be determined. The availability of homogeneous preparations of these receptor proteins will enable us to better understand their function.

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