Cyclic Adenosine 3',5'-Monophosphate-dependent Protein Kinase of Human Erythrocyte Membranes*

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SUMMARY
A cyclic AMP (adenosine 3', 5'-monophosphate)-dependent protein kinase has been found in membranes derived from human erythrocytes which accounts for greater than 70% of the total cyclic AMP-dependent protein kinase activity of these cells. Components of the erythrocyte membranes were phosphorylated by membrane-bound protein kinase, but stimulation of the rate of phosphorylation by cyclic AMP was observed only when histones or protamine served as substrates. The membrane-associated cyclic AMP-dependent protein kinase resembles many soluble protein kinases; it requires 20 μM Mg2+ for maximal activity, has apparent dissociation constant of 3.3 nM for the "membrane-cyclic AMP complex." Cyclic AMP-independent protein kinase activity was dissociated from the membrane by treatment with 1 mM NH4Cl, but the cyclic AMP-binding activity was retained by the particulate fraction. These findings suggest that the cyclic AMP-binding moiety is firmly integrated into the membrane structure, whereas the catalytic moiety may only be loosely associated with the membrane.

EXPETIMENTAL PROCEDURE

Materials
Adenosine 3',5'-monophosphate, guanosine 3',5'-monophosphate, inosine 3',5'-monophosphate, uridine 3',5'-monophosphate, thymidine 3',5'-monophosphate, and adenosine 2',3'-monophosphate were purchased from Boehringer Mannheim. Tubercidin 3',5'-monophosphate was a gift from Dr. Arthur R. Hanze, Upjohn Co., Kalamazoo, Mich., and the 3'-cyclic ester of 9-(5'-deoxy-5-dihydroxyphosphill!-lrlletllyl-P-D-ribofuranosyl)-adenine was generously supplied by Dr. J. G. Moffat, Syntex Institute of Molecular Biology, Palo Alto, Calif.

Proteinase was purchased from Eli Lilly, arginine- and lysine-rich histones were from Sigma; and casein was obtained from Nutritional Biochemical Co.

Radioisotopes—[γ-32P]ATP (20.8 Ci per mmole) was purchased from Amer sham-Searle; cyclic [3H]AMP (16.3 Ci per mmole) from Schwarz-Mann.

Cyclic Nucleotide Phosphodiesterase—A partially purified preparation of beef heart cyclic nucleotide phosphodiesterase (specific activity = 1 amole of cyclic AMP hydrolyzed per min per mg of protein at 30°) was obtained from Dr. Elihu Goren of this laboratory.

Most of the available information about the structure and properties of these protein kinases has been derived from studies of soluble protein kinases (7-13). Weller and Rodnight (14) have described protein kinases in ox brain microsomes and synaptosomes which catalyze the phosphorylation of serine residues in unidentified membrane proteins and in exogenous substrates like casein and phosphitin. Phosphorylation of membrane components was stimulated as much as 100% by cyclic AMP, but the cyclic nucleotide had no effect upon phosphorylation of the exogenous substrates. Rat brain synaptic membrane vesicles, and membrane fragments also exhibited considerable cyclic AMP-dependent protein kinase activity when histone was used as substrate (15). Recently, Lemaire et al. (16) reported that 50% of the cyclic AMP-dependent protein kinase activity in bovine anterior pituitary glands was associated with microsomal and nuclear fractions. The association of cyclic AMP-dependent protein kinases with cellular membranes may be a general phenomenon. The studies reported here are concerned with some of the properties of cyclic AMP-dependent protein kinase in membranes of human erythrocytes.

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Protein Kinase Assay—Erythrocyte ghosts were prepared from 15 ml of fresh heparinized blood according to the procedure of either Dodge et al. (17) or Post et al. (18). Unless otherwise indicated, the method of Dodge et al. was used to prepare the membranes. Membranes could be stored at -20° for 2 months without significant loss of activity.

For assay of protein kinase activity the reaction mixture (0.2 ml) contained 0.05 M potassium phosphate buffer (pH 7.0), 0.02 M MgSO₄, 50 μM [γ-32P]ATP (15,000 cpm per nmole), 2.0 μM cyclic AMP (where indicated), 0.01 M dithiothreitol, 0.25 mg of protamine, 0.5 mg of bovine serum albumin, and 20 to 40 units of protein kinase. A unit of kinase activity was defined as the amount of enzyme required to catalyze the transfer of 1 pmole of 32P from [γ-32P]ATP to protamine (or other substrates) per min at 37°. Incubations were performed at 37° for 5 min unless otherwise noted. The amount of 32P transferred to protamine was determined by a modification (12) of the method of De Lange et al. (19). Phosphorylation of membrane components in the enzyme preparation was measured in parallel assays in which protamine was omitted and all data on phosphorylation of exogenous proteins were corrected for endogenous membrane phosphorylation. The bovine serum albumin used in the assay was not a substrate for the kinase. Background radioactivity was determined in assays containing boiled membranes. The 32P incorporated into protamine was stable to treatment with hot trichloroacetic acid, but was completely released from the protein by boiling in 1 N NaOH for 5 min.

Cyclic AMP-binding assays were carried out in 0.05 M potassium phosphate buffer, pH 7.0, containing 10 mM Mg²⁺, a saturating amount of cyclic [H]AMP and 30 to 60 μg of membrane protein in a final volume of 0.2 ml. After a 90-min incubation at 0° (completion of the binding reaction was attained in 45 min at 0°), 1 ml of 0.02 M potassium phosphate buffer, pH 6.0, was added and the contents of the assay tubes were quantitatively transferred to Millipore or glass fiber filters by rinsing with the phosphate buffer. The filters were washed with an additional 10 ml of buffer and dried in scintillation vials. Each vial received 10 ml of scintillation fluid (4 g of Omnifluor per liter of toluene) and radioactivity was determined in a liquid scintillation spectrometer. Control assays were carried out with boiled membranes.

Binding assays performed on solubilized protein kinase (see Table VI) were carried out according to the method of Gilman (20), except that 0.05 M potassium phosphate buffer, pH 7.0, was substituted for 0.02 M sodium acetate, pH 4.0.

Protein was assayed by the method of Lowry et al. (21) with bovine serum albumin as the standard. Hemoglobin was determined by the procedure of Dodge et al. (17). Glucose 6-phosphate dehydrogenase was assayed according to the method of Glock and McLean (22).

RESULTS

The protein kinase associated with membranes prepared by two different procedures (17, 18) accounted for greater than 70% of the total erythrocyte kinase activity and was stimulated 3- to 8-fold by the addition of 2 μM cyclic AMP (Table I). Phosphorylation of endogenous substrate (or substrates) was not enhanced by cyclic AMP and comprised approximately 20 to 25% of the total kinase activity.

The erythrocyte membranes were apparently not contaminated with nonspecifically adsorbed soluble proteins as judged by the absence of hemoglobin and glucose 6-phosphate dehydrogenase activity in these preparations.

**Table I**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Proteine kinase activity</th>
<th>Percentage of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>11.8</td>
<td>15.2</td>
</tr>
<tr>
<td>-</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>134</td>
<td>734</td>
</tr>
<tr>
<td>-</td>
<td>30.8</td>
<td>34.7</td>
</tr>
</tbody>
</table>

25% of the kinase activity observed with protamine in the absence of cyclic AMP. Cyclic AMP-independent phosphorylation of human erythrocyte membranes was also catalyzed by a homogeneous preparation (13) of a soluble cyclic AMP-dependent protein kinase from bovine heart muscle. The soluble erythrocyte protein kinase had a lower specific activity than the membrane-bound enzyme and was only slightly stimulated by cyclic AMP (Table I).

The rate of phosphorylation of protamine was constant for 10 min and was directly proportional to the concentration of membrane protein up to 500 μg per ml in the presence and absence of cyclic AMP.

Effect of Divalent Metal Ions—Membrane-associated cyclic AMP-dependent protein kinase requires Mg²⁺ for maximal activity and exhibits activity in the absence of divalent cations. Of the divalent metals tested, Co²⁺ supported the highest level of basal activity at concentrations of 2 and 20 mM (Table II). In the presence of cyclic AMP, a 4- to 5-fold stimulation of protein kinase activity was observed with either 2 or 20 mM Mg²⁺, while no stimulation was seen with Co²⁺ or Zn²⁺. Only Mn²⁺ functioned as a partial substitute for Mg²⁺, but the degree of stimulation by cyclic AMP in the presence of Mn²⁺ was low (50 to 70%). When Fe²⁺ was utilized in the reaction mixture, considerable basal protein kinase activity was discernible, but the addition of cyclic AMP led to slight inhibition of phosphorylation. No protein kinase activity was detected in the presence of Ca²⁺.

Substrate Specificity—Casein and the basic proteins protamine, arginine- and lysine-rich histones were good substrates for the membrane-bound protein kinase (Table III). Upon the addition of 2 μM cyclic AMP, the protein kinase activity was stimulated 4- to 5-fold with protamine or the histones as substrates. No enhancement of kinase activity by cyclic AMP was found with casein which, however, yielded the highest basal activity (Table III).

The effect of cyclic AMP on the activity of membrane-bound protein kinases derived from the erythrocytes of a patient with hemolytic anemia (reticulocytes count 7%) had the same specific activity as the enzyme present in membranes derived from normal individuals, suggesting that the activity measured in these studies was not due to contamination of the erythrocyte preparation by reticulocytes.
protein kinase was determined over the range 5 nM to 5 μM and the apparent $K_m$ for cyclic AMP was estimated to be 28 nM.

**Cyclic Nucleotide Specificity**—A variety of cyclic 3',5'-nucleotides and analogs were tested at two concentrations for their ability to stimulate the membrane-associated protein kinase (Table IV). Cyclic AMP was the most effective compound at both concentrations. At a concentration of 2 μM, only cyclic IMP (90%) and cyclic tubercidin 3') 5'-monophosphate (75%) approached the level of stimulation produced by cyclic AMP, while cyclic GMP was half as effective. When a higher concentration (20 μM) was employed, cyclic AMP, cyclic IMP, and cyclic GMP were nearly equal in their stimulatory capacities and the cyclic 3',5'-pyrimidine monophosphates, cyclic UMP, and cyclic CMP became moderately good activators of membrane-bound kinase. These findings are in agreement with those of others (7, 23, 24) in that high levels of many natural and synthetic cyclic 3',5'-ribonucleotides activate a broad spectrum of soluble protein kinases. The inability of either cyclic TMP or cyclic 2',3'-AMP to stimulate the membrane protein kinase is in accord with the apparent requirements for a free 2'-hydroxyl group and a cyclic 3', 5'-diester (24, 25). The absence of significant stimulation by the 5'-methylene phosphonate may be a unique feature of the erythrocyte enzyme, since this compound activates a number of soluble mammalian protein kinases (23), including beef heart protein kinase prepared in this laboratory.

**$K_m$ Values**—The apparent $K_m$ values for protamine, ATP, and Mg$^{2+}$ were determined by measuring initial velocities and plotting the data according to the method of Lineweaver and Burk (26). Values of 26 pg per ml, 8.3 μM, and 3 mM were obtained for protamine, ATP, and Mg$^{2+}$, respectively. Cyclic AMP had no effect on these apparent $K_m$ values but stimulated protein kinase activity by increasing the velocity of the phosphorylation reaction 3- to 8-fold.

**Binding of Cyclic AMP to Membranes**—Human erythrocyte membranes bound cyclic AMP with a high affinity and exhibited a maximum binding capacity of 12 pmol per mg of membrane protein (Fig. 1). A reciprocal plot of the data yielded a dissociation constant of 3.3 nM for the “membrane-cyclic AMP complex.” The binding reaction appeared to be specific for cyclic AMP, since neither a 100- to 1000-fold molar excess of 5'-AMP nor an equal concentration of cyclic GMP decreased the binding of cyclic [3H]AMP (Table V). The bound radioactivity was com-
Binding of cyclic AMP by erythrocyte membranes

Binding assays contained 30 μg of membrane protein and a saturating concentration (20 μM) of cyclic [3H]AMP (16.3 Ci per mmole). Preliminary incubation denotes the treatment of the reaction mixture for the stated time period prior to the addition of membrane protein; postincubation refers to treatment of the reaction mixture following the standard 90 min incubation. Incubations with 50 μg of cyclic nucleotide phosphodiesterase (Diesterase) were carried out at 30°.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cyclic AMP bound</th>
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<tbody>
<tr>
<td>None</td>
<td>435</td>
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<tr>
<td>Membranes omitted from reaction mixture</td>
<td>8</td>
</tr>
<tr>
<td>5'-AMP (20 μM)</td>
<td>457</td>
</tr>
<tr>
<td>5'-AMP (200 μM)</td>
<td>425</td>
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<tr>
<td>Cyclic GMP (20 nM)</td>
<td>439</td>
</tr>
<tr>
<td>Diesterase (membranes omitted from reaction mixture)</td>
<td>23</td>
</tr>
<tr>
<td>Diesterase, 30 min preliminary incubation</td>
<td>40</td>
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<tr>
<td>Diesterase, 30 min postincubation</td>
<td>345</td>
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<tr>
<td>Nonradioactive cyclic AMP (20 nM), 60 min postincubation</td>
<td>443</td>
</tr>
<tr>
<td>Nonradioactive cyclic AMP (200 nM), 60 min postincubation</td>
<td>446</td>
</tr>
</tbody>
</table>

Separation of catalytic and cyclic AMP-binding activities after NH4Cl treatment

Samples of erythrocyte membranes (600 μg, 731 units per mg) were extracted by incubation with 500 μl of 1 M NH4Cl containing 1% bovine serum albumin for 2 hours at 4°. The particulate (membrane) and soluble fractions were then separated by centrifugation for 30 min at 30,000 × g. The membrane fraction was resuspended in 250 μl of 0.05 M potassium phosphate buffer, pH 7.0, and 20-μl aliquots were assayed for protein kinase activity. Assays were also performed with 40-μl aliquots of the soluble fraction, following dialysis against 500 volumes of 0.05 M potassium phosphate buffer, pH 7.0, for 4 hours.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein kinase activity</th>
<th>Cyclic AMP bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyclic AMP</td>
<td>12 μM Cyclic AMP</td>
</tr>
<tr>
<td>Soluble</td>
<td>4.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Particulate</td>
<td>8.0</td>
<td>30.7</td>
</tr>
<tr>
<td>NH4Cl-soluble</td>
<td>27.8</td>
<td>29.9</td>
</tr>
<tr>
<td>NH4Cl-particulate</td>
<td>9.0</td>
<td>13.4</td>
</tr>
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</table>

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<th>Sample</th>
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<tr>
<td>NH4Cl-particulate</td>
<td>9.0</td>
<td>13.4</td>
</tr>
</tbody>
</table>

* Membranes extracted with 0.05 M potassium phosphate buffer, pH 7.0, containing 1% bovine serum albumin.

The solubilized protein kinase activity was not stimulated by cyclic AMP and had very little cyclic AMP-binding capacity (Table VI). The sum of the activities observed in the soluble and particulate fractions of NH4Cl-treated membranes consistently exceeded the original activity. A possible explanation for this phenomenon is that the solubilized catalytic moiety may have a greater specific activity than a maximally stimulated catalytic moiety associated with the membrane. The membrane fraction retained less than half of the original cyclic AMP-dependent protein kinase activity and 100% of the original cyclic AMP-binding activity.

Discussion

Many of the kinetic properties of the membrane-associated cyclic AMP-dependent protein kinase of the erythrocyte resemble those of soluble mammalian kinases (7, 12, 29). Both kinds of protein kinases have similar divalent metal requirements, substrate specificities, Kₐ values for cyclic AMP, Kₐ values for protamine and ATP, and cyclic nucleotide specificities. Erythrocyte membranes also avidly bind cyclic AMP as evidenced by a dissociation constant of 3.3 nM for the membrane-cyclic AMP complex. The binding reaction appears to be specific for cyclic AMP, since neither an equal concentration of cyclic GMP nor a 100- to 1000-fold molar excess of 5'-AMP inhibited the binding of cyclic [3H]AMP at 0°. The cyclic nucleotide in the "membrane-cyclic [3H]AMP complex" was not readily displaced by the addition of an excess of nonradioactive cyclic AMP at 0° or hydrolyzed by cyclic nucleotide phosphodiesterase (Table V), two characteristics which have been observed with the cyclic AMP-binding component of soluble protein kinases (20, 30). The difference between the Kₐ for stimulation and the Kₐ for cyclic AMP binding may reflect the temperature dependence of the equilibrium constant, since the catalytic and binding measurements were carried out at 37 and 0°, respectively.

Protein kinase activity could be dissociated from the membrane by treatment with NH4Cl, NaCl, or MgSO4, with concomitant losses of cyclic AMP dependence and cyclic AMP-binding capacity in the solubilized enzymes. The particulate fraction, however, retained essentially all of the original binding activity suggesting that there had been a selective release of the catalytic component of the enzyme. It is of interest to consider this dissociation of a cyclic AMP-independent kinase from the membrane-associated cyclic AMP-binding protein in terms of the hypothesis proposed by Lemaire et al. (16). These authors suggested that particulate fractions derived from bovine anterior pituitaries contained a protein kinase activity which resulted from the combination of catalytic subunits of a soluble, cytoplasmic protein kinase with protein substrates in the various membranes. Although the catalytic moiety of human erythrocyte membrane protein kinase may phosphorylate specific membrane proteins independently of cyclic AMP when it resides in the membrane, it appears to be at least partially controlled by a specific membrane-associated cyclic AMP-binding protein in the presence of soluble protein substrates.

* This protein kinase activity could also be quantitatively recovered in the supernatant fluid following centrifugation at 100,000 × g for 60 min.
The function of the membrane-associated protein kinase in human erythrocytes is not established. The possibility that it may play a role in phosphorylating certain membrane proteins seems reasonable. Phosphorylation of membranes by endogenous protein kinases has been described for ox brain (14), rat synaptosomal (15), and bovine pituitary (16, 31) membrane systems. Changes in the concentration of plasma or erythrocyte cyclic AMP could lead to alterations in the local cyclic nucleotide concentration at the cyclic AMP-binding sites in the membrane which, in turn, might lead to modification of erythrocyte function mediated by protein kinase action. Additionally, the cyclic AMP-binding portion of the membranes could serve as a site for transporting and storing cyclic AMP.

The membrane-associated cyclic AMP-dependent protein kinase of the human erythrocyte provides a model system for further studies on the interaction between the functionally distinct catalytic and cyclic AMP-binding components. In particular, it would be of interest to determine whether the mechanism of activation of the membrane-associated kinase by cyclic AMP is similar to that of the soluble enzyme or whether certain limitations and restrictions are imposed by association with the cellular surface. Since the catalytic and binding activities can be dissociated, they may also be studied as separate entities and compared to the cyclic AMP binding and catalytic subunits isolated from soluble protein kinases. In a more general sense, membrane-associated cyclic AMP-dependent protein kinase might also be exploited as an "endogenous probe" in investigating membrane structure if the phosphorylation of specific endogenous substrates alters membrane structure and function.

Note Added in Proof—In more recent studies we have noted a consistent 25 to 50\% stimulation of the phosphorylation of endogenous substrates by cyclic AMP.

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Cyclic Adenosine 3',5'-Monophosphate-dependent Protein Kinase of Human Erythrocyte Membranes
Charles S. Rubin, Jack Erlichman and Ora M. Rosen


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