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Specific Photoaffinity Labeling of the Adenosine 3′:5′-Cyclic Monophosphate Receptor in Intact Ghosts from Human Erythrocytes

((ethyl 2-diazomalonyl)cAMP)

C. EARL GUTHROW§*, HOWARD RASMUSSEN*, DAVID J. BRUNSWICK†, AND BARRY S. COOPERMAN†‡

* Department of Biochemistry and † Department of Chemistry, University of Pennsylvania, Philadelphia, Pa. 19174

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ABSTRACT **[^{3}H]N^6-(Ethyl 2-diazomalonyl)-adenosine 3′:5′-cyclic monophosphate is incorporated into intact ghosts from human erythrocytes on photolysis at 253.7 nm. Incorporation is blocked in the presence of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and does not occur in the absence of irradiation. Sodium dodecyl sulfate disc gel electrophoresis of solubilized ghosts shows that one protein is labeled. The position of this protein on the gel corresponds exactly to that previously found [*J. Biol. Chem.* 247, 8145 (1972)] for the endogeneous protein substrate of the endogenous, cyclic AMP-dependent, protein kinase.**

Photoaffinity labeling has recently attracted wide attention because of its potential as a general tool for specifically labeling biological receptor sites, even in quite complex systems (1). In previous, separate, publications we have demonstrated the presence of an adenosine 3′:5′-cyclic monophosphate (cAMP)-dependent protein kinase in human erythrocyte ghosts that catalyzes the phosphorylation of an endogenous acceptor (2), and described the synthesis of three potential photoaffinity reagents for cAMP (3, 6). We now wish to report on the use of one of these photoaffinity labels, N^6-(ethyl 2-diazomalonyl)-cAMP, to specifically label the cAMP receptor site in intact human erythrocyte ghosts.

MATERIALS AND METHODS

Human erythrocyte ghosts were prepared from freshly drawn blood as described previously (2). Ghosts were stored at −20° in 20 mOsM sodium phosphate buffer (pH 6.5) and were used within 1 week of preparation. Prior to use ghosts were thawed and refrozen to assure penetration of substrates (4). N^6-(ethyl 2-diazomalonyl)cAMP (EDMcAMP) and [^{3}H]EDMcAMP (24.1 Ci/mmol) were prepared as described elsewhere (3, 6). cAMP was purchased from Sigma. [^{3}H]cAMP was purchased either from Schwarz Bio-Research or New England Nuclear Corp. [^{3}H]N^6-butyryl cAMP was prepared by alkaline hydrolysis of [^{3}H]N^6,$O^{2'}$-dibutyryl cAMP (New England Nuclear Corp.) All other chemicals were reagent grade.

Protein kinase activity at pH 6.5 and 30° was measured as previously described (2). Noncovalent cyclic nucleotide binding at pH 6.5 and 4° was measured as previously described (2), except that $Mg(OAc)_2$, NaF, and ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) were omitted from the reaction mixture. Covalent incorporation experiments were performed at 4° as follows: Freeze-thawed ghosts equilibrated at pH 6.5 were brought to pH 5.5¶ with 0.1 M sodium acetate buffer (pH 4.6) and immediately transferred to a quartz tube. Further additions were made such that the final reaction mixture (1.6 ml) contained 68 μmol of dithiothreitol, 2.2–2.5 mg of ghost protein, and variable amounts of various cyclic nucleotides. Photolyses were performed in a Rayonet photochemical reactor at 253.7 nm with vigorous stirring of the suspension by means of a magnetic stirring bar. For the amount of ghost used, the suspensions are highly turbid and photolysis is presumed to take place at or close to the wall of the tube. After photolysis, 0.2 μmol of nonradioactive cAMP and 6 ml of Buffer A [20 mOsM sodium phosphate buffer (pH 7.4) containing 5 mM dithiothreitol] were added and the photolyzed ghosts were sedimented at 3000 × g for 20 min.

Samples that were to be fractionated were washed three additional times in Buffer A by resuspension and centrifugation. The final pellet was drained, dissolved, and subjected to sodium dodecyl sulfate–polyacrylamide disc-gel electrophoresis, and the resulting gel was fixed, stained, and sliced as previously described (2). Each slice was dissolved in basic hydrogen peroxide (5) and the radioactivity was measured in 10 ml of a toluene:methylcellosolve 5:3 (v/v)-based scintillation mixture in a Packard Tricarb scintillation spectrometer.

Samples that were to be counted for total incorporation were washed three times essentially as described above and then resuspended and precipitated with 10% trichloroacetic acid. The precipitated ghosts were dissolved in warm 0.2 M NaOH and after cooling 1 ml of this solution was added to 9 ml of a toluene:Triton X-100 1:1 (v/v) emulsion-based scintillation mixture. After the addition of the alkaline solution, 0.3 ml of 1 M acetic acid was added to quench chemiluminescence, and the sample was counted for radioactivity in an Intertechnique scintillation spectrometer.

RESULTS

Noncovalent Binding of EDMcAMP to Erythrocyte Ghosts. EDMcAMP stimulates the activity of the protein kinase of erythrocyte ghosts (Fig. 1). The concentration of EDMcAMP needed to give one-half of the maximal stimulation was about 0.75 μM. The corresponding value for activation by cAMP (2) was 0.23 μM. The maximum activity obtained with EDMcAMP (82 pmol of phosphate per mg of protein per 5 min) is somewhat less than that obtained with cAMP (93 pmol of phosphate per mg of protein per 5 min).

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; EDMcAMP, N^6-(ethyl 2-diazomalonyl)-adenosine 3′:5′-cyclic monophosphate.

‡ To whom requests for reprints should be addressed.

§ Present address: Department of Medicine, Duke University Medical Center, Durham, N.C. 27710.

¶ Photolyses were done at pH 5.5 to minimize formation of the 1,2,3-triazole anion isomer of EDMcAMP, which is formed reversibly in a pH-dependent equilibrium having an apparent pKa of 6 (6).

TABLE 1. *Displacement by EDMcAMP of [^{3}H]cAMP bound to erythrocyte ghosts*

[^{3}H]cAMP (μM)	EDMcAMP (μM)	pmol [^{3}H]cAMP bound per mg of ghost protein
0.066	—	6.3
0.066	0.80	4.9
0.066	8.0	1.65
0.066	80.0	0.43
0.091	—	6.2
0.091	0.20	5.8
0.091	0.80	4.6

A second demonstration of noncovalent binding is provided by the results in Table 1, showing that EDMcAMP can displace bound [^{3}H]cAMP from erythrocyte ghost. In similar experiments, the binding of N^6,$O^{2'}$-di(ethyl 2-diazomalonyl)-cAMP and $O^{2'}$-(ethyl 2-diazomalonyl)cAMP were found to be much weaker.

Covalent Incorporation on Photolysis of EDMcAMP in the Presence of Erythrocyte Ghosts. The results of several incorporation experiments are summarized in Table 2. It can be seen that incorporation is specific for the cAMP site [(*a*) vs. (*b*), I–III] and that irradiation is required for incorporation [II (*a*) vs. II (*d*)]. In a previous study on the photoaffinity labeling of rabbit muscle phosphofructokinase with $O^{2'}$-(ethyl 2-diazomalonyl)adenosine 3′:5′-cyclic monophosphate we found incorporation to proceed exclusively via carbene formation (3). By contrast, the labeling we see in the present study appears to result from a second photolytic process in addition to the formation of carbene. Evidence for this second process comes from a comparison of III (*c*) and III (*b*), from which it is seen that EDMcAMP that has been pre-photolyzed in solution for 3 min in the absence of erythrocyte ghost, is still capable, on further irradiation in the presence of erythrocyte ghost, of giving substantial labeling. As the 3-min pre-photolysis is sufficient for virtually total loss of the diazo group (and concommitant formation of solvent insertion products from the intermediate carbene) this labeling cannot arise from carbene formation. Evidence for labeling via carbene formation comes from a comparison of III (*a*) and III (*c*), from which it is seen that more than twice as much labeling results from irradiation of erythrocyte ghost is the presence of EDMcAMP as in the presence of pre-photolyzed material.

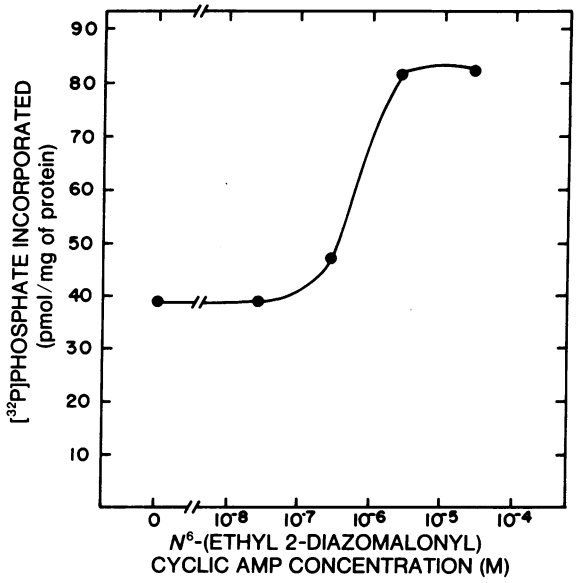

FIG. 1. Activation of protein kinase from human erythrocytes by N^6-(ethyl 2-diazomalonyl)cAMP.

Elsewhere (6) we have shown that on irradiation of EDMcAMP at 253.7 nm, the initial rapid loss of diazo group is followed by a slower photolytic process which appears to be typical of N^6-acylated adenosine derivatives. It seems reasonable to suppose that incorporation obtained with pre-photolyzed EDMcAMP is due to the formation of some reactive intermediate during this second process. This hypothesis is supported by the data in series IV and V, showing that specific photolytic labeling of the cAMP site can be obtained with [^{3}H]N^6-butyryl cAMP but not with [^{3}H]cAMP. For the shorter photolysis times employed in series I and II, labeling due to this secondary process would be expected to be less important.

Gel Electrophoresis. A sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiment on erythrocyte ghost photolyzed for 1 min at pH 5.6 in the presence of 4 nM [^{3}H]-EDMcAMP is shown in Fig. 2. The pattern of protein peaks is similar to that seen earlier (2) and follows the numbering system proposed by Fairbanks *et al.* (7). A single ^{3}H-labeled band is observed, falling between protein peaks IV and V. Control gels were not run on erythrocyte ghost samples pro-

TABLE 2. *Photolytic labeling of erythrocyte ghosts*

Series	Compound	Concentration (nM)	Irradiation time (min)	Concentration of labeled sites (nM) (*a*) On irradiation	(*b*) On irradiation in the presence of 0.1 mM cAMP	(*c*) On irradiation with EDMcAMP replaced by prephotolyzed EDMcAMP*	(*d*) With no irradiation
I	[^{3}H]EDMcAMP	3.6	1	0.15	0.035	—	—
II	[^{3}H]EDMcAMP	3.6	3	0.16	0.041	—	0.021
III	[^{3}H]EDMcAMP	0.94	12	0.11	0.011	0.047	—
IV	[^{3}H]N^6-butyryl cAMP	13	12	0.20	0.083	—	0.051
V	[^{3}H]cAMP	6.4	12	0.020	0.021	—	0.005

* Prepared by photolyzing EDMcAMP in solution for 3 min.

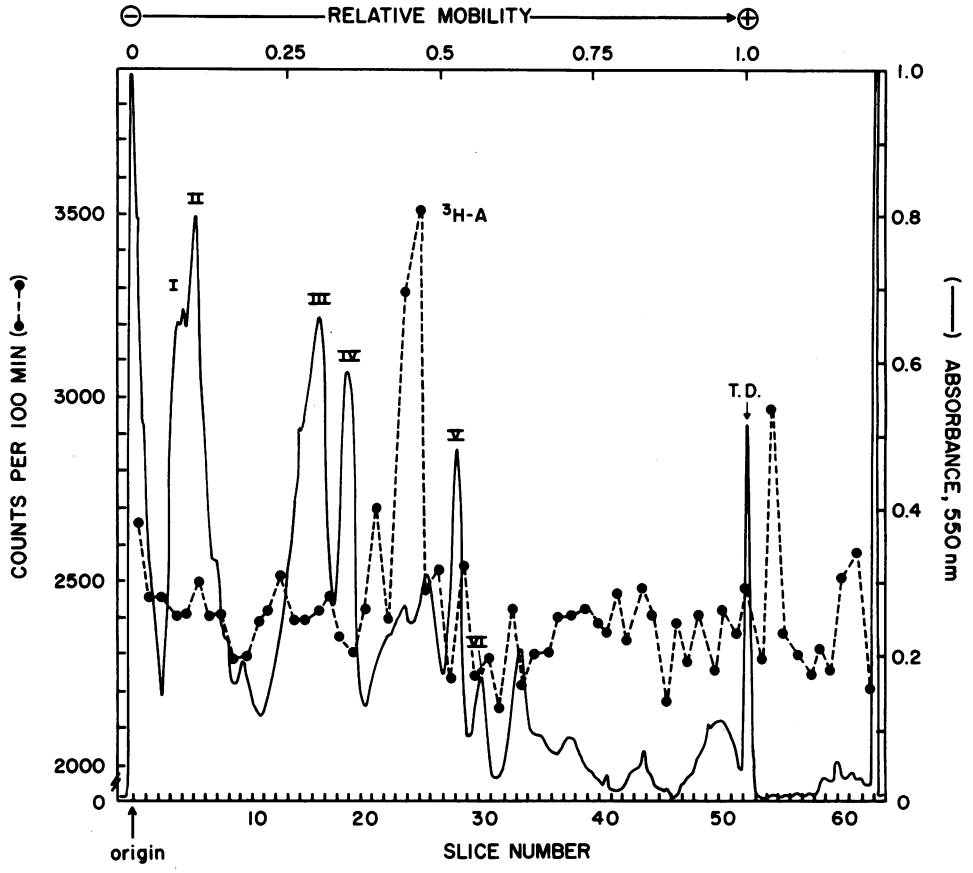

FIG. 2. Electrophoretogram of human erythrocyte ghosts photolyzed in the presence of [^{3}H]N^6-(ethyl 2-diazomalonyl)cAMP. The single protein peak having radioactivity significantly above background is labeled *^{3}H-A*. *T.D.* denotes the position of the tracking dye. The peak of radioactivity coming after the tracking dye does not correspond to protein and might be due to photolyzed N^6-(ethyl 2-diazomalonyl)cAMP that was noncovalently bound to the ghosts.

tected from labeling by cAMP because, as seen in Table 2, I (*b*)–III (*b*), too few counts were incorporated to give meaningful results. However, the presence of cAMP must greatly decrease ^{3}H incorporation into the peak marked ^{3}H-A because in the absence of cAMP >75% of the counts applied to the gel are found in ^{3}H-A, while in the presence of cAMP the total counts incorporated into erythrocyte ghost is reduced by 75–90% [Table 2, I–III, (*a*) vs. (*b*)]. Previously we demonstrated that the erythrocyte ghost contained an endogenous substrate for the ghost protein kinase whose phosphorylation was totally dependent on the presence of cAMP (2). On sodium dodecyl sulfate gel electrophoresis this protein, labeled with ^{32}P (from [γ-^{32}P]ATP), migrated to exactly the same position as the ^{3}H-labeled peak in Fig. 2.

DISCUSSION

The results of Table 2 and Fig. 2, taken together, provide a clear demonstration that EDMcAMP is, on photolysis, forming a covalent bond specifically with the cAMP receptor site in erythrocyte ghost membranes. The function of the receptor protein remains to be determined. However, it is interesting to note that the soluble cAMP dependent protein kinase from heart muscle catalyzes the phosphorylation of its own cAMP binding (regulatory) subunit (Rosen, O. M., Rubin, C. & Erlichman, J., manuscript in preparation). The coincidence of the ^{3}H- and ^{32}P-labeled protein on the sodium dodecyl sulfate gel strongly suggests that, in a totally analogous manner, the protein labeled by N^6-(ethyl 2-diazomalonyl)cAMP is the regulatory subunit of the membrane-bound cAMP-dependent protein kinase.

This study shows that EDMcAMP and other photoaffinity labels for cAMP (3, 6) have great potential usefulness for identifying cAMP receptor sites in complex, intact structures. Slime mold aggregation (8) and contact inhibition (9) are examples of problems of great current interest for which such an approach could yield useful information.

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