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A Study of Adenosine 3′–5′ Cyclic Monophosphate Binding Sites of Human Erythrocyte Membranes Using 8-Azidoadenosine 3′–5′ Cyclic Monophosphate, A Photoaffinity Probe

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An earlier report (1a) has shown the utility of 8-N_3cAMP (8-azidoadenosine-3′, 5′-cyclic monophosphate) as a photoaffinity probe for cAMP binding sites in human erythrocyte membranes. The increased resolution obtained using a linear-gradient SDS polyacrylamide gel system now shows that: 1) both cAMP and 8-N_3cAMP stimulate the phosphorylation by [γ-^{32}P]-ATP of the same red cell membrane proteins; 2) the protein of approximately 48,000 molecular weight whose phosphorylation by [γ-^{32}P]-ATP is stimulated by cAMP and 8-N_3cAMP migrates at a slower rate than the protein in the same molecular weight range which is heavily photolabeled with [^{32}P]-8-N_3cAMP; 3) other cyclic nucleotide binding sites exist besides those initially reported; 4) the variation in the ratio of incorporation of [^{32}P]-8-N_3cAMP into the two highest affinity binding sites appears to be the result of a specific proteolysis of the larger protein.

Key words: cAMP binding sites; photoaffinity probe, cAMP; membranes, human erythrocyte; membrane phosphorylation

INTRODUCTION

The ubiquity of the nucleotides cAMP and cGMP and the number of functions which they are known to mediate have led biochemists to devote increasing amounts of time to the mechanism of their action. Thus, the second messenger hypothesis of Sutherland (2) has been extended to include the concept of cyclic nucleotides activating protein kinases (3) or affecting phosphoprotein phosphatase activity (4a,b). It has been suggested that there may be more than one cAMP stimulated protein kinase in a single cell or tissue (5) or two or more cyclic nucleotide-binding subunits per protein kinase catalytic subunit (6). Protein kinase activity may be regulated by numerous mechanisms, e.g. the cAMP-induced dissociation of regulatory and catalytic subunits, the presence or absence of a proteinaceous inhibitor, or modulator, and stimulation of activity by substrate binding (reviewed in Ref. 8). Also, an autophosphorylation reaction may be occurring which modifies the cAMP binding-dissociation sequence (4a,b). Considerable data has been accumulated which implies that cAMP and cGMP control opposing metabolic functions, i.e. Goldberg's Yin-Yang hypothesis (7). To understand the mechanisms through which cAMP

and cGMP effect biological changes, information will be needed on the binding sites of these nucleotides. We propose that biologically active photoaffinity analogs of cAMP and cGMP when used properly will give such information.

8-N_3cAMP has been shown to be a very good mimic of cAMP in that it stimulates the phosphorylation by [γ-^{32}P]-ATP of the same red cell membrane proteins as does cAMP. 8-N_3cAMP, on photolysis, is also effective at photolabeling cAMP binding sites (1a). Using [^{32}P]-8-N_3cAMP, data has been obtained which indicates that not all of the cAMP-binding proteins are phosphorylated by [γ-^{32}P]-ATP and that still another regulatory mechanism may be possible, i.e. modification of the cAMP-binding subunit by a membrane-bound protease.

METHODS

Human erythrocyte membranes were prepared as previously described (1a). [^{32}P]-8-N_3cAMP was synthesized using a modification of an earlier reported procedure (1a), and will be described in detail elsewhere (1b). On the day of use membranes were thawed and used immediately. Solutions were photolyzed for 1 min at 0°C and at 1 cm distance using a UVS 11 mineral light. Membrane solutions were prepared for electrophoresis by dissolving in an equal volume of protein solubilizing solution (PSM) which consists of 25% sucrose, 2.5% SDS (sodium laurylsulfate), 2.5 mg% Pyronin Y, 25 mM Tris-HCl buffer (pH 8.0), 2.5 mM EDTA, and 15.4 mg dithiothreitol per ml or 0.25 ml 2-mercaptoethanol per 10 ml. Solubilized membranes were boiled for 5 min in a covered tube before electrophoresis. A slab gel electrophoresis system was used, employing a linear gradient (6 to 12%) of polyacrylamide with a 4% stacking gel. Both chambers of the gradient-making apparatus contained 0.36 M Tris-HCl buffer (pH 8.7), 0.1% SDS, 0.13 vol.% N,N,N′,N′-tetramethylethylenediamine and 0.01 wt.% ammonium persulfate. For a 6 to 12% gel the first and second chambers contained 5.9 and 12.2 wt.% acrylamide (1.5 wt.% bisacrylamide), respectively, with the second also containing 0.64 M sucrose. The stacking gel contained 0.1% SDS, 62.4 mM Tris-HCl (pH 6.7), 4 wt.% acrylamide, 0.05 vol.% N,N,N′,N′-tetramethylethylenediamine and 0.15 wt.% ammonium persulfate. Gradient gel dimensions were 16.5 × 13.3 cm × 1 mm; stacking gel 16.5 × 2 cm × 1 mm. Electrophoresis was done at 35 ma for about 4.5 hr or until the tracking dye reached the bottom of the gel. After electrophoresis gels were removed from the form, washed and fixed for 1 hr in 1ℓ aqueous 5% acetic acid, 10% 2-propanol. Staining was done for 1 hr in 250 ml of a solution containing 25% 2-propanol, 10% acetic acid and 0.05% Coomassie Brilliant Blue. The gels were destained for about 1.5 hr with fixing solution in the presence of several pieces of synthetic packing foam to soak up the stain. All these steps were carried out at 50°C with shaking. Gels were dried on a Hoefer Scientific Instruments Model SE 540 slab gel dryer at 80° for 2 hr. Autoradiography was done as previously described (1). An Ortec Model 4310 densitometer was used for scanning dried gels and autoradiographs. Dried gels were sliced and counted in Beckman Ready-Solv Solution VI with a Beckman LS-250 Liquid Scintillation System.

RESULTS

The gel system reported herein gave improved separation and visualization of proteins from human erythrocyte membranes. The linearity of the polyacrylamide gradient was checked by adding a radioactive compound to the chamber of the gradient-making apparatus containing the high percentage of acrylamide, making the gel, then slicing it

vertically and counting these slices. Figure 1 shows the results of this determination and the position of the major bands on the gradient, with their apparent molecular weights as determined from a plot of migration distance vs log molecular weight of standard proteins.

A representative gel showing the numbering of the bands is shown in Fig. 2. The numbering system follows the decimal notation of Steck (10) although the bands may not be identical due to the use of the linear gradient and improved resolution. For example, the approximate molecular weight of the protein most heavily labeled with [^{32}P]-8-N_3 cAMP was previously reported to be 55,000 (1a) but with the linear gradient gel system it appears to be 48,000 ± 5%. Some of the bands visualized by CBB stain may be a result of proteolysis, e.g. many of those between 6.1 and 7 and between 7 and hemoglobin seem to be derived from higher molecular weight proteins since they appear in larger amounts with increased incubation time at 37°. The intensity of these bands may therefore be useful in estimating the integrity of the membrane.

At 3.8 μM concentrations both cAMP and 8-N_3cAMP stimulate phosphorylation by [γ-^{32}P]-ATP of the same membrane proteins to approximately the same degree (Fig. 3). The cAMP-stimulated phosphorylation of bands 2.1, 2.4, 3, and 4.7 has been reported before (11). We have detected three other bands exhibiting this behavior, i.e. one immediately above band 4.7 and two more towards the bottom of the gel, with approximate molecular weights of 29,000 and 15,500. An unequivocal assignment of these ^{32}P-labeled bands to Coomassie blue-staining protein bands cannot be made at the present time.

Photolysis of membranes in the presence of relatively high concentrations of [^{32}P]-8-N_3cAMP (0.51 μM or greater) resulted in label being found in bands 2, 2.1, 3, 4.3, 4.5, 4.6 (sometimes split into 2 bands in which case the top one is labeled), 4.7 and 6. This is observable in Fig. 4, slots 1, 3, 5 and 7. The extent of labeling is 4.7 >> 6 >> than all others. Saturation studies of [^{32}P]-8-N_3cAMP binding to the erythrocyte membrane

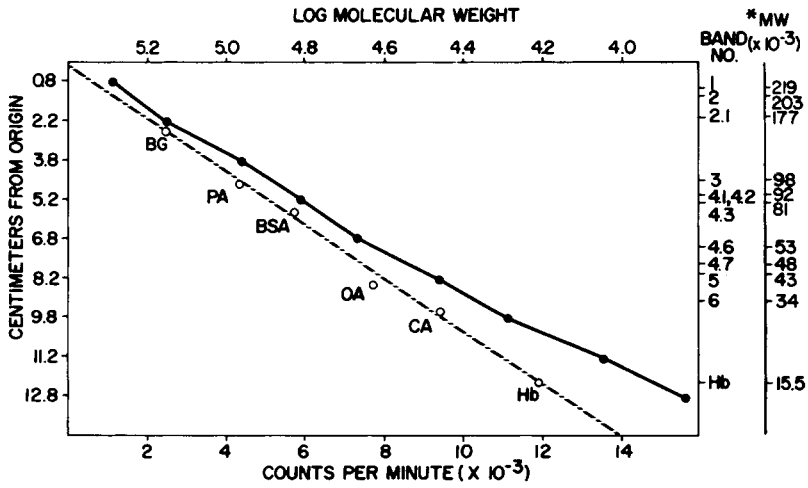

Fig. 1. Linearity of the polyacrylamide gradient (_____). Log of standard protein molecular weight vs their migration distance on a 6 to 12% gel (–•–•–). Standard proteins: β-galactosidase, BG, 130,000; phosphorylase a, PA, 100,000; bovine-serum albumin, BSA, 68,000; ovalbumin, OA, 43,000; carbonic anhydrase, CA, 29,000; hemoglobin (human) Hb, 15,500 (9). Numbers on immediate right correspond to erythrocyte membrane proteins. Vertical displacement indicates position on the gel. *Approximate molecular weights (±5%) as determined from the standard curve.

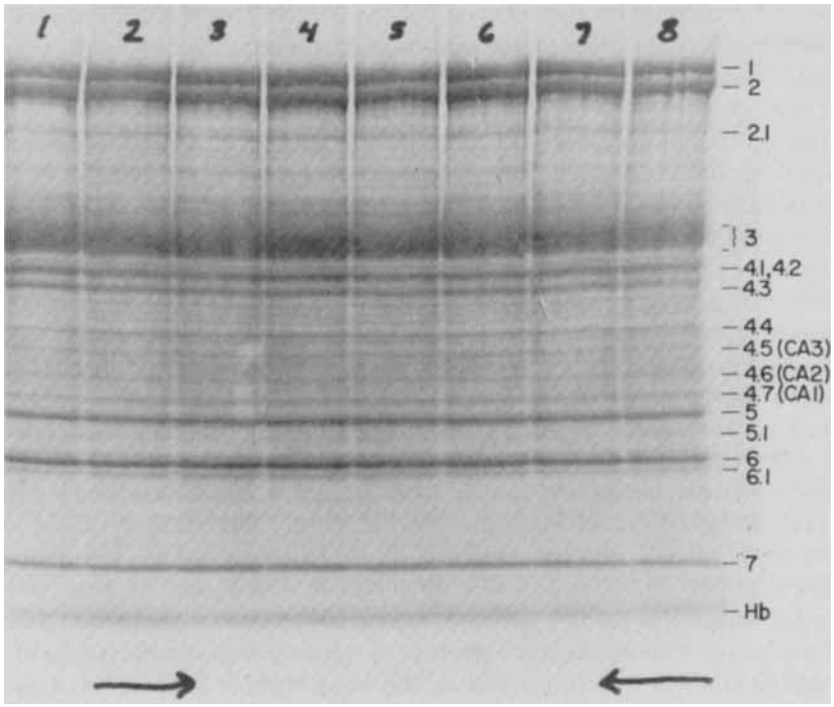

Fig. 2. Coomassie-blue stained gel of erythrocyte membrane proteins indicating band numbering and location of the three high affinity cAMP-binding proteins: CA1, CA2 and CA3.

indicate that bands 3 and 6 start to be labeled at about 0.3 μM which is about four times the concentration (0.075 μM) at which the photolabeling of major cAMP-binding protein (band 4.7) is saturated. At lower concentrations (0.010 to 0.090 μM) only protein in bands 4.5, 4.6 and 4.7 appear to be labeled.

The experiment, as described in the legend of Fig. 4, resulted in several bands being photolabeled which were not phosphorylated by [γ-^{32}P]-ATP. Since there are several of these proteins, we have presently limited our investigation to the proteins photolabeled at low (less than 0.075 μM) [^{32}P]-8-N_3cAMP concentrations.

An earlier report showed that one of the photolabeled proteins coincided with a glycoprotein on a 5.7% polyacrylamide gel (1a). Using a 6 to 12% gel the same results were obtained. Polyacrylamide gel electrophoresis on a 3 to 12% gel, with the protein migration occurring perpendicular to the gel gradient, gave evidence that the photolabel coincided with CBB band 4.7 and a PAS-positive band only at specific acrylamide concentrations (Fig. 5). The slope of the migration curve of the photolabeled protein relative to other protein bands indicates that it is not a glycoprotein. Because of the evidence that the photolabel coincides with CBB bands 4.5, 4.6, 4.7 and a PAS-positive band only at specific acrylamide concentrations, the photolabeled bands will subsequently be referred to as CA3, CA2, CA1, respectively.

Competition studies with cAMP and cGMP indicate that CA1, CA2 and CA3 all bind cAMP very tightly. Cyclic AMP (0.39 μM or less) completely prevents photolabeling of all three proteins by 0.16 μM [^{32}P]-N_3cAMP (Fig. 6). Under the same conditions 0.80 μM cGMP appears to have much less protective effect with all the proteins but appears to

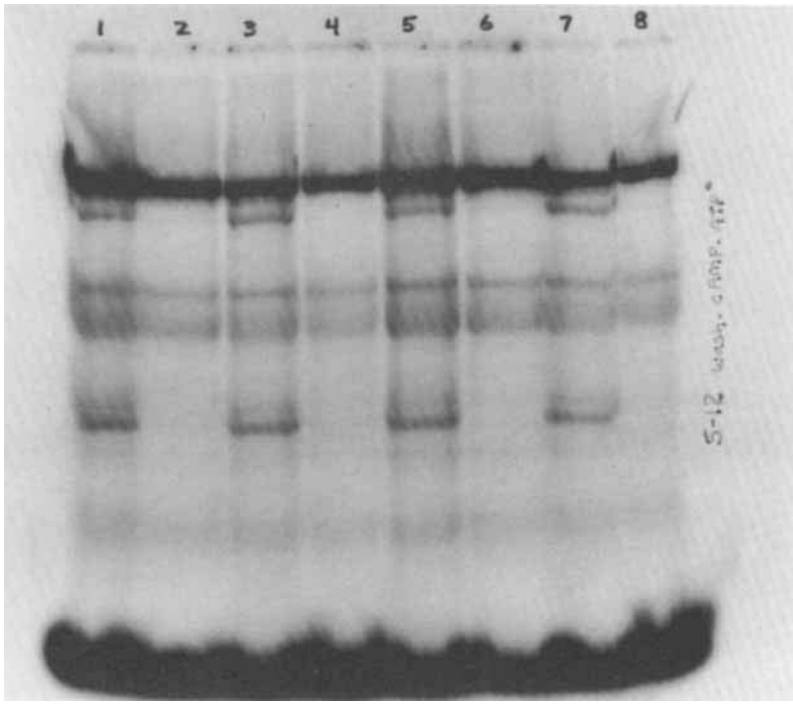

Fig. 3. Autoradiograph of 6% gel showing phosphorylation of erythrocyte membrane components with γ-^{32}P-ATP in the presence of 3.2 μM cAMP (5,7) or, no cyclic nucleotides (2,4,6,8). After phosphorylation membranes were precipitated with ice-cold 6% perchloric acid, washed once with distilled water, then solubilized with PSM. Reaction conditions: 40 μM ATP, 0.2 mM EDTA, 31 mM NaCl, 16 mM KCl, 5.9 mM $MgCl_2$ and 16 mM Tris-HCl buffer, pH 7.4 at 25°C.

protect CA2 the best. The protective effect of cGMP on CA1 is unusual in that it effects an approximate 45 to 50% decrease in photolabeling by 0.16 μM [^{32}P]-8-N_3cAMP at several concentrations between 0.64 and 16.0 μM (Owens and Haley, unpublished results). Therefore, only approximately half of the CA1 sites may be protected by cGMP. Cyclic AMP and cGMP seem to have little effect on photolabeling of the protein in band 6. However, 0.1 μM NADH greatly reduces photolabeling of this protein (a subunit of glyceraldehyde-3-phosphate dehydrogenase [12]) in the presence of 0.44 μM [^{32}P]-8-N_3cAMP.

The divalent metal ions Ca^{2+} and Mg^{2+} at concentrations from 0 to 3.9 mM had no effect on photolabeling of any band. In addition, there was no observable effect of pH in the range of 6.8 to 8.4 on the photolabeling of any band but 6, which shows maximal labeling at about pH 8. Photolabeling of band 6 also increases with increasing ionic strength (Owens and Haley, unpublished results).

A previous paper (1a) demonstrated labeling of two proteins designated as band A (here identified as CA1) and band B (which does not coincide with any stained band but appears between bands 5 and 5.1 as a doublet due to the increased resolution of the gradient gel) and stated that the labeling of band B varied considerably. Several comparisons of radioactivity in band A and band(s) B before and after timed incubations at 37° indicates a reciprocal relationship between the counts lost in band A and those gained in band(s) B (Fig. 7). These results indicate that the B band(s), with approximate molecular

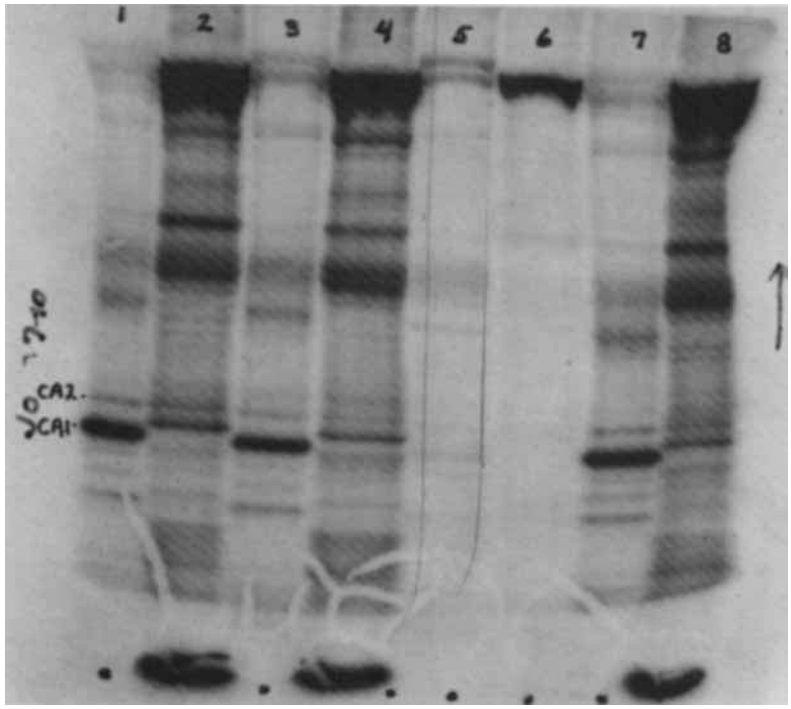

Fig. 4. Erythrocyte membranes photolabeled with 0.78 μM [^{32}P]-8-N_3cAMP (1,3,5,7) or phosphorylated at 25°C with 20 μM γ-^{32}P-ATP in the presence of 0.78 μM 8-N_3cAMP (2,4,6,8). Membranes were washed immediately prior to use with 0.1 mM EDTA, 15 mM NaCl, 1.5 mM Tris-HCl, pH 7.4 (1,2), distilled H_2O (3,4), 0.5% Triton in 0.25 mM EDTA, 40 mM NaCl, 20 mM KCl, 1.25 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4 (5,6; see Fig. 3), or the same solution as in 5 and 6 but without Triton (7,8). Reaction conditions as in Fig. 3.

weights of 41,000 and 39,000, are derived from CA1 when an endogenous protease clips off polypeptides of approximately 9,500 and 7,500 molecular weight. The observed variations in "labeling" of B may therefore be due to variations in times and conditions of incubation. Tryptic digestion of erythrocyte membranes show that CA1 is readily converted to a protein of almost identical molecular weight as band B (Fig. 7).

The question of whether or not cAMP-binding proteins (presumably regulatory subunits of protein kinases) are phosphorylated has been the object of some study (4a; 8; 13a, b,c). In many of our experiments the [^{32}P]-8-N_3cAMP photolabeled protein CA1 and the protein phosphorylated by [γ-^{32}P]-ATP in the presence of cyclic nucleotide migrate identically. Occasionally, however, they can be separated. This does not allow us to state unequivocally whether the major cAMP binding protein is phosphorylated. But, if the cAMP-binding protein and phosphoprotein are the same then phosphorylation appears to cause decreased mobility under certain conditions (Fig. 4). It also appears that ATP may be phosphorylating proteins which migrate identically with CA2 and CA3 (Fig. 4). Whether or not these are the same protein photolabeled with [^{32}P]-N_3cAMP remains to be determined.

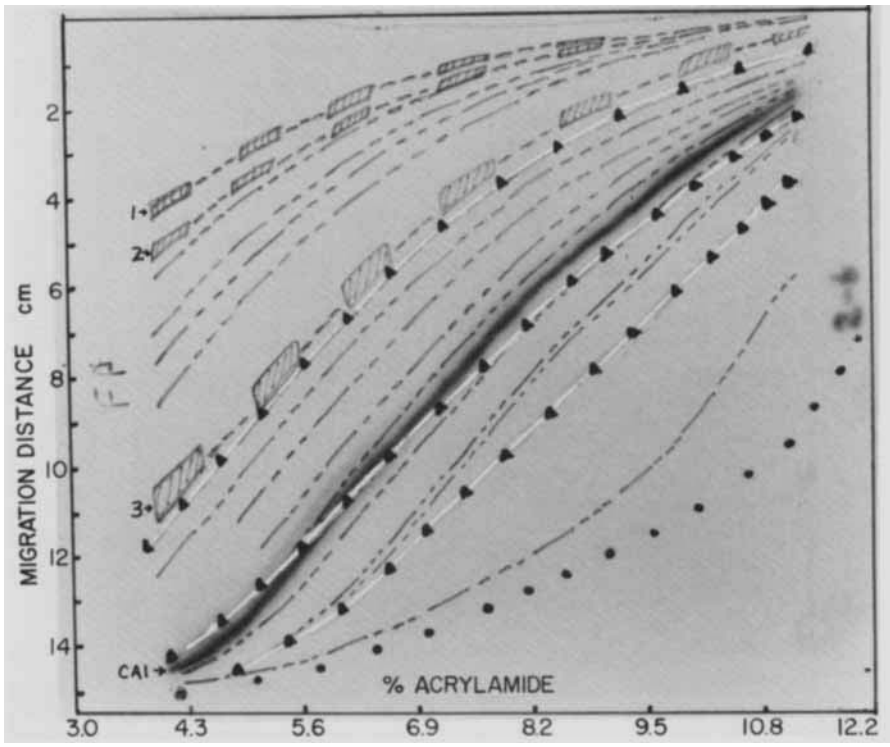

Fig. 5. Autoradiograph showing that the major cAMP-binding protein (CA-1; broad, black, continuous line) does not coincide with CBB-staining proteins (– – – –) or with glycoproteins stained by PAS procedure (white line with triangles) at most acrylamide concentrations. Procedure: SDS-polyacrylamide gel electrophoresis was run perpendicular to the 3 to 12% slab gradient gel. Dots along bottom of autoradiograph indicate final position of tracking dye. The gel was first stained for glycoprotein and the bands marked with ink. Then staining for protein using Coomassie Brilliant Blue was done. Following location of the protein and glycoprotein bands the gel was dried under vacuum and autoradiographed. Using a light box the orientation of the stained bands were located and drawn on the autoradiograph.

DISCUSSION

Our data indicate that 8-N_3cAMP is a good biological substitute for cAMP because it mimics the action of cAMP on stimulating membrane protein phosphorylation by [γ-^{32}P]-ATP. Also, it is a chemotactic agent for Dictyostelium discoideum (29).

However, it is important to realize that every protein photolabeled by [^{32}P]-8-N_3 cAMP is not necessarily a specific cAMP binding protein. Proteins which ordinarily bind cGMP, ATP, NADH, etc., or proteolytic products of these proteins may also be labeled. For example, cAMP does not effectively prevent photolabeling by [^{32}P]-8-N_3cAMP of protein in band 6, identified as a subunit of glyceraldehyde-3-phosphate dehydrogenase (12), but NADH does. This points out that competition studies between 8-N_3cAMP and the respective nucleotides are necessary to give additional confirmation of the nature of the binding sites. (Note: The addition of an azido group in the 8-position probably changes

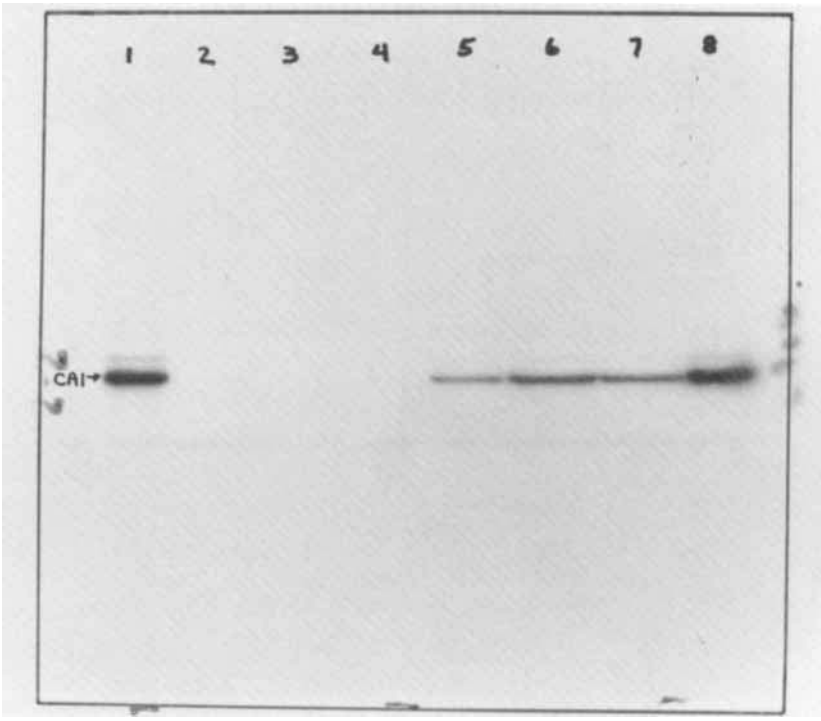

Fig. 6. Autoradiograph indicating effects of cyclic nucleotides on photolabeling with [^{32}P]-8-N_3 cAMP (0.16 μM). Samples 1 and 8 contained only 0.16 μM [^{32}P]-8-N_3cAMP; samples 2,3, and 4 contained, in addition, 0.16, 0.40 and 0.80 μM cAMP, respectively; samples 5,6, and 7 contained 0.80, 1.6, and 2.4 μM cGMP in addition to [^{32}P]-8-N_3cAMP. Reaction conditions as given in Fig. 3.

the equilibrium between the syn and anti forms of the nucleotide. Thus, 8-N_3cAMP, which probably exists predominantly in the syn form, may mimic the adenosine moiety of NADH better than cAMP.)

The observation that cGMP decreases differentially the photo incorporation of [^{32}P]-8-N_3cAMP into proteins CA1, CA2 and CA3 and has a saturating effect at approximately 50% protection of CA1 sites implies unusual interactions of cGMP with these cAMP binding proteins. Whether or not this is of biological importance remains to be determined. A potential photoaffinity analog of cGMP (proposed to be 8-N_3cGMP) has been synthesized and may give added information with regards to cGMP interactions in this model system (28).

At 0.51 μM concentrations [^{32}P]-8-N_3cAMP also labels proteins that are thought to possess ATP binding sites, e.g. 2 and 2.1, and the protein reported to be the anion transport channel (band 3) (14,24,25). This labeling is not greatly decreased by equal concentrations of cAMP. Therefore, these proteins are probably not specific for cAMP. For example, some labeling of the anion transport protein would be expected simply because 8-N_3cAMP is an anion. This may be of physiological importance if some means of cAMP transport in and out of cells is necessary.

Slight membrane proteolysis, not detectable by a change in CBB-staining profile, may also give confusing results when using photoaffinity analogs. Proteolysis of cAMP-binding protein CA1 has been found responsible for the variation in the quantity of

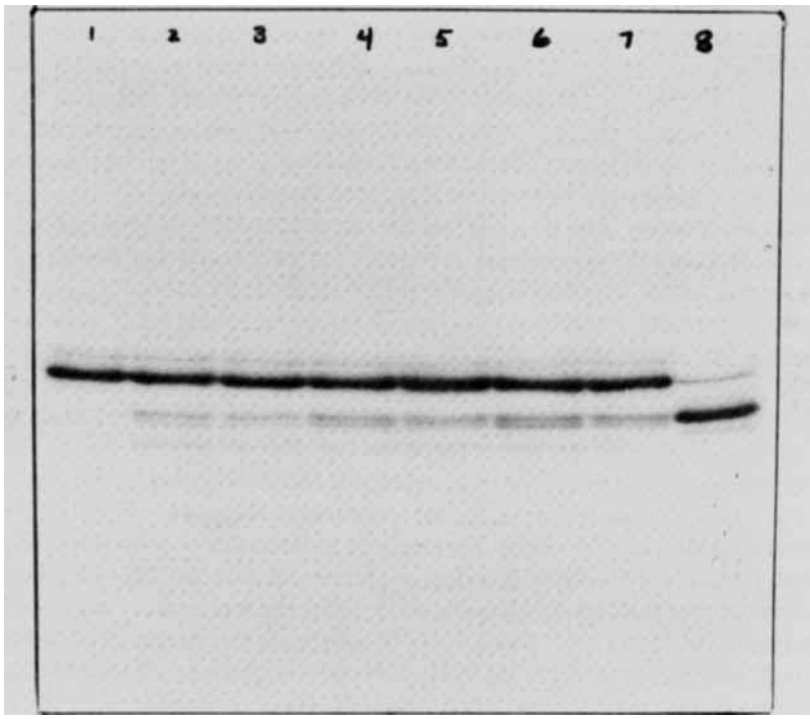

Fig. 7. Autoradiograph indicating limited proteolysis of CA-1 by endogenous protease. Membranes were photolabeled with 0.16 μM [^{32}P]-8-N_3cAMP then incubated at 37°C for 3 (slots 2 and 3), for 6 (slots 4 and 5), or for 12 hr (slots 6 and 7). Sample 1 was dissolved in PSM immediately after photolabeling and boiled for 5 min. Sample 8 was photolabeled then incubated with 0.5 μg ml^{-1} trypsin for 2 min at 37°C.

radioactivity appearing in proteins migrating between bands 5.0 and 5.1. This may represent another regulatory mechanism for protein kinases in which the altered cAMP-binding subunit may inhibit or modulate kinase or other enzyme activity. Whether the protease responsible for degradation of CA1 is the same as the neutral, membrane-bound protease reported by Morrison and Neurath (15) is unknown. A contaminating protease from leucocytes is unlikely because of the method of membrane preparation so the protease is probably indigenous to the membrane. Studies are currently under way to determine the specificity and conditions for maximal activity of this protease since it may be possible that nucleotide binding or phosphorylation affects the availability of some membrane proteins for degradation by endogenous proteases.

Erythrocyte membranes show a remarkable degree of phosphorylation by [γ-^{32}P]-ATP, especially with the addition of cAMP. The demonstration of two (possibly three) cAMP-binding proteins of high affinity in combination with various competition experiments, support the hypothesis that there is more than one specific cAMP-binding protein in the erythrocyte membrane. This, with the observed cAMP-stimulated phosphorylation of several proteins (rather than only one) indicates the presence of a protein kinase with different regulatory subunits or several different protein kinases.

It is known that phosphorylation of certain membrane proteins affects transport of glucose in fat cells (16) and Ca^{++} in cardiac microsomes (17a,b), permeability (18), and

perhaps adenylate cyclase activity (19), all of which have some significance in mature erythrocytes. (Adenylate cyclase has been recently reported to occur in human erythrocytes [20a,b]). Also, it has been proposed that a correlation between various forms of muscular dystrophy and altered erythrocyte membrane phosphorylation exists (21a,b). (The question of whether this correlates with altered cAMP binding characteristics obviously needs to be explored.) Due to these facts alone it would not be surprising to find several phosphoproteins, and protein kinases, in the membrane.

The absence of any effects by Mg^{++} or Ca^{++} on 8-N_3cAMP and, presumably, cAMP binding, complements the experiments of Williams (11), Fairbanks and Avruch (22), and of Guthrow et al. (23a), who have shown that Ca^{++} abolishes phosphorylation of bands 2 and 3 and inhibits cAMP-stimulated phosphorylation of 2.1, 3 and 4.5 It would seem, then, that the inhibitory activity of Ca^{++} is due to interference with ATP utilization, not with cAMP binding. It may be of considerable importance to note that both cAMP and 8-N_3cAMP stimulate the phosphorylation of proteins in bands 2.0 and 2.1 which are thought to be Ca^{++}-stimulated ATPases (26, 27).

Our present data do not allow us to determine the identity and function of the 29,000 and 15,500 (approximate) molecular weight units whose phosphorylation is stimulated by cAMP and 8-N_3cAMP. They may be artifacts due to proteolytic breakdown products of other phosphorylated proteins, or phospholipids rather than proteins. It should be noted that the data of Guthrow et al. (23a) show phosphorylation in this region that is attributed to incorporation of ^{32}P into lipids, i.e. di- and tri-phosphoinositides, phosphatidic acid and an unidentified lipid. They did not observe cAMP-stimulated phosphorylation. However, a report of cAMP-stimulated phosphorylation of the polyphosphoinositides in a canine kidney membrane fraction has been reported (26). That these two bands may be membrane proteins with definite function cannot be ruled out, since phosphorylation of two membrane proteins of approximate molecular weights 22,000 and 16,000 are thought to be involved in regulation of insulin-stimulated glucose transport in fat cells (16).

The evidence for the existence of a cAMP-dependent protein kinase in erythrocyte membranes (23a,b,c; 13a) and the apparent molecular weight of the major cAMP-binding protein (48,000 ± 5%) suggests that this protein may be the regulatory subunit of a cAMP-dependent protein kinase. Greengard's laboratory has shown that a cAMP-binding protein of approximate molecular weight 49,000 occurs in a large variety of tissues and that its phosphorylation by [γ-^{32}P]-ATP is stimulated by cAMP (4a,b). It has been shown that the cAMP-binding subunits of some protein kinases are also phosphorylated (27a; 13b). Guthrow et al. (27b) suggested that since a soluble cAMP-dependent protein kinase from heart muscle catalyzes the phosphorylation of its own regulatory subunit, this may also be the case in erythrocyte membranes. But is has been shown that the membrane puts certain restraints on availability of substrate; once the erythrocyte protein kinase is freed from the membrane its action may become quite indiscriminate (22).

According to Rubin (13a), the cAMP binding activity in human erythrocyte membranes can be completely separated from the phosphoprotein in question. The electrophoretic separation of the highest affinity cAMP-binding protein, CA1, and a phosphorylated protein of similar mobility (Fig. 4) can be interpreted in two ways, i.e. the proteins are identical and phosphorylation causes a decrease in mobility, or they are different and the cAMP-binding protein is not phosphorylated. Decreased mobility of phosphorylated

relative to non-phosphorylated protein has been reported (13b). Our data do not unequivocally support either idea. However, the facts that CBB stain and ^{32}P label coincide throughout the gel, and that CBB band positions are unchanged by [^{32}P]-8-N_3cAMP photolabeling or phosphorylation with [γ-^{32}P]-ATP indicate that the cAMP-binding protein and phosphoprotein are different. The protein which is phosphorylated is not visible after staining with CBB either because of insufficient quantity or because of the presence of attached carbohydrate. There is a possibility that an erythrocyte protein kinase, whose regulatory subunits behave as noted above, may constitute a third type, distinct from those represented by the protein kinases from beef heart and from rabbit skeletal muscle (13b). Two distinct types of regulatory subunits, one binding cAMP and one being phosphorylated, could characterize this protein kinase. This may be represented as $R_1R_2(C)_2$ where only R_1 binds cAMP and only R_2 may be phosphorylated.

The experiments in this paper demonstrate the utility of 8-N_3cAMP as a chemical probe for cAMP sites. 8-N_3cAMP biochemically substitutes for cAMP and, on photolysis, forms a covalent bond with proteins that bind it. There are some pitfalls in using this approach to identify cAMP binding proteins. Various levels of non-specificity for cAMP sites, and proteolysis of labeled proteins are two of these. However, carefully correlated experimentation can eliminate most of the problems and the use of 8-N_3cAMP will give added insight into the biological phenomena involving cAMP-macromolecular interactions.

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