Journal of Supramolecular Structure 9:57–68 (1978)
Transmembrane Signaling 149–160

# Use of Photoaffinity Nucleotide Analogs to Determine the Mechanism of ATP Regulation of a Membrane-Bound, cAMP-Activated Protein Kinase

James R. Owens and Boyd E. Haley

*Division of Biochemisty, University of Wyoming, Laramie, Wyoming 82070*

Using a radioactively tagged, photoaffinity analog of cAMP, 8-azidoadenosine-3′,5′-cyclic monophosphate (8-$N_3$cAMP), and [$\gamma^{32}$P]ATP, the membrane-binding properties of both the regulatory and catalytic subunits of the cAMP-activated protein kinase of human erythrocyte membranes were investigated. [$^{32}$P]8-$N_3$cAMP was used to locate and quantify regulatory subunits. Increased phosphorylation of specific membrane proteins by [$\gamma^{32}$P]ATP was used to determine the presence of the catalytic subunit. The data support a mechanism which operates through a tight membrane-bound regulatory subunit and a catalytic subunit that is released from the membrane when cAMP is present and the Mg•ATP concentration is below approximately 10 $\mu$M. The catalytic subunit is not required for the Mg•ATP inhibition of 8-$N_3$cAMP binding. Experiments with a photoaffinity analog of ATP, 8-azidoadenosine triphosphate (8-$N_3$ATP), support the hypothesis that ATP hydrolysis and phosphorylation are not involved in the regulation. The data indicate that the regulatory subunit contains an ATP regulatory site which inhibits 8-$N_3$cAMP binding and the release of the catalytic subunit. These results indicate that the membrane-bound type I enzyme (type IM) differs significantly from the soluble (type IS) enzyme studied in other tissues. These enzymes are compartmentalized by being in different cellular locations and are regulated differently by Mg•ATP.

**Key words: protein kinase, cAMP, ATP, phosphorylation, membranes, photoaffinity**

Recent reports have supported the concept that cAMP-activated protein kinases may be divided into two major types [1–3]. In general, type II does not bind Mg•ATP tightly but does undergo autophosphorylation of the regulatory subunit which affects cAMP-stimulated dissociation of the subunits [4–7]. Type I binds Mg•ATP tightly and does not undergo autophosphorylation [2, 8, 9]. Previous reports have shown the utility of [$^{32}$P]8-$N_3$cAMP as a probe for cAMP binding proteins such as the regulatory subunit of protein kinases (10–12, 14, 15). Using erythrocyte membranes as a model system, [$^{32}$P]8-$N_3$cAMP as a photoprobe for the regulatory subunit, and increased phosphorylation of specific membrane protein by [$\gamma^{32}$P]ATP as a measure of the presence of an active catalytic subunit, we have

Received April 18, 1978; accepted July 3, 1978.

0091-7419/78/0901-0057$02.30 

studied the mechanism of cAMP and ATP regulation of this membrane–enzyme system. The data indicate that the major, membrane-bound, cAMP-activated protein kinase of human erythrocytes is an example of type I protein kinase and that Mg·ATP as well as cAMP regulate its activity. Using [$\beta,\gamma^{32}$P] 8-$N_3$ ATP, which mimics the regulatory action of ATP with this enzyme, a site on the regulatory subunit was photolabeled at 8-$N_3$ ATP concentrations which caused rapid dissociation of 8-$N_3$ cAMP. Brief accounts of this work have been previously presented [18,19].

## METHODS AND MATERIALS

[$^{32}$P] 8-$N_3$ cAMP and [$\beta,\gamma^{32}$P] 8-$N_3$ ATP were synthesized by a previously reported procedure [11, 16]. [$\gamma^{32}$P] ATP was purchased from ICN. All other nucleotides were purchased from Sigma Chemical Company except adenylyl imido-diphosphate (AMP·PNP) which was generously supplied by Dr. R. Yount. Preparation of human erythrocyte membranes, polyacrylamide gel electrophoresis, and autoradiography were done as described earlier [12].

Removal of the regulatory and catalytic subunits was attempted by washing an amount of membranes containing 700 μg protein two times at 0° with 5 ml of a solution containing 80 mM NaCl, 50 mM KCl, 0.5 mM ethylenediamine tetraacetate (EDTA), 2.5 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.4, ± cAMP or 8-$N_3$ cAMP. Washing involved vortexing the membranes for 1 minute in the above solution followed by centrifugation (for 10 minutes at 25,000g) and then aspirating off the supernatant. The presence of an active catalytic subunit was determined by increased $^{32}$P incorporation into membrane proteins known to be endogenous substrates for the cAMP-activated protein kinase. Membrane protein phosphorylations were done in 0.2 ml of wash solution containing 50 μM [$\gamma^{32}$P] ATP ± cAMP at 4°. The presence of regulatory subunit in the membrane preparations was determined by photolysis of membranes with [$^{32}$P] 8-$N_3$ cAMP present. To check for the regulatory subunit in the wash solutions, the initial eluate was mixed with [$^{32}$P] 8-$N_3$ cAMP in a quartz cuvette, photolyzed for 5 minutes at 2 cm distance, mixed with 700 μg membrane protein (to assure a large enough pellet on PCA precipitation), and the protein precipitated by addition of 5 ml of 6% PCA (perchloric acid) at 0°C. $^{32}$P incorporation was determined by liquid scintillation counting in Beckman Ready-Solv Solution VI.

## RESULTS

The autoradiograph of Figure 1 shows that the catalytic subunit may be removed from the membrane by washing with cAMP present. In this experiment membranes were divided into three portions. One portion was not washed, one was washed without cAMP, and one was washed with 1 μM cAMP present. These portions were then split and incubated with 50 μM [$\gamma^{32}$P] ATP ± 1 μM cAMP. The proteins of unwashed membrane, separated in slots 1 and 2, indicate that cAMP stimulates phosphorylation of proteins near Coomassie stained bands 2.1, 2.4, 3, 4.6, 4.7, and two more towards the bottom of the gel (approximate molecualr weights of 29,000 and 15,500) as previously reported for this gel system [12]. Membranes washed without cAMP present, slots 3 and 4, gave results identical with the unwashed membranes. However, membranes washed with cAMP present, slots 5 and 6, show that the cAMP-stimulated phosphorylation is completely absent. Similar results were obtained if membranes were washed with 8-$N_3$ cAMP.

The data shown in the autoradiograph in Figure 2 indicate that the regulatory subunit,

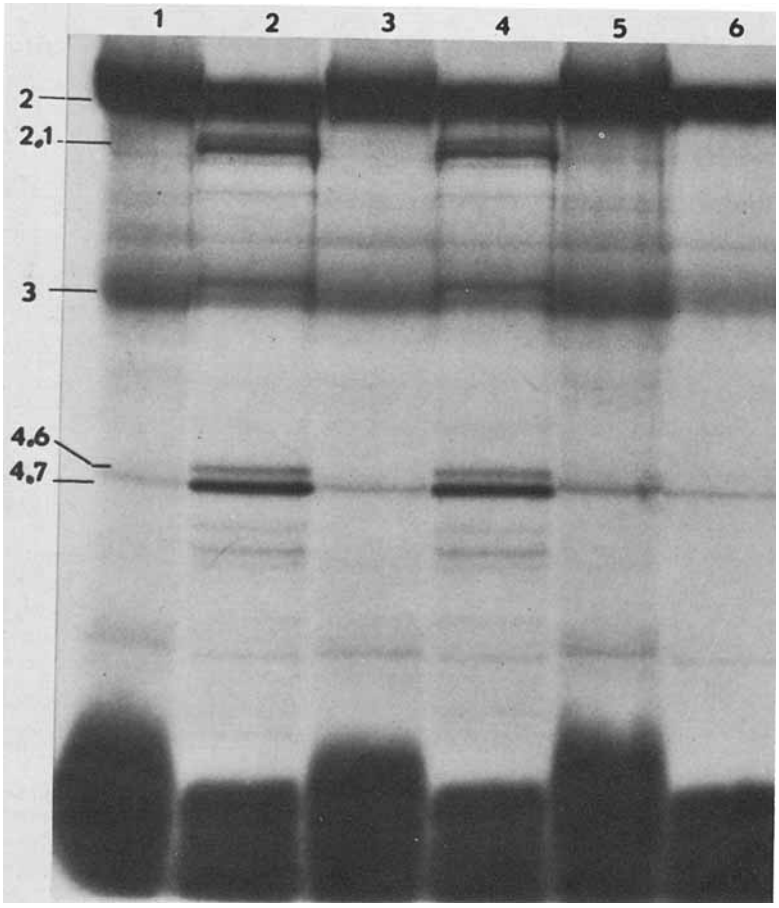


Fig. 1. Autoradiograph of a SDS polyacrylamide slab gel on which membrane proteins were separated. Prior to electrophoresis membrane proteins were phosphorylated by [$\gamma^{32}$P] ATP with (slots 2,4, and 6) and without (slots 1, 3, and 5) 1 $\mu$M cAMP present. Slots 1 and 2 contain unwashed membranes, slots 3 and 4 contain membranes washed without cAMP present, and slots 5 and 6 are membranes washed with 1 $\mu$M cAMP present. Numbers to the left of the figure represent the Coomassie blue stained protein bands which coincide with $^{32}$P incorporation (see text).

labeled CA-1, remained tightly membrane-bound before and after binding 8-$N_3$cAMP. The radioactivity incorporated into CA-1 of slots 3 and 5 (85 and 92 cpm, respectively) was approximately 2% of the radioactivity that was found in equivalent portions of washed membranes (slots 2 and 4 with 4,560 and 4,356 cpm, respectively). The difference in these two sets of values represents the amount of regulatory subunit removed by washing.

The data seem to indicate that washing with cAMP plus ATP did not significantly release the regulatory unit, either, since the CA-1 band of slot 7 contained less than 5% of the counts observed in the CA-1 band of slot 6. However, it is probable that CA-1 may be released from the membrane by Mg·ATP, but that the released form, with Mg·ATP bound, will not photoincorporate [$^{32}$P] 8-$N_3$cAMP to any significant extent [13]. Comparison of the level of radioactivity in Ca-1 found in slots 1, 2, and 4 versus 6 indicated that ATP by some manner prevented photolabeling by 8-$N_3$cAMP. Washing with [$^{32}$P] 8-$N_3$cAMP plus

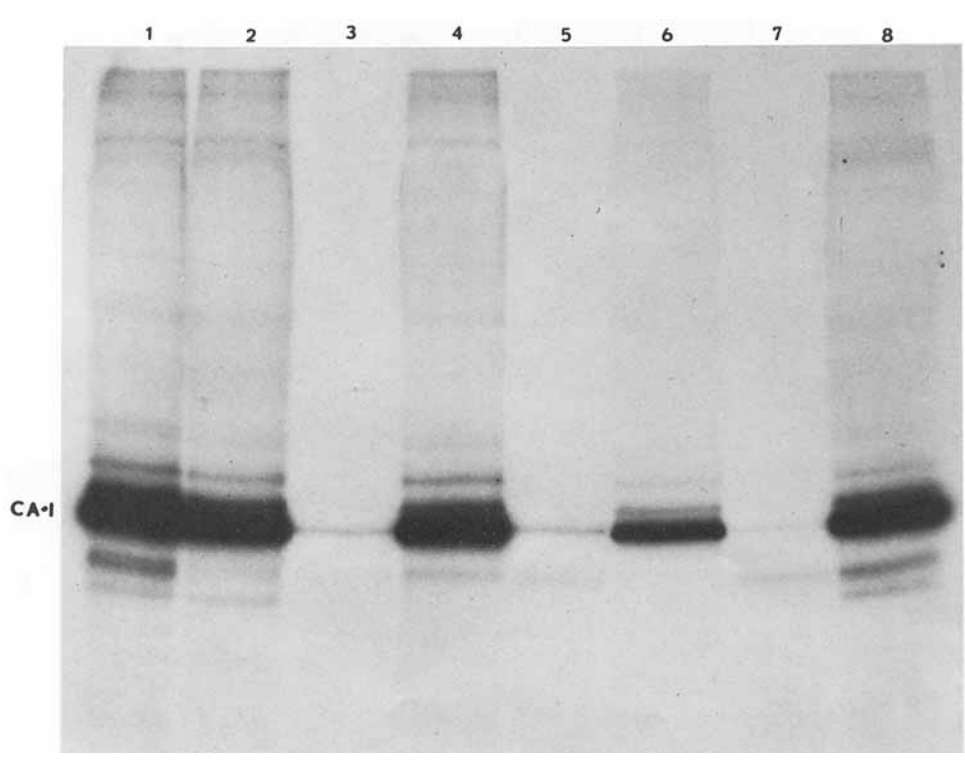


Fig. 2. Autoradiograph of a SDS-gradient polyacrylamide gel on which membrane proteins photolabeled with [$^{32}$P] 8-$N_3$cAMP have been separated. Slot 1 contains unwashed membranes which were incubated 5 minutes with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP, then photolyzed. Slot 2 contains membranes washed first, then incubated with [$^{32}$P] 8-$N_3$cAMP and photolyzed. Slot 4 contains membranes washed with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP present, then photolyzed. Slot 6 contains membranes washed with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP and 0.4 mM Mg·ATP present, and then photolyzed. Slot 8 contains unwashed membranes preincubated 5 minutes with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP at 4°, then incubated for 30 seconds with 0.4 mM Mg·ATP, photolyzed, and then washed. Slots 3, 5, and 7 are the first-wash eluate of membranes in slots 2, 4 and 6, respectively, which were mixed with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP and photolyzed as given in the text. The CA-1 bands contained 4,986; 4,560; 85; 4,357;92;1,220;75; and 4,206 cpm in slots 1–8, respectively.

0.4 mM ATP, both nucleotides being added simultaneously (slot 6), reduced the photoincorporation into band CA-1 by approximately 75%. Addition of ATP and immediate photolysis, after incubation with [$^{32}$P] 8-$N_3$cAMP (slot 8), did not decrease photoincorporation nearly as much (< 30%). Other experiments, not described here, indicate that Mg·ATP decreases the tight, but reversible binding of [$^{32}$P] 8-$N_3$cAMP to these membranes. Therefore, the decrease in photoincorporation is due to a decrease in binding affinity for the cyclic nucleotide and not to a decrease in the efficiency of the photolabeling process.

Figure 3 shows the concentration effects of Mg·ATP on the photoincorporation of [$^{32}$P] 8-$N_3$cAMP into CA-1. Approximately 75 $\mu$M Mg·ATP gave a maximum decrease in photoincorporation of about 72–84% depending upon the membrane preparation. Fifty percent of the maximum decrease in photoincorporation was obtained by approximately 5–10 $\mu$M Mg·ATP. The actual concentration is difficult to determine since ATP is being hydrolyzed by other membrane enzymes, eg the $Mg^{2+}$·ATPase. Also, experiments we have

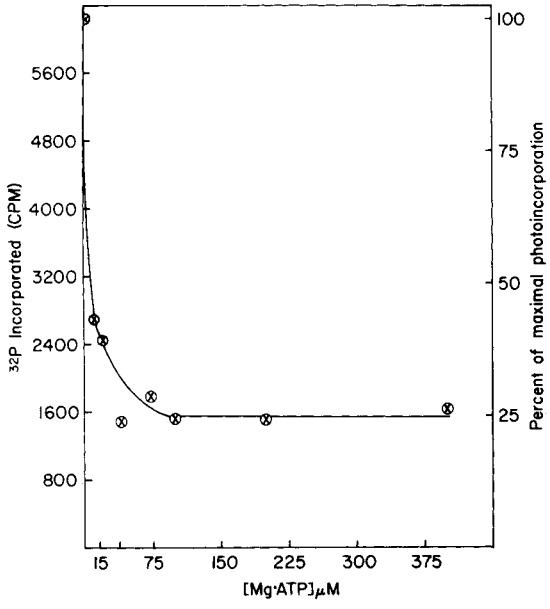


Fig. 3. [$^{32}$P] 8-$N_3$cAMP (1 μM) was preincubated with membranes for 5 minutes at 4° prior to the addition of Mg·ATP. After 10 minutes of incubation with Mg·ATP the membrane solutions were photolyzed and the proteins separated on SDS polyacrylamide gels. The CA-1 band was sliced out and counted by liquid scintillation.

performed indicate that the effects of Mg·ATP are very dependent on ionic strength [13].

The unique cyclic nucleotide binding properties of CA-1 led to development of an experimental technique we call "cold trap." In effect, whichever cyclic nucleotide, 8-$N_3$cAMP or cAMP, first occupies the regulatory site may be trapped into that site by lowering the temperature to below 4°. For example, at 37° there is good exchange between cAMP and 8-$N_3$cAMP on the regulatory protein. However, at 4° the off rate of the occupying cyclic nucleotide is so slow that it is barely detectable after 1 hour incubation and this off rate is not noticeably enhanced by additions of large amounts of the other cyclic nucleotide, including cAMP.

Figure 4 shows the effect of adding ATP, Mg·ATP, and cAMP before and after incubation of the membranes with [$^{32}$P] 8-$N_3$cAMP at 4°. The results show that 8 μM cAMP is a much better protector against photolabeling than 200 μM Mg·ATP when the protector is added before the 8-$N_3$cAMP. However, if 8-$N_3$cAMP is allowed to occupy the cyclic nucleotide site first and the temperature is below 4°, then Mg·ATP decreases photoincorporation more effectively than cAMP at the concentrations given. For example, after 20 minutes of preincubation with [$^{32}$P] 8-$N_3$cAMP, the results show that additions of 0.5 mM ATP (without $Mg^{2+}$) had no effect and 8.0 μM cAMP only slightly decreases the binding (9–10% after 15 minutes), whereas Mg·ATP gave a maximum inhibition of about 80% within 5 minutes. Neither $Mg^{2+}$ or $Ca^{2+}$ added alone had any effect on [$^{32}$P] 8-$N_3$cAMP photoincorporation [12].

The object of the experiment resulting in the autoradiogram of Figure 5 was to determine if the catalytic subunit was required for the Mg·ATP inhibition of photoincorporation of [$^{32}$P] 8-$N_3$cAMP into CA-1, the membrane-bound regulatory subunit. With

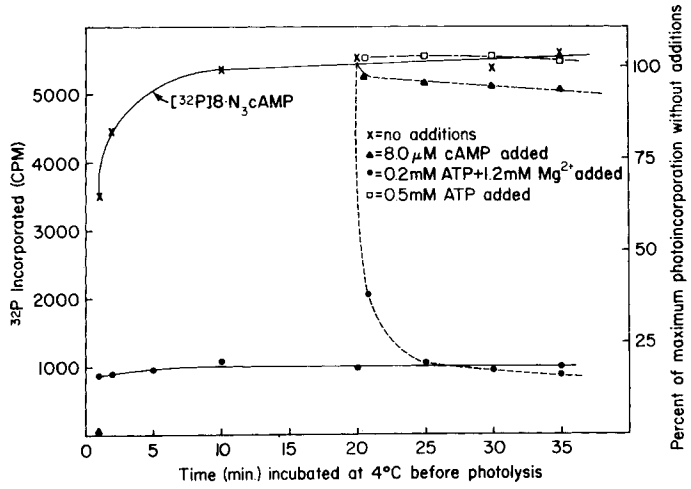


Fig. 4. Membranes were incubated at 4° for various times with 79 nM [$^{32}$P] 8-$N_3$cAMP then photolyzed (—x—x—) or incubated for 20 minutes, other nucleotides added as indicated, and the solution photolyzed after ½, 5, 10 and 15 minutes of incubation. The bottom solid line (—•—) shows the effect of adding 0.2 mM Mg·ATP before adding [$^{32}$P] 8-$N_3$cAMP. The solid triangle in the lower left corner represents the effect of adding 8 μM cAMP before adding [$^{32}$P] 8-$N_3$cAMP.

regard to the activity of the catalytic subunit, the membrane proteins of slot 5 (unwashed) show a phosphorylation profile which is consistent with that observed when the catalytic subunit is active. However, slot 6 (washed with 8-$N_3$cAMP present) shows endogenous phosphorylation consistent with that observed when the catalytic subunit is not active (ie the catalytic subunit is restrained or, as is the case here, it has been removed from the system). Slot 7 contained membrane proteins treated identically to those in slot 6 with the exception that a portion of the first-wash eluate was added back to the membranes (ie, the catalytic subunit was washed away and then replaced). The phosphorylation profile of slot 7 is similar to that observed when cAMP is added to unwashed membranes. This proves that the catalytic subunit is in the wash solution and may be added back giving a cAMP-like stimulated phosphorylation.

[$^{32}$P] 8-$N_3$cAMP was photoincorporated into the CA-1 protein of slots 1, 2, and 8 to level of 1,035 cpm ± 5%, again indicating that washing did not remove the cAMP-binding protein. The photoincorporation of radioactivity in CA-1 protein of slot 3 (unwashed, Mg·ATP added) and slot 4 (washed with 8-$N_3$cAMP present, Mg·ATP added) was 266 and and 203 cpm. This indicates that the Mg·ATP-induced decrease in photoincorporation of [$^{32}$P] 8-$N_3$cAMP did not require one-to-one regulatory subunit binding with the catalytic subunit, since the catalytic subunit was present in membranes of slot 3 but enzymatically undetectable in those of slot 4.

The data in Figure 6 show the effect of the presence of catalytic subunit on the concentration of Mg·ATP required to given maximum dissociation of [$^{32}$P] 8-$N_3$cAMP from the regulatory site. These data indicate that while catalytic subunit is not absolutely required, it greatly decreases the concentration of Mg·ATP required for equal decrease in [$^{32}$P] 8-$N_3$cAMP photoincorporation.

Figure 7 shows the effect of various nucleotide triphosphates (NTPs) on inhibition of [$^{32}$P] 8-$N_3$cAMP photoincorporation. Neither Mg·AMP·PNP nor Mg·GTP was very

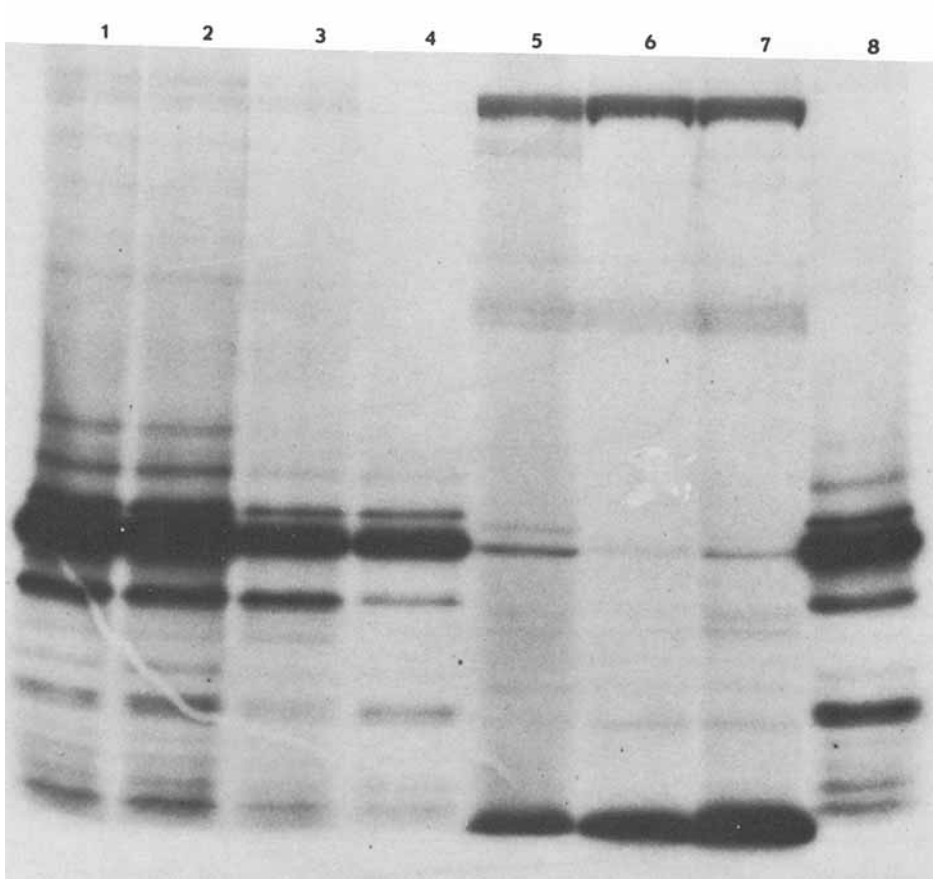


Fig. 5. Autoradiograph of a SDS gradient polyacrylamide gel on which membrane proteins were separated. Membranes used were either unwashed after preparation (slots 1, 2, 3, and 5), washed without [$^{32}$P] 8-$N_3$cAMP present (slot 8), or washed with 1 $\mu$M [$^{32}$P] 8-$N_3$cAMP present (slots 4, 6, and 7). The membrane solutions were then either photolyzed to incorporate [$^{32}$P] 8-$N_3$cAMP (slots 1, 2, 3, 4, and 8 with 3 and 4 having 0.4 mM Mg·ATP added 5 minutes before photolysis) or mixed with [$\gamma^{32}$P] ATP and cAMP to determine the level of activity of the cAMP activated protein kinase (slots 5, 6, and 7).

effective. Mg·8-$N_3$ATP was very similar to Mg·ATP in decreasing photoincorporation. Using [$\beta, \gamma^{32}$P] 8-$N_3$ATP, we have completed experiments showing that it is not a substrate for the cAMP-activated protein kinases of human erythrocyte membranes but that it is photoincorporated into regulatory subunits, at concentrations which effect inhibition of 8-$N_3$cAMP photoincorporation. Other experiments with $Ca^{2+}$·ATP and $Ca^{2+}$·GTP indicated that neither affected photoincorporation to a significant degree.

## DISCUSSION

Other researchers have reported the inhibition of soluble cAMP-activated protein kinases by Mg·ATP and the fact that Mg·ATP promotes the dissociation of [$^3$H] cAMP from its binding site on the regulatory subunit [2, 8, 9]. These reports raise questions about the location of the Mg·ATP binding site which causes the dissociation; ie is it on the

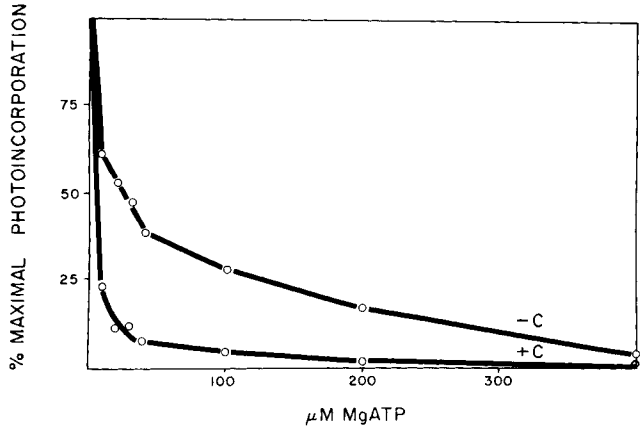


Fig. 6. Two portions of membranes were incubated at 4° for 10 minutes with 1 μM [$^{32}$P] 8-$N_3$cAMP in a solution of 80 mM NaCl, 50 mM KCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$ and 20 mM Tris·HCl, pH 7.4. One portion was washed two times with this solution to remove the catalytic subunit. Aliquots of the washed and unwashed membranes were then incubated for 5 minutes with the indicated concentrations of Mg·ATP and photolyzed as given in the text. Membrane proteins were separated on SDS·gradient polyacrylamide gels and the major photolabeled band (CA-1) was sliced out and counted by liquid scintillation.

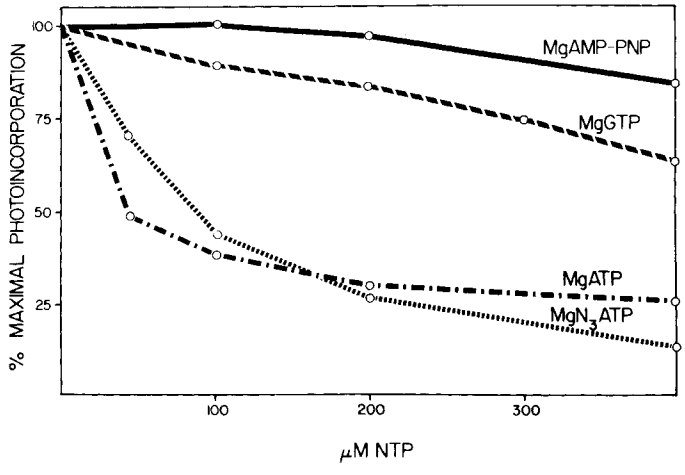


Fig. 7. Membranes were incubated at 4° for 10 minutes with 79 μM [$^{32}$P] 8-$N_3$cAMP with subsequent addition of other nucleotide triphosphates at the concentrations indicated. After 10 minutes of incubation with the nucleotide triphosphates, the solutions were photolyzed as given in the text. Membranes proteins were separated on SDS·gradient polyacrylamide gels and the major photolabeled band (CA-1) was sliced out and counted by liquid scintillation.

catalytic or regulatory subunit, or both, or is it the same site as the catalytic Mg·ATP site involved in protein phosphorylation? Also, does the catalytic subunit affect the dissociation of cAMP? Is catalytic subunit presence absolutely required for Mg·ATP to promote dissociation?

A report on soluble, type I protein kinases indicates that catalytic subunit is required for Mg·ATP to cause dissociation of cAMP [24]. Research described herein shows that the catalytic subunit of the membrane-bound enzyme is not absolutely required although its presence does appear to decrease the concentration of Mg·ATP required for equivalent cAMP dissociation. We believe that this discrepancy may be explained by ionic-strength effects or by the existence of two forms of type I cAMP protein kinases. These may be defined as type I soluble (type IS; these may be isolated from cells or tissues without the use of detergents) and type I membrane-bound (type IM; these require detergents to be removed from membranes or cellular material). Human erythrocyte membranes as isolated in this report contain primarily type IM. We have observed only type IS in pigeon heart tissue but two forms of type II cAMP protein kinases, ie type IIS and type IIM [13].

Very little is known about the quaternary structure or mode of action of membrane-bound, cAMP-activated protein kinases. For example, they may not exist in the $R_2C_2$ form as do both soluble forms of type I and type II, but they may exist in an RC form as reported for a cAMP-activated kinase purified from the particulate fraction of bovine cerebral cortex [21]. We have no data to support the ratio of R to C in the type IM enzyme and will depict it as RC since this is the simplest possible form. What this report does show is that the regulatory protein (R = CA-1) is attached to the membrane by strong, presumably hydrophobic, forces and may be removed only by detergent action as reported by others [13, 20]. Attachment of the catalytic subunit (C) is through R, and it is freely released by the binding action of cAMP or 8-$N_3$cAMP. Figure 8 shows four plausible mechanisms for cAMP activation of type IM protein kinase. Mechanisms 1 and 2 propose that C is either bound to R or to $P_S$ (membrane protein substrate) and that the specificity of $P_S$ phosphorylation is controlled by proximity to C since C is never free to migrate off the membrane. Data in Figures 1 and 5 show that this is not the case. Mechanisms 3 and 4 propose that cAMP causes the free release of C and that the specificity of protein phosphorylation is dependent on C recognition of $P_S$. Mechanism 3 also proposes that R would be freely released on binding cAMP, which Figure 2 shows is not the case. Data presented in Figures 1, 2, and 5 indicate that type IM, R, and C subunits are controlled as indicated in mechanism 4. Using a different experimental approach and rabbit heart homogenates, Corbin et al [23] have reported a similar mechanism.

Our data indicate that cAMP (or 8-$N_3$cAMP) is bound by type I regulatory subunit at temperatures below 4° in such a way that it is not readily accessible to exchange with a 100-fold excess of subsequently added cAMP or 8-$N_3$cAMP. This is supported by the very slow off rate for bound cAMP observed by many other laboratories and by the high percentage of reversibly bound [$^{32}$P] 8-$N_3$cAMP which is covalently incorporated on photolysis with one short (2–5 minutes) ultraviolet exposure. We routinely observe photoincorporation into about 85% of the total membrane binding sites. Others have reported the use of [$^{32}$P] 8-$N_3$cAMP to quantitate cAMP receptor proteins in the cytosol of various tissues [17]. However, with similar systems other nucleotide photoaffinity analogs used in our laboratory, eg 8-$N_3$ATP, 8-$N_3$GTP, and the 8-azidoadenosine derivative of 3′-dephopho CoA, photoincorporate at best into only 10% of the total binding sites. This indicates that 8-$N_3$cAMP is being bound such that the nitrene formed by photolysis does not have solvent or solute molecules in close proximity. Thus, because of the slow off rate, a

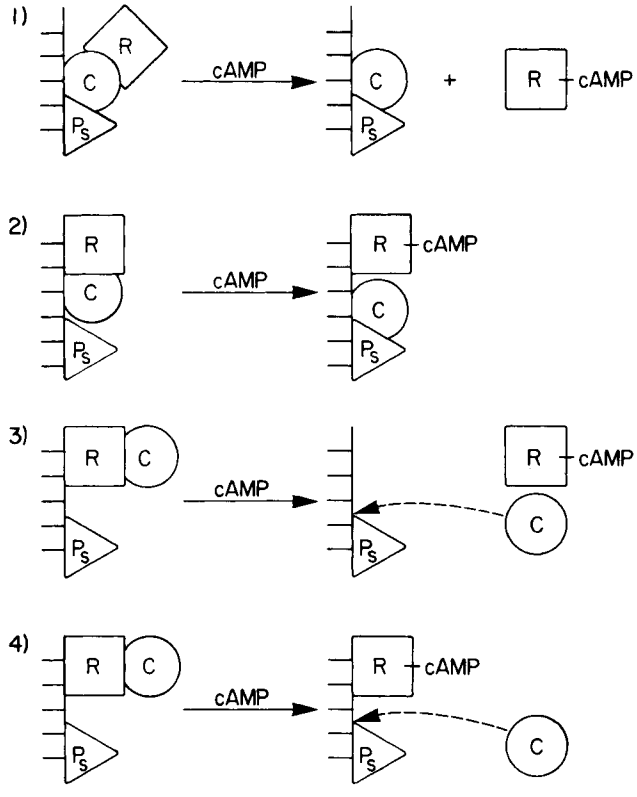


Fig. 8. Mechanisms describing the possible binding properties of Type IM catalytic (C) and regulatory (R) subunits to their respective biological membranes and protein substrates ($P_S$) after addition of cAMP.

highly efficient reaction takes place between the nitrene and an amino acid residues in the binding site. This is also supported by our observation that complete proteolysis of CA-1 photolabeled with [$^{32}$P] 8-$N_3$cAMP gives over 90% of the $^{32}$P-label in two polypeptides of identical molecular wieght which may be separated by isoelectric focusing. The two peptides exist on an approximately 1:1 ratio and the separation is indicative of a single charge difference [13]. In a less tightly or rigidly bound system, a more random labeling would be expected. The highly specific and efficient photoincorporation and the very slow off rate (or exchange rate) at 4° indicate that [$^{32}$P] 8-$N_3$cAMP is rigidly held in a solvent–solute free pocket of the regulatory protein which is unaffected by addition of cAMP, ATP without $Mg^{2+}$, and Mg·GTP. However, addition of Mg·ATP (or Mg·8-$N_3$ATP) rapidly effects dissociation of either cAMP or 8-$N_3$cAMP from regulatory protein under like conditions, with or without catalytic subunit present. Therefore, an allosteric site for Mg·ATP on the regulatory protein must be proposed which is entirely different from the cAMP binding site. Figure 9 describes a working model of the nucleotide regulation of type IM enzyme. It indicates that the R subunit (ie, CA-1) contains both Mg·ATP and cAMP binding sites and proposes that the binding of these two nucleotides has opposing effects on the restraint and inhibition of the catalytic subunit. The Mg·ATP binding site is proposed to be on R

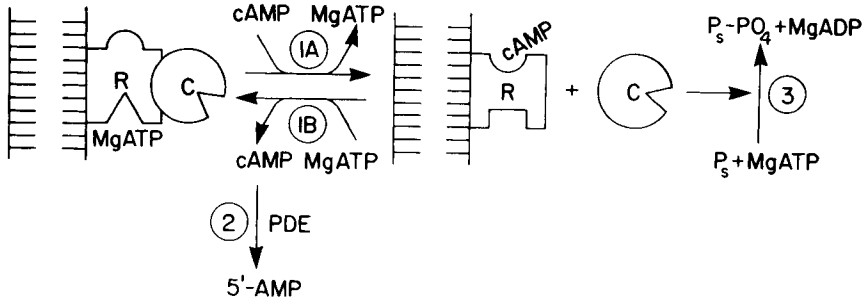


Fig. 9. Mechanism proposing binding sites on the regulatory subunit for cAMP, Mg·ATP, and catalytic subunit. Regulatory Mg·ATP site is distinct from Mg·ATP site on the catalytic subunit.

because of the ability of Mg·ATP and Mg·8-$N_3$ATP to effect dissociation of cAMP without catalytic subunit present (Figs. 5 and 7) and the photoincorporation of [$\beta$, $\gamma^{32}$P] 8-$N_3$ATP onto R at concentrations which cause dissociation of [$^{32}$P] 8-$N_3$cAMP from R. We propose that the cAMP and Mg·ATP sites on R are at different locations because of the "cold trap" experiments (see text and Fig. 4). Also, autophosphorylation is eliminated because experiments with [$\beta$, $\gamma^{32}$P] 8-$N_3$ATP showed that it was not a substrate for the catalytic subunit and did not phosphorylate any proteins of molecular weights near those of R or C.

We have observed that the presence of C appears to decrease the Mg·ATP concentration required for maximum decrease of [$^{32}$P] 8-$N_3$cAMP photoincorporation (Fig. 6). This indicates a cooperative relationship between Mg·ATP and C with regard to the cAMP binding properties of R of type IM protein kinase. We propose that coupling of reactions 1B and 2 (Fig. 9) could represent the "turn off" switch for certain hormone stimulated actions, mediated by type I protein kinases, which result in the increased production of ATP. Also, this mechanism may allow type II protein kinases to be active in the same cell or tissue where type I protein kinases are "turned off." Our observations with pigion heart tissue support this theory in that increasing Mg·ATP concentrations decreased photoincorporation of [$^{32}$P] 8-$N_3$cAMP onto the type I regulatory subunit but had no effect on photoincorporation on to the type II regulatory subunit [13]. However, the autoradiograms of Figures 2 and 5 show photoincorporation of [$^{32}$P] 8-$N_3$cAMP into two proteins whose approximate molecular weights (54,000 and 59,000, respectively) correspond to that observed for the dephosphorylation and phosphorylated forms of type II protein kinase regulatory subunit. Addition of Mg·ATP clearly decreases photoincorporation into these proteins also. This indicates that cAMP binding to type IIM protein kinase regulatory subunits may also be under MgATP regulation.

The tentative mechanism represented in Figure 9 is overly simplified. For instance, without Mg·ATP present a 25–35% increase in photoincorporation of [$^{32}$P] 8-$N_3$cAMP is observed on going from 8.0 mM to 400 mM $Na^+$ or $K^+$. More recent data show that the Mg·ATP inhibition of photoincorporation of [$^{32}$P] 8-$N_3$cAMP is very dependent on ionic strength, with Mg·ATP being much more effective at lower values [13]. This indicates that the association of the regulatory and catalytic subunits may also be controlled by ionic strength, as suggested by Fairbanks and Avruch [22]. Also, our results may explain the ionic-strength effects reported by Corbin et al [23] in which omitting NaCl reduced the amount of particulate-bound cAMP in rabbit heart tissue.

We also feel the detailed mechanism of sequestering of C subunit by R subunit will require further research. Figure 9 indicates that the R subunit never leaves the membrane. However, recent results indicate that Mg·ATP effects the release of R subunit in a manner similar to the cAMP-stimulated release of C subunit [13]. Experiments to elucidate the effects of Mg·ATP and Mg·8-$N_3$ATP on R subunit binding to the membrane are currently underway.

Considering the above observations, we suggest that experimentally measured cellular concentrations of cAMP may not be sufficient to predict cAMP-stimulated effects in vivo. The crux of the matter may be which proteins bind cAMP in the presence of the existing concentrations of endogenous constituents such as ATP, cGMP, GTP, and metal cations. We have observed an example of each of these substances affecting [$^{32}$P] 8-$N_3$cAMP photolabeling of certain proteins. We propose that the use of this analog will resolve many of the complex problems concerning physiological events mediated by cAMP. Use of this compound has already shown what has to be considered cellular compartmentalization of cAMP binding proteins [14].

## ACKNOWLEDGMENTS

This work was supported by NIH grant GM-21998.

## REFERENCES

1. Corbin JD, Keely SL, Park CR: J Biol Chem 250:218–225, 1975.
2. Hofmann F, Beavo JA, Bechtel PJ, Krebs EG: J Biol Chem 250:7795–7801, 1975.
3. Hosey MM, Tao M: J Biol Chem. 252:102–109, 1977.
4. Rosen OM, Erlichman J: J Biol Chem 250:7788–7794, 1975.
5. Rangel-Aldoa R, Rosen O: J Biol Chem 251:3375–3380, 1975.
6. Malkinson AM, Krueger BK, Rudolph SA, Casnellie JE, Haley BE, Greengard P: Metabolism 24:331–341, 1975.
7. Rangel-Aldao R, Rosen O: J Biol Chem 251:7526–7529, 1976.
8. Haddox MK, Newton NE, Hartle DK, Goldberg ND: Biochem Biophys Res Commun 47:361–364, 1972.
9. Beavo JA, Bechtel PJ, Krebs EG: Proc Natl Acad Sci USA 71:3580–3583, 1974.
10. Haley B: Biochemistry 14:3852–3855, 1975.
11. Haley B: In Jacoby, Wilchek (eds): "Methods in Enzymology." New York: Academic Press, 1977, vol 46, pp 339–346.
12. Owens JR, Haley B: J Supramol Struct 5:91–102, 1976.
13. Owens JR, Haley B: (Unpublished results).
14. Skare K, Black JL, Pancoe WL, Haley BE: Arch Biochem Biophys 180:409–415, 1977.
15. Pomerantz A, Rudolph SA, Haley B, Greengard P: Biochemistry 14:3858, 1975.
16. Haley B, Hoffman J: Proc Natl Acad Sci 71:3367, 1974.
17. Walter U, Uno I, Liu A, Greengard P: J Biol Chem 252:6494–6500, 1977.
18. Owens J, Haley B: J Supramol Struct (Suppl 1) 7:151, 1977.
19. Owens J, Haley B: J Supramol Struct (Suppl 2) 8:117, 1978.
20. Rubin CS, Erlichman J, Rosen OM: J Biol Chem 247:6135–6139, 1972.
21. Uno I, Ueda T, Greengard P: J Biol Chem 252:5164–5174, 1977.
22. Fairbanks G, Avruch J: Biochemistry 13:5514–5521, 1974.
23. Corbin J, Sugden P, Lincoln T, Keely, S: J Biol Chem 252:3854–3861, 1976.
24. Brostrom D, Corbin J, King C, Krebs EG: Proc Natl Acad Sci USA 68:2444–2447, 1971.