Adenosine Cyclic 3′,5′-Monophosphate Uptake and Regulation of Membrane Protein Kinase in Intact Human Erythrocytes†

Takuji Tsukamoto, Kimita Suyama, Patricia Germann, and Martin Sonenberg*

ABSTRACT: The uptake of adenosine cyclic 3′,5′-monophosphate (cAMP) and stimulation of membrane-associated protein kinase in mature human erythrocytes were investigated. cAMP transport across the membrane was temperature dependent, and cAMP binding to the isolated membrane had less temperature dependence. More than 99% of the [^{3}H]-cAMP taken up by erythrocytes was nonmembrane bound. Maximal stimulation of membrane protein kinase and maximal occupancy of membrane cAMP binding sites by extracellular cAMP occurred at 30 °C within 30 min after initiation of the incubation of erythrocytes with cAMP. The concentration of extracellular cAMP that gave half-maximal stimulation of membrane protein kinase was 5.4×10^{-4} M, a value consistent with the concentrations of cAMP (5.2×10^{-4} M) found to occupy half-maximally the membrane cAMP binding sites in erythrocytes. Extracellular cAMP and to a lesser extent guanosine cyclic 3′,5′-monophosphate and inosine cyclic 3′,5′-monophosphate stimulated membrane protein kinase in erythrocytes. The cAMP uptake by human erythrocytes as well as cAMP binding to membranes in the erythrocyte was blocked by an inhibitor [4,4′-bis(isothiocyano)stilbene-2,2-disulfonate] of the anion channel. These studies indicate that cAMP can be transported across membranes into human erythrocytes and can bind to membranes to activate membrane protein kinase. It appears that there is a shared transport channel for cAMP and anion transport.

There are numerous studies of the phosphorylation of human erythrocyte membranes. It has been demonstrated that human erythrocyte membrane phosphorylation is mainly mediated by membrane-associated cAMP-dependent[1] and cAMP-independent protein kinases (Guthrow et al., 1972; Rubin & Rosen, 1973; Avruch & Fairbanks, 1974a,b). The properties of cAMP binding to human erythrocyte membranes and that of cAMP activation of membrane-associated cAMP-dependent protein kinase have been well characterized by using isolated membranes (Guthrow et al., 1972; Rubin et al., 1972). To our knowledge there are no detailed studies with respect to cAMP uptake by erythrocytes and activation of membrane-associated cAMP-dependent protein kinase using intact human erythrocytes.

Although it is commonly thought that cyclic nucleotides cannot enter intact cells, there are various systems where effects have been observed upon addition of extracellular cyclic nucleotides [summarized in Ryan & Hendrick (1974)]. With regard to intact human erythrocytes, micromolar concentrations of cAMP added extracellularly changed the paramagnetic resonance spectrum of a membrane-bound spin-labeled fatty acid (Kury & MConnell, 1975). Exogenous cAMP (0.1–10 nM) increased the degree of hypotonic hemolysis of human erythrocytes (Rasmussen et al., 1975). At a concentration of 0.2 mM, exogenous cAMP stimulated membrane phosphorylation probably through activation of membrane-bound and/or cytoplasmic cAMP-dependent protein kinase in intact human erythrocytes (Plut et al., 1977). In addition, studies of the effects of cAMP (5 mM) on glycolysis in human erythrocytes suggest that extracellular cAMP may be the source, in part, of intracellular cAMP which acts as a regulatory agent (Ford & Omachi, 1972).

The present study shows that cAMP added extracellularly to intact human erythrocytes was transported across the membranes and bound to the membranes to activate membrane protein kinase.[2] This study suggests that human erythrocytes can serve as a useful model to investigate the mechanism of the pharmacological effects of cAMP. Since it is possible to load human erythrocytes with specified concentrations of cAMP, it may be possible to correlate stimulation of membrane-associated protein kinase with other biochemical events.

Experimental Procedures

Materials. cAMP, cIMP, cGMP, cCMP, cUMP, cTMP, adenosine cyclic 2′,3′-monophosphate, 5′-AMP, ATP, and bovine serum albumin were obtained from Schwarz/Mann Co. and Sigma Chemical Co. Protamine sulfate was purchased from Sigma. [γ-^{32}P]ATP (20–25 Ci/mmol) and [^{3}H]cAMP (13.7 Ci/mmol) were obtained from International Chemical and Nuclear Corp. Hank's balanced salt solutions were obtained from Grand Island Biological Co. DIDS was purchased from Pierce Chemical Co. Cytochalasin B was obtained from Aldrich Chemical Co.

Preparation of Erythrocytes and Erythrocyte Membranes. Blood from young healthy male donors between the ages of 19 and 31 years was collected directly into heparinized vacutainer tubes. The blood was centrifuged at 4 °C and washed 5 times with 10 mM Tris buffer containing 150 mM NaCl (pH 7.8). Each erythrocyte preparation was monitored with a cell count of erythrocytes, reticulocytes, and leukocytes including a leukocyte differential count. The average (±SEM) residual cell counts in the erythrocyte preparations were 0.013 ± 0.002% leukocytes and 0.066 ± 0.021% reticulocytes. Membranes were prepared from washed erythrocytes essentially by a modification (Hanahan & Ekholm, 1974) of the method of Dodge et al. (1963). Hemolysis was initiated by thoroughly mixing 2.0 mL of a washed erythrocyte suspension

† From the Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York 10021. *Received August 24, 1979.* Supported in part by Grants CA-08748, AM-15773, and AM-18759 of the National Institutes of Health.

[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-monophosphate; cIMP, inosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; cCMP, cytidine cyclic 3′,5′-monophosphate, cUMP, uridine cyclic 3′,5′-monophosphate; cTMP, thymidine cyclic 3′,5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DIDS, 4,4′-bis(isothiocyano)stilbene-2,2-disulfonate.

[2] Since submission of this manuscript a similar study has appeared (Thomas et al., 1979) which is in accord with our reported findings.

0006-2960/80/0419-0918$01.00/0

(50% hematocrit) with 20 mL of 10 mM Tris buffer (pH 7.8) and letting it stand for 10 min at 0 °C. Membranes obtained from intact erythrocytes incubated with cAMP or isolated membranes incubated with cAMP were processed in an identical fashion. The membranes were sedimented at 29000*g* for 30 min (noted as membranes washed once). Membranes obtained by subsequent washes are noted in succession as membranes washed twice (pink colored), membranes washed 3 times (slightly pink colored), and membranes washed 4 times (milky white). In these studies, the membranes were used after four washes.

Determination of [^3H]cAMP Uptake by Intact Human Erythrocytes. Isolated human erythrocytes (35–40% hematocrit) in the incubation buffer (Hank's balanced salt solution without Ca or Mg) were incubated with 10^{-5} M [^{3}H]cAMP (650–750 cpm/pmol) between 0 and 30 °C and 0 and 150 min, as indicated in the figure legends. At the end of the incubation, aliquots (0.2 mL) were removed and added to 10 mL of 10 mM Tris buffer containing 150 mM NaCl at 0 °C. Erythrocytes were collected by centrifugation for 8 min at 1240*g* in a refrigerated centrifuge (4 °C) and washed one more time with the buffer. Cold 10% trichloroacetic acid (1.5 mL) was added to the final pellet, and the mixture was mixed rapidly on a vortex mixer, allowed to stand for 15 min at 0 °C, and then centrifuged at 1240*g* for 15 min. A 0.2-mL amount of final clear supernatant was added directly to 10 mL of scintillation fluid (Aquasol-2, New England Nuclear), and the radioactivity was determined in a Packard 3380 liquid scintillation spectrometer. The procedure using 10% trichloroacetic acid to extract [^{3}H]cAMP from human erythrocytes recovered 95 ± 0.3% (n = 5) of membrane-bound [^{3}H]cAMP in addition to nonmembrane-bound [^{3}H]cAMP.

Determination of [^3H]cAMP in Membranes of Intact Human Erythrocytes. Membranes were prepared after incubating erythrocytes with 10^{-5} M [^{3}H]cAMP and separating the erythrocytes from free [^{3}H]cAMP. A 0.5-mL amount (1.8–2.2 mg of membrane protein) of the third washed membrane suspension was placed in a scintillation vial and dissolved with 0.3 mL of 10% sodium dodecyl sulfate. Each vial contained 10 mL of scintillation fluid (Aquasol-2), and radioactivity was determined in a liquid scintillation spectrometer. Only tightly bound cAMP was studied (no displacement of [^{3}H]cAMP by 10^{-5} M cAMP during the washing procedure).

Protein Kinase Assay after Isolation of Membranes from Erythrocytes Incubated with cAMP. Protein kinase activity was determined by measuring the amount of ^{32}P incorporated into an exogenous protein substrate (protamine) in the presence and absence of cAMP. The protein kinase activity assayed in the presence of cAMP is considered as total protein kinase activity. The reaction mixture (0.3 mL) contained 40 mM potassium phosphate buffer (pH 7.0), 8 mM $MgSO_4$, 3.5×10^{-6} M [γ-^{32}P]ATP (1500–2200 cpm/pmol), and 300 μg of protamine sulfate. The reaction was initiated by the addition of an aliquot (25 μL) of membranes (75–105 μg of membrane protein). When cAMP was present in the reaction mixture, its concentration was 3.3×10^{-6} M. Incubations were performed at 0 °C for 90 min. The reaction was terminated by adding 100 μL of reaction mixture to 2.5 mL of ice-cold 11% trichloroacetic acid together with 20 μL of 2.5% bovine serum albumin solution. The amount of ^{32}P transferred to protamine and membranes was determined by the method of Erlichman et al. (1971) except that 5% trichloroacetic acid was replaced by 10% trichloroacetic acid. The amount of ^{32}P transferred to membranes was determined in parallel assays in which protamine was omitted. All results on phosphorylation of exogenous protein substrates were corrected for endogenous membrane phosphorylation unless otherwise noted. As blanks, boiled membranes were used in the assays. The assay error in duplicates was within 5%. In this study, protein kinase activity is expressed as the amount of transferred phosphate from ATP to exogenous protein substrate in 90 min at 0 °C. In other cases, the protein kinase activity ratio is used, i.e., the ratio of protein kinase activity in the absence to that in the presence of added cAMP (Keely et al., 1975).

[^3H]cAMP Binding Assay Using Isolated Human Erythrocyte Membranes. The cAMP binding assay was performed essentially by the method of Rubin et al. (1972). The assay mixture (0.3 mL) contained 40 mM potassium phosphate buffer (pH 7.0), 8 mM $MgSO_4$, 40 nM [^{3}H]cAMP (8500–8800 cpm/pmol), and 100–120 μg of membrane protein (40 nM [^{3}H]cAMP was a sufficient amount to saturate membrane cAMP binding sites using isolated membranes). After a 90-min incubation at 0 °C (unless otherwise noted), 0.1 mL of reaction mixture was added to 2.0 mL of 20 mM potassium phosphate buffer (pH 6.0). The diluted reaction mixture was quantitatively filtered through a 24-mm cellulose ester (Millipore) filter with 0.45-μm pore size which had previously been soaked in 20 mM potassium phosphate buffer (pH 6.0). The filters were washed with 10 mL of the buffer and dried in scintillation vials. The membrane-bound [^{3}H]-cAMP in the filters was determined in 10 mL of scintillation fluid [10 mL of toluene containing 10% (v/v) Bio-Solv BBS-3 solubilizer (Beckman) and 0.4% (w/v) omnifluor (New England Nuclear)] by using a Packard 3380 liquid scintillation spectrometer. Control assays were carried out with boiled membranes.

cAMP Binding and Stimulation of Membrane Protein Kinase Using Intact Human Erythrocytes. Human erythrocytes, washed 5 times with 10 mM Tris buffer containing 150 mM NaCl (pH 7.8), were additionally washed 2 times with the incubation buffer (Hank's balanced salt solution without Ca and Mg) and mixed in a 30 °C shaking water bath (140 cycles/min) with a small portion of the incubation buffer containing various concentrations of cAMP. The incubation time was 60 min unless otherwise noted. At the end of the incubation, the incubation medium was diluted 10 times with ice-cold incubation buffer and immediately centrifuged at 3020*g* for 5 min at 4 °C. Erythrocyte pellets were resuspended in 10 mM Tris buffer containing 150 mM NaCl (pH 7.8) and washed 3 times with a buffer volume 20 times that of the packed erythrocyte volume.

Erythrocyte membranes were then prepared. cAMP binding to membranes and protein kinase activity associated with membranes were determined after freezing and thawing the membranes. The membrane proteins in each assay tube were carefully adjusted to the same concentration.

Chemical Analysis. Protein was determined by the method of Lowry et al. (1951). Hemoglobin associated with membranes was measured by using the cyanomethemoglobin method (Cannan, 1958). The membrane hemoglobin was subtracted from the total protein to give the nonhemoglobin protein (membrane protein). Nonhemoglobin protein in membranes was also determined by the method of Bodemann & Passow (1972).

Thin-layer chromatography of [^{3}H]cAMP before and after incubation of [^{3}H]cAMP with erythrocytes and erythrocyte membranes was performed by using Eastchromagram cellulose sheets (Eastman Kodak Co.). These were developed at room temperature by using 1.0 M ammonium acetate–95% ethanol (30:75 v/v) as the solvent system according to the method of

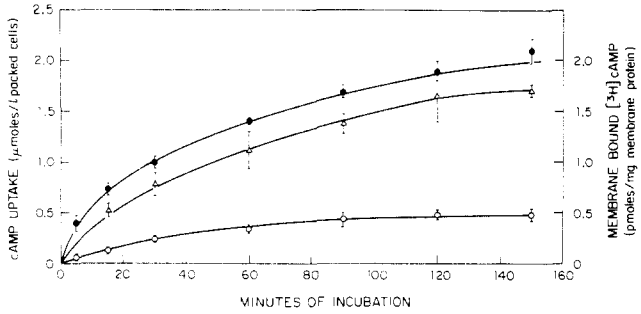

FIGURE 1: Uptake of extracellular cAMP by human erythrocytes as a function of time. Isolated erythrocytes (37% hematocrit) were incubated in Hank's balanced salt solution at 30 (●) or 0 °C (○) for the time indicated in the presence of 10^{-5} M [^{3}H]cAMP; (△) binding of extracellular cAMP to membranes isolated from intact erythrocytes incubated for various periods of time at 30 °C. Values are the means ± SEM of replicate determinations from four experiments.

Tao (1974). Radioactive materials taken up by erythrocytes as well as membrane-bound radioactive materials were extracted by boiling water or 10% trichloroacetic acid (Tao, 1974). There was no detectable degradation of [^{3}H]cAMP by isolated purified membranes during the course of the binding assay using thin-layer chromatography.

Statistical Methods. All the results are expressed as mean percent stimulation ± SEM (standard error of the mean) unless otherwise noted. Statistical significance was determined by Student's *t* test based on paired data (Zar, 1974). Statistical significance was assigned if $p < 0.05$.

Results

Kinetics of Extracellular cAMP Uptake by Human Erythrocytes. Extracellularly added [^{3}H]cAMP (10^{-5} M) was taken up by erythrocytes as a function of time and temperature (Figure 1). Fifty percent of the maximum uptake occurred within the first 30 min of incubation at 30 °C. After 30 min of incubation, the uptake process was slower and an apparent equilibrium was achieved in 120–150 min at 30 °C (Figure 1). At this time 1.9 μmol of [^{3}H]cAMP per L of packed

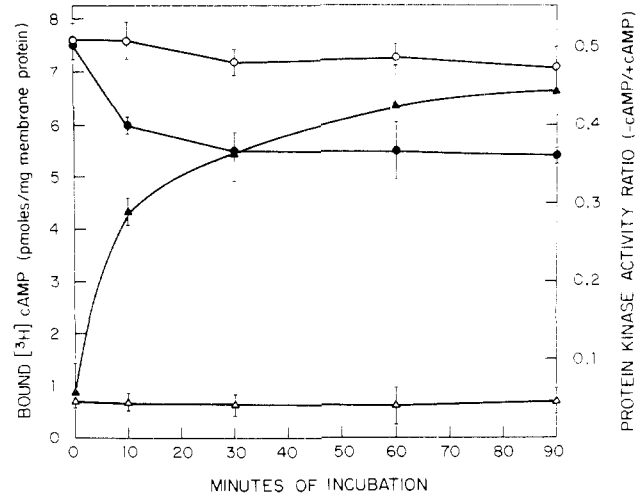

FIGURE 2: Effects of extracellular cAMP on the membrane cAMP binding sites in human erythrocytes as a function of time. Isolated erythrocytes (40% hematocrit) were preincubated in Hank's balanced salt solution at 30 °C for the time indicated in the absence (control, ○) or presence of 5×10^{-4} M cAMP (●). Then [^{3}H]cAMP binding to the membranes isolated from the erythrocytes was determined. Values are the means ± SEM of replicate determinations from four experiments. (△ and ▲) Effect of extracellular cAMP on membrane protein kinase in human erythrocytes as a function of time. Isolated erythrocytes (40% hematocrit) were preincubated in Hank's balanced salt solution at 30 °C for the time indicated in the absence (control, △) or presence of 5×10^{-4} M cAMP (▲). Preparation of membranes for protein kinase assay is described under Experimental Procedures. Values are the means ± SEM of replicate determinations from five experiments.

cAMP binding by extracellular cAMP (1 mM) was 24% at 10 °C, 59% at 22 °C, and 55% at 30 °C. There was no significant reduction in [^{3}H]cAMP binding to membranes isolated from erythrocytes incubated with 1 mM cAMP at 0 °C (Figure 3A).

For determination of whether cAMP uptake by erythrocytes or binding to membranes or both were temperature dependent, extracellular [^{3}H]cAMP uptake by erythrocytes as well as

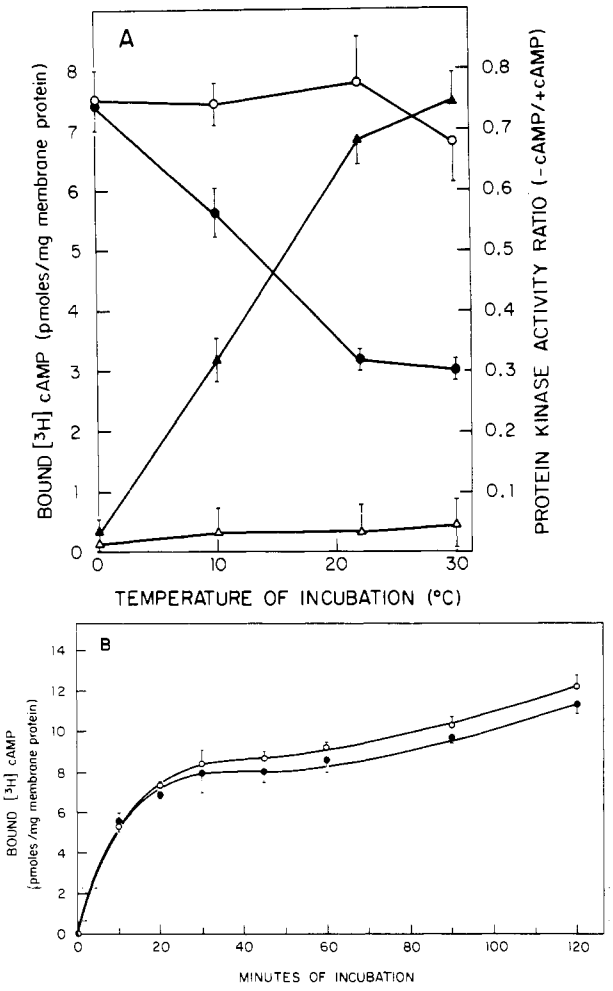

FIGURE 3: (A) Temperature dependence of the extracellular cAMP effect on the membrane cAMP binding sites and the stimulation of membrane protein kinase in human erythrocytes. Isolated erythrocytes (40% hematocrit) were preincubated in Hank's balanced salt solution at the temperature indicated for 60 min in the absence (control) or presence of 1 mM cAMP. Preparation of membranes for the [^{3}H]cAMP binding and the protein kinase assays is described under Experimental Procedures. ○ and ● represent results of [^{3}H]cAMP binding to membrane isolated from erythrocytes incubated in the absence and presence of 1 mM cAMP, respectively. △ and ▲ represent results of the protein kinase assay with respect to membranes isolated from erythrocytes incubated in the absence and presence of 1 mM cAMP, respectively. Each point represents the mean ± SEM of values of replicate determinations from three experiments. (B) Rates of [^{3}H]cAMP binding to isolated human erythrocyte membranes. ● and ○ represent results at 30 and 0 °C, respectively. Values are the means ± SEM of replicate determinations from three experiments.

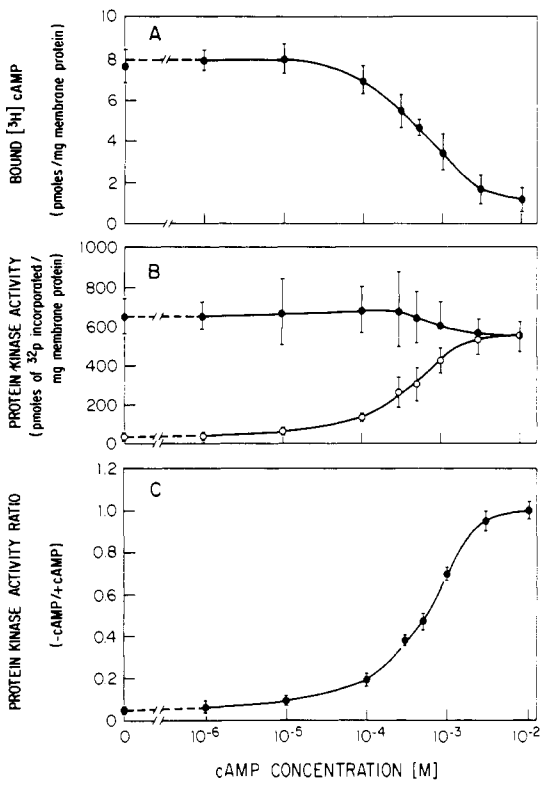

FIGURE 4: (A) Effect of extracellular cAMP concentration on the membrane cAMP binding sites in human erythrocytes. Erythrocytes (42% hematocrit) were preincubated for 60 min at 30 °C with Hank's balanced salt solution containing the indicated concentration of cAMP. Preparation of membranes for the [^{3}H]cAMP binding assay is described under Experimental Procedures. Each point represents the mean ± SEM of values of replicate determinations from five experiments. (B) Effect of extracellular cAMP concentration on erythrocyte membrane protein kinase activity assayed in the absence (open circles) and the presence (closed circles) of 3.3×10^{-6} M cAMP. Erythrocytes (42% hematocrit) were preincubated for 60 min at 30 °C with Hank's balanced salt solution containing the indicated concentration of cAMP. Preparation of membranes for protein kinase assay is described under Experimental Procedures. Each point represents the mean ± SEM of values of replicate determinations from five experiments. (C) Effect of extracellular cAMP concentration on the erythrocyte membrane protein kinase activity ratio. The activity ratio was determined as described under Experimental Procedures.

The level of protein kinase activity (assays after incubating with intact erythrocytes were performed in the absence and in the presence of cAMP) increased in response to increasing concentrations of extracellularly added cAMP (Figure 4B). Assays were made with membranes isolated from erythrocytes exposed to various concentrations of cAMP (60 min at 30 °C). The protein kinase activity (assayed in the absence of 3.3 μM cAMP) increased in proportion to the concentration of extracellular cAMP and reached a maximum at 10^{-2} M. Total membrane protein kinase activity (protein kinase activity assayed in the presence of 3.3 μM cAMP after incubation) was not significantly changed by incubating erythrocytes with increasing concentrations of cAMP up to 1 mM. However, at a concentration of 10^{-2} M, extracellular cAMP decreased the total membrane protein kinase activity (Figure 4B). Figure 4C shows the dose–response curve for the effect of extracellular cAMP on the membrane protein kinase activity ratio. Although the dose–response curve for the extracellular cAMP influence on the membrane protein kinase activity ratio essentially paralleled the protein kinase activity, there was less variance of the data than in the case of protein kinase activity. By use of the protein kinase activity ratio, significant stimulation ($p < 0.05$) of membrane protein kinase by 10^{-5} M cAMP was noted (Figure 4C). The concentration of extracellular cAMP required to stimulate membrane protein kinase half-maximally was graphically obtained to be $\sim 5.4 \times 10^{-4}$ M.

Cyclic Nucleotide Specificity. Various cyclic 3′,5′-nucleotides were tested for their effect on cAMP binding to membrane and the membrane protein kinase by using a concentration of 3×10^{-4} M with human erythrocytes (Figure 5). With respect to occupancy of the membrane cAMP binding sites in human erythrocytes by externally added cyclic nucleotides, only cAMP was effective (Figure 5). Further, cAMP was the most effective compound to stimulate membrane protein kinase. At a concentration of 3×10^{-4} M, stimulation of membrane protein kinase by cAMP was 7.7-fold, while stimulation by cGMP and cIMP, which were the only other effective cyclic nucleotides tested here, was 2.2- and 1.6-fold, respectively (Figure 5). In addition to cyclic 3′,5′-

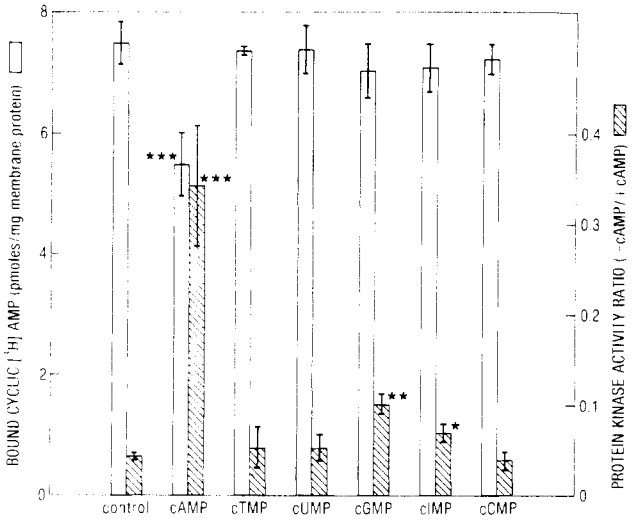

FIGURE 5: Response of membrane cAMP binding sites and membrane protein kinase activity in human erythrocytes to various cyclic nucleotides. Erythrocytes (42% hematocrit) were preincubated for 60 min at 30 °C with Hank's balanced salt solution containing 3×10^{-4} M 3′,5′-nucleotide compound. Preparation of membranes for the cAMP binding assay and the protein kinase assay is described under Experimental Procedures. Values are the means ± SEM of replicate determinations from five experiments. Significant effects of extracellular cyclic 3′,5′-nucleotide on the membrane protein kinase activity are indicated as (*) $p < 0.05$, (**) $p < 0.005$, and (***) $p < 0.001$.

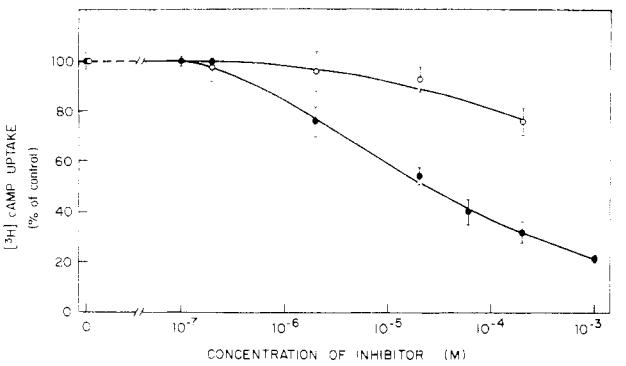

FIGURE 6: Effects of DIDS and cytochalasin B on the cAMP uptake by human erythrocytes. Human erythrocytes (37% hematocrit) were incubated with 10^{-5} M [^{3}H]cAMP in the presence of the indicated concentrations of DIDS (●) or cytochalasin B (○) for 120 min at 30 °C. Values are the means ± SEM of replicate determinations from three experiments.

nucleotide compounds, cyclic 2′,3′-AMP and 5′-AMP were also tested by using a concentration of 3×10^{-4} M. Neither compound had an effect on the membrane protein kinase or the occupancy of the membrane cAMP binding sites in human erythrocytes.

Effects of DIDS and Cytochalasin B on cAMP Uptake by Human Erythrocytes. For evaluation of the mechanism of cAMP transport across the membranes in intact human erythrocytes, [^{3}H]cAMP uptake studies were undertaken with two types of inhibitors. DIDS, a known inhibitor of anion transport (Sachs et al., 1975), blocked the uptake of [^{3}H]-cAMP as a function of DIDS concentration (Figure 6). The concentration of DIDS required to inhibit half-maximally the [^{3}H]cAMP uptake by erythrocytes was 2.4×10^{-5} M. Although cytochalasin B, a modifier of membrane structure (Plagemann et al., 1977), reduced the uptake of [^{3}H]cAMP by erythrocytes to some extent, the concentration of cytochalasin B required to inhibit the [^{3}H]cAMP uptake to the same extent as DIDS was much greater than DIDS (Figure 6).

The effect of DIDS on the decrease in [^{3}H]cAMP binding to membranes by extracellular cAMP in human erythrocytes was also studied. DIDS inhibited the decrease in [^{3}H]cAMP binding to membranes by extracellular cAMP in proportion to the DIDS concentration. The concentration of DIDS required to inhibit half-maximally the decrease in [^{3}H]cAMP binding to membranes by extracellular cAMP was 1.6×10^{-5} M. No effect on [^{3}H]cAMP binding was noted when DIDS or cytochalasin B was incubated with membranes instead of intact erythrocytes.

Discussion

Although evidence for extracellular cAMP effects on human erythrocytes has been accumulating, no detailed studies of extracellular cAMP stimulation of membrane protein kinase have been reported. Further, there has been no direct demonstration of cAMP transport across the membrane in intact human erythrocytes. In the presently described model studies, we have found that cAMP extracellularly added to human erythrocytes was taken up by cells, bound to membranes, and activated membrane protein kinase.

The process of extracellular cAMP transport across the membrane in intact human erythrocytes was temperature dependent (Figure 1), whereas the binding of cAMP to isolated membranes was minimally influenced by temperature (Figure 3B). Therefore, the larger reduction of membrane cAMP binding by extracellular cAMP at 30 °C as opposed to 0 °C appears to be due to accelerated cAMP transport rather than increased binding of cAMP to membranes. It has been shown that cAMP did not bind to right side out ghosts and erythrocytes but bound to inside out and permeable ghosts, indicating that cAMP binding sites were localized on the inner surface of the plasma membrane (Rubin et al., 1973; Kant & Steck, 1973). Further, using the various membrane preparations mentioned above, Rubin et al. (1973) have shown that both the catalytic and cAMP binding components of protein kinase are localized on the inner surface of the plasma membrane. It should be noted that these experiments (Rubin et al., 1973; Kant & Steck, 1973) were done at 0 °C as the incubation temperature in cAMP binding studies. However, at 30 °C, cAMP binding to membranes in intact erythrocytes was observed (Figure 1). From the observations of the present study and other investigators (Rubin et al., 1973; Kant & Steck, 1973), it can be concluded that cAMP is transported across the membrane temperature dependently and cAMP taken up by erythrocytes binds to the inner surface of the membrane presumably with less temperature dependence.

The uptake of cAMP by human erythrocytes was time dependent and began to reach an apparent equilibrium steady state in 120–150 min at 30 °C. This result is slightly different from a recent report (Holman, 1978) that cAMP readily diffuses into resealed human erythrocyte ghosts and reaches equilibrium within 60–90 min. However, it is difficult to compare those studies (Holman, 1978) to the present study using intact human erythrocytes because of different membrane and cytosol constituents and the different permeabilities between resealed ghosts and intact human erythrocytes (Shwoch & Passow, 1973). Further detailed studies are necessary in order to obtain the kinetic parameters for cAMP influx and efflux in human erythrocytes. There might be a substantial difference between the mode of intracellular cAMP binding to membranes in erythrocytes and its binding to isolated membranes using the standard assay method being currently employed by other investigators (Guthrow et al., 1972; Rubin & Rosen, 1973; Rubin et al., 1973; Kant & Steck, 1973; Zail & Van den Hoek, 1975). cAMP (40 nM) produced

~10 pmol of membrane-bound cAMP per mg of membrane protein after 90 min of incubation at 30 °C by using isolated membranes (Figure 3B). In contrast, a very small portion of cAMP was found to be membrane bound when the cAMP was taken up by erythrocytes instead of isolated membranes (Figure 1). For example, after a 60-min incubation of erythrocytes with 10^{-5} M extracellular cAMP, the intracellular cAMP concentration in erythrocytes was approximately ~1.4 μM (<0.58% of the total uptake was membrane bound) and increased to ~1.9 μM after 150 min of incubation. Therefore, after incubation times between 60 and 150 min, there must be at least 0.98 μM cAMP (considering that 70% of cAMP uptake by erythrocytes is unaltered) intracellular nonmembrane-bound cAMP (Figure 1). Notwithstanding, the amount of membrane-bound cAMP observed after 150 min of incubation was only 1.7 pmol/mg of membrane protein (Figure 1). This cannot be explained by the metabolism of cAMP taken up by erythrocytes since most of the cAMP (>70%) incorporated into the cell was recovered and identified as unaltered cAMP. Although further studies are required to resolve this problem, some interpretations can be suggested: (a) the difference of the ionic environment and components (ATP, ADP, etc.) between the erythrocytes and the assay medium for cAMP binding to isolated membranes may cause the discrepancy; (b) the availability of cellular cAMP for cAMP binding sites may be much less than that in the binding studies using isolated membranes, e.g., compartmentalization of intracellular cAMP; (c) there may be a difference of cAMP accessibility under the two distinct assay conditions; (d) part of the cAMP binding sites may be buried in membranes, and the hemolysis and membrane preparation procedures may result in exposure of some of the previously unavailable sites.

No information is available about the mechanism of cAMP transport across membranes in intact human erythrocytes. It has been proposed that cytochalasin B is an apparent competitive inhibitor of cAMP exit in resealed erythrocyte ghosts (Holman, 1978). Cytochalasin B is a well-known inhibitor for D-glucose transport in erythrocytes (Taverna & Langdon, 1973) and is considered to act on hydrophobic regions of several integral membrane proteins (Graff et al., 1977). We have found that high concentrations of cytochalasin B (10^{-4}–10^{-3} M) blocked the uptake of cAMP by erythrocytes to some extent (20–40%) as shown in Figure 6. However, because lesser concentrations (2 μM) of cytochalasin B can almost completely inhibit the glucose (30 mM) transport in human erythrocytes (Taverna & Langdon, 1973), the effect of cytochalasin B appears to be due to some kind of interaction with membrane components resulting in perturbation of cAMP transport regions rather than specific interaction with the cAMP transport system. Interestingly, the cAMP uptake by human erythrocytes was markedly blocked by an inhibitor (DIDS) of the anion channel (Figure 6). The concentration of DIDS required to inhibit half-maximally cAMP transport is 3×10^{-5} M. A similar effect of an anion channel blocker on the cAMP regulation of membrane flexibility in human erythrocytes has been observed (Kury & McConnell, 1975). There may be a shared transport channel for cAMP and anion transport. The above data suggest that a carrier-mediated transport system for cAMP exists in intact human erythrocytes. Since the concentrations inside and outside the erythrocytes indicate that cAMP is not transported against a concentration gradient, active transport is not a likely mechanism in the present study. The blocking effect of DIDS on cAMP transport may be indirect, i.e., secondary to a change in membrane potential. More extensive studies of cAMP uptake are required to characterize cAMP transport.

In addition to information about overall transport of cAMP into intact erythrocytes, it would be important to characterize the topological relation of cAMP transport sites to cAMP binding sites. In this context, the intact human erythrocyte should prove useful in understanding the mechanism of cAMP transport across the membrane and membrane binding of cAMP.

cAMP interaction with human erythrocytes resulted in increased membrane protein kinase activity assayed in the absence of cAMP and did not produce any significant increase in membrane protein kinase activity assayed in the presence of cAMP (Figure 4B). This also indicates that extracellular cAMP is transported across the membrane to bind to membrane-associated cAMP-dependent protein kinase. The dose–response curves of membrane cAMP binding site occupancy and stimulation of membrane cAMP-dependent protein kinase correlated well with each other in the region of extracellular cAMP concentrations between 10^{-5} and 10^{-2} M (parts A and C of Figure 4). The concentration of extracellular cAMP required to obtain half-maximal stimulation of membrane cAMP-dependent protein kinase was 5.4×10^{-4} M, a value consistent with the concentration of extracellular cAMP (5.2×10^{-4} M) which half-maximally occupied the membrane cAMP binding sites. High concentrations (~10^{-3}–10^{-2} M) of cAMP decreased significantly ($p < 0.05$) the total membrane protein kinase activity (Figure 4B). This may be due to the dissociation of catalytic subunits of activated protein kinase in the membrane during hemolysis and/or washing. It is not clear at present whether the dissociation of catalytic components of activated membrane protein kinase occurs in the intact erythrocyte.

The results obtained with extracellular cAMP on membrane binding and stimulation of membrane cAMP-dependent protein kinase in human erythrocytes are well correlated with each other: e.g., kinetics (Figure 2), temperature dependence (Figure 3), and dose–response (parts A and C of Figure 4). It is clear that cAMP is taken up by erythrocytes and binds to membranes (Figure 1). Therefore, it is likely that the decrease in the membrane [^{3}H]cAMP binding is simply the result of the increase in binding of cAMP to membranes. However, it is likely that the decrease in membrane binding of [^{3}H]cAMP may not be accounted for solely by the total amount of membrane-bound cAMP. At this time one cannot exclude the possibility that membrane phosphorylation stimulated by cAMP may directly or indirectly modify membrane cAMP binding. It may be that part of the extracellular cAMP taken up by erythrocytes will be associated with some membrane proteins or pooled in the membrane space, e.g., water tightly bound to membranes in addition to cAMP binding components of membrane-associated protein kinase. This may occur in the transport channel which is destroyed in permeable membranes. Because of the above reasons, determination of the occupancy of membrane cAMP binding sites was extensively employed to compare to extracellular cAMP stimulation of membrane protein kinase in erythrocytes in this study. In order to understand the process of cAMP transport and the fate of cAMP taken up by cells, it will be necessary to analyze in detail the membrane components with which cAMP is associated by using intact erythrocytes as well as isolated permeable membranes.

Acknowledgments

The authors gratefully acknowledge the excellent technical assistance of John Q. Landers, Margaret Priddle, and Loredana Fermeglia. The authors also thank Dr. Allan S.

Schneider for his helpful discussions during the preparation of this manuscript.

References

Avruch, J., & Fairbanks, G. (1974a) *Biochemistry 13*, 5507.
Avruch, J., & Fairbanks, G. (1974b) *Biochemistry 13*, 5514.
Bodemann, H., & Passow, H. (1972) *J. Membr. Biol. 8*, 1.
Cannan, R. K. (1958) *Am. J. Clin. Pathol. 30*, 211.
Dodge, J. T., Mitchell, C. D., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119.
Erlichman, J., Hirsch, A. H., & Rosen, O. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 731.
Ford, D. L., & Omachi, A. (1972) *Biochim. Biophys. Acta 279*, 587.
Graff, J. C., Wohlhueter, R. M., & Plagemann, P. G. W. (1977) *J. Biol. Chem. 252*, 4185.
Guthrow, C. E., Jr., Allen, J. E., & Rasmussen, H. (1972) *J. Biol. Chem. 247*, 8145.
Hanahan, D. J., & Ekholm, J. E. (1974) *Methods Enzymol. 30* (Part A), 168.
Holman, G. D. (1978) *Biochim. Biophys. Acta 508*, 174.
Kant, J. A., & Steck, T. L. (1973) *Biochem. Biophys. Res. Commun. 54*, 116.
Keely, S. L., Corbin, J. D., & Park, C. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1501.
Kury, P. G., & McConnell, H. M. (1975) *Biochemistry 14*, 2798.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Plagemann, P. G. W., Graff, J. C., & Wohlhueter, R. M. (1977) *J. Biol. Chem. 252*, 4191.
Plut, D. A., Hosey, M. M., & Tao, M. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 320.
Rasmussen, H., Lake, W., & Allen, J. E. (1975) *Biochim. Biophys. Acta 411*, 63.
Rubin, C. S., & Rosen, O. M. (1973) *Biochem. Biophys. Res. Commun. 50*, 421.
Rubin, C. S., Erlichman, J. S., & Rosen, O. M. (1972) *J. Biol. Chem. 247*, 6135.
Rubin, C. S., Rosenfeld, R. D., & Rosen, O. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3735.
Ryan, W. L., & Hendrick, M. L. (1974) *Adv. Cyclic Nucleotide Res. 4*, 81.
Sachs, J. R., Knauf, P. A., & Dunham, P. B. (1975) in *The Red Blood Cell* (Surgenor, D. M., Ed.) Vol. II, p 613, Academic Press, New York.
Shwoch, G., & Passow, H. (1973) *Mol. Cell. Biochem. 2*, 197.
Tao, M. (1974) *Methods Enzymol. 38* (Part C), 155.
Taverna, R. D., & Langdon, R. G. (1973) *Biochim. Biophys. Acta 323*, 207.
Thomas, E. L., King, L. E., Jr., & Morrison, M. (1979) *Arch. Biochem. Biophys. 196*, 459.
Zail, S. S., & Van den Hoek, A. K. (1975) *Biochem. Biophys. Res. Commun. 66*, 1078.
Zar, J. H. (1974) in *Biostatistical Analysis*, p 121, Prentice-Hall, Englewood Cliffs, NJ.

Kinetic Studies on the Dissociation of Adenosine Cyclic 3′,5′-Monophosphate from the Regulatory Subunit of Protein Kinase from Rabbit Skeletal Muscle†

Vincent Chau, Laura C. Huang,‡ Guillermo Romero, Rodney L. Biltonen, and Ching-hsien Huang*

ABSTRACT: The exchange rate of unlabeled adenosine 3′,5′-monophosphate (cAMP) with labeled [^{3}H]cAMP in the dimeric regulatory subunit–cAMP complex of cAMP-dependent protein kinase, type I, purified from rabbit skeletal muscle is described by using the equilibrium isotope exchange technique. Results indicate that the rate of exchange carried out in the absence of the catalytic subunit (C) is rather slow with a half-life of ~870 s. This slow exchange rate is not affected by the presence of MgATP (50 μM). However, when both MgATP (50 μM) and C (1–13 nM) are present, the rate of isotope exchange is observed to increase markedly. Furthermore, less than stoichiometric amounts of C are required for the increase in the rate of cAMP exchange, indicating that the effect of C on the rate enhancement is a catalytic process. These results indicate that in the presence of MgATP, a ternary complex between C and regulatory subunit–cAMP complex must be formed, and a dynamic equilibrium between the ternary complex and its dissociable species must be reached within seconds. On the basis of our kinetic data, it is proposed that the formation of this ternary complex intermediate allows the rapid activation or the inactivation of cAMP-dependent protein kinase following changes in the cellular cAMP levels.

Adenosine 3′,5′-monophosphate (cAMP)[1] dependent protein kinase from mammalian skeletal muscle is a relatively well understood tetrameric enzyme composed of two pairs of dissimilar subunits, R_2C_2. Its function is to serve as a receptor for cAMP and to phosphorylate one or more specific serine or threonine residues in a number of protein or enzyme substrates (Krebs, 1972; Rubin & Rosen, 1975; Cohen, 1978). Since cAMP is a general and common "second messenger" for many hormones and since phosphorylation of enzymes often results in the alteration of many enzymatic activities, cAMP-dependent protein kinase thus plays an important role in the hormonal regulation of glycogen metabolism in mam-

† From the Departments of Biochemistry and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 10, 1979.* Portions of this paper are derived from the dissertation of V.C. presented in partial fulfillment of the requirements for a Ph.D. degree in Biophysics, University of Virginia (1979). A preliminary account of this work has been presented at the ASBC/AAI Meeting, June 4–8, 1978, Atlanta, GA. This research was supported in part by U.S. Public Health Service Grants GM-22430, GM-20637, and GM-17452.

‡ The recipient of Career Development Award 1K04-AM-00212 from the U.S. Public Health Service.

[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-monophosphate; R_2, dimeric regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit monomer of cAMP-dependent protein kinase.

0006-2960/80/0419-0924$01.00/0