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Effect of cAMP on Membrane-bound Calcium

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Summary

In order to investigate the functional relationship between calcium (binding and transport) and membrane phosphorylation, the effects of extracellular cAMP on membrane-bound calcium and membrane phosphorylation were tested using human erythrocytes as a model system.

To make the study feasible, the intracellular calcium concentration of human erythrocytes was increased by the divalent cation ionophore, A23187. cAMP interaction with these erythrocytes resulted in stimulation of membrane phosphorylation and an increased amount of membrane-bound calcium. The maximum increase in the amount of membrane-bound calcium obtained by 2 mM extracellular cAMP was 60 %. Under the same conditions, 80 % stimulation of membrane phosphorylation was observed. The apparent Ka for the cAMP effect on the amount of membrane-bound calcium was 0.30 mM. This value was consistent with the concentration of cAMP found to stimulate membrane phosphorylation half-maximally.

Thus, the cAMP effect on erythrocyte function observed by several investigators may involve a causal relationship between membrane phosphorylation and calcium binding to erythrocyte membranes.

Introduction

It has been noted that calcium plays an important role in the maintenance of membrane structure and a number of membrane functions. With respect to human erythrocytes, of which membranes have been most frequently used for various studies as a model system, cellular calcium has been found to regulate deformability of cells[18] and transport of ions across the membrane[8]. It has been shown that calcium accumulates in human erythrocytes as a result of depletion of intracellular ATP† and this has also been found to affect deformability[18,2]. Although it has been suggested that a Ca^{2+}-activated ATPase[5] and/or membrane-bound protein kinase[4] may play a role in these phenomana, the mechanism of calcium action on membrane structure and function is still rather obscure. A specific calcium binding site may be important for cell deformability and cell shape control in human erythrocytes[17].

† Abbreviations used in this paper : ATP, adenosine 5′-triphosphate ; cAMP, adenosine cyclic 3′, 5′-monophoshpate ; 5′-AMP, adenosine 5′-monophosphate.

The evidence for the regulation of human erythrocyte cell deformability and shape control through membrane phosphorylation has been accumulating[13,14]. Recent observations suggest that cAMP, when applied extracellularly, may influence the deformability of human erythrocytes[9,10] and the quality and distribution of aggregates of the membrane-bound protein in erythrocytes[6]. Since it has been found that extracellular cAMP is transported across the membranes and stimulates membrane-bound protein kinase[15], the cAMP effect on erythrocyte membrane function observed by several investigators may be due to the stimulation of a membrane-bound cAMP-dependent protein kinase resulting in an increased state of membrane phosphorylation.

These observations suggest certain interrelationships among the effects of cAMP, membrane phosphorylation and calcium on human erythrocyte functions. We therefore have studied the effect of extracellular cAMP on calcium uptake by erythrocytes and membrane bound calcium.

Materials and Methods

Materials. cAMP, bovine serum albumin and 5'-AMP were obtained from Schwartz/Mann Co. Protamine sulfate was purchased from Sigma Chemical Co. ATP was a generous gift from Kyowa Hakko Kogyo Co. A23187 was kindly supplied by Mr. Kenji Toda, Eisai Co. [^{3}H] cAMP and [^{32}P] orthophosphoric acid were purchased from Amersham Co. Other chemicals were obtained through Katayama Chemical Industries Co. LTD.

Preparation of erythrocytes and erythrocyte membranes. Blood was collected directly into heparinized vacutainer tubes from young healthy male blood donors between the ages of 21 and 25 yr. The blood was centrifuged at 4°C and washed three times with 10 mM Tris buffer containing 150 mM NaCl (pH 7.8). Membranes were prepared from washed erythrocytes essentially by a modification[3] of the method of Dodge et al[1]. Hemolysis was initiated by thoroughly mixing 2.0 ml of washed erythrocyte suspension (50 % hematocit) with 20 ml of 10 mM Tris buffer (pH 7.8) for 10 min at 0°C. The membranes were sedimented at 29,000 g for 30 min (noted as first washed membranes). Membranes obtained by subsequent washes were noted in succession as second washed membranes, third washed membranes and fourth washed membranes. The membranes used in these studies are described in the *Results*.

[^{3}*H*] *cAMP binding assay.* The [^{3}H] cAMP binding assay was performed essentially by the method of Rubin et al[11]. The assay mixture (0.3 ml) contained 40 mM potassium phosphate buffer (pH 7.0), 8 mM $MgSO_4$, 40 nM [^{3}H] cAMP (saturable amount) and 100 −120 μg of membrane proteins. After a 90 min incubation at 0°C, 0.1 ml of the 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 0.45μm 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 using

a liquid scintillation spectrometer. Control assays were carried out with boiled membranes.

Thin-layer chromatography of [^{3}H] cAMP before and after incubation of [^{3}H] cAMP with erythrocyte membranes proved no detectable degradation of [^{3}H] cAMP during the course of the [^{3}H] cAMP binding assay.

Determination of cAMP binding using intact human erythrocytes. Well washed human erythrocytes were washed two additional times with the incubation buffer†† and mixed with a small portion of the buffer containing cAMP in a 30°C shaking water bath. At the end of the 60 min incubation period, the incubation medium was diluted 10 times with ice cold incubation buffer and immediately centrifuged at 3,000 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 three times with a buffer volume 20 times that of the packed erythrocyte volume. Erythrocyte membranes were then prepared. The cAMP binding site occupancy in the membranes was determined after the membranes were freeze-thawed. The membrane proteins in each assay tube were carefully adjusted to the same concentration.

Membrane phosphorylation of intact erythrocytes. Washed erythrocytes were resuspended in the incubation buffer (30−40 % hematocrit) containing ^{32}Pi as $H_3{}^{32}PO_4$. In a typical experiment, 0.2 ml of the erythrocyte suspension contained 2−5 μCi of ^{32}P. After incubation for 2 hr at 30°C, various concentrations of cAMP were added to the incubation medium and the incubation was continued for another hour. At the end of incubation, the incubation medium was diluted 30 times with ice cold phosphate-buffered saline and immediately centrifuged at 3,000 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 once with the buffer, and then erythrocyte membranes were prepared. The amount of ^{32}P transferred to membranes was determined as described previously[16].

Uptake of Calcium by erythrocytes in the presence of ionophore A-23187. To determine Ca^{2+} uptake, erythrocytes were resuspended in the incubation buffer (30−40 % hematocrit) containing various concentrations of $CaCl_2$ in the presence of an appropriate amount of A-23187 with or without cAMP. After incubation for 30 min at 30°C, 1.0 ml aliquots were removed and added to 9 ml of 10 mM Tris buffer containing 150 mM NaCl and 20 μM La_2O_3 (pH 7.8) at 0°C. Erythrocytes were collected by centrifugation for 10 min at 1,000 g in a refrigerated centrifuge and washed two more times with La_2O_3-containing buffer. After two washings of erythrocytes, membranes were prepared as previously described except the hemolyzing and washing buffers contained 20 μM La_2O_3. Membrane-bound Ca^{2+} was extracted with an equal volume of 1.5 N HNO_3 containing 2.5 % Triton X -100. Intracellular free Ca^{2+} (non membrane-bound Ca^{2+}) was extracted with an equal volume of 20 % (w/v) trichloroacetic acid.

Determination of Calcium. The amount of Ca^{2+} was determined using an atomic absorption spectrophotometer (Perkin-Elmer Model 303) with $CaCO_3$ as the standard.

†† The standard incubation buffer used in this study was 20 mM Tris buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose, pH 7.4.

Chemical analysis. Protein content was determined by the method of Lowry et al[7]. Thin-layer chromatography of [^{3}H] cAMP was performed by using Eastchromatogram cellulose sheets (Eastman Kodak Co.). These were developed at room temperature using 1.0 M ammonium acetate-95 % ethanol (30 : 75 v/v) as the solvent system.

Radioactivity determination. The radioactivity ([^{3}H] cAMP and ^{32}P) was determined in an Aloka liquid scintillation spectrometer, LSC-900 using 10 ml of toluene containing 15 % (v/v) Triton X-100 and 0.4 % (w/v) omnifluor (New England Nuclear) as the scintillation fluid.

Results

Effect of extracellular cAMP on membrane [^{3}H] cAMP binding sites in human erythrocytes.

The available [^{3}H] cAMP binding sites of membranes isolated from erythrocytes incubated with various concentrations of cAMP were decreased in proportion to the extracellular cAMP concentration and almost diminished at 10 mM extracellular cAMP. The concentration of extracellular cAMP required to produce a half-maximal decrease in binding of [^{3}H] cAMP to membranes was 0.52 mM (Fig. 1).

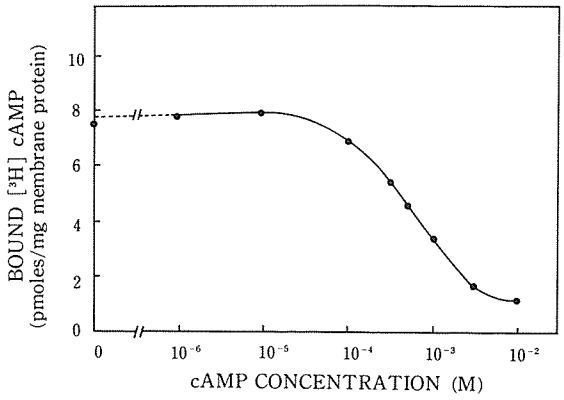

Fig. 1 Effects of extracellular cAMP concentration on membrane [^{3}H] cAMP binding sites in human erythrocytes. Each point represents the mean value of triplicate determinations from three experiments. Preparation of membranes (fourth washed membranes) for the [^{3}H] cAMP binding assay is described under Materials and Methods.

Membrane phosphorylation using intact human erythrocytes in the presence of various concentrations of extracellular cAMP.

The direct phosphorylation of membranes of intact human erythrocytes was performed by incubating erythrocytes in inorganic ^{32}P with various concentrations of cAMP. Membrane phosphorylation increased in proportion to the concentration and reached a maximum at 2 mM cAMP (Fig. 2). The lowest concentration of extracellular cAMP

which stimulated membrane phosphorylation in intact erythrocytes appeared to be 0.1 mM. The concentration of extracellular cAMP required to stimulate membrane phosphorylation half-maximally was graphically obtained to be approximately 0.32 mM.

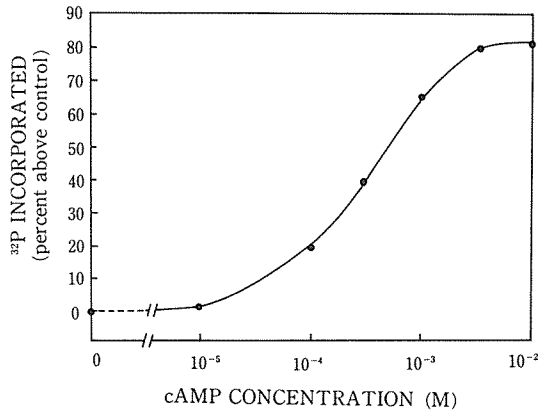

Fig. 2 Membrane phosphorylation using intact human erythrocytes in the presence of various concentrations of extracellular cAMP. Each point represents the mean value of duplicate determinations from two experiments.

Effect of extracellular cAMP on membrane-bound calcium in intact human erythrocytes.

The effect of 2 mM extracellular cAMP on membrane-bound Ca^{2+} was tested using intact human erythrocytes with and without extracellular Ca^{2+} in the absence of ionophores. Since 30 min at 30°C was employed as the incubation condition to see the effect of extracellular cAMP, membranes (third washed membranes) isolated from erythrocytes incubated without extracellular cAMP for 30 min at 30°C were used as controls. The amount of membrane-bound Ca^{2+} in control membranes was 21.2 nmol per membrane equivalent of 1 ml of the packed erythrocytes in the absence of extracellular Ca^{2+}. This value corresponds to 1.5×10^{6} atoms per cell (Table 1). On the other hand, cellular Ca^{2+} concentration of control membranes was 50 nmol per ml packed erythrocytes. Thus, about

Table 1 The effects of extracellular cAMP and calcium on membrane-bound calcium in intact human erythrocytes.

Conditions	Membrane-bound Ca^{2+} (million atoms/cell)
Control	1.50
+0.1 mM Ca^{2+}	1.50
+2 mM cAMP	1.45
+0.1 mM Ca^{2+}, 2 mM cAMP	1.69

Values are the means of duplicate determinations from two experiments.

40 % of the total Ca^{2+} in intact human erythrocytes appeared to be present in the membrane.

The existence of 0.1 mM Ca^{2+} in the incubation medium without extracellular cAMP had no significant effect upon the amount of membrane-bound Ca^{2+} or cellular Ca^{2+} levels (Table 1). Nor did extracellular cAMP alter the amount of membrane-bound Ca^{2+} in the absence of extracellular Ca^{2+}. Although the membrane-bound Ca^{2+} appeared to be increased by 13 % in the presence of extracellular 2 mM cAMP with 0.1 mM Ca^{2+}, the effect of cAMP was too little to be analyzed in detail (Table 1). Furthermore, no change in intracellular non membrane-bound Ca^{2+} was observed in the presence of extracellular

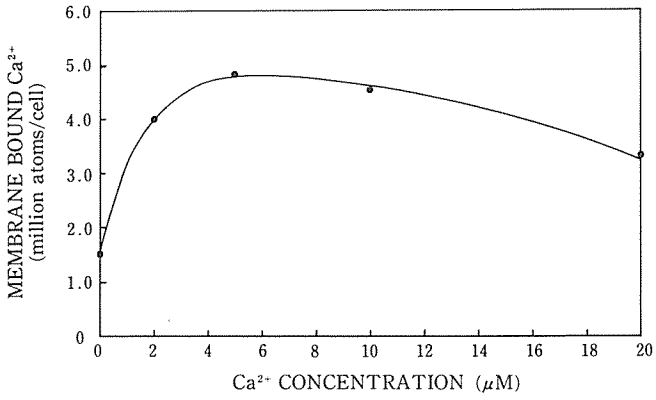

Fig. 3 Effect of extracellular calcium concentration on the uptake of calcium by membranes in human erythrocytes in the presence of 2 μM A23187. Each point represents the mean value of duplicate determinations from three experiments.

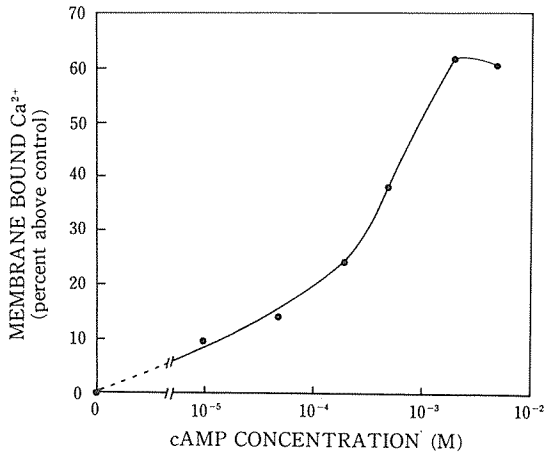

Fig. 4 Effect of extracellular cAMP on membrane-bound calcium in human erythrocytes in the presence of extracellular 5 μM calcium and 2 μM A23187. Each point represents the mean value of duplicate determinations from four experiments.

cAMP with Ca^{2+}.

Effect of extracellular cAMP on membrane-bound calcium in the presence of extracellular calcium and A23187.

The effect of extracellular cAMP on membrane-bound calcium was investigated using human erythrocytes loaded with Ca^{2+}. The divalent cation ionophore, A23187 was employed as a means of loading fresh erythrocytes with Ca^{2+}. In the presence of A23187, the uptake of extracellular Ca^{2+} by membranes in human erythrocytes was dependent upon the concentration of extracellular Ca^{2+} as shown in Fig. 3. Maximum uptake of extracellular Ca^{2+} by membranes occurred around 5 μM Ca^{2+} in the presence of 2 μM A23187. Under the same conditions, intracellular Ca^{2+} concentration was 128 nmol per ml packed cells. Extracellular Ca^{2+} concentration higher than 5 μM resulted in less membrane-bound Ca^{2+}.

Extracellular cAMP increased the amount of erythrocyte membrane-bound Ca^{2+} in the presence of extracellular 5 μM Ca^{2+} and 2 μM A23187. An increase in membrane-bound Ca^{2+} seemed to occur with as low as 10 μM extracellular cAMP and was proportional to the concentration until 2 mM, at which concentration the membrane-bound Ca^{2+} reached a maximum (Fig. 4). The concentration of cAMP that gave one-half maximal increase in membrane-bound Ca^{2+} was determined to be about 0.3 mM.

On the other hand, the intracellular Ca^{2+} accumulation did not increase significantly even at 2 mM cAMP concentration.

Discussion

In order to evaluate the relationship between cAMP, membrane phosphorylation and Ca^{2+} effects on human erythrocytes, effects of cAMP on membrane phosphorylation and membrane-bound Ca^{2+} in the erythrocyte were investigated. First of all, effects of extracellular cAMP on membrane [^{3}H] cAMP binding sites in human erythrocytes were studied. [^{3}H] cAMP binding to membranes was decreased in proportion to extracellular cAMP concentration (Fig. 1). This observation clearly indicates that cAMP transports across the membranes in intact human erythrocytes since cAMP binding sites have been well known to be localized on the inner surface of plasma membrane[12]. Extracellular cAMP interaction with human erythrocytes resulted in increased membrane phosphorylation (Fig. 2). The dose-response curves of membrane cAMP binding site occupancy and stimulation of membrane phosphorylation were comparable to each other in the region of extracellular cAMP concentrations between 10 μM and 10 mM (Figs. 1 and 2). Thus, it is conceivable that extracellular cAMP stimulation of membrane phosphorylation occurred through activation of membrane cAMP-dependent protein kinase. Extracellular cAMP appeared to increase the amount of membrane-bound Ca^{2+} in intact human erythrocytes which were not loaded with Ca^{2+} (Table 1). However, it was quite difficult to obtain reproducible results in those experiments due to the limitation of the resolution of the atomic absorption spectrophotometer used in this study. A means to overcome this problem might be to increase the amount of membranes in each assay. However, the available human erythrocytes to be used for preparing membranes were limited. From the reasons mentioned above, intact human erythrocytes were loaded with Ca^{2+} using the divalent cation iono-

phore, A23187. In this system, there is less variance (within 10 %) in the data. Extracellular cAMP increased the amount of membrane-bound Ca^{2+} in human erythrocytes loaded with Ca^{2+}, without significant alteration in intracellular Ca^{2+} concentration.

This may suggest that extracellular cAMP, namely cAMP incorporated and bound to membranes, stimulates intracellular Ca^{2+} binding to the membranes without greatly changing transport of extracellular Ca^{2+} across the membranes. In Ca^{2+}-loaded erythrocytes under our experimental conditions, the amount of membrane-bound Ca^{2+} and the amount of intracellular Ca^{2+} were 63 nmol per membrane equivalent of 1 ml of the packed erythrocytes and 128 nmol per 1 ml of the packed erythrocytes, respectively. Therefore, a 60 % increase in membrane-bound Ca^{2+} under the influence of cAMP should reflect a 29.5 % decrease in intracellular Ca^{2+} if the increase in membrane-bound Ca^{2+} was solely due to the intracellular Ca^{2+} binding to membrane. Nevertheless, the amount of intracellular Ca^{2+} remained nearly constant in the presence of cAMP. This may suggest that the equilibrium between extracellular and intracellular Ca^{2+} in the presence of A23187 will not be influenced by the amount of membrane-bound Ca^{2+}.

The stimulation of erythrocyte membrane phosphorylation and increase in membrane-bound Ca^{2+} appear to be specific for cAMP because other similar compounds (adenosine 2′, 3′-cyclic monophosphate and 5′-AMP) failed to provide the same response (data not shown). The small amount of leukocyte (0.02 ± 0.004 %) and reticulocyte (0.06 ± 0.021 %) contamination in our human erythrocyte preparation is not likely to account for the cAMP stimulation of membrane phosphorylation and calcium binding to the membranes. The extracellular cAMP concentration required to obtain a half-maximal increase in membrane-bound calcium was found to be about 0.3 mM, a value consistent with the concentration of extracellular cAMP which stimulated membrane phosphorylation half-maximally (Figs. 2 and 4).

Results obtained in this study alone will not be able to provide a clear model for the relationship between membrane phosphorylation and calcium binding to membranes. However, it can be concluded that cAMP stimulates calcium binding to membranes through increased phosphorylated membrane components resulting from stimulation of membrane cAMP-dependent protein kinase. Since it has been suggested that membrane-bound calcium may play an important role in the regulation of erythrocyte deformability and shape, it might be that membrane phosphorylation plays a central role in these physiological phenomena in human erythrocytes.

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膜結合カルシウムに及ぼすcAMPの影響

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摘　　要

カルシウム（輸送および結合）と膜リン酸化との機能的つながりについて研究するために，モデル細胞として人赤血球を使用し，細胞外cAMPの膜結合カルシウムと膜リン酸化に及ぼす影響を調べた．

本研究を容易にするため，2価のカチオンのIonophoreであるA23187を用いて人赤血球の細胞内カルシウム量を上昇させる方法を採用した．これらの赤血球とcAMPの相互作用によっ

て，膜リン酸化は上昇し，同時に膜結合カルシウム量も増加した．膜結合カルシウム量の最大増加は2mM cAMPで得られ，その増加量は60%であった．同条件下において，膜リン酸化の上昇は80%であった．この膜結合カルシウムに及ぼすcAMP効果の見掛け上のKaは0.30mMであり，この濃度のcAMPによって膜リン酸化の50%最大刺激が引き起こされた．上記の結果から，今まで数人の研究者によって観察されたcAMPの赤血球機能への効果は，赤血球膜リン酸化およびカルシウム膜結合に及ぼすcAMP効果によってもたらされる可能性が考えられる．