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RELATION BETWEEN PHOSPHORYLATION AND ADENOSINE TRIPHOSPHATE-DEPENDENT Ca^{2+} BINDING OF SWINE AND BOVINE ERYTHROCYTE MEMBRANES

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Summary

The correlation between the ATP-dependent Ca^{2+} binding and the phosphorylation of the membranes from swine and bovine erythrocytes was studied. The Ca^{2+} binding was measured by using $^{45}CaCl_2$, and the phosphorylation by [γ-^{32}P]ATP was studied with the technique of SDS polyacrylamide gel electrophoresis. 200 mM NaCl and KCl markedly repressed the Ca^{2+} binding of swine erythrocyte membranes. The radioactivity of ^{32}P-labelled membranes was revealed mainly in 250 000 dalton protein and a lipid fraction. NaCl and KCl also repressed the phosphorylation of the lipid which was identified as triphosphoinositide by paper chromatography. The membranes prepared from trypsin-digested erythrocytes completely retained the Ca^{2+}-binding activity, and lost 30% of (Ca^{2+} + Mg^{2+})-ATPase activity. The Ca^{2+}-binding and ATPase activity of isolated membranes decreased to 55% and to 0%, respectively, by tryptic digestion. Neither the Ca^{2+} binding nor the phosphorylation of polyphosphoinositides were detected in bovine erythrocyte membranes.

These results suggest that the formation of triphosphoinositide rather than the (Ca^{2+} + Mg^{2+})-ATPase of membranes is linked to the ATP-dependent Ca^{2+} binding of erythrocyte membranes.

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Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol bis-(β-aminoethylether)-*N*,*N*'-tetra-acetic acid.

Introduction

The concentration of Ca^{2+} in erythrocytes is about 10 μM [1—3], which is much lower than that of the extracellular fluid [4]. The existence of Ca^{2+}-stimulated Mg^{2+}-ATPase (in this paper, this ATPase is referred to as (Ca^{2+} + Mg^{2+})-ATPase) in erythrocyte membranes was demonstrated by Dunham and Glynn [5] and the extrusion of Ca^{2+} from resealed ghost was shown by Schatzmann [6]. After these findings, the correlation between the (Ca^{2+} + Mg^{2+})-ATPase and the outward Ca^{2+} transport of erythrocytes has been studied and an active transport mechanism in which (Ca^{2+} + Mg^{2+})-ATPase participates has been suggested [7].

Cha et al. [8] reported that isolated erythrocyte membranes took up Ca^{2+} from the medium in the presence of ATP and Mg^{2+} and that this Ca^{2+} uptake was intimately linked to (Ca^{2+} + Mg^{2+})-ATPase of erythrocyte membranes. On the other hand, Buckley and Hawthorne [9] showed that Ca^{2+} binding of isolated erythrocyte membranes increased with membrane-bound polyphosphoinositides which were formed in the presence of ATP and Mg^{2+}, as did (Ca^{2+} + Mg^{2+})-ATPase activity, and suggested that polyphosphoinositides might be involved in the regulation of intracellular Ca^{2+}. Subsequently, based on the preincubation effects of ATP, Buckley suggested that ATP caused the formation of inside-out vesicles of erythrocyte membranes which could accumulate Ca^{2+} on the responsibility of (Ca^{2+} + Mg^{2+})-ATPase and that the polyphosphoinositides were not dynamically involved in the accumulation of Ca^{2+} [10].

In the present work the relation between the ATP-dependent Ca^{2+} binding and the phosphorylation of membranes from swine and bovine erythrocytes has been studied. Our results show that the 250 000 dalton protein and the polyphosphoinositide of swine erythrocyte membranes were phosphorylated by ATP, and only the phosphorylation of a lipid is connected with the Ca^{2+} binding and that neither Ca^{2+} binding nor the phosphorylation of polyphosphoinositide was observed in bovine erythrocyte membranes.

Materials and Methods

Preparation of erythrocyte membranes. Fresh swine and bovine blood was collected at a local slaughterhouse a few minutes after the animals had been stunned by electric shocks. The collected erythrocytes were washed three times with the solution of 130 mM NaCl and 20 mM Tris-HCl, pH 7.4. During the washing the white buffy-layer was removed as completely as possible. The washed erythrocytes were lysed with the solution (6 vols) of 0.1 mM EDTA and 20 mM Tris-HCl, pH 7.4. Erythrocyte membranes collected by centrifugation were washed twice with the solution of 0.1 mM EDTA/20 mM Tris-HCl, pH 7.4, twice with 20 mM Tris-HCl, pH 7.4, then three times with 40 mM Tris-HCl, pH 7.4. These procedures were performed at 4°C. The final pellet was stored at −25°C before use. Protein concentration was determined by the method of Lowry et al. [11].

Measurement of Ca^{2+} binding. The reaction was started by adding $^{45}CaCl_2$ and ATP, and terminated by cooling the reaction mixture in an ice bath. The constitution of the reaction mixture is specified in the appropriate figures and

tables. After cooling, erythrocyte membranes obtained by centrifugation were washed five times with 40 mM Tris-HCl, pH 7.4, and dissolved in 1 ml of 2% SDS. The radioactivity of ^{45}Ca in the SDS solution was measured with Packard liquid scintillation spectrometer, Model 3310.

Measurement of ATPase activity. Mg^{2+}-ATPase activity was assayed at 37°C in 4 ml of the reaction mixture containing 5 mM $MgCl_2$, 0.1 mM ouabain, 1 mM ATP, 40 mM Tris-HCl, pH 7.4, 1 mM EGTA and 4—8 mg membrane protein. The reaction was started by adding ATP, and stopped by adding trichloroacetic acid. The liberated P_i was determined by the method of Allen modified by Nakamura [12]. $(Ca^{2+} + Mg^{2+})$-ATPase was estimated by subtracting Mg^{2+}-ATPase activity from the activity with 0.5 mM $CaCl_2$ substituted for 1 mM EGTA of the reaction mixture for the Mg^{2+}-ATPase.

Tryptic digestion of intact erythrocytes and isolated erythrocyte membranes. 10 ml of packed erythrocytes were suspended in 20 ml of 130 mM NaCl and 20 mM Tris-HCl, pH 7.4. 10 mg trypsin, dissolved in 1 ml of 20 mM Tris-HCl, was added to this suspension at 30°C. 20 min later, 20 mg of trypsin inhibitor was added to stop the digestion. The digested erythrocytes were washed five times with the solution of 130 mM NaCl and 20 mM Tris-HCl, pH 7.4 at temperature below 4°C. Isolated erythrocyte membranes prepared from 10 ml of packed cells were digested by the same procedure except 40 mM Tris-HCl, pH 7.4, was used instead of 130 mM NaCl/20 mM Tris-HCl.

Phosphorylation of erythrocyte membranes. The constitution of the reaction mixture is specified in the respective figures. The reaction was started by adding [γ-^{32}P]ATP and $MgCl_2$ at 20°C, then terminated by adding one-fourth of the volume of ice-cold 40% trichloroacetic acid containing 1 mM ATP and 0.5 mM P_i. ^{32}P-labelled membranes were washed five times with 1 mM ATP/0.5 mM P_i/4% trichloroacetic acid. After washing, the membranes were dissolved with 1 ml of 100% formic acid, and radioactivity was measured. Aliquots of membranes dissolved in SDS were used for SDS polyacrylamide gel electrophoresis. The method of Avruch and Fairbanks [13] was followed in electrophoresis except that 4% gel and chromotope 2R was used as a tracking dye. γ-globulin, albumin, ovalbumin, chymotrypsinogen and cytochrome *c* were used as molecular weight references. After the phoresis, gels were stained with Coomassie blue. Duplicate unstained gels were sliced into 3-mm thick sections, and the radioactivity in each slice was measured. Phospholipids from the phosphorylated membranes extracted with acidic chloroform/methanol were chromatography according to Steiner and Lester's method [14].

Chemicals. Chemicals used were of analytical grade. $^{45}CaCl_2$ and $^{32}P_i$ were purchased from the Japan Radioisotope Association. [γ-^{32}P]ATP was made according to the method of Glynn and Chapell [15]. Authentic tri-, di- and monophosphoinositides were supplied by Dr. K. Hayashi, Department of Biochemistry, Medical School, Gunma University. Trypsin (Type I) and trypsin inhibitor (Type I-S) were purchased from Sigma Chemical Co. Cyclic AMP was purchased from Boehringer Mannheim-Yamanouchi K.K.

Results

Swine erythrocyte membranes bound ^{45}Ca in the presence of ATP and Mg^{2+}. This Ca^{2+} binding depended on the reaction temperature showing its maximal

activity at 40°C. The membranes bound 0.2 nmol Ca^{2+}/mg membrane protein in 30 min at 37°C. In the absence of ATP the amount of Ca^{2+} binding was only 1/10 of this value. Temperature had no effect on the ATP-independent Ca^{2+} binding.

Fig. 1 shows the effect of Na^+ and K^+ on the ATP-dependent Ca^{2+} binding. Almost no binding was observed when these ions were present when the reaction was initiated. Na^+ and K^+ which were added in the middle of the reaction stopped the Ca^{2+} binding immediately, and no decrease of bound Ca^{2+} was observed. These ions had no effect on Ca^{2+} binding in the absence of ATP.

The effect of Na^+ and K^+ on the release of ^{45}Ca from membranes was examined. ^{45}Ca was bound to isolated membranes by ATP, and free ^{45}Ca in the medium was eliminated by washing the membranes. Na^+ and K^+ added into the medium containing the ^{45}Ca-bound membranes did not induce the release of ^{45}Ca from the membranes whether ATP was present or not.

These results show that Na^+ and K^+ inhibit the reaction which is directly connected with the Ca^{2+} binding, and induce no release of bound Ca^{2+}. Ca^{2+} seems to bind the membrane stably.

As shown in Fig. 2, the membranes which were preincubated with ATP and Mg^{2+} at 37°C prior to the addition of Ca^{2+} increased the Ca^{2+} binding as the preincubation time was prolonged.

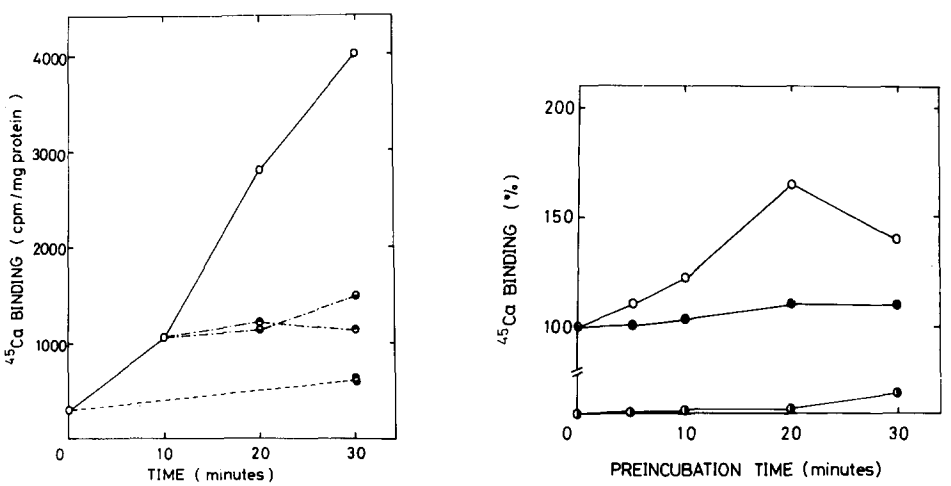

Fig. 1. Effect of NaCl and KCl on the ATP-dependent Ca^{2+} binding of swine erythrocyte membranes. The reaction mixture contained 10 mM $MgCl_2$, 5 μM $^{45}CaCl_2$, 0.1 mM ouabain, 1 mM ATP, 40 mM Tris-HCl (pH 7.4) and 17.4 mg protein of swine erythrocyte membranes. Ca^{2+} binding without NaCl and KCl (○———○), with 200 mM KCl (●· · · · ·●), or 200 mM NaCl (●· · · · ·●). 10 min after the reaction without NaCl and KCl was started, 200 mM KCl (●· — · — ·●) or 200 mM NaCl (●· — · — ·●) were added.

Fig. 2. Effect of the preincubation of swine erythrocyte membranes and ATP on the Ca^{2+} binding. Membranes were preincubated with ATP for appropriate times at 37°C in the medium containing 0.1 mM ouabain, 1 mM ATP, 40 mM Tris-HCl (pH 7.4) and 21.6 mg membrane protein with 10 mM $MgCl_2$ (○, ◑) or without $MgCl_2$ (●). After the preincubation, the Ca^{2+} binding was assayed in the medium of 10 mM $MgCl_2$, 5 μM $^{45}CaCl_2$, 0.1 mM ouabain, 1 mM ATP and 40 mM Tris-HCl (pH 7.4) for 30 min at 37°C (○, ●) or at 0°C (◑). The Ca^{2+} binding of non-preincubated membranes at 37°C for 30 min was indicated as 100%. Volumes of the preincubation and the binding reaction mixture were 5.4 ml and 6 ml, respectively.

No increase of the binding was observed when the reaction was performed at 0°C.

To ascertain the role of membrane proteins on the Ca^{2+} binding, two kinds of trypsin-digested membrane were prepared. One was obtained by tryptic digestion of isolated membranes. As shown in Table I, the membranes prepared from trypsin-digested erythrocytes completely retained the binding activity, and lost 30% of $(Ca^{2+} + Mg^{2+})$-ATPase activity. On the other hand, in the isolated membranes digested with trypsin, Ca^{2+} binding and ATPase activity decreased to 55% and to 0%, respectively. Trypsin penetrates the isolated membranes obtained by hemolysis, but not the membranes of intact erythrocytes. The results obtained with the digested membranes indicate that in intact erythrocytes Ca^{2+} binds the membranes on the cytoplasmic side. The protein part of ATPase is considered to be embedded in and to protrude from both sides of the membrane. Decrease of ATPase activity which was observed in both of the digested membranes may be due to the digestion of the exposed parts of the enzyme. Repression of the Ca^{2+}-binding activity by the digestion suggests either that Ca^{2+} anchors to proteins of the membranes, directly or indirectly, or that there exist some enzymes which participate in the Ca^{2+} binding and utilize ATP as the substrate. The results that the digested membranes retained 55% of the binding activity and none of $(Ca^{2+} + Mg^{2+})$-ATPase activity excludes the possibility of this enzyme being $(Ca^{2+} + Mg^{2+})$-ATPase.

The phosphorylation of erythrocyte membranes by terminal phosphate of ATP was examined. Erythrocyte membranes were incubated with [γ-^{32}P]ATP in the presence of Mg^{2+}, and the reaction was stopped at appropriate times. ^{32}P-labelled membranes were analyzed with SDS-polyacrylamide gel electrophoresis. Fig. 3 shows the protein and radioactivity patterns of SDS polyacrylamide gel electrophoresis of ^{32}P-labelled membranes. Bands 1, 2, 3, 4, 5 and 6 in gel correspond to proteins having molecular weights of 250 000, 230 000, 100 000, 70 000, 50 000 and 40 000, respectively. The radioactivity appeared mainly in two fractions: a protein fraction and lipids. The position of the phosphorylated protein corresponds to a protein band (band 1 and/or band 2) of an apparent molecular weight of 250 000. The other radioactivity peak, which

TABLE I

ATPases AND THE ATP-DEPENDENT Ca^{2+} BINDING OF TRYPSIN-DIGESTED MEMBRANES OF SWINE ERYTHROCYTES

Two kinds of trypsin-digested membrane were prepared as described in Materials and Methods. The conditions of the Ca^{2+} binding were the same as in Fig. 1. Values are expressed as relative activity (%).

Membranes	Activities		
	Mg^{2+}-ATPase	$(Ca^{2+} + Mg^{2+})$-ATPase	Ca^{2+}-binding
Control *	100	100	100
Membranes from trypsin-digested erythrocytes	73	67	104
Trypsin-digested membranes	13	1	55

* Nondigested isolated membranes.

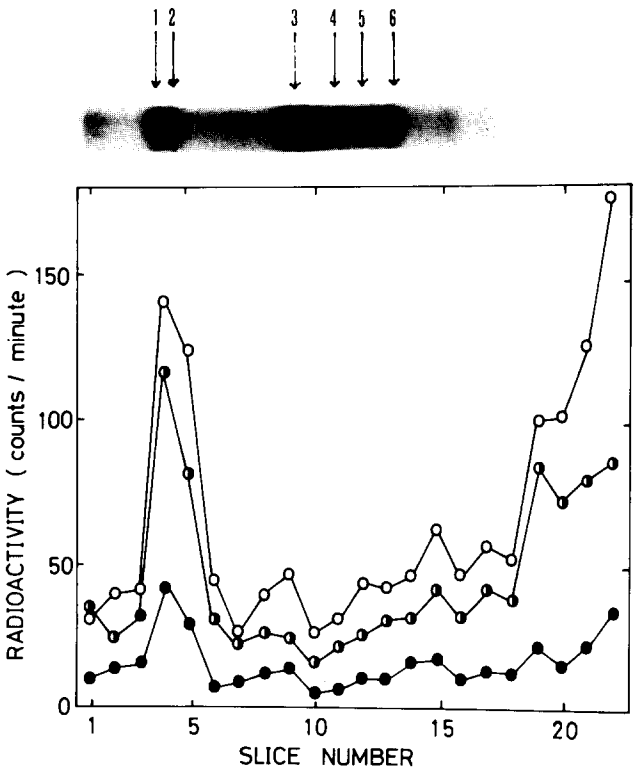

Fig. 3. SDS polyacrylamide gel electrophoresis of ^{32}P-labelled swine erythrocyte membranes. The phosphorylation was carried out at 20°C for 1 min (●), 3 min (◑) or 5 min (○) in the medium containing 10 mM $MgCl_2$, 1 mM EGTA, 1 mM ouabain, 5 μM [γ-^{32}P]ATP, 40 mM Tris-HCl (pH 7.4) and 10.4 mg membrane protein/ml. Above is the protein band pattern.

appeared after slice number 20, corresponds to a lipid fraction. The lipids of ^{32}P-labelled membranes were extracted with acidic chloroform/methanol and the radioactivity of ^{32}P was collected only in the chloroform phase. On analysis of polyphosphoinositides the radioactivity in the chloroform phase was found in the position of triphosphoinositide on the paper chromatogram (Fig. 4). Buckley and Hawthorne reported that no other lipid among the lipids of swine erythrocyte membranes, except for polyphosphoinositide, was phosphorylated by ATP [9]. Their results also showed that the radioactivity of ^{32}P was found in both di- and triphosphoinositides, and a higher activity was revealed in diphosphoinositide. As shown in Fig. 4, no radioactivity was detected in diphosphoinositide position in this experiment. Some activity of ^{32}P was recorded in triphosphoinositide after the phosphorylation at 4°C.

To know the relation between the ATP-dependent Ca^{2+} binding and the phosphorylation of the membranes, Na^+ and K^+, which were shown as potent inhibitors of Ca^{2+} binding, were added to the reaction mixture for the phosphorylation. As shown in Fig. 5, 200 mM of NaCl and KCl markedly repressed the incorporation of ^{32}P into the lipid fraction. The protein pattern of the electrophoretogram was similar to that of Fig. 3. The phosphorylation of the

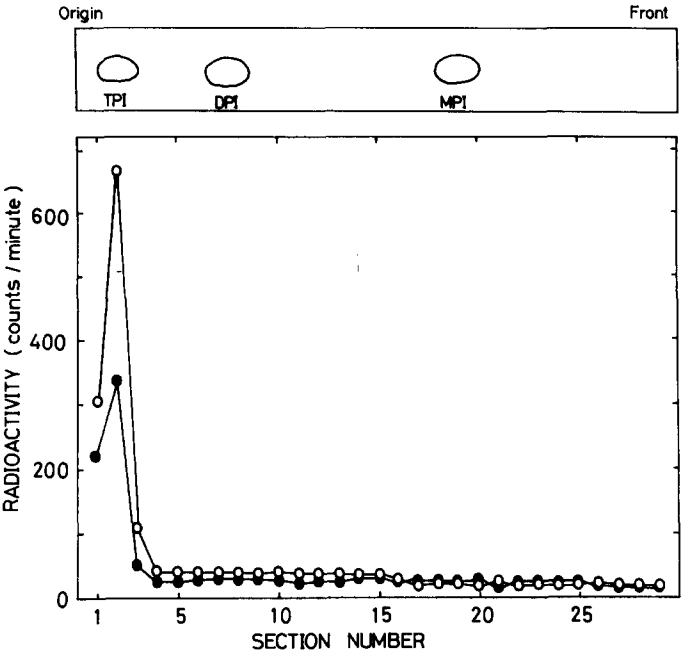

Fig. 4. Chromatogram of ^{32}P-labelled phospholipids of swine erythrocyte membranes. The reaction medium for the phosphorylation was the same as Fig. 3. The phosphorylation was carried out for 5 min at 0°C (●) or 20°C (○). The upper diagram shows the migration of authentic tri-, di- and monophosphoinositide.

250 000 dalton protein, however, was not inhibited by these ions. These results strongly suggest that the ATP-dependent Ca^{2+} binding is linked to triphosphoinositide formation on the erythrocyte membranes.

By adding unlabelled ATP during the phosphorylation reaction, the increase

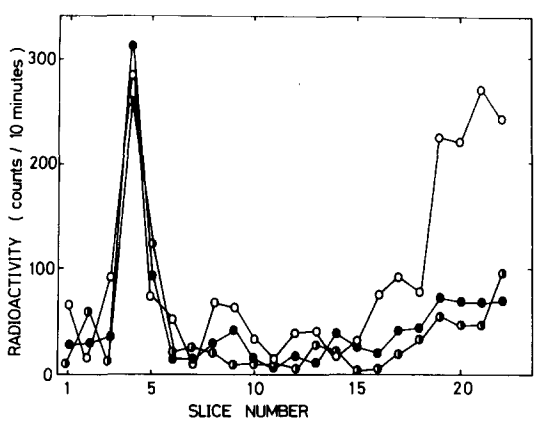

Fig. 5. SDS polyacrylamide gel electrophoresis of swine erythrocyte membranes phosphorylated in the presence of NaCl or KCl. Swine erythrocyte membranes were phosphorylated for 5 min at 20°C in the same medium as Fig. 3 except for the use of 6.4 mg membrane protein. Without the addition of NaCl and KCl (○), with 200 mM NaCl (●) or with 200 mM KCl (◑).

of the radioactivity of the membranes was stopped immediately, but no decrease of the radioactivity of ^{32}P incorporated in the membranes was observed.

Bovine erythrocyte is known as 'low-K^+ erythrocyte' because it contains a lower concentration of K^+ than Na^+ in contrast to human or swine [16]. A reason for this descrepancy may be due to the difference in the activity of ($Na^+ + K^+$)-ATPase of these membranes [17]. Some other properties of bovine erythrocyte membranes may also differ from those of swine. Therefore, the ATP-dependent Ca^{2+} binding and the phosphorylation of bovine erythrocyte membranes were studied. Bovine erythrocyte membranes bind only a small amount of Ca^{2+} even in the absence of Na^+ and K^+. Cyclic AMP showed no effect on the Ca^{2+} binding.

Fig. 6 shows the distribution of the radioactivity on the gel after SDS polyacrylamide gel electrophoresis of ^{32}P-labelled bovine erythrocyte membranes. Molecular weight of proteins corresponding to numbered bands are equal to those of Fig. 3. The major activity was collected in the 250 000 dalton protein part and the second largest peak appeared in a 100 000 dalton protein. In the lipid fraction, however, almost no radioactivity was found.

Results obtained from bovine erythrocyte membranes strongly support the proposal that the Ca^{2+} binding induced by ATP is connected with the phosphorylation of polyphosphoinositide.

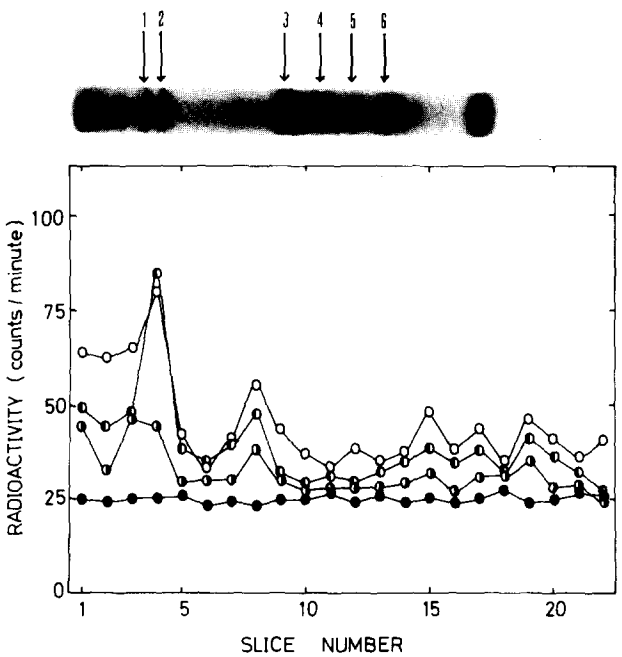

Fig. 6. SDS polyacrylamide gel electrophoresis of bovine erythrocyte membranes. Bovine erythrocyte membranes were phosphorylated at 37°C for 0 min (●), 1 min (◑), 3 min (◐) or 5 min (○) in the same medium as Fig. 3 except for the use of 11.8 mg bovine erythrocyte membrane protein. The photograph is the band pattern of the proteins and the graph presents the distribution of the radioactivity.

Discussion

It has been shown that erythrocytes have a Ca^{2+}-pump which required ATP, and isolated erythrocyte membranes bind Ca^{2+} in the presence of ATP and Mg^{2+}. Cha et al. [8] reported that by altering the experimental conditions, (Ca^{2+} + Mg^{2+})-ATPase activity changed in a manner parallel to the change of the ATP-dependent Ca^{2+} uptake. Based on these results they concluded that the Ca^{2+} uptake was intimately linked to (Ca^{2+} + Mg^{2+})-ATPase. Although it was not known whether the Ca^{2+} taken up by isolated erythrocyte membranes was transported into vesicles or was bound to membranes, they suggested that Ca^{2+} was bound to membranes in the presence of ATP through the mechanisms associated with Ca^{2+} transport system [8]. Buckley and Hawthorne reported that membranes that increased Ca^{2+} binding with elevated polyphosphoinositide level had higher (Ca^{2+} + Mg^{2+})-ATPase than membranes with normal levels [9].

Our results with the trypsin-digested membranes, however, clearly showed that there was no parallelism in the activities of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} binding.

A major polyphosphoinositide detected in this experiment was triphosphoinositide contrary to diphosphoinositide in the results of Buckley and Hawthorne. The discrepancy between their results on polyphosphoinositide and ours may be caused by the difference in the preparation of the isolated membranes. As shown in Fig. 2, membranes which were preincubated with ATP at 37°C bound no Ca^{2+} at 0°C. This result indicates that the fluidity of the membranes may influence the Ca^{2+} binding.

Avruch and Fairbanks reported that in the presence of 5—20 mM $MgCl_2$, the radioactivity from [γ-^{32}P]ATP transferred to two protein fractions and a lipid fraction of erythrocyte membranes [18]. They also presented that higher concentrations of NaCl and KCl inhibited the phosphorylation of 105 000 dalton protein and the lipid fraction, and activated the phosphorylation of 215 000 dalton protein. Our results agree well with theirs except that the phosphorylation of 105 000 dalton protein was very low even in the absence of NaCl and KCl.

Cha and Lee regarded the phosphorylated components of erythrocyte membranes as the reaction intermediate of (Ca^{2+} + Mg^{2+})-ATPase without identification of the components, and presented an enzymatic sequence of the Ca^{2+}-transport mechanism of erythrocytes [19]. According to their proposed sequence, Mg^{2+} phosphorylates and Ca^{2+} dephosphorylates the (Ca^{2+} + Mg^{2+})-ATPase. However, Garraham and Rega have recently shown that Ca^{2+} is required for the step of phosphorylation of (Ca^{2+} + Mg^{2+})-ATPase [20]. Avruch and Fairbanks reported that in the presence of 3 mM $MgCl_2$ and 120 mM KCl, the terminal phosphate of ATP transfers mainly into 215 000 dalton protein and lipids [18]. Therefore, under the experimental conditions of Cha and Lee, the amount of the phosphorylated intermediate of the Ca^{2+}-pump ATPase, if any, would be very low.

Results presented in this paper suggest that triphosphoinositide formation rather than (Ca^{2+} + Mg^{2+})-ATPase is intimately associated with the ATP-dependent Ca^{2+} binding. The participation of polyphosphoinositides in the

active transport of cations has been suggested because of their rapid turnover and their strongly negative charge. In this study, however, no evidence was obtained which might indicate that the Ca^{2+} binding connected with triphosphoinositide formation has anything to do with active cation transport.

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