Biochimica et Biophysica Acta, 330 (1973) 294–301

BBA 76513

CALCIUM BINDING BY THE ERYTHROCYTE MEMBRANE

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(Received August 20th, 1973)

SUMMARY

Calcium binding to isolated erythrocyte membranes was stimulated by ATP. This stimulatory effect of ATP required Mg^{2+}.

Ethacrynic acid and ruthenium red inhibited the stimulatory effect of ATP.

About 80% of the bound Ca^{2+} was associated with the membrane protein.

The strongly bound Ca^{2+} was confined to two high molecular weight membrane proteins.

Increasing amounts of Ca^{2+} bound to the membrane inhibited Na^{+} binding in the presence of ATP.

INTRODUCTION

Ca^{2+} bound to membranes exert physical and physiological effects which include excitation–contraction coupling in muscle, stimulus–secretion coupling in endocrine glands, maintenance of cell shape, and control of membrane transport[1]. Although Ca^{2+} binding to and release from the membranes are likely to be involved in these processes, the precise mechanism of Ca^{2+} action is still rather obscure.

We therefore decided to use the erythrocyte membrane as a model system to study factors controlling Ca^{2+} binding and to investigate the molecular groups responsible for this binding.

MATERIALS

Double glass distilled water was used in the preparation of all reagents. Plastic containers were used to store the calcium-containing solutions in order to avoid adsorption on to glass.

ATP, cyclic AMP, *N*-acetylneuraminic acid, neuraminidase (Type V) and phospholipase C (Type 1) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Trypsin and ruthenium red was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and ethacrynic acid from Merck Sharp and Dohme Ltd., Hoddesdon, Herts, U.K.

^{45}Ca (10–25 mCi/mg Ca^{2+}) and ^{22}Na (carrier free) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Soluene™ 100 and Insta Gel were

supplied by Packard Instrument Company Inc., 2200 Warrenville, Downers Grove, Illinois 60515, U.S.A.

The erythrocytes were prepared from recently expired acid–citrate–dextrose blood obtained from the Clinical Pathology Dept., Manchester Royal Infirmary.

METHODS

Preparation of erythrocyte membranes

The erythrocytes were washed 3 times with isotonic choline chloride, taking care to remove the plasma and white cells as completely as possible. A stock buffer solution containing 0.2 M NaCl, 0.075 M Na_2HPO_4 and 0.025 M KH_2PO_4 (pH 7.2) was diluted to give buffers of 60, 30 and 20 mOsm concentrations. EDTA was added to the diluted buffers to give a final concentration of 1 mM. The cells or ghosts were washed twice with each of the above diluted buffers using 8 times the original cell volume. After 6 washings with EDTA-containing buffers, the membranes were washed 3 times in 5 mM histidine imidazole buffer to remove the EDTA.

Measurement of Ca^{2+} binding

In the standard assay erythrocyte membranes (2 mg membrane protein) were incubated in a reaction mixture containing 120 mM KCl, 30 mM histidine imidazole buffer (pH 7.0), 5 mM $MgCl_2$, with and without 1 mM ATP and 10 μM $CaCl_2$ (0.05 μCi ^{45}Ca). The mixture (total volume 1 ml) was incubated at 37 °C for 20 min in a shaking water bath. The reaction was stopped by adding 5 ml of ice-cold 0.5 mM histidine imidazole buffer pH 7.0. The membranes were then centrifuged at $6000 \times g$ for 15 min at 0–4 °C. Membranes were resuspended in the same washing buffer and centrifuged a further two times before preparation for radioactive counting.

Measurement of the Na^+ binding

The Na^+ binding procedure was carried out as described above for the binding of Ca^{2+} except that the reaction mixture contained the following: 20 mM KCl, 50 mM histidine imidazole buffer (pH 7.0), with and without 1 mM ATP, 1 mM NaCl (0.5 μCi ^{22}Na) and 2 mg membrane protein, in a volume of 1 ml.

Separation of membrane lipid- and protein-bound Ca^{2+}

The membrane pellet containing ^{45}Ca was extracted 3 times with chloroform–methanol (1:1, v/v) by the method of Burger *et al.*[2], and the insoluble protein precipitate was removed by centrifugation. The lipid extract was combined with chloroform and water to give chloroform–methanol–water proportions of 10:5:1 (by vol.). After centrifugation at $1085 \times g$ for 10 min, an aqueous upper phase and an organic lower phase formed. The organic phase was taken to contain the phospholipid-bound Ca^{2+}, the aqueous phase the unbound Ca^{2+} and the precipitate the protein-bound Ca^{2+}.

Enzymic modification of isolated erythrocyte membranes

The membranes were incubated separately with trypsin, phospholipase C and neuraminidase in 10 ml Tris–HCl buffer (pH 7.0). The mixture contained 0.1 mg enzyme protein per mg of membrane protein in a total volume of 1 ml. The reaction

was terminated by the addition of 5 ml ice-cold 0.5 mM histidine imidazole buffer (pH 7.0). The membranes were centrifuged at 6000 × *g* for 20 min at 0–4 °C and washed a further 2 times with this buffer.

Electrophoretic separation of membrane proteins

The erythrocyte membrane proteins were separated by polyacrylamide gel electrophoresis using the buffer system developed by Neville[3]. Gels containing 3% (stacking gel) and 10% (separation gel) were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of *N,N'*-bis(methylene)acrylamide. Both gels contained 0.1% sodium dodecylsulphate and were polymerised chemically by the addition of 0.025% by volume of tetramethylethylenediamine and ammonium persulfate. 5-cm gels were prepared in glass tubes of a total length of 7.5 cm with an internal diameter of 6 mm. The stacking gel length was 1 cm. The buffers used were: upper reservoir 0.04 M boric acid–0.041 M Tris (pH 8.6), stacking gel 0.0267 M H_2SO_4–0.0541 M Tris (pH 6.1), separating gel and lower reservoir 0.0308 M HCl–0.4244 M Tris (pH 9.18). The membranes (50–75 μg protein) were suspended in a total volume of 0.2 ml containing the following: 0.0625 M Tris–HCl (pH 6.8), 2% sodium dodecylsulphate, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. The proteins were completely dissociated by immersing the mixture for 3 min in boiling water. Electrophoresis was carried out at a current of 2 mA/tube until the bromophenol blue band reached a mark about 0.5 cm from the end of the gel.

After electrophoresis the gels were stained with 1% amido black in methanol–acetic acid–water (50:7:43, by vol.). Destaining was carried out with 3–5 changes of methanol–acetic acid–water (same proportions as above) for 24–36 h. After destaining the gels were scanned with a Joyce–Loebl 'chromoscan' recording densitometer.

Location of ^{45}Ca in gels

After electrophoresis the gels were immediately sectioned with a gel slicer or sharp blade. The sections were solubilised with 0.3 ml of 30% H_2O_2 overnight at 50 °C.

Determination of radioactivity

Membrane ^{45}Ca: The membranes containing ^{45}Ca were oven-dried and then dissolved in 0.5 ml Soluene™. The ^{45}Ca was counted with a Nuclear Chicago liquid scintillation counter using 0.5% 2,5-diphenyloxazole (PPO) in toluene as scintillant.

Aqueous solutions of ^{45}Ca: The scintillant used here was 0.5% PPO in toluene mixed with Triton X-100 (2:1, v/v).

^{45}Ca in polyacrylamide gels: Scintillant used was Insta Gel.

$^{22}Na^+$ was counted with a Wallach sodium iodide crystal detector.

Analytical methods

Protein was determined by the method of Lowry *et al.*[4], using crystalline bovine serum albumin as standard.

Sialic acid was determined by the method of Aminoff[5], using *N*-acetylneuraminic acid as standard.

Phosphate was determined by the method of Atkinson[6], using KH_2PO_4 as standard.

RESULTS

Ca^{2+} binding by the isolated membranes

Ca^{2+} binding by the isolated membranes in the presence of ATP was stimulated about 3-fold after 15–20 min incubation (Fig. 1). In the presence of 10 μM Ca^{2+} and 5 mM Mg^{2+} the optimum ATP concentration was about 1 mM (Fig. 2). Stimulation by ATP of Ca^{2+} binding required Mg^{2+}, the maximum effect being obtained at a concentration of 2 mM (Fig. 3). In the absence of ATP increasing [Mg^{2+}] decreased Ca^{2+} binding.

Effect of ion transport inhibitors on Ca^{2+} binding

Ruthenium red and ethacrynic acid inhibited Ca^{2+} binding in the presence of ATP (Fig. 4), the concentrations for 50% inhibition being about 10^{-5} M and $5 \cdot 10^{-5}$M respectively. Neither of these compounds had any effect on Ca^{2+} binding in the absence of ATP. Ouabain (10^{-4} M) had no effect on Ca^{2+} binding in the presence or absence of ATP.

Distribution of Ca^{2+} within the membrane components

After extraction of the membrane lipids with chloroform–methanol, over 80% of the bound Ca^{2+} was retained in the protein fraction (Table I). The lipid phase contained 10 to 15% of the Ca^{2+}, while 4 to 5% appeared in the aqueous phase or to

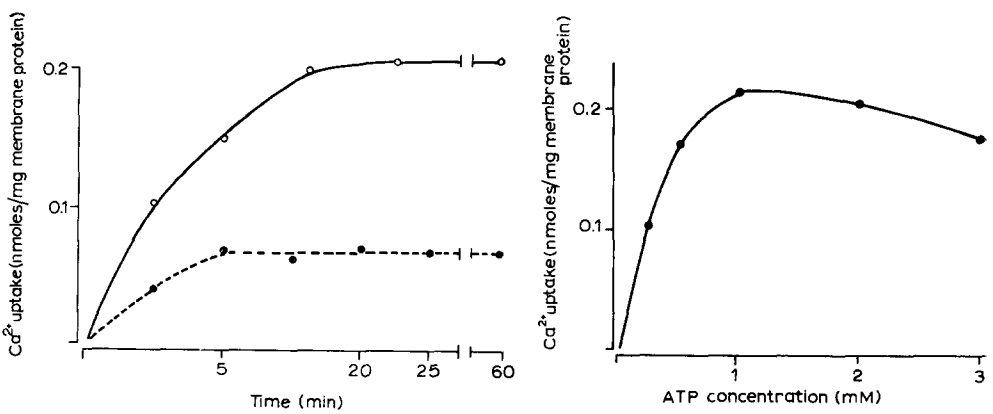
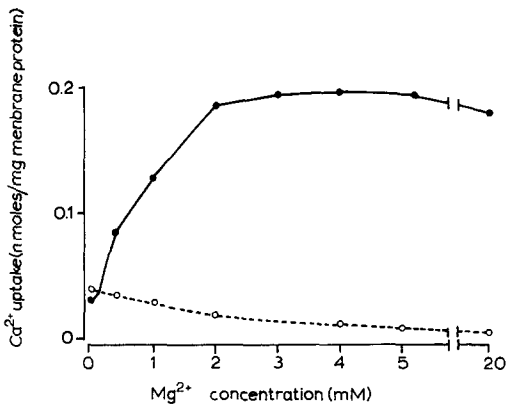


Fig. 1. Time course of the binding of Ca^{2+} in the presence and absence of ATP. The incubation mixture contained 2 mg membrane protein, 120 mM KCl, 30 mM histidine imidazole buffer (pH 7.0), 5 mM $MgCl_2$ with and without 1 mM ATP and 10 μM $CaCl_2$ (0.05 μCi ^{45}Ca) in a total volume of 1 ml. Incubation was carried out at 37 °C for various time intervals. After incubation the membranes were washed 3 times with 5 ml 0.5 mM histidine-imidazole buffer (pH 7.0). ○—○, 1 mM ATP present; ●---●, control.

Fig. 2. Binding of Ca^{2+} in the presence of various concentrations of ATP. The incubation system consisted of 2 mg membrane protein, 120 mM KCl, 30 mM histidine imidazole buffer (pH 7.0), 10 μM $CaCl_2$ (0.05 μCi ^{45}Ca) and varying concentrations of ATP.



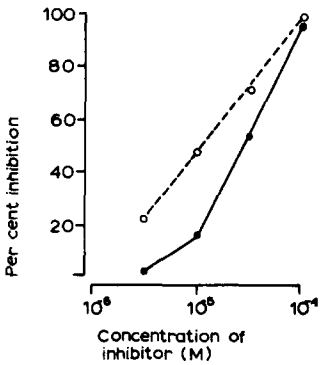


Fig. 3. Effect of varying the concentration of Mg^{2+} on Ca^{2+} binding in the presence and absence of ATP. Ca^{2+} was bound to the membranes in the presence and absence of 1 mM ATP by the procedure described in Methods except that the concentration of Mg^{2+} was varied. ●—●, no ATP present; ○---○, 1 mM ATP present.

Fig. 4. Effect of inhibitors on the ATP stimulated Ca^{2+} binding. Ca^{2+} was bound to the membranes in the presence of 1 mM ATP by the standard procedure described in Methods except that varying concentration of inhibitor was added to the assay mixture. ○---○, ruthenium red; ●—●, ethacrynic acid.

TABLE I

DISTRIBUTION OF CALCIUM WITHIN THE MEMBRANE COMPONENTS

Membranes were labelled with ^{45}Ca in the presence and absence of ATP, by the standard procedure described in Methods. After binding the membranes were washed free of unreacted Ca^{2+} and the lipid extracted with chloroform–methanol as described above. Values are means of 3 experiments carried out in duplicate, ±S.E. The recovery of protein and phospholipid in their respective fractions was 95% and 87%, respectively.

Fraction	*Amount recovered*			
	No ATP added		*In presence of ATP*	
	nmoles/g protein	*% of total*	*nmoles/g protein*	*% of total*
Protein	49.1 ± 2.3	80.0	164.2 ± 5.6	84.2
Lipid	9.3 ± 1.6	15.2	18.7 ± 1.8	9.6
Aqueous	2.9 ± 0.7	4.8	12.1 ± 0.9	6.2

be unbound. The unbound Ca^{2+} may have been derived from inter- or intra-membrane vesicular spaces or it may have been released from a weak ligand during the fractionation procedure. The presence of Ca^{2+} in the protein fraction does not preclude the possibility of the ion forming protein–phospholipid bridges in the intact membrane.

The effect of enzyme modification of the membrane on Ca^{2+} binding

The effect of some commercially available enzymes on the release of Ca^{2+} bound in the presence of ATP is shown in Table II. Trypsin which hydrolysed about 25% of

the membrane protein displaced 45% of the Ca^{2+}. Phospholipase C, while removing 69% of phospholipid phosphorus from the membrane, released 27% of the bound Ca^{2+}. Neuraminidase, although cleaving 84% of the sialic acid residues from the membranes, had no significant effect on the Ca^{2+} release.

TABLE II

EFFECT OF ENZYMES ON THE RELEASE OF Ca^{2+}

Ca^{2+} was bound to the membrane by suspending 2 mg membrane protein in an incubation mixture which contained 120 mM KCl, 5 mM $MgCl_2$, 30 mM histidine imidazole buffer (pH 7.0), 1 mM ATP and 10 μM $CaCl_2$ (0.05 μCi ^{45}Ca) in a total volume of 1 ml. After incubation at 37 °C for 20 min the membranes were freed of unreacted Ca^{2+} by washing 3 times in 0.5 mM histidine imidazole buffer (pH 7.0). They were then treated with the hydrolytic enzymes for 1 h at 37 °C, washed as above and the ^{45}Ca remaining in the membranes determined. Values are average of 3 experiments carried out in duplicate ±S.E.

Enzyme	*% Decrease in bound calcium (untreated= 100)*
Trypsin	44.9±4.7
Phospholipase-C	26.7±2.8
Neuraminidase	0.7±0.1

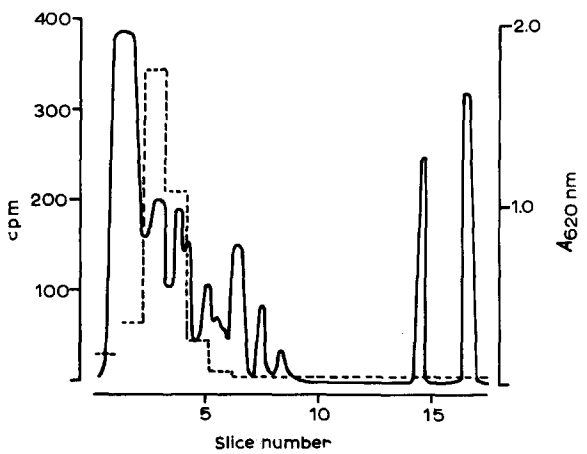


Fig. 5. Separation of erythrocyte membrane proteins and determination of ^{45}Ca content of each. The membrane proteins were separated on 10% polyacrylamide gels as described in text. After electrophoresis the gels were either stained with 1% amido black and scanned on a densitometer or sliced into 3-mm gel sections for ^{45}Ca determination of the separated protein bands. ———, $A_{620\ nm}$; - - - - - - -, ^{45}Ca cpm.

Electrophoretic separation of calcium binding proteins

A densitometer tracing of the erythrocyte membrane proteins after polyacrylamide gel electrophoresis and staining by amido black is shown in Fig. 5. Most of the recovered ^{45}Ca was located in two high molecular weight proteins.

In order to obtain sufficient counts of ^{45}Ca after electrophoresis the specific activity of the radioactive calcium used in the incubation mixture was ten times

greater than that described for the standard binding procedure and the gels were sliced without prior staining after electrophoresis to avoid loss of ^{45}Ca. Only about 8% of the ^{45}Ca was recovered in the two gel slices containing the calcium binding proteins.

Effect of membrane-bound calcium on Na^+ binding to isolated membranes

In the absence of ATP, increasing the amount of membrane-bound Ca^{2+} had no effect on Na^+ binding. In the presence of 1 mM ATP about twice as much Na^+ was bound (2.08-fold increase, average of 3 experiments) but this additional Na^+ binding was progressively inhibited by increasing the membrane-bound Ca^{2+} (Fig. 6). When the membranes were incubated for 1 h at 37 °C with 1 mM EDTA the amount of bound calcium was reduced by 85% and the ATP activating effect on Na^+ binding was restored to 76% of its original value.

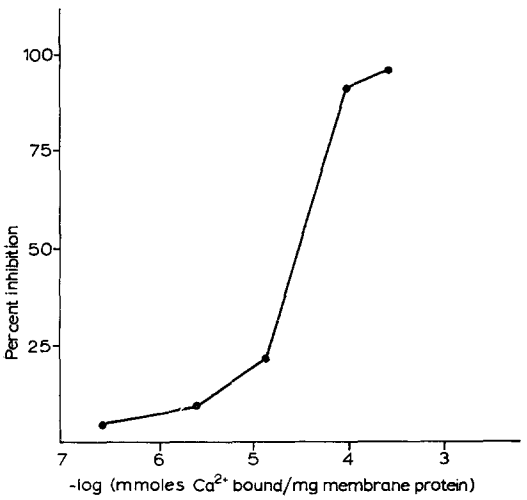


Fig. 6. Effect of membrane-bound Ca^{2+} on Na^+ binding in the presence of ATP. Membranes were loaded with Ca^{2+} by adding varying concentration of Ca^{2+} to the assay mixture. Unreacted Ca^{2+} was removed by washing 3 times in 0.5 mM histidine–imidazole buffer (pH 7.0). ^{22}Na binding was then carried out on the Ca^{2+}-loaded membranes using an incubation system that contained 2 mg membrane protein, 20 mM KCl, 50 mM histidine imidazole buffer (pH 7.0), 1 mM NaCl (0.5 μCi ^{22}Na) and 1 mM ATP in a final volume of 1 ml. After 20 min incubation at 37 °C the membranes were washed 3 times with 0.5 mM histidine imidazole buffer.

DISCUSSION

The amount of calcium bound by the erythrocyte membranes in the present investigation was of the order of that found for retinal rod disk membranes[7]. Also the degree of stimulation of Ca^{2+} uptake by ATP was roughly in agreement with that in isolated retinal rod disk membranes[7] and fibroblast membranes[8]. Forstner and Manery[9] have previously reported that ATP has no stimulatory effect on Ca^{2+} binding by isolated erythrocyte membranes. These investigators did not, however, include Mg^{2+}. In contrast, our results clearly show that Mg^{2+} is necessary for the activating effect of ATP on Ca^{2+} binding.

Separation of membrane lipid and protein fractions showed that over 80%

of the Ca^{2+} taken up was bound to the latter fraction. In support of this result was the finding that trypsin, which hydrolysed about 25% of the membrane protein, released 45% of the bound calcium. The results are thus in agreement with those of Forstner and Manery[10] who also showed most of the calcium in the erythrocyte membrane to be protein-bound, but they disagree with Shlatz and Marinetti[22] who reported that the calcium in liver plasma membranes was largely bound to phospholipid. Treatment with neuraminidase suggested that sialic acid residues were not involved in Ca^{2+} binding in the present investigation. These results show the limitations of the model phospholipid membranes which have been widely used for studying the nature of the Ca^{2+}-membrane interaction[12,13].

The physiological significance of the results presented here is as yet unknown. Further work is required to ascertain whether the ATP stimulated Ca^{2+} binding is related to Ca^{2+}-ATPase. The observation that most of the membrane Ca^{2+} was protein bound is in agreement with the fact that most soluble Ca^{2+}-binding-molecules previously isolated are of protein nature[14–16]. A Ca^{2+}-protein interaction is more likely to perform a direct and specific biological action than reaction of the cation with a phospholipid molecule.

ACKNOWLEDGEMENT

This work was supported by the Cystic Fibrosis Research Trust.

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