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Calcium Binding by Human Erythrocyte Membranes

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1. The characteristics of Ca^{2+} binding to haemoglobin-free human erythrocyte membranes were investigated by using ^{45}Ca and centrifugation partition of 'ghosts' from their external incubation medium. Equilibrium of 'ghosts' with external Ca^{2+} required less than 15min. 2. The binding did not vary with temperature in the range 0–37°C. 3. At pH7.4 'ghosts' bound a maximum of 283μmol of Ca^{2+}/g of 'ghost' protein, equivalent to 6.85×10^7 Ca^{2+} ions per cell. 4. Increasing the ionic strength from 0.01 to 0.46 diminished Ca^{2+} binding, as did ATP in concentrations ranging from 0 to 15mM in the incubation medium. 5. An increase of the pH from 3.0 to 9.3 caused a marked increase in the amount of Ca^{2+} bound. 6. Extraction of ^{45}Ca-labelled 'ghosts' with chloroform–methanol showed that the distribution of Ca^{2+} was: 79% protein-bound, 16% lipid-bound, 5% in the aqueous phase, presumably non-bound. Most of the lipid-bound Ca^{2+} (about 80%) was associated with a phospholipid fraction containing phosphatidylserine, phosphoinositides and phosphatidylethanolamine, giving a molar Ca^{2+}: phosphorus ratio of about 1:2.

Ca^{2+} ions bound to and contained within cellular membrane systems exert profound physical and physiological effects, which include excitation–contraction coupling in muscle, stimulus–secretion coupling in endocrine glands, intercellular communication and adhesion, as well as preservation of cell shape and cell permeability barriers (Manery, 1966, 1969). Ca^{2+} ions appear to bind to isolated membranes, forming a Ca^{2+}–membrane complex, as shown for sarcoplasmic reticulum (Ebashi & Lipmann, 1964; Martonosi & Feretos, 1964), erythrocyte 'ghosts' (Gent, Trounce & Walser, 1964; Kwant & Seeman, 1969), liver microsomal fraction (Carvalho, Sanui & Pace, 1963) and renal microsomal fraction (Palmer & Posey, 1970). Some insight into the nature of Ca^{2+}–membrane interaction has been provided by studies on simple model phospholipid membranes (Hendrickson & Fullington, 1965; Hauser, Chapman & Dawson, 1969; Hauser & Dawson, 1967; and others). These studies suggest that in model and, by inference, in biological membranes, Ca^{2+} binds via an electrostatic attachment to acidic phospholipid phosphate groups, causing molecular packing, increased electrical resistance (Ohki & Goldup, 1968) and relative impermeability to water and small cations (van Breemen, 1968). On the other hand, with only a few exceptions (see, e.g., Joos & Carr, 1969), the possibility of membrane protein–Ca^{2+} interaction has been largely unexplored to date.

In the present paper the factors governing Ca^{2+} binding by erythrocyte 'ghosts' are described. Also, results are presented which show that almost 80% of the bound Ca^{2+} is associated with the protein, and approx. 20% with the lipid component of the membrane.

EXPERIMENTAL

Solutions. The water used for all solutions was rendered Ca^{2+}-free by passage through a Barnstead ion-exchanger, redistilled in an all-Pyrex glass distillation apparatus (Corning, model AG-2), and demineralized in a standard mixed-bed resin (Bantam, no. 0802) in a Barnstead demineralizer. Ca^{2+}-free water and all aqueous solutions were stored in Nalgene containers, which were initially rinsed with this triply purified water. Reagent-grade chemicals were used throughout.

A standard stock solution of $CaCl_2$ (100mM) was prepared by dissolving anhydrous $CaCO_3$ (1.02g) in M-HCl (20ml). After heating to drive off CO_2 the solution was diluted to 100ml with 0.01M-tris–HCl buffer; the final pH was 7.4. The concentration of the solution was checked by using an emission flame attachment to a Zeiss PMQ II spectrophotometer.

Preparation of haemoglobin-free 'ghosts'. These were prepared from outdated whole human blood in ACD ('acid–citrate–dextrose') preservative, washed in 0.155M-tris–HCl buffer, pH7.4, and haemolysed in 0.01M-tris–HCl buffer, pH7.4. The remaining haemoglobin was determined by the method of Dodge, Mitchell & Hanahan (1963). The average residual haemoglobin was 0.014% of the mean corpuscular haemoglobin, indicating that the 'ghosts' were essentially haemoglobin-free.

'Ghost' protein. The concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951)

with crystalline bovine serum albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) as a reference standard. Dry weights of 'ghosts' were obtained by difference weighing at 100°C of 3ml samples of 'ghosts' suspended in water. The average protein concentration by weight was 40% (total range 38–42%) of the total dry weight in three separate 'ghost' samples (nine estimates).

Ca^{2+}-binding procedure. 'Ghosts' containing 4mg of protein were suspended in 0.01M-tris–HCl buffer, pH7.4, and mixed with $CaCl_2$ (100mM) containing 0.5μCi of $^{45}CaCl_2$. The final suspension was 10ml in volume, and contained $CaCl_2$ at a final concentration of 5 or 10mM as indicated in the Results section. The specific radioactivity of $^{45}CaCl_2$ was approx. 8000d.p.m./μmol and was determined separately in each experiment. After incubation of the 'ghosts' for 1h at 22°C with intermittent mixing, the samples were centrifuged at 39000*g* for 10min in a Sorvall RC 2B refrigerated centrifuge. Exactly 9ml of supernatant was removed by aspiration and the remaining 'ghosts', in a volume of 1ml in buffer, were thoroughly mixed. The 'ghosts' were washed three times in exactly the same fashion with 9ml of Ca^{2+}-free 0.01M-tris–HCl buffer, pH7.4, and samples were taken from the remaining 1ml for ^{45}Ca and protein determinations. Before radioactivity counting, the 'ghost' samples were partially dried in an oven at 100°C, solubilized in 1ml of NCS (Nuclear–Chicago Solubilizer, a quaternary ammonium base, 0.6M in toluene) solution by the procedure of Dryden & Manery (1970), and mixed with 15ml of scintillation fluid (see '^{45}Ca-counting procedure'). The specific radioactivity of the incubation medium (d.p.m./μmol) was used to calculate the number of μmol of Ca^{2+} bound/g of protein.

^{45}Ca-counting procedure. A standard quench curve for channels-ratio counting of ^{45}Ca radioactivity was constructed with a Nuclear–Chicago Mark I liquid-scintillation counter. Quenched standards were prepared with the use of acetone added in increasing amounts (0 to 0.4ml) to 0.02ml of a dilute solution of $^{45}CaCl_2$ (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) containing 24000d.p.m. and 0.05ml of $CaCl_2$ non-radioactive carrier solution (100mM). To each vial was added 1ml of NCS plus 15ml of scintillation fluid, prepared by mixing 4g of 2,5-diphenyloxazole, 100mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 1l of toluene. The solubilizing agent (NCS) was added to the standards to validate comparisons with dried ^{45}Ca-labelled 'ghosts' which were solubilized in NCS before radioactivity counting (see 'Ca^{2+}-binding procedure'). The efficiency of counting ranged from 75 to 85% in standard solutions, and from 65 to 85% in 'ghost' samples. Suitable corrections for radioisotope decay were made, as well as corrections for quenching.

ATP–'ghost'–Ca^{2+} incubations. The effect of ATP on Ca^{2+}-binding by 'ghosts' was tested in two ways. (*a*) 'Ghosts' were incubated with 5mM-$CaCl_2$ as described in the 'Ca^{2+}-binding procedure' except for the addition of small volumes of a stock solution of tris–ATP (500mg of ATP/10ml in 0.01M-tris–HCl buffer, adjusted to pH7.4 with M-NaOH). The final ATP concentrations of the incubation media were varied from 0 to 15mM. (*b*) 'Ghosts' were first incubated with 5mM-$CaCl_2$, centrifuged and washed as described in the 'Ca^{2+}-binding procedure' section. The ^{45}Ca-labelled 'ghosts' were then re-incubated in 10ml of 0.01M-tris–HCl buffer, pH7.4, containing 0–15mM-tris–ATP for 1h at 22°C. After both types of incubations, the 'ghosts' were centrifuged and washed three times as described above, and measurements were made of the bound Ca^{2+}.

Lipid extraction of ^{45}Ca-labelled 'ghosts'. Several samples of ^{45}Ca-labelled 'ghost' suspensions were prepared as described under '^{45}Ca-binding procedure', by using an external $CaCl_2$ concentration of 10mM to saturate Ca^{2+}-binding sites on the 'ghost'. The 'ghost' samples were combined, centrifuged into a firm pellet, and all supernatant fluid was removed by aspiration. The final radioactive pellet contained 20–40μmol of Ca^{2+}, 50–100mg of protein and 50–100μmol of total P. The pellet was extracted three times with chloroform–methanol (1:1, v/v) by the method of Burger, Fujii & Hanahan (1968) and the insoluble protein residue was removed by centrifugation. It was heated at 90°C in 1–3ml of M-NaOH for 1h to convert it into a fine suspension, and then cooled and left for protein and ^{45}Ca determinations. The lipid extract was combined with chloroform and water to give chloroform/methanol/water proportions 10:5:1 (by vol.) by the method of Folch, Lees & Sloane-Stanley (1957). After centrifugation at 1085*g* for 10min, an aqueous upper phase and an organic lower phase formed. No interface material was noted.

Phospholipid phosphorus determination. Samples for phosphorus determination were dried under a stream of nitrogen and digested in 1.5ml of hot 70% (v/v) $HClO_4$, under a fume hood. After digestion, the method of Bartlett (1959) was followed for subsequent analysis. A reagent blank and reference standards (0–0.24μmol) prepared from a stock 1mM-KH_2PO_4 solution were subjected to the entire procedure.

Extraction of phosphoinositides from insoluble protein residue. The methods of Eichberg & Hauser (1967) and Gonzalez-Sastre & Folch-Pi (1968) were used to extract the phosphoinositides that were held as contaminants within the insoluble protein residue left after chloroform–methanol extraction of ^{45}Ca-labelled 'ghosts'. Standards of tri- and mono-phosphoinositides were subjected to the procedure together with experimental samples. The phosphoinositides were identified by ascending t.l.c. on silica gel H.R. (Brinkman Instruments, Toronto, Canada) in 1% (w/v) potassium oxalate. The solvent system was chloroform–methanol–4M-NH_3 (9:7:2, by vol.).

Silicic acid chromatography of lipid extract. The lipid extract obtained from chloroform–methanol extraction of ^{45}Ca-labelled 'ghosts' was evaporated to dryness, redissolved in 1ml of chloroform and passed through a column (5cm×0.5cm) of silicic acid. The latter was prepared from heat-activated silicic acid (Mallinckrodt Chemical Works, Montreal, Canada) containing 50% (w/w) Celite Flow Aid (Johns–Manville Co., New York, N.Y., U.S.A.) dissolved in chloroform. In general, there was less than 50μg of lipid phosphorus in each sample put on a column. Lipid was eluted from the column by the methods of Hanahan, Dittmer & Warashina (1957) or Blumenstein (1964) with 30ml of chloroform to obtain neutral lipid, then 30ml of chloroform–methanol (4:1, v/v) to obtain acidic phospholipid, followed by 30ml of chloroform–methanol (3:2, v/v) to obtain neutral phospholipid, and finally 30ml of methanol, which eluted no lipid if the previous elutions had been efficient.

Thin-layer chromatography of phospholipids. Plates were coated with silica gel H.R. by using a slurry of 30g of gel in 100ml of water; they were activated before use by heating at 100°C for 1h followed by cooling in a desiccator at 22°C. Each phospholipid sample (15μg of total phosphorus) was contained in 0.1ml of chloroform–methanol (2:1, v/v), and was applied as a band at the origin. Chromatograms were developed in an ascending neutral system of chloroform–methanol–water (65:35:4, by vol.) for 30–45min. Purified reference standards of phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, mono- and tri-phosphoinositides were also applied and R_F values compared with those of 'ghost' phospholipid samples. The phospholipids were coloured by exposure to iodine vapour; ninhydrin–butanol spray was used to identify amino-group-containing phospholipids, and the spray described by Dittmer & Lester (1964) was used to show the absence of contaminating non-lipid organic phosphates from the plates.

RESULTS

Characteristics of binding of Ca^{2+} to 'ghosts'. The influence of several environmental factors on Ca^{2+} binding by 'ghosts' was studied in an attempt to gain some understanding of the nature of the binding process and to establish the optimum conditions for maximum binding before proceeding to the identification of Ca^{2+}-binding sites within 'ghosts'.

Owing to the centrifugation binding technique employed, the shortest incubation time-interval investigated was 15min, and by this time full equilibration of 'ghosts' and Ca^{2+} had apparently been reached (Fig. 1). In subsequent experiments incubation periods of 1h were chosen for convenience. Table 1 indicates that Ca^{2+} binding did not vary with temperature in the range 0–37°C.

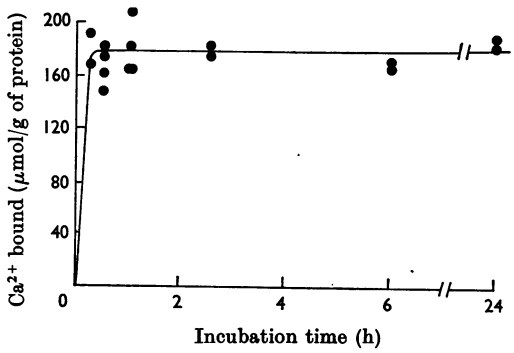

Fig. 1. Effect of incubation time on Ca^{2+} binding to 'ghosts'. 'Ghost' suspensions in 0.01M-tris–HCl buffer, pH7.4, containing 4mg of protein, were incubated with 5mM-$CaCl_2$ containing 0.5μCi of $^{45}CaCl_2$ in the same buffer at 22°C in a total volume of 10ml. At various time-intervals they were centrifuged and washed three times in the same buffer (Ca^{2+}-free) (see the Experimental section). Each value is the mean of duplicate analyses performed on a single 'ghost' sample.

The effect of ionic strength is illustrated in Fig. 2. As the ionic strength in the initial incubation medium was increased from 0.014 to 0.465, Ca^{2+} binding was decreased by 73%. Using a somewhat different procedure, Gent *et al.* (1964) observed a decrease in binding of 74% as the ionic strength was increased from 0.01 to 0.06. The curve in Fig. 2 is biphasic in nature, which may indicate the existence of two binding species. One of these, accounting for 40% of the bound Ca^{2+}, is very sensitive to ionic strength, and the other, which is less sensitive, accounts for 60%.

In all of the binding experiments described below, the ionic strength of the incubation medium varied between 0.024 and 0.039, which are the calculated ionic strengths in 0.01M-tris–HCl buffer with 5 and 10mM-calcium chloride respectively. Since this range is close to the steep part of the curve in Fig. 2, small changes in ionic strength would be expected to alter the Ca^{2+}-binding capacity of the 'ghosts' considerably. This possibility was kept in mind in each experimental design.

Since traces of Ca^{2+} may normally be associated with the erythrocyte membranes (Harrison & Long, 1968; Weed, La Celle & Merrill, 1969), it was considered necessary to eliminate the possibility that our measurements of Ca^{2+} binding to 'ghosts' partly reflected simple exchange of Ca^{2+} between endogenous 'ghost' Ca^{2+} and $^{45}Ca^{2+}$ in the external medium. To test this, 'ghosts' were incubated in EDTA (0–5mM) at pH7.4 to remove endogenous calcium. The 'ghosts' were subsequently washed and re-incubated with ^{45}Ca in the usual fashion. As shown in Table 2, the amount of Ca^{2+} bound did not differ between control and EDTA-treated samples. Since the Ca^{2+} content of erythrocyte 'ghosts' is small and EDTA (5mM) has been reported to remove 90% of erythrocyte Ca^{2+} at pH7.4 (Harrison & Long, 1968) we concluded that exchange of $^{45}Ca^{2+}$ with endogenous Ca^{2+} in the membrane was negligible in these experiments. Thus in this work 'ghosts' were not pre-treated with EDTA.

Fig. 3 illustrates the linear correlation between the amount of Ca^{2+} bound and 'ghost' protein concentration, showing that binding is dependent on the total number of 'ghost' receptor sites available for Ca^{2+} ions. The amount of Ca^{2+} bound is therefore expressed as units per g or mg of 'ghost' protein.

In some membrane systems, such as sarcoplasmic reticulum (Martonosi & Feretos, 1964), mitochondria (Lehninger, 1966) and renal microsomal fraction (Palmer & Posey, 1970), ATP has been found to facilitate Ca^{2+} uptake. In erythrocyte

Table 1. *Effect of incubation temperature on* Ca^{2+} *binding to 'ghosts'*

In a final incubation volume of 10ml, 'ghost' suspensions containing 4mg of protein were incubated at various temperatures with 5mM-$CaCl_2$ containing 0.5μCi of $^{45}CaCl_2$ in 0.01M-tris–HCl buffer, pH7.4. After 60min the 'ghosts' were washed three times at the appropriate temperature and bound Ca^{2+} was measured (see the Experimental section). An analysis of variance performed on the results showed no significant differences between values at the three temperatures studied.

Temperature (°C)	No. of experiments	Ca^{2+} bound (μmol/g of protein)	
		Mean	Range
0	4	150	133–164
25	21	150	130–170
37	4	135	105–167

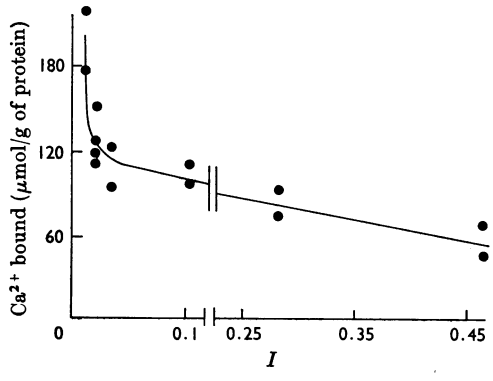

Fig. 2. Effect of ionic strength on Ca^{2+} binding to 'ghosts'. 'Ghost' suspensions containing 4mg of protein were incubated in a final volume of 10ml with 4.2mM-$CaCl_2$ (containing 0.5μCi of $^{45}CaCl_2$) at 22°C for 1h in tris–HCl buffer, pH7.4, of concentrations in the range 0.001–0.500M. The 'ghosts' were centrifuged, washed three times with the appropriate Ca^{2+}-free buffer and the Ca^{2+} bound was measured (see the Experimental section). Each value represents the mean of duplicate analyses on a single 'ghost' sample.

Table 2. *Effect of EDTA pretreatment on subsequent* Ca^{2+} *binding by 'ghosts'*

'Ghost' suspensions in 0.01M-tris–HCl buffer, pH7.4, containing 4mg of 'ghost' protein were incubated in a final volume of 10ml with 0–5mM-EDTA (disodium salt) for 1h at 22°C, centrifuged at 39000*g* for 10min and washed three times with the same buffer to remove EDTA. The 'ghosts' were subsequently incubated in 5mM-$CaCl_2$ (containing 0.5μCi of $^{45}CaCl_2$) (see the Experimental section). Each value shown represents the mean of duplicate analyses on individual 'ghost' samples.

Concn. of EDTA in preincubation medium (mM)	Ca^{2+} bound (μmol/g of protein)
0	175, 174, 148, 201
2	132, 178, 128, 215
3	177, 177
5	203, 173

'ghosts', however, no enhancement was observed (Fig. 4). In fact, ATP decreased Ca^{2+} binding when Ca^{2+}, 'ghosts' and ATP were incubated simultaneously (curve *a*) and also when ATP was added to 'ghosts' that had been previously labelled with ^{45}Ca (curve *b*). A similar effect of ATP was observed by Sanui & Pace (1967*b*) in liver microsomal fractions. Since ATP added to the incubation medium would be expected to raise the ionic strength of the medium, and also to chelate Ca^{2+} ions, calculations (Table 3) were made to try to account for the observed decrease in Ca^{2+} binding on the basis of increased ionic strength (Fig. 2) or of decreased Ca^{2+} concentration. In incubations with 5mM-ATP the decrease of 3.5% in Ca^{2+} binding (column 3) predicted from the increased ionic strength, and the observed (28%) decrease in binding, are significantly different. At 10 and 15mM-ATP the observed decrease of 55% is much greater than that expected (11.6 and 27.6%). Undoubtedly much of the decrease in binding caused by the presence of ATP can be attributed to a marked decrease in Ca^{2+} concentration (column 5). In fact, in the presence of 10 and 15mM-ATP the Ca^{2+} concentrations (0.16 and 0.07mM) are so low that almost no binding should have occurred (see Fig. 5 of the present paper and Fig. 2 of Kwant & Seeman, 1969). Thus Ca^{2+} binding was greater than expected from consideration of either ionic strength or free Ca^{2+} ions, which suggests indirectly that the binding was somehow facilitated. This phenomenon would be consistent with the proposal of Abood and co-workers that a Ca^{2+}–ATP–membrane ternary complex is formed (Abood, 1966; Abood, Kurahasi & Perez del Carro, 1967). However, this possibility was not explored further by us, since it was clear from our experiments that, as a source of metabolic energy, ATP was definitely not required for optimum Ca^{2+}-binding conditions.

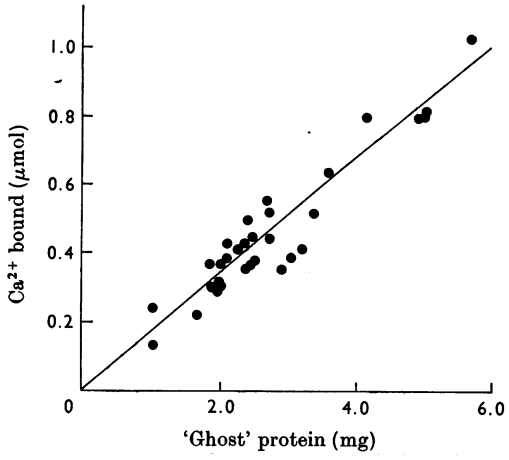

Fig. 3. Effect of 'ghost' protein concentration on Ca^{2+} binding by 'ghosts'. 'Ghost' suspensions of different protein concentrations were incubated with 5mM-$CaCl_2$ containing 0.5μCi of $^{45}CaCl_2$ as described in the Experimental section. Ca^{2+} bound was measured in duplicate for each incubation mixture. Each point represents a separate experiment.

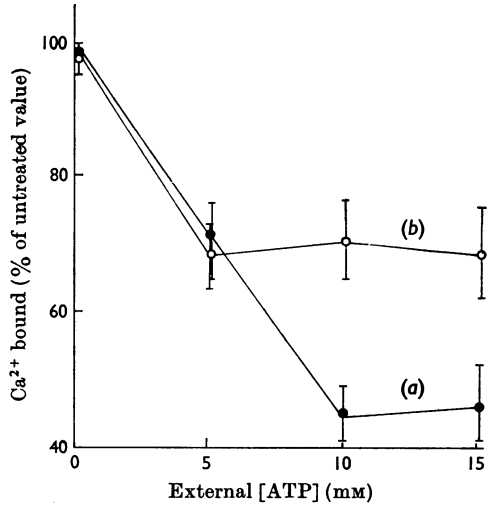

Fig. 4. Effect of ATP on Ca^{2+} binding to 'ghosts'. 'Ghosts' containing 4mg of protein were incubated in 5mM-$CaCl_2$ containing 0.5μCi of $^{45}CaCl_2$ at pH7.4 in 0.01M-tris–HCl buffer for 1h at 22°C. (*a*) Tris–ATP was added to give a final concentration of 0–15mM. Total volume of each incubation was 10ml. After incubation the 'ghosts' were washed three times with Ca^{2+}-free buffer and Ca^{2+} bound (μmol/g of protein) was determined. In (*b*) the 'ghosts' were centrifuged after Ca^{2+} binding, washed three times and then reincubated in tris–ATP (final concentration 0–15mM) with the same buffer at pH7.4 for 1h (see the Experimental section). Each value represents the mean of four separate experiments with each analysis performed in duplicate. The results are expressed as a percentage of total Ca^{2+} bound in 'ghosts' not treated with tris–ATP (100% = 160μmol of Ca^{2+}/g of protein). The bars indicate the total range of values obtained.

At pH7.4, maximum binding of Ca^{2+} ions by 'ghosts' was 283μmol of Ca^{2+}/g of 'ghost' protein (Fig. 5). Above a concentration of 10mM-calcium chloride in the incubation medium, no further binding took place, indicating that all possible sites had been saturated. Despite the fact that the 'ghosts' were washed three times with Ca^{2+}-free media, much Ca^{2+} remained firmly bound. The saturation value is in the same range as those found for 'ghosts' by Gent *et al.* (1964), Kwant & Seeman (1969) and Carvalho *et al.* (1963) using different binding techniques. The saturation value of 283μmol/g of 'ghost' protein is equivalent to 0.113mol of Ca^{2+}/kg 'ghost' dry weight, calculated on the basis that 'ghost' protein equals 40% of 'ghost' dry weight (see the Experimental section). If the dry weight of one 'ghost' is 1pg (calculated from Engelman, 1969), it can also be calculated that, at saturation, there are 6.85×10^7 Ca^{2+} ions bound per 'ghost'

[0.113mol/kg = 0.113mol/10^{15} 'ghosts' = $(6.06 \times 10^{23} \times 0.113)/10^{15} = 6.85 \times 10^7$ Ca^{2+} ions/'ghost'].

The influence of pH on Ca^{2+} binding is shown in Fig. 6. As the pH was increased from 3.0 to 6.5, 70μmol of Ca^{2+} was taken up, which is about 22% of that bound at pH9.3; between pH6.5 and 8.0, another 31% (100μmol) and between pH8.0 and 9.3 another 47% (155μmol) was bound. It was difficult to assign pH values at which half of the total bound Ca^{2+} was taken up, but the dependence of binding on pH suggests that Ca^{2+} binds ionically and that several types of dissociable anionic membrane groups may be responsible (see the Discussion section).

Distribution of Ca^{2+} *within membrane components.* The relative importance, quantitatively, of protein and lipid as Ca^{2+}-receptor components in the 'ghosts' was tested in a series of experiments in which lipid was extracted from Ca^{2+}-saturated 'ghosts' and the partition of the Ca^{2+} determined (see the Experimental section). Extractions with chloroform–methanol (1:1 and 2:1, v/v) yielded three phases in each of which protein, total phosphorus and ^{45}Ca radioactivity were measured

Table 3. *Effect of ATP on* Ca^{2+} *binding by 'ghosts'*

Values shown for the observed decrease in bound Ca^{2+} are taken from Fig. 4, curve (*a*). The total ionic strengths of the incubation solutions (legend to Fig. 4) were calculated by using the equation $I = 0.5cz^2$ (Clark, 1952), where *c* and *z* refer to concentration and charge of each ion respectively. The concentrations of free Ca^{2+}, 'free ATP' and Ca^{2+}–ATP were estimated by using the formation constant of $10^{3.77}$ M^{-1} for Ca^{2+}–ATP (Epstein & Whittam, 1966). The decrease in Ca^{2+} bound (column 3) predicted from the increase in ionic strength was estimated from Fig. 2.

Incubation solution		Decrease in Ca^{2+} bound (%)		Equilibrium concentration (mM)		
Tris–ATP (mM)	I	Expected because of increased I	Observed	Free Ca^{2+}	Free ATP	Ca^{2+}–ATP
0	0.220	0.0	0	5.00	0.0	0.0
5	0.044	3.5	28	0.84	0.84	4.16
10	0.092	11.6	55	0.16	5.16	4.84
15	0.145	27.6	55	0.07	10.07	4.93

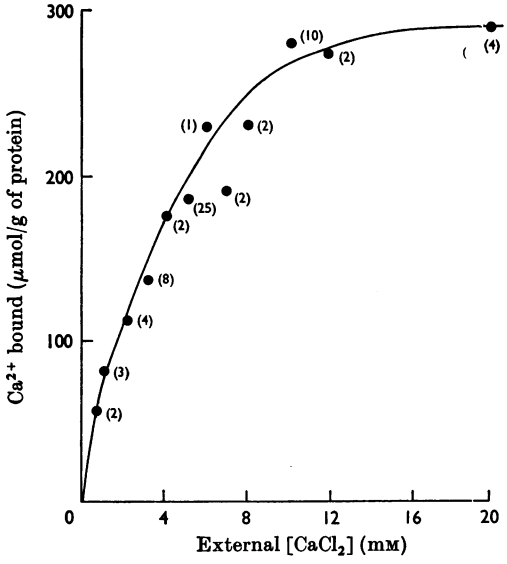

Fig. 5. Effect of external $CaCl_2$ concentration on Ca^{2+} binding by 'ghosts'. 'Ghosts' were incubated in 0.01 M-tris–HCl buffer, pH 7.4, for 1 h at 22°C in increasing (0–20 mM) concentrations of $CaCl_2$ containing 1 μCi of $^{45}CaCl_2$. After three washings, the amount of Ca^{2+} bound was measured (see the Experimental section). Mean values are plotted and the numbers in parentheses indicate the total number of experiments performed at each concentration of $CaCl_2$. Analyses of protein and ^{45}Ca were done in duplicate.

(Table 4). Only a small fraction (5%) of the total Ca^{2+} was found in the aqueous phase; this Ca^{2+} was probably derived from the inter- and intra-vesicular spaces of the original 'ghost' suspension and may therefore largely represent non-bound Ca^{2+}. Surprisingly, 79% of the total Ca^{2+} was found in the insoluble protein residue, which contained 94% of the membrane protein, whereas only 16% of the Ca^{2+} was found in the lower lipid-containing phase. This distribution was unexpected, since it has been demonstrated by many authors that phospholipids of biological membranes possess a high affinity for bivalent cations (for references see Manery, 1966, 1969; Rothstein, 1968).

Since a small amount of 'ghost' lipid phosphorus (1–2%) remained in the protein residue (Table 4) after lipid extraction, it was considered possible that protein-bound Ca^{2+} might be associated with highly acidic phosphoinositide contaminants of the residue (Hauser & Dawson, 1967). Accordingly, the protein residue was extracted three times with chloroform–methanol–HCl (as described in the Experimental section) to remove phosphoinositides. Portions of the extract were removed for phosphorus determination and for identification by t.l.c. with suitable mono- and tri-phosphoinositide standards. The phosphoinositides which were extracted from 68 mg of insoluble protein residue contained 0.77 μmol of phospholipid phosphorus, an amount that could bind, at maximum, only 0.385 μmol of Ca^{2+}, assuming a molar ratio of Ca^{2+} to phosphorus of 1:2. This would account for only 2.36% of the total protein-bound Ca^{2+}. Thus the Ca^{2+} contained in the protein residue was indeed protein-bound, and not associated with lipid contaminants.

The possibility that the chloroform–methanol extraction itself caused changes in Ca^{2+} distribution was considered, i.e. that Ca^{2+} migrated from the non-polar lipid environment to the more polar protein environment during the extraction. The following calculation shows that even at its maximum binding capacity the lipid component could not account for more than about one-quarter of the Ca^{2+} bound to the membrane. Assuming that Ca^{2+} can only bind to acidic phospholipids (and, perhaps, in small amounts to phosphatidylethanol-

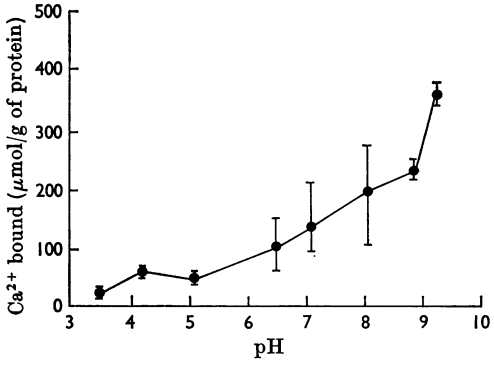

Fig. 6. Effect of pH on Ca^{2+} binding by 'ghosts'. 'Ghosts' were incubated with 5mM-$CaCl_2$ containing 0.5μCi of $^{45}CaCl_2$ for 1h at 22°C in media of different pH values. Buffers used for the pH ranges 1.0–7.0 and 6.0–9.3 were 0.01M-succinate–NaOH and 0.01M-tris–HCl respectively. Subsequent washing steps were performed in the same buffer solutions (Ca^{2+}-free) as used in each incubation. Each value plotted represents the mean of four experiments and the bars indicate the total range of values obtained.

amine; Joos & Carr, 1967), the total concentration of Ca^{2+}-binding lipid (including phosphatidylethanolamine) in 1g of 'ghost' dry weight is 0.048g (i.e. 1g 'ghost' dry weight contains 12% by weight of phospholipid, of which 40% is in the form of phosphatidylethanolamine, phosphatidylserine and phosphoinositides; van Deenen & de Gier, 1964). If the average molecular weight of phospholipid is taken as 750, 0.048g equals 64μmol of phospholipid. Assuming that each Ca^{2+} ion is bound via two phosphate groups at a maximum, then the phospholipids contained in 1g of 'ghost' dry weight could bind a maximum of 32μmol of Ca^{2+}. Under saturation binding conditions (i.e. 283μmol of Ca^{2+} bound per g of 'ghost' protein, which is equivalent to 113.2 μmol of Ca^{2+}/g 'ghost' dry weight) this represents only 28% of the total Ca^{2+} bound to the whole membrane.

It is clear that there are not enough acidic phospholipids to account for the amount of Ca^{2+} that binds to 'ghosts' at pH7.4. From the chloroform–methanol-extraction studies only 16% of the total bound Ca^{2+} was recovered in the lipid phase. Thus if migration of Ca^{2+} from phospholipid to protein had occurred during chloroform–methanol extraction, the extent of migration would have been no greater than about $28-16=12\%$ of the total Ca^{2+} bound. This calculation suggests that the phenomenon of migration, even if present, would not produce a serious distortion of the Ca^{2+}-distribution results.

The distribution results reported in the present paper were confirmed by experiments showing that

Table 4. *Distribution of* Ca^{2+} *within membrane components separated by lipid extraction*

'Ghost' suspensions were incubated for 1h with 10mM-$CaCl_2$ containing 1μCi of $^{45}CaCl_2$. After removal of the supernatant the 'ghost' pellet was extracted with chloroform–methanol and the resultant aqueous, protein and lipid phases were analysed for protein, total P and ^{45}Ca (see the Experimental section). The total recoveries of protein, ^{45}Ca and P were 95%, 87 and 89% respectively. The percentage distribution of the recovered ^{45}Ca, protein and P are given as mean values of six separate 'ghost' extractions. Each analysis was performed in duplicate. Values in parentheses represent the S.E.M.

	Amount recovered (% of total)		
	Ca^{2+}	Protein	Phosphorus
Upper (aqueous) phase	5 (±2)	3 (±1)	7 (±2.5)
Protein residue	79 (±4)	94 (±5)	1 (±1)
Lower (organic) phase	16 (±3)	3 (±1)	92 (±4)

protein carboxyl groups play a major role in Ca^{2+} binding. These have been reported briefly (Forstner & Manery, 1970).

Because 16% of the Ca^{2+} was associated with the lipid phase of the membrane (Table 4), attempts were made to identify the specific classes of lipid that bound Ca^{2+} ions. The lipids were therefore fractionated by silicic acid column chromatography (see the Experimental section) and each resulting fraction was sampled for ^{45}Ca, total phosphorus analysis and t.l.c. to identify the lipid classes. Of the total Ca^{2+} recovered from the column, 77% was found in fraction 2 (Table 5) containing phosphatidylserine, phosphatidylethanolamine and phosphoinositides. The calcium/phosphorus molar ratio was 1.0:2.3, suggesting that within the lipid, at a molecular level, one Ca^{2+} ion binds to two lipid phosphate groups.

DISCUSSION

Ca^{2+}-binding procedure. The binding technique used throughout these experiments was chosen because three washes of the 'ghost' pellet in Ca^{2+}-free media would be expected to remove the cations loosely associated with the membrane and leave only the Ca^{2+} ions that were firmly bound. Fractionation experiments show that this condition was largely fulfilled, since 95% of the total 'ghost' Ca^{2+} was recovered bound firmly to 'ghost' lipid and protein, whereas only 5% was present in the aqueous phase. Also Fig. 5 demonstrates that despite three washes of the 'ghost' pellet, a saturation level indicative of high Ca^{2+}-binding capacity was reached when the initial incubation medium contained about 10mM-calcium chloride. Since the

Table 5. *Silicic acid chromatography of 'ghost' lipids*

After incubation of 'ghosts' (4mg of protein) with $CaCl_2$ (5mM) and $^{45}CaCl_2$ and extracting them with chloroform–methanol (1:1, v/v), the lipid fraction was eluted from silicic acid columns with chloroform–methanol solutions of various proportions (for details see the Experimental section). The components of the lipid fraction were identified by t.l.c. Samples of each of the four resulting fractions were analysed for ^{45}Ca and total P; the results are expressed as percentages of recovered Ca^{2+} and P respectively. Each value is the mean of three separate experiments. All analyses were performed in duplicate. Total recovery of P and Ca^{2+} from silicic acid columns was 94 and 101% respectively.

Fraction no.	Identity	Ca^{2+} (% of total)	Phosphorus (% of total)	Ca/P (molar ratio)
1	Neutral lipid	10.2	0.57	1.0:0.1
2	Phosphatidylserine, phosphatidylethanolamine and phosphoinositides	77.3	59.9	1.0:2.3
3	Phosphatidylcholine and sphingomyelin	12.5	39.5	1.0:21.3
4	No lipid detected	0	0	0

washing steps may have introduced new equilibrium conditions, no estimate of the free Ca^{2+} concentration at true equilibrium could be made. Thus the method did not permit calculation of the affinity constant of the Ca^{2+}–membrane complex (Edsall & Wyman, 1958).

The experiments relating to the effect of environmental variables on Ca^{2+} binding to 'ghosts' indicate that the binding is an electrostatic interaction of Ca^{2+} with anionic membrane groups. Metabolic activity and energy, as ATP, do not seem to be required for optimum binding. In fact ATP appeared to decrease binding by chelating free Ca^{2+} ions from the solution or the membrane. Sanui & Pace (1967*a*,*b*) observed similar effects of ATP on Ca^{2+} ions bound by a liver microsomal fraction.

Although the pH curve (Fig. 6) does not precisely mirror the H^+-dissociation curve of 'ghosts' obtained by Sanui & Pace (1962), dissociable membrane anions with different p*K* values are likely to be important in Ca^{2+} binding. Below pH 6.5 possible binding groups are acidic phospholipid carboxyl and phosphate groups, since these all have p*K* values below pH 4.6 (Hendrickson & Fullington, 1965; Feinstein, 1964; Joos & Carr, 1967; Papahadjopoulos, 1968; Hauser & Dawson, 1967). Phosphatidylcholine, although present in high concentration in 'ghosts' (van Deenen & de Gier, 1964), did not appear to be responsible for the majority of the Ca^{2+} bound (Table 5), probably because at pH 7.4 it exists as an internally neutralized zwitterion (Santis & Rojas, 1969). Fatty acids at high pH values (>9.0) are able to bind Ca^{2+} ions (Yamauchi, Matsubara, Kimizuka & Abood, 1968) but their concentration in 'ghosts' is too low (van Deenen & de Gier, 1964) to be of quantitative significance.

N-Acetylneuraminic (sialic) acid carboxyl groups, with a p*K* of 2.6 (Eylar, Madoff, Brody & Oncley, 1962; Svennerholm, 1957; Haydon & Seaman, 1967), are good potential Ca^{2+}-binding sites. In our experiments all of the 'ghost' sialic acid would have been contained within the protein residue after chloroform–methanol extraction. The role of sialic acid and the important Ca^{2+}-binding function of side-chain carboxyl groups of aspartic acid and glutamic acid have been investigated in detail and reported briefly (Forstner & Manery, 1970).

Other groups might be involved in Ca^{2+} binding. Above pH 6.5 the dissociation of imidazole groups of histidine (p*K* 6.0–7.0) and amino groups (p*K* 7.5–10.8) may play an important role in Ca^{2+} binding. Deprotonation of amino groups would be expected to enhance the net negative charge on the membrane, possibly then allowing increased Ca^{2+} interaction with anions. Alternatively, amino-group N atoms may be directly involved in forming protein–metal chelation complexes (Freeman, 1967). Cysteine with its thiol groups (p*K* 8–10.5) might also be considered as a possible Ca^{2+} receptor site in 'ghosts', although Gurd & Wilcox (1956) have reported that S atoms do not form good ligands for Ca^{2+} ions.

The physiological significance of the observed distribution of Ca^{2+} within membranes is as yet unknown. Since both lipid and protein appear to bind Ca^{2+} ions, it is tempting to speculate that, physiologically, Ca^{2+} may bridge lipid phosphate and protein carboxyl groups, thereby stabilizing membrane structure. The extent of the binding of Ca^{2+} to protein suggests that, for future studies of the mechanism of Ca^{2+}-regulated membrane events, such as drug–membrane interaction, cell adhesion, hormone secretion and muscle contraction, attention should be paid to the influence of Ca^{2+} ions on

the properties of the membrane protein, protein–lipid interaction and the distribution of Ca^{2+} between protein and lipid. It has been shown by Weed *et al.* (1969) that Ca^{2+} ions within erythrocyte membranes exert a profound effect on the deformability characteristics of the cell, an effect that is thought to be due to induced changes in the physical nature of the protein at the inner aspect of the cell membrane. The well-known Ca^{2+}-binding properties of membrane phospholipids, derived from model membrane systems, may therefore represent only a part of the total functional change accompanying Ca^{2+}–membrane interaction.

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