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CALCIUM BINDING TO THE RAT LIVER PLASMA MEMBRANE

L SHLATZ* AND G. V. MARINETTI

Department of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y. (U S A)

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SUMMARY

Calcium binding to isolated rat liver plasma membranes shows saturation kinetics, has a pH optimum of 7.8–8.0, and is independent of metabolic energy. Scatchard analysis shows two classes of calcium binding sites. The higher affinity sites, with an association constant of $4.0 \cdot 10^3$ M^{-1}, accomodate 22 ± 2 (S.D.) nmoles of calcium/mg membrane protein. The lower affinity sites with an association constant of $3.2 \cdot 10^2$ M^{-1} accomodate 120 ± 16 (S.D.) nmoles of calcium/mg membrane protein. Mg^{2+} competes with Ca^{2+} only for the low affinity binding sites. K^+ and Na^+ do not inhibit calcium binding. EDTA decreases the amount of calcium bound to the membranes.

The ATP effect on calcium binding depends on the calcium concentration. At 1 mM calcium, ATP at 0.3 mM inhibits binding but at 3 mM calcium, ATP stimulates binding. ADP and AMP at 0.3 mM have no effect on calcium binding at 1 mM calcium but ADP stimulates binding at 3 mM calcium. Cyclic AMP at 10^{-3} M increases calcium binding at both 1 and 3 mM levels of calcium.

Phospholipases, neuraminidase and proteases were used to determine the role of phospholipids, neuraminic acid and proteins in the binding of calcium. Of the total extrapolated maximum calcium binding sites (142 ± 18 (S.D.) nmoles/mg membrane protein), acidic phospholipids accounted for approximately 100 nmoles/mg membrane protein while neuraminic acid residues accounted for approximately 50 nmoles/mg membrane protein.

Treatment of the membrane with a variety of functional group reagents showed that agents which react with amino groups or hydroxyl groups of proteins have a small or no effect on calcium binding. However, SH agents increase calcium binding but only when the calcium concentration in the medium was 1 mM or greater.

Sodium dodecyl sulfate (0.1 %) increases calcium binding very markedly, Triton X-100 (0.1 %) gave a 3-fold increase in calcium binding and Lubrol (0 1 %) had no influence on calcium binding. Triton may unmask certain acidic phospholipids. Sodium dodecyl sulfate is believed to become incorporated into the membrane and convert it to a highly charged form which now binds calcium. This detergent may also unmask acidic phospholipids.

* Present address: Mt. Sinai School of Medicine, Physiology Dept, New York, N.Y., U.S.A.

INTRODUCTION

Ca^{2+} binds to artificial and natural membranes and alters the permeability, excitability, and adhesiveness of these structures[1,2]. Mitochondria and sarcoplasmic reticulum bind calcium passively and, in addition, accumulate the cation actively, utilizing ATP as an energy source[3,4]. Reynafarje and Lehninger[5] have demonstrated that non-respiring mitochondria possess at least two classes of metabolism-independent calcium binding sites. Three types of passive calcium binding sites have been distinguished on membranes of the sarcoplasmic reticulum[6]. Gent *et al.*[7] have shown that calcium binding to red cell membranes was dependent on the pH and ionic strength of the medium but independent of any metabolic process. Liver microsomes have been shown to have similar cation binding characteristics[8].

Rasmussen[9] has discussed the relationship between Ca^{2+}, hormones and cyclic AMP and has postulated a model to integrate these interrelations. The model considers Ca^{2+} as mediators of the action of certain hormones. It is postulated that certain hormones increase the influx of Ca^{2+} into the cell or require extracellular Ca^{2+} for activating adenyl cyclase. The increase in Ca^{2+} then moderates the activities of certain enzymes which had previously been stimulated by cyclic AMP. A relatively impressive body of evidence is presented by Rasmussen[9] which shows the role of calcium in a fairly large number of cyclic AMP-dependent processes. Calcium efflux from cells is required to restore the calcium concentration within the cell. A specific Ca^{2+}-dependent ATPase appears to play an important role in this process.

This report describes the properties of calcium binding to isolated rat liver plasma membranes, the number and relative affinities of calcium binding sites and the nature of the functional groups involved in the binding process.

EXPERIMENTAL SECTION

Materials

Type HAWP Millipore filters, 0.45 μm pore size, were obtained from Millipore Corporation. ^{45}Ca was purchased from International Chemical and Nuclear Corporation as $CaCl_2$ in 0.5 M HCl (5.96 mCi/mg calcium). ATP, ADP, AMP and cyclic AMP were obtained from P-L Biochemicals Inc. Trypsin was obtained from Worthington Biochemical Corp., chymotrypsin from Nutritional Biochemicals Corp., and Pronase from Calbiochem. Neuraminidase from Vibrio cholerae (500 units/ml) was obtained from General Biochemicals. Phospholipase A, from *Naja naja* snake venom was purchased from the Miami Serpentarium. Phospholipase C was from C. F. Boehringer und Soehne. The following chemical agents were used: azobenzene-2-sulfenyl bromide (Nutritional Biochem.); 5,5'-dithiobis-2-nitrobenzoic acid (K and K Labs); phenylmethylsulfonyl fluoride (Sigma), succinic anhydride (Eastman Organic), iodoacetamide (Sigma); mercaptoethanol (Eastman Organic), *p*-chloromercuribenzoate (Sigma).

Methods and reagents

Preparation of plasma membranes

The plasma membranes were isolated by the modification[10] of the Neville method[11]. After isolation, the membrane preparations were washed with 0.1 M Tris–HCl buffer (pH 7.5) followed by 1.0 mM EDTA. The washed pellets obtained by

centrifugation at 3000 rev./min were resuspended in 0.1 M Tris buffer (pH 7.5) to a concentration of 300–400 μg membrane protein/ml. Protein concentration was determined by the method of Lowry *et al.*[12].

Measurement of calcium binding

Millipore filtration. Samples containing 60–80 μg of membrane protein in 0.2 ml of Tris–HCl buffer (pH 7.5), 0.7 ml of 0.1 M Tris–HCl (pH 7.5), 5 μl containing 0.5 μCi of $^{45}CaCl_2$, varying concentrations of cold $CaCl_2$, and any reagent noted in the legends were incubated at 37 °C for 10 min in a shaking water bath. The final volume of the incubation medium was 1.0 ml Unless otherwise specified the test agents were made in 0.1 M Tris–HCl buffer (pH 7.5). Using a Pasteur pipette the samples were placed in a Millipore suction apparatus containing a 0.45 μm, type HAWP Millipore filter (25-mm diameter) which had previously been rinsed with 250 mM KCl and washed with glass-distilled water. The mixture was completely filtered by vacuum within 5 s and then washed twice with 5.0-ml aliquots of 0.1 M Tris–HCl buffer (pH 7.5). The filters were removed, allowed to dry and placed in scintillation vials containing 10 ml of Bray's scintillation cocktail[13]. Controls containing all reagents except the membranes were run simultaneously in each experiment to correct for the amount of $^{45}Ca^{2+}$ bound nonspecifically to the filters. Standards of the $^{45}CaCl_2$ solutions were prepared by spotting 5 μl (0.5 μCi) of each radioactive solution on Millipore filters, drying and counting them under the same conditions as the membrane samples. Samples and standards were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314.

Equilibrium dialysis. Calcium binding to the membrane was measured by use of a Multi-Micro Equilibrium Dialysis Apparatus (Interscience, Philadelphia, Pa.). The two plastic chambers were separated by dialysis tubing No. 18 (Union Carbide Corp.) which had previously been soaked in 0.1 M Tris–HCl buffer (pH 7.5) and blotted dry. Each chamber contained a total volume of 1 ml and possessed an injection port.

To 2 ml of the membrane suspension were added 8 ml of 0.1 M Tris–HCl buffer (pH 7.5) to give a final membrane protein concentration of 80–100 μg/ml. 1 ml of this membrane suspension was injected into the left chamber. To the right chamber was added 0.99 ml of the Tris–HCl buffer and 10 μl of $CaCl_2$ solution containing 1.0 μCi of $^{45}Ca^{2+}$. The final calcium solutions varied in concentration from $5 \cdot 10^{-7}$ to $5 \cdot 10^{-3}$ M. The temperature was maintained at 5 °C.

After equilibrium was established (42 h as determined by time studies) 10-μl aliquots in triplicate from each chamber were removed and placed into scintillation vials containing 10 ml of Bray's scintillation cocktail. 10-μl (1.0 μCi) aliquots of each stock $^{45}CaCl_2$ solution, which served as standards, were placed into scintillation vials containing 10 ml of Bray's scintillation cocktail. The samples and standards were counted as stated above.

RESULTS

Millipore filtration versus equilibrium dialysis for determining Ca^{2+} binding

Millipore filtration measures calcium binding in short time intervals whereas equilibrium dialysis requires a long time interval (42 h). The results in Fig. 1 show the amount of calcium bound as a function of calcium concentration Equilibrium

dialysis gave higher values than the Millipore method but the latter values are believed to be more meaningful since the membrane is altered during the binding studies to a minimal extent. During the 42 h required for equilibrium dialysis the membrane is susceptible to alteration and proteolytic degradation.

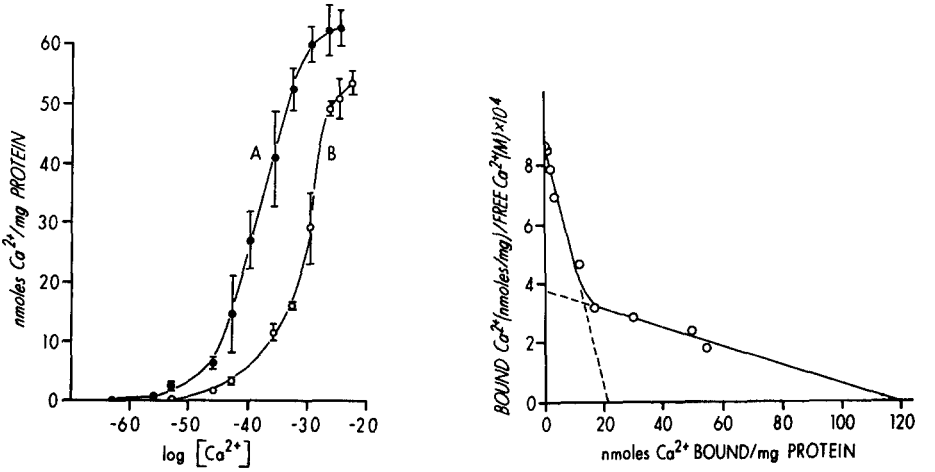


Fig 1 Concentration dependence of calcium binding. The concentration dependence of calcium binding was determined by equilibrium dialysis, Curve A and Millipore filtration, Curve B. Experimental details are given in the text. Each point represents the mean ± S D. of duplicate determinations of four separate experiments

Fig 2 Scatchard plot of calcium binding The results shown in Fig 1 for the Millipore data are depicted as a Scatchard plot in the figure

TABLE I

NUMBER AND AFFINITY OF CALCIUM BINDING SITES

The figures were extrapolated graphically from intercepts on the ordinate (nK) and abscissa (n) of the linear portions of the Scatchard plots in Fig 1 (for the Millipore filtration method) The numbers were obtained from the mean values in Fig 1 and thus represent the mean extrapolated maximum binding sites ± S D

Method of determination	*Number of binding sites (nmoles/mg)*	*Association constant (l/mole)*
Equilibrium dialysis	72 ± 3	$4.8 \cdot 10^3$
Millipore filtration	22 ± 2	$4.0 \cdot 10^3$
	120 ± 16	$3.2 \cdot 10^2$

Scatchard[14] derived a method for plotting data on the binding of small molecules to macromolecules from which the relative affinities and number of binding sites can be extrapolated. Fig. 2 shows the Scatchard plot of the Millipore data presented in Fig. 1. The plot is biphasic indicating the presence of two classes of binding sites. The extrapolated intercepts of the linear segments of the plot are used to calculate the number of binding sites and the association constant for each type of binding site. The intercept on the abscissa (n) represents the number of binding sites per mg of

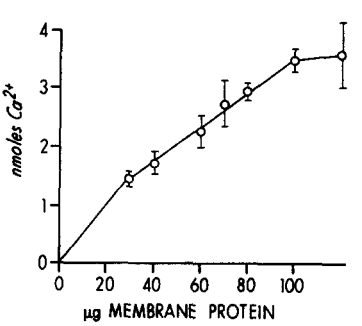
membrane protein. The intercept on the ordinate is nK where K is the association constant of the Ca^{2+} binding sites. The higher affinity binding sites for Ca^{2+} with an association constant of $4.0 \cdot 10^3$ M^{-1} accommodated 22 ± 2 (S.D.) nmoles of Ca^{2+}/mg membrane protein. The lower affinity sites had an association constant of $3.2 \cdot 10^2$ M^{-1} and accommodated 120 ± 16 (S.D.) nmoles of Ca^{2+}/mg membrane protein.These results are summarized in Table I. The maximum amount of Ca^{2+} bound to the membranes would be 4.3 μg Ca^{2+}/mg membrane (this takes into account the plasma membrane consisting of 75 % protein and 25 % lipid[10,14]).

Optimal conditions for calcium binding

The membranes used in these studies were prepared by the Ray modification[10] of the Neville method[11]. Since the Ray method uses Ca^{2+} to increase the membrane yield, a significant amount of endogenous Ca^{2+} in the rat liver membrane preparation would exchange with the added $^{45}CaCl_2$, thus the apparent amount of Ca^{2+} bound would be lowered as a result of isotope dilution. The membranes were therefore washed with EDTA before use. When the membrane preparations were washed with 1.0 mM EDTA, and then with 0.1 M Tris–HCl buffer (pH 7.5), the amount of Ca^{2+} bound was increased by 85 % as compared to control membranes not washed with EDTA. Thus, the binding measurements were routinely carried out with EDTA-washed then buffer-washed membranes.

The binding of Ca^{2+} as a function of membrane concentration was studied. The binding increased linearly over the concentration range of 30 to 100 μg of membrane. (Fig. 3).

The binding of Ca^{2+} was fairly rapid and was dependent on pH and ionic strength but was independent of temperature in the range of 0–37 °C. After 2 min of incubation at 37 °C, 64 % of maximal binding was attained (Fig. 4). After 10–15 min the amount of Ca^{2+} bound had attained saturation. The pH optimum was shown to be between 7.8 and 8.0 (Fig. 5).



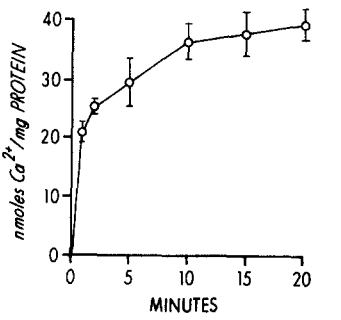


Fig. 3 Calcium binding as a function of membrane concentration. The incubation system consisted of 1.0 mM $CaCl_2$ containing 0.5 μCi of ^{45}Ca, 0.1 M Tris–HCl buffer (pH 7.5) and varying amounts of membrane. The final volume was 1.0 ml. Incubation was carried out at 37 °C for 10 min. The samples were analyzed by Millipore filtration as explained in the text. Each point represents the mean ± S.D. of duplicate determinations of three separate experiments.

Fig 4 The time course of calcium binding. The incubation system contained 60–80 μg of membrane protein and 1.0 mM $^{45}CaCl_2$ (0.5 μCi) in 0.1 M Tris–HCl buffer, pH 7.5 in a final volume of 1.0 ml. After 1, 2, 5, 10, 15 and 20 min incubation at 37 °C the samples were analyzed by Millipore filtration as explained in the text. Each point represents the mean ± S.D. of duplicate determinations of three separate experiments

Effect of EDTA and temperature on calcium binding

The data in Table II show that calcium binding was markedly inhibited by EDTA and by boiling the membrane. However, calcium binding was not influenced significantly over the temperature range of 0 to 37 °C.

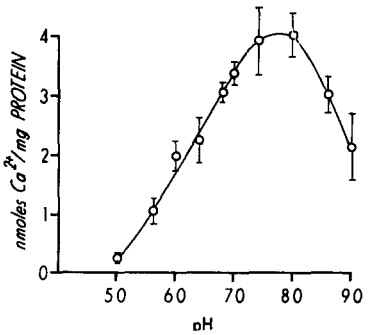


Fig. 5 The effect of pH on calcium binding. Membranes (80–100 μg protein) were subjected to equilibrium dialysis against $5 \cdot 10^{-6}$ M $CaCl_2$ containing 1.0 μCi of ^{45}Ca over a pH range of 5.0–9.0. In the 5.0–5.6 pH range 0.1 M acetic acid–sodium acetate buffer was used. 0.02 M KH_2PO_4–Na_2HPO_4 buffer was used from pH 5.6–6.8 and 0.1 M Tris–HCl buffer was employed to obtain a pH range of 7.0–9.0. Each point represents the mean ± S.D of duplicate determinations of four separate experiments. Equilibrium dialysis was carried out as explained in the text.

TABLE II

THE EFFECT OF EDTA AND TEMPERATURE ON CALCIUM BINDING

Membranes (60–80 μg protein) suspended in 0.1 M Tris–HCl buffer (pH 7.5) were incubated for 10 min with $5 \cdot 10^{-4}$ M $^{45}CaCl_2$ (0.5 μCi) under the conditions listed. The amount of calcium bound to the membrane was determined by Millipore filtration. Each point represents the mean of duplicate determinations of two separate experiments (± S.E.).

Conditions	*Amount of calcium bound (nmoles/mg membrane protein)*
Control, 37 °C	16.2 ± 0.8
0 °C	15.8 ± 0.7
22 °C	16.5 ± 0.7
Membranes boiled prior to incubation	4.2 ± 0.1
10^{-3} M EDTA	2.4 ± 0.6

Cation competition for calcium binding sites

Table III shows the results of competition studies with various cations. Neither 150 mM K^+ nor 150 mM Na^+ interfered with Ca^{2+} binding to the plasma membranes to any appreciable extent. In the presence of 1.5 mM Mg^{2+}, however, Ca^{2+} binding was inhibited. At an added Ca^{2+} concentration of 3.0 mM, Mg^{2+} decreased Ca^{2+} binding by 43 % even though the Mg^{2+}/Ca^{2+} ratio was 0.5.

Scatchard plot analysis of the Mg^{2+} competition for Ca^{2+} binding sites revealed that Mg^{2+} did not alter the binding of Ca^{2+} to the high affinity sites. However, the mean number of low affinity sites available for Ca^{2+} binding was decreased from 120 to 46 nmoles Ca^{2+} per mg of membrane protein. The extrapolated maximum binding is thus decreased by 68 % as contrasted to the 45 % decrease in the measured non-maximum binding.

The effect of nucleotides on calcium binding

The isolated rat liver plasma membranes have been shown to possess Mg^{2+}-activated ATPase, ATP pyrophosphohydrolase, 5'-nucleotidase and adenyl cyclase activity[10,15]. Since there is a wide array of nucleotide metabolism which occurs at the cell surface and since nucleotides bind Ca^{2+}, the effect of nucleotides on Ca^{2+} binding was examined.

In Table IV it is shown that 0.3 mM ADP or AMP had no effect on the amount of Ca^{2+} bound at 1 mM Ca^{2+} in the medium. At 3.0 mM added Ca^{2+}, ADP stimulated Ca^{2+} binding by 16 %. ATP, which is known to be a strong divalent cation complexing agent, gave a 51 % decrease in the amount of Ca^{2+} bound when the added Ca^{2+} concentration was 1.0 mM. At 3.0 mM added Ca^{2+}, ATP stimulated Ca^{2+} binding by 27 %.

Scatchard plot analysis of these results showed that ATP decreased the number of high affinity Ca^{2+} binding sites from 22 to 10 nmoles of Ca^{2+}/mg membrane protein but led to an increase in binding sites with low affinity.

Rasmussen[9] has discussed the relationships between cyclic AMP, calcium and cell membranes. Ca^{+2} is required either for the stimulus which activates adenyl cyclase or for the physiological action of cyclic AMP within the cell. It therefore

TABLE III

EFFECT OF CATIONS ON CALCIUM BINDING TO THE PLASMA MEMBRANE

The incubation system consisted of 60–80 μg of membrane protein in 0.1 M Tris–HCl buffer (pH 7.5) containing either 150 mM NaCl, 150 mM KCl or 1.5 mM $MgCl_2$ and either 1 mM or 3 mM $CaCl_2$ (0.5 μCi of ^{45}Ca) Incubation was carried out at 37 °C for 10 min. Each point represents the mean ± S.D of duplicate determinations of three separate experiments. Binding was determined by Millipore filtration as explained in the text.

	nmoles Ca^{2+} bound/mg membrane protein	
	1 mM Ca^{2+}	*3 mM Ca^{2+}*
Control	29 ± 5	51 ± 3
NaCl, 150 mM	30 ± 3	49 ± 2
KCl, 150 mM	31 ± 2	46 ± 3
$MgCl_2$, 1.5 mM	25 ± 2	29 ± 1

TABLE IV

EFFECT OF NUCLEOTIDES ON CALCIUM BINDING TO THE PLASMA MEMBRANE

The incubation system contained 60–80 μg membrane protein, 0.1 M Tris–HCl buffer (pH 7.5), either 1 mM or 3 mM $CaCl_2$ containing 0.5 μCi of ^{45}Ca and either ATP, ADP, AMP or cyclic AMP at the concentrations given in the table The samples were incubated for 10 min at 37 °C and then analyzed by Millipore filtration as explained in the text Each point represents the mean ± S.D of duplicate determinations of three separate experiments

	nmoles Ca^{2+} bound/mg membrane protein	
	1 mM Ca^{2+}	*3 mM Ca^{2+}*
Control	29 ± 5	51 ± 3
ATP, 0.3 mM	14 ± 1	65 ± 3
ADP, 0.3 mM	29 ± 3	59 ± 4
AMP, 0.3 mM	28 ± 1	50 ± 7
Cyclic AMP, 1 mM	58 ± 3	94 ± 4

seemed of interest to see if cyclic AMP influenced calcium binding to the membrane. It can be seen in Table IV that cyclic AMP increased calcium binding at both the 1 mM and 3 mM level of calcium in the medium.

The results of Scatchard analyses of the effect of cyclic AMP on calcium binding over a wide range of calcium concentration showed that the main effect of cyclic AMP was to increase the number of low affinity sites. At an added Ca^{2+} concentration of 1.0 mM, 1.0 mM cyclic AMP increased the amount of Ca^{2+} bound to the membrane by 100 %. At 3 mM Ca^{2+} the increase was 86 %. Scatchard analysis showed that cyclic AMP increased the number of high affinity sites from 22 to 30 nmoles of Ca^{2+}/mg membrane protein and increased the number of low affinity sites from 120 to 240 nmoles of Ca^{2+}/mg membrane protein

The effect of enzymatic modification of the membrane on calcium binding

The effect of proteolytic enzymes. To determine which components in the plasma membrane were involved in the binding, the effect of treating the membrane with various modifying agents was observed. Proteolytic hydrolysis of the plasma membrane by chymotrypsin, trypsin or Pronase produced a 14–25 % decrease in the amount of Ca^{2+} bound (Table V) when the added calcium concentration was 5 0 mM. These enzymes had no effect when the added calcium concentration was 1.0 mM. Benedetti and Emmelot[16] found that when liver plasma membranes were treated with trypsin, the overall thickness of the trilamellar membrane was reduced from a normal value of 80 Å to about 60 Å. It would appear that the decrease in Ca^{2+} binding to the plasma membrane after proteolytic hydrolysis is due primarily to a structural modification of the membrane system

The effect of neuraminidase Since the carboxyl groups of neuraminic acid residues are possible binding sites for Ca^{2+}, the effect of their removal by neuraminidase was studied. The results are shown in Fig. 6. A 26 % decrease in Ca^{2+} binding was observed. Scatchard plot analysis of these results revealed that the mean number of high affinity binding sites decreased only slightly from 22 to 18 nmoles of Ca^{2+}/mg membrane protein. The low affinity binding sites decreased in number from 120 to 74

TABLE V

EFFECT OF PROTEOLYTIC ENZYMES ON CALCIUM BINDING TO THE PLASMA MEMBRANE

Membranes (2–3 mg protein) suspended in 4 0 ml of 0 1 M Tris–HCl buffer (pH 7 5) were preincubated with 100 μg of either trypsin, chymotrypsin or Pronase for 45 min at 37 °C The proteolytic enzyme was omitted in the control samples The mixtures were centrifuged and the pellets washed with Tris–HCl buffer and recentrifuged The resulting pellets were resuspended in 4 0 ml of Tris–HCl buffer Aliquots of the membrane suspensions (60–80 μg protein) were incubated with either 1 mM or 5 mM $CaCl_2$ containing 5 μCi of ^{45}Ca Binding was determined by Millipore filtration as explained in the text Each point represents the mean ± S D of duplicate determinations of three separate experiments

	nmoles Ca^{2+} bound/mg membrane protein	
	1 mM Ca^{2+}	*5 mM Ca^{2+}*
Control	29 ± 5	53 ± 2
Trypsin	32 ± 1	41 ± 3
Chymotrypsin	25 ± 3	46 ± 2
Pronase	26 ± 1	40 ± 1

nmoles/mg membrane protein. The total decrease in Ca^{2+} binding sites by neuraminidase treatment was 50 nmoles/mg membrane protein. This corresponds closely to the amount of neuraminic acid (51 nmoles/mg membrane protein) shown to be present in the plasma membrane[10], although only 70 % of the total neuraminic acid residues are susceptible to neuraminidase. This finding indicates that the carboxyl groups of the neuraminic acid residues play an important role in Ca^{2+} binding to the plasma membrane and that the major part of these carboxyl groups are available for binding.

The effect of phospholipases. The role that phospholipids play in binding Ca^{2+} to the plasma membrane was investigated by the use of phospholipases A_1, C, and D. Fig. 7 shows that phospholipase D was effective in increasing (by 34 %) Ca^{2+} binding, while the phospholipase C caused a 49 % decrease in the amount of Ca^{2+} bound. Since phospholipase D cleavage results in the formation of phosphatidic acid, the resultant polar phosphate group is capable of binding Ca^{2+}. Phospholipase C, however, removes the entire polar head of the phospholipid and would be expected to decrease the number of binding sites available to Ca^{2+}. Scatchard plot analysis (Table VI) revealed that phospholipase C treatment of the membranes reduced the extrapolated maximum total number of Ca^{2+} binding sites from 142 to 38 nmoles/mg membrane protein. It

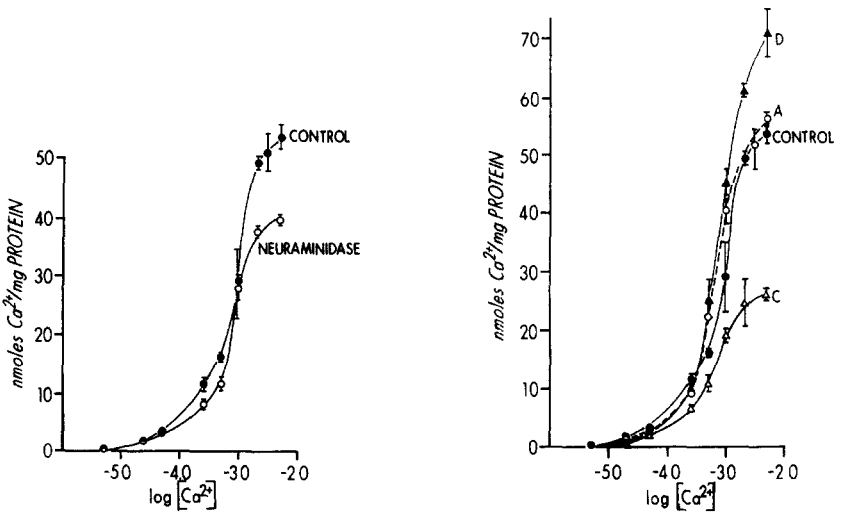


Fig. 6. The effect of neuraminidase on calcium binding. Membranes (2–3 mg protein) suspended in 4 0 ml of 0 1 M Tris–HCl buffer (pH 7 5) were preincubated with 10 units of neuraminidase and 1 mM $CaCl_2$ for 45 min at 37 °C. Neuraminidase was omitted in the control samples. The mixtures were centrifuged and the pellets washed with 4 ml of Tris–HCl buffer containing 1 mM EDTA and recentrifuged. The resulting pellets were resuspended in 4 0 ml of Tris–HCl buffer. Aliquots of the membrane suspension (60–80 μg protein) were incubated with varying concentrations of $^{45}CaCl_2$ (0 5 μCi) for 10 min at 37 °C and then analyzed by Millipore filtration. Each point represents the mean of duplicate determination of three separate experiments

Fig 7 The effect of phospholipases on calcium binding. Membranes (2–3 mg protein) suspended in 4 0 ml of 0.1 M Tris–HCl buffer (pH 7 5) were preincubated with 200 μg of either phospholipase C, D or A_1 for 45 min at 37 °C as explained in the text Phospholipase was omitted in the control samples. The mixtures were centrifuged and the pellets washed with 4 0 ml of Tris–HCl buffer and recentrifuged. The resulting pellets were resuspended in 4.0 ml of Tris–HCl buffer. Aliquots of the membrane suspensions (60–80 μg protein) were incubated with varying concentrations of $^{45}CaCl_2$ (0.5 μCi) for 10 min at 37 °C and then analyzed by Millipore filtration Each point represents the mean of duplicate determinations of three separate experiments

has been shown that isolated rat liver plasma membranes contain about 780 nmoles of total phospholipid P per mg of protein[10]. The acidic phospholipids account for approximately 35 % of the total phospholipids[17] or approximately 270 nmoles of phospholipids/per mg of protein. It appears that not all of these acidic phospholipids in the membrane are available for binding Ca^{2+} since phospholipase C reduced the maximum number of Ca^{2+} sites by approximately 100 nmoles/mg membrane protein, and moreover the maximum total number of Ca^{2+} binding sites is 142 which is close to half the 270 nmoles of acidic phospholipids/mg of membrane protein. This might suggest that in part each Ca^{2+} forms an ionic bridge with two phospholipid molecules.

TABLE VI

THE EFFECT OF PHOSPHOLIPASES ON THE NUMBER AND AFFINITY OF CALCIUM BINDING SITES

The figures were extrapolated graphically from the intercepts on the ordinate and abscissa of the linear portions of the Scatchard plots derived from the data presented in Fig 7. Mean values are shown in the table.

Prior treatment	*Number of binding sites*	*Association constant (l/mole)*
None	22	$4\,00 \cdot 10^3$
	120	$3\,17 \cdot 10^2$
Phospholipase D	24	$2\,92 \cdot 10^3$
	136	$4\,41 \cdot 10^2$
Phospholipase A	18	$4\,17 \cdot 10^3$
	90	$6\,56 \cdot 10^2$
Phospholipase C	4	$1\,60 \cdot 10^4$
	34	$1\,00 \cdot 10^3$

TABLE VII

EFFECT OF FUNCTIONAL GROUP AGENTS ON CALCIUM BINDING TO THE PLASMA MEMBRANE

Membranes (2–3 protein) suspended in 4.0 ml of 0.1 M Tris–HCl buffer, pH 7 5, were incubated for 45 min at 37 °C with the test agent at the concentration given in the table The chemical agents were omitted in the control samples. Acetate buffer, 0 2 M (pH 5 0) was substituted for Tris buffer when testing azobenzenesulfenylbromide The mixtures were centrifuged and the pellets washed with Tris–HCl buffer and recentrifuged. The resulting pellets were resuspended in 4.0 ml of Tris–HCl buffer Aliquots of the membrane suspensions (60–80 μg protein) were incubated with 5 mM $CaCl_2$ containing $^{45}CaCl_2$ (0 5 μCi) for 10 min at 37 °C and then analyzed by Millipore filtration as explained in the text Each point represents the mean ± S.D of duplicate determinations of three separate experiments

Agent added	*nmoles Ca^{2+} bound/mg membrane protein*
None, control	53 ± 2
5,5-Dithiobis-2-nitrobenzoic acid, 1 mM	60 ± 4
Iodoacetamide, 1 mM	72 ± 2
Azobenzene-2-sulfenylbromide, 10 mM	78 ± 2
p-Chloromercuribenzoate, 0 1 mM	72 ± 2
Mercaptoethanol, 0 1 mM	75 ± 4
Succinic anhydride, 0 5 mM	61 ± 2
Phenylmethylsulfonylfluoride, 0.1 mM	66 ± 2

Effect of chemical agents on calcium binding

The effects of SH agents and acylating agents on calcium binding are shown in Table VII. These agents had little or no effect on calcium binding at calcium concentrations of less than 1 mM but increased Ca^{2+} binding at 5.0 mM calcium. The percent increase varied from 24–47 %.

Effect of detergents on calcium binding

The effects of three detergents, triton, lubrol and sodium dodecyl sulfate on calcium binding are shown in Fig. 8. Sodium dodecyl sulfate markedly increased calcium binding. Triton gave nearly a 3-fold increase whereas lubrol did not influence calcium binding. These detergent effects are believed due in part to masking or unmasking phospholipids in the membrane, or in the case of sodium dodecyl sulfate, the incorporation of this anionic detergent in the membrane.

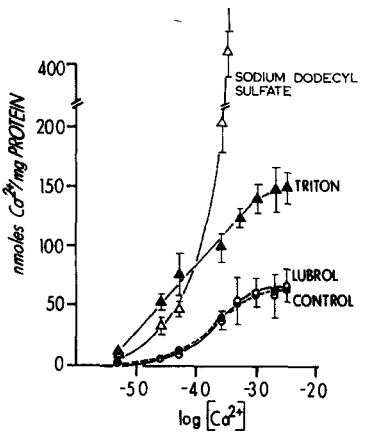


Fig 8 The effect of detergents on calcium binding. Membranes (80–100 μg protein) were suspended in 1.0 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing 0.1% of either Triton X-100, sodium dodecyl sulfate or Lubrol PX The membrane suspensions were dialyzed to equilibrium for 42 h at 5 °C against identical detergent–buffer solutions with varying concentrations of $CaCl_2$ containing 1.0 μCi of ^{45}Ca The control samples were identical except that the detergents were omitted Each point represents the mean ± S.D. of duplicate determinations of three separate experiments. Experimental details are given in the text.

DISCUSSION

Isolated plasma membranes bind a considerable amount of Ca^{2+} without the intervention of metabolic energy. The binding of Ca^{2+} is a fairly rapid process which is influenced by pH and ionic strength but not affected appreciable by temperature in the range of 0–37 °C. The biphasic nature of the Scatchard plot of the Ca^{2+} binding data indicates the presence of two classes of binding sites with different affinity constants. The high affinity sites for Ca^{2+} ($K_{assoc} = 4.0 \cdot 10^3\ M^{-1}$) were small in number accommodating 22 nmoles of Ca^{2+}/mg membrane protein, while the low affinity sites ($K_{assoc} = 3.2 \cdot 10^2\ M^{-1}$) were greater in number accommodating 120 nmoles of Ca^{2+}/mg membrane protein The possibility of some cooperative interaction between the two classes of sites or that another set of binding sites with an intermediate affinity exists could not be excluded. A small number of very high affinity sites also cannot be excluded since Ca^{2+} binding was not studied below 10^{-8} M.

Competition studies with various cations revealed that the monovalent ions, K^+ and Na^+, did not compete with Ca^{2+} for the binding sites on the plasma membrane. Mg^{2+}, however, was capable of decreasing the amount of Ca^{2+} bound to the membrane. Mg^{2+} did not alter the binding of Ca^{2+} to the high affinity sites; but, the number of low affinity Ca^{2+} binding sites was decreased from 120 to 46 nmoles Ca^{2+}/mg membrane protein.

Since the binding of Ca^{2+} by the plasma membranes exhibited saturation kinetics and was influenced by the presence of other cations, the plasma membranes appeared to be capable of behaving as ion exchange membranes. These properties of Ca^{2+} binding to the plasma membrane resemble the metabolism-independent binding of Ca^{2+} to other membranes. Gent *et al.*[7] have demonstrated that $^{45}Ca^{2+}$ was adsorbed to the red cell membranes in a manner dependent on the pH and ionic strength of the surrounding medium but independent of any metabolic process since neither cooling, dinitrophenol, ouabain, or hydrocortisone altered the binding. Liver cell microsomes have also been shown to behave as ion exchange membranes[8]. Reynafarje and Lehninger[5] have shown that rat liver mitochondria, in which respiration is inhibited by antimycin A and rotenone, possess two major classes of Ca^{2+} binding sites. Three types of Ca^{2+} binding sites have been distinguished on membranes of the sarcoplasmic reticulum[6]. Therefore, it would appear that Ca^{2+} can bind to both external membranes of cells and membranes of subcellular organelles in the absence of metabolic energy.

The presence of ATP was shown to decrease Ca^{2+} binding at the lower Ca^{2+} concentration range. When the added Ca^{2+} was above 1.0 mM the effect produced by ATP was reversed, resulting in an increase in Ca^{2+} binding. Thus ATP (and ADP) appear to alter the membrane structure such that more binding sites are made available. Some of the groups may be phospholipid polar moieties. ATP has been shown to behave like EDTA in reducing the divalent cation binding of liver microsomes[18]. The interaction of Ca^{2+} with ATP may provide a mechanism by which Ca^{2+} facilitates the regulation of membrane structure and function. Abood[19] has proposed the existence of a lipoprotein–Ca–ATP complex which regulates the permeability characteristics of nerve membranes.

At 1 mM cyclic AMP, the measured Ca^{2+} binding was increased by 100 %. Since cyclic AMP caused a 2-fold increase in the extrapolated maximum number of low affinity binding sites, it would appear that the nucleotide in some manner induced a conformational change over a relatively large portion of the membrane resulting in a modification of the Ca^{2+} binding pattern. Kafka and Pak[20] have shown that cyclic AMP was capable of facilitating the adsorption of Ca^{2+} to monomolecular films of monooctadecyl phosphate. The physiological significance of the effect of cyclic AMP on the plasma membrane binding pattern is questionable since the concentration required to alter the Ca^{2+} binding was high. Cyclic AMP may make available certain masked polar groups of phospholipids in the plasma membrane. The total number of extrapolated binding sites for Ca^{2+} in the presence of 10^{-3} M cyclic AMP is 270 nmoles/mg membrane protein, a number identical to the 270 nmoles of acidic phospholipids present per mg membrane protein.

Three molecular components of the plasma membrane, *i.e.* proteins, phospholipids and neuraminic acid are capable of binding Ca^{2+}. Structural integrity of the plasma membrane was essential for optimal Ca^{2+} binding since both proteolytic and

lipolytic alteration of the membrane resulted in a decrease in the amount of Ca^{2+} bound. Of the total Ca^{2+} extrapolated maximum binding sites (142 ± 16 nmoles/mg membrane protein), acidic phospholipids account for approximately 100 nmoles/mg membrane protein while neuraminic acid residues account for approximately 50 nmoles/mg membrane protein. The phospholipids account for sites with low binding affinity. Long and Mouat[21] have shown that the binding of Ca^{2+} to erythrocytes decreased linearly with the removal of *N*-acetylneuraminic acid by neuraminidase. Palmer and Posey[22] have demonstrated that phospholipase C-pretreated renal microsomes bound 40 to 60 % less Ca^{2+} than untreated membranes. The results also suggested that Ca^{2+} binding to membrane proteins was relatively small in comparison to Ca^{2+} binding to acidic phospholipids and neuraminic acid residues. Koketsu *et al.*[23] have shown that when membrane fragments capable of binding radioactive Ca^{2+} were extracted by organic solvents the protein-containing residue would not bind Ca^{2+}, whereas the extracted lipids did bind Ca^{2+}.

The binding of calcium to the liver plasma membrane can be contrasted to the binding of calcium to calsequestrin as reported by MacLennon and Wong[24]. Calsequestrin, a protein isolated from rabbit sarcoplasmic reticulum, has a molecular weight of 44000 and binds 43 nmoles Ca^{2+}/mole protein or 970 nmoles/mg protein. The binding constant for Ca^{2+} is $2.5 \cdot 10^4$ M^{-1}. The pH profile for binding shows a relatively small but steady increase from pH 6.0 to 9.0 One type of binding site was observed. In our studies on the plasma membrane, there are at least two types of binding sites for Ca^{2+}, the pH profile shows a peak between 7.8–8.0, and binding constants are slightly smaller. In our membrane system phospholipids and neuraminic acid (not proteins) are quantitatively more important for binding Ca^{2+}.

Inasmuch as the neuraminic acid residues are exposed on the membrane and that proteins do not play a major role in binding, one must conclude that phospholipids are the important components which bind Ca^{2+} but a large number of these acidic phospholipids are masked. Therefore those agents such as nucleotides (ATP, ADP, AMP, cyclic AMP), SH agents, and certain detergents which increase Ca^{2+} binding probably do so by unmasking these phospholipids or in the case of sodium dodecyl sulfate this detergent may become incorporated into the membrane and convert it to a highly anionic form *via* its sulfate groups which can bind Ca^{2+} ions.

In a previous publication[25] we reported the phosphorylation of the plasma membrane by the cyclic AMP-dependent protein kinase and showed that the phosphorylated membrane bound more calcium than the control membrane. It was further demonstrated that glucagon and epinephrine could substitute for cyclic AMP for stimulation of the protein kinase phosphorylation of the plasma membrane since these hormones are capable of stimulating the membrane-bound adenyl cyclase which produces cyclic AMP.

Another action of hormones on the plasma membrane is to modify the binding of Ca^{2+}. It was observed[26] that glucagon, epinephrine and hydrocortisone at levels of 10^{-8} M increased the binding of calcium to plasma membranes whereas insulin decreased calcium binding. ATP at 0.3 mM inhibited the effects of epinephrine, glucagon and hydrocortisone and reversed the effect of insulin on calcium binding.

These observations indicate that hormones may modify the membrane structure and function by influencing directly the amount of bound calcium or by acting indirectly through adenyl cyclase and protein kinase to convert the membrane to a

phosphorylated state which has greater binding capacity for Ca^{2+}. The low calcium and high calcium states of the membrane can be expected to influence membrane properties such as permeability and excitability.

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