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CALCIUM BINDING PROPERTIES OF SARCOPLASMIC RETICULUM MEMBRANES

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SUMMARY

Sarcoplasmic reticulum from rabbit skeletal muscle demonstrates two types of ATP-independent calcium binding. About 10% of the total calcium-binding sites are very little influenced by ionic strength and are readily inactivated by trypsin proteolysis. The remaining calcium-binding sites are relatively heat stable and are not inactivated by trypsin proteolysis. Calcium binding by these sites is dependent on the ionic strength and is completely inhibited by 0.01 M $MgCl_2$, 0.6 M KCl or 0.6 M NaCl. Total calcium-binding capacity as tested in media of low ionic strength is 50 nmoles of calcium bound per mg of sarcoplasmic reticulum protein. Both binding systems have an apparent dissociation constant of 40 μM and are not influenced by hydrolysis of the membrane phospholipids by phospholipase C.

INTRODUCTION

Active calcium uptake by muscle sarcoplasmic reticulum has been thoroughly studied[1-5]. Hydrolysis of one molecule of ATP causes the translocation of two atoms of calcium across the reticulum membrane and concentration gradients of up to 3000 are achieved[6]. ATP-independent calcium binding to sarcoplasmic reticulum membranes was noted by several authors[7,8]. An extensive study of the binding of calcium and other ions to sarcoplasmic reticulum was carried out by CARVALHO[9] and CARVALHO AND LEO[10]. However, little is known about the relation of ATP-independent calcium binding to membrane components, ATPase activity and active calcium uptake. Using a highly sensitive radioisotope assay for calcium binding, the present report demonstrates that the sarcoplasmic reticulum in the absence of ATP reveals two types of calcium binding. The properties of these two binding systems are reported, and their possible function in Ca^{2+} transport is discussed.

MATERIALS AND METHODS

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle as described in an accompanying paper[12].

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Trypsin-treated sarcoplasmic reticulum

Sarcoplasmic reticulum suspensions of 4 mg protein/ml in a solution of 100 mM KCl and 5 mM imidazole–HCl buffer (pH 7.0), was incubated with two times crystallized trypsin (EC 3.4.4.4) 0.4 mg/ml. After 30 min at 30° the reaction was terminated by the addition of soybean trypsin inhibitor, 0.1 mg/ml. The trypsin-treated sarcoplasmic reticulum was recovered by centrifugation for 20 min at 200000 × *g* and washed once with an equal volume of 0.3 M sucrose.

Phospholipase C-treated sarcoplasmic reticulum

Sarcoplasmic reticulum suspensions of 15 mg protein/ml in a solution of 100 mM KCl 5 mM imidazole–HCl buffer (pH 7.0) and 0.5 mM $CaCl_2$ was incubated with phospholipase C from *Clostridium welchii* (EC 3.1.4.3) 1 mg/ml. After 30 min at 30° the reaction was terminated by the addition of ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) to bind the free calcium ions. The phospholipase C-treated sarcoplasmic reticulum was recovered by centrifugation for 20 min at 200000 × *g* and was washed twice with an equal volume of 0.3 M sucrose.

Analytical methods

Total calcium determination. Determination of total calcium in sarcoplasmic reticulum and the reagents used in this study was performed in the Perkin–Elmer atomic absorption spectrometer model 290. Interference by phosphate was overcome by the addition of 1% La^{3+} (ref. 13).

Assay of calcium binding. Sarcoplasmic reticulum preparations (2–6 mg protein) were equilibrated for 10 min with 10 ml of 5 mM Tris–HCl buffer (pH 7.4) and unless otherwise indicated, containing 10 μM, $^{45}CaCl_2$ with a specific activity of about $5 \cdot 10^6$ counts/min per μmole. The sarcoplasmic reticulum was recovered by centrifugation for 30 min at 200000 × *g* and the amount of bound calcium was calculated from radioactivity measurements of an aliquot of the sarcoplasmic reticulum sediment suspended in water[14]. Two types of controls were used to calculate the net amount of bound calcium. One control had the same composition as the reaction mixture but the sarcoplasmic reticulum preparation was first heated in a boiling water bath for 10 min. The second control contained a high concentration of unlabeled calcium (usually 0.01 M) but the same amount of $^{45}CaCl_2$ per ml.

When sarcoplasmic reticulum with bound ^{45}Ca was equilibrated with unlabeled calcium, practically all of it was found to be rapidly exchangeable. The amount of calcium binding depends on the concentration of Ca^{2+} regardless of whether it is added as $^{45}CaCl_2$ or as $^{45}Ca_2$EGTA buffer and the amount of Ca^{2+} calculated according to WEBER *et al.*[7]. Equilibration of sarcoplasmic reticulum with $^{45}CaCl_2$ for up to 1 h prior to centrifugation gave no additional binding.

Radioactivity measurements

^{45}Ca was determined in a Packard scintillation counter using the scintillation mixture described by BRAY[14].

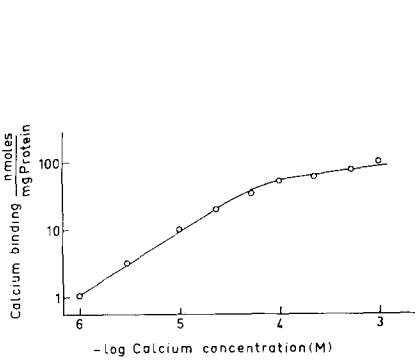
ATPase activity and active calcium uptake were determined as described in an accompanying paper[12]. Protein was determined by the method of LOWRY *et al.*[15]. Adenine nucleotide was determined as described by ESTABROOK *et al.*[16].

Materials

Trypsin, 2 times crystallized and *Cl. welchii* phospholipase C were obtained from Worthington. $^{45}CaCl_2$ was purchased from the Radiochemical Centre. EGTA was a product of Fluka. All other reagents were of analytical grade.

RESULTS

As can be seen from Fig. 1 the binding of calcium to sarcoplasmic reticulum membrane has a typical saturation curve. At low Ca^{2+} concentrations, there is a linear relationship between the specific binding of calcium and calcium concentration. At saturation, when the concentration of calcium in the medium was 0.1 mM the capacity for calcium binding is 50 nmoles/mg of sarcoplasmic reticulum protein with a small increase at higher concentrations.



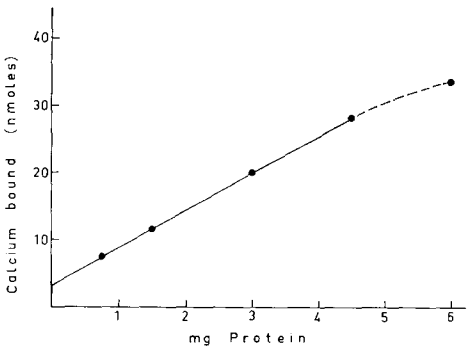


Fig. 1. Binding of calcium to sarcoplasmic reticulum at various calcium concentrations. Binding of calcium was measured as described under MATERIALS AND METHODS employing the indicated concentration of $CaCl_2$ and 0.3 mg/ml of sarcoplasmic reticulum protein.

Fig. 2. Calcium binding at various concentrations of sarcoplasmic reticulum protein. Calcium binding was measured as described under MATERIALS AND METHODS, employing the indicated amounts of sarcoplasmic reticulum protein per 10 ml and a concentration of 10 μM $CaCl_2$.

Essentially the same binding curves are obtained at 0° and at 25° (*cf.* Fig. 3). Since the amount of calcium taken up by active uptake is also not influenced by temperature, an assay of ATP[16] contamination in sarcoplasmic reticulum was performed. It was found that the sarcoplasmic reticulum contained less than 0.1 nmole ATP per mg of reticulum protein.

Assuming that each binding site has the same intrinsic affinity for calcium and each site is uninfluenced by its neighbors, the equation of KLOTZ was used to calculate the calcium dissociation constant[17]. A total calcium-binding capacity of 50 nmoles/mg of reticulum protein (see Fig. 2) was used to tabulate the amount of calcium binder. It can be seen that the experimental points in the plot shown in Fig. 4 fall on a straight line. This fact supports the assumption that under the conditions studied interaction between different sites is negligible, and the slope of the line can be used to calculate the dissociation constant between sarcoplasmic reticulum and calcium. The value of 40 μM thus obtained is in good correlation with the dissociation constant reported by CARVALHO[9].

Table I shows that calcium binding tested at low ionic strength is inhibited by mono- and divalent cations. Both magnesium and strontium inhibited calcium binding to a similar extent and on a molar basis are much more efficient than potassium and sodium.

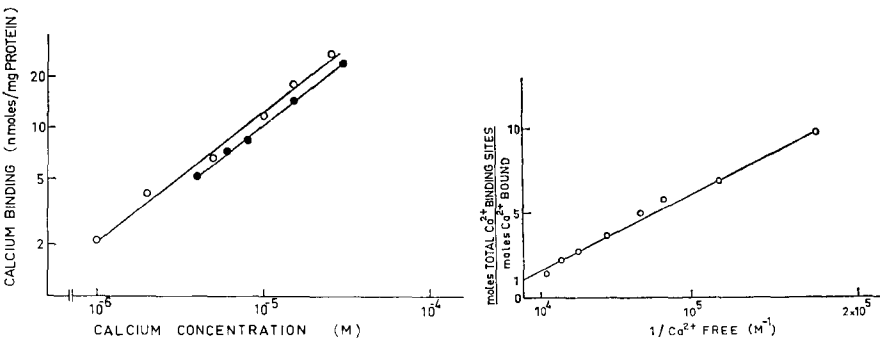


Fig. 3. The effect of temperature on calcium binding. Calcium binding was measured at 0° (●—●) and at 25° (○—○) in the stand assay for calcium binding.

Fig. 4. Klotz plot of calcium binding at different calcium concentrations. The reciprocal value of moles calcium bound per moles of available calcium binding sites (taken as 50 nmoles/mg protein, from Fig. 1) is plotted against the reciprocal value of free calcium concentration. The slope of the line is used to calculate the dissociation constant[17]. The apparent dissociation constant is 40 μM.

TABLE I

THE EFFECT OF MONOVALENT AND DIVALENT CATIONS ON CALCIUM BINDING

Calcium binding was measured in the presence of 5 mM Tris–HCl buffer (pH 7.4) and various concentrations of $CaCl_2$. When indicated, KCl, NaCl, $MgCl_2$ or $SrCl_2$ was added to the assay system.

Salt added	$CaCl_2$ *concn.*	*Calcium binding (μmoles/mg protein)*		
		1 μM	*10 μM*	*100 μM*
No addition		0.6	9	50
KCl (0.1 M)		0.20	1.3	10
NaCl (0.1 M)		0.25	2.0	12
$MgCl_2$ (1 mM)		0.29	1.9	6.5
$SrCl_2$ (1 mM)		0.13	1.0	5.5

The inhibition of calcium binding by other ions can be analyzed in terms of the Michaelis–Menten equation[18,19]. When the reciprocal value of specific calcium binding is plotted against the concentration of the inhibitory ion as shown in Fig. 5, a straight line is obtained for each concentration of Ca^{2+}. The intersection of these lines gives the value of $-K_i$. Calculated from Fig. 5 the K_i for the inhibition of calcium by potassium is 0.03 M. The K_i for sodium calculated from a similar plot (not shown) is 0.05 M.

Calcium binding at different pH values shows an asymmetrical pH optimum curve (Fig. 6). There is a sharp rise at the acid side of the curve and a moderate slope at the basic side. At pH 5.0 there is essentially no binding of calcium, while at pH 7.0 there is already maximal binding. When calcium binding is tested in the presence of

increasing concentrations of KCl there is a progressive decrease in calcium binding, leveling off at 0.6 M KCl. As can be seen from Fig. 7 about 10% of the calcium-binding sites are not affected by KCl. These sites can also be distinguished by trypsin proteolysis which specifically inactivates the KCl-insensitive calcium-binding sites

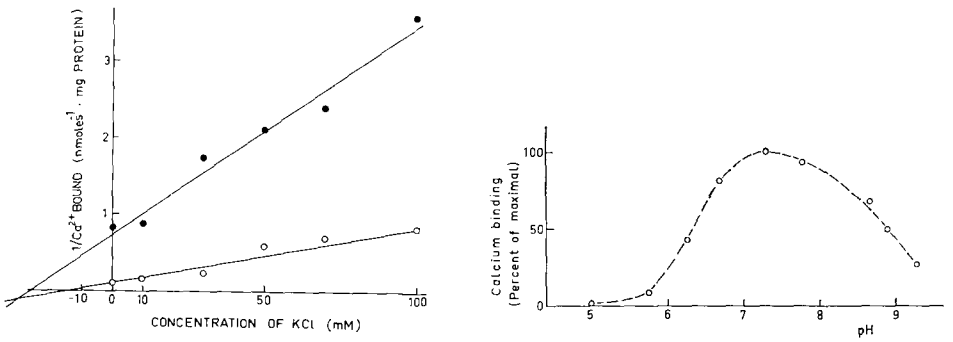


Fig. 5. The effect of KCl on calcium binding at two different concentrations of calcium. The indicated concentration of KCl was included in the standard assay for calcium binding containing 100 μM $CaCl_2$ (O—O) and 1 μM $CaCl_2$ (●—●).

Fig. 6. pH-optimum curve of calcium binding. The assay medium containing $CaCl_2$ (1 μM) was adjusted to the indicated pH values by the following buffers to give final concentrations of 5 mM: acetate buffer at pH 5.0 and 5.6, phosphate buffer at pH 6.2, 6.5 and 7.2. Tris–HCl buffer at pH 7.8, 8.6, 8.8 and 9.2. Calcium binding was measured by the procedure described under MATERIALS AND METHODS.

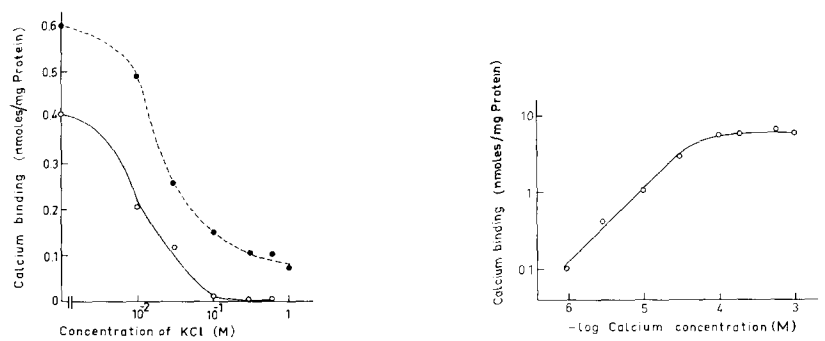


Fig. 7. The effect of KCl on calcium binding. The effect of KCl on calcium binding by trypsin-treated sarcoplasmic reticulum (O—O) and untreated control (●----●) was measured in the standard assay of calcium binding containing the indicated concentration of KCl and 1 μM $CaCl_2$. Trypsin treatment was carried out as described under MATERIALS AND METHODS.

Fig. 8. Calcium binding in the presence of 0.6 M KCl. Calcium binding was measured in the presence of 0.6 M KCl in the standard assay system for calcium binding and the indicated concentration of $CaCl_2$.

(*cf.* Fig. 7). Incubation of sarcoplasmic reticulum suspension at 70° for 10 min also causes specific inactivation of the KCl-insensitive calcium-binding sites (*cf.* Table II).

The properties of the trypsin-sensitive calcium binder were tested in the presence of 0.6 M KCl in the assay system. At this concentration of KCl essentially the only contribution to calcium binding is by the trypsin-sensitive calcium binder, which is only slightly affected by KCl.

The relationship between Ca^{2+} concentration and calcium binding is shown in Fig. 8. It can be seen that a saturation curve is also obtained for the KCl-insensitive calcium binding. Using the value of 5 nmoles calcium bound per mg protein, as total KCl-insensitive calcium-binding sites, the apparent dissociation constant was calculated from the plot shown in Fig. 9. Surprisingly, practically the same apparent dissociation constant was determined for the KCl-insensitive trypsin-inhibited calcium binding (*i.e.*, 35 μM) as for the total calcium binding measured at low ionic strength (40 μM).

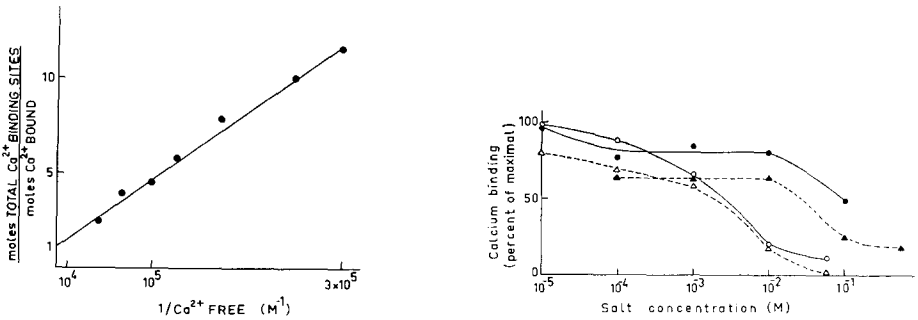


Fig. 9. Klotz plot of the KCl-insensitive calcium binding. Calcium binding was measured in the presence of 0.6 M KCl in the assay system. A value of 5 nmoles calcium bound per mg protein was used at the number of available calcium-binding sites at 0.6 M KCl concentration. This value is obtained from the saturation curve shown in Fig. 8. The apparent dissociation constant calculated from the slope of the curve is 35 μM.

Fig. 10. The effect of Mg^{2+} and Sr^{2+} on the KCl-insensitive calcium binder. Calcium binding was measured in the standard assay system in the presence of 0.6 M KCl and various concentrations of $MgCl_2$ or $SrCl_2$ as indicated in the abscissa of the Figure. ▲, 10 μM $CaCl_2$ + $MgCl_2$; △, 10 μM $CaCl_2$ + $SrCl_2$; ●, 100 μM $CaCl_2$ + $MgCl_2$; ○, 100 μM $CaCl_2$ + $SrCl_2$.

Two divalent cations were tested for their effect on the trypsin-sensitive calcium binding (Fig. 10). Magnesium which is essential for active calcium uptake has little effect on calcium binding up to a concentration of 0.01 M $MgCl_2$, where it causes a sharp decrease in calcium binding. Strontium, which is known to inhibit both the

TABLE II

THE EFFECT OF VARIOUS TREATMENTS ON CALCIUM BINDING BY SARCOPLASMIC RETICULUM

Calcium binding was measured at the indicated concentration of $CaCl_2$ in the presence and absence of 0.6 M KCl. Trypsin and phospholipase C treatments were carried out as described under MATERIALS AND METHODS. Heat treatment was the incubation of sarcoplasmic reticulum suspension for 10 min at 70°.

Treatment	*ATPase activity (units)*	*Calcium binding (nmoles/mg protein)*			
		$CaCl_2$(1 μM)	*$CaCl_2$(1 μM) + KCl (0.6 M)*	*$CaCl_2$(100 μM)*	*$CaCl_2$(100 μM) + KCl (0.6 M)*
Untreated	1.7	0.60	0.07	50	5.0
Phospholipase C	0.2	0.50	0.07	52	—
Trypsin	0.1	0.40	<0.01	40	<0.1
Trypsin + ATP	2.7	0.41	<0.01	—	<0.1
Heat	0.1	0.56	<0.01	—	—

rate and extent of active calcium uptake, inhibits calcium binding at much lower concentrations where comparable concentrations of magnesium have very little effect. Fig. 11 shows that the effect of strontium on the KCl-insensitive calcium binding is competitive inhibition, with an inhibition constant of $K_i = 2$ mM.

The effects of various treatments on the properties of the two calcium-binding systems are summarized in Table II. Of special interest is the relationship between ATPase activity and calcium binding. Phospholipase C treatment which causes inhibition of about 90 % of the ATPase activity has no effect on calcium binding, where-

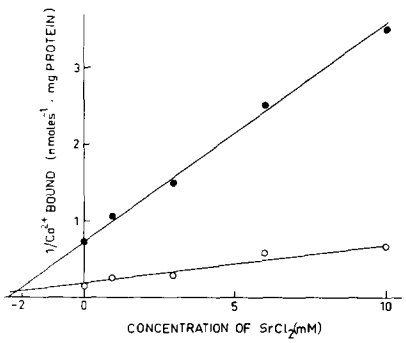


Fig. 11. The inhibition of calcium binding by $SrCl_2$. The indicated concentrations of $SrCl_2$ were included in the standard assay for calcium binding containing 100 μM $CaCl_2$ (○—○) and 10 μM $CaCl_2$ (●—●).

as trypsin proteolysis in the presence of ATP which causes an activation of ATPase activity, completely abolishes the specific calcium binder. These results rule out a suggestion that the calcium binder might be identical with the ATPase enzyme[8].

DISCUSSION

The present report essentially confirms the values reported by CARVALHO[9] for the dissociation constants of calcium, potassium and sodium measured at low ionic strength and neutral pH. The competition between potassium and calcium for binding sites in the sarcoplasmic reticulum membrane noted by CARVALHO[9] and the sensitivity of the assay for calcium binding used in the present work made it possible to demonstrate two types of calcium binding. About 90 % of the calcium-binding capacity was found to be inhibited at high ionic strength, whereas the remaining capacity could not be inhibited by increasing the salt concentration further.

The distinction between two types of calcium binding is supported by the fact that proteolysis by trypsin specifically inhibits those calcium-binding sites that are not affected by salts. A leveling off of calcium binding on further increasing the concentration of potassium can also be seen in the experiments reported by CARVALHO[9]. Accordingly his interpretation is that calcium, magnesium and potassium are bound to the same sites. However, the non-exchangeable, bound calcium which amounts to about 5–10 % of the maximum binding capacity[9] (in the present work a value of 10 % was found, *cf.* Fig. 7) was assumed to be an intrinsic part of the membrane structure.

In agreement with CARVALHO[9] we also found that calcium binding at low ionic strength is nonspecific (Table I). Potassium, sodium, magnesium and strontium inhibit calcium binding; the divalent cations, on an equivalent basis, are stronger inhibitors than the monovalent cations. The trypsin-sensitive calcium binder, on the other hand, shows a high specificity towards calcium. It is not inhibited by magnesium even at a magnesium to calcium molar ratio of 100 (Fig. 10). At as high a concentration of 0.1 M $MgCl_2$ the inhibition might be due to a nonspecific effect on conformation. Trypsin-sensitive calcium binding has a specificity similar to that of calcium uptake. Strontium, which is also actively transported and known to inhibit calcium transport, is also a competitive inhibitor of trypsin-sensitive calcium binding.

A further indication for a possible role of the trypsin-sensitive calcium binder in active transport is the report of MARTONOSI[11] that trypsin proteolysis of sarcoplasmic reticulum in the presence of ATP greatly abolishes active calcium uptake, whereas the ATPase activity is not reduced. As shown in Table II this treatment also destroys the calcium specific binder.

Current models of active transport systems postulate a carrier molecule in equilibrium between two states; one in which the transported molecule is strongly bound, and a second in which it is loosely bound. It is also assumed that ATP affects the equilibrium between the two states[21,22]. Active transport takes place when a thermodynamically favored and structurally oriented reaction causes an oscillation of the carrier between its two forms, with concomitant transport of material against a concentration gradient.

The ability of the carrier to bind and release transported material is generally explained by a change in affinity[23]. This view may be extended to include an increased affinity towards another ion, in effect, a changed specificity. The result would be a displacement of the transported ion into the internal medium.

The present work demonstrates the existence of two systems which bind calcium, each of which has the same affinity for calcium, but differs in its affinity towards other ions (Table II). It is tempting to speculate that these two systems are in effect the two forms of the carrier and that calcium is displaced by magnesium from the nonspecific form.

Trypsin serves to locate the two binders. As would be expected from active transport models, the specific calcium binder, which is presumably on the outer surface of the reticulum vesicles, is destroyed by trypsin, whereas the nonspecific calcium binder resists trypsin action, possibly by virtue of its location on the inner surface of the vesicles.

MARTONOSI[8] and MARTONOSI *et al.*[20] showed that phospholipase C from *Cl. welchii* hydrolyzed practically all the lecithin in the membranes of the sarcoplasmic reticulum and is without effect on other phospholipids. He also showed that phospholipase C treatment causes little, if any, change in the membrane proteins, as both ATPase activity and active calcium uptake were recovered on the addition of micellar dispersions of lecithin or lysolecithin. In the present study phospholipase C treatment was essentially without any effect on either type of calcium binding. This finding, together with the inactivation caused by trypsin proteolysis, strongly suggests that the specific calcium binder is a protein. The possibility, however, is not excluded that calcium binding may also be due to other phospholipids which are not hydrolyzed by phospholipase C.

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