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COMPARATIVE STUDIES ON Ca^{2+}- AND Mg^{2+}-BINDING OF SARCOPLASMIC RETICULUM AND CHROMAFFIN GRANULE MEMBRANES

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Abstract—The binding of calcium and magnesium ions to sarcoplasmic reticulum (SR) and chromaffin granule membranes was comparatively studied. The SR membranes are equipped with equal quantities of binding sites for both calcium and magnesium ions. The binding sites in presence of ATP combine specifically with calcium ions, while in the absence of ATP the binding sites react unspecifically with both ions. The trace amount of magnesium present in the SR membranes preparations is sufficient to drive ATP dependent calcium accumulation. Magnesium binding, however, is not affected by ATP. The chromaffin granule membranes bind calcium and magnesium in the same concentration range as observed for the SR membranes. Magnesium binding, however, is two times higher than that of calcium binding. In the absence of ATP, calcium and magnesium ions mutually compete. In the presence of ATP, magnesium binding values increase 3–5-fold, while the calcium binding isotherm remains unchanged. The appreciable contribution of the lipid phase to ions binding has been investigated, but was found to be of minor importance in this study.

In recent years strong evidence has been furnished showing that the storage of a great variety of substances is accomplished by ATP driven active transport processes. The membranes of the sarcoplasmic reticulum of the muscle and the adrenalin storing granules of the adrenal gland belong to the most extensively studied structures. Inside the cisternal enlargements of the SR membranes high concentrations of calcium ions are stored by an ATP supported process—involving phosphorylation and dephosphorylation of the transport-protein [13, 16].

The much weaker membrane bound ATP dependent transport system involved in the uptake of catecholamine into chromaffin granule is mediated by a proton motive force generated by ATP-splitting [2, 20, 21, 34].

The two systems differ considerably with respect to their kinetics and their energetics. As a common feature, the activities of both transport systems require the presence of ionized magnesium. We have, therefore, to assume that the membranes do not only interact with the respective transport substrate calcium or catecholamine but also with magnesium ions at cytoplasmic concentrations.

While considerable knowledge concerning the process of storage has accumulated, comparatively little is known about the mechanism by which the stored, substances are released. Many observations indicate that calcium ions at low concentrations are involved in the release process [3, 6, 9, 12, 17, 30, 37].

Hence, it can be expected that calcium binding sites are present in these membranes whose dissociation constants are in the range of the cellular calcium concentration [29]. While the interaction of calcium with the sarcoplasmic reticulum membranes has been studied quite extensively, only a few aspects of calcium binding to the membranes of the chromaffin granules have been investigated. Native chromaffin granules contain considerable quantities of calcium and magnesium [5, 18, 43]. Although intact granular membranes appear to be only slightly permeable for calcium, magnesium and even monovalent cations, binding studies performed with intact granules indicate that the organelles can bind calcium [5, 18, 12].

Calcium binding studies performed by measuring the electrophoretic mobility of the organelles furnished estimates concerning the number and the affinity for the externally located calcium binding sites [8]. In the following the results of a comparative study will be presented in which the binding of calcium and magnesium ions to sarcoplasmic reticulum and chromaffin granule membranes have been studied in order to gain information concerning the mutual interactions of these two ions with the membranes. The external cytoplasmic surfaces of both membrane systems are equipped with binding sites for calcium and magnesium ions at similar concentrations and affinities. In the sarcoplasmic reticulum membranes the magnesium binding sites can be identified as constituents of the ATP-dependent calcium transport system. In both membranes the low affinity sites for calcium can become occupied only at calcium concentrations transiently present in the cytoplasm when calcium ions invade the cell during excitation.

MATERIALS AND METHODS

Preparations of membranes

Sarcoplasmic reticulum vesicular membranes. Sarcoplasmic reticulum vesicular membranes were prepared from skeletal muscle of rabbits. The vesicles were fractionated by centrifugation in 0.5 and 1.0 M

sucrose according to the method of Hasselbach [15]. The vesicles were washed with 0.1 M KCl, sedimented by centrifugation (100,000 *g* for 60 min) and finally resuspended in 0.1 M KCl.

Membranes of chromaffin granules from bovine adrenal medulla

The membranes of chromaffin granules are prepared by the method of Smith and Winkler [40] and Foldes *et al* [11] as modified by Balzer and Khan [4] chromaffin granules were from a 1.8 M sucrose fraction; see Schneider [38] and Huber *et al* [19]. Bovine adrenals were obtained from the slaughter house and transported in ice to the laboratory. All subsequent steps were carried out at 4°. The medulla was carefully removed and homogenized with 0.3 M sucrose. The homogenate was centrifuged for 30 min at 1000 *g*. The supernatant was filtered through cheese-cloth and the filtrate was re-centrifuged for 30 min at 15,000 *g*. The sediment containing granules was homogenized with 0.3 M sucrose and subjected to sucrose gradient centrifugation. Eight millilitres of 0.3 M sucrose suspension were layered over 20 ml of 1.8 M sucrose solution and centrifuged at 105,000 *g* for 30 min. According to Schneider [38] the application of 1.8 M sucrose yields a preparation practically free of mitochondrial contaminations.

A pink sediment was formed in the 1.8 M sucrose layer, clearly separated from light brownish material which formed a band above the sediment. The granules contained in the 1.8 M sucrose sediment were lysed in 50 vol. of 0.1 M KCl. The membranes were sedimented by centrifugation at 105,000 *g* for 30 min and resuspended in 0.1 M KCl (~2% soluble proteins in the lysed membranes) [42]. Membranes lysed further by hypoosmotic shock showed now significant changing in binding-values.

Determination of Ca^{2+}- *and* Mg^{2+}-*binding*

Membrane preparations equivalent to 0.2 mg/ml of protein (in some experiments with chromaffin granule membranes 0.1 mg protein/ml) were incubated for 2 min at 22° in histidine buffer (pH 7.0, 0.02 M) with increasing concentrations of radioactively labelled Ca^{45} or Mg^{28}, respectively. Incubation media containing calcium concentrations $<10\ \mu M$ were supplemented with calcium EGTA buffers according to Makinose and Hasselbach [26] using $K_{diss} = 2 \times 10^{-7}$ M [27, 39] as dissociation constant. Calcium binding in the presence of magnesium was studied at a total magnesium concentration of 5 mM. The same concentration of calcium was applied to study its effect on magnesium binding. Energy dependent ion binding was analyzed in the presence of 5 mM ATP. For the calculation of the concentrations ionized calcium and magnesium in the ATP containing media $K_{diss} = 10^{-3}$ M and $K_{diss} = 10^{-4}$ M [1] were used as dissociation constants for the calcium ATP and magnesium ATP complex, respectively. The membranes were separated from 2.0 ml of the incubation medium by ultrafiltration through Sartorius filters (0.45 μm) according to Paulus [33] and Fiehn *et al.* [10]. The filters retained quantitatively the vesicular protein, since no protein could be detected in the filtrate. The filters were washed twice with 1 ml of distilled water and subsequently transferred to scintillation vials. The scintillation fluid contained 100 g naphthalene, 100 mg POPOP, 14 g PPO, 200 ml methanol and dioxane giving a total volume of 2000 ml. To account for residual radioactivity trapped in the filters, controls containing no membrane protein were filtered.

If the vesicular membranes were permeable for calcium and magnesium the associated amounts determined by filtration technique comprise two

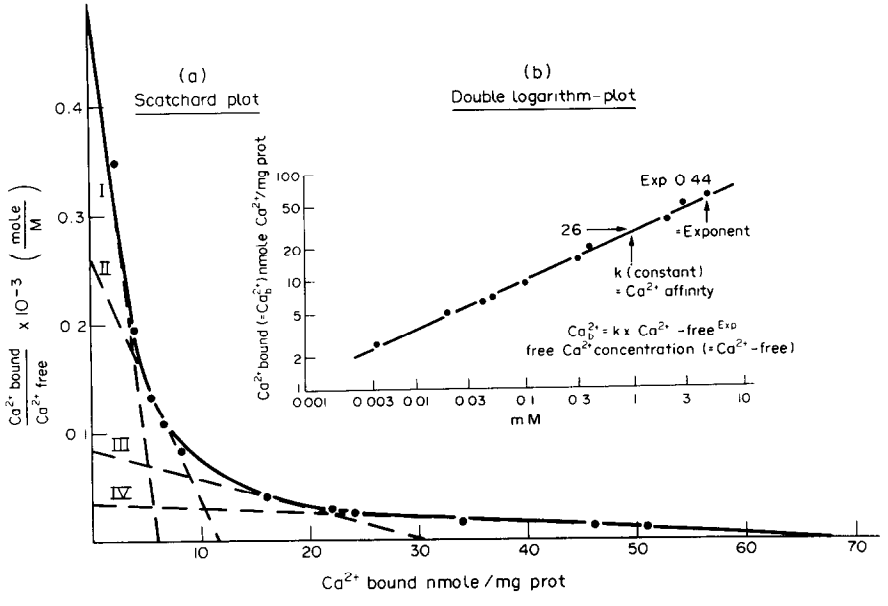

Fig. 1. Comparative schematic representation of binding experiment calculations by Scatchard plot (A) and double-logarithmic plot method (B).

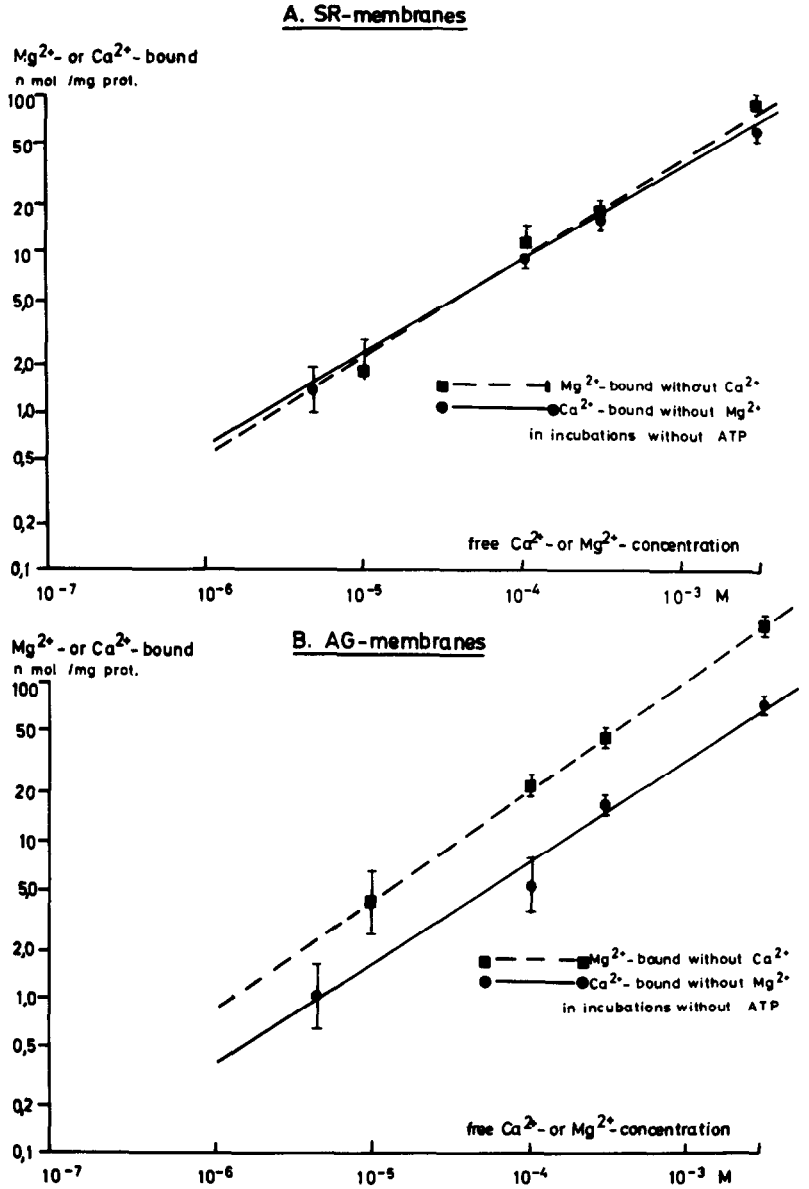

Fig. 2. Mg^{2+}- and Ca^{2+}-binding in (A) SR-sarcoplasmic reticulum- and (B) AG-adrenal granule-membranes. (9–12 experiments for each mean value ± S.E.M.)

fractions, one fraction is bound to the membrane protein, the other is trapped inside the vesicular volume. In order to avoid any possible error from this latter the membranes were washed twice with distilled H_2O, thus Ca^{2+}- or Mg^{2+}-determination refer to membrane bound ions only. The content of Mg^{2+}- or Ca^{2+}-contamination traces in the membranes or incubation medium was measured by atomic absorption.

In double logarithmic plots the binding isotherms appeared as straight lines covering large concentration intervals. The curves flattened near complete saturation which is only achieved for ATP-dependent calcium uptake (see Fig. 1, Scatchard plot and log/log plot) and the literature about the misinterpretation of the Scatchard plot [8, 23, 31].

The results were in the log/log plot preferable to the Scatchard plot for clarity of determining values and also for avoiding arbitrariness and errors in calculation.

The log/log plot was chosen in order that the concentrations tested should cover the full range of physiological possibilities. The values measured were based on the mathematical double log equation.

$$Ca_b^{2+} = K \times Ca^{2+}\text{-free}^{Exp.} \qquad (1)$$

(see Fig 1). The value Ca^{2+} bound is equal to $K = Ca^{2+}$ bound at 1.0 mM Ca^{2+}-free concentration (coefficient) and the magnitude of the exponent of the curve. The mathematical background of the function is the equation (1) and the theoretical justification for the values found in straight line of the double log plot showing the mathematical dependence of the values found. That means $K = Ca^{2+}$-

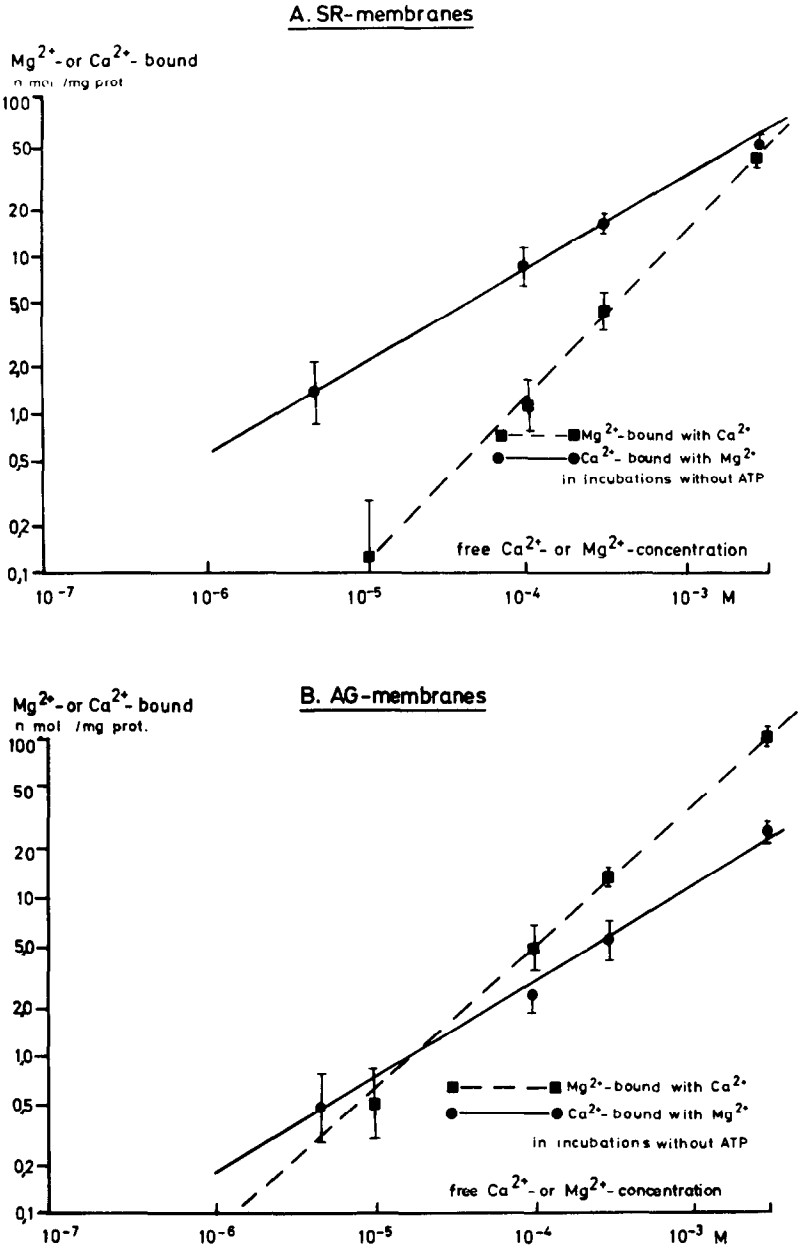

Fig. 3. Comparison of Mg^{2+}- and Ca^{2+}-binding in (A) SR-sarcoplasmic reticulum- and (B) AG-adrenal granule membranes with mutual interaction of the other ion by the additional presence of 5 mM $CaCl_2$ in the incubation medium. (9–12 experiments for each mean value ± S.E.M.)

affinity multiplied by the Ca^{2+}-free concentration gives the factor for the ionic binding capacity. The exponent of the equation (1), is the factor for the saturation of the binding sites declining by the increase of the Ca^{2+}-free concentration, when the exponent is Exp < 1.0.

Materials. Calcium[45] was purchased from Amersham Buchler GmbH. Braunschweig, and Magnesium[28] from NEN Chemicals GmbH., Dreieich, Germany. Mg^{28} with high specific activity was produced in U.S.A. (obtained from NEN Chemicals, Boston) and directly sent from Frankfurt airport to the institute.

RESULTS

Passive binding

In the following, the expression "binding" is used as a technical term to describe the association of the ions with the vesicular membranes. Passive binding refers to the results obtained in the absence of ATP from the incubation medium. The possible compartmental distribution of the bound ions will be considered in connection with the discussion of Figs. 2 and 5.

Figures 2(A) and (B) illustrate that both types of membranes possess binding sites for magnesium and

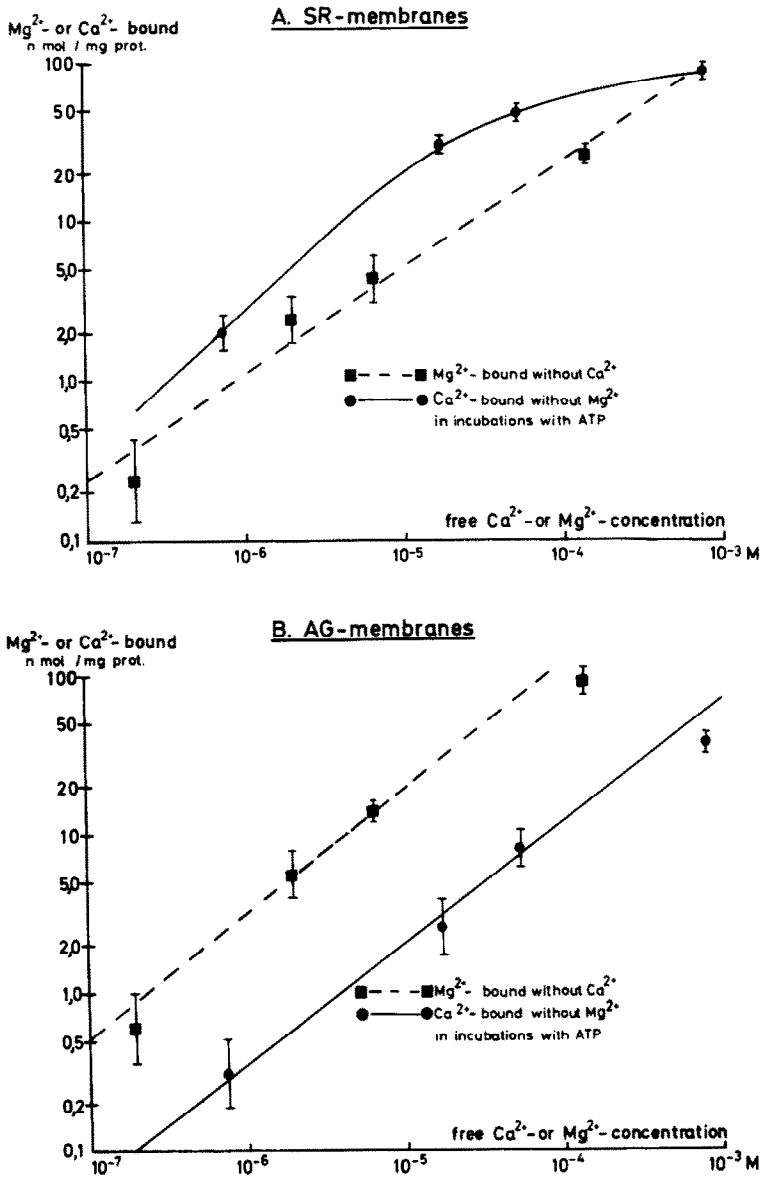

Fig. 4. Mg^{2+}- and Ca^{2+}-binding in (A) SR-sarcoplasmic reticulum- and (B) AG-adrenal granule-membranes by the additional presence of 5 mM ATP in the incubation medium. (9–12 experiments for each mean value ± S.E.M.)

calcium ions which are occupied at micro- to millimolar concentrations. Furthermore, both membranes have similar binding capacities of 50–100 nmoles/mg protein. The data are plotted double logarithmically in order to cover the large range of free ion concentrations and the corresponding binding values. Since the experiments were performed in media of low ionic strength, one can expect that all cationic binding sites present in the membranes have been titrated.

Since the sarcoplasmic reticulum membranes as well as the membranes of the chromaffin granules retain their physiological sites, the quantities of ions bound mainly reflect the association of ions with the cytoplasmic surface of the membranous vesicles.

It is illustrated by Fig. 2(B) that at all concentrations in chromaffin granule membranes the number of magnesium binding sites exceed that of calcium ions. The apparent preference of the chromaffin granule membranes for magnesium ions is substantiated by the results obtained in the presence of 5 mM calcium.

As shown in Fig. 3(B), this high concentration of calcium ions affects only slightly the binding of magnesium ions in the millimolar range, whereas in the micromolar range magnesium binding is significantly reduced indicating that high affinity calcium binding sites can be occupied by magnesium ions (compare Fig. 2B). On the other hand, the binding of calcium is little affected by high concentrations of magnesium ions.

A very different pattern of ion binding characterizes the sarcoplasmic reticulum membranes (Fig. 3A). Calcium ions can effectively displace magne-

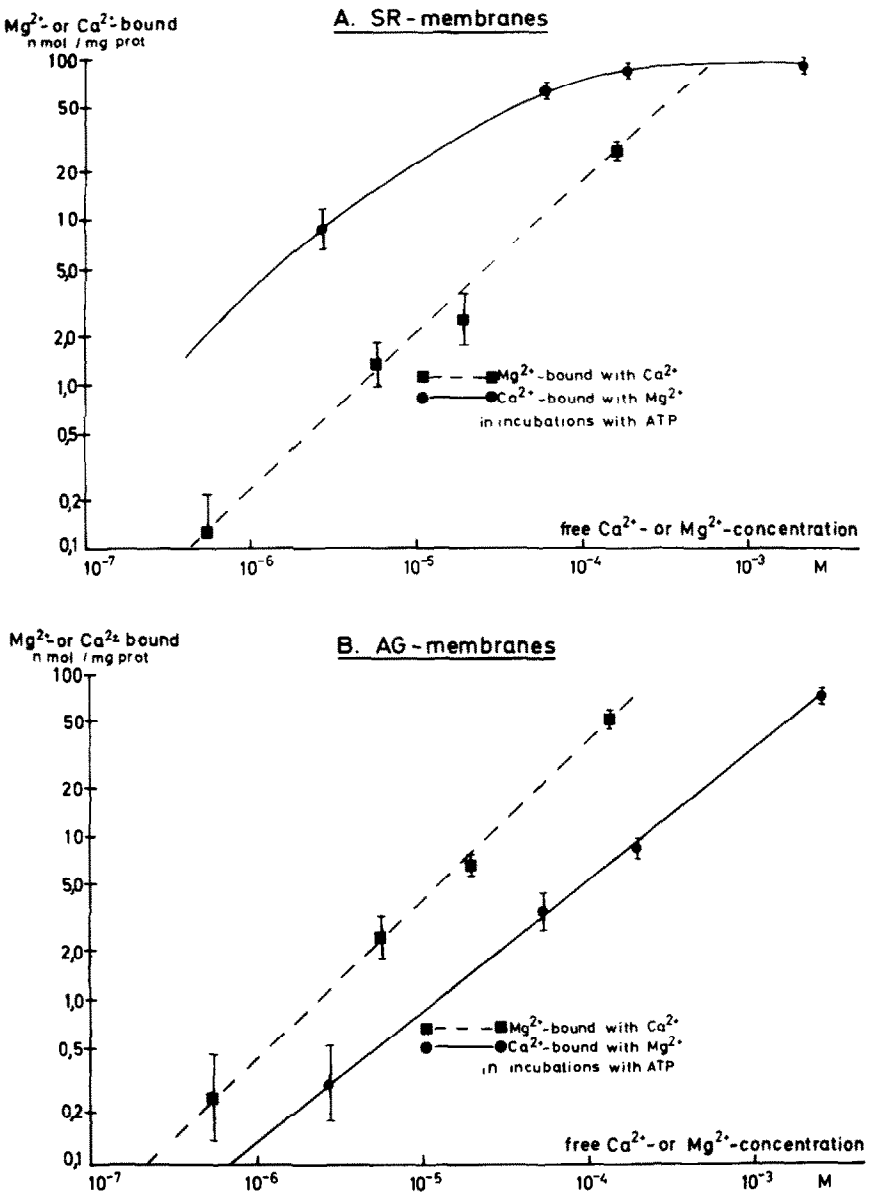

Fig. 5. Comparison of Mg^{2+}- and Ca^{2+}-binding in (A) SR-sarcoplasmic reticulum- and (B) AG-adrenal granule-membranes with mutual interactions of the other ion in the presence of ATP. The addition in the incubation medium was 5 mM $CaCl_2$, resp. $MgCl_2$ and 5 mM ATP. (9–12 experiments for each mean value ± S.E.M.)

sium ions while magnesium ions do not considerably influence calcium binding. Evidently, magnesium ions combine relatively unspecifically with the binding sites from which they can be displaced by calcium ions. On the other hand, ionized calcium at low concentration combined with specific sites which have a relatively low affinity for magnesium ions.

Calcium and magnesium binding in the presence of ATP

Calcium binding. The interaction of transporting membranes with ATP can interfere with cation binding in quite a different way. As demonstrated for the sodium–potassium transporting membranes the binding of ATP can modify sodium and/or potassium binding [35]. Furthermore, under conditions where the terminal phosphate group of ATP is transferred to the protein, the affinity of the membranes for the respective ions can be altered. From these effects of ATP the ATP supported accumulation of the ions must be distinguished. The ATP dependent calcium storage of sarcoplasmic reticulum membranes in the presence and the absence of added magnesium ions is illustrated by Figs. 4(A) and 5(A) respectively.

The effect of ATP is most clearly seen at low concentrations of free calcium ions. At high concentration of calcium, ATP does not increase calcium storage.

These results must be considered as a coincidence, because in the absence of ATP only external binding

sites are occupied while ATP dependent calcium uptake leads to the saturation of internal calcium binding sites (Figs. 4 and 5). At the external calcium concentration of 0.5 mM, when 100 nmoles/mg calcium are combined with the vesicles, approximately 20 nmoles calcium/mg are bound to the external vesicular surface as can be estimated from Fig. 2. Therefore, the remaining 80 nmoles calcium are confined to the interior surface of the membranes. On account of the relatively low affinity of the internal sites located in the calcium transport protein [15] and in calsequestrin [25] approximately 30–40 nmoles calcium/mg protein remain unliganded corresponding to a concentration of soluble calcium of 5–8 mM.

The uptake of calcium in the absence of added magnesium shown in Fig. 4(A) needs some comment, since it is well established that an active transport of calcium ions needs magnesium ions as activator. Evidently, the small amount of magnesium ions which is bound to the membranes [22] at the usual total concentration of contaminating magnesium is sufficient to support a low calcium uptake which leads during the incubation period of 2 min to the observed accumulation.

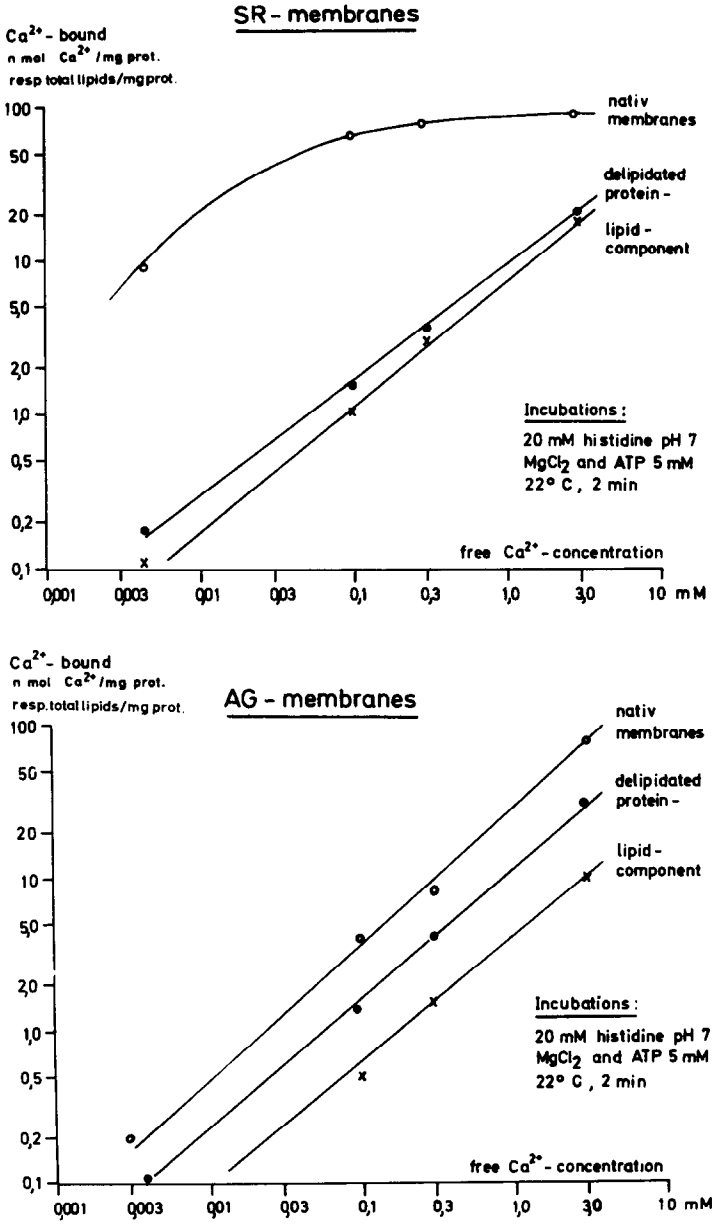

Fig. 6. Ca^{2+}-binding of SR- and AG-membranes with delipidated proteins and lipid components. Delipidation of proteins: The products of hydrolysis after phospholipase A digestion were removed by albumin and 0.1 M KCl washings (Fiehn and Migala [10]. The lipids were extracted with chloroform–methanol 2:1 from the native membranes and sonicated. Both lipidated proteins and total lipids were calculated to the equivalent content to 1 mg protein and incubated at the indicated incubation medium.

The binding of calcium by the chromaffin granule membranes is comparatively little affected by ATP either in the presence or in the absence of magnesium ions (Figs. 4 and 5) (compare Figs. 4 with 2 and 5 with 3).

Magnesium binding

In contrast to calcium binding of the sarcoplasmic reticulum membranes magnesium binding is only slightly affected by ATP. In the absence of calcium the addition of 5 mM ATP caused a small increase in magnesium binding. In the presence of calcium and ATP (Fig. 5) the magnesium binding isotherm deviated only slightly from that observed in the absence of both ATP and calcium (Fig. 2A). Evidently the competitive effect of calcium observed in the absence of ATP (Fig. 3A) is very much reduced in the presence of ATP because chelation of calcium by ATP lowers the free calcium concentration in the incubation medium (Figs. 4 and 5).

In contrast to the sarcoplasmic reticulum membranes, magnesium binding of the chromaffin granule membranes is significantly enhanced by ATP. It produces a considerable enhancement of magnesium binding at low as well as at high concentrations (Figs. 2B and 4B). This enhancement is largely reduced by the addition of 5 mM calcium.

In Table 1 the values found in the log/log plot are summarized.

The Mg^{2+}-binding show in the SR-membranes as well as in the AG-chromaffin granule membranes similar exponents with a high binding capacity in presence of ATP. The exponents increased to values near 1.0 (linearity) in presence of Ca^{2+} with and without ATP.

The behaviour of Ca^{2+} in the SR-membranes shows in presence of ATP the active Ca^{2+}-transport with Ca^{2+}-binding on the out- and inside of the membranes. These values do not follow the double log plot equation but result in a curve.

In the AG-membranes the Ca^{2+}-binding show no similar ATP effect. The increased Mg^{2+}-binding capacity in the AG-membranes in presence of ATP seems not to be an ion-penetration from one side of the membrane to the other, but is more likely due to an ATP effect on the binding dynamics.

DISCUSSION

The results demonstrated in Fig. 2 show that the membranes display a binding characteristic for magnesium ions which appears very similar to that for calcium ions. However, while the binding of calcium is not significantly affected by high concentration of magnesium, this ion, magnesium, is displayed from the binding sites by calcium ions. This corresponds to the behaviour of the calcium binding sites in troponine from which Ca^{2+} cannot be displaced by Mg^{2+} [36]. The low permeability of the membranes for the ions do not allow for penetration of measurable amounts into the vesicles in the absence of ATP [7, 28].

The Ca^{2+}-binding curve found in the presence of ATP are in good agreement with most results reported in the literature. In the presence of ATP the binding capacity of the sarcoplasmic reticulum membranes becomes elevated and reaches 50–100 nmoles Ca^{2+}/mg protein at a free Ca^{2+}-concentration of 0.1 mM in the solution. These Ca^{2+}-binding sites which combine with Ca^{2+} are mostly located inside the sarcoplasmic reticulum vesicles and are saturated as the result of the ATP-dependent calcium accumulation [14]. It has remained a matter of controversy which component of the sarcoplasmic reticulum vesicles constitutes these calcium binding sites [24, 25].

The observation that Mg^{2+} does not affect the ATP dependent calcium-accumulation has two implications: As mentioned above the traces of Mg^{2+} that contaminate the membranes are sufficient to activate ATP-dependent Ca^{2+}-translocation. The binding of large quantities of Ca^{2+} in the presence of ATP, but in the absence of added Mg^{2+} clearly indicates that Ca^{2+} is not taken up in exchange for Mg^{2+}—as suggested by Tada *et al.* [41].

The concept of a Mg–Ca exchange also disagrees with the fact that Mg^{2+}-binding is not reduced under conditions where Ca^{2+} is accumulated. The fact that in the presence of high concentrations of Ca^{2+} which elevates the level of phosphoprotein, magnesium binding is invariant, indicates that phosphorylation evidently does not change the magnesium affinity of the membranes.

The chromaffin granule membranes combine with

Table 1.

		SR-membranes						AG-membranes					
		Without ATP			With ATP			Without ATP			With ATP		
		Free concn. (M)			Free concn. (M)			Free concn. (M)			Free concn. (M)		
		10^{-6}	10^{-4}	10^{-3}	10^{-6}	10^{-4}	10^{-3}	10^{-6}	10^{-4}	10^{-3}	10^{-6}	10^{-4}	10^{-3}
Mg^{2+}-binding (nmoles/mg protein)	Without Ca^{2+}	0.55	9	40	1.1	23	110	0.8	20	100	3.2	120	725
		(Exp. 0.63)			(Exp. 0.66)			(Exp. 0.70)			(Exp. 0.78)		
	With Ca^{2+}	0.01	1.3	14	0.24	17	135	0.08	5	38	0.46	40	360
		(Exp. 1.03)			(Exp. 0.92)			(Exp. 0.89)			(Exp. 0.95)		
Ca^{2+}-binding (nmoles/mg protein)	Without Mg^{2+}	0.6	9	38	2.8	57	85	0.35	7	33	0.36	12	70
		(Exp. 0.59)			(curve)			(Exp. 0.65)			(Exp. 0.76)		
	With Mg^{2+}	1.3	9	24	3.6	70	90	0.2	3	12	0.13	5	32
		(Exp. 0.44)			(curve)			(Exp. 0.60)			(Exp. 0.79)		

Ca^{2+} and Mg^{2+} in the same concentration range as the sarcoplasmic reticulum membranes. In general, the chromaffin granule membranes are equipped with nearly twice the binding sites for Mg^{2+} than for Ca^{2+}. In the absence of ATP the Ca^{2+} and Mg^{2+} ions can mutually compete for their binding sites.

In the presence of ATP much larger amounts of magnesium were found to be associated with the membranes. At a concentration of 0.1 mM ionized magnesium, which corresponds to the free magnesium concentration in the cytoplasm, the membrane associated amount of magnesium is increased by the factor of 4, i.e. from 15 to 60 nmoles/mg protein. The present results do not allow to decide if this increase of magnesium association is brought about by an ATP dependent binding reaction on the outside of the membrane or by the active uptake of magnesium into the vesicular lumen and also binding inside of the membrane due to change in membrane dynamics or permeability. In the case of the sarcoplasmic reticulum membranes various proteins with specific cation binding properties have been identified [32]. Such a designation is not possible for the chromaffin granule membranes. An associated binding of the lipid phase can be ignored in the binding experiments showing that the extracted total lipids under the same conditions bind approximately 10–50 times less Ca^{2+} than the native membranes (at 0.1 mM free concentration the sarcoplasmic reticulum-lipids bind only 1.0 vs 70 nmoles; chromaffin granule membrane-lipids 0.45 vs 5.0 nmoles) (Fig. 6).

As to the significance of calcium and magnesium binding by sarcoplasmic reticulum membranes, its functional involvement in the calcium concept of excitation concentration coupling is well established. In the less precise concept of excitation secretion coupling a key role has also been assigned to calcium ions. For the suprarenal gland, it is well established that acetylcholine affects catecholamine release by inducing an influx of calcium into the cell. It has repeatedly been reported that calcium ions induce catecholamine release by directly promoting the aggregation of catecholamine vesicles with the plasma membrane [17, 44]. Such a direct calcium effect requires the presence of calcium binding sites on the external surface of the chromaffin granules and/or the internal surface of the plasma membrane. The affinity of these sites should be high and fall into the range observed in this study.

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REFERENCES

1. R. Adolfsen and E. N. Moudrianakis, *J. biol. Chem.* **253**, 4378 (1978).
2. D. K. Apps, *Fedn. Proc.* **41**, 2775 (1982).
3. P. F. Baker and D. E. Knight, *Nature* **276**, 620 (1978).
4. H. Balzer and A. R. Khan, *Naunyn-Schmiedeberg's Arch. Pharmac.* **291**, 319 (1975).
5. J. L. Borowitz, *J. Cell. Physiol.* **69**, 305 (1967).
6. S. W. Carmichael, *Trends Pharmac. Sci.* **3**, 308 (1982); *J. Autonomic Nervous System* **7**, 7 (1983).
7. J. Chevallier and R. A. Butow, *Biochemistry* **10**, 2733 (1971).
8. Ph. M. Dean and E. K. Matthews, *Biochem. J.* **142**, 637 (1974).
9. W. W. Douglas, *Br. J. Pharmac.* **34**, 475 (1968).
10. W. Fiehn and A. Migala, *Eur. J. Biochem.* **20**, 245 (1971).
11. A. Foldes, P. L. Jeffrey, B. N. Preston and L. Austin, *Biochem. J.* **126**, 1209 (1972).
12. M. J. Geisow and R. D. Burgoyne, *J. Neurochem.* **38**, 1735 (1982).
13. W. Hasselbach and M. Makinose, *Biochem. Z.* **333**, 518 (1961).
14. W. Hasselbach, *Ann. N. Y. Acad. Sci.* **137**, 1041 (1965).
15. W. Hasselbach, in *Molecular Bionergetics and Macromolecular Biochemistry*, pp. 149–171. Springer, Berlin (1972).
16. W. Hasselbach, in *Topics in Current Chemistry*, Vol. 78, pp. 1–56. Springer, Berlin (1979).
17. D. H. Haynes, M. A. Kolber and S. J. Morris, *J. theor. Biol.* **81**, 713 (1979).
18. N. A. Hillarp, in *Adrenergic Mechanisms* (J. Vane, G. Wolstenholme and M. O'Connor, Eds.). CIBA, London (1960).
19. E. Huber, P. König, G. Schüler, W. Aberer, H. Plattner and H. Winkler, *J. Neurochem.* **32**, 35 (1979).
20. R. G. Johnson, S. Carty and A. Scarpa, *Fedn. Proc.* **41**, 2746 (1982).
21. R. G. Johnson and A. Scarpa, *J. biol. Chem.* **254**, 3750 (1979).
22. H. R. Kalbitzer, D. Stehlik and W. Hasselbach, *Eur. J. Biochem.* **82**, 245 (1978).
23. I. M. Klotz, *Science* **217**, 1247 (1982).
24. D. H. MacLennan, *J. biol. Chem.* **245**, 4508 (1970).
25. D. H. MacLennan, *J. biol. Chem.* **248**, 980 (1974).
26. M. Mackinose and W. Hasselbach, *Biochem. Z.* **343**, 360 (1965).
27. A. E. Martell, in *Stability Constants of Metal-Ion-Complexes*, pp. 647–655. The Chem. Soc., London (1971).
28. H. Miyamoto and M. Kasai, *J. Biochem.* **85**, 765 (1979).
29. S. J. Morris and R. Schober, *Eur. J. Biochem.* **75**, 1 (1977).
30. St. J. Morris, Th. C. Südhof and D. H. Haynes, *Biophys. J.* **37**, 117 (1982).
31. J. G. Nørby, P. Ottolenghi and J. Jørgen, *Analyt. Biochem.* **102**, 318 (1980).
32. T. J. Ostwald and D. H. MacLennan, *J. biol. Chem.* **249**, 5867 (1974).
33. H. Paulus, *Analyt. Biochem.* **32**, 91 (1969).
34. Ch. J. Pazoles, *Fedn. Proc.* **41**, 2769 (1982).
35. R. Post, S. Kume and F. N. Rogers, in *Mechanisms in Bioenergetics* (G. F. Azone *et al.*, Eds), pp. 203–218. Academic Press, New York (1973).
36. J. D. Potter, B. Nagy, H. Collins, J. C. Seidel, P. Laevis, S. S. Lehrer and J. Gergely, *Molecular Basis of Motility* (L. M. G. Heilmeyer, J. Rüegg and T. Wieland, Eds.), pp. 93–106. Springer, Berlin (1976).
37. H. J. Schümann and A. Philippu, *Naunyn-Schmiedeberg's Arch. exp. Path. u. Pharmak.* **244**, 366 (1963).
38. F. H. Schneider, *Biochem. Pharmac.* **21**, 2627 (1972).
39. G. Schwarzenbach, *Die komplexometrische Titration*. F. Enke, Stuttgart (1960).
40. A. D. Smith and H. Winkler, *Biochem. J.* **103**, 480 (1967).
41. M. Tada, T. Yamamoto and Y. Tonomura, *Physiol. Rev.* **146**, 1 (1978).
42. G. Taugner, *Naunyn-Schmiedeberg's Arch. Pharmac.* **274**, 299 (1972).
43. H. Winkler and E. Westhead, *Neuroscience* **5**, 1803 (1980).
44. H. Winkler, *Scand. J. Immunol.* **15**, Suppl. 9, 75 (1982).