Low Affinity Calcium Binding Sites of the Calcium Transport ATPase of Sarcoplasmic Reticulum Membranes

Wilhelm Hasselbach and Vera Koenig

Max-Planck-Institut für medizinische Forschung, Abteilung Physiologie, Jahnstr. 29, D-6900 Heidelberg

Z. Naturforsch. **35 c,** 1012–1018 (1980); received August 27, 1980

Sarcoplasmic Reticulum, Calcium Binding, Low Affinity Binding Sites

Calcium binding sites having low affinity constants of $< 10^3\ M^{-1}$ were titrated in native sarcoplasmic reticulum vesicles as well as in lipid deprived membranes and in the isolated calcium transport ATPase. Short time calcium binding measurements and the determination of the calcium binding heat allow to distinguish low affinity calcium binding sites located on the external surface of the sarcoplasmic reticulum membranes from those present in the section of the transport molecule directed to the vesicular space. The same number of internal binding sites was found for preparations deprived of their lipid content as well as of preparations depleted of their lipids and of their accessorial proteins. Magnesium interferes with calcium binding to the external as well as to the internal low affinity calcium binding sites. The implications of the existence of the low affinity calcium binding sites in the internal section of the calcium transport ATPase are discussed.

Introduction

The existence of a variety of calcium binding proteins has been demonstrated in the membranes of the sarcoplasmic reticulum [1–5]. It has been shown that high affinity binding sites for calcium are present in the section of the calcium transport protein facing the cytoplasmic surface of the vesicles. The activation of calcium transport depends on the occupation of these sites [3, 6]. The majority of the calcium binding sites, however, have a low affinity. A considerable fraction of these are located on the external surface of the membranes [7]. Yet, most of the low affinity binding sites are thought to reside in extrinsic proteins, located in the interior of the sarcoplasmic reticulum vesicles [4, 5, 8]. These proteins are not involved in calcium transport and are considered to increase the calcium storing capacity of the reticulum. While there is general agreement concerning the existence of high affinity calcium binding sites in the calcium transport ATPase, the occurrence of internally located low affinity sites in the molecule is a matter of conjecture [9–12]. Since the release of calcium into the interior of the vesicles during active calcium accumulation requires that at least transiently the affinity of the transport protein for calcium is reduced, it has been suggested that the low affinity sites are transiently formed when the transport protein is phosphorylated by ATP [9, 10]. Internal low affinity calcium binding sites in the unphosphorylated protein are generally considered as nonexistent. On the other hand, it has been demonstrated that in the absence of ATP the presence of calcium concentrations in the millimolar range in the interior of the vesicles causes changes in the affinity of the calcium transport protein for magnesium and inorganic phosphate [13-17]. This effect of internal calcium indicates the presence of permanent, low affinity calcium binding sites on the internal surface of the reticular membrane. In this study we attempted to find out whether such sites can be established and differentiated from the sites residing in the accessorial proteins. For this purpose, we studied calcium binding of native vesicles and of the purified lipoprotein complex of the isolated ATPase and that of partially delipidated ATPase preparations.

Abbreviation: EGTA, ethyleneglycol bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid.

Enzymes: Phospholipase A_2 or phosphatide 2-acylhydrolase (EC 3.1.1.4); Ca^{2+}-dependent ATPase, ATP phosphohydrolase (EC 3.6.1.3).

Reprint requests to Prof. Dr. Wilhelm Hasselbach.

0341-0382/80/1100-1012 $ 01.00/0

Materials and Methods

The sarcoplasmic reticulum vesicles were prepared from white skeletal muscle according to the procedure described by Hasselbach and Makinose [18] as modified by de Meis and Hasselbach [19]. The extrinsic proteins of the membranes were re-

moved by incubating the sarcoplasmic vesicles 20 mg · ml^{-1} for 10 min at 4 °C in a solution containing 0.3 M sucrose, 0.4 M KCl, 0.1 mM dithiothreitol, 20 mM histidine, 0.01 mM phenylmethylsulfonylfluoride and 6.0 mg · ml^{-1} sodium deoxycholate. After dilution with 4 volumes of 0.1 M KCl, the membranes were precipitated by centrifugation in a Beckman Ti 60 rotor at 50 000 × g · 1 h. 21 ± 2% ($\bar{x} \pm$ S.D. $n = 8$) of the starting protein remained in the supernatant. The pellet was resuspended in 0.1 M KCl and recentrifuged. Subsequently the preparation was dispersed in 0.1 M KCl. Lipid depletion was performed as described [20]. The preparations which were used for the determination of the heat developed when the low affinity calcium binding sites were occupied, were dialyzed for 12–18 h in a solution containing 0.1 M KCl, 20 mM imidazole pH 7.0, 0.1 mM $CaCl_2$, 1 mM sodium azide and, when required, 10 mM $MgCl_2$. Calcium binding was determined by the use of ^{45}Ca which rapidly exchanged with the endogenous traces of membrane bound calcium [1, 2]. In a series of experiments the vesicles were separated from the incubation medium containing 0.1 M KCl, 20 mM imidazole pH 7.0, 0.1–10 mM $CaCl_2$, with or without 10 mM $MgCl_2$. The protein concentration was 15 mg · ml^{-1} by high speed centrifugation in a Beckman air fuge 132 000 × g 20 min. The bound calcium was determined as the difference between the calcium concentration in the suspension and the protein free supernatant as described by Kometani and Kasai [21]. All experiments were performed in triplicates. For fast separation of the membrane from the solution filtration technique was used; aliquots of the suspension containing 0.5 mg protein were sucked through double filters. The membranes are collected on the upper filter. It contains the membrane bound calcium and the calcium trapped by the filter. The quantity of the latter was determined as the radioactivity found in the protein free lower filter. This method yields in addition to the membrane bound calcium the calcium trapped in the vesicular space. The latter quantity has been determined according to [7] and [23]. As long as the vesicles were incubated for 1–2 min, the intravesicular calcium content remained small. Gel electrophoretic separation was performed on 7.5% polyacrylamide gels containing 0.1% sodium dodecylsulfate, 0.1 M Tris bicine pH 8.2. The electrode chambers were filled with the same fluid.

Heat measurements were performed in a batch microcalorimeter of LKB 10700-2 which was equipped with two twin cells with chambers of 6 and 2 ml. The protein solution containing 10 mg protein · ml^{-1} of which 5.6 ml were filled into the first large chamber. The second large chamber was filled with 5.6 ml dialyzing fluid. The two small chambers were filled with 2 ml dialyzing fluid containing the required calcium concentrations. Care was taken that all experiments were performed at the same protein concentration.

The reaction was started by rotating the calorimeter cells. The heat burst lasted 2–3 min. Temperature equilibrium was reached after 10–15 min. The experiments were performed at 20 °C.

Results

The low permeability of the sarcoplasmic membranes for calcium allows titration of the calcium binding sites on the cytoplasmic surface of the vesicles [21–24]. It is assumed that calcium bound to these sites is quickly and completely removed by EGTA, while calcium occupying internal sites is released only very slowly [20, 23]. Fig. 1 illustrates the results of experiments in which vesicles were incubated in solutions containing 1 mM calcium in the presence and absence of 10 mM magnesium. As indicated on the abscissa, excess EGTA was added and the vesicles were removed from the solution by filtration through Sartorius filters 0.45 μm. The calcium retained by the vesicles, which were collected on the millipore filter, is shown on the ordinate. The half time which calcium needs to equilibrate with the internal space amounts to approximately 30 minutes. This time is in quite good agreement with the permeation time found by Kometani and Kasai [21]. The fact that calcium permeation is not affected by magnesium, which was present in the assay prior to the addition of calcium, shows that internal magnesium does not facilitate calcium entry. As a consequence of the slow permeation of calcium ions across the sarcoplasmic membranes only external calcium binding sites are saturated when the vesicles are incubated for ~ two minutes in calcium containing solutions. Fig. 2 illustrates the saturation of external binding sites which was determined after the vesicles had been incubated for two minutes in solutions containing 0.1–10 mM calcium. This binding capacity is significantly

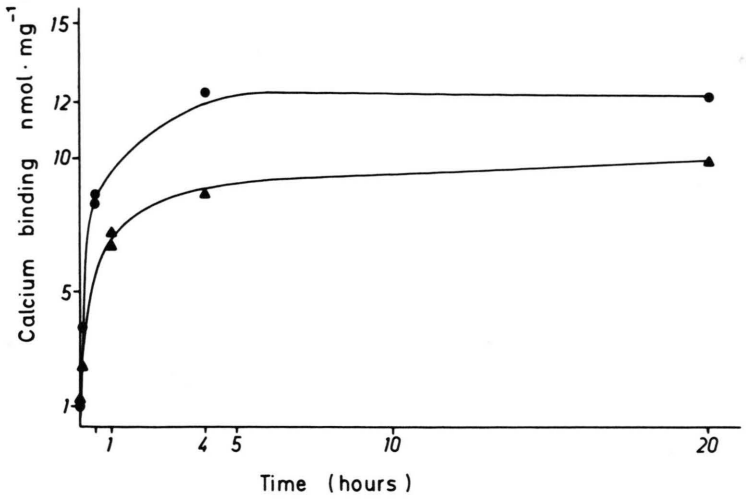

Fig. 1. Time course of calcium uptake by native sarcoplasmic reticulum vesicles. The vesicles were incubated in a solution containing 0.1 M KCl, 20 mM imidazole pH 7.0, 1 mM $CaCl_2$, 0.5 mg protein · ml^{-1} ●, and in addition 10 mM $MgCl_2$ ▲. After the time indicated on the abscissa the suspension was diluted with 5 volumes of a 0.1 M KCl solution containing 1 mM EGTA and filtered through Sartorius filter 0.45 µM, immediately followed by a rinse with 5 ml of the calcium free solution. Calcium bound to the external binding sites is rapidly removed from the vesicles by EGTA [7, 23].

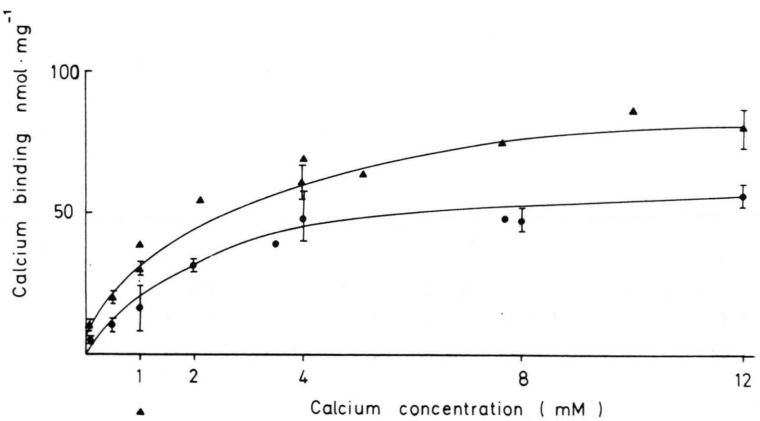

Fig. 2. Calcium binding to native vesicles. The incubation medium contained 0.25 mg protein · ml^{-1}, 0.1 M KCl, 20 mM imidazole pH 7.0, and no ▲, ▲ or 10 mM $MgCl_2$ ●, ●, respectively. ●, ▲ vesicles were removed by centrifugation, ●, ▲ vesicles were removed by filtration, means ± SD, $n = 3-5$. Calcium binding was terminated after 1–2 min by filtration through double filters (Sartorius 0.45 µM). The bound calcium was calculated from the difference between the radioactivity in the upper and the lower filter (comp. Materials and Methods).

Table I. Low affinity calcium binding sites of native sarcoplasmic reticulum vesicles and isolated ATPase determined by short term incubation. The experiments were performed as described in Materials and Methods. The results of Figs. 2 and 3 were used for extrapolation in Scatchard plots. The number of low affinity sites were obtained by subtracting the number of sites titrated at 0.1 mM calcium from the total number of sites. In the calorimetric measurements only low affinity sites are recorded because the protein preparations were previously equilibrated with 0.1 mM calcium. A specific binding heat of 1000 cal per mol (Fig. 7) was used to calculate the number of calcium binding sites from the developped binding heat. In the absence and in the presence of 10 mM magnesium the Scatchard plots yielded dissociation constants for the low affinity sites of 2–3 mM for the intact vesicles and for ATPase preparations of 4.5 mM and 7 mM, respectively.

Calcium binding sites determined by	Low affinity calcium binding			
	of closed vesicles		of purified ATPase	
	no Mg^{2+}	10 mM Mg^{2+}	no Mg^{2+}	10 mM Mg^{2+}
	[nmol Ca^{2+} · mg protein^{-1}]			
^{45}Calcium binding	90 ∓ 2	63 ∓ 10	165 ∓ 3	122 ∓ 12
^{45}Calcium binding corrected for the presence of 21% protein not accessible for external calcium	112	79	–	–
Binding heat measurements	–	56 ∓ 4	–	96 ∓ 2
Binding heat measurements corrected for the presence of 21% protein not accessible for external calcium	–	70	–	–

reduced when the assay is supplemented with 10 mM magnesium. Evidently in addition to the above mentioned high affinity calcium binding sites, which are saturated at 100 μM calcium, a considerable number of low affinity sites are located on the external surface of the sarcoplasmic vesicles. While the location of the high affinity sites can be confined to the transport ATPase, the participation of other membrane constituents in low affinity calcium binding cannot be ruled out.

Possible calcium binding sites located on the internal section of the calcium transport ATPase should be rapidly accessible in purified calcium transport ATPase preparations as well as in preparations where the lipid matrix has been destroyed and reduced by partial enzymatic delipidation. Figs. 3 and 4 demonstrate that in both preparations, the number of easily accessible low affinity calcium binding sites has significantly increased in the absence as well as in the presence of magnesium ions. The finding that the increment in calcium binding was found to be identical for ATPase preparations with a complete lipid matrix and for that of lipid deprived preparations, indicates that the lipids do not essentially contribute to calcium binding. It should be also noted that the lipid deprived preparation contains only the transport ATPase protein, as shown by the gel electropherogram in Fig. 5. The increase of the number of calcium binding sites does not result in a significant change in the half satura-

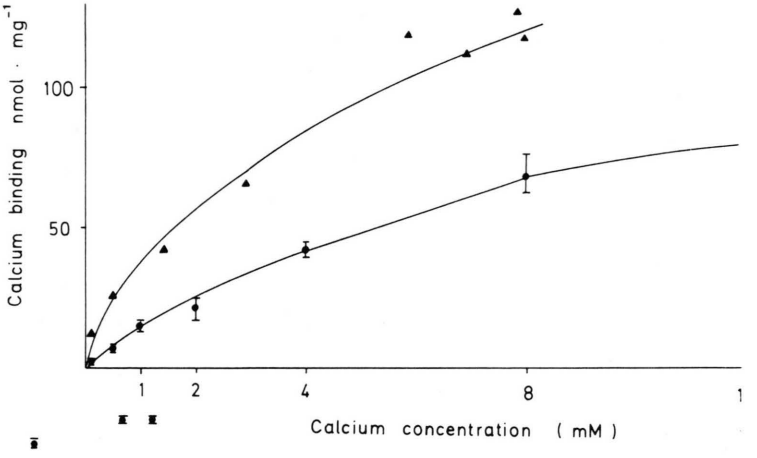

Fig. 3. Calcium binding by purified calcium transport ATPase. The experiments were performed under the same conditions as described in Fig. 2. ▲ no $MgCl_2$, ● 10 mM $MgCl_2$ was present.

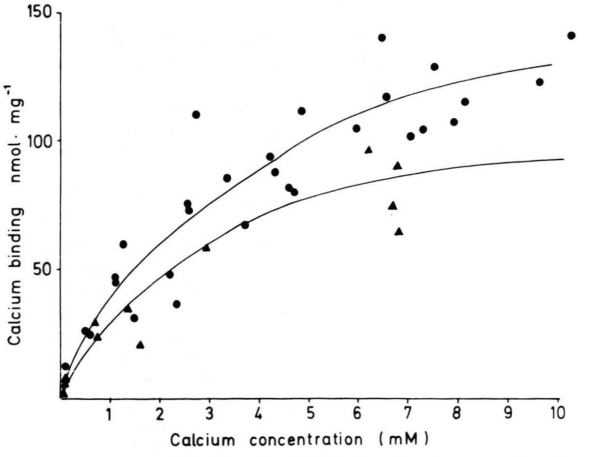

Fig. 4. Calcium binding by lipid deprived sarcoplasmic reticulum membranes. The experiments were performed as described in Fig. 1. ● no $MgCl_2$, ▲ 10 mM $MgCl_2$ was present. The protein was removed by centrifugation.

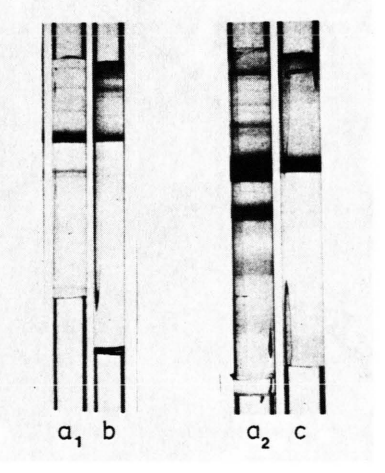

Fig. 5. Gel electropherograms of native sarcoplasmic reticulum membranes a_1, a_2, purified ATPase b, and lipid deprived sarcoplasmic reticulum membranes. The amounts of protein applied were 50 μg in a_1, b_1 and 100 μg in a_2 and c.

tion concentration. The total number of calcium binding sites of these preparations is markedly reduced by the presence of 10 mM magnesium. A comparison of calcium binding of preparations where all binding sites are accessible with the calcium binding of closed vesicles allows one to estimate the number of low affinity calcium binding sites facing the luminal space of the vesicles.

The evaluation of the binding isotherms of the different preparations in Scatchard or Lineweaver-Burk plots yield maximal binding values which are collected in the Table. The figures show that the maximum calcium binding capacity of the ATPase exceeds that of closed vesicles by ~ 60 and 75 nmol calcium · mg protein^{-1} in the presence and the absence of 10 mM magnesium, respectively. However, these figures have to be corrected because the accessorial proteins which are removed from the vesicles for the isolation of the ATPase do not contribute to calcium binding during short term incubation due to their internal location. Hence, the higher calcium binding capacity of the ATPase preparations is in part due to the removal of the nonbinding protein fraction of approximately 20% (Materials and Methods).

To verify these results, calcium binding of closed vesicles and purified ATPase preparations was additionally monitored by measuring the enthalpy change accompanying calcium binding. As shown in Fig. 6, closed vesicles develop less heat than ATPase preparations. The developped heat originates in both preparations only from the occupation of the low affinity binding sites because the high affinity sites had been previously saturated by 0.1 mM calcium. Since the burst of heat following the mixing of the vesicles with the calcium containing solution lasts only 1–2 minutes, only externally located calcium binding sites contribute to heat development.

When the developped heat is related to the amount of calcium bound at the corresponding free calcium concentration in the medium, the enthalpy of calcium binding increases linearly until 45 nmol calcium · mg protein^{-1} and 65 nmol calcium · mg protein^{-1} are bound in native vesicles and ATPase preparations in the presence of 10 mM magnesium (Fig. 7). In the absence of magnesium the breaks occur at an occupation of 70 nmol · mg protein^{-1} and 130 nmol · mg protein^{-1} for native vesicles and ATPase preparations, respectively. The enthalpy change related to the occupation of one binding site is relatively small and ranges between 900 and 1000 cal · mol^{-1} for native vesicles and purified ATPase preparations in the presence of 10 mM magnesium. A somewhat higher value of 1500 cal · mol^{-1} was

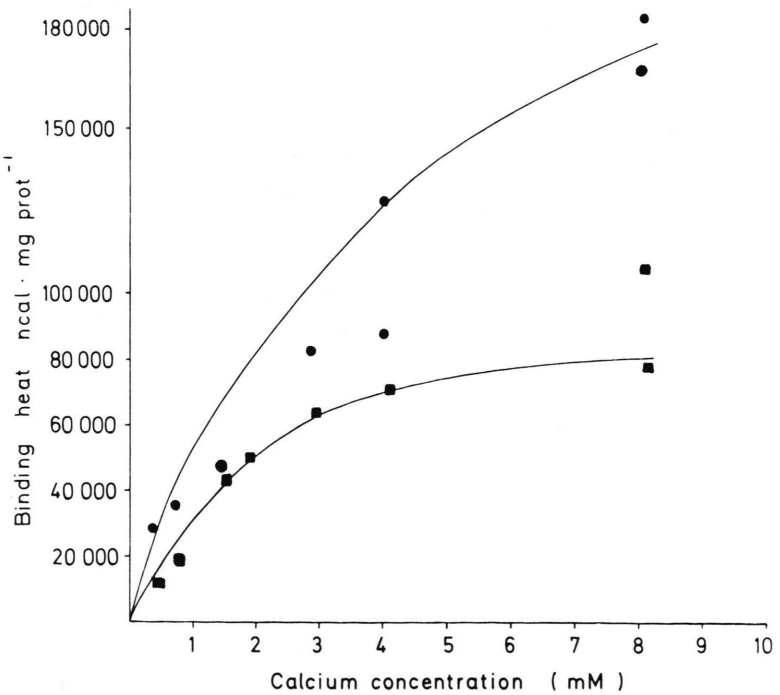

Fig. 6. The dependence of heat development accompanying calcium binding to low affinity binding sites of sarcoplasmic reticulum vesicles and purified ATPase. Sarcoplasmic reticulum vesicles ■ and isolated ATPase ● were dialyzed for 12 – 18 h against solutions containing 0.1 M KCl, 0.02 M imidazole pH 7.0, 1 mM sodium azide, and 0.1 mM $CaCl_2$ at 4 °C. The heat measurements were performed as described in Materials and Methods. The dialyzed vesicular suspension and dialyzing fluid contained in the large calorimeter chambers were mixed with 2 ml of dialyzing fluid containing the calcium concentrations indicated on the abscissa after the calorimeter had reached thermal equilibrium which requires 60–90 min after filling. The calorimeter was calibrated after each experiment by applying a known current to the resistance built in the calorimeter cell. Corresponding experiments were performed in the presence of 10 mM $MgCl_2$ (not shown). Under these conditions sarcoplasmic reticulum vesicles and ATPase preparations maximally develop 56000 and 96000 ncal · mg protein^{-1}, respectively.

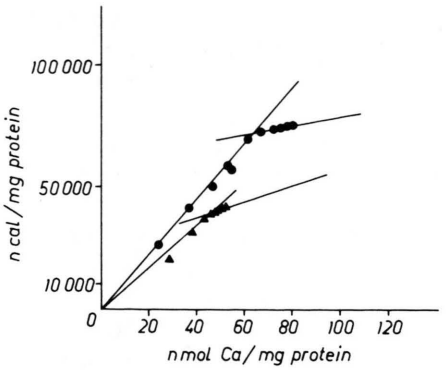

Fig. 7. The dependence of binding heat on the occupancy of the low affinity calcium binding sites of sarcoplasmic reticulum vesicles and purified ATPase in the presence of 10 mM magnesium. ▲ sarcoplasmic reticulum vesicles, ● purified ATPase. The titration graphs were obtained by combining the results of heat measurements with calcium binding measurements depicted in Figs. 2 and 3. The quantities of calcium bound to the high affinity sites at 0.1 mM calcium were substrated from the totally bound calcium. Binding and heat values were related to the free calcium concentration in the respective assays.

observed for the ATPase preparation in the absence of magnesium. When more calcium is bound, the enthalpy change per mol of calcium becomes definitely smaller, indicating that another population of calcium binding sites becomes saturated.

Discussion

The increment in calcium binding by ATPase preparations as compared to closed vesicles, revealed that in ATPase preparations an additional quantity of at least 30–50 nmol calcium · mg protein^{-1} low affinity binding sites is present. The enthalpy change accompanying the binding also indicates that in the preparation of the ATPase, the opening of the vesicular structure makes additional low affinity calcium binding sites accessible. Evidently the intact vesicular structure prevents calcium from reacting with the low affinity sites at the luminar surface during incubation periods lasting only a few minutes. The slow penetration of calcium across the sarcoplasmic reticulum membranes is a well established fact [21–24] and allows to titrate externally located calcium binding sites during short term incubation. The permeation rate for inward directed calcium movement is in agreement with the values found by Kometani and Kasai [21] which were obtained by monitoring osmotically induced light scattering changes. The total amount of externally located calcium binding sites which were occupied during the incubation period of approximately one minute comprises 8–12 nmol high and approximately 90 nmol of low affinity sites per mg protein. While the location of the high affinity sites in the calcium transport ATPase is undisputed, the membrane constituents carrying the low affinity sites are not well established. In the presence of 10 mM magnesium the number of low affinity calcium binding sites is significantly reduced (Table). If magnesium would compete for calcium at these sites, this difference should have disappeared when the number of binding sites occupied at infinitely high calcium concentration was extrapolated. Evidently magnesium binding induces a change in the protein which reduces the number of sites accessible for calcium. On account of these findings it is unlikely that phospholipids are involved because their ability to discriminate between calcium and magnesium ions is less specific. The possibility that one of the accessorial proteins contributes to the external low affinity sites cannot definitely be excluded.

The significantly greater binding capacity of ATPase preparations and of delipidated preparations revealed by short term binding experiments has several implications.

1. The accessorial proteins, possibly located in the interior of the vesicles, cannot contribute to this binding increment because they were removed during the isolation procedure.

2. Lipids are also excluded as binding sites as the lipid depletion does not affect calcium binding.

3. Therefore, the additional low affinity calcium binding sites titrated in the ATPase preparations must reside in the section of the molecule directed to the vesicular lumen.

The quantity of internal calcium binding sites in the ATPase preparation has to be considered as a minimum number, since our estimate relies on the assumption that during the isolation procedure of the ATPase a number of low affinity binding sites exposed on the outer surface is not diminished. Should this occur, which is to be expected when these sites reside in accessorial proteins which are lost during the isolation procedure, a corresponding number of new internal calcium sites must have been exposed to compensate for this loss. This means that we would have to add ~ 80 nmol sites per mg protein which corresponds to the number of

calcium binding sites residing in the fraction of the accessorial proteins, mainly calsequestrin. We now would have $\sim 100-130$ nmol internal calcium binding sites per mg protein in the ATPase. To obtain the number of all low affinity calcium binding sites in the native vesicles, these figures have again to be increased by the number of external low affinity sites, thus yielding a total of $\sim 200-260$ nmol · mg protein^{-1} of low affinity calcium binding sites in the absence of magnesium and $\sim 150-180$ nmol · mg protein^{-1} in its presence (10 mM). Similar high values for calcium binding of native vesicles were extrapolated by Miyamoto and Kasai [7] and Chiu and Haynes [26]. Evidently a more accurate estimate of the number of internal calcium binding sites of the ATPase requires better knowledge concerning the arrangement of the calcium binding proteins in the membranes. Depending on our assumptions 3–13 low affinity calcium binding sites would be located in the internal section of the ATPase molecules. The latter quite high figure agrees well with the number of β_3 sites determined by Miyamoto and Kasai [7] which the authors could not assign to any membrane constituent. However, the calcium affinity of the sites identified by the authors is considerably lower than that of the internal sites found in the isolated protein. Whatever the precise number of low affinity calcium binding sites in the molecule is, these sites do not need the presence of ATP or another phosphate donor for their formation as is implied in most reaction schemes. The role of these phosphate independent low affinity sites, demonstrated in this study, remains to be established: 1. They may provide additional calcium binding sites in the interior of the vesicles. 2. They may act as regulatory sites which, when occupied, suppress the activity of the calcium transport ATPase of the calcium pump. 3. They may be identical with the sites from which calcium is released when the calcium pump accumulates calcium ions or to which calcium ions are bound before they cross the membrane during ATP synthesis. Hence, during energy dependent calcium translocation a mechanism must be operative by which calcium is translocated between preexisting low and high affinity calcium binding sites (*cf.* [27]).

Acknowledgement

The authors are grateful to Dr. Nestruck-Goyke for critical reading of the manuscript.

[1] W. Fiehn and A. Migala, Eur. J. Biochem. **20,** 245–248 (1971).
[2] J. Chevallier and R. A. Butow, Biochemistry **10,** 2733–2737 (1971).
[3] N. Ikemoto, J. Biol. Chem. **249,** 649–651 (1974).
[4] D. H. MacLennan and P. T. Wong, Proc. Nat. Acad. Sci. **68,** 1231–1235 (1971).
[5] T. J. Ostwald and D. H. MacLennan, J. Biol. Chem. **249,** 974–979 (1974).
[6] W. Hasselbach and M. Makinose, Biochem. Z. **339,** 94–111 (1963).
[7] H. Miyamoto and M. Kasai, J. Biochem. **85,** 765–773 (1979).
[8] A. P. Carvalho, J. Cell. Physiol. **67,** 73–84 (1966).
[9] L. de Meis and A. L. Vianna, Ann. Rev. Biochem. **48,** 275–292 (1979).
[10] G. Inesi, in Membrane Transport in Biology, pp. 357–393, Springer-Verlag Berlin-Heidelberg-New York, 1979.
[11] C. Coan, S. Verjovski-Almeida, and G. Inesi, J. Biol. Chem. **254,** 2968–2974 (1979).
[12] N. Ikemoto, J. F. Morgan, and S. Yamada, J. Biol. Chem. **253,** 8027–8033 (1978).
[13] L. de Meis, J. Biol. Chem. **251,** 2055–2062 (1976).
[14] F. U. Beil, D. von Chak, and W. Hasselbach, Eur. J. Biochem. **81,** 151–164 (1977).
[15] R. Prager, C. Punzengruber, N. Kolossa, F. Winkler, and J. Suko, Eur. J. Biochem. **97,** 239–250 (1979).
[16] C. Punzengruber, R. Prager, N. Kolossa, F. Winkler, and J. Suko, Eur. J. Biochem. **92,** 349–359 (1978).
[17] W. Hasselbach, in Topics in Current Chemistry **78,** pp. 1–56, Springer-Verlag Verlin-Heidelberg-New York 1979.
[18] W. Hasselbach and M. Makinose, Biochem. Z. **339,** 94–111 (1963).
[19] L. de Meis and W. Hasselbach, J. Biol. Chem. **246,** 4759–4763 (1971).
[20] G. Swoboda, J. Fritzsche, and W. Hasselbach, Eur. J. Biochem. **95,** 77–88 (1979).
[21] T. Kometani and M. Kasai, J. Membr. Biol. **41,** 295–308 (1978).
[22] G. Meissner and D. McKinley, J. Membr. Biol. **30,** 79–98 (1976).
[23] M. Chiesi and G. Inesi, J. Biol. Chem. **254,** 10370–10377 (1979).
[24] W. Hasselbach, Biochem. of Intracell. Structures (ed. L. Wojtczak *et al.*) pp. 145–152, PWN Warszawa, 1969.
[25] G. Meissner, G. E. Conner, and S. Fleischer, Biochim. Biophys. Acta **298,** 246–269 (1973).
[26] V. C. K. Chiu and D. H. Haynes, Biophys. J. **18,** 3–22 (1977).
[27] P. J. Garrahan and R. P. Garay, in Current Topics in Membrane and Transport, (eds. F. Bronner and A. Kleinzeller), **vol. 8,** pp. 29–97, Academic Press, 1976.