Biochimica et Biophysica Acta, 1066 (1991) 43–53

ADONIS 000527369100255Q

BBAMEM 75256

Interaction of alkaline metal ions with Ca^{2+}-binding sites of Ca^{2+}-ATPase of sarcoplasmic reticulum: ^{23}Na-NMR studies

Igor M. Timonin, Sergei N. Dvoryantsev, Victor V. Petrov, Enno K. Ruuge and Dmitri O. Levitsky

USSR Cardiology Research Center, Academy of Medical Sciences, Moscow (U.S.S.R.)

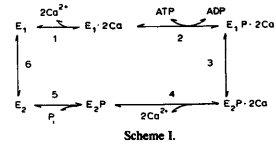
(Received 7 February 1991)

Key words: Sarcoplasmic reticulum; ATPase, Ca^{2+}-; Alkaline metal ion; NMR, ^{23}Na-; (Skeletal muscle)

The analysis of the ^{23}Na-NMR signal shape variations in the presence of vesicles of light sarcoplasmic reticulum (SR) shows the existence of sodium sites on the membranes with K_d values of about 10 mM. Other monovalent cations displace Na^+ from SR fragments in a competitive manner according to the row $K^+ > Rb^+ > Cs^+ > Li^+$. Calcium ions also reduce Na^+ binding, the Na^+ desorption curve being of a two-stage nature, which, as suggested, indicates the existence of two types of Ca^{2+}-sensitive Na^+ binding sites (I and II). Sites of type I and II are modified by Ca^{2+} in submicromolar and millimolar concentrations, respectively. Analysis of sodium (calcium) desorption produced by calcium (sodium) allowed us to postulate the competition of these two cations for sites I and identity of these sites to high-affinity Ca^{2+}-binding ones on the Ca^{2+}-ATPase. Sites I weakly interact with Mg^{2+} ($K_{app_{Mg}} \approx 30$ mM). Reciprocal effects of sodium and calcium on binding of each other to sites II cannot be described by a simple competition model, which indicates nonhomogeneity of these sites. A portion of sites I ($\approx 70\%$) interacts with Mg^{2+} ($K_{app_{Mg}} = 3$–4 mM). The pK_a value of sites II is nearly 6.0. The number of sites II is three times greater than that of sites I. In addition, sites with intermediate affinity for Ca^{2+} were found with K_d values of 2–5 μM. These sites were revealed due to the reducing of the sites II affinity for Na^+ upon Ca^{2+} binding to SR membranes. It can thus be concluded that in nonenergized SR there are binding sites for monovalent cations of at least three types: (1) sites I (which also bind Ca^{2+} at low concentrations), (2) magnesium-sensitive sites II and (3) magnesium-insensitive sites II.

Introduction

The transfer of calcium ions through sarcoplasmic reticulum membrane is provided by their binding to high affinity sites on the Ca^{2+}-ATPase cytoplasmic domain and by the subsequent ATP-dependent transport of bound Ca^{2+} into the lumen of SR. The latter process is accompanied by a considerable reduction of the sites' affinity for calcium. Nominally, the Ca^{2+}-ATPase function is described satisfactory by a cyclic scheme (Scheme I) postulating the existence of two conformations of the enzyme (E_1 and E_2) with high and low affinity for Ca^{2+}, respectively [1,2].



Scheme I.

Partial Ca^{2+}-ATPase reactions are subjected to regulatory effects of ATP, calcium, magnesium and monovalent cations. Potassium ions, for example, are supposed to activate formation of the enzyme phosphoprotein intermediate (step 2) [3–6], stimulate the liberation of tightly bound Ca^{2+} (step 4) [7] or of P_i from the enzyme (step 5) [4–9], inhibit the ATPase phosphorylation in the presence of P_i [10] and ATP formation from ADP and P_i [10,11], affect isomerization stages (steps 3 and 6) [4,12,13]. The multiple effects of monovalent

Abbreviations: SR, sarcoplasmic reticulum; Mops, 3-(*N*-morpholino)propanesulphonic acid; $C_{12}E_8$, octaethylene glycol dodecyl ether.

Correspondence: D.O. Levitsky, USSR Cardiology Research Center, 3rd Cherepkovskaya str., 15a, Moscow 121552, U.S.S.R.

cations on the enzyme saturated by Ca^{2+} result in the stimulation of ATPase activity and increase in Ca^{2+}-transport rate [14–16]. The activating effects are observed when potassium or sodium ion concentrations are in the range of 5 to 50 mM. On the other hand, when present at concentrations higher than 100 mM and/or when Ca^{2+} concentrations are below those saturating the enzyme, alkaline metal ions suppress the ATPase activity [11,14,17].

The binding of monovalent cations to Ca^{2+}-ATPase is usually estimated by their influence on the kinetic characteristics of the ATPase reaction and Ca^{2+} transport. Taking account of the previous data, one can suppose the presence on SR membranes of more than one type of sites for monovalent cation. Thus, according to the data obtained by Medda et al. [12] K^+ affects at least four stages of the ATPase cycle. Ribeiro and Vianna [17] postulated the existence of two K^+-binding sites providing ATPase activation. It has been suggested that K^+-binding sites, 'activating' and 'inhibiting' Ca^{2+}-ATPase, differ from Ca^{2+}-binding sites [11,17]. So far, however, the binding sites for the monovalent cations on SR membranes have not been investigated by direct methods with the exception of Li^+-binding sites [18]. In this connection it should be noted, that Li^+ affects the Ca^{2+}-ATPase function in the least extent as compared to other alkaline metal ions [14,11,8].

The aim of this work was to study binding sites for alkaline metal cations in SR and reciprocal relation of these sites with the Ca^{2+}-binding ones. For this purpose, the ^{23}Na-NMR method was used. Due to the presence of a quadrupolar moment in the ^{23}Na nucleus the ^{23}Na-NMR signal shape undergoes marked changes when Na^+ is bound by a macromolecular ligand. The natural content of the Na^+ magnetic isotope is close to 100%. Therefore, the ^{23}Na-NMR method is characterized by a relatively high sensitivity [23]. Moreover, ^{23}Na-NMR method permits, as it was shown in the experiments on Ca^{2+}-binding proteins, to study not only the binding of Na^+ but to investigate Ca^{2+}-binding sites as well [21,22].

In this paper we demonstrate the existence of two types of Na^+-binding sites on the SR membranes that do not differ in the affinity for the cation. At the same time they differ in pK_a and in their ability to interact with Ca^{2+} and Mg^{2+}. Some of the sites seem to be identical to the high-affinity Ca^{2+}-binding sites.

Materials and Methods

Membrane preparation

Crude fraction of SR membranes was isolated from white muscles of rabbit hind legs as described earlier [24]. The muscle tissue was minced by a homogenizer 'Virtis 45' in a medium containing 0.1 M KCl, 10 mM $NaHCO_3$ (pH 7.8). The KCl-microsomes were precipitated by two-fold centrifugation in a medium containing 0.6 M KCl, 10 mM Tris-HCl (pH 7.0). Heavy SR fraction enriched by terminal cisternae was precipitated for 20 min at $15000 \times g$. Light SR fraction used in the experiments was obtained by precipitating the rest of vesicles at $80000 \times g$ for 40 min. As evidenced by SDS-electrophoresis in a polyacrylamide gel, the Ca^{2+}-ATPase content in the light SR was not less than 90%.

Liposome preparation

The fraction of SR lipids was isolated according to the method of Folch et al. [25]. To prepare liposomes, the final suspension was sonicated by a Lab-Line Ultratip at maximum power for 5 seconds in a medium containing 20 mM NaCl, I mM EGTA, 50 mM Tris-Mops (pH 7.0) and $CaCl_2$ at different concentrations. The phospholipid concentration was measured in the presence of 10 mM $C_{12}E_8$ by ^{31}P-NMR method using phosphoric asid as a standard [26].

The ^{23}Na-NMR measurements

The ^{23}Na-NMR spectra were obtained at 52.9 MHz on a Bruker CXP-200 spectrometer. For signal accumulation 45°-pulses were used. The sweep width was 5000 Hz; interval between pulses was 0.2 s. To stabilize resonance conditions, D_2O was added to a sample (10% of the volume). For a separate determination of two characteristic times of transverse relaxation T_2' and T_2'' a decrease curve of transverse magnetization was approximated by the superposition of two exponents using a nonlinear least-squares method.

The measurements were performed at 20°C in 2.5 ml of the incubation medium usually containing 25 mg SR protein, 20 mM NaCl, 1 mM EGTA and different concentrations of $CaCl_2$, 50 mM Tris-Mops (pH 7.0). The free calcium concentration was calculated using an apparent dissociaton constant of the Ca-EGTA complex [27].

Calcium binding measurements

Passive calcium binding to SR membranes was measured with $^{45}Ca^{2+}$. Vesicles of SR (200 μg protein) were incubated for 10 min in 1 ml of the medium containing 50 mM Tris-Mops (pH 7.0), 0.1 mM $^{45}CaCl_2$ and different concentrations of NaCl and EGTA. No EGTA was added to the medium at free Ca^{2+} concentrations higher than 0.1 mM. The SR fragments were precipitated on HA-45 Millipore nitrocellulose filters. The amount of $^{45}Ca^{2+}$ bound to SR protein was calculated as a difference between the total $^{45}Ca^{2+}$ absorbed on the filter and that bound nonspecifically. The latter was determined separately. Preliminary estimates showed that the calcium nonspecific binding depended on the amount of water retained on the

filter rather than on NaCl and $CaCl_2$ concentrations in the medium. Radioactivity of the filters was measured in Bray scintillator.

Sodium binding measurements

Sodium binding was measured with ^{22}Na. The SR membranes (1 mg protein) were incubated in 1 ml of the medium containing 1 mM EGTA, 50 mM Tris-Mops (pH 7.0), 3 mM $^{22}NaCl$ and various concentrations of nonlabelled NaCl. The SR vesicles were precipitated on filters as described for ^{45}Ca assay.

Materials

Sucrose, Tris, Mops, EGTA, proteinase (type 14), calcium ionophore A23187 were obtained from Sigma. Metal chlorides were purchased from Merck. All chemicals were of analytical grade. Alamethicin was a gift from V.B. Ritov (Moscow State University).

Theory

The ^{23}Na nucleus has a spin $I = 3/2$ and a quadrupolar moment which considerably affects the relaxation parameters of ^{23}Na, provided the ion is found in a nonsymmetric environment [28].

The relaxation equations for the spin $I = 3/2$ in the presence of chemical exchange between free and bound states were proposed by Bull [29]. These equations are simplified for the cases when the exchange rate is higher as compared to the relaxation rate [23]. For two-position exchange

$$Na^+ \text{ in solution} \rightleftharpoons Na^+ \text{ bound} \tag{1}$$

time dependence of transverse magnetization has the form

$$M(t) = M(0)\cdot\left[\frac{3}{5}\exp\left(-\frac{t}{T_2'}\right)+\frac{2}{5}\exp\left(-\frac{t}{T_2''}\right)\right] \tag{2}$$

where

$$\frac{1}{T_2'} = \frac{1-P_{Na}}{T_2^0}+\frac{\pi^2}{5}P_{Na}\chi^2\left(\tau_c+\frac{\tau_c}{1+\omega^2\tau_c^2}\right) \tag{3}$$

and

$$\frac{1}{T_2''} = \frac{1-P_{Na}}{T_2^0}+\frac{\pi^2}{5}P_{Na}\chi^2\left(\frac{\tau_c}{1+\omega^2\tau_c^2}+\frac{\tau_c}{1+4\omega^2\tau_c^2}\right) \tag{4}$$

are rapidly and slowly decreasing components of transverse magnetization, respectively; $T_2^0 = (\Delta\nu_{1/2}^0)^{-1}$ is a relaxation time of sodium ions in an aqueous solution; χ is a quadrupolar constant for ^{23}Na in bound state; ω is the resonance frequency of ^{23}Na nucleus; τ_c is the correlation time characterizing the fluctuations of an electric field gradient near a cation-binding site; P_{Na} is a sodium ion portion bound to the membrane

$$P_{Na} = [B\cdot Na]/[Na^+]$$

where B is the number of binding sites.

As follows from Eqns. 2–4, the Fourier transform of the function of decreasing transverse magnetization in time results in a superposition of two Lorentz lines with the same resonance frequency and different widths. The wide signal corresponds to the value $1/T_2'$ and the narrow one to the value $1/T_2''$.

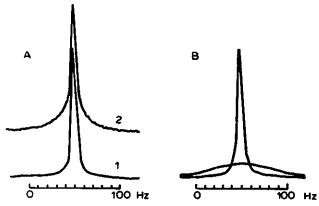


Fig. 1. The shape of ^{23}Na-NMR signals. (A) The signals were obtained in the absence (1) and presence (2) of SR vesicles. (B) Schematic decomposition into two components of the ^{23}Na-NMR signal in a sample containing SR vesicles. The medium contains 10 mg SR protein/ml, 10 mM NaCl, 1 mM EGTA, 50 mM Tris-Mops (pH 7.0).

Results

Sodium binding to SR membranes

The ^{23}Na-NMR spectrum of a NaCl aqueous solution is a single Lorentz line with $\Delta\nu_{1/2}^0 = 7$ Hz at 20 °C (Fig. 1A, curve 1). Upon the addition of SR fragments to the NaCl solution the ^{23}Na-NMR spectrum can be represented as a superposition of two Lorentz lines with various widths and the same resonance frequency (Fig. 1B). The time decrease of transverse magnetization is, accordingly, nonexponential in this case and consists of rapidly and slowly decreasing components with weighting factors of approx. 0.6 and approx. 0.4, respectively. This shows that the description of Na^+ behaviour in SR membrane suspension does not contradict to a two-position model (Eqn. 1).

We have analyzed the ^{23}Na-NMR lineshape at various concentrations of sodium ions and the constant concentration of SR fragments. For a wide signal the width $\Delta\nu_{1/2}'$ varies upon changes of Na^+ concentration and may reach several hundred Hz (Fig. 2). For a narrow signal the $\Delta\nu_{1/2}''$ value is 10–15 Hz and only slightly depends on Na^+ concentration.

The dependence of the value on sodium concentration being analyzed, the affinity of the cation-binding

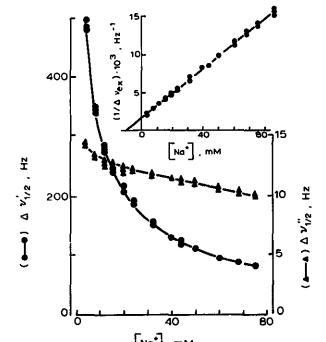


Fig. 2. Half-width of wide ($\Delta\nu'_{1/2}$) and narrow ($\Delta\nu''_{1/2}$) components of the ^{23}Na-NMR signal at different Na^+ concentration. (Inset) The dependence of $1/\Delta\nu'_{ex}$ (where $\Delta\nu'_{ex} = \Delta\nu'_{1/2} - \Delta\nu^0_{1/2}$ is proportional to Na^+ portion bound to SR membranes) on Na^+ concentration in the medium. The abscissa intercept corresponds to K_d value of Na^+-binding sites. The ordinate intercept is proportional to K_d/B where B is the number of binding sites. Explanation is given in the text. Medium composition as in Fig. 1.

site for Na^+ can be quantitavely evaluated. Indeed, for this system in the interval of $[Na^+]$ from 5 to 100 mM the following condition is to be fulfilled

$$P_{Na} \ll 1 \tag{5}$$

Therefore, for evaluating changes in the portion of bound ions it is convenient to use the value $\Delta\nu'_{ex} = \Delta\nu'_{1/2} - \Delta\nu^0_{1/2}$. This value as evidenced by Eqn. 3 under the condition 5 is proportional to the portion of bound ions

$$\Delta\nu'_{ex} \approx [B{\cdot}Na]/[Na^+] \tag{6}$$

On the other hand, the relation $\Delta\nu'_{ex} = f([Na^+])$ is hyperbolic (Fig. 2), thus $1/\Delta\nu'_{ex}$ depends on $[Na^+]$ linearly (Fig. 2, inset). Hence, we have a relation

$$\Delta\nu'_{ex} \approx B/(K + [Na^+]) \tag{7}$$

or, taking into account Eqn. 6,

$$[B{\cdot}Na]/[Na^+] \approx B/(K + [Na^+]) \tag{8}$$

In Eqn. 8, which is analogous to that of Benesi-Hildebrand, the parameter K corresponds to the dissociation constant of sodium binding sites (K_{dNa}) and B reflects the number of the binding sites. K_{dNa} and B values can be found by reconstruction of the curve on Fig. 2 in semi-inverse coordinates (inset in Fig. 2). Under these conditions K_{dNa} is equal to 11 mM and the B value is, in relative units, $6.8 \cdot 10^3$ Hz mM per 25 mg SR protein. The K_{dNa} value is apparent since the effect of a proton is not taken into account. In case alkaline metals, Ca^{2+}, or Mg^{2+} are present in the medium besides H^+, the K_{dNa} value is 'double' apparent and it will be designated differently as K_{appNa}.

The condition in Eqn. 5 is the primary requirement which must be fulfilled for the system to be expressed by the Benesi-Hildebrand equation (Eqn. 8). The percentage of the bound Na^+ (P_{Na}) was independently estimated by means of ^{22}Na assay. Using the method of membrane precipitation on the nitrocellulose filters it was found that the quantity of Na^+ bound to SR membranes did not exceed 15% of the Na^+ quantity nonspecifically bound to the filter with radioactive solution (data not shown). In turn, the quantity of solution bound to the filter is about 5% of filtered radioactive solution. Thus, not more than 0.7–0.8% of total Na^+ is bound to the membranes and the condition in Eqn. 5 is fulfilled in a wide range of protein and sodium concentrations. At the same time, it is necessary to note that estimation of the number of Na-binding sites on SR membranes by means of the ^{22}Na assay is very difficult to carry out. The fact is that the systematic errors inherent to the ^{22}Na assay (5–7%) are commensurable even with the maximal level of the useful signal (15%). Thus, as to the signal/noise ratio ^{23}Na-NMR method is by two orders more sensitive than the ^{22}Na assay.

When SR vesicles are incubated with a non-specific proteinase for an hour, the $\Delta\nu'_{ex}$ value decreases practically to the level of the narrow signal component of about 20 Hz. For a liposome suspension prepared from SR lipids (20 mg/ml) and at Na^+ concentrations from 10 to 80 mM the $\Delta\nu'_{ex}$ value is of the same order of magnitude (data not shown). This agrees with the data obtained by Papahadjopoulos et al. [30] who estimated the affinity of liposomes from phosphatidylcholine and phosphatidylserine mixture to Na^+ to be very low ($K_d \approx 1$ M). Thus, the Na^+-binding sites seem to locate on protein components of the SR membranes.

The addition of 20 μM calcium ionophore A23187 or alamethicin (200 μg/ml) into the medium does not change sodium ion binding to SR membranes in the presence of other alkaline ions and Ca^{2+}. Therefore, all these ions can interact during the spectrum accumulation time (5–10 min) with the sites located on both sides of the SR membrane. One can not also exclude the possibility for the sites to be located only on the external membrane side.

Effects of monovalent cations and Ca^{2+} on the ^{23}Na-NMR lineshape

In the presence of K^+, Rb^+, Cs^+, and Li^+ the $\Delta\nu'_{ex}$ value decreases, which corresponds to diminishing in

the amount of Na^+ bound to SR membranes. The curves of $\Delta\nu'_{ex}$ dependence on the concentrations of monovalent cations (Me^+) can be described by a hyperbola and the $1/\Delta\nu'_{ex}$ dependence on $[Me^+]$ is linear (Fig. 3). This is in agreement with a competition model for binding sites on the SR membranes. For example, the value K_{appK} is 40 mM. The value of K_{dK} (13 mM, a dissociation constant in the absence of Na^+) derived from the relationship

$$K_{appK} = K_{dK}(1+[Na^+]/K_{dNa})$$

does not depend on Na^+ concentration. In an analogous way, the K_d values for other monovalent cations were defined. The K_d values increase in a row:

$$Na^+ (10\ mM), K^+ (13\ mM), Rb^+ (30\ mM),$$
$$Cs^+ (70\ mM), Li^+ (120\ mM) \qquad (9)$$

The ^{23}Na-NMR signal is modified by Ca^{2+} in quite a different manner. The form of the curve of Na^+ displacement by Ca^{2+} indicates the existence of two types of Ca^{2+} binding sites on the membranes (Fig. 4). This may also indicate the presence of two types of Ca^{2+}-sensitive Na^+-binding sites. The latter suggestion will be further subjected to consideration. Two types of sites (affected by Ca^{2+} in submicromolar and millimolar concentrations) are denoted in this paper as sites I and II, respectively.

Interaction of Ca^{2+} and Na^+ with sites I

The values of Ca^{2+} concentrations required for half-maximal inhibition of sodium binding to sites I

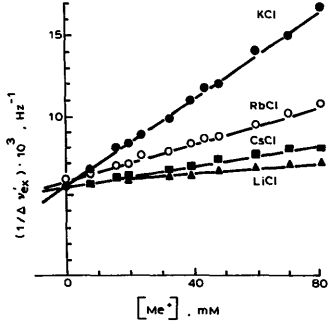


Fig. 3. Sodium binding to SR membranes ($\Delta\nu'_{ex}$) in the presence of alkaline metal ions (Me^+). Linear regression is done in coordinates ($1/\Delta\nu'_{ex}$, $[Me^+]$). The abscissa intercept is equal to the apparent K_d value of the corresponding ion. Medium contains 10 mg SR protein/ml, 20 mM NaCl, 1 mM EGTA, 50 mM Tris-Mops (pH 7.0).

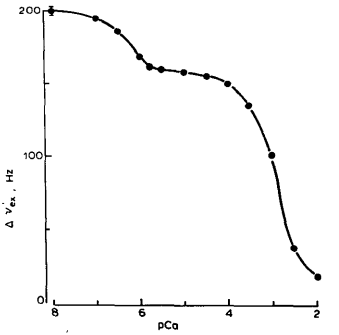


Fig. 4. Effect of Ca^{2+} on Na^+ binding to SR membranes. Medium composition as in Fig. 3.

(Fig. 5A) linearly (with a significance level α equal to 0.05 according to the χ^2-criterion) depend on Na^+ concentration in the range from 15 to 80 mM (Fig. 5B), this being in agreement with a competitive model. Abscissa intercept corresponds to K_{dNa} (14 mM) and the ordinate intercept gives K_{dCa} (0.16 μM). As seen from the Table, the relative amount of sites I does not practically vary as $[Na^+]$ increases.

To check a suggestion that Ca^{2+} and Na^+ compete for sites I, the binding of $^{45}Ca^{2+}$ at submicromolar concentrations at various $[Na^+]$ was studied. Calcium binding to high-affinity sites is inhibited by increasing sodium concentration (Fig. 6A). The Fig. 6B shows the dependence of the $[Ca^{2+}]_{1/2}$ value (corresponding to K_{appCa}) on $[Na^+]$. The Na^+ concentrations being equal to 20 and 40 mM, the $[Ca^{2+}]_{1/2}$ values were obtained by plotting curves 1 and 2 from Fig. 6B in Hill coordinates. At higher Na^+ concentrations of 70, 100, and 150 mM the Scatchard plots were used. It is seen that the experimental points are well approximated by a linear function. Thus, K_{Na} obtained as abscissa intercept equals to 13 mM, and K_{dCa} obtained as ordinate intercept is 0.15 μM. The K_{dCa} and K_{dNa} values of sites I calculated from Na^+ binding experiments and the analogous values of high-affinity sites calculated from the measurements of Ca^{2+} binding coincide well in pairs. This indicates the identity of sites I and high-affinity Ca^{2+} binding sites in the SR membranes.

Interaction of Ca^{2+} and Na^+ with sites II

As noted above, K_d of the whole pool of Na^+-binding sites in a calcium-free medium is about 10 mM. Since sites II bind 4–5-times more Na^+ than sites I,

the constant predominantly reflects the Na^+ binding to sites II (with accurancy not worse than 20%).

The curve reflecting the dependence K_{appNa} on pCa (Fig. 7, curve 1) is biphasic. First, the K_{app} increases with the increase of $[Ca^{2+}]$ in micromolar range, then, after an outlining plateau, a further growth at submillimolar concentrations is observed. Increasing K_{appNa}

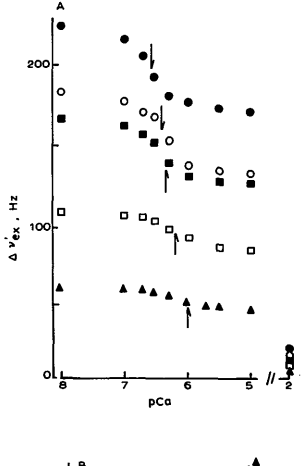


Fig. 5. Sodium binding to sites I. (A) Dependence of a Na^+ relative portion ($\Delta\nu'_{ex}$) bound to sites I on pCa at 15 mM (●——●), 20 mM (○——○), 25 mM (■——■), 40 mM (□——□), and 80 mM (▲——▲) NaCl. Calcium concentrations providing half-maximal inhibition of Na^+ binding are marked by the arrows. (B) Dependence of Ca^{2+} concentrations providing half-maximal inhibition of Na^+ binding to sites I ($[Ca^{2+}]_{1/2}$) on Na^+ concentration. Symbols as in Fig. 5A. The ($[Ca^{2+}]_{1/2}$) values were obtained by reconstracting curves in Fig. 5A in the Hill coordinates. The medium contains 10 mg SR protein/ml, 1 mM EGTA, 50 mM Tris-Mops (pH 7.0).

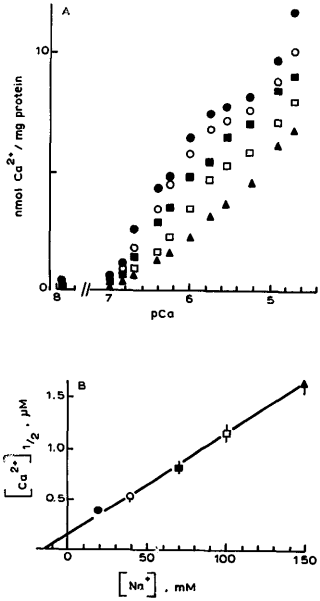


Fig. 6. Passive $^{45}Ca^{2+}$ binding to high-affinity sites on SR membranes. (A) Calcium binding in the presence of 20 mM (●——●), 40 mM (○——○), 70 mM (■——■), 100 mM (□——□), and 150 mM (▲——▲) NaCl. (B) Concentrations of Ca^{2+} providing half-maximal saturation of the high-affinity sites at different Na^+ concentrations. The medium composition as in Fig. 5.

in the range of 30–1000 μM Ca^{2+} may result from the competition between Ca^{2+} and Na^+ for sites II. To verify this assumption, curve 1 (Fig. 7) was plotted in the appropriate coordinates (K_{appNa}, $[Ca^{2+}]$) and linear regression in the range $[Ca^{2+}] = 30$–1000 μM (not shown) gave values $K_{dCa} = 1.3$ mM and $K_{dNa} = 23$ mM. However, the hypothesis of this dependence linearity having rather low significance level ($0.005 < \alpha < 0.01$ according to the χ^2-criterion), the values K_{dCa} and K_{dNa} for sites II can be accepted only as a rough estimate. The relative number of sites II only slightly depends on $[Na^+]$ (Table I).

Calcium at concentrations up to 10 μM fails to compete with sodium for sites II. The Ca^{2+} concentration being varied in this range, a change in K_{appNa} may

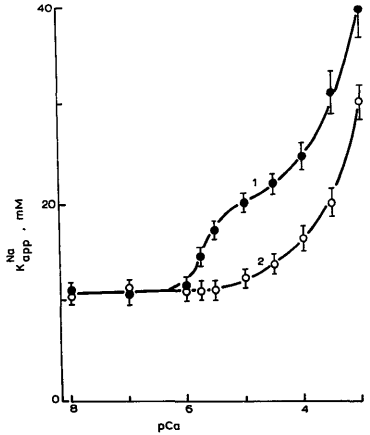


Fig. 7. Dependence of apparent K_d of the Na^+-binding sites on pCa in the absence (1) and in the presence (2) of deoxycholate. For each Ca^{2+} concentration K_{appNa} was calculated as shown in the inset of the Fig. 2. Deoxycholate to SR protein ratio was 1:10 (w/w). Medium composition as in Fig. 5.

be due to an allosteric effect on sites II of bound Ca^{2+}. In the presence of a detergent $C_{12}E_8$ (not shown) or deoxycholate the marked increase in the K_{appNa} value in the range of Ca^{2+} concentrations from 1 to 10 μM does not take place (Fig. 7, curve 2).

Experiments were also performed on $^{45}Ca^{2+}$ binding to the low-affinity sites on SR vesicles at various Na^+ concentrations (Fig. 8A). The K_{appCa} values increase with $[Ca^{2+}]$ increasing in the medium (Fig. 8B). Linear regression gives values $K_{dNa} = 34$ mM and $K_{dCa} = 0.7$ mM, respectively. It should be noted, that experimental points lie on a straight line much worse if compared to the case of sites I. At the same time, the calculated amount of the binding sites varies insignificantly (Table I).

The fact that the dependence $K_{appNa} = f([Ca^{2+}])$ and $K_{appCa} = f([Na^+])$ are monotonous but not quite linear, may indicate both competition of Ca^{2+} and Na^+ and their allosteric effects on the binding with low affinity of each other. Thus, in spite of close values of K_{dCa} (and K_{dNa}) estimated in the experiments on $^{45}Ca^{2+}$ binding and ^{23}Na-NMR studies we cannot state with confidence that sites II and low-affinity calcium sites are indentical.

Interaction of sites I and II with hydrogen ions

Fig. 9 shows displacing of Na^+ from sites I by calcium ions at various pH values. The $[Ca^{2+}]_{1/2}$ value is seen to increase with a decrease in pH. Since three parameters, Ca^{2+} and proton concentrations as well as the amount of Na^+ bound to the membranes in this experiment change simultaneously, it is hardly possible to make a definite conclusion about the type of interaction of these ions with sites I.

On the other hand, one can estimate pK_a values for sites II since it is possible to measure Na^+-binding in a calcium-free medium. Fig. 10 (curve 1) shows $\Delta\nu'_{ex}$ variation in a wide pH range. As evidenced from the shape of this curve, the protonation of chemical groups

TABLE I

Relative amount of cation-binding sites and cooperativity of Ca^{2+} binding at different Na^+ concentration

$[Na^+]$ (mM)	Relative amount of sites I [a] (Hz mM per 25 mg protein)	Hill coeff. for sites I	Number of high-affinity sites (nmol per mg protein)	Hill coeff. for high-affinity sites	Relative amount of sites II [b] (Hz mM per 25 mg protein)	Number of low-affinity sites (nmol per mg protein)
15	1540	1.4	–	–	5540	–
20	1680	1.5	8.0	1.7	5030	250
25	1520	1.4	–	–	5420	–
40	1430	1.2	7.8	1.5	4850	242
70	–	–	8.3	1 [c]	–	250
80	1400	1.0	–	–	4530	–
100	–	–	7.4	1 [c]	–	268
150	–	–	6.0	1 [c]	–	320

[a] The value was calculated as a product of $(K_{dNa} + [Na^+])$ and a difference between relative amounts of bound Na^+ $(\Delta\nu'_{ex})$ at $[Ca^{2+}] = 10^{-8}$ M and $[Ca^{2+}] = 10^{-5}$ M.

[b] The value was calculated as a product of $(K_{dNa} + [Na^+])$ and a difference between relative amounts of bound Na^+ $(\Delta\nu'_{ex})$ at $[Ca^{2+}] = 10^{-5}$ M and $[Ca^{2+}] = 10^{-2}$ M.

[c] The value is close to unity since the experimental curves are easily linearized in the Scatchard plots.

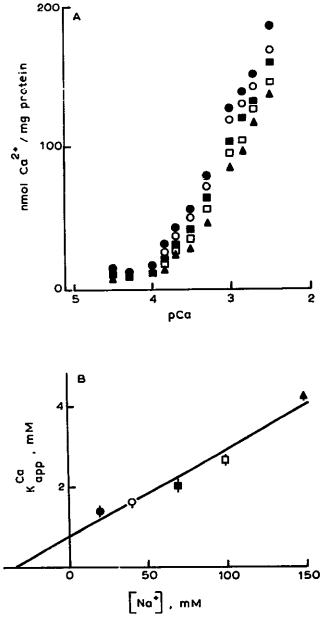


Fig. 8. Binding of $^{45}Ca^{2+}$ to low-affinity sites of SR. (A) Calcium binding in the presence of 20 mM (●——●), 40 mM (○——○), 70 mM (■——■), 100 mM (○——○), and 150 mM (▲——▲) NaCl. (B) Dependence of Ca^{2+} concentrations providing half-maximal saturation of the low-affinity sites on Na^+ concentration. The ($[Ca^{2+}]_{1/2}$) value were obtained by reconstructing curves in Fig. 8A. in the Scatchard coordinates. The medium contained 10 mg protein/ml, 50 mM Tris-Mops (pH 7.0).

with pK_a in the range of 5.9–6.1 leads to Na^+ desorption from the greater part of sites II. As follows from Fig. 10 (curve 2), the K_{appNa} increases with $[H^+]$ increase, but the relative amount of sites II is not practically altered (curve 3). It is noteworthy that this amount expressed in relative units well coincides with an analogous value obtained from Ca^{2+} interaction with sites II (Table I). The dependence $K_{appNa} = f([H^+])$ seems to be power function, that may indicate to the participation of several similar ionized groups in Na^+ coordination at sites II. The fact that pK_a value approximates 6.0, points to the participation of carboxyl groups and possibly histidine residues in the formation of sites II.

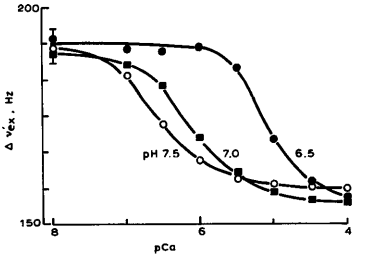


Fig. 9. Sodium binding to sites I at various pH. The medium composition as in Fig. 5.

Thus, Na^+ binding Ca^{2+}-sites of carp parvalbumins containing carboxylates have pK_a values in the range from 4.8 to 6.5 [31] and analogous sites of troponin C have two pK_a values, 4.6 and 6.0, respectively [32].

Thus, sites I and II differ in their sensitivity to pH, the pK_a value of sites II being lower than that of sites I.

Interaction of sites I and II with Mg^{2+}

Magnesium ions displace Na^+ from 50 to 70% of the binding sites in the presence of both low (Fig. 11, curve 2), and high (Fig. 11, curve 1) concentrations of calcium. At $[Ca^{2+}] = 10^{-5}$ M high-affinity Ca^{2+}-binding sites are saturated and Na^+ is bound only to sites II. The K_{appMg} value obtained by reconstructing curve

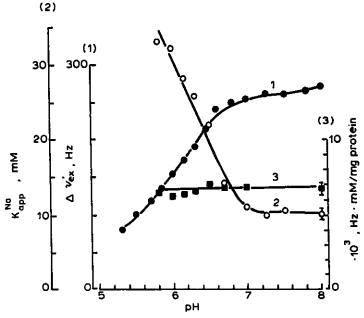


Fig. 10. Dependence of sodium binding to SR membranes on pH. (Curve 1) The Na^+ relative portion ($\Delta\nu'_{ex}$) bound to membranes. The NaCl concentration was 12 mM. (Curve 2) The K_{appNa} value of Na^+-binding sites. (Curve 3) A relative amount of Na^+-binding sites. The Ca^{2+} concentration was $3 \cdot 10^{-8}$ M.

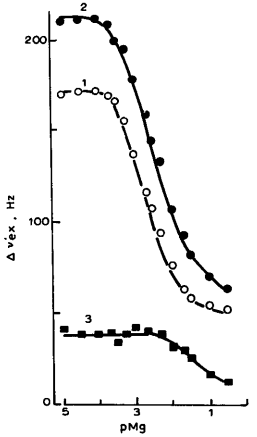


Fig. 11. Effect of Mg^{2+} on Na^+ binding to SR membranes. (Curve 1) A Na^+ relative portion ($\Delta\nu'_{ex}$) bound to sites II. (Curve 2) A Na^+ relative portion ($\Delta\nu'_{ex}$) bound to sites I and II. (Curve 3) Difference between curves 2 and 1 corresponding to a Na^+ portion bound to sites I. Medium composition as in Fig. 3.

1 in the Hill coordinates is 3–4 mM and the Hill coefficient is close to unity. At $[Ca^{2+}] = 10^{-8}$ M, when sites I and sites II are both exposed to magnesium ions, K_{appMg} is 5 mM, i.e. only slightly differs from K_{appMg} for sites II. The difference between curves I and 2 (curve 3) corresponding to the amount of Na^+ bound to sites I changes with the change in Mg^{2+} concentration in the range of 10–100 mM. This indicates that sites I unlike sites II interact with Mg^{2+} very weakly ($K_{appMg} \approx 30$ mM).

Discussion

The linearity of the $(\Delta\nu'_{ex})^{-1} = f([Na^+])$ dependence is observed in a wide range of Na^+ concentrations (4–100 mM). First, the above linearity enables us to assume the existence of Na^+-binding sites characterized by the same value of dissociation constant. Second, of two possible schemes of Na^+ interaction with binding sites (B) characterized by a single K_d value

$$B + Na^+ = [B \cdot Na]$$

$$B + nNa^+ = [B \cdot nNa],\ n > 2$$

it allows us to choose the first upon analyzing. Consequently, Na^+ binds to the corresponding sites in the stoichiometric ratio 1:1. An analogous conclusion can be made from the experiments on displacing Na^+ from the binding sites by other monovalent cations.

However, the influence of calcium ions on the sodium signal revealed two types of sites (I and II) for sodium binding which may differ in their ability to interact with calcium. Close K_d values for Na^+ (and Ca^{2+}) obtained by treating ^{23}Na-NMR data and the curves of $^{45}Ca^{2+}$ displacing by sodium ions within the frame of Benesi-Hildebrand formalism allow us to assume sodium and calcium (in submicromolar concentrations) competition for the same binding sites (sites I) in the stoichiometric ratio 1:1. In other words, Na^+-binding sites I are identical to the high-affinity Ca^{2+}-binding sites on Ca^{2+}-ATPase described previously [33–35]. This is confirmed by the fact that the value of Ca^{2+} concentration providing half-maximal inhibition of Na^+ binding increases from 0.2 μM at pH 7.5 to 0.5 μM (pH 7.0) and 7 μM (pH 6.5). These values are close to those obtained in the experiments on the pH effects on Ca^{2+} saturation of high-affinity sites in SR membranes [36]. Besides, practicaly the same degree of cooperativity in Ca^{2+} binding characterizes both sites I (Table I) and high-affinity sites (Table I; see also [37]). The degree of the cooperativity of Ca^{2+}-binding sites I decreases with an elevation of Na^+ concentration which resembles the change in the cooperativity of Ca^{2+} binding to high affinity Ca^{2+}-ATPase sites with a decrease in pH [36,38]. Both the cases can be explained by a competitive way of displacement Ca^{2+} from its binding sites by H^+ and Na^+. This displacing of Ca^{2+} may lead to a disorder in the conformation state of the protein which is necessary for exposing cooperative properties.

The greater part of Na^+-binding sites, sites II, is modified at high Ca^{2+} concentrations. The apparent dissociation constant for Na^+ at pH 7.0 increases from 20 to 40 mM upon Ca^{2+} concentration increase from 0.01 to 1 mM. On the other hand, K_{app} value for Ca^{2+} also increases with the increase in Na^+ concentration. Thus, sites II and low-affinity Ca^{2+}-binding sites are interconnected and the saturation of one of them leads to changes in cation-binding properties of the other. Undoubtedly, the simplest explanation of this mutual influence results from an assumption about the identity of sites II and low-affinity Ca^{2+}-binding sites. However, the analysis of their cation-binding properties performed within the frame of traditional Benesi-Hildebrand construction and showing the nonlinearity of the dependence $K_{appNa} = f([Ca^{2+}])$ prevented us from finally making such a conclusion.

The calculated number of sites II is much less than the number of low-affinity Ca^{2+}-binding sites. Indeed, if sites I are similar to high-affinity Ca^{2+}-binding sites,

their amount should be about 7 nmol/mg SR protein (Table I). The number of sites I is about 1/3 of that of sites II, hence the amount of the latter is 20–25 nmol/mg SR protein. At the same time the number of low-affinity calcium sites affected by sites II is nearly 250 nmol/mg SR protein. Thus the major part of sites II and low-affinity calcium sites should be connected in an allosteric manner.

It should be noted that when Na^+ and Ca^{2+} interact with the binding sites of other Ca^{2+}-binding proteins, both the presence and the absence of the competitive interaction are observed. Thus, it is shown for parvalbumins and calmodulin and its trypsin fragments that Na^+ and Ca^{2+} compete for the Ca^{2+}-binding sites [21,20,22]. As established for troponin C and its trypsin fragments, Na^+ and Ca^{2+} compete only for low-affinity Ca^{2+}-binding sites [21]. Sodium binding to high-affinity sites of troponin C is inhibited by Ca^{2+} allosterically.

The value of K_{appNa} varies in the presence of Ca^{2+} at micromolar concentrations. This we believe to be due to Ca^{2+} binding to some sites possessing a K_{appCa}, as roughly estimated from Fig. 7, of 2 to 5 μM. Being far enough from K_{appCa} values of sites I (approx. 0.4 μM) and sites II (approx. 1 mM), this K_{app} may characterize a new type of sites with intermediate affinity to Ca^{2+}. We failed to detect intermediate-affinity sites both in ^{23}Na-NMR and $^{45}Ca^{2+}$ experiments in amounts comparable to that of sites I (high-affinity sites). Nevertheless, it is noteworthy that previous data [37] indicate as well the existence of a small number of the intermediate-affinity sites located on SR membranes.

Calcium binding to sites with intermediate affinity leads to decreasing the affinity of sites II (which as we assume are located on each Ca^{2+}-ATPase molecule) for Na^+. According to our data the amount of 'intermediate' sites is insignificant (evidently, less than 20% of sites I or high-affinity sites; in this case they cannot be recorded because of scattering in experimental data). Therefore one intermediate-affinity site should be formed with a participation of several enzyme molecules, i.e. it may belong to a Ca^{2+}-ATPase oligomer. Other data confirm this conclusion since modifying influence of micromolar calcium on sites II is removed in the presence of detergents at low concentrations. Under these conditions detergent molecules are inserted into a lipid SR bilayer causing no solubilization of the latter though destroying protein-to-protein interaction [39].

If rested on the postulate of the existence of two type sites for binding monovalent ions, the multiple action of monovalent ions on SR functions could be explained. Sodium binding to sites I may lead (due to Ca^{2+} displacement in a competitive manner) only to the inhibition of Ca^{2+}-ATPase and Ca^{2+} transport activities. According to our data, sites II are the only candidates on the role of 'activating' sites. In favour of this assumption is the fact that sites II affinity for the different alkaline metal ions (Eqn. 9) changes in the same manner as the stimulating rows of the ATPase activity, Ca^{2+} transport [15,14] and the rate of phosphoprotein decomposition [8]. Besides, sites II, as well as 'activating' sites postulated by Ribeiro and Vianna [17], could be subdivided into two pools. The sites belonging to one of them are not specific for alkaline metal ions and interact with Mg^{2+}.

As noted above, concentrations of alkaline metal ions ensuring Ca^{2+}-ATPase inhibition are considerably higher than those activating the enzyme. At the same time, K_d values of sites I and II for sodium are close to each other and equal to 10–15 mM. This discrepancy may be due to the presence of micromolar Ca^{2+} able under these conditions to interact with the sites I, but not with the sites II. In this case, K_{appNa} of the sites I (reflecting the inhibitory constant) increases markedly if compared to that for sites II. Consequently, it should also be expected that in the presence of Ca^{2+} in the range of 1 to 100 μM one can not observe any inhibition of Ca^{2+}-ATPase activity by alkaline metal ions at all. This results, indeed, was obtained (in case of K^+) by Duggan [15].

References

1 Inesi, G. (1985) Annu. Rev. Physiol. 47, 573–604.
2 Jencks, W.P. (1989) J. Biol. Chem. 264, 18855–18858.
3 Yamada, S. and Ikemoto, N. (1980) J. Biol. Chem. 255, 3108–3119.
4 Shigekawa, M., Doughterty, J.P. and Hasselbach, W. (1978) J. Biol. Chem. 253, 1442–1450.
5 Shigekawa, M. and Doughterty, J.P. (1978) J. Biol. Chem. 253, 1451–1457.
6 Chaloub, R.M. and De Meis, L. (1980) J. Biol. Chem. 255, 6168–6172.
7 Beeler, T. and Keffer, J. (1984) Biochim. Biophys. Acta 773, 99–105.
8 Shigekawa, M. and Pearl, L. (1976) J. Biol. Chem. 251, 6947–6952.
9 Inao, S. and Kanazawa, T. (1986) Biochim. Biophys. Acta 857, 28–37.
10 Martin, D.W. and Tanford, C. (1984) FEBS Lett. 177, 146–149.
11 Sande-Lemos, M.P. and De Meis, L. (1988) J. Biol. Chem. 263, 3795–3798.
12 Medda, P., Fassold, E. and Hasselbach, W. (1987) Eur. J. Biochem. 165, 251–259.
13 Shigekawa, M. and Akowitz, A.A. (1978) J. Biol. Chem. 254, 4726–4730.
14 The, R. and Hasselbach, W. (1972) Eur. J. Biochem. 30, 318–324.
15 Duggan, P.F. (1977) J. Biol. Chem. 252, 1620–1637.
16 Jones, L.R., Besch, H.R. and Watanabe, A.M. (1977) J. Biol. Chem. 252, 3315–3323.
17 Ribeiro, J.M. and Vianna, A.I. (1978) J. Biol. Chem. 253, 3151–3157.
18 Stephens, E. and Grisham, G. (1977) Biochemistry 18, 4876–4885.
19 Grandjean, J., Laszlo, P. and Gerday, C. (1977) FEBS Lett. 81, 376–380.
20 Gerday, C., Grandjean, J. and Laszlo, P. (1979) FEBS Lett. 105, 384–385.

21 Delville, A., Grandjean, J., Laszlo, P., Gerday, C., Grabarek, Z. and Drabikowski, W. (1980) Eur. J. Biochem. 105, 289–295.
22 Delville, A., Grandjean, J., Laszlo, P., Gerday, C., Brzeska, H. and Drabikowski, W. (1980) Eur. J. Biochem. 109, 515–522.
23 Forsen, S. and Lindman, B. (1978) Chem. Brit. 14, 29–35.
24 Levitsky, D.O., Aliev, M.K., Kuzmin, A.V., Levchenko, T.S., Smirnov, V.N. and Chazov, E.I. (1976) Biochim. Biophys. Acta 443, 262–284.
25 Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226–497–501.
26 Yeagle, P.L., Langdon, R.G. and Martin, R.B. (1977) Biochemistry 16, 3487–3491.
27 Fabiato, A. and Fabiato, F. (1979) J. Physiol. 75, 463–505.
28 Hubbard, P.S. (1970) J. Chem. Phys. 53, 985–987.
29 Bull, T.E. (1972) J. Magn. Reson. 8, 344–353.
30 Kurland, R., Newton, C., Nir, S. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 137–147.
31 Birdsall, W.J., Levine, B.A., Williams, R.J.P. et al. (1979) Biochemie 67, 741–750.
32 Anderson, T., Drakenberg, T., Forsen, S. and Thulin, E. (1981) FEBS Lett. 125, 39–44.
33 Chevallier, J. and Butow, B.A. (1971) Biochemistry 10, 2733–2737.
34 Ikemoto, N. (1974) J. Biol. Chem. 249, 649–651.
35 Ostwald, T.J. and MacLennan, D.M. (1974) J. Biol. Chem. 249, 974–979.
36 Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C. and Inesi, G. (1981) Biochemistry 20, 6617–6625.
37 Inesi, G., Kurzmack, M., Coan, C. and Lewis, D.E. (1980) J. Biol. Chem. 255, 3025–3031.
38 Hill, T.L. and Inesi, G. (1982) Proc. Natl. Acad. Sci. USA 79, 3979–3982.
39 Anderson, J., Le Maire, M., Kragh-Hansen, U., Champeil, P. and Moller, J. (1983) Eur. J. Biochem. 134, 205–214.