Biochimica et Biophysica Acta, 1145 (1993) 75–84

BBAMEM 75835

In squid axons the Ca_i^{2+} regulatory site of the Na^+/Ca^{2+} exchanger is drastically modified by sulfhydryl blocking agents. Evidences that intracellular Ca_i^{2+} regulatory and transport sites are different

Reinaldo DiPolo [a] and Luis Beaugé [b,c]

[a] *Centro de Biofísica y Bioquímica, IVIC, Caracas (Venezuela),* [b] *Instituto de Investigación Médica M. y M. Ferreyra, Córdoba (Argentina) and* [c] *Marine Biological Laboratory, Woods Hole, MA (USA)*

(Received 14 July 1992)

Key words: Sodium–calcium ion exchange; Squid axon; Sulfhydryl blocking agent

We have explored the effect of the sulfhydryl group blocker *p*-chloromercuryphenylsulfonic acid (PCMBS) on Ca^{2+} and Na^+ interactions with the Na^+/Ca^{2+} exchanger in squid giant nerve fibers. Steady-state Na_o^+-dependent Ca^{2+} efflux (forward) and Na_i^+-dependent Ca^{2+} influx (reverse) were measured in internally dialyzed, voltage clamped squid axons. External PCMBS (0.5 mM, for 25–35 min) has no effect on the activation of Ca^{2+} efflux by Na_o^+, and Ca_o^{2+} or on the activatory external monovalent cation site. In contrast, when applied internally it drastically reduces the affinity of the Na^+/Ca^{2+} exchanger towards Ca_i^{2+} ions without affecting its maximal rate of transport; in the presence of MgATP the $K_{0.5}$ for Ca_i^{2+} activation of forward Na^+/Ca^{2+} exchange increases from 1.5 μM to 95 μM; likewise the apparent affinity of the Ca_i^{2+} stimulation of the reversal exchange decreases 100-fold. Interestingly, no effect of PCMBS was found on the interactions between Na_i^+ and Ca_i^{2+} ions with the internal transport site(s) (inhibition of Na_o^+ and Ca_o^{2+}-dependent Ca^{2+} efflux by Na_i^+). On the other hand, Na_i^+ ions do not modify the interactions of Ca_i^{2+} with that site. Two important characteristics of the Ca_i^{2+} regulatory site are uncover in this work: (i) sulfhydryl groups are important in maintaining the integrity of the Ca^{2+} binding domain of the Ca_i^{2+} regulatory site and (ii) Na_i^+ and Ca_i^{2+} regulatory, or Na_i^+ and Ca_i^{2+} transporting sites, are different entities.

Introduction

The Na^+/Ca^{2+} exchange is one of the major plasma membrane bound mechanism responsible for both net Ca^{2+} extrusion from and Ca^{2+} entry into the cell. The physiological relevance of this system can be inferred from its role in regulating cardiac and arterial muscle contraction, photoreception, neural-transmission and secretion [1]. There is now evidences of the existence of more than one Na^+/Ca^{2+} exchange molecule with different characteristics. Thus, the retinal rod exchanger requires the presence of potassium ions for activity (4 Na^+/(1 Ca^{2+} + 1 K^+) stoichiometry) [2,3], while no such requirement exist for the cardiac and squid exchanger (3 Na^+/1 Ca^{2+}) [4,5].

A complete understanding of this countertransport system requires information at the molecular level: i.e., detail of the structure-function relationship. An important step towards this goal has been the recent cloning and expression of functional Na^+/Ca^{2+} exchange from cardiac and retinal rods [6,7]. In principle two complementary studies can provide information about the regions of the molecule at which the different ligands bind: (a) selective mutagenesis in the case of the cloned and expressed carriers, and/or, (b) chemical block of specific groups in vivo. In the case of the squid Na^+/Ca^{2+} exchanger which has not yet been cloned, the second approach seems a logical approach to identify functionally important chemical groups. The dialyzed squid axon, which allows complete control of the intra and extracellular environment and membrane potential while accurately measuring ionic fluxes is an ideal preparation to study the sidedness of chemical modifiers.

In this work we found that PCMBS has no effect on Na_o^+, Ca_o^{2+} and monovalent activatory cation affinities. On the other hand, it induces a drastic reduction in the apparent affinity of the intracellular Ca_i^{2+} regulatory site without affecting the maximal rate of Na^+/Ca^{2+} exchange. These results suggest that sulfhydryl groups are structural part or at least influence the Ca_i^{2+} regulatory binding site. In contrast they do not seem relevant for any of the extracellular binding sites of the exchanger. In addition, the differential effect of

Correspondence to: R. DiPolo, Centro de Biofísica y Bioquímica, IVIC, Apartado 21827, Caracas 1020A, Venezuela.

PCMBS on the intracellular Ca^{2+} regulatory and Na^+ and Ca^{2+} transport sites suggests that the regulatory and transport loci are different entities. A preliminary report of part of this work has been presented elsewhere [8].

Materials and Methods

Experimental preparation

The experiments were carried out on live specimens of *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA, and with the tropical squid *Loligo plei* at the Instituto Venezolano de Investigaciones Cientificas, IVIC, Caracas, Venezuela. For Ca^{2+} efflux measurements, the axons were mounted in a dialysis chamber designed to dialyze and voltage clamp the axon [9]. For Ca^{2+} influx we used a similar efflux chamber modified in order to create a well defined region of external isotope solution where the axon was immersed [10]. Dialysis capillaries were from regenerated cellulose fibers with a molecular cut off of 9000 Da (150 μm o.d.; 141 μm i.d.; Spector, Los Angeles, CA). For axons of smaller diameter such as those of *L. plei* (mean diameter 420 μm) control of axoplasmic constituents could be effectively achieved with cellulose acetate capillaries (120 μm o.d., 100 μm i.d.; Fabric Research, MA). The dialysis capillaries contained a 75 μm platinized platinum iridium wire (20% iridium) for passing current. Prior to the addition of internal or external radioactive solutions, the axons were routinely pre-dialyzed for at least 45 min with an isotope free internal dialysis medium containing 1–3 mM EGTA and no ATP.

Solutions

The standard dialysis medium had the following composition (mM): Tris-Mops, 385; NaCl, 40; $MgCl_2$, 5; glycine, 285; Tris-EGTA, 1–3; pH 7.3 (17–18°C). The osmolarity of the solutions was adjusted at 940 mosmol/l. The estimation of the $[Ca^{2+}]_i$ was based on a dissociation constant of 0.15 μM for CaEGTA [11]. The standard sodium sea water (NaSW) had the following composition (mM): Na^+, 440; Ca^{2+}, 0.5; Mg^{2+}, 60; Cl^-, 567; Tris-Cl, 10; pH 7.6 (17–18°C). Removal of external Na^+ was compensated with isosmolar amounts of Tris or Li ions. The external solutions always contained 1 mM NaCN and 200 nM tetrodotoxin. The axons were voltage clamped at their resting membrane potential which under the above ionic conditions ranged from -5 to $+10$ mV. The leak current was measured by applying 20 mV hyperpolarizing pulses of 20 ms duration. The membrane resistance of axons bathed in these solutions ranged from 1500 to 3500 ohm cm^2. All reagents used were of analytical grade. *p*-Chloromercuryphenylsulfonic acid (PCMBS) was from Sigma. In the present experiments we have used a PCMBS concentration ranging from 0.1–0.5 mM. At these concentrations this compound has no effect on the membrane resistance nor on the 'leak' of $^{45}Ca^{2+}$ (see Figs. 1 and 5 as examples). Radioactive solutions were made by adding solid $^{45}CaCl_2$ to the dialysis or external medium. In efflux experiments the external medium was delivered at a rate of 1.5 ml/min; in influx determinations the rate was 0.1 ml/min. The flow through the dialysis capillary was about 0.001 ml/min in both instances. Radioactive samples collected every 3 min were mixed with 5 ml of scintillation liquid and counted in a liquid scintillation counter.

In all experiments each axon served as its own control since steady-state Ca^{2+} efflux and influx were always measured before and after a given treatment.

Results

The effect of external PCMBS on the Na_o^+-dependent and Ca_o^{2+}-dependent Ca^{2+} efflux components

Fig. 1 shows an experiment in which the effect of external PCMBS was explored on both Na_o^+- and Ca_o^{2+}-dependent Ca^{2+} efflux components (Na_o^+/Ca_i^{2+}

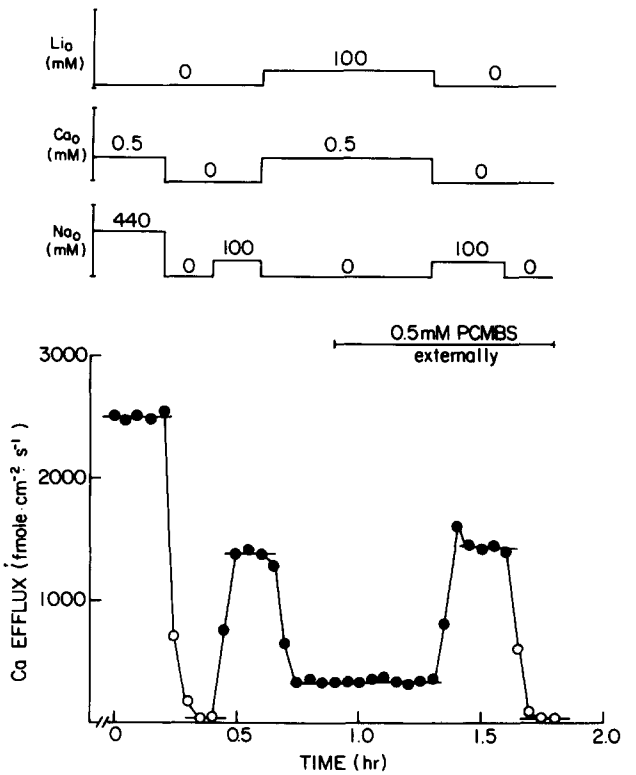

Fig. 1. The effect of external PCMBS on the Na_o^+- and Ca_o^{2+}-dependent Ca^{2+} efflux components. Ordinate: Ca^{2+} efflux in fmol/cm^2 per s. Abscissa: time in hours. All concentrations are in millimolar. The axon was dialyzed for 45 min with the radioactive dialysis medium, at time zero the external medium was counted at 3 min intervals. Notice that both the Na_o^+ and Ca_o^{2+} components are not affected by the presence of PCMBS. Axon diameter 650 μm, temperature 17°C.

and Ca_o^{2+}/Ca_i^{2+} exchanges). The axon was predialyzed for about 45 min with 1 mM EGTA and no ATP prior to the addition of the same solution containing radioactive $^{45}Ca^{2+}$ at a saturating $[Ca^{2+}]$ of 100 μM. An eventual effect of PCMBS could be on the maximal rate of transport, the apparent affinity for ligands (Na_o^+, Ca_o^{2+} and/or Li_o) or both. Therefore, Na_o^+-dependent and Li_o-activated Ca_o^{2+}-dependent Ca^{2+} efflux components were determined at non saturating concentrations of these cations. In the standard NaSW Ca^{2+} efflux reached a steady state value of 2550 fmol/cm^2 per s. Upon removal of external Na^+ and Ca^{2+}, Ca^{2+} efflux dropped to background levels. Addition of 100 mM Na_o^+ in the absence of Ca_o^{2+} brought the Na_o^+-dependent Ca^{2+} efflux to about 1400 fmol/cm^2 per s. Withdrawal of Na_o^+ in the presence of 0.5 mM Ca_o^{2+} and 100 mM Li_o induced a Ca_o^{2+}-dependent Ca^{2+} efflux of 310 fmol/cm^2 per s. The addition of 0.5 mM PCMBS to the external medium had no effect on the Ca_o^{2+}/Ca_i^{2+} exchange or the Na_o^+-dependent components since their magnitude were the same as those observed before the addition of the chemical blocker. Fig. 1 also shows that, at the concentration used, PCMBS has no detectable effect on the 'leak' of Ca^{2+} measured in the absence of external Na^+ and Ca^{2+} or the membrane resistance which in this particular experiment was 3275 ohm cm^2 before and 3230 ohm cm^2 after the addition of PCMBS.

The effect of internal PCMBS on the Na_o^+-dependent Ca^{2+} efflux

To investigate whether PCMBS from the cytoplasmic side has any effect on the kinetic parameters of the Na^+/Ca^{2+} exchange, we explored its effect on the Na_o^+-dependent Ca^{2+} efflux both at submicromolar (0.7 μM) and saturated (200 μM) $[Ca^{2+}]_i$. In the experiment of Fig. 2, after the efflux of Ca^{2+} has reached a steady value of 78 fmol/cm^2 per s, removal of Na_o^+ and Ca_o^{2+} brings the efflux to background levels. This drop corresponds mostly (> 90%) to the Na_o^+-dependent component since Ca_o^{2+}-dependent Ca^{2+} efflux is very small under this particular condition [12]. Subsequent addition of external Na^+ and Ca^{2+} brought back the efflux to the initial levels. The arrow shows the time at which 0.5 mM PCMBS were added to the dialysis medium. This produced initially a small increase in Ca^{2+} efflux which was followed by a significant (89 ± 4%, $n = 8$) inhibition of the Na_o^+-dependent component. When Ca_i^{2+} was raised from 0.7 to a saturating concentration of 200 μM the Na_o^+-dependent component increased to about 2200 fmol/cm^2 per s. Interestingly, the levels of the Na_o^+-dependent Ca^{2+} efflux attained at a saturating Ca_i^{2+} concentration in the presence of PCMBS are similar to those observed in axons dialyzed with high Ca_i^{2+} but without PCMBS (not shown); this suggest that the blocker has no effect on the maximal rate of the Na^+/Ca^{2+} ex-

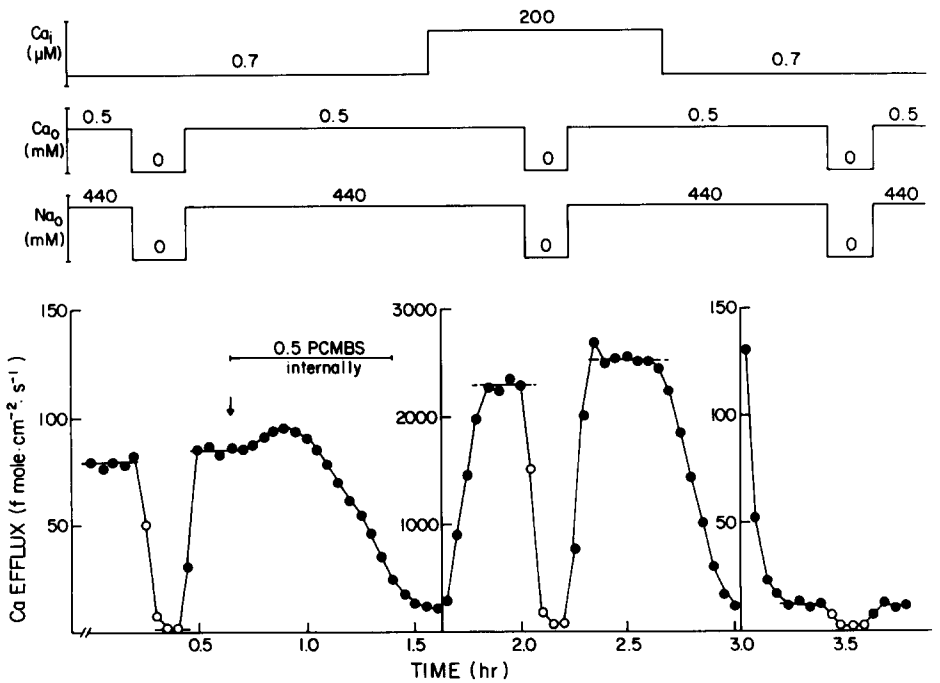

Fig. 2. The effect of internal PCMBS on the Na_o^+-dependent Ca^{2+} efflux. Ordinate: Ca^{2+} efflux in fmol/cm^2 per s. Abscissa: time in hours. •, Ca^{2+} efflux in standard NaSW (see Materials and Methods). ○, Ca^{2+} efflux in 0 Na_o^+, 0 Ca_o^{2+}. At the arrow 0.5 mM PCMBS was introduced into the dialysis medium. All concentrations are in millimolar except the $[Ca^{2+}]_i$ which is in μM. Axon diameter 500 μm. Temperature 17°C.

change. In addition, PCMBS acts irreversibly since its removal does not modify the levels of fluxes attained after its application. The fact that PCMBS at 0.5 mM has no effect on the Na_o^+-dependent Ca^{2+} efflux when applied from the extracelular compartment indicates that this coumpound is relatively impermeable to the squid axon membrane. A similar situation occurs in barnacle muscle fibers in which the permeable sulfhydryl reagent *N*-ethylmaleimide (NEM) inhibits Na^+/Mg^{2+} exchange and releases Ca^{2+} from intra-

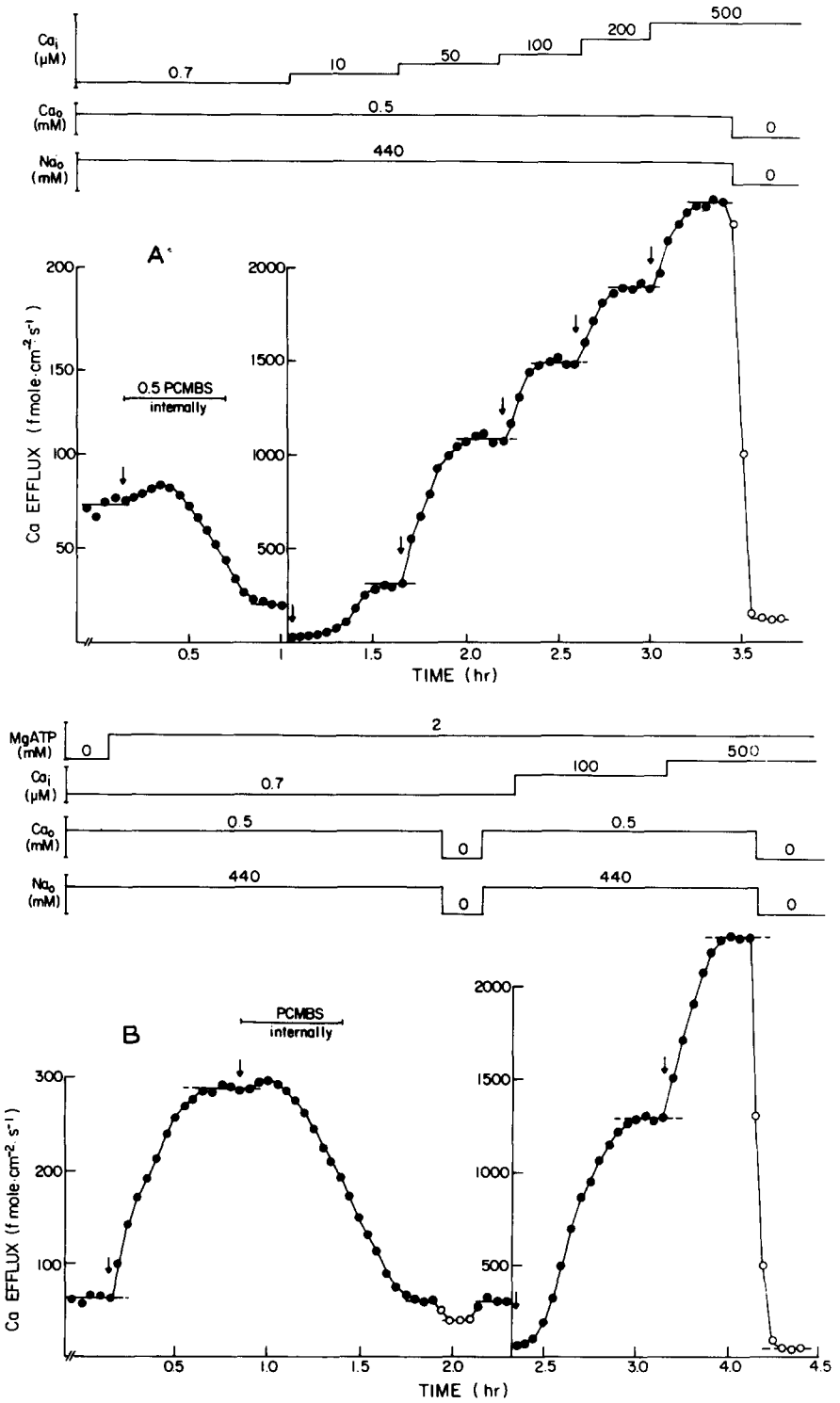

Fig. 3. (A) The activation of Na_o^+-dependent Ca^{2+} efflux by Ca_i^{2+} in the absence of MgATP and in the presence of 0.5 mM internal PCMBS. Ordinate: Ca^{2+} efflux in fmol/cm^2 per s. Abscissa: time in hours. •, Ca^{2+} efflux in standard NaSW. Notice the change in scale for Ca^{2+} efflux. Axon diameter 425 μm. Temperature 18°C. (B) The activation of Na_o^+-dependent Ca^{2+} efflux by Ca_i^{2+} in the presence of 2 mM MgATP and 0.25 mM internal PCMBS. Axon diameter 495 μm. Temperature 18°C.

cellular stores, contrary to external PCMBS which has no effect [13,14].

The effect of PCMBS on the transport and regulatory Ca_i^{2+} sites

The next step was to explore the possibility that PCMBS modifies only the affinity of the exchanger towards internal Ca^{2+}. To that aim we analyzed the Ca_i^{2+} activation of the Na_o^+-dependent Ca^{2+} efflux in axons dialyzed without (Fig. 3A) and with (Fig. 3B) MgATP. Fig. 3A shows the inhibition of Ca^{2+} efflux by PCMBS at 0.7 μM Ca_i^{2+} and its progressive restoration to normal efflux values when Ca_i^{2+} is increased stepwise up to 500 μM. It can be observed that the amount of Ca^{2+} required to take that activation to half maximal value is much higher than in untreated axons (about 12 μM, see below). Fig. 3B shows that at 0.7 μM Ca_i^{2+} all ATP stimulated Na_o^+-dependent Ca^{2+} efflux is abolished by PCMBS. Similar to the experiment of Fig. 3A the Na_o^+-dependent Ca^{2+} efflux can be completely restored by increasing internal Ca^{2+} concentration. Fig. 4 summarizes the results on different axons. In the absence of PCMBS, the $K_{0.5}$ for Ca_i^{2+} with or without ATP determined in two axons coincides with the values reported previously from our laboratory (1.5 $\pm$ 0.3 μM with ATP ($n = 15$) and 12 $\pm$ 1.3 μM without ATP ($n = 10$)). One of the main characteristics of the MgATP effect is a marked increase in Ca_i^{2+} affinity with no change in the maximal fluxes [15]. In axons treated with PCMBS, Ca_i^{2+} stimulation of Na_o^+-dependent Ca^{2+} efflux is similar in the presence and absence of ATP. Therefore, in the presence of PCMBS the axons behaves as if they have not seen MgATP at all. Comparing with untreated axons, PCMBS decreases the apparent affinity for Ca_i^{2+} about 10-fold in the absence of MgATP but one hundred fold in its presence.

In squid axons as well as in cardiac and barnacle muscle, Ca^{2+} ions from the cytoplasmic side are essential for the proper functioning of all partial reactions of the Na^+/Ca^{2+} exchanger [16–18]. The fact that PCMBS largely reduces the affinity for Ca_i^{2+} without modifying the maximal rate of transport might indicate an alteration of the Ca_i^{2+} regulatory site without modification of that from which Ca_i^{2+} is transported. In the experiments described above (see Fig. 4) it is not possible to discern whether the sulfhydryl blocker affects the Ca_i^{2+} transport, the Ca_i^{2+} regulatory site, or both. Examination of the Ca_i^{2+} regulatory site can be done taken advantage of the fact that Na_i^+ inhibits the Na_o^+- and Ca_o^{2+}-dependent Ca^{2+} efflux. At 0.7 μM Ca_i^{2+} inhibition is complete with 100 mM Na_i^+; when Ca_i^{2+} is 200 μM, 200 mM Na_i^+ are required [19,20]. Under high Na_i^+ conditions and with no external Na^+, Ca_i^{2+} ions activate in a saturable fashion (no Ca^{2+} exit through the Na^+/Ca^{2+} exchanger) the Na_i^+/Ca_o^{2+} ex-

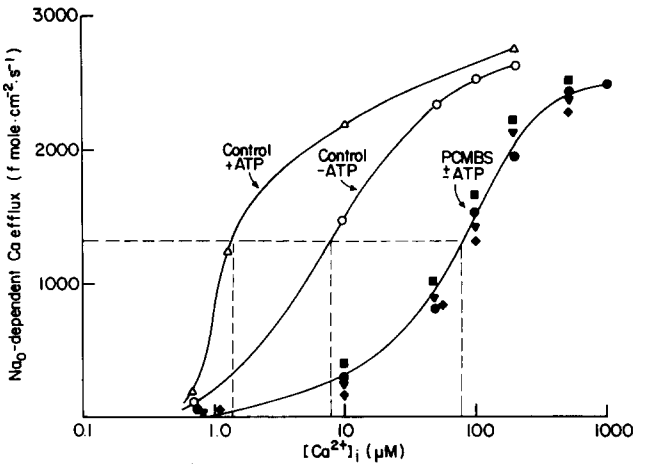

Fig. 4. Activation curves of the forward Na_o^+/Ca_i^{2+} exchange in control axons (with and without MgATP) and in axons treated with internal PCMBS (with and without MgATP). Ordinate: steady-state Na_o^+-dependent Ca^{2+} efflux in fmol/cm^2 per s. Abscissa: $[Ca^{2+}]_i$ in the dialysis solution in μM. ●,■, axons treated with PCMBS (with MgATP). ◆, ▼, axons treted with PCMBS (without MgATP). ○, control axons without MgATP. △, control axons with 2 mM MgATP. Notice that PCMBS only affects the affinity for Ca_i^{2+} ions and not the V_{max} of the exchanger. The mean temperature in these experiments was 17.5°C.

change through the Na^+/Ca^{2+} exchanger; this stimulation follows the binding of Ca_i^{2+} ions to the regulatory site [16]. Fig. 5 shows the Ca_i^{2+} activation of the Na_i^+-dependent Ca^{2+} influx under conditions in which all Ca_o^{2+}/Ca_i^{2+} exchange is absent. The external medium contained Li ions (200 mM) to activate the external monovalent cation site. In the first part of the experiment the axon was dialyzed with 100 mM Na_i^+ no Ca^{2+} (3 mM EGTA and no added calcium; $[Ca^{2+}]$

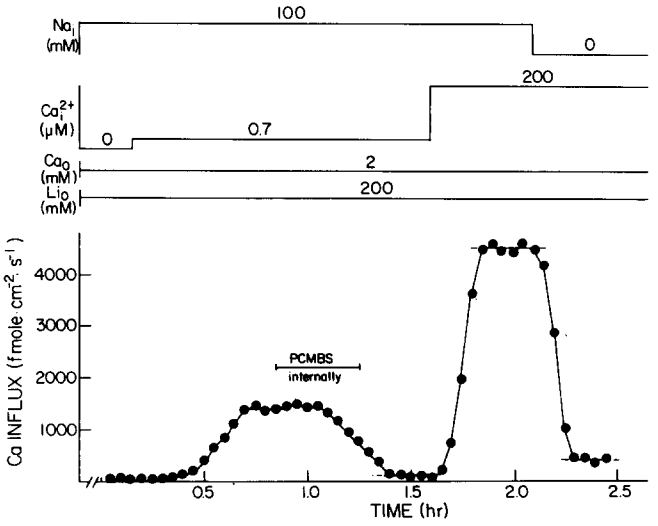

Fig. 5. The effect of internal PCMBS on the Na_i^+-dependent Ca^{2+} influx at 0.7 and 200 μM Ca_i^{2+}. Ordinate: Ca^{2+} influx in fmol/cm^2 per s. Abscissa: time in hours. Notice that PCMBS reduces the influx of Ca^{2+} to a very low value at 0.7 μM Ca_i^{2+}. At 200 μM Ca_i^{2+} the Ca^{2+} influx recovers to a normal value. Axon diameter 600 μm. Temperature: 18°C.

$< 10^{-9}$ M) and no MgATP. Under these conditions Ca^{2+} influx is very low. Increasing $[Ca^{2+}]_i$ to 0.7 μM, a value close to the $K_{0.5}$ of the regulatory site caused a significant increase in Ca^{2+} influx to a steady value of about 1400 fmol/cm^2 per s. Addition of 0.25 mM PCMBS inhibited most of the Na_i^+-dependent Ca^{2+} influx. Increasing the $[Ca^{2+}]_i$ from 0.7 to 200 μM augmented the Na_i^+-dependent Ca^{2+} influx to values similar to that seen in untreated nerve fibers, thus indicating that PCMBS has no effect on the V_{max} of the reverse exchange. Finally, removal of Na_i^+ brought the influx of Ca^{2+} to about 400 fmol/cm^2 per s which corresponds to the magnitude of the Ca_o^{2+}/Ca_i^{2+} exchange component.

Fig. 6 summarizes the results on the Ca_i^{2+} activation of the Na_i^+-dependent Ca^{2+} influx in the presence of 200 mM Na_i^+ and 2 mM MgATP. It can be observed that the $K_{0.5}$ for Ca^{2+} of the Ca_i^{2+} regulatory site shifted from 0.63 μM to about 60 μM in the presence of PCMBS.

Sodium and calcium interactions at the intracellular transporting sites

In squid axons, internal Na^+ and Ca^{2+} interactions on the transport sites can be adequately explained by a competition model in which three Na^+ ions and one calcium can attach to the carrier molecule. The $K_{0.5}$ for Na_i^+ inhibition of Na_o^+- and Ca_o^{2+}-dependent Ca^{2+} efflux is 25 mM and 54 mM at 0.7 and 200 μM Ca_i^{2+}, respectively (Ref. 20; see also Fig. 11).

From the experiments described so far it is clear that PCMBS affects essentially the Ca_i^{2+} regulatory

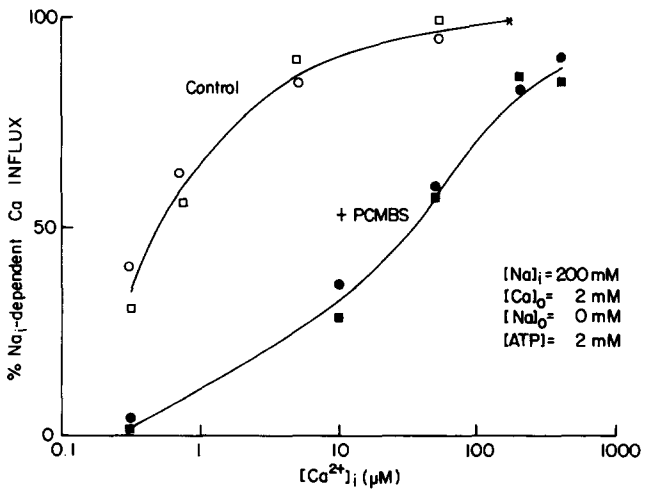

Fig. 6. The effect of internal PCMBS on the apparent affinity of the Ca_i^{2+} regulatory site of the Na^+/Ca^{2+} exchanger. The activation of the Na_i^+-dependent Ca^{2+} influx (reverse Na^+/Ca^{2+} exchange) by Ca_i^{2+} ions was carrier out in the presence of 200 mM Na_i^+ and 2 mM MgATP. The external Ca_o^{2+} was 2 mM. ●, ■, +PCMBS. ○, □, −PCMBS. The apparent K_{Ca} is 0.63 μM in control axons and 60 μM in PCMBS-treated axons.

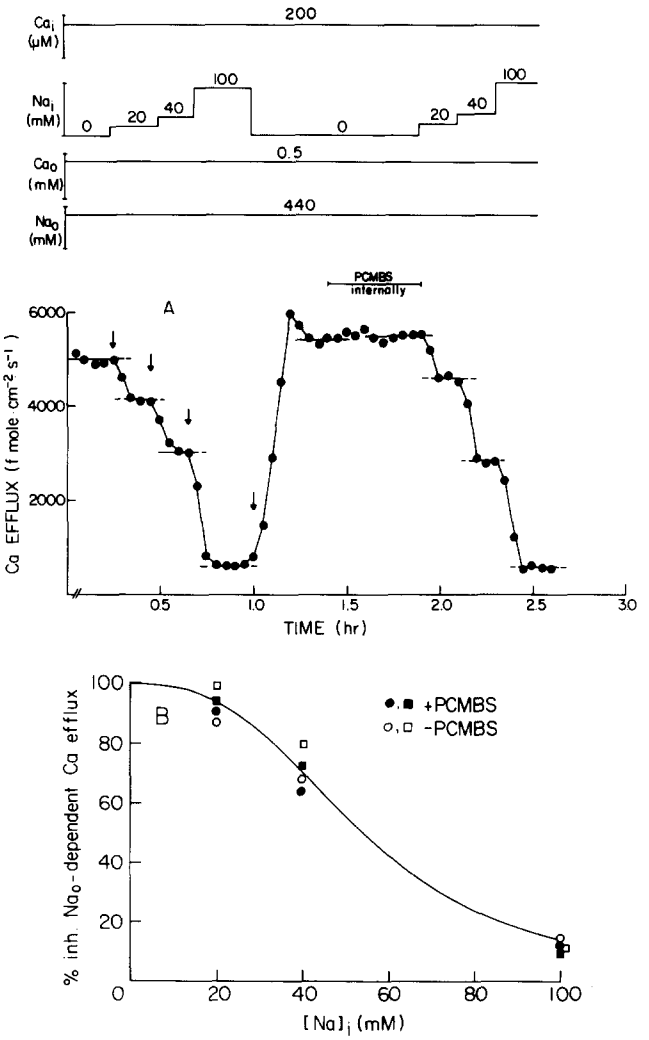

Fig. 7. (A) The effect of internal PCMBS on the inhibition of the Na_o^+-dependent Ca^{2+} efflux by Na_i^+ at a saturating Ca_i^{2+} concentration. Ordinate Ca^{2+} efflux in fmol/cm^2 per s. Abscissa: time in hours. Notice that the inhibition of Ca^{2+} efflux by internal Na^+ is similar before and after the PCMBS treatment. Axon diameter 550 μm. Temperature 16.8°C. (B) Relative inhibition of the Na_o^+-dependent Ca^{2+} efflux by Na_i^+ in four different axons untreated and treated with PCMBS. The line through the points were fitted with the equation derived from the model shown in Fig. 11. The dissociation constants for Na_i^+ were: $K_1 = 265$ mM; $K_2 = 36$ mM and $K_3 = 2$ mM [20]. The dissociation constant for Ca_i^{2+} was 25 μM [24].

site of the Na^+/Ca^{2+} exchanger. Therefore, an important point to explored is whether PCMBS also affects Na_i^+-Ca_i^{2+} interactions at the transport sites. Fig. 7A describes the inhibition of the Na_o^+-dependent Ca^{2+} efflux by internal Na^+ at saturating Ca_i^{2+} (200 μM) in the absence and presence of PCMBS. It is clear that the inhibition by Na_i^+ is the same with or without the chemical modifier. The data points in Fig. 7B are taken from experiments similar to that described in Fig. 7A. The line through the points corresponds to a model of 3 Na^+ and 1 Ca^{2+} binding site where the binding of one Na^+ is sufficient to displace Ca^{2+} (see Fig. 11 and

Discussion). The dissociation constants for Na_i^+ and Ca_i^{2+} were obtained from previously reported data by simultaneously fitting Na_i^+ inhibition at two Ca_i^{2+} concentrations (0.7 and 200 μM, [20]).

If the inhibition of Ca^{2+} efflux and the stimulation of Ca^{2+} influx by Na_i^+ takes place on the same site, PCMBS should not affect the stimulation of Ca^{2+} influx by Na_i^+ at saturating Ca_i^{2+} concentrations. These expectations were borne out. Fig. 8 shows that the activation of the Ca^{2+} influx by Na_i^+ in four different axons dialyzed with a saturating Ca_i^{2+} concentration (500 μM) is the same with or without PCMBS. The line through the data points correspond to the theoretical equation used in Fig. 7B employing the same Na_i^+ and Ca_i^{2+} dissociation constants.

Internal Na^+ and the Ca_i^{2+} regulatory site

For a system where the Ca_i^{2+} regulatory and transport sites are different entities, one would expect a different sensitivity of each site towards inhibition by internal Na^+ ions. We have already shown (Fig. 7A and B) that Na_i^+ competitively inhibits Ca^{2+} efflux. In this section we attempted to study the possible interactions of intracellular Na^+ with the Ca_i^{2+} regulatory site. In order to do so, we have chosen the conditions in which only the Na_i^+-dependent Ca^{2+} influx is in operation (no exit of Ca^{2+} through the Na^+/Ca^{2+} exchange); therefore, any effect of Na_i^+ will reflect an interaction that takes place at the Ca_i^{2+} regulatory site. If cytoplasmic Na^+ competed with Ca_i^{2+}, increasing Na^+ concentration should displace Ca^{2+} ions from the regulatory site and therefore inhibit Na_i^+/Ca_o^{2+} exchange. Fig. 9A describes an experiment in which internal Na^+ was varied at a constant submicromolar

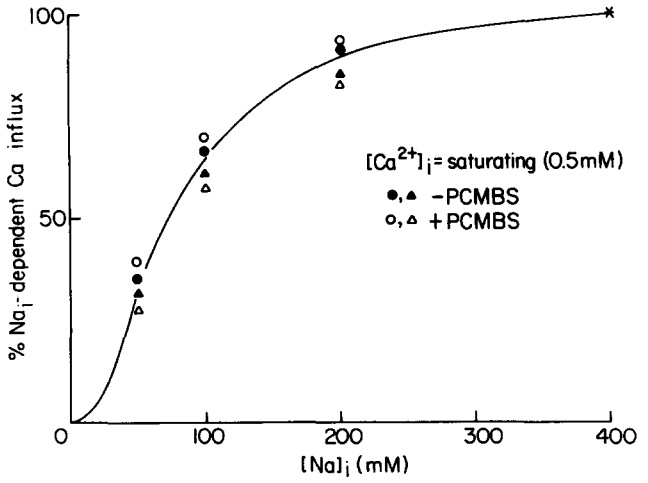

Fig. 8. Activation of the reverse Na_i^+/Ca_o^{2+} exchange by Na_i^+ at a saturating $[Ca^{2+}]_i$ in the presence and absence of PCMBS. Notice the absence of effect of PCMBS on the binding and transport of Na^+ ions through the exchanger. The line through the points were fitted to the theoretical equation depicted in the model of Fig. 11.

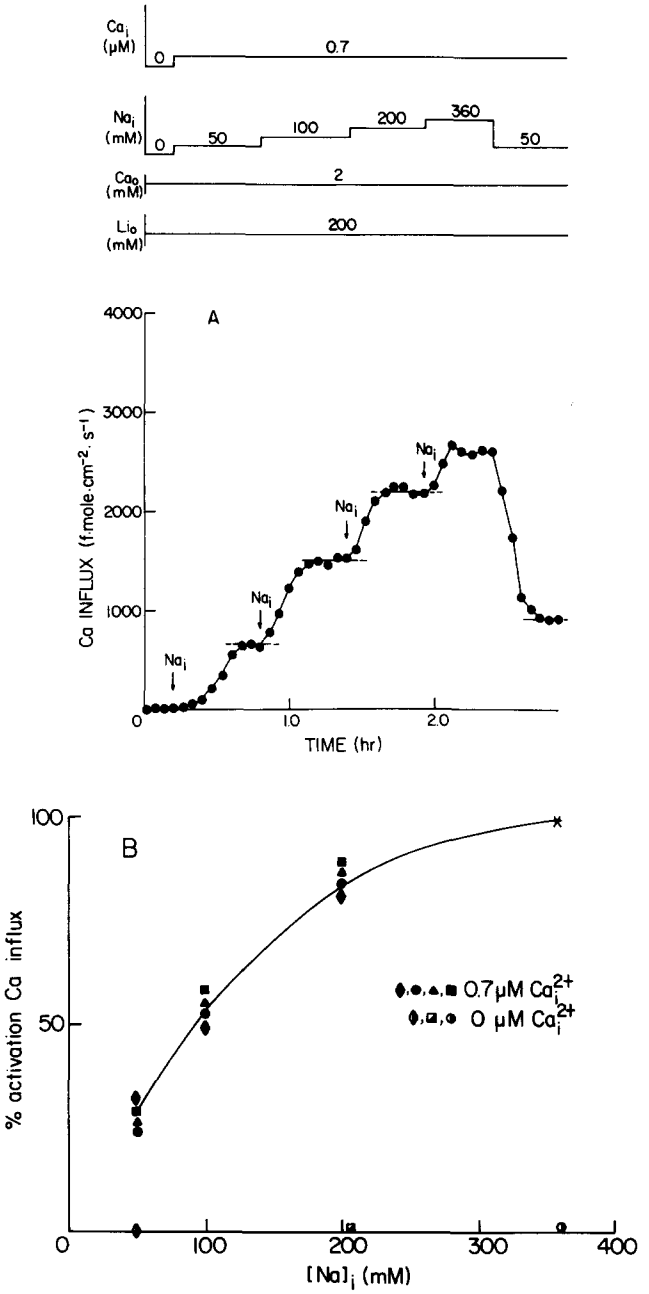

Fig. 9. (A) The effect of step increases in the $[Na^+]_i$ on the Ca^{2+} influx in an axon dialyzed with a submicromolar $[Ca^{2+}]_i$. Ordinate: Ca^{2+} influx in fmol/cm^2 per s. Abscissa: time in hours. Notice at the beginning of the experiment that in the absence of internal Na_i^+ and Ca_i^{2+} Ca^{2+} influx is closed to background. Axon diameter 600 μm. Temperature 17°C. (B) Activation of Na_i^+-dependent Ca^{2+} influx by Na_i^+ ions at a submicromolar $[Ca^{2+}]_i$ (0.7 μM). Notice that Na_i^+ always activates Ca^{2+} influx even at extreme high Na_i^+ concentrations. The experimental points on the abscissa represent three different experiments in which the effect of Na_i^+ was tested in the complete absence of Ca^{2+} ions (3 mM EGTA no added Ca^{2+}).

Ca_i^{2+} concentration of 0.7 μM. This allowed us to used Na_i^+ concentrations as low as 50 mM without any carrier mediated Ca^{2+} efflux [20]. The results of several experiments of these type are shown in Fig. 9B.

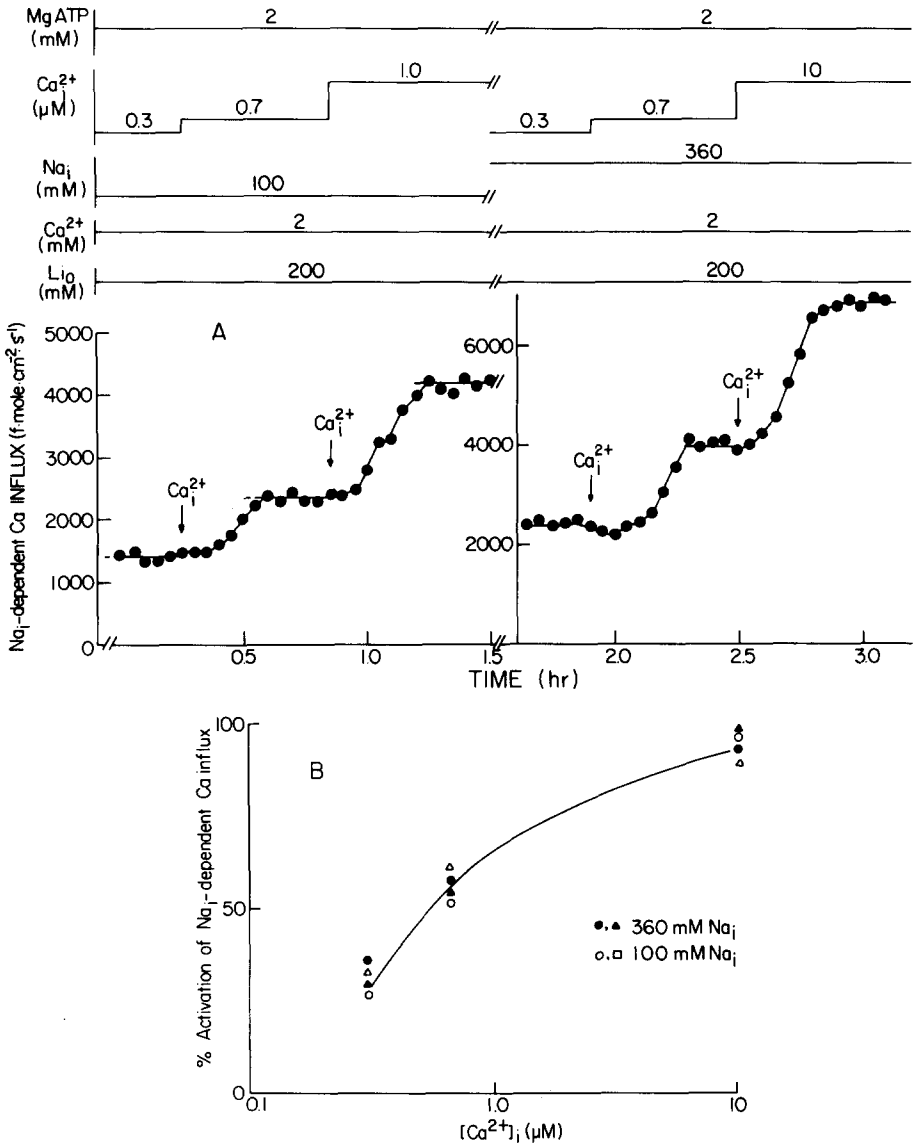

Fig. 10. (A) The effect of step increases in $[Ca^{2+}]_i$ (0.3, 0.7 and 10 μM) on Ca^{2+} influx at two different Na_i^+ concentrations (100 and 360 mM) in an axon dialyzed with 2 mM MgATP. Ordinate: Na_i^+-dependent Ca^{2+} influx in fmol/cm^2 per s. Abscissa: time in hours. Notice the change in the ordinate scale upon changing Na_i^+ from 100 to 360 mM. (B) Activation of Na_i^+-dependent Ca^{2+} influx by Ca_i^{2+} ions (0.3, 0.7, 10 μM) at two different $[Na^+]_i$ (100 and 360 mM) in axons dialyzed with 2 mM MgATP. The $K_{1/2}$ for Ca_i^{2+} is close to 0.5 μM. Notice that even at this submicromolar Ca_i^{2+} concentrations Na_i^+ ions do not displace Ca_i^{2+} from the regulatory site.

The relevant feature is the fact that Na_i^+ at high concentrations not only do not inhibit, but monotonically stimulate Ca^{2+} influx ($K_{0.5}$ of about 100 mM). Fig. 9B also shows that Ca_i^{2+} behaves as an essential activator of the exchanger since under these conditions no Na_i^+-dependent Ca^{2+} influx exist in the absence of internal Ca^{2+} ions.

We also explored the Ca_i^{2+} activation of the Na_i^+-dependent Ca^{2+} influx under conditions where there was no Ca^{2+} efflux. Fig. 10A describes one experiment and Fig. 10B summarizes several, where Ca_i^{2+} was varied between 0.3 and 10 μM at two fixed and different Na_i^+ concentrations (100 and 360 mM). It is clear from the figures that the activation of Na_i^+-dependent Ca^{2+} influx by the binding of Ca^{2+} ions to the intracellular regulatory site takes place with the same apparent affinity at the two Na_i^+ concentrations used. The most reasonable explanation for the results of Figs. 9 and 10 is that internal sodium ions do not have access to the intracellular Ca^{2+} regulatory site.

Discussion

In this work we have examined the functional changes produce in the squid axon Na^+/Ca^{2+} exchanger by the relatively impermeant *p*-chloromercuryphenylsulfonic acid (PCMBS), a specific sulfhydryl group blocker [21]. Mercurial compounds are highly specific sulfhydryl reagents. and among them, the organomercurial *p*-chloromercurybenzoate has

been widely used. PCMBS binds to sulfhydryl groups with exceedingly high affinity with a dissociation constant of about 10^{-20} M [22]. Therefore, a reasonable expectation is that this compound will bind only to those sulfur-containing amino acids (cysteine and methionine). The amino acid sequence of the squid Na^+/Ca^{2+} exchanger is not yet known. However, if a substantial homology exist between the cardiac and squid Na^+/Ca^{2+} exchanger, one can expect the presence of cysteine groups in the highly acidic region of the cytoplasmic (f-loop) squid antiporter. This domain which has been suggested to be involved in cation binding [6] has two cystein residues groups (one surrounded by acidic aminoacids (position 762) and the other very near by (position 770)) [6]. Nevertheless, no experimental data exist that this corresponds to the Ca_i^{2+} binding site.

The fact that externally applied PCMBS has no effect on the Na_o^+- and Ca_o^{2+} dependent Ca^{2+} efflux as well as on the external monovalent activatory cation site could mean either that cystein residues are not part of the site structure or that they are not accessible to the chemical blocker.

The intracellular effect of PCMBS is very marked and consist in a tremendous decrease in the MgATP induce affinity change of the Ca_i^{2+} regulatory site and the disapperance of the MgATP effect. By linking these findings we could conclude that the MgATP effect take place primarily through a modification of the Ca_i^{2+} regulatory site. In fact, we have already shown that MgATP largely increases the affinity of the Ca_i^{2+} regulatory site for calcium ions [19]. A crucial feature of the effect is that with PCMBS the ability of the Na^+/Ca^{2+} exchanger to translocate ions across the membrane remains intact since the maximal rate of exchange is not affected. This is strong evidence against an unspecific pharmacological effect of the sulfhydryl blocker.

There are three pieces of evidence that support the notion of a structural difference between the Ca_i^{2+} regulatory and the Na_i^+ transport site: (1) PCMBS at the concentration that modifies the affinity of the Ca_i^{2+} regulatory site has no effect either on the inhibition of Ca^{2+} efflux or activation of Ca^{2+} influx by Na_i^+; (2) at the same concentration of Ca_i^{2+} at which Na_i^+ strongly inhibits the efflux of Ca^{2+}, it stimulates the influx of Ca^{2+}. (3) At Na_i^+ concentrations which completely block Ca^{2+} efflux, Ca^{2+} ions still bind to the intracellular regulatory site with an apparent affinity independent of the Na_i^+ concentration. The present results are consistent with studies in cardiac Na^+/Ca^{2+} exchanger in which proteolysis of the exchanger at the cytoplasmic surface abolished Ca_i^{2+} regulation without inhibiting Na^+/Ca^{2+} exchange [23].

The unexpected finding that Na_i^+ did not affect the Ca_i^{2+} regulatory site, in contrast to the strong interaction with the Ca^{2+} transport site, might be explained with a simple kinetic scheme model shown in Fig. 11. According to this model Ca_i^{2+} regulatory and Ca^{2+} and Na^+ transport sites are different and in order to be transported Na^+ and Ca^{2+} ions compete for the same carrier species. PCMBS only modifies, while Na^+ ions do not interact with, the Ca_i^{2+} regulatory site. The function of the Ca_i^{2+} regulatory site is to allow the carrier to bind Na^+ or Ca^{2+} ions for transport. In addition, the binding of one Na^+ or 1 Ca^{2+} ion to the transport site is sufficient to displace the other ion from it. Therefore, we can treat kinetic data on Na_i^+ and Ca_i^{2+} interactions ignoring the Ca_i^{2+} regulatory site. We have done this with early data [19,20] obtaining the following values for the dissociation constant: $K_1 = 265 \pm 66$ mM; $K_2 = 36 \pm 9$ mM and $K_3 = 1.9 \pm 2$ mM. The dissociation constant for the Ca_i^{2+} transport site was experimental determined to be 25 μM in the absence of ATP [24]. Figs. 7B and 8 shows that the model and the constants obtained adequately fit the data reported here.

The main conclusion from the differential effects of PCMBS and Na_i^+ is that Ca_i^{2+} regulatory site most be different from the Na_i^+ transport site. Regarding the identity of the loci for Ca_i^{2+} regulation and transport

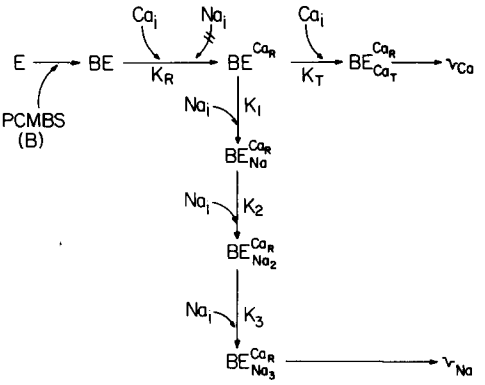

Fig. 11. Kinetic model of Na^+ and Ca^{2+} interaction at the intracellular sites of the Na^+/Ca^{2+} exchange carrier (E). The figure shows the case where PCMBS (B from blocker) binds to the carrier (BE) and modifies the interaction of Ca^{2+}(i) with the intracellular Ca^{2+} regulatory site. Other Na^+ and Ca^{2+} interactions with the carrier remain unaltered (this work). The following additional assumptions were made: (i) binding of Ca_i^{2+} to the regulatory site is essential to allow the interactions of Na_i^+ ions (three) and Ca_i^{2+} ions (one) with their transporting sites; (ii) Na^+ ions do not have access to the Ca_i^{2+} regulatory site; (iii) binding of one Na^+ ion is enough to displace Ca^{2+} from its transporting site; (iv) rapid equilibrium. The resulting equations, ignoring the Ca_i^{2+} interactions with the regulatory site, are: for Ca^{2+} efflux (via Ca_i^{2+}-Na_o^+ or Ca_o^{2+}/Ca_i^{2+} exchanges): $v/V = [Ca]/((K_c(1 + [Na]/K_1 + [Na]^2/K_1K_2 + [Na]^3/K_1K_2K_3)) + [Ca])$. For Na^+ efflux or Ca^{2+} influx (via Na_i^+/Ca_o^{2+} reversal exchange): $v/V = [Na]^3/(K_1K_2K_3(1 + [Ca]/K_c) + K_2K_3[Na]^2 + [Na]^3)$. Taking K_c equal to 25 μM [24] the fitting of old data [20] gave the following values for the Na^+ dissociation constants: K_1, 265 ± 66 mM; K_2, 36 ± 9 mM; K_3, 1.9 ± 2 mM.

we face the following options: (i) if the Ca^{2+} regulatory and the Ca^{2+} transport sites are different then Na^+ and Ca^{2+} interactions reflect a competition for the same carrier conformation, which may or may not indicate identical sites; (ii) if the Ca^{2+} regulatory and transport sites are the same then the place at which Na_i^+ binds in order to be transported most be on a different structure.

Acknowledgements

We wish to thank Ted Begenisch and Sherrill Spires for their helpful guidance on the used of sulfhydryl reagents. We wish to thank the director and staff of the Marine Biological Laboratory and the staff of the Centro de Biofisica y Bioquimica in charge of the squid supply. We wish to thank Dhuwya Otero for the drawings and Lilian Palacios for the secretarial help. This work was supported by CONICIT (S1–2231), CONICET and National Science Foundation (BNS 9120177).

References

1 Blaustein, M.P., DiPolo, R. and Reeves, J. (1991) Ann. N.Y. Acad. Sci. 639, 253–667.
2 Cervetto, L., Lagnado, R., Perry, D., Robinson, W. and McNaughton, P.A. (1989) Nature 337, 740–743.
3 Scknetkamp, P.P.M., Basu, D.K. and Szerencsei, R.T. (1989) Am. J. Physiol. 257, C153–C157.
4 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3184–3190.
5 Condrescu, M., Rojas, H., Gerardi, A., DiPolo, R. and Beaugé, L. (1990) Biochim. Biophys. Acta 1024, 198–202.
6 Nicoll, D., Longoni, S. and Philipson, K. (1990) Science 250, 562–565.
7 Reilander, H., Achilles, A., Friedel, U., Maul, G., Lottspeich, F. and Cook, J.N. (1992) EMBO J. 11, 1689–1695.
8 DiPolo, R. and Beaugé, L. (1992) Biophys. J. 61, A388.
9 DiPolo, R., Bezanilla, F., Caputo, C. and Rojas, H. (1985) J. Gen. Physiol. 86, 457–478.
10 DiPolo, R. (1979) J. Gen. Physiol. 73, 91–113.
11 DiPolo, R., Requena, J., Brinley, F.J., Mullins, L.J., Scarpa, T. and Tieffert, T. (1976) J. Gen. Physiol. 67, 433–467.
12 DiPolo, R. and Beaugé, L. (1984) J. Gen. Physiol. 84, 895–914.
13 Ashkey, C.C. and Ellory, J.C. (1972) J. Physiol. 226, 653–674.
14 Ashley, C.C., Ellory, J.C. and Hainaut, K. (1974) J. Physiol 242, 255–272.
15 Blaustein, M. (1977) Biophys. J. 20, 70–111.
16 DiPolo, R. and Beaugé, L. (1986) Biophys. Biochim. Acta 854, 298–306.
17 Kimura, J., Miyamae, S. and Noma, A. (1987) J. Physiol. 384, 199–222.
18 Rasgado Flores, H., Santiago, E.M. and Blaustein, M.P. (1989) J. Gen. Physiol. 93, 1219–1241
19 DiPolo, R. and Beaugé, L. (1991) Ann. N.Y. Acad. Sci. 639, 110–111.
20 Beaugé, L. and DiPolo, R. (1991) Ann. N.Y. Acad. Sci. 639, 157–155.
21 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day, San Francisco.
22 Wong, S.S. (1991) Chemistry of Protein Conjugation and Cross-Linking, CRC Press, Boca Raton.
23 Hilgemann, D.W. (1990) Nature 344, 242–245.
24 DiPolo, R. and Beaugé, L. (1990) J. Gen. Physiol. 95, 819–835.