Biochimica et Biophysica Acta, 946 (1988) 289–298
Elsevier

BBA 74239

Na^+–Ca^{2+} exchange in squid optic nerve membrane vesicles is activated by internal calcium

Madalina Condrescu, Andrés Gerardi and Reinaldo DiPolo

Instituto Venezolano de Investigaciones Científicas (IVIC), Centro de Biofísica y Bioquímica, Caracas (Venezuela)

(Received 11 July 1988)

Key words: Sodium–calcium ion exchange; Membrane vesicle; Calcium ion, intracellular; (Squid optic nerve)

The role of intracellular Ca^{2+} as essential activator of the Na^+–Ca^{2+} exchange carrier was explored in membrane vesicles containing 67% right-side-out and 10% inside-out vesicles, isolated from squid optic nerves. Vesicles containing 100 μM free calcium exhibited a 2-fold increase in the initial rate of Na_i^+-dependent Ca^{2+} uptake as compared with vesicles where intravesicular calcium was chelated by 2 mM EGTA or 10 mM HEDTA. The activatory effect exerted by intravesicular Ca^{2+} on the reverse mode of Na^+–Ca^{2+} exchange (i.e. Na_i^+–Ca_o^{2+} exchange) is saturated at about 100 μM Ca_i^{2+} and displays an apparent $K_{1/2}$ of 12 μM. Intravesicular Ca^{2+} produced activation of Na_i^+–Ca_o^{2+} exchange activity rather than an increase in Ca^{2+} uptake due to Ca^{2+}–Ca^{2+} exchange. The presence of Ca_i^{2+} was essential for the Na_i^+-dependent Na^+ influx, a partial reaction of the Na^+–Ca^{2+} exchanger. In fact, the Na^+ influx levels in vesicles loaded with 2 mM EGTA were close to those expected from diffusional leak while in vesicles containing Ca_i^{2+} an additional Na^+–Na^+ exchange was measured. The results suggest that in nerve membrane vesicles Ca^{2+} at the inner aspect of the membrane acts as an activator of the Na^+–Ca^{2+} exchange system.

Introduction

The Na^+–Ca^{2+} exchange mechanism is a carrier mediated transport system in which the transmembrane Na^+ electrochemical gradient is used to fuel net Ca^{2+} movements across the plasma membrane [1–4]. Recent experiments in dialyzed squid axons have shown that Ca^{2+} entry through the exchange mechanism (reverse Na_i^+–Ca_o^{2+} exchange) not only requires the presence of sodium and calcium ions inside and outside the cell, but also the presence of micromolar amounts of intracellular free calcium [5]. This observation is in line with the identification in Na^+-loaded myocytes [6] and in squid axons [7] of an outwardly directed ionic current caused by the electrogenic Na^+–Ca^{2+} exchange which did not develop in the absence of intracellular Ca^{2+} ions. Further evidence obtained in dialyzed squid axons on the role of Ca_i^{2+} as an essential activator of the exchanger indicates that intracellular Ca^{2+} is not only required for the reverse reaction (Ca_o^{2+}-dependent Na^+ efflux) but also for the Na^+–Na^+ exchange mode (Na_o^+-dependent Na^+ efflux) [8].

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetracetic acid, HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; PMSF, phenylmethylsulfonyl fluoride; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Correspondence: M. Condrescu, Instituto Venezolano de Investigaciones Científicas (IVIC), Centro de Biofísica y Bioquímica, Apartado 21827, Caracas 1020A, Venezuela.

Although a great deal of information on the operation of the Na^+–Ca^{2+} exchange has been provided by work with cardiac sarcolemmal vesicles [9–11] it has become apparent that the exchanger of intact cells exhibits a number of features which do not seem to persist in membrane preparations. Thus, the vesicle exchanger appears to be symmetrical, displaying a similar affinity for Ca^{2+} on both sides of the membrane [12]. In addition, intravesicular Ca^{2+} between 0.1 and 0.5 mM has been recently shown to elicit stimulation of the exchanger in heart sarcolemmal vesicles [26]. However, this differs by about one order of magnitude from the concentrations of Ca^{2+} that stimulate Na_i^+-dependent Ca^{2+} influx in living cells in the absence of ATP [5,6,7,15]. Furthermore, no evidence has been reported so far for a regulatory effect of micromolar amounts of Ca_i^{2+} on the exchange rate.

One problem inherent in vesicle exchanger studies is the unhomogeneity of the preparations with respect to sidedness. In addition, due to the reduced vesicular volume, the intravesicular concentration of the transported cation (Ca^{2+}, Na^+) very rapidly attains values in the millimolar range. The present study was carried out to investigate whether Ca_i^{2+} is an essential activator of Na^+–Ca^{2+} exchange in a preparation of squid nerve membrane vesicles mainly consisting of right-side-out vesicles [13,14]. To overcome the difficulty of controlling intravesicular Ca^{2+} concentration we prepared vesicles containing EGTA or HEDTA as well as known concentrations of free calcium (as Ca · EGTA or Ca · HEDTA) in their interior.

Evidence is provided that the presence of micromolar concentrations of intravesicular Ca^{2+} stimulates Ca^{2+} uptake by the vesicles, an effect that is due to activation of Na_i^+–Ca_o^{2+} exchange activity and not to an increase in Ca^{2+}–Ca^{2+} exchange. In addition, micromolar concentrations of intravesicular Ca^{2+} are also needed to observe Na^+–Na^+ exchange in these vesicles. These observations support the view that internal Ca^{2+} behaves as an activator of the Na^+–Ca^{2+} exchange carrier as is the case in living cells (Barnacle muscles [15], cardiac cells [6] and squid axons [3,5,8]). Some of these results have been presented previously in abstract form [16].

Materials and Methods

Preparation of nerve membrane

Nerve membrane vesicles were isolated from optic nerves of the tropical squid *Sepiotheutis sepioidea* using the method described previously [14]. Optic nerves were homogenized (Teflon-glass homogenizer followed by Dounce-glass homogenizer) in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.3) containing 1 mM PMSF. The suspension was centrifuged 10 min at $2000 \times g$ in a Sorvall refrigerated centrifuge, the supernatant was separated and the pellet was washed twice. Combined supernatants were then centrifuged at $12000 \times g$, the resulting supernatant was separated and the pellet was washed twice. The microsomal fraction was obtained by centrifugation of the combined supernatants proceeding from the $12000 \times g$ step, for 45 min at $100000 \times g$. This pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.3) and layered on top of 1.12 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.3). The gradient was spun down for 90 min at $65000 \times g$ in a Beckman SW 25.2 rotor. The white band centered at the interphase was collected, diluted four times with 10 mM Tris (pH 7.3) and sedimented at $100000 \times g$. At the end of the isolation procedure the vesicles were suspended at a concentration of 5 mg protein/ml in 100 mM NaCl, 100 mM KCl and 100 mM Na-Mops (pH 7.3) (except ions and the presence of calcium buffers are otherwise indicated in text and figure legends) frozen in liquid N_2 and stored frozen at $-70\,°C$. The entire procedure was performed at 4 °C. The nerve membrane vesicles prepared as described above exhibited a sodium deoxycholate-activated, ouabain-inhibitable Na^+/K^+-ATPase activity of 90.3 ± 5.2 μmol P_i/mg protein per h ($n = 10$). In this preparation approx. 67% of the vesicles are oriented right-side-out and 10% inside-out while the resting 23% are leaky vesicles [13].

Measurement of intravesicular EGTA concentration

In order to investigate the modulation of Na^+–Ca^{2+} exchange activity by Ca^{2+} we intended in this study to maintain intravesicular free calcium at known concentrations by the use of EGTA or HEDTA. However, membranes are not permeable to EGTA and HEDTA and a freeze-thaw cycle

TABLE I

EGTA CONCENTRATION IN VESICLES ORIGINALLY RESUSPENDED IN A MEDIUM CONTAINING 2 mM ^{14}C-EGTA (EGTA-CONTAINING VESICLES) OR IN A MEDIUM WITHOUT EGTA AND FURTHER INCUBATED WITH 2 mM ^{14}C-EGTA (CONTROL VESICLES)

Membranes were originally resuspended (at approx. 5 mg protein/ml) in (mM): 100 NaCl, 100 KCl, 100 Na-Mops, 2 $CaCl_2$ and 2 ^{14}C-EGTA (EGTA-containing vesicles) or in (mM): 100 NaCl, 100 KCl, 100 Na-Mops (Control vesicles), and quickly frozen in liquid N_2. 100-μl aliquots of EGTA-containing and control vesicles were thawed at room temperature, centrifuged 45 min at 100000 × g (at 4°C), pellets were resuspended in 100 μl of the ^{14}C-EGTA containing medium and incubated 5 min at 4°C. At the end of this period 12 ml of (mM); 100 NaCl, 100 KCl, 100 Na-Mops and 10 EGTA were added and the tubes were centrifuged 45 min at 100000 × g. The pellets were resuspended in 0.5 ml 3% sodium dodecyl sulfate (SDS) and 0.4 ml aliquots were counted. Corrections were made for the 23% population of leaky vesicles. Results expressed as means ± S.E.

Preparation	Intravesicular EGTA concentration (mM)
EGTA-containing vesicles	2.09 ± 0.05 (n = 6)
Control vesicles	0.08 ± 0.04 (n = 4)

has been used to introduce these compounds within vesicles. This procedure was previously utilized to render cardiac sarcolemma vesicles permeable to ouabain [17]. To ensure that using this treatment the intravesicular calcium buffer concentration was identical to that in the resuspension medium, the experiment detailed in Table I was carried out using ^{14}C-EGTA. The results show that vesicles originally resuspended in a medium comprising 2 mM ^{14}C-EGTA really have in their interior this concentration of EGTA. In contrast, control vesicles, which were resuspended in a medium without EGTA and were incubated in the presence of ^{14}C-EGTA after being thawed, only have a negligible quantity of EGTA. Moreover, when the ^{14}C-EGTA-containing vesicles were treated with 0.2% deoxycholate (DOC) and washed, they released intravesicular EGTA, remaining 0.11 mM ^{14}C-EGTA attached to the membrane (not shown). These results could be extrapolated to the case of HEDTA.

Measurement of Na_i^+-dependent Ca_o^{2+} uptake

(a) Effect of K^+ on Na_i^+–Ca_o^{2+} exchange

Na_i^+-dependent $^{45}Ca_o^{2+}$ uptake was measured as previously described [14]. The nerve membrane vesicles were originally suspended in 100 mM NaCl, 100 mM Na-Mops (pH 7.3) and variable concentrations (0–150 mM) of KCl. To maintain osmolarity and ionic strength constant, different concentrations (150–0 mM) of *N*-methylglucamine-Cl were added. The vesicles were thawed at room temperature and preincubated at 25°C for 5 min. The Na_i^+-dependent $^{45}Ca_o^{2+}$ uptake was initiated by adding approx. 20 μg of Na^+-loaded vesicle protein to the uptake mixture in a final volume of 250 μl containing 100 μM $^{45}CaCl_2$ (0.05 μCi/nmol), 100 mM *N*-methylglucamine-Cl, 100 mM *N*-methylglucamine-Mops (pH 7.3), variable concentrations of KCl (0–150 mM) and *N*-methylglucamine-Cl (150–0 mM), in the presence of 0.5 μM valinomycin. The mixture was then incubated at 25°C and Ca^{2+} uptake was arrested after 10 s by adding EGTA (2 mM, final concentration). Aliquots of 100 μl were filtered through 0.45 μm Millipore filters under suction. Filters were washed twice with a 5 ml aliquot of 200 mM KCl, 100 mM K-Mops (pH 7.3) to displace externally bound Ca^{2+}. The filters were then dried and the amount of $^{45}Ca^{2+}$ taken up by the vesicles was determined by counting them in a liquid scintillation counter. In all experiments vesicles were used in parallel which were suspended in 100 mM KCl and 100 mM K-Mops instead of NaCl and Na-Mops (pH 7.3) to estimate the passive diffusion and unspecific Ca^{2+} binding.

(b) Activatory effect of intravesicular free Ca^{2+} on Na_i^+–Ca_o^{2+} exchange, i.e. the reverse mode of Na^+–Ca^{2+} exchange

In order to estimate the effect of intravesicular Ca^{2+} on Na_i^+–Ca_o^{2+} exchange, the nerve membrane vesicles were suspended in: 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3), and either 2 mM EGTA or 10 mM HEDTA (control vesicles) or in: 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3), and either 2 mM EGTA or 10 mM HEDTA plus enough $CaCl_2$ to obtain variable concentrations of free Ca^{2+} (experimental

vesicles). The nominal ionized calcium concentrations for high ionic strength conditions were based on a Ca · EGTA dissociation constant of 0.15 μM and a Ca · HEDTA dissociation constant of 5 μM [18]. The Na_i-dependent $^{45}Ca_o^{2+}$ uptake was initiated by diluting Na^+-loaded vesicles (20 μg of protein) in a final volume of 250 μl containing enough $^{45}CaCl_2$ (0.05 μCi/nmol) as to obtain 100 μM Ca^{2+} in the extravesicular medium and, 100 mM *N*-methylglucamine-Cl, 100 mM KCl and 100 nM *N*-methylglucamine-Mops (pH 7.3) in the presence of 0.5 μM valinomycin. The $CaCl_2$ concentration of the dilution media was adjusted taking into account the presence of free EGTA (or HEDTA) in the control vesicles. This mixture was incubated at 25°C for different time intervals (2.5–30 s), then the reaction was terminated, aliquots filtered and washed as described. The quantity of $^{45}Ca^{2+}$ remaining in the filtered vesicles was determined in a liquid scintillation counter.

Measurement of Ca_i^{2+}-dependent $^{45}Ca_o^{2+}$ uptake (Ca^{2+}–Ca^{2+} exchange)

Ca^{2+}–Ca^{2+} exchange activity was estimated either in the absence of Na^+ or in the presence of 200 mM intra- and extravesicular Na^+. Thus no Na^+ gradient was present across the vesicle membrane in any of these conditions. In order to determine Ca^{2+}–Ca^{2+} exchange in the absence of Na^+, vesicles were originally suspended in 100 mM *N*-methylglucamine-Cl, 100 mM KCl, 100 mM *N*-methylglucamine-Mops (pH 7.3) and 2 mM EGTA plus 100 μM free calcium. In this case the dilution medium contained 100 mM *N*-methylglucamine-Cl, 100 mM KCl, 100 mM *N*-methylglucamine-Mops (pH 7.3) plus 100 μM $^{45}CaCl_2$ and 0.5 μM valinomycin. Vesicles containing 100 mM *N*-methylglucamine-Cl, 100 mM KCl, 100 mM *N*-methlglucamine-Mops (pH 7.3) and 2 mM EGTA were used in parallel to estimate passive Ca^{2+} diffusion into the vesicles and unspecific Ca^{2+} binding. The magnitude of Ca^{2+}–Ca^{2+} exchange was also determined in the presence of Na^+: for this purpose the membrane vesicles were suspended in a medium containing 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3), 2 mM EGTA and 100 μM free calcium, and the dilution medium contained 100 μM $^{45}CaCl_2$, 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3) and 0.5 μM valinomycin. All other procedures were similar to those described above. The passive Ca^{2+} uptake and unspecific binding was estimated as stated above, using vesicles containing 2 mM EGTA.

Measurement of Na_i^+-dependent $^{22}Na^+$ uptake (Na^+–Na^+ exchange)

In order to determine the magnitude of Na^+–Na^+ exchange, the membrane vesicles were suspended in 100 mM Na-Mops, 100 mM *N*-methylglucamine-Mops, 100 mM K-Mops (pH 7.3), 0.5 μM tetrodotoxin and 2 mM EGTA (control) or 2 mM EGTA plus variable concentrations of free calcium (experimental).

The vesicles were then diluted in 100 mM ^{22}Na-Mops (0.01 μCi/mmol), 100 mM *N*-methylglucamine-Mops, 100 mM K-Mops (pH 7.3) in the presence 0.1 mM ouabain and 0.5 μM tetrodotoxin, and the $^{22}Na^+$ uptake at 25°C was estimated by the Millipore filtration technique.

The rate of passive diffusion of $^{22}Na^+$ was determined by measuring the influx of $^{22}Na^+$ into vesicles suspended in 200 mM *N*-methylglucamine-Mops, 100 mM K-Mops (pH 7.3), 2 mM EGTA and diluted in the above dilution medium.

Other assays

Na^+/K^+-ATPase activity was assayed as previously reported [14]. The protein content of the nerve membrane was determined by the method of Lowry et al. [19].

Materials

$^{45}CaCl_2$ (4–50 mCi/mg calcium), $^{22}NaCl$ (100–1800 mCi/mg sodium) and ^{14}C-EGTA (5 mCi/mmol) were purchased from New England Nuclear. Valinomycin was a product of Calbiochem. Ouabain, tetrodotoxin, sodium deoxycholate, *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA), ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), *N*-methyl-D-glucamine, 4-morpholinepropanesulfonic acid (Mops), phenylmethylsulfonyl fluoride (PMSF) and tris(hydroxymethyl)aminomethane (Tris) were from Sigma Chemical. Other chemicals and reagents were of analytical grade. All data are presented as means ± S.E.

Results

Effect of K^+ on Na_i^+–Ca_o^{2+} exchange

It has been previously demonstrated that Na^+–Ca^{2+} exchange activity generates a membrane potential in a vesicle preparation which becomes self-limiting for further Na^+–Ca^{2+} exchange because of the build up of charge across the vesicle membrane [20]. The potassium specific ionophore valinomycin allows the charge to dissipate and thereby enhances the rate of exchange. Therefore we decided to explore the modulation of the exchanger activity by intravesicular Ca^{2+} in the presence of K^+-valinomycin. This raises the question of how K^+ per se affects Na_i^+–Ca_o^{2+} exchange activity in our system since it has been previously shown that this ligand ion interacts with the carrier and chemically activates it [21,22].

We investigated the effect of potassium on Na_i^+–Ca_o^{2+} exchange in the absence of an electric gradient across the vesicle membrane to avoid activation of the carrier. Fig. 1 shows the stimulation of Na_i^+-dependent Ca^{2+} uptake by different concentrations of potassium. Flux measurements were performed in the presence of valinomycin and in the presence of equal potassium concentrations in the intra- and extravesicular medium. The uptake of Ca^{2+} in exchange of intravesicular Na^+ increased with increasing concentrations of K^+, reaching plateau at 100 mM K^+. The $[K^+]$ required to achieve half-maximal activation of the exchange was 82 mM under these conditions. The passive diffusion of calcium (using *N*-methylglucamine-loaded vesicles) was not affected by potassium (lower trace in Fig. 1).

Therefore potassium per se stimulates Na^+–Ca^{2+} exchange in squid optic nerve membrane vesicles, in agreement with the chemical activation of the exchanger by K_i^+ which has been reported by DiPolo and Beaugé [23] in dialyzed axons and by Coutinho et al. in brain synaptosomes [22]. Taking into account this effect, we carried out subsequent experiments in the presence of 100 mM KCl in both intra- and extravesicular media.

Effect of intravesicular Ca^{2+} on Na_i^+-dependent calcium uptake

Na_i^+–Ca_o^{2+} exchange activity of nerve membrane vesicles originally suspended in media containing EGTA and Ca · EGTA (or HEDTA and Ca · HEDTA) was investigated.

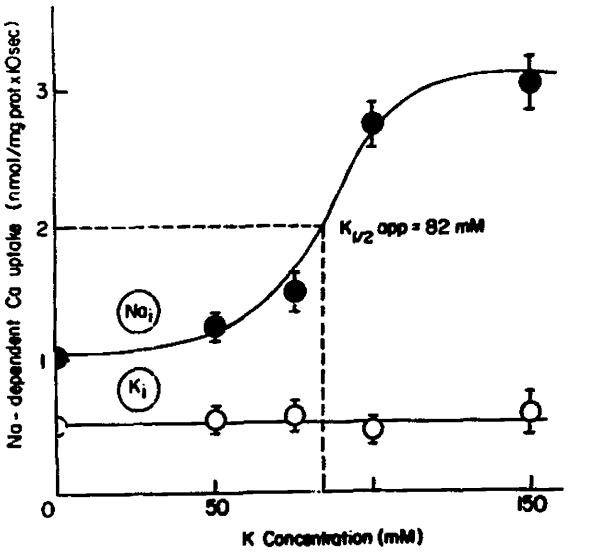

Fig. 1. Effect of potassium on Na^+-dependent Ca^{2+} uptake by Na^+-loaded (●———●) or K^+-loaded (○———○) vesicles. Vesicles were originally suspended in: 100 mM NaCl, 100 mM Na-Mops (pH 7.3) and variable concentrations of KCl (0–150 mM) and *N*-methylglucamine-Cl (150–0 mM), respectively. Vesicles were thawed at room temperature and preincubated 5 min at 25°C. Na^+–Ca^{2+} exchange was initiated by diluting vesicles 20 times in a medium containing 100 μM $^{45}CaCl_2$ (0.05 μCi/nmol), 100 mM *N*-methylglucamine-Cl, 100 mM *N*-methylglucamine-Mops (pH 7.3) variable concentrations of KCl (0–150 mM) and additional *N*-methylglucamine-Cl (150 mM–0 mM), respectively, in the presence of 0.5 μM valinomycin. The mixture was incubated at 25°C and $^{45}Ca^{2+}$ uptake was arrested at 10 s by adding 2 mM EGTA (final concentration). 100 μl aliquots (containing 25 μg protein, approximately) were filtered through 0.45 μm Millipore filters. Filters washed twice with 5 ml of 200 mM KCl, 100 mM K-Mops (pH 7.3). The radioactivity retained on the filters was determined by liquid scintillation counting. The passive diffusion of ^{45}Ca was estimated by using K^+-loaded vesicles instead of Na^+-loaded vesicles. The points represent means ± S.E. of three determinations from three different membranes (each in duplicate). Valinomycin was added from a 100 μM stock solution in dimethyl sulfoxide; solvent alone had no effect on exchange activity.

Fig. 2 depicts the effect of intravesicular free calcium on the time course of Na_i^+-induced Ca^{2+} influx in these vesicles. It is shown in Fig. 2A that the uptake of Ca^{2+} in exchange for intravesicular Na^+ proceeded rapidly during the first 30 s in both EGTA-containing (2 mM EGTA) and Ca · EGTA-containing (2 mM EGTA, 100 μM free calcium) vesicles. However, the extent of Na_i^+-de-

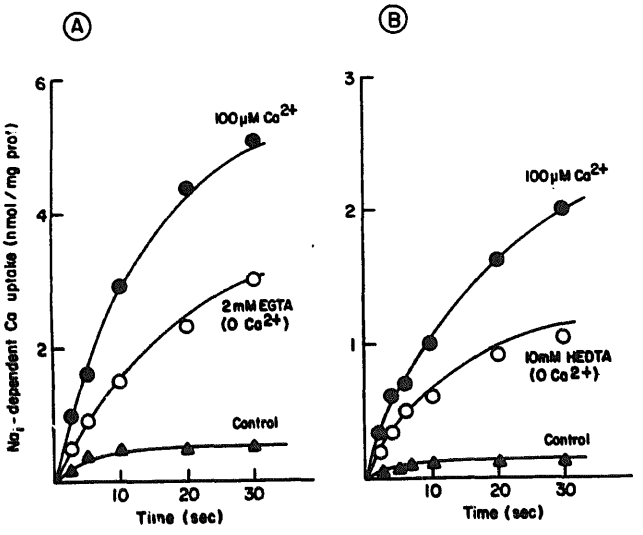

Fig. 2. Time-course of Na^+-dependent Ca^{2+} uptake in vesicles containing EGTA and vesicles containing Ca·EGTA (A) (or HEDTA and Ca·HEDTA (B)). Vesicles originally suspended in: 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3), 2 mM EGTA (or 10 mM HEDTA) (o——o) or in: 100 mM NaCl, 100 mM KCl, 100 mM Na-Mops (pH 7.3), 2 mM EGTA and 2.1 mM $CaCl_2$ (or 10 mM HEDTA plus 10 mM $CaCl_2$) (●——●). Vesicles were thawed at room temperature and preincubated 5 min at 25°C. The Na^+-dependent $^{45}Ca^{2+}$ uptake was initiated by diluting vesicles 20 times in a medium comprising 100 μM $^{45}Ca^{2+}$ (0.05 μCi/nmol), 100 mM *N*-methylglucamine-Cl, 100 mM KCl and 100 mM *N*-methylglucamine-Mops (pH 7.3) in the presence of 0.5 μM valinomycin. The mixture was incubated at 25°C for various time intervals. Additional details described under materials and Methods. The points represent means ± S.E. of ten determinations from five different membrane preparations.

pendent $^{45}Ca_o^{2+}$ uptake was higher in calcium containing vesicles. A comparable situation is observed in Fig. 2B. In this case vesicles were prepared containing 10 mM HEDTA (control) or 10 mM HEDTA plus enough $CaCl_2$ as to obtain 100 μM intravesicular Ca^{2+} (experimental). Here the presence of 10 mM HEDTA diminishes the absolute value of Na_i^+-dependent calcium uptake compared to 2 mM EGTA-containing vesicles. This seems to be rather an effect of the calcium buffer concentration than of HEDTA per se since no difference with Fig. 2A was obtained when vesicles were prepared with 2 mM HEDTA (not shown). However, an acceleration of Na_i-dependent calcium uptake is noticed in calcium containing vesicles compared to control vesicles, which is similar to that of Fig. 2A. It seems reasonable to conclude that the vesicles are not leaky to Ca^{2+} in the absence of intravesicular Ca^{2+} since the passive diffusion and unspecific binding of Ca^{2+} (using K^+- or *N*-methylglucamine-loaded vesicles instead of Na^+-loaded vesicles) was not different in EGTA or Ca·EGTA containing vesicles (data not shown). Therefore, intravesicular free calcium activates $Na_i^+-Ca_o^{2+}$ exchange in this vesicle preparation. The dependence of this effect on intravesicular free calcium concentration is shown in Fig. 3. HEDTA (10 mM) was used to buffer intravesicular calcium allowing an adequate control of the intravesicular cation concentration during the 10 s time-course of the Ca^{2+} uptake reaction. Stimulation of Na_i^+-dependent Ca^{2+} uptake increase with increasing concentrations of intravesicular free calcium until a plateau is reached around 100 μM Ca^{2+}, with about 90% stimulation of $Na_i^+-Ca_o^{2+}$ exchange. This effect has a high statistical significance ($P < 0.001$) and half-maximal stimulation was obtained with $Ca_i^{2+} = 12$ μM.

Stimulation by Ca_i^{2+} of $Na_i^+-Ca_o^{2+}$ exchange vs. $Ca^{2+}-Ca^{2+}$ exchange

At this point it is important to discern if the increase in Ca^{2+} uptake by vesicles containing Ca·EGTA is a result of $Na_i^+-Ca_o^{2+}$ exchange stimulation by intravesicular Ca^{2+} or whether it

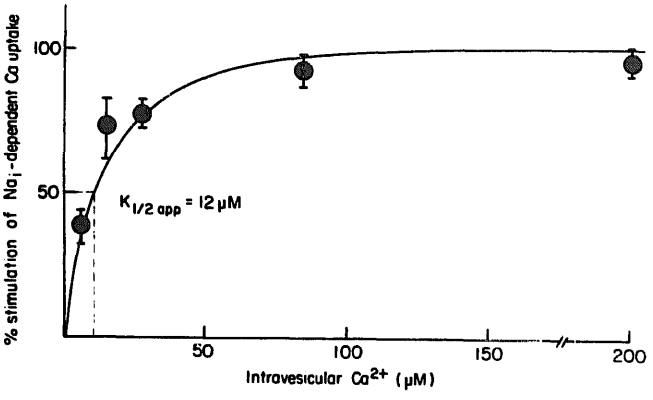

Fig. 3. Dependence of Na^+-Ca^{2+} exchange stimulation on the intravesicular free calcium concentration. Vesicles containing 10 mM HEDTA and 10 mM HEDTA plus variable concentrations of $CaCl_2$ were originally prepared. The intravesicular free calcium concentration was calculated on the basis of a Ca·HEDTA dissociation constant of 5 μM [17]. % stimulation of Na_i-dependent Ca uptake by intravesicular Ca^{2+} was calculated from 10 s ^{45}Ca uptake data. Additional details described under Materials and Methods. The points represent means ± S.E. of eight determinations from four different membrane preparations.

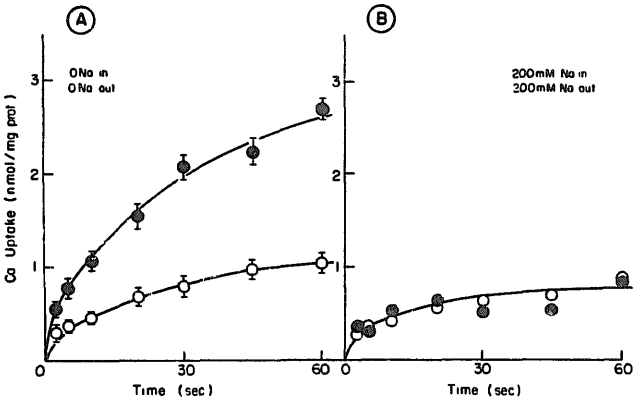

Fig. 4. Estimation of Ca^{2+}–Ca^{2+} exchange between intra and extravesicular Ca^{2+} in the absence of Na^+ (A) and in the presence of 200 mM intra- and 200 mM extravesicular Na^+ (B). Passive $^{45}Ca^{2+}$ uptake determined using vesicles containing 2 mM EGTA, (no free calcium) and with Na^+ replaced by K^+ (○———○). Ca^{2+}–Ca^{2+} exchange between intravesicular free Ca^{2+} (100 μM) and extravesicular calcium (●———●). Additional details given under Materials and Methods. The points represent means ± S.E. of ten determinations from five different membrane preparations.

reflects a Ca^{2+}–Ca^{2+} exchange between intra- and extravesicular Ca^{2+}. To clarify this question we determined the Ca^{2+}–Ca^{2+} exchange activity in the absence of internal and external Na^+ and subsequently we investigated how this exchange is affected by the presence of internal and external Na^+ (200 mM). The results are shown in Fig. 4. Fig. 4A shows the Ca^{2+}–Ca^{2+} exchange measured in the absence of Na^+: the time-course of Ca^{2+} uptake in vesicles containing 2 mM EGTA (control, open circles) indicates that in the absence of intravesicular calcium and with 100 μM Ca^{2+} in the extravesicular medium there is a passive diffusion and unspecific binding of Ca^{2+} which is similar to that determined above by abolishing the Na^+ gradient. In contrast, when 100 μM Ca^{2+} is present inside the vesicles (Ca · EGTA, closed circles) a Ca^{2+}–Ca^{2+} exchange between intra- and extravesicular space starts operating, attaining a magnitude of about 3 nmol/mg protein per min. Nevertheless, this Ca^{2+}–Ca^{2+} exchange becomes totally inhibited in the presence of 200 mM Na^+ inside and outside the vesicles, i.e. in the absence of a Na^+ gradient. It is shown in Fig. 4B that under these conditions the time-course of Ca^{2+} uptake by vesicles containing 100 μM Ca^{2+} (closed circles) is identical to the passive diffusion and unspecific binding of Ca^{2+} in vesicles containing only 2 mM EGTA (open circles). This agrees with the observation of Slaughter et al. that in cardiac sarcolemmal vesicles Na^+ concentrations higher than 10 mM inhibit Ca^{2+}–Ca^{2+} exchange, which these authors interpret as due to competitive interference with Ca^{2+}-binding sites on the carrier [24,25]. These results indicate that almost all of the activation of Ca^{2+} uptake by intravesicular Ca^{2+} is on the Na_i^+-dependent component and not on the Ca^{2+}–Ca^{2+}-dependent one.

Effect of intravesicular Ca^{2+} on Na^+–Na^+ exchange

Previous evidence obtained in dialyzed squid axons indicates that internal Ca^{2+} not only

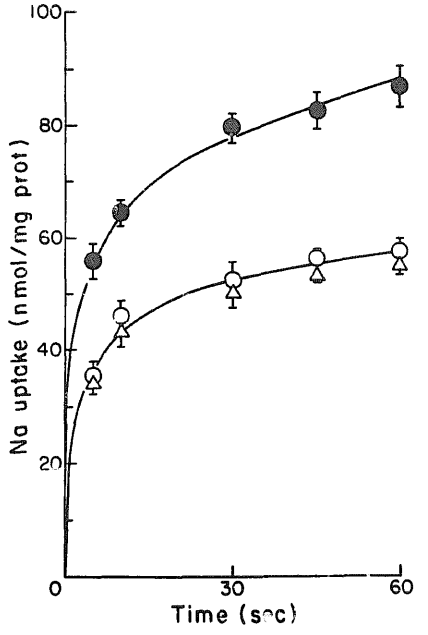

Fig. 5. Time-course of Na_i-dependent $^{22}Na_o^+$ uptake. Vesicles were suspended in 100 mM Na-Mops, 100 mM *N*-methylglucamine-Mops, 100 mM K-Mops (pH 7.3), 0.5 μM tetrodotoxin and 2 mM EGTA (○———○) or 2 mM Ca·EGTA (Ca^{2+} = 25 μM) (●———●), were frozen in liquid N_2 and kept at −70 °C. Prior the experiment was performed, vesicles were thawed at room temperature and preincubated for 5 min at 15 °C. In order to estimate Na^+–Na^+ exchange, vesicles were diluted 20 times in 100 mM ^{22}Na-Mops (0.01 μCi/nmol), 100 mM *N*-methylglucamine-Mops, 100 mM K-Mops (pH 7.3) in the presence of 0.1 mM ouabain and 0.5 μM tetrodotoxin, and $^{22}Na^+$ uptake at 15 °C was measured by Millipore filtration. Other details under Materials and Methods. The rate of passive diffusion of $^{22}Na^+$ into vesicles suspended in 200 mM *N*-methylglucamine-Mops, 100 K-Mops, 0 mM Na-Mops and 2 mM EGTA and diluted 20 times in the above dilution medium. (△———△). The points represent means ± S.E. of eight determinations from four different membranes.

activates a Ca_o^{2+}-dependent Na_i^+ efflux component (Na_i^+–Ca_o^{2+} exchange) but also a Na_o-dependent Na_i efflux (Na_i^+–Na_o^+ exchange). Since several experimental evidences indicate that the Ca_i^{2+}-activated Na_o^+-dependent Na_i^+ efflux is a mode of operation of the Na^+–Ca^{2+} exchange mechanism [5,8] we decided to explore its dependence on intravesicular calcium. In order to lower passive Na^+ fluxes which are too large in vesicular preparations as compared to Ca^{2+} fluxes (Ref. 10 and unpublished observations in our laboratory) we replaced chloride by the impermeant anion Mops, we added 0.5 μM tetrodotoxin to both intra- and extravesicular media and we measured the fluxes at 15 °C instead of 25 °C. The results of this type of experiment are shown in Fig. 5. The time-course of $^{22}Na_o^+$ uptake by vesicles containing 100 mM Na-Mops, 100 mM *N*-methylglucamine-Mops, 100 mM K-Mops and 2 mM EGTA and diluted 20 times in 100 mM ^{22}Na-Mops, 100 mM *N*-methylglucamine-Mops and 100 mM K-Mops appears in the lower trace of this figure (open circles). It is striking that in these conditions $^{22}Na^+$ uptake is identical to the passive Na^+ influx measured by diluting in the same medium vesicles containing 200 mM *N*-methylglucamine-Mops, 100 mM K-Mops, no Na-Mops and 2 mM EGTA (open triangles). In contrast with this finding, the presence of 25 μM free calcium inside the vesicles accelerates Na^+ uptake (closed circles). This indicates that Na^+–Na^+ exchange is dependent on the presence of intravesicular calcium. Increasing intravesicular Ca^{2+} concentration above 50 μM not only has no further activatory effect on Na^+–Na^+ exchange, but gradually inhibits this activation, possibly due to competition for Na^+-binding sites on the carrier.

Discussion

The results presented in this study indicate that micromolar concentrations of intravesicular free calcium accelerate the reverse mode of Na^+–Ca^{2+} exchange (i.e. Na_i^+–Ca_o^{2+} exchange) as well as Na^+–Na^+ exchange in Na^+-loaded nerve membrane vesicles. This finding in membrane vesicles appears to be a manifestation of the previous observation that cytoplasmic Ca^{2+} stimulates Na_i^+-dependent Ca^{2+} influx in dialyzed squid axons [3,5,8], barnacle muscle cells [15] and heart cells [6], suggesting that a Ca^{2+}-modulating site on the carrier resides on the inner side of the membrane.

The population of vesicles used in this work consists of approx. 10% inside-out and 67% right-side-out vesicles [13]. These vesicles were prepared containing Na^+ + EGTA or Na^+ + Ca · EGTA in their interior (EGTA was replaced by HEDTA in some experiments), and the intravesicular Na^+-extravesicular Ca^{2+} exchange was measured. This means that in EGTA (or HEDTA)-containing vesicles the extravesicular Ca^{2+} is expected to stimulate a basal level of Na_o^+–Ca_i^{2+} exchange activity (direct mode of Na^+–Ca^{2+} exchange) in the inside-out vesicles present in the preparation and representing 13% of the total sealed vesicles participating in the uptake measurements. This is shown in Fig. 2 where an exchange activity is observed in the absence of intravesicular Ca^{2+}. In vesicles containing Ca · EGTA (or Ca · HEDTA), an additional Na_i^+–Ca_o^{2+} exchange activity (reverse mode of Na^+–Ca^{2+} exchange), activated by intravesicular free calcium and presumably due to right-side-out vesicles will appear (see Fig. 2). However, in these experiments about 50% of the Na^+–Ca^{2+} exchange activity persists in the absence of intravesicular calcium. This high basal activity can not be totally ascribed to a 13% population of inside-out vesicles. One could interpret that it results from partial activation of rightside-out vesicle Na^+–Ca^{2+} exchange in the absence of internal calcium. However, Na^+–Na^+ exchange experiments (Fig. 5) do not favor this view since this is not observed unless intravesicular calcium is present. In this case there is no contribution of inside-out vesicles because calcium is chelated in the extravesicular medium and therefore only the right-side-out population of vesicles is being studied. An alternative explanation is that the presence of relatively high concentrations of EGTA (or HEDTA) in the membrane resuspension solution might induce an increased ratio of inside-out/right-side-out vesicles with respect to that previously reported [13].

It could be argued that the exchanger is not activated by free calcium but by the couple Ca · EGTA or Ca · HEDTA. Although experiments in the absence of calcium buffers were not conducted

in vesicles because of our interest to control intravesicular Ca^{2+} concentration in the range from 0 to 100 μM, this seems not to be the case. In fact, DiPolo and Beaugé have recently observed in dialyzed axons an effect of internal calcium on the reverse of the exchange, even in the absence of EGTA (unpublished results).

Stimulation of Na_i^+–Ca_o^{2+} exchange activity in nerve membrane vesicles occurs at micromolar concentrations of Ca_i^{2+} (half-maximal activation at 12 μM Ca^{2+}) in the absence of ATP and this value is similar to the concentration of Ca^{2+} required to elicit exchange half-maximal stimulation in squid axons dialyzed without ATP [5,8]. This finding in squid optic nerve membranes agrees with the observation in cardiac membrane vesicles by Reeves and Poronnik [26] who reported a stimulation of the Na^+–Ca^{2+} exchange activity by preincubation of sarcolemmal vesicles with Ca^{2+} (0.1–0.5 mM). However, it should be pointed out that the stimulatory effect of Ca_i^{2+} reported here occurs at lower concentrations of intravesicular calcium, attaining saturation around 100 μM Ca^{2+}.

There is an alternative interpretation for the stimulation of Na_i^+–Ca_o^{2+} exchange by intravesicular Ca^{2+}: it is possible that the vesicles are leaky to Ca^{2+} in the absence of intravesicular Ca^{2+} (vesicles containing EGTA or HEDTA only) and become tightly sealed as Ca^{2+} concentration rises in their interior. If this were the case, Na^+–Ca^{2+} exchange activity would be underestimated in vesicles containing EGTA (or HEDTA) only. However, this does not seem to be the case since we did not detect any difference in Ca^{2+} passive permeability between vesicles containing EGTA and those containing Ca · EGTA (data not shown). In addition, the same level of passive Ca^{2+} permeability was also detected in vesicles prepared without EGTA or Ca · EGTA in their interior (see Fig. 1). Moreover, addition of 2 mM external EGTA or even 10 mM HEDTA to vesicles loaded with Ca^{2+} did not induce release of the internal calcium, indicating that these concentrations of EGTA or HEDTA, respectively, do not promote a leak pathway for Ca^{2+} (not shown).

The stimulation of Ca^{2+} uptake by intravesicular free calcium most probably represents an effect on Na^+–Ca^{2+} exchange activity rather than Ca^{2+}–Ca^{2+} exchange. This is inferred from the fact that in the presence of 200 mM Na^+, Ca^{2+}–Ca^{2+} exchange is inhibited and Ca^{2+} uptake is reduced to passive diffusion of the cation (Fig. 4).

Another important finding reported here is the activation by intravesicular Ca^{2+} of Na^+–Na^+ exchange in nerve membrane vesicles. A Na_o^+-dependent Na^+ efflux with several features suggesting that it represents the Na^+–Ca^{2+} exchange system operating in a Na^+–Na^+ exchange mode has been demonstrated in cardiac membrane vesicles by Reeves and Sutko [28]. Recently, DiPolo and Beaugé showed the existence of this component in an intact preparation; in dialyzed squid axons they measured in the absence of ATP an ouabain-insensitive Na_o^+-dependent Na^+ efflux which was activated by internal alkalinization in the presence of Ca_i^{2+}, which was inhibited by Mg_i and finally, was activated by Ca_i^{2+} with a low apparent affinity ($K_{1/2}$ in the micromolar range) [8]. These arguments strongly favor a common origin of the Ca_i^{2+} – activated, Na_o^+-dependent Na^+ efflux and the Na^+–Ca^{2+} exchange mechanism. In the vesicle preparation, we have been able to measure an intravesicular Na^+-dependent Na_o^+ uptake in the presence of intravesicular calcium. Interestingly, Na^+ uptake in vesicles containing 2 mM EGTA and 200 mM Na^+ was identical to the passive diffusion of Na^+ into vesicles containing no sodium (Fig. 5). Activation of Na^+–Na^+ exchange was maximal with intravesicular free calcium concentrations of 25 to 50 μM; higher concentrations of Ca^{2+} gradually diminished the activatory effect.

In summary, we have shown that Ca^{2+} at the intracellular side of the membrane exerts a modulatory effect on the different modes of operation of the Na^+–Ca^{2+} exchange carrier. It has recently been proposed that Na^+–Ca^{2+} exchange activity may be regulated by the distribution of charged groups between the inner and outer surfaces of the membrane [26,27]. This could explain the mechanism of stimulation of exchange activity by Ca^{2+}, quinacrine and tetraphenylphosphonium since the binding of these agents to the membrane involves a local electrostatic effect [27]. However, the stimulatory effect of Ca^{2+} and probably of quinacrine and tetraphenylphosphonium seem to be exerted at the inner side of the membrane and

shows a remarkable degree of specificity. This probably implies the existence of specific sites on the carrier present on one side of the membrane and conferring to the molecule a marked asymmetry.

Comparison between Na^+–Ca^{2+} exchange from membrane vesicle preparations and intact cells leaves the impression that some features exhibited by the intact exchanger may be lost during vesicle purification [29]. The results obtained in this study suggest that the modulatory effect of internal Ca^{2+} on the Na^+–Ca^{2+} exchanger, described in living cells, has been preserved in the squid optic nerve vesicle preparation.

Acknowledgments

We are grateful to Dr. Guillermo Whittembury for critical reading of the manuscript. We also thank Miss Elizabeth González for typing the manuscript, Mrs. Dhuwya Palma for the figures and the squid supply staff at IVIC and Mochima. This work was supported by Grant S1-1934 from CONICIT, Venezuela, by Fundación Polar, Venezuela and by Grant 1R01 HL 39243-01 from NIH, U.S.A. and Grant RGBC 87-84 from The Third World Academy of Sciences, Italy.

References

1 Baker, P.F. and Blaustein, M.P. (1968) Biochim. Biophys. Acta 150, 167–170.
2 Reuter, H. and Seitz, N. (1968) J. Physiol. (Lond) 195, 451–470.
3 DiPolo, R. (1979) J. Gen. Physiol. 73, 91–113.
4 Mullins, L.J. (1977) J. Gen. Physiol. 70, 681–696.
5 DiPolo, R. and Beaugé, L. (1986) Biochim. Biophys. Acta 854, 298–306.
6 Kimura, J., Noma, A. and Irisawa, H. (1986) Nature 319, 596–597.
7 DiPolo, R., Bezanilla, F., Caputo, C. and Rojas, H. (1985) J. Gen. Physiol. 86, 457–478.
8 DiPolo, R. and Beaugé, L. (1987) J. Gen. Physiol. 90, 505–525.
9 Bers, D.M., Philipson, K.D. and Nishimoto, A.Y. (1980) Biochim. Biophys. Acta 601, 358–371.
10 Pitts, B.R.J. (1979) J. Biol. Chem. 254, 6232–6235.
11 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 590–594.
12 Philipson, K.D. and Nishimoto, A.Y. (1982) J. Biol. Chem. 257, 5111–5117.
13 Osses, L., Condrescu, M. and DiPolo, R. (1986) Biochim. Biophys. Acta 860, 583–591.
14 Condrescu, M., Osses, L. and DiPolo, R. (1984) Biochim. Biophys. Acta 769, 261–269.
15 Rasgado-Flores, H., Santiago, E. and Blaustein, M.P. (1986) Biophys. J. 49, 546a.
16 Condrescu, M., Gerardi, A. and DiPolo, R. (1987) Biophys. J. 51, 10a.
17 Van Alstyne, E., Burch, R.M., Knickelbein, R.G., Webb, J.G., Hungerford, R.T., Gower, E.J., Poe, S.L. and Lindenmayer, G.E. (1980) Biochim. Biophys. Acta 602, 131–143.
18 DiPolo, R., Requena, J., Brinley, F.J., Mullins, L.J., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433–467.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Reeves, J.P. and Sutko, J.L. (1980) Science 208, 1461–1464.
21 Allen, T.J.A. and Baker, P.F. (1983) J. Physiol. (Lond.) 345, 80P.
22 Coutinho, O.P., Carvalho, C.A.M. and Carvalho, A.P. (1984) Brain Res. 290, 261–271.
23 DiPolo, R. and Beaugé, L. (1984) J. Gen. Physiol. 84, 895–914.
24 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3183–3190.
25 Reeves, J.P. and Sutko, J.L. (1983) J. Biol. Chem. 258, 3178–3182.
26 Reeves, J.P. and Poronnik, P. (1987) Am. J. Physiol. 252, C17–C23.
27 De la Peña, P. and Reeves, J.P. (1987) Am. J. Physiol. 252, C24–C29.
28 Reeves, J.P. and Sutko, J.L. (1979) Fed. Proc. 38, 1199.
29 Baker, P.F. and Allen, T.J.A. (1986) in Intracellular Calcium Regulation (Baker, H., Gietzen, K., Rosenthal, J., Rüdel, R., Wolf, H., eds), pp. 35–46, Manchester University Press, Manchester, U.K.