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Stoichiometry of the Sodium–Calcium Exchanger in Nerve Terminals†

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ABSTRACT: The stoichiometry of the Na^+–Ca^{2+} exchanger from synaptic plasma membranes was studied in both native and reconstituted preparations. In kinetic experiments performed with the native preparation, initial rates of Na^+ gradient-dependent Ca^{2+} influx were compared to Ca^{2+}-dependent Na^+ efflux. These experiments showed that 4.82 Na^+ ions are exchanged for each Ca^{2+} ion. A thermodynamic approach in which equilibrium measurements were made with the reconstituted preparation resulted in a similar (4.76) stoichiometry. The effects of membrane potential generated by valinomycin-induced K^+ fluxes could be demonstrated in the reconstituted preparation. In addition, the direct contribution of the Na^+–Ca^{2+} exchanger to the membrane potential across the reconstituted vesicle membrane could be demonstrated by using the lipophilic cation tetraphenylphosphonium.

The sodium–calcium exchanger (or antiporter) is one of the major calcium-transporting molecules found in most, if not all, excitable and secretory cells (Reuter & Seitz, 1968; Baker et al., 1969; Kaczorowski et al., 1984). It transports calcium across the membrane in exchange for sodium ions. The amount and direction of calcium flux depend on the direction and magnitude of sodium and calcium gradients and the membrane potential (Mullins, 1977). From experiments performed in the squid giant axon (Mullins, 1977; Blaustein & Russell, 1975), the heart (Mullins, 1975; Reeves & Sutko, 1980; Ledvora & Hegyvary, 1983), and synaptosomes (Blaustein & Ector, 1976), it is known that the sodium–calcium antiporter is electrogenic. The number of sodium ions that are reported to be exchanged for each calcium ion varies from three to six (or even more) (Mullins, 1977; Reeves & Sutko, 1980; Ledvora & Hegyvary, 1983; Blaustein & Ector, 1976; Horackova & Vassort, 1979). This difference is not trivial at all, since it determines the range of membrane potential at which the exchanger will be active, the direction of its activity, and its capability to regulate cytoplasmic free Ca^{2+} ion concentration (Mullins, 1981a). The variability in the stoichiometry of the Na^+–Ca^{2+} exchanger may be due to genuine differences among different biological systems, or, alternatively, it also might reflect the fact that in most preparations studied, including membrane vesicles derived from native sarcolemma (Pitts, 1979; Reeves & Hale, 1984), many other unrelated ionic permeabilities were present; these permeabilities may be responsible for some of the fluxes attributed to the sodium–calcium exchanger or may affect the exchanger indirectly by acting on the membrane potential and thus on the driving force acting on sodium–calcium antiporter.

In this work, we have measured the stoichiometry of Na^+–Ca^{2+} antiport both in a native synaptic plasma membrane (SPM)[1] vesicle preparation and in a functionally reconstituted preparation of the synaptic plasma membrane sodium–calcium exchanger. The reconstituted preparation eliminates most of the unrelated ionic permeabilities for two reasons. First, during reconstitution, a great excess of lipids is provided; in the native vesicles, the protein:phospholipid ratio is 1:1.4, while in the reconstituted vesicles this ratio is 1:50. Thus, the probability of having the exchanger and another channel (or transporter) in the same vesicle is greatly reduced. Second, the reconstituted vesicles have a smaller diameter (550 Å) compared to the native vesicles (850 Å). The surface area decreases with the square of the diameter, to 42%. Therefore, most of the proteins that were in the same native vesicles before reconstitution are probably separated and localized in different vesicles after the reconstitution. Thus, the combination of higher phospholipid content, which also imposes a hydrophobic barrier, and the fact that different permeabilities reside in different veiscles leads to the expectation that measurements of sodium–calcium antiport activity will have a better "signal to noise" ratio and offer a biochemical preparation which might yield more meaningful measurements from the physiological viewpoint.

Experimental Procedures

Preparation of synaptic plasma membranes (SPM) was done essentially as described (Rahamimoff & Spanier, 1979; Erdreich & Rahamimoff, 1983), except that a three-step ficoll gradient (2%, 8%, and 12%) instead of a five-step one was used. Reconstitution was performed as described in our previously published procedure (Barzilai et al., 1984) with minor modifications. The method consists of solubilization of SPM in 2% cholate in the presence of a 50-fold excess (by

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[1] Abbreviations: SPM, synaptic plasma membrane; TPP^+, tetraphenylphosphonium; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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weight) of purified brain phospholipids. Dialysis is performed against a Na^+-containing buffer, the exact composition of which is dictated by the needs of the experiments to be performed, in hollow biofibers (Bio-Rad) or artificial kidney units, CDAK-0.6 (Cordis Dow) or CF 12-11 (Travenol) depending on the amount of material to be reconstituted. The reconstituted vesicles were collected by centrifugation at 100000*g* for 4.5 h at 6 °C and suspended in a minimal volume of the same buffer as used for the dialysis.

For some experiments, such as when $^{22}Na^+$ was introduced as the intravesicular medium, a different method of reconstitution was used. It involved cholate removal by gel filtration. This was done by passing the solubilized protein–phospholipid mixture on a Sephadex G50 mini-column. Tuberculin syringes were filled with the gel previously preequilibrated with the medium of the desired internal composition to be introduced into the reconstituted vesicles. The syringes were centrifuged for 2 min at about 1000*g*. This step led to partial drying and shrinking of the gel. Careful introduction of the solubilized protein–phospholipid mixture (up to 200 μL/1-mL syringe) into the gel bed led to its reswelling to the original size. Centrifugation of these syringes under conditions *exactly* identical with those employed before led to vesicle formation, concentration, and collection in a minimal volume that has been excluded from the gel. In order to achieve efficient cholate removal and vesicle resealing, this procedure was repeated 3 times. The Na^+ gradient dependency of Ca^{2+} uptake and its extent were identical with those of the reconstituted vesicles formed by hollow fiber dialysis.

Transport activity of both the native and the reconstituted preparations ($^{22}Na^+$, $^{45}Ca^{2+}$, or [^{3}H]TPP$^+$) was performed by using Dowex 50 mini-columns (Gasko et al., 1976). Usually 3–5 μL of the vesicles was diluted into 100–245 μL of the test medium, the composition of which and the exact quantities used are given in the legends to the relevant figures or tables.

The internal volume of the reconstituted vesicles was measured by preequilibrating a Sephadex G50 mini-column with 1 mM [^{3}H]glucose in addition to the Na^+-containing solution. The trapped glucose in the reconstituted vesicles was measured following a second transfer of the reconstituted vesicles through an identical Sephadex G50 column equilibrated with the buffered Na^+-containing solution (without the glucose solution) to ensure that no glucose bound to the vesicles. No losses of [^{3}H]glucose from the vesicles occurred by this procedure.

Brain phospholipids were prepared by a modification of the Bligh and Dyer extraction procedure (Sheltawy & Dawson, 1969), followed by chromatography on a potassium oxalate washed column of silicic acid. The column was washed by chloroform to remove neutral lipids, followed by extraction of phospholipids with methanol–chloroform (80:20).

Protein was determined by the method of Lowry et al. (1951); high lipid-containing samples were assayed in the presence of SDS (sodium dodecyl sulfate). Phospholipid phosphate was determined as described (Ames, 1974).

All the biochemical reagents were purchased from Sigma, Israel; the chemical reagents were analytical grade.

RESULTS

Stoichiometry of the Na^+–Ca^{2+} Exchanger in Native Vesicles. The stoichiometry of the Na^+–Ca^{2+} exchanger has been investigated in several ways: In the experiment shown in Figure 1A,B, the kinetic approach was adopted. Native synaptic plasma vesicles were preloaded with either $^{22}NaCl$ or unlabeled NaCl by preincubation for 3 h at 37 °C. The vesicles were collected by centrifugation and suspended in a minimal volume of the same medium. The reaction was started by rapid dilution of these vesicles into an isoosmotic KCl solution containing labeled or unlabeled $CaCl_2$ (depending on whether $^{22}Na^+$ efflux or $^{45}Ca^{2+}$ influx was measured) which resulted in activation of the Na^+–Ca^{2+} antiport. The initial rates of $^{45}Ca^{2+}$ influx and $^{22}Na^+$ efflux are compared in Figure 1A,B. Control experiments in which Na^+ gradient independent $^{45}Ca^{2+}$ influx or $^{22}Na^+$ efflux in the absence of externally added Ca^{2+} were also done (Figure 1A,B). The ratio of sodium ions exchanged for each calcium ion is 4.82 to 1 when early time points (up to 30 s) are taken and the Na^+ gradient or Ca^{2+}-independent components are subtracted. Already these experiments point out that the coupling ratio between Na^+ and Ca^{2+} is probably at least 5.

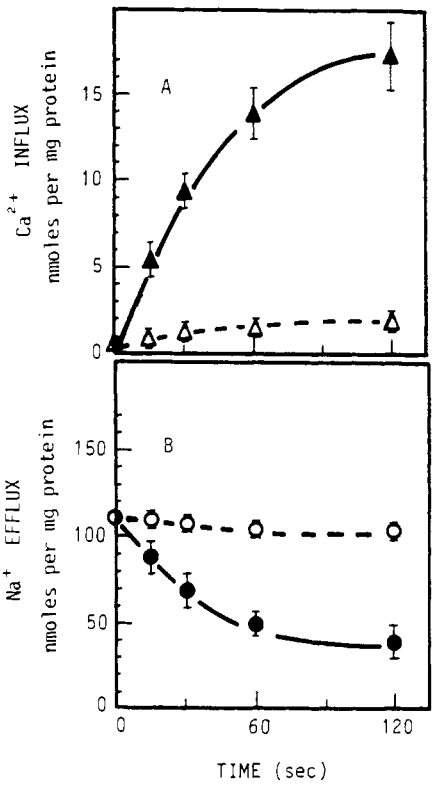

FIGURE 1: Stoichiometry of Na^+–Ca^{2+} antiport. (A) Three microliters (30 μg of protein) of SPM vesicles preloaded in buffered 0.15 M NaCl was diluted into either 100 μL of 0.15 M KCl, 0.01 M Tris-HCl, pH 7.4, and 50 μM $^{45}CaCl_2$ (▲) or 100 μL of 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4 (△). $^{45}Ca^{2+}$ influx was measured at the time points specified. Three microliters (30 μg of protein) of SPM vesicles preloaded in buffered 0.15 M $^{22}NaCl$ was diluted into 100 μL of 0.15 M KCl and 0.01 M Tris-HCl, pH 7.4, with (●) or without (○) 50 μM $^{40}CaCl_2$. $^{22}Na^+$ efflux was measured at the time points specified. The data presented are means of five different experiments; each point was done in triplicate. The bars represent standard deviations. The initial three points of each graph were extrapolated to 60 s, and the ratio between Na^+ efflux and Ca^{2+} influx after subtraction of the Na^+ gradient and Ca^{2+}-independent fluxes was found to be 4.82.

Several reasons led us to reexamine this finding in other ways. One major reason was, that if other Na^+ or Ca^{2+} permeabilities were present in the preparation, with rates much faster than Na^+–Ca^{2+} antiport, our measurements would be grossly distorted.

Effect of Valinomycin in Native and Reconstituted Vesicles. Another reason that led us to try and reexamine the question of stoichiometry of Na^+–Ca^{2+} antiport stemmed from the experiment presented in Figure 2A. In this experiment, the possible effects of the membrane potential on the antiport were examined. Figure 2A shows the results obtained when sodium-loaded native synaptic plasma membrane vesicles were diluted into a KCl solution containing calcium, in the presence

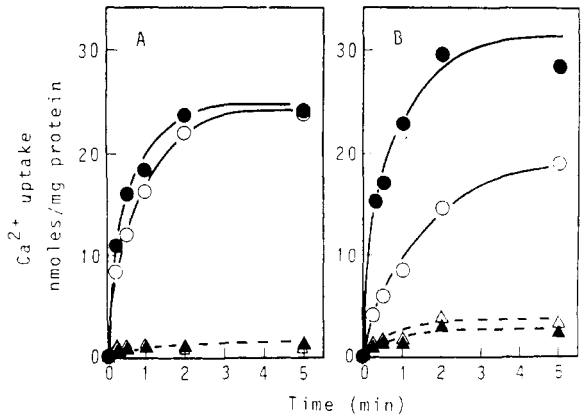

FIGURE 2: Effect of membrane potential on Na^+ gradient dependent Ca^{2+} influx. (A) Five microliters of native SPM vesicles (30 μg protein) was preloaded in 0.15 M NaCl and 0.01 M Tris-HCl buffer, pH 7.4, and diluted into 245 μL of 0.15 M KCl, 0.01 M Tris-HCl, pH 7.4, and 50 μM $^{45}CaCl_2$ with (●) or without (○) 20 μM valinomycin. (B) The same type of experiment as in (A) except that reconstituted SPM vesicles were used and the amount of protein was 5 μg/5 μL. The effect of valinomycin on Na^+ gradient independent Ca^{2+} uptake is also shown, in the presence (▲) and in the absence (△) of added valinomycin.

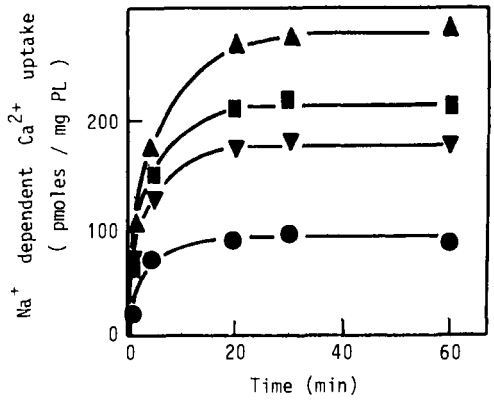

FIGURE 3: Time dependence of Na^+ gradient dependent Ca^{2+} uptake in reconstituted SPM vesicles at different external Na^+ concentrations. SPM vesicles (15 μg of protein) were preloaded in 0.12 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.03 M KCl during reconstitution. Five microliters of these vesicles was diluted into 200 μL of a solution containing varying amounts of NaCl, balancing amounts of choline chloride, 0.03 M KCl, 0.01 M Tris-HCl, pH 7.4, 50 μM $^{45}CaCl_2$, and 20 μM valinomycin. Ca^{2+} uptake was measured at 0, 0.5, 5, 20, and 30, and 60 min at 23°C. Na^+ gradient independent Ca^{2+} uptake has been subtracted. External [Na^+] used was 0.11 (●), 0.1 (▼), 0.09 (■), and 0.08 M (▲).

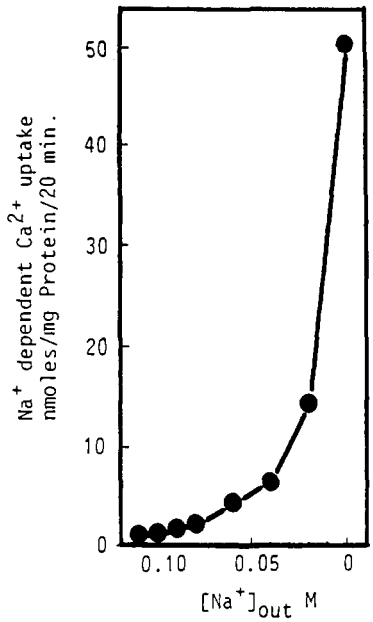

FIGURE 4: Effect of external Na^+ concentration on Na^+ gradient dependent Ca^{2+} uptake into reconstituted vesicles. SPM vesicles were preloaded in 0.12 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.03 M KCl during reconstitution. Five microliters of these vesicles (10 μg of protein) was diluted into 200 μL of a solution containing varying amounts of NaCl, balancing amounts of choline chloride, 0.03 M KCl, 0.01 M Tris-HCl, pH 7.4, 50 μM $^{45}CaCl_2$, and 20 μM valinomycin. Ca^{2+} uptake was measured after 20 min at 23 °C. Na^+ gradient independent Ca^{2+} uptake has been subtracted.

or in the absence of valinomycin. From the stoichiometry measured (see Figure 1), we expected that if no other ions were cotransported via the Na^+–Ca^{2+} exchanger in the course of the antiport reaction or in parallel with it, the addition of valinomycin to the system should have a considerable stimulatory effect on Ca^{2+} influx. Although always consistently observed, the stimulatory effect of valinomycin was rather small. The reason is probably the fact that the native synaptic plasma membrane vesicles were highly permeable to potassium even in the absence of valinomycin as indicated by measurements done employing ^{86}Rb (not shown here). Therefore, we decided to repeat this experiment, except that this time employing reconstituted vesicles. In this preparation, we knew (from measuring $^{86}Rb^+$ fluxes) that the passive K^+ permeability was very small. Figure 2B shows such an experiment which is identical with the one shown in Figure 2A except that reconstituted SPM vesicles are used. The addition valinomycin had a very pronounced effect on the Na^+ gradient dependent Ca^{2+} uptake.

Stoichiometry in Reconstituted Vesicles. The experiment in Figure 2B demonstrates the considerable benefits offered by the reconstituted preparation for studying the stoichiometry of the Na^+–Ca^{2+} antiport process. It demonstrated that positive inside polarization of the membrane increases considerably the rate of Na^+ gradient dependent Ca^{2+} influx, as expected for a process in which more Na^+-derived charges are exchanged than Ca^{2+}-derived charges. We therefore decided to reexamine the stoichiometry of the reaction with this preparation and this time by employing the equilibrium approach. To do so, we started by measuring the time dependence of Na^+ gradient dependent Ca^{2+} influx at different Na^+ gradients. Figure 3 shows that even when a shallow Na^+ gradient is performed across the reconstituted vesicles' membrane (0.12 M NaCl inside and 0.11–0.08 M NaCl outside), equilibrium is reached in 20 min. Figure 4 shows the effect of external sodium concentration on the Na^+ gradient dependent Ca^{2+} influx. The reconstituted vesicles were preloaded as described under Experimental Procedures in a buffered 0.12 M NaCl and 0.03 M KCl solution during their formation. They were diluted into an external isoosmotic solution containing varying amounts of NaCl, a balancing amount of choline chloride, 0.03 M KCl, and valinomycin. Calcium influx was measured under conditions of equilibrium (20 min of influx), zero (or close to zero) membrane potential, and a variable sodium gradient. The calcium associated with the vesicles in the absence of a sodium gradient was determined and subtracted. (This value never exceeded 10% of the values obtained in the presence of a sodium gradient.) As shown in Figure 4, the relation between the external sodium concentration and the amount of calcium transported exhibits a more than linear behavior.

In order to calculate the respective Na^+ and Ca^{2+} gradients across the vesicles' membrane, two types of control experiments were performed. In the experiment shown in Figure 5, the dissipation of the Na^+ gradient during the 20 min of Ca^{2+} uptake was measured. The experimental conditions were

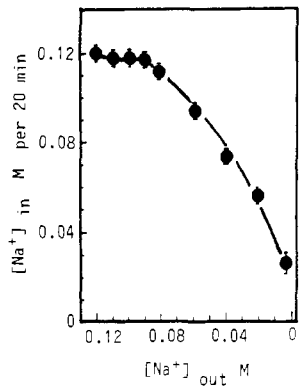

FIGURE 5: Retention of internal Na^+ concentration by reconstituted vesicles at different external Na^+ concentrations. SPM vesicles were preloaded in medium identical with that in Figure 4 and diluted into a solution containing varying amounts of buffered NaCl, balancing amounts of choline chloride, 0.03 M KCl, and 20 μM valinomycin. $^{22}NaCl$ was used instead of unlabeled NaCl. $^{22}Na^+$ content of the vesicles was measured for 20 min. The bars represent standard deviations measured in two different reconstitution experiments, and each point was done in quadruplicate.

similar to those in Figure 4 except that $^{22}NaCl$ was used and Ca^{2+} was omitted from the external medium. The $^{22}Na^+$ content of the vesicles was measured for 20 min at all external Na^+ concentrations specified. It can be seen (Figure 5) that practically no $^{22}Na^+$ loss occurs from the vesicles as long as the external Na^+ concentration is maintained above 0.08 M.

The second type of control experiment was designed to try and estimate the proportion of free vs. bound Ca^{2+} on the internal face of the vesicles. This was done by diluting NaCl-preloaded reconstituted vesicles (internal composition as in Figure 4) into an identical NaCl-containing medium in the presence of $^{45}Ca^{2+}$ and the Ca^{2+} ionophore A23187. Following 20 min of equilibration, the $^{45}Ca^{2+}$ content of the vesicles was measured, and the average internal Ca^{2+} concentration was calculated. In different experiments (up to 10 mM external Ca^{2+}), the intravesicular total Ca^{2+} concentration exceeded only by 12–17% the external Ca^{2+} concentration. This excess represented the bound Ca^{2+} in equilibrium with the free Ca^{2+}. The same type of experiment was repeated also with an external KCl-containing medium, and the results obtained were similar. Addition of 0.1% Triton X-100 led to almost complete Ca^{2+} loss from the vesicles.

From these and similar experiments, both the Ca^{2+} and the respective Na^+ gradients can be calculated with reasonable confidence and inserted into the equation described in the legend of Figure 6. Therefore, plotting the natural logarithm of the Na^+ and Ca^{2+} gradients should yield a straight line (see legend of Figure 6). The slope of this line is the required "coupling" ratio between Na^+ and Ca^{2+} ions. Figure 6 shows these data. The first four points (marked by solid circles) are experimental data; the next two points (marked by solid triangles) are experimental data corrected for $^{22}Na^+$ loss. The solid line connecting the points represents the linear regression equation. The calculated slope of this curve is 4.61.

In principle, using the equilibrium approach, one should be able to calculate r—the coupling ratio between Na^+ and Ca^{2+} ions from both the slope and the intercept with the ordinate (see legend to Figure 6). In practice, however, we find the slope to be a much more reliable measure for several reasons: In experiments such as those shown in Figures 4 and 6, it is reasonable to assume that equal internal and external K^+ concentrations and addition of valinomycin to the external medium should "short circuit" the electrogenic action of the exchanger and the membrane potential should be zero (or very

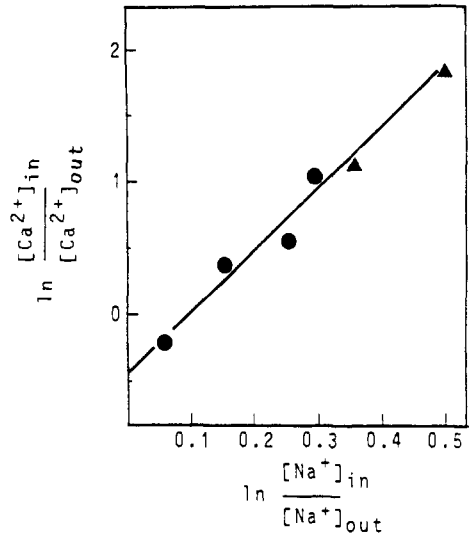

FIGURE 6: Relationship between the natural logarithms of the respective Ca^{2+}–Na^+ gradients. The natural logarithm of the calculated internal to external calcium concentration is plotted against the natural logarithm of the internal to external Na^+ concentration. The internal volume of the vesicles as determined from [^{3}H]glucose space (see Experimental Procedures) is 1.35 μL/mg of phospholipid. The data points are taken from an experiment identical with that presented in Figure 4 directly (●) or from Figure 4 and corrected by Figure 5 (▲); all other experimental conditions are identical as well. The electrochemical equilibrium equation $\ln([Ca^{2+}]^{in}/[Ca^{2+}]^{out}) = r \ln([Na^+]^{in}/[Na^+]^{out}) - (r-2)(F/RT)$ can be simplified under our experimental conditions ($\psi = 0$); r is the coupling ratio between the number of Na^+ ions exchanged for each Ca^{2+} ion. It is also the slope of the straight line obtained. (Slope = 4.61.)

close to it). However, even a very small residual membrane potential, due to unspecified surface charges, for example, will have a major effect on the intercept. In six experiments such as those presented here, the average intercept obtained was −0.62 (SD = 0.192). The average slope, on the other hand, was 4.766 (SD = 0.57). The variability in the slope is therefore much smaller than the variability of the intercept. In two additional experiments (data not shown) under conditions of positive inside membrane polarization, the average slope was 5.185 while the average intercept was −1.55 (0.15 SD). In addition, as long as the Ca^{2+} binding sites within the vesicles are not saturated (below 10 mM Ca^{2+}) and the proportion of free Ca^{2+} in equilibrium with the bound one remains constant, the slope of the straight line obtained in Figure 6 will remain the same.

These data show that the minimal integer coupling factor between Na^+ ions transported in one direction and calcium ions transported in the opposite direction is 5.

Contribution of the Na^+–Ca^{2+} Exchanger to the Membrane Potential. The stoichiometry of close to 5 (Figures 1 and 6) and the effects of positive inside membrane polarization on the Na^+–Ca^{2+} exchanger (Figure 2B) indicated that one should be able to demonstrate charge movements resulting from Na^+ gradient dependent Ca^{2+} uptake. The reconstituted preparation, which is relatively impermeable to passive monovalent ion fluxes and in which the Na^+–Ca^{2+} exchanger is probably reconstituted into different liposomes than other Na^+- or Ca^{2+}-carrying proteins, was chosen for this purpose. The experiment shown in Table I demonstrates the contribution of the Na^+–Ca^{2+} exchanger itself to the membrane potential. Reconstituted SPM vesicles preloaded with 0.14 M NaCl, 0.01 M KCl, and 0.01 M Tris buffer during their formation were diluted into 0.01 M KCl and 0.01 M Tris-HCl, pH 7.4, in 0.14 M choline chloride. The medium also contained unlabeled Ca^{2+} and the lipophilic cation TPP^+. It can be seen that in

Table I: Contribution of Na^+–Ca^{2+} Exchanger Activity to Changes in Membrane Potential in Reconstituted Vesicles

time (s)	[medium]^{out} composition[a] choline chloride (M)	NaCl (M)	$CaCl_2$ (μM)	EGTA (μM)	TPP^+ uptake (nmol/mg of PL)	Ca^{2+} uptake (nmol/mg of PL)
0	0.14	0	50	0	0.183 ± 0.1	0.012
15	0.14	0	50	0	0.671 ± 0.08	0.39 ± 0.08
0	0.14	0	0	100	0.107 ± 0.024	
15	0.14	0	0	100	0.195 ± 0.05	
0	0	0.14	50	0	0.159 ± 0.024	0.014
15	0	0.14	50	0	0.166 ± 0.014	0.068 ± 0.01

[a] 0.01 M KCl, 0.035 mM TPP^+, and 0.01 M Tris-HCl, pH 7.4, were present in all experiments. The ± values represent standard deviations. Five microliters of reconstituted vesicles (15 μg of protein) containing 0.14 M NaCl, 0.01 M KCl, and 0.01 M Tris-HCl, pH 7.4, was diluted into 200 μL of external medium as specified. In the experiments measuring [^{3}H]TTP uptake (0.04 μCi/200 μL), the Ca^{2+} (when added) was unlabeled while in the parallel experiments in which Ca^{2+} uptake was measured, $^{45}Ca^{2+}$ (0.1 μCi/200-μL reaction mixture) was used, and the TPP^+ added was unlabeled. PL = purified brain phospholipids.

the absence of valinomycin-induced K^+ fluxes (external choline chloride) a considerable amount of TPP^+ is taken up by the vesicles upon activation of the Na^+-Ca^{2+} antiporter (addition of Ca^{2+}). Under conditions when the Na^+–Ca^{2+} exchanger is not activated such as in the absence of added external Ca^{2+} and in the presence of EGTA, in the presence of Ca^{2+} and the absence of the driving Na^+ gradient, only a small amount of TPP^+ is taken up by the vesicles. This amount represents probably the amount of TPP^+ solubilized in the membrane and in equilibrium with the outside. In parallel experiments, Na^+ gradient dependent Ca^{2+} uptake was measured by using the same reaction medium as that used for TPP^+ uptake, except that the Ca^{2+} was labeled and the TPP^+ unlabeled.

These experiments demonstrate that, in the reconstituted vesicles, the "signal to noise" ratio is large enough to show the contribution of the Na^+ gradient dependent Ca^{2+} uptake activity to the changes in the membrane potential.

Discussion

The importance of the Na^+–Ca^{2+} exchanger in regulating intracellular Ca^{2+} ion concentration in nerve cells and thus neurotransmitter liberation from nerve terminals stems from several reasons. The activity of the antiporter is controlled by at least five parameters: intra- and extracellular sodium and calcium ion concentrations and the membrane potential. Physiological experiments mainly from the squid giant axon show that below the exchanger's reversal potential (−25 mV in the squid) it extrudes Ca^{2+} from the cell. Hyperpolarization of the membrane leads to an increase in the amount of Ca^{2+} extruded from the cell (Mullins, 1981b). Depolarization of the membrane, on the other hand, above its reversal potential causes the exchanger to serve as a calcium entry pathway into the cell. Therefore, in neuronal preparations that are continuously undergoing large changes in membrane polarization the Na^+–Ca^{2+} exchanger can serve in multiple roles. Moreover, under conditions of steady-state depolarization, when ionic channels inactivate (Mullins, 1981b; Baker et al., 1973), the exchanger might assume a unique role in controlling Ca^{2+} influx. In view of all this, the question of the exchanger's stoichiometry is of considerable importance. Theoretical considerations (Mullins, 1977, 1981a,b) based on measurements of intra- and extracellular Na^+ and Ca^{2+} levels in the squid, and the assumption that $[Ca^{2+}]_{in}$ is controlled exclusively by the exchanger, demand that at least four Na^+ ions should be exchanged for each Ca^{2+} ion. Much higher numbers than 4 were also proposed (Horackova & Vassort, 1979; Lederer & Nelson, 1981).

In the present work, we have explored the advantages of the reconstituted Na^+–Ca^{2+} exchanger derived from brain synaptic plasma membranes for studying its stoichiometry. Quantitative measurements based on initial rate measurements in the native membrane vesicle and equilibrium values obtained in the reconstituted preparation point to a stoichiometry of at least five Na^+ exchanged for each Ca^{2+}. Kinetic and equilibrium measurements performed previously (Pitts, 1979; Reeves & Hale, 1984) employing native sarcolemmal vesicles from heart yielded a ratio of three Na^+ ions exchanged for each Ca^{2+}. It is quite possible that this difference stems from inherent differences between sarcolemmal vesicles and synaptic plasma membrane ones.

One should bear in mind two limitations of this system: One of them is that contrary to physiological preparations of single neuronal cells we are dealing with a heterogeneous population of nerve endings. We cannot determine whether the stoichiometry of 5 obtained here applies to all nerve terminals or is a result of mixed SPM Na^+–Ca^{2+} exchangers, some of them with coupling ratios below 5 and others above it.

The preparation of SPM vesicles followed by reconstitution of the membrane proteins into a highly phospholipid-enriched vesicle leads not only to removal of cytoplasmic components that might be of importance in regulating the activity of the Na^+–Ca^{2+} exchanger (Baker et al., 1973; Caroni & Carafoli, 1983) but also to possible separation between the exchanger protein itself and some regulatory subunit that might be of relevance to the activity when present in the native membrane. It will be possible to overcome these difficulties, however, when an identification of the Na^+–Ca^{2+} exchanger protein is successfully done. Then, one should be able to reconstitute the "functional" protein entity used in the present work with its possible "regulatory" parts and study the role of added cytoplasmic components in modulating their combined activity.

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Registry No. Na, 7440-23-5; Ca, 7440-70-2.

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A Differential Scanning Calorimetric Study of the Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Linear Saturated Fatty Acyl Chains†

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ABSTRACT: The thermotropic phase behavior of a series of 1,2-diacylphosphatidylcholines containing linear saturated acyl chains of 10–22 carbons was studied by differential scanning calorimetry. When fully hydrated and thoroughly equilibrated by prolonged incubation at appropriate low temperatures, all of the compounds studied form an apparently stable subgel phase (the L_c phase). The formation of the stable L_c phase is a complex process which apparently proceeds via a number of metastable intermediates after being nucleated by incubation at appropriate low temperatures. The process of L_c phase formation is subject to considerable hysteresis, and our observations indicate that the kinetic limitations become more severe as the length of the acyl chain increases. The kinetics of L_c phase formation also depend upon whether the acyl chains contain an odd or an even number of carbon atoms. The L_c phase is unstable at higher temperatures and upon heating converts to the so-called liquid-crystalline state (the L_α phase). The conversion from the stable L_c to the L_α phase can be a direct, albeit a multistage process, as observed with very short chain phosphatidylcholines, or one or more stable gel states may exist between the L_c and L_α states. For the longer chain compounds, conversions from one stable gel phase to another become separated on the temperature scale, so that discrete subtransition, pretransition, and gel/liquid-crystalline phase transition events are observed. We have also examined some aspects of the hysteresis in the pretransition, and our observations indicate that the behavior can be approximated by a reversible process which is subject to some modest kinetic limitations, these limitations again becoming more severe for the longer chain phosphatidylcholines.

Lipid bilayers composed of phosphatidylcholines (PCs)[1] containing two identical linear saturated fatty acyl chains are the most thoroughly studied of the model membrane systems. Such studies have employed a variety of physical techniques and have tended to concentrate on a few members of the homologous series, with the result that the thermotropic phase behavior of some of these PCs (especially DPPC) is relatively well understood. From the extensive literature on these compounds, it is expected that when fully hydrated and thoroughly equilibrated by prolonged incubation at low temperatures, the longer chain PCs like DPPC ($n \geq 16$) form a highly ordered, condensed, crystallike phase (the L_c phase) in which the hydrocarbon chains are in a fully extended, all-trans conformation (Cameron & Mantsch, 1982) with their long axes tilted to the bilayer normal (Ruocco & Shipley, 1982). In that phase, the phosphate head groups are relatively immobile (Fuldner, 1981; Lewis et al., 1984) and are assumed to lie parallel to the bilayer plane (Pearson & Pascher, 1979; Griffin et al., 1978), and the interfacial region of the lipid bilayer is partially dehydrated (Camerson & Mantsch, 1982). Upon heating, the L_c phase

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[1] Abbreviations: DSC, differential scanning calorimetry; DTA, differential thermal analysis; NMR, nuclear magnetic resonance; PC, phosphatidylcholine (in this paper, a phosphatidylcholine is usually designated by the notation *n*:0-PC, where *n* is the number of carbon atoms per acyl chain with the zero indicating the absence of double bonds); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; HS-DSC, high-sensitivity differential scanning calorimetry.

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