Biochimica et Biophysica Acta, 1066 (1991) 208–218

ADONIS 0005273691002846

BBAMEM 75298

Na^+-Ca^{2+} exchange activity in synaptic plasma membranes derived from the electric organ of *Torpedo ocellata*

Michela Tessari * and Hannah Rahamimoff

Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem (Israel)

(Received 17 December 1990)

Key words: Sodium–calcium ion exchange; Calcium ion regulation; Calcium ion transport; Synaptic membrane; Electric organ; (*T. ocellata*)

Synaptic plasma membranes obtained by hypo-osmotic treatment of purified *Torpedo ocellata* synaptosomes, contain an electrogenic Na^+-Ca^{2+} exchange system. The dependence of the initial reaction rate on $[Ca^{2+}]$ reveals a single binding site for Ca^{2+} with an average apparent K_m of 13.66 (S.D. = 12.07) μM $[Ca^{2+}]$ and maximal reaction velocity of V_{max} = 11.33 (S.D. = 5.93) nmol/mg protein per s. The dependence of the initial rate of the Na^+ gradient dependent Ca^{2+} influx on the internal $[Na^+]$ exhibits a sigmoidal curve which reaches half-maximal reaction rate at 170.8 (S.D. = 19.9) mM $[Na^+]$. Addition of ATPγS does not change the $K_{0.5}$ to Na^+. The average Hill coefficient is 3.09 (S.D. = 0.86) indicating that 3–4 Na^+ ions are exchanged for each Ca^{2+}. Na^+ gradient dependent Ca^{2+} uptake in *Torpedo* SPMs takes place also in the absence of K^+ suggesting that K^+ co-transport is not obligatory. The temperature dependence of the initial and steady-state rates of Na^+ gradient dependent Ca^{2+} influx reveal that maximal reaction velocities of the *Torpedo* exchanger are attained between 15 and 20 °C. The energy of activation between 0 and 20 °C is 20826 cal/mol. In comparison, rat brain synaptic plasma membrane Na^+-Ca^{2+} exchanger reaches maximal reaction rates between 30 and 40 °C. Reconstitution of Torpedo or rat brain Na^+-Ca^{2+} exchangers into a membrane composed of either *Torpedo* or brain phospholipids, does not alter the temperature dependence of the native *Torpedo* or rat brain Na^+-Ca^{2+} exchangers; inspite of considerable differences in the composition of the fatty acyl chains that are esterified to brain and *Torpedo* phospholipid head groups and differences in membrane fluidity that were detected. An ATP-dependent Ca^{2+} pump, which is insensitive to FCCP, is also present in the same synaptic membrane.

Introduction

Intracellular calcium ion concentration is of key importance in the regulation of neuronal cell function [1]. The Na^+-Ca^{2+} exchanger is one of the major Ca^{2+} regulating molecules found in all excitable and many nonexcitable cells [2]. In this work we examine the existence and properties of the Na^+-Ca^{2+} exchanger in synaptic plasma membranes isolated from nerve terminals (synaptosomes) of the electric fish *Torpedo ocellata*. We consider this study to be of interest for three independent reasons. First, the *Torpedo* electric organ is a pure cholinergic synapse and there is no previous knowledge of Na^+-Ca^{2+} exchange process in the cholinergic nervous system. Second, the *Torpedo* electric organ has been for many years the choice biochemical model to study synaptic transmission. Thus, in this system acetylcholine metabolism [3] storage in vesicles [4,5] and its release [6] were studied in detail. Recently, some of the proteins present in synaptic vesicles were identified [7,8] although their role has yet to be resolved. This is also the site where vesicular ionic channels were found [9]. The third reason why we consider

* On leave from the Department of Pharmacology, University of Padova, Italy. Present address: Department Farmacologia SN, GLAXO, via Fleming 2, 37135 Verona, Italy.

Abbreviations: SPM(s), synaptic plasma membrane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; DPH, 1,6-diphenyl-hexa-1,3,5-triene; NaP_i, sodium phosphate buffer; KP_i, potassium phosphate buffer; BPL(s), calf brain phospholipid(s); TPL(s), *Torpedo* electric organ phospholipid(s); FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; βME, β-mercaptoethanol; ATPγS, adenosine (5'-O^3)-1-thiotriphosphate.

Correspondence: H. Rahamimoff, Department of Biochemistry, Hebrew University-Hadassah Medical School, P.O.Box 1172, Jerusalem 91010, Israel.

this study of the Na^+-Ca^{2+} exchanger to be of interest is the variability of the properties of Na^+-Ca^{2+} exchangers in different cell preparations. These variabilities are manifested as different stoichiometries of the process [10–13], different K^+ dependence [14–17], variable regulation by ATP [18,19] and its variable kinetic parameters especially in membrane vesicles [20]. Some of these differences might indicate that different molecular entities are involved in Na^+-Ca^{2+} exchange in different tissues. Synaptic membranes isolated from rat or guinea pig brain [12,21–25] which served for previous studies on the neuronal Na^+-Ca^{2+} exchanger are a heterogeneous source derived from different neuronal cells. In addition, they are a rather poor source of the exchanger protein with low site density [25] resulting in an apparently 50-fold lower V_{max} than sarcolemmal membranes [20]. The *Torpedo ocellata* Na^+-Ca^{2+} exchanger described in this work, offers a neuronal preparation of high maximal velocity, comparable to that of the cardiac Na^+-Ca^{2+} exchanger, that originates from a homogeneous cholinergic preparation.

Materials and Methods

Preparation of synaptic membranes. Synaptosomes were prepared from freshly excised electric organs of *Torpedo ocellata* a member of the Torpedinidae elasmobranch fish family which can be caught between January and June off the Mediterranean coast of Israel. The fish were used within several days of capture. They were anaesthetized by chilling on ice prior to removal of the electric organs. The method developed by Michaelson and Sokolovsky [26] to obtain purified synaptosomes was used without any modification. In principle this involved homogenization of the organs at 15% (w/v) in 0.8 M glycine, 1 mM EGTA (pH 6.8), centrifugation at $1000 \times g$ for 10 min, collection of the supernatant (S1) which was centrifuged for 45 min at $20000 \times g$ to obtain the pellet 'P2'. The P2 pellet was fractionated by a discontinuous sucrose gradient from which the material banding at the interface between 0.15 and 0.3 M sucrose (a_2) contained purified synaptosomes. The synaptosomes were diluted 3–4-fold with 0.8 M glycine/1 mM EGTA (pH 6.8) and recentrifuged at $27000 \times g$ for 20 min, after which they were lysed by exposure to 10 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA at 4°C for 20 min. The synaptic membranes were collected by sedimentation at 27000 $\times g$ for 15 min. Each membrane preparation consisted of 2–4 fish depending on their size which varied. The membranes were frozen in small aliquots in liquid N_2 and kept at $-80\,^\circ C$.

Na^+ gradient dependent Ca^{2+} transport and ATP dependent Ca^{2+} transport activities were stable for over one year.

Characterization of the synaptic membrane fraction. Subfractionation of the P2 pellet on the discontinuous sucrose gradient (see previous paragraph) revealed that Na^+-Ca^{2+} exchange activity was detected only in membranes which were prepared from the material collected from the interface between 0 and 0.15 M sucrose (a_1, lowest density) and from the interface between 0.15 and 0.3 M sucrose (a_2, the density from which synaptosomes are harvested). The total amount of material in the low density sucrose fraction was very small and it was not used in this study. Specific ^{125}I-α-bungarotoxin binding (assayed as described by Schmidt and Raftery [27] was 0.00141–0.00158 nmol/mg protein in the P2 pellet. Among the sucrose gradient fractions, the highest α-bungarotoxin binding (0.011–0.022 nmol/mg protein) was obtained in fractions of densities higher than 0.3 M where no Na^+-Ca^{2+} exchange activity was detected. The range of ^{125}I-α-bungarotoxin binding in membranes obtained from the synaptosomal fraction (0.15 M–0.3 M sucrose interface) was between 0.00198 and 0.00568 nMoles/mg protein, indicating some post synaptic contamination. Since, however, fractions of high α-bungarotoxin binding capacity had no Na^+-Ca^{2+} exchange activity, this contamination was of no considerable importance. In a similar fashion to other plasma membranes, the synaptic membrane fraction used in this study had a 5'-AMP nucleotidase activity of 2.75–5.38 nmol P_i/mg protein per min. The ATP-dependent $^{45}Ca^{2+}$ uptake activity which was present in the same membrane fraction as the Na^+ gradient dependent Ca^{2+} transport activity (see Results) was insensitive to the addition of FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone), indicating that it was not a mitochondrial contaminate. To test whether contamination from synaptic vesicles could be responsible for the Ca^{2+} transport activities detected, a purified preparation of *Torpedo* synaptic vesicles was prepared as described by Tashiro and Stadler [5]. The ATP-dependent Ca^{2+} transport activity in the synaptic vesicle fraction ranged between 5 and 10 nmol/mg protein per 10 min and their Na^+-Ca^{2+} exchange activity did not exceed 2 nmol/mg protein per 10 min.

Determination of Ca^{2+} uptake in native membrane vesicles. Na^+ gradient dependent Ca^{2+} uptake was determined following preloading of the membrane vesicles in high Na^+ containing solutions. This was done by their incubation with a 40–50-fold volume excess of the desired loading solution (usually 0.4 M NaCl, 10 mM Tris-HCl (pH 7.5) or 0.4 M NaP_i (pH 7.5)) at 37 °C for 20 min, after which they were concentrated by centrifugation at $27000 \times g$ for 20 min at 4 °C and resuspended in a minimal volume of the same high Na^+ containing solution to give a protein concentration of about 3–5 mg per ml. The exact composition of the loading solutions is described in the legends to

the appropriate figures or tables. Usually 3 μl of Na^+-loaded vesicles was diluted rapidly into 250 μl of Na^+-free isoosmotic solution containing 50 μM $^{45}Ca^{2+}$ to initiate Na^+ gradient dependent Ca^{2+} uptake. Zero-time measurements and Ca^{2+} uptake when external [Na^+] was equal to internal one (no Na^+ gradient) were determined as well. The reactions were terminated by dilution with 2 ml of iso-osmotic KCl solution and filtration through Schleicher and Schuell 0.45 μm nitrocellulose filters. The filters were rinsed two more times with the same KCl solution, dried and counted in a liquid scintillation counter. The measurements were carried out in triplicates and each type of experiment was repeated at least five times with different batches of membranes.

ATP-dependent Ca^{2+} uptake was determined after preloading the vesicles in either 0.4 M KCl, 10 mM Tris-HCl (pH 7.5) or 0.4 M KP_i (pH 7.5) and dilution of 10 μl of these vesicles into 100 μl of identical medium to the internal one, except that it contained also 100 μM $^{45}Ca^{2+}$, 5 mM $MgCl_2$ and 2 mM ATP-Tris. Zero-time controls and measurements of the Ca^{2+} associated with the vesicles in the absence of added ATP were also done. The reactions were terminated as described for Na^+ gradient dependent Ca^{2+} uptake.

Na^+-dependent Ca^{2+} efflux was determined in the following manner. Vesicles were loaded up with $^{45}Ca^{2+}$ in an ATP-dependent manner (see above) for 30 min, at the end of which they were collected by centrifugation for 20 min at 27000 × *g* at 4 °C. The vesicles were resuspended in a minimal volume of the same uptake medium and their specific $^{45}Ca^{2+}$ content was determined. Ca^{2+} efflux was initiated by diluting 3 μl of the vesicles into 250 μl of either 0.4 M NaCl, 10 mM Tris-HCl (pH 7.5) (to determine the Na^+ gradient dependent component of the Ca^{2+} efflux), or into 250 μl of 0.4 M KCl, 10 mM Tris-HCl (pH 7.5) (to determine the Na^+ gradient independent component of Ca^{2+} efflux) for time point specified. The reactions were terminated as described for Ca^{2+} influx measurements.

Na^+ gradient dependent Ca^{2+} uptake in reconstituted synaptic membranes. Torpedo SPMs were added at a weight ratio of 1:30 (membrane protein/added phospholipid) to a solution containing either purified calf brain phospholipids (BPL) of *Torpedo* electric organ derived phospholipids (TPL) in 2% sodium cholate, 0.4 M NaP_i (pH 7.5), 5 mM β-ME and 0.1 mM EDTA. The membranes were incubated in the solubilizing solution for 20 min at 37 °C, after which the insoluble residues were separated from the solubilized membranes by centrifugation at 27000 × *g* for 20 min. Reconstitution was carried out by passing the solubilized membrane-phospholipid mixture through three consecutive Sephadex G-50 minicolumns pre-equilibrated with 0.4 M NaP_i, 5 mM β-ME and 1 mM EDTA as described in detail [12]. 3 μl of reconstituted membrane vesicles were diluted into 250 μl of Na^+ free solution (0.4 M KCl, 0.01 M Tris-HCl (pH 7.4)) containing 50 μM $^{45}Ca^{2+}$ exactly as described for native membranes. The transport reactions were stopped by passing the reaction mixtures through a Dowex 50 minicolumn to separate intravesicular $^{45}Ca^{2+}$ from extravesicular one [28]. Zero-time controls and the amount of $^{45}Ca^{2+}$ associated with the vesicles in the absence of Na^+ gradient (0.4 M NaCl containing solution) were determined as well. When rat brain SPMs were used for comparison, the osmolarity of the

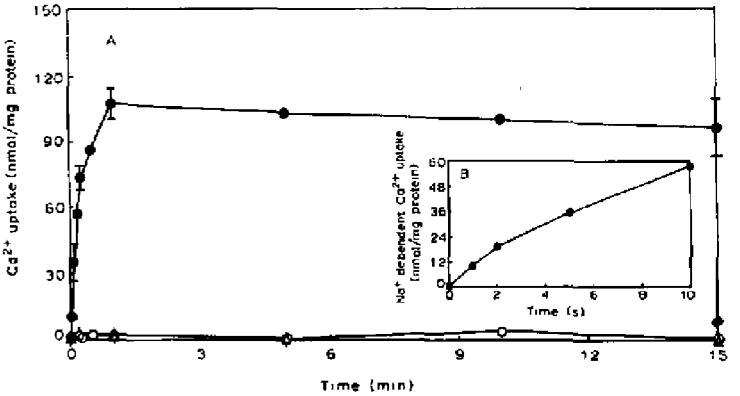


Fig. 1. Na^+ gradient dependent Ca^{2+} uptake in *Torpedo* SPMs. (A) 3 μl of *Torpedo* SPMs (about 10 μg protein) preloaded with 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) (see Materials and Methods) were diluted into 250 μl of: 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4), 50 μM $^{45}CaCl_2$ (0.1 μCi) (●), or the same medium except that it contained also 10 μM nigericin (△), or 10 μM A23187 (◆). 3 μl of the same vesicles were also diluted into 250 μl of 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4), 50 μM $^{45}CaCl_2$ (0.1 μCi) (○). The reactions were terminated as described in Materials and Methods at the time points indicated. The results shown are an average calculated from three different experiments using the same SPM preparation. Each time point was done in triplicate. The bars represent S.D. (B) Expanded scale of the initial time points of the Na^+ gradient dependent component of Ca^{2+} uptake.

solutions was 0.2 M; otherwise, all the experimental details were identical to those used with *Torpedo* membranes.

Preparation of phospholipids. Phospholipids were extracted from calf brains or *Torpedo* electric organs by the Bligh and Dyer extraction procedure as described previously [12]. Further purification involved silicic acid column chromatography, removal of neutral phospholipids by chloroform washes and extraction of the phospholipids by methanol/chloroform (75 : 25, v/v) as described [29].

Quantitative analysis of the phospholipids was carried out by two-dimensional thin-layer chromatography [30]. The spots located by exposure to I_2 were scraped from the plates and their phospholipid phosphate was determined by the method of Ames [31]. The fatty acid composition of the phospholipids used was determined by gas chromatography using a Tracor 540 instrument and Sillar 10C column following transesterification to their corresponding methyl esters using Meth-Prep commercial kit from (Alltech, Deerfield, Ill.).

Polarized fluorescence studies. Changes in reconstituted membrane fluidity were determined by measuring polarized fluorescence [32]. DPH (1,6-diphenylhexa-1,3,5-triene) was used as a probe. Equilibrium fluorescence studies were carried out in a Perkin-Elmer LS-5 spectrofluorometer with a xenon power supply. The measuring chamber was connected to a bath circulator (Haake FK) to obtain the desired temperature. The spectrofluorometer was connected to an L-shaped polarization unit with KSW38 polarizers (Polarex E. Kasemann). The temperature in the fluorometer chamber was monitored by a thermistor probe (Yellow Springs Inst. Co. Inc. YSI-423) connected to a YSI thermometer (Model 425C).

Protein determination. Protein was determined by the method of Lowry et al. [33].

Chemicals. $^{45}CaCl_2$ was purchased from Amersham, International, Amersham, U.K.; ATPγS (adenosine (5′-O^3)-1-thiotriphosphate) was purchased from Boehringer, Mannheim, F.R.G.; Biochemicals were purchased from Sigma, Israel; all reagents were analytical grade reagents.

Results

Torpedo nerve endings contain Na^+ gradient dependent and ATP-dependent Ca^{2+} transport systems in the same membrane

To demonstrate the presence of a Na^+-Ca^{2+} exchange system in *Torpedo* SPMs, they were preloaded with a high Na^+ containing solution (NaP_i or NaCl) and diluted into a Na^+-free solution (KCl or choline chloride) containing $^{45}Ca^{2+}$, establishing thus an outward oriented Na^+ gradient. The Na^+ gradient driven Ca^{2+} influx was determined by measuring the $^{45}Ca^{2+}$ content of the vesicles. The time course of Ca^{2+} influx into NaCl-loaded SPM vesicles is shown in Fig. 1. The Ca^{2+} taken up by the vesicles in the absence of a Na^+ gradient, $[Na^+]^{in} = [Na^+]^{out}$ or, in the presence of a high inside Na^+ gradient and nigericin (which dissipates the driving Na^+ gradient by exchanging internal Na^+ with external K^+) or, in the presence of the Ca^{2+} ionophore A23187, is also shown. It can be seen, that *Torpedo* SPMs contain a Na^+ gradient driven Ca^{2+} influx system. At 25 °C, the time course is quite rapid and initial rates can be measured up to 2 s (see Fig. 1B). Thereafter the rate declines and at 2 min already steady-state rates of Na^+ gradient driven Ca^{2+} uptake are obtained. Considerable differences were detected in the specific Na^+ gradient dependent Ca^{2+} transport activities measured in the different SPM preparations during the two fishing seasons in which *Torpedo* membranes were studied. In the experiments summarized in Fig. 1, membranes from preparation of 'intermediate' specific Na^+ gradient dependent Ca^{2+} transport activities were used. The ranges of steady-state transport activity detected in different preparations of *Torpedo* membranes which were all prepared from fresh tissue were between 50 and 200 nmol Ca^{2+} per mg of membrane protein in 10 min. Freezing one of the two electric organs in liquid N_2, even without prolonged storage prior to preparation of membranes, led to about 50% loss in transport activity as compared to the other electric organ from which membranes were prepared immediately after excision from the fish.

In addition to the Na^+ gradient driven Ca^{2+} transport system, the same membrane preparation takes up $^{45}Ca^{2+}$ also in the absence of a driving Na^+ gradient, if ATP is added to the reaction mixture. Fig. 2 shows *Torpedo* SPM vesicles preloaded in KP_i buffer (pH 7.4) diluted into the same external medium, to which ATP, Mg^{2+} and $^{45}Ca^{2+}$ were added. It can be seen, that addition of ATP to the reaction medium activates a

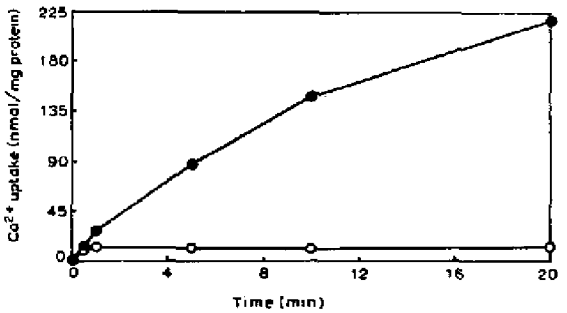


Fig. 2. ATP-dependent Ca^{2+} uptake in *Torpedo* SPMs. 3 μl of *Torpedo* SPMs (about 10 μg protein) were preloaded with 0.4 M KP_i buffer (pH 7.4). They were diluted into 250 μl medium of identical composition except that it contained also 2 mM ATP, 5 mM $MgCl_2$ and 100 μM $^{45}CaCl_2$ (0.1 μCi) (●), or the same medium without ATP (○).

Ca^{2+} transport system which operates in the absence of any monovalent ionic gradient. In the absence of ATP, very little Ca^{2+} is associated with the vesicles. Addition of FCCP (not shown) has no effect on the ATP dependent Ca^{2+} uptake.

The Na^+-driven and the ATP-driven Ca^{2+} transport systems dwell in the same vesicle

To determine whether both systems are present within the same membrane or they are within different membranes that co-sediment during the preparative procedure, the *Torpedo* SPM vesicles were preloaded with $^{45}Ca^{2+}$ in an ATP-dependent fashion as in the experiment shown in Fig. 2. After 30 min of Ca^{2+} uptake, the Ca^{2+}-loaded vesicles were separated from the uptake medium by centrifugation (as described in Materials and Methods), their $^{45}Ca^{2+}$ content was determined and they were diluted into an external isoosmotic NaCl containing medium to initiate Na^+-dependent Ca^{2+} efflux. The Na^+-independent component ('dilution-dependent component') of Ca^{2+} efflux was determined by diluting the vesicles into an isoosmotic KCl containing medium. The results of this experiment (shown in Fig. 3A and 3B) demonstrate that Ca^{2+} introduced into the vesicles via the ATP-dependent Ca^{2+} pump can be released from the vesicles via the Na^+ gradient driven Ca^{2+} transporter, indicating that both transporters are present within the same vesicular structure. In the experiment presented in Fig. 3, the $^{45}Ca^{2+}$ content of the vesicles after 30 min of ATP-dependent Ca^{2+} loading was 260 nmol/mg protein. As also with Na^+ gradient dependent Ca^{2+} influx, the Ca^{2+} content of the vesicles following ATP-dependent Ca^{2+} loading varied between different preparations of *Torpedo* membranes. The initial rate of Na^+ gradient dependent Ca^{2+} efflux in a similar manner to the Na^+ gradient dependent Ca^{2+} influx was rapid and linear till about 2 s, after which it declined. It can be seen that in 20 min 67% of the vesicles' total $^{45}Ca^{2+}$ content is lost, of this 64% is lost in a Na^+ gradient dependent manner. Although in the efflux experiment shown in Fig. 3 only 'inside out' vesicles participate, it demonstrates that the *Torpedo* Na^+ gradient dependent Ca^{2+} transporter is capable of transporting Ca^{2+} across the membrane in both directions.

Characterization of the Torpedo Na^+-Ca^{2+} exchanger

To characterize the *Torpedo* Na^+-Ca^{2+} exchanger, some of the kinetic properties of the transporter were determined. The dependence of the initial rate of internal high Na^+ gradient dependent Ca^{2+} influx on the external [Ca^{2+}] was measured between 5 and 200 μM. The total Ca^{2+} content of the solutions (in addition to the $^{45}CaCl_2$ that was used) was determined by atomic absorption spectrophotometric measurement and the final concentrations used were calculated. The

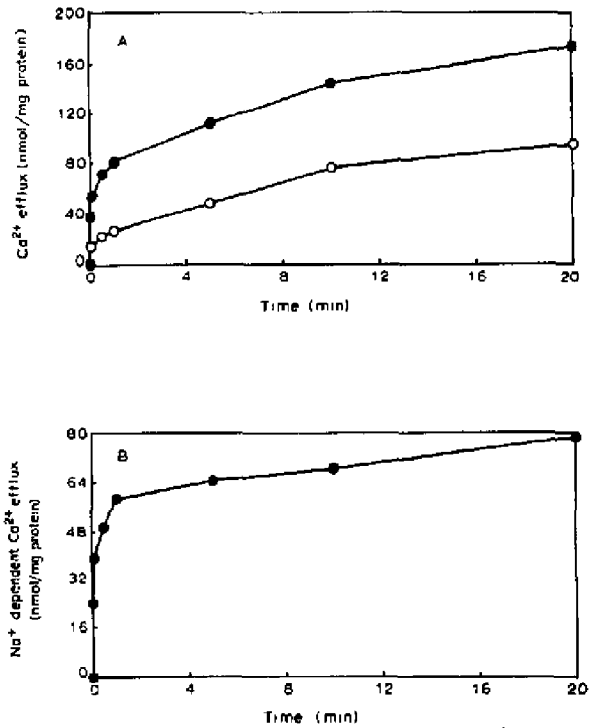


Fig. 3. Ca^{2+} efflux from *Torpedo* SPMs preloaded with Ca^{2+} in an ATP-dependent manner. (A) *Torpedo* SPM vesicles were preloaded with $^{45}Ca^{2+}$ in the presence of ATP-Mg^{2+} as described for Fig. 2. After 30 min of uptake the vesicles were concentrated by centrifugation and suspended in a minimal volume of the uptake medium (see Materials and Methods). 3 μl of these vesicles (about 10 μg protein) were diluted into either 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) (●), or 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4) (○) for the time points specified. The reactions were terminated as described and their $^{45}Ca^{2+}$ content determined. (B) The calculated net Na^+ dependent component of Ca^{2+} efflux obtained by subtracting the amount of Ca^{2+} lost from the vesicles by dilution into the KCl containing medium in Fig. 3A from the total amount of $^{45}Ca^{2+}$ lost from the vesicles by dilution into the NaCl containing medium in Fig. 3A.

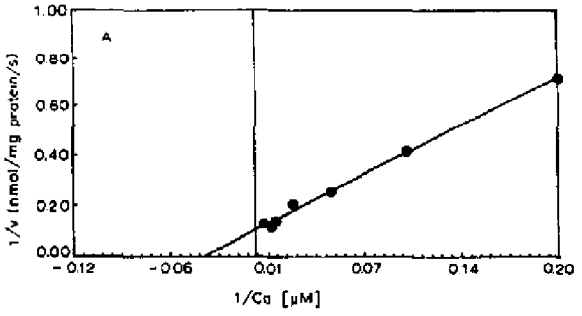
kinetic behaviour exhibited a Michaelis-Menten type curve, indicating that a single binding site for Ca^{2+} was involved. The linear form (Lineweaver-Burk plot) of such an experiment is shown in Fig. 4A. In cardiac sarcolemmal vesicles [34] and in rat brain SPM vesicles [25,35], a wide range of the apparent K_m values to Ca^{2+} were detected. This was also found in different preparations of *Torpedo* SPMs. The average values and the range of the different kinetic parameters of the *Torpedo* Na^+-Ca^{2+} exchanger are summarized in Table 1.

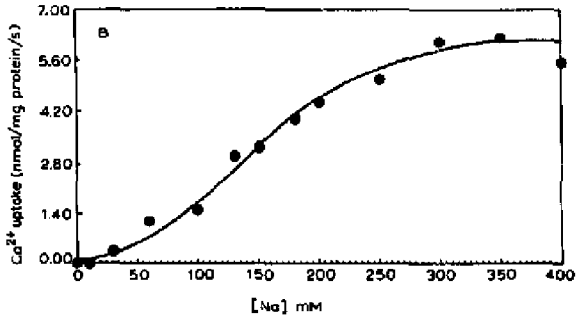
The initial rate of Ca^{2+} uptake, exhibited a sigmoidal dependence on [Na^+] (Fig. 4B), indicating, that more than one binding site of Na^+ was involved in the process. The half-maximal initial velocity ($K_{0.5}$) to Na^+ of the *Torpedo* Na^+-Ca^{2+} exchanger reached an aver-

age value of 170.8 mM [Na^+]; This value was within a narrow range (S.D. = 19.9). The corresponding Hill plot is shown in Fig. 4C. The values of Hill coefficients obtained (see Table I) indicate, that at least 3–4 Na^+ ions are exchanged for each Ca^{2+}.

In the squid giant axon [36–38] the $K_{0.5}$ to Na^+ in the absence of ATP is 110–300 mM. Addition of ATP [37,38] decreases this value to about 50 mM [Na^+]. ATPγS [39] can substitute for ATP in activating Na^+-Ca^{2+} exchange in the squid giant axon. It was of interest to examine if the $K_{0.5}$ to Na^+ decreases also in *Torpedo* SPMs when ATPγS is included in the reaction mixture.

Fig. 5 shows an experiment in which *Torpedo* SPM vesicles were preloaded with varying concentrations of





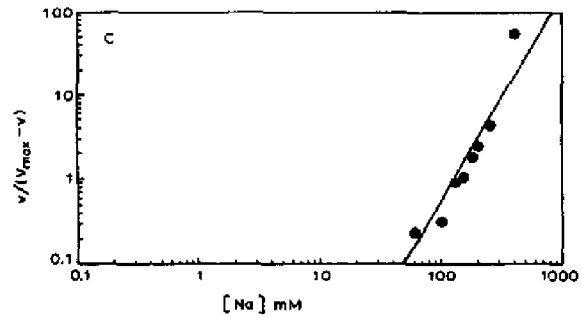


TABLE I

Kinetic parameters of the Torpedo Na^+-Ca^{2+} exchanger

[a] *Torpedo* SPMs were preloaded with 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4). Three μl of these vesicles (10 μg protein) were diluted into an external medium composed of 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4) or 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) and 5–200 μM $^{45}Ca^{2+}$ (0.2–0.1 μCi ^{45}Ca). For details see Fig. 4A.

[b] *Torpedo* SPMs were preloaded with 0–0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) and either balancing amounts of choline chloride or LiCl. 3 μl of these vesicles (10–15 μg protein) were diluted into an external medium containing 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$ (0.1 μCi). For details see Fig. 4B and Fig. 4C.

Torpedo Na^+-Ca^{2+} exchanger	Range	Average (S.D.)	No. of experiments
K_m Ca^{2+} (μM) [a]	1–30	13.66 (12.01)	8
V_{max} (nmol/mg protein per s)	5–26.5	11.33 (5.93)	11
$K_{0.5}$ Na^+ (mM) [b]	150–190	170.8 (19.9)	6
Hill coefficient	2.34–4.15	3.09 (0.85)	6

NaCl (0–0.4 M) with and without 1 mM ATPγS. LiCl was used to balance the osmolarity. To initiate Ca^{2+} uptake, the Na^+-loaded vesicles were diluted into a KCl-containing medium. When the vesicles were preloaded with Na^+ in the presence of 1 mM ATPγS the external medium contained ATPγS as well. When ATPγS was omitted from the vesicles internal medium, it was omitted from the uptake medium as well. All media contained 4 mM $MgCl_2$. It can be seen, that the

Fig. 4. [Ca^{2+}] and [Na^+] dependence of Na^+-Ca^{2+} exchange in *Torpedo* SPMs. (A) Lineweaver-Burk plot of [Ca^{2+}] dependence of Na^+ gradient dependent Ca^{2+} uptake. 3 μl *Torpedo* SPM vesicles (about 10 μg protein) were preloaded with 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4). They were diluted into 250 μl of medium composed of either: 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4) and 5–200 μM $^{45}CaCl_2$ (0.2–0.1 μCi), or 0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) and 5–200 μM $^{45}CaCl_2$ (0.2–0.1 μCi). The reactions were terminated after 1 s as described in Materials and Methods. The Na^+-dependent component of Ca^{2+} influx was determined. The Ca^{2+} uptake at each [Ca^{2+}] was carried out in quadruplicate. The line connecting the data points was calculated by linear regression. In this experiment the K_M to Ca^{2+} is 29.5 μM and V_{max} 9.7 nmol/mg protein per s. (B) Internal [Na^+] dependence of Na^+ gradient dependent Ca^{2+} influx. *Torpedo* SPM vesicles were preloaded with 0–0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4) and balancing amounts of LiCl (0.4–0 M). 3 μl of these vesicles (about 10 μg protein) were diluted into 250 μl of an external medium composed of 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$ (0.1 μCi). The reactions were terminated after 1 s. The $^{45}Ca^{2+}$ content of the vesicles was determined. The amount of Ca^{2+} associated with the vesicles in the absence of internal Na^+ (0.4 M LiCl) was subtracted from the Ca^{2+} content of the vesicles in the presence of internal NaCl. Each data point is an average obtained from quadruplicate measurements. (C) Hill plot of internal [Na^+] dependence of Ca^{2+} influx. The data obtained in Fig. 4B were used to calculate the Hill plot. The line connecting the data points was obtained by linear regression analysis. The slope of this line is 2.44.

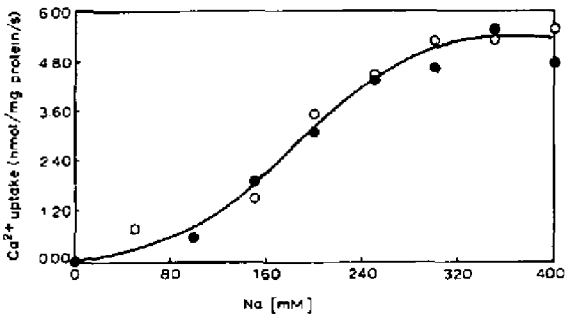


Fig. 5. The effect of ATPγS on the internal $[Na^+]$ dependence of Na^+ gradient dependent Ca^{2+} influx. *Torpedo* SPM vesicles were preloaded with 0–0.4 M NaCl, 0.01 M Tris-HCl (pH 7.4), 4 mM $MgCl_2$, balancing amounts of LiCl (0.4–0 M) with 1 mM ATPγS (●) or without (○). 3 μl of these vesicles (about 10 μg protein) were diluted into 250 μl of an external medium composed of 0.4 M KCl, 0.01 M Tris-HCl (pH 7.4), 4 mM $MgCl_2$ and 50 μM $^{45}CaCl_2$ (0.1 μCi). When the vesicles were preloaded in ATPγS containing internal medium the uptake medium contained 1 mM ATPγS as well (●). Na^+ gradient dependent Ca^{2+} uptake was determined as in Fig. 4B.

sigmoidal dependence of the Na^+-dependent Ca^{2+} uptake on internal $[Na^+]$ is not altered when ATPγS is included in the media; nor was the $K_{0.5}$ to Na^+.

In a similar fashion to the squid giant axon [40], cardiac [41,42] and brain [12] Na^+-Ca^{2+} exchangers, the *Torpedo* electric organ Na^+-Ca^{2+} exchanger is electrogenic and more Na^+ derived charges cross the membrane in one direction than Ca^{2+} derived charges cross the membrane in the opposite direction. Consequently, the rate of internal Na^+ gradient dependent Ca^{2+} influx into vesicles is retarded as negative inside membrane polarization builds up. Hence, entry of positive charge into the vesicles stimulates Na^+ gradient dependent Ca^{2+} influx. This stimulation is especially noticeable when the native carrier is reconstituted into a high phospholipid containing membrane for two reasons: First, the membranes have a high phospholipid content they constitute a better permeability barrier against unspecific cationic 'leaks' than the native membrane. Second, reconstitution of the exchanger into a membrane composed of high phospholipid to protein weight ratio (30:1) leads presumably to separation between the Na^+-Ca^{2+} exchanger and many of the unrelated channels and transporters present in the native membrane into different vesicular structures. Table II demonstrates that a stimulation between 2.5- and 3.2-fold of the initial rate of Na^+ gradient dependent Ca^{2+} influx is obtained either when valinomycin (K^+ ionophore) is added to the uptake medium that contains K^+ or, when FCCP (protonophore) is added in the presence and in the absence of K^+, to permit the free passage of H^+.

The experiment shown in Table II also demonstrates, that Na^+ gradient dependent Ca^{2+} uptake in *Torpedo* SPMs is not dependent on the presence of K^+

TABLE II

The effect of FCCP and valinomycin on Na^+ gradient dependent Ca^{2+} uptake in Torpedo synaptic plasma membranes

Torpedo SPMs were loaded during reconstitution (see Materials and Methods) with 0.39 M NaP_i, 0.01 M choline chloride (pH 7.4); 5 μl of these vesicles (about 2 μg protein) were diluted into 250 μl of a solution which contained 50 μM $^{45}CaCl_2$ (0.1 μCi) and the external medium as specified in Table II, or the same additions and either 0.39 M NaCl, 0.01 M choline chloride or 0.39 M NaCl, 0.01 M KCl (respective 'no Na^+ gradient'-media). The reactions were terminated as described in Materials and Methods and the $^{45}Ca^{2+}$ associated with the vesicles in the absence of a Na^+ gradient which was always less than 5% of the total $^{45}Ca^{2+}$ taken up by the vesicles and zero-time controls were subtracted.

External medium	Additions	Na^+ dependent Ca^{2+} uptake (nmol/mg protein per 15 s)
0.4 M choline Cl	–	9.1
0.4 M choline Cl	10 μM FCCP	28.7
0.4 M choline Cl	10 μM valinomycin	8.5
0.39 M choline Cl, 0.01 M KCl	–	11.3
0.39 M choline Cl, 0.01 M KCl	10 μM FCCP	28.3
0.39 M choline Cl, 0.01 M KCl	10 μM valinomycin	27.4

since the same amount of Ca^{2+} is associated with the vesicles in the absence of K^+ (choline chloride medium) as in its presence (choline chloride and K^+ medium). Moreover, similar stimulation of Na^+ gradient dependent Ca^{2+} uptake is obtained by the addition of FCCP to the choline chloride containing medium to that obtained by valinomycin added to the K^+-containing medium. These data indicate, that K^+ stimulation of Na^+ gradient dependent Ca^{2+} influx does not constitute a specific requirement of Na^+-Ca^{2+} exchange in *Torpedo* SPMs for K^+, but occurred due to the entry of positive charge.

The temperature dependence of the Torpedo Na^+-Ca^{2+} exchanger

The experiments presented show, that the apparent initial specific transport activity of *Torpedo* SPMs and the apparent V_{max} were considerably higher than that of rat brain SPMs. Since the experiments were carried out at 25°C and since the *Torpedo* is a poikilothermic animal, the difference in kinetic parameters could reflect either a difference in temperature dependence of the two transport proteins, a difference in site density or both.

To distinguish between these possibilities, the temperature dependence of Na^+ gradient dependent Ca^{2+} influx of rat brain and *Torpedo* SPMs were compared. In Fig. 6A the temperature dependence of the initial rate of Ca^{2+} transport in *Torpedo* SPMs in the pres-

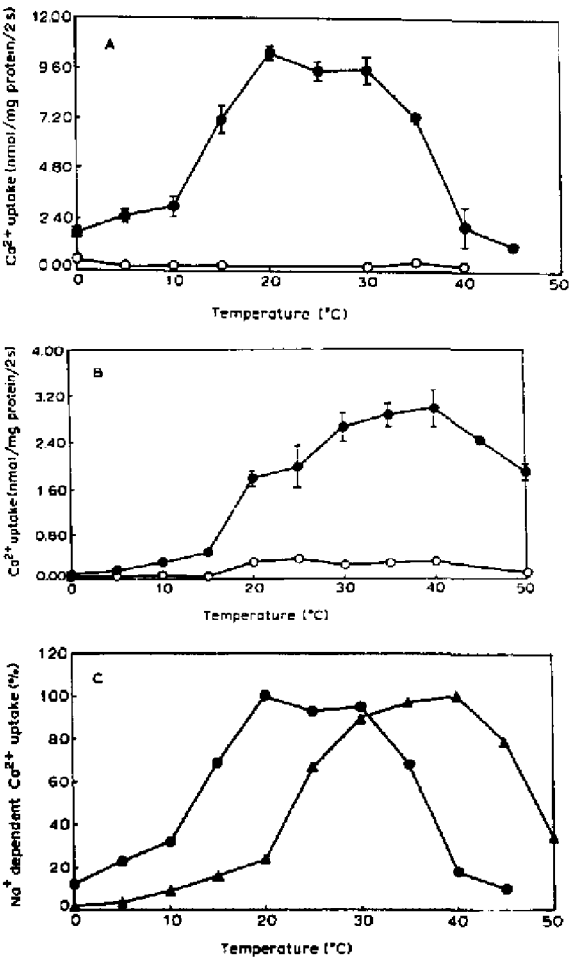


Fig. 6. Temperature dependence of Na^+ gradient dependent Ca^{2+} influx on native *Torpedo* and rat brain SPMs. (A) 3 μl of *Torpedo* SPM vesicles (about 10 μg protein) preloaded with 0.4 M NaCl, 0.01 M Tris-HCl (pH 7) were diluted into 0.4 M KCl (●) or 0.4 M NaCl (○) containing also 0.01 M Tris-HCl (pH 7.4) and 50 μM $^{45}CaCl_2$. The reactions were terminated as described. The averaged data presented in this figure (the bar represents S.D.) were obtained from three different experiments using the same SPM preparation. (B) The experiment was identical to Fig. 6A except that rat brain SPMs were used (20–30 μg/3 μl) and the osmolarity of internal NaCl and external KCl containing media was 0.2 M. (C) The data in this figure were compiled from three different preparations each, of *Torpedo* and rat brain SPMs. To permit comparison, the highest value of Na^+ gradient dependent Ca^{2+} uptake in each individual experiment was taken as 100%, and all other data points were normalized accordingly.

ence of a driving Na^+ gradient and in its absence are shown, in Fig. 6B the same experiment is presented except that rat brain synaptic plasma membranes are used, and in Fig. 6C for comparison, the normalized values (the highest extent of the initial rate of Na^+ gradient dependent Ca^{2+} uptake is taken as 100%) of both preparations are presented on the same scale. It can be seen, that *Torpedo* SPMs reach the highest activity of Na^+ gradient dependent Ca^{2+} uptake between 15 and 20°C. On the other hand, rat brain Na^+-Ca^{2+} exchanger reaches its optimal steady-state level of transport activity between 30 and 40°C. Further increase in temperature, results in a gradual loss of the transport activity. It should be noted that at 20°C, when the *Torpedo* Na^+-Ca^{2+} exchanger already reaches its maximal activity, the rat brain Na^+-Ca^{2+} exchanger exhibits only about 24% of its maximal transport activity. At 25°C, however, the temperature at which the maximal reaction velocity of the rat brain Na^+-Ca^{2+} exchanger was determined [22] 67% of its optimal activity is reached. Thus, the different temperature dependence of rat brain and *Torpedo* SPM Na^+-Ca^{2+}

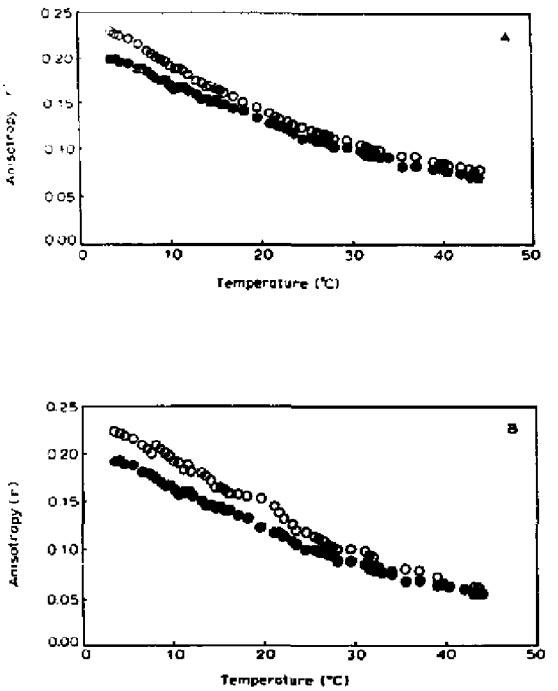


Fig. 7. Changes in steady-state fluorescence anisotropy of DPH introduced into liposomes composed of TPLs or BPLs and proteoliposomes composed of *Torpedo* SPMs reconstituted into TPLs or BPLs. Reconstituted membranes containing either phospholipids only or *Torpedo* SPMs and phospholipids at a weight ratio of 1:30 (protein to phospholipid) were prepared as described in Materials and Methods. The liposomes or proteoliposomes were diluted in the same medium as their internal one (0.4 M NaP_i buffer (pH 7.4), 5 mM βME) to a concentration of 1 mM phospholipid. DPH in tetrahydrofuran was added to a final concentration of 1 μM. Polarized fluorescence was measured as described in Materials and Methods between 4.5 and 44°C. Anisotropy of DPH (r) was calculated from the equation $r=(I_{\parallel}-I_{\perp})/(I_{\parallel}+2I_{\perp})$, where $I_{\parallel}$ is the vertical component of the fluorescence excited by a vertical polarized light (parallel component) and $I_{\perp}$ is the horizontal component of the above fluorescence (perpendicular component). The expression $F=I_{\parallel}+2I_{\perp}$ is the total fluorescence for a rod like molecule. ●, TPLs; ○, BPLs.

TABLE III

The composition of Torpedo ocellata electric organ phospholipids and their fatty acyl chains

Phospholipid head group content was determined by the method of Yavin and Zutra, [30]; fatty acid content was determined by gas chromatography (for details see Materials and Methods). The results presented were obtained from measurements carried out on four different phospholipid preparations extracted from 4–6 different electric organs each.

Phospholipid head group	Range (%)		
Phosphatidylethanolamine [a]	24–38		
Phosphatidylcholine	35–43		
Phosphatidylserine	8–19		
Phosphatidylinositol	2– 5.5		
Sphingomyelin	13–18		
Saturated fatty acids	Range (%)	Unsaturated fatty acids	Range (%)
14:0	2	14:1	1
16:0	25–27	16:1	3–5
18:0	12–16	16:2	2–3
		18:1	14–16
		18:2	0.5–1
		18:3	1
		20:4	4.5–5
		22:1	1
		22:2	1
		22:3	1
		22:4	1
		22:5	2
		22:6	23–29

[a] Contains also lysophosphatidylethanolamine.

exchangers can not explain alone the difference in the maximal reaction velocities of the two transporters. Similar temperature dependence of both *Torpedo* and rat brain Na^+-Ca^{2+} exchangers is maintained also when steady-state rates of transport are compared.

Calculating the activation energy (E_a) of Na^+ gradient dependent Ca^{2+} uptake between 0 and 20 °C from the slope of the straight line (not shown), which was obtained by plotting the logarithm of the maximal reaction rate at each temperature ($\log V_{max}$) against each corresponding reciprocal temperature (Arrhenius plot), resulted in $E_a = 20826$ cal/mol. This value is similar to the activation energy calculated for the cardiac Na^+-Ca^{2+} exchanger between 10 and 37 °C [42] $E_a = 18000$ cal/mol.

To distinguish whether the different temperature dependence of the rat brain and *Torpedo* Na^+-Ca^{2+} exchangers resulted from difference in the composition of their respective lipid membranes and hence in membrane fluidity or, reflected differences between the two proteins the following experiments were done: *Torpedo* and rat brain SPMs were reconstituted each into calf brain and *Torpedo* electric organ phospholipids. The temperature dependence of the activity profile was determined. Calf brain phospholipids (BPLs) were used rather than rat brain ones since their composition is similar [43] and larger quantities can be easily prepared. In addition, quantitative analysis of *Torpedo* phospholipid (TPL) head groups and their fatty acyl chain content was carried out (Table III) and compared to calf brain phospholipids [43] and the fluidity of each reconstituted membrane was determined.

Reconstitution involved solubilization of the native synaptic membranes in 2% cholate addition of a 30-fold weight excess (relative to membrane protein) of the exogeneous phospholipids tested as described [12]. The temperature dependence of Na^+ gradient dependent Ca^{2+} uptake of the reconstituted transporters was determined. Each one of the reconstituted transporters retained its 'native' optimal temperature dependence (not shown). Changes in the 'bulk' composition of the membrane into which the transporters are reconstituted have no effect on their temperature dependence. Analysis of the phospholipid head group composition of brain and *Torpedo* electric organ phospholipids revealed, that the distribution is quite similar. The two phospholipid mixtures vary, however, considerably in their fatty acid composition: *Torpedo ocellata* electric organ phospholipids contain about 63% unsaturated fatty acids as compared to 33% in calf brain phospholipids [43]. Moreover, in calf brain phospholipids, arachidonic acid (20:4), is the fatty acid with the highest unsaturation detected. In *Torpedo ocellata* phospholipids docosahexanoic acid (22:6) reached between 23 and 29% of the total fatty acids in different preparations and other C_{22} unsaturated fatty acids

about 6% more. Since these would have considerable effects on the fluidity of the respective membranes, this parameter was tested by measuring the steady-state fluorescence anisotropy of the fluorescent probe DPH (1,6-diphenylhexa-1,3,5-triene) in the different reconstituted membranes. The results of these measurements are shown in Fig. 7A and Fig. 7B.

In Fig. 7A the changes in anisotropy (r) of DPH in liposomes composed of TPLs or BPLs was measured between 4.5 and 44°C. No proteins were added to the reconstitution mixtures, otherwise the preparative procedures were identical to those used to prepare proteoliposomes (see Materials and Methods). In Fig. 7B the changes in anisotropy of DPH over the same temperature range are shown except that proteoliposomes containing *Torpedo* SPMs and either TPLs or BPLs were used. It can be seen, that liposomes composed of TPLs and BPLs differ in their fluidity (Fig. 7A). The anisotropy of DPH in liposomes composed of TPLs at each temperature below 30°C is lower than the anisotropy of DPH in BPL-containing liposomes. The difference in anisotropy of DPH between the two types of liposomes increases as the temperature is lowered. Reconstitution of *Torpedo* SPM protein into phospholipid membranes composed of either TPLs or BPLs (Fig. 7B) does not alter the difference in anisotropy of DPH measured in protein-free liposomes (Fig. 7A). A similar pattern of increased difference in anisotropy of DPH below 30° is maintained between the two reconstituted membranes. Similar profile of changes in anisotropy of DPH with changes in temperature is obtained also when rat brain SPMs are reconstituted into TPLs or BPLs (not shown).

It seems that both transporters maintain their 'native' temperature dependence independently of the phospholipid membrane into which they are reconstituted, although these membranes differ in their bulk fluidity.

Discussion

This work characterizes the Na^+-Ca^{2+} exchange activity in synaptic plasma membranes from the *Torpedo* electric organ. This neuronal preparation has a high exchange reaction velocity which is comparable in magnitude to that of the mammalian sarcolemmal Na^+-Ca^{2+} exchanger. Reeves and Philipson, [20] summarized the maximal Na^+-Ca^{2+} exchange activities in different preparations and have shown, that the highest activity is measured in cardiac sarcolemmal vesicles (25 ± 18 nmol/mg protein per s) while in most other preparations the activity is 1–2 orders of magnitude lower. The value reported here of 11.33 (S.D. = 5.93) indicates that SPMs from the *Torpedo* electric organ are one of the preparations where the exchanger is most active.

There are two properties at least that distinguish this exchanger from that in rat brain SPMs or that from sarcolemmal vesicles: the low apparent affinity to Na^+ and the relatively low temperature at which optimal transport activity is attained.

The difference in the apparent affinity to Na^+ is substantial. While in cardiac membranes [34] and in rat brain SPMs [44] the [Na^+] needed to reach half-maximal reaction velocity ($K_{0.5}$) is 20–30 mM, in the *Torpedo* electric organ it is 170 mM (Table 1). These differences in the affinity to Na^+ in isolated vesicles support the notion that different proteins are involved in the exchange process in *Torpedo* and in brain.

The *Torpedo* electric organ Na^+-Ca^{2+} exchanger is not the only case where high $K_{0.5}$ for Na^+ is found. High $K_{0.5}$ to Na^+ (110–300 mM), was found also in the squid giant axon when it was poisoned by CN^- [37], or ATP depleted by dialysis [38]. Addition of ATP decreased the $K_{0.5}$ to Na^+ to values of about 50 mM. Substitution of ATP by ATPγS led to increase in the affinity of the squid Na^+-Ca^{2+} exchanger to both Ca^{2+} and Na^+ [39,18] suggesting that kinase mediated phosphorylation of the exchanger protein or of another regulatory protein might be involved in the process. In excised patches of cardiac myocytes [19], some evidence was obtained for stimulation of Na^+-Ca^{2+} exchange currents by ATP. This regulation was lost upon treatment of the preparation with chymotrypsin. Attempts to reproduce ATP regulation of Na^+-Ca^{2+} exchange in membrane vesicles were unsuccessful [20], with one exception [45], where activation of Na^+-Ca^{2+} exchange by Ca^{2+}, Mg^{2+} and ATP or ATPγS was obtained following pretreatment of the membranes with phosphorylase phosphatase. It could be, that preparation of membrane vesicles results in loss of ATP regulation of Na^+-Ca^{2+} exchange due to activation of endogenous proteolytic activity or another unknown reason. Therefore it is not surprising that we observed no changes in the affinity of the *Torpedo* SPM Na^+-Ca^{2+} to Na^+ following treatment with ATPγS. It is of interest, that in poikilothermic marine animals such as squid and *Torpedo*, when the intact membrane is ATP depleted or when membrane vesicles are prepared, the Na^+-Ca^{2+} exchangers exhibit a high $K_{0.5}$ to Na^+ as compared to similar preparation from rat brain and mammalian cardiac sarcolemma.

The second line of evidence which suggests that *Torpedo* Na^+-Ca^{2+} exchanger differs from rat brain one is the temperature dependence profile of the two carriers. While the *Torpedo* Na^+ gradient dependent Ca^{2+} uptake reaches optimal activity between 15 and 20°C, the rat brain one reaches optimal activity between 30 and 40°C. Evidence suggests, that membrane composition and fluidity are of importance in modulating the activity of transport proteins [46,47]. TPLs and BPLs differ in fatty acid composition of the phospho-

lipid head groups which is manifested by different membrane fluidity. The different temperature dependence of Na^+-Ca^{2+} exchange in SPMs from *Torpedo* electric organ and rat brain seems however to be independent from the bulk lipid composition of the membrane since it is retained following reconstitution of each carrier into either one of the phospholipids. It is impossible to rule out that each transporter carries over its specific annular lipids also following solubilization in detergent and reconstitution. But even if this is the case, and the annular lipids contribute to the specific microenvironment which regulates the temperature dependence of rat brain and Torpedo Na^+-Ca^{2+} exchangers, these have to be different and reflect probably differences in the protein backbone to which they are attached.

Acknowledgements

Part of this work was supported by the U.S.-Israel Binational Science Foundation. We would like to thank Professor R. Rahamimoff for reading our manuscript and for his helpful comments; Mrs. Beryl Levene for unfailing help in preparing it.

References

1 Augustine, G.J., Charlton, M.P. and Smith, S.J. (1987) Ann. Res. Neurosci. 10, 633–693.
2 Allen, T.J., Noble, D. and Reuter, H. (1989) Na^+-Ca^{2+} Exchange, Oxford University Press, Oxford.
3 Israel, M. and Tucek, S. (1974) J. Neurochem. 22, 487–491.
4 Wagner, J.A., Carlson, S.S. and Kelly, R.B. (1978) Biochemistry 17, 1199–1206.
5 Tashiro, T. and Stadler, H. (1978) Eur. J. Biochem. 90, 479–487.
6 Dunant, Y. (1986) Prog. Neurobiol. 26, 55–92.
7 Sudhof, T.C., Baumert, M., Perin, M.S. and Jahn, R. (1989) Neuron 2, 1475–1481.
8 Linial, M., Miller, K. and Scheller, R.H. (1989) Neuron 2, 1265–1273.
9 Rahamimoff, R., De Riemer, S.A., Sakmann, B., Stadler, H. and Yakir, N. (1988) Proc. Natl. Acad. Sci. USA 85, 5310–5314.
10 Mullins, L.J. and Brinley, F.J. Jr. (1975) J. Gen. Physiol. 65, 135–152.
11 Reeves, J.P. and Hale, C.C. (1984) J. Biol. Chem. 259, 7333–7739.
12 Barzilai, A. and Rahamimoff, H. (1987) Biochemistry 26, 6113–6118.
13 Lagnado, L. and McNaughton, P.A. (1990) J. Membr. Biol. 113, 177–191.
14 Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W. and McNaughton, P.A. (1989) Nature 337, 740–743.
15 Schnetkamp, P.P.M., Basu, O.K. and Szerencsei, R.T. (1989) Am. J. Physiol. 257, C153–C157.
16 Crespo, L.M., Grantham, C.J. and Cannell, M.B. (1990) Nature 345, 618–621.
17 Dahan, D., Spanier, R. and Rahamimoff, H. (1991) J. Biol. Chem. 266, in press.
18 DiPolo, R. and Beaugé, L. (1990) J. Gen. Physiol. 95, 819–835.
19 Hilgemann, D.W. (1990) Nature 344, 242–245.
20 Reeves, J.P. and Philipson, K.D. (1989) Na^+-Ca^{2+} exchange activity in plasma membranes vesicles, in Na^+-Ca^{2+} Exchange (Allen, T.J., Noble, D. and Reuter, H., eds.), pp. 27–53, Oxford University Press, Oxford.
21 Rahamimoff, H. and Spanier, R. (1979) FEBS Lett. 104, 111–114.
22 Rahamimoff, H. and Spanier, R. (1984) Biochim. Biophys. Acta 773, 279–289.
23 Michelis, M.L. and Michaelis, E.K. (1981) Life Sci. 28, 37–45.
24 Gill, D.L., Grollman, E.F. and Kohn, D.L. (1981) J. Biol. Chem. 256, 184–192.
25 Rahamimoff, H. (1989) The molecular biochemistry of Na^+-Ca^{2+} exchange, in Na^+-Ca^{2+} Exchange (Allen, T.J., Noble, D. and Reuter, H., eds.), pp. 153–177, Oxford University Press, Oxford.
26 Michaelson, D.M. and Sokolovsky, M. (1978) J. Neurochem. 30, 217–230.
27 Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349–354.
28 Gasko, D.D., Knowles, A.F., Shertzer, H.C., Suolinna, E.H. and Racker, E. (1976) Anal. Biochem. 72, 57–65.
29 Sheltawy, A. and Dawson, R.M.C. (1969) Chromatographic and Electrophoretic Techniques (Smith, I., ed.), Vol. 1, pp. 450–493, Heinemann Medical Books, London.
30 Yavin, E. and Zutra, A. (1977) Anal. Biochem. 80, 430–437.
31 Ames, B. (1974) Methods Enzymol. 8, 115–117.
32 Lakowicz, J.R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York.
33 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
34 Reeves, J.P. and Sutko, J.L. (1983) J. Biol. Chem. 258, 3178–3182.
35 Erdreich, A. (1987) Ph.D. thesis approved by the Senate of the Hebrew University.
36 DiPolo, R. (1974) J. Gen. Physiol. 64, 503–517.
37 Baker, P.F. and McNaughton, P.A. (1976) J. Physiol. 259, 103–144.
38 Blaustein, M.P. (1977) Biophys. J. 20, 79–110.
39 DiPolo, R. and Beaugé, L. (1987) Biochim. Biophys. Acta 897, 347–353.
40 Baker, P.F. (1972) Prog. Biophys. Mol. Biol. 24, 177–223.
41 Mullins, L.J. (1981) Ion Transport in Heart, Raven Press, New York.
42 Reeves, J.P. and Sutko, J.L. (1980) Science 208, 1461–1464.
43 Hermoni-Levine, M. and Rahamimoff, H. (1990) Biochemistry 29, 4940–4950.
44 Barzilai, A. (1987) Ph.D. thesis approved by the Senate of the Hebrew University.
45 Caroni, P. and Carafoli, E. (1983) Eur. J. Biochem. 132, 451–460.
46 Deuticke, B. and Haest, C.W.M. (1987) Annu. Rev. Physiol. 49, 221–235.
47 Carruthers, A. and Melchior, D.L. (1988) Annu. Rev. Physiol. 50, 221–235.