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Characterization of the ATP-promoted aspect of Na^+-Ca^{2+} exchange present in squid retinal nerve axolemma

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Using an in vitro system which consists of an axolemma-rich vesicle fraction prepared from squid retinal nerve fibers, an Na^+-Ca^{2+} exchange process has been characterized and appears identical with that reported in squid giant axon. This exchange is absolutely dependent on the establishment of an Na^+ gradient, shows monovalent and divalent cation specificity and is highly sensitive to monensin, A23187 and valinomycin but not to ouabain, digitoxigenin, vanadate, pentylenetetrazole, tetrodotoxin or tetraethylammonium. Furthermore, it was found that the exchange process is enhanced by the addition of ATP. This ATP-promoted aspects of Na^+-Ca^{2+} exchange shares many similar characteristics with Na^+-Ca^{2+} ATP hydrolysis and may indicate a common mechanism for both activities via a protein phosphorylation-dephosphorylation event.

Introduction

The giant axons of squids have been used to obtain valuable data regarding the physiological properties of excitable membrane for many years. One function of the axonic membrane is to maintain a low intracellular level of ionized calcium. In the case of the squid giant axon, this value has been determined to be on the order of 20–100 nM [1–6]. Since the body fluid of squids contain approx. 4 mM calcium [7], there exists a calcium concentration gradient greater than 10^5-fold across the axonic membrane. The maintenance of this electrochemical gradient is vital to the normal function of these axons. Nevertheless, calcium is known to diffuse into the axon via passive transport and influx during excitation through various channel and exchange mechanisms [8,9]. Therefore, there must be a mechanism(s) by which Ca^{2+} is actively extruded from the axon.

A major calcium extrusion mechanism known to exist in squid giant axons is the Na^+-Ca^{2+} exchange system originally reported by Baker et al. [10,11]. In this system, 3 or 4 mole equivalents of Na^+ outside is apparently exchanged for 1 mole equivalent of Ca^{2+} inside [11–13]. Preliminary calculations [13,14] indicate that the gradient energy provided by 3 to 4 mole equivalents of Na^+ is sufficient to extrude Ca^{2+} from the axon.

On the other hand, there is some question as to a possible role of ATP in this process. Baker and Glitsch [15] showed that reduction of intracellular ATP by apyrase, an ATP-hydrolyzing enzyme, greatly reduced Na^+-dependent extrusion of Ca^{2+} from squid axons. Blaustein [14] detailed the Na^+-Ca^{2+} exchange system in squid giant axons and found that ATP increases the affinity of both calcium and sodium ions at the inside (Ca_i^{2+}) and outside surfaces (Na_o^+) of the axolemma, respectively. Other similar findings have been reported in barnacle muscle fibers [16], dog heart sarco-

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Abbreviations: A23187, calcium ionophore (calimycin).

lemma [17] and calf heart sarcolemma [18].

Because of the difficulties encountered with intact squid giant axons in controlling ATP concentrations, particularly at low concentration (e.g., micromolar ranges and below) [19,9], and the problems associated with obtaining an uncontaminated axolemma fraction from this preparation [20], we have adopted an in vitro system which consists of an axolemma-rich vesicle fraction prepared from the squid retinal nerve fibers [21–25]. Using this preparation, we previously reported the existence of a number of ATP hydrolyzing systems which were activated by addition of various inorganic ions. In particular, we characterized a ouabain-insensitive Na^+-Ca^{2+} ATP hydrolysis in which Na^+ (Li^+), Ca^{2+} and K^+ ions selectively stimulated hydrolysis of micromolar concentrations of ATP to ADP and inorganic phosphate [24,25]. Furthermore, it was shown that this ATP hydrolysis apparently occurred via a protein phosphorylation-dephosphorylation process which was highly dependent on calcium concentration [23]. We have now determined the existence of a Na^+-Ca^{2+} exchange process in retinal nerve fibers of squid that appears identical to that reported in the squid giant axon. This Na^+-Ca^{2+} exchange process also has many characteristics similar to those reported by us for Na^+-Ca^{2+} ATP hydrolysis (i.e., previously, ($Na^+ + Ca^{2+}$)-ATPase activity, [25]) and we now report the results.

Materials and Methods

Isolation and purification of retinal nerve membranes. Squids, *Loligo pealei*, were captured and kept alive by the Marine Biological Laboratory, Woods Hole, MA, during the summer months of 1981-1982. Retinal axons were dissected out in 750 mM sucrose and stored at −20°C as previously described by Matsumura and Clark [23]. An axolemma-rich preparation from these retinal axons was prepared using the original method of Fisher et al. [26] and Marcus et al. [20] as modified by Matsumura and Clark [25].

Determination of Na^+-Ca^{2+} exchange in retinal nerve membranes. Calcium influx and efflux experiments were carried out using the rapid filtration method developed by Gill et al. [27]. Physiological buffer solution were prepared exactly as outlined by Blaustein [14] except Hepes was replaced with 30 mM Tris base.

For calcium influx experiments, a portion of the axolemma vesicle preparation was taken up into an appropriate standard solution (usually NaCl pre-equilibrium, Table I) so that a 10 μl aliquot would contain approx. 31 μg protein as determined by the method of Lowry et al. [28]. Vesicles were pre-equilibrated for 2 h at 4°C. A 10 μl aliquot was added to 160 μl of a potassium depolarizing solution (i.e., Ca-loading solution) which contained 0.01 mM $CaCl_2$ and 0.3 μCi of

TABLE I

STANDARD SOLUTIONS FOR Na^+-Ca^{2+} EXCHANGE

In addition to the components listed below, all solutions contained 2 mM KCN, 0.1 mM ouabain, 0.7 μg/ml oligomycin, 150 mM glucose and 30 mM Tris base. Standard solutions were diluted to volume with double distilled, deionized water and the pH of each adjusted to 7.8 with maleic acid at 20°C.

Solution	Composition (mmol/l)					
	NaCl	KCl	Choline chloride	LiCl	$CaCl_2$	EGTA
Pre-equilibration	425	10	–	–	–	–
Ca-loading	–	435	–	–	0.01	–
Na_o-efflux	(425 to 0)	10	(0 to 425)	–	–	0.5
Li_o-efflux	–	10	–	425	–	0.5
Ca_o-efflux	–	10	415	–	10	0.5
$Li_o + Ca_o$-efflux	–	10	–	415	10	0.5
Choline	–	10	425	–	–	0.5

$^{45}CaCl_2$ (specific activity for $^{45}CaCl_2$ was 0.2 Ci/mmol) unless otherwise stated in text. Calcium influx continued for a specified amount of time (usually 5 min) at 20°C.

A 120 μl aliquot was added to 8 ml of a non-effluxing solution (i.e., choline solution) at 20°C for 2 min. Zero-time control tubes were included to verify the integrity of vesicles by choline solution. The entire suspension was vacuum filtered on pre-washed 0.45 μm cellulose acetate filters (Millipore Corp., Bedford, MA 01730) and rinsed once with 10 ml ice-cold choline solution. The washed filters were removed from their holders and dissolved in liquid scintillation fluid. The amount of $^{45}Ca^{2+}$ remaining on the filter was determined by liquid scintillation means. All filters were corrected for background radiation which remained on filters that received no vesicles.

For calcium efflux experiments, vesicles were loaded with $^{45}CaCl_2$ exactly as for calcium uptake experiments except that upon completion of loading, a 120 μl aliquot was placed into 8 ml of an appropriate efflux solution. Cation-specific efflux (usually 2 min) was determined as the difference between the $^{45}Ca^{2+}$ remaining on filters (after background correction) from samples effluxed against choline solution (i.e., non-effluxing) versus samples effluxed against Na_o-, Li_o-, or Ca_o-efflux solutions (see Table III).

Determination of Na^+-Ca^{2+} ATP hydrolysis. Microsomal vesicles were prepared and pre-equilibrated with NaCl exactly as for Na^+-Ca^{2+} exchange experiments. To determine Na^+-Ca^{2+} ATP hydrolysis during calcium uptake, a 10 μl aliquot of vesicles (31 μg protein) was added to 160 μl of Ca-loading solution which contained 0.1 mM Tris-ATP fortified with 0.9 μCi of tetra(triethylammonium) [γ-^{32}P] ATP at a specific activity of 60 mCi/mmol. Control tubes had choline chloride pre-equilibrated vesicles to correct for non Na^+-Ca^{2+} stimulated ATP hydrolysis. Incubation was continued for 5 min with constant agitation at 20°C. ATP hydrolysis was stopped by addition of 50 μl of ice-cold trichloroacetic acid (10% w/v) and placing the reaction vessel into an ice bath. A mixture of bovine serum albumin (1.0 mg) and KH_2PO_4 (1.35 mg) in 0.1 ml of distilled water was added and the mixture equilibrated for 5.0 min. The precipitate was collected by centrifugation ($3000 \times g$ for 3 min) and the supernatant transferred to a clean test tube. Activated charcoal (100 mg) was added and the solution vortexed. Ethanol (0.2 ml) was used to rinse the sides of the tube and the mixture centrifuged ($3000 \times g$ for 3 min). A 0.5 ml aliquot which contained inorganic [^{32}P] P_i was taken for liquid scintillation determination of radioactivity.

To determine Na^+-Ca^{2+} ATP hydrolysis during calcium efflux, vesicles were pre-equilibrated with NaCl and loaded with calcium as above but in the absence of ATP. A 120 μl aliquot was added to 8 ml of an appropriate efflux solution (usually Na_o-efflux solution) which contained 0.1 mM Tris-ATP fortified with [γ-^{32}P]ATP as above. At the end of the 2 min efflux period, ATP hydrolysis was stopped by addition of 2.5 ml of ice-cold trichloroacetic acid (10% w/v). To correct for non Na^+-Ca^{2+} stimulated ATP hydrolysis, control vesicles were effluxed against choline solution. The work-up for the determination of [^{32}P]P_i was exactly as described above for ATP hydrolysis during calcium uptake experiments.

In all experiments where ATP was utilized, 10 μM vanadate and 10 μM digitoxigenin was added in addition to 2 mM KCN, 0.1 mM ouabain and 0.7 μg/ml oligomycin to eliminate ATP hydrolysis by mitochondrial and adenosine triphosphatase contamination.

Chemicals. Tris-adenosine 5′-triphosphate (ATP, A0270), ouabain, oligomycin, A23187 (calimycin), sodium vanadate, digitoxigenin, $LaCl_3$, tetrodotoxin, tetracaine, pentylenetetrazole, valinomycin, monensin, tetraethylammonium, and KCN were purchased from Sigma Chemical Company, St. Louis, MO. Tetra(triethylammonium) [γ-^{32}P] ATP and $^{45}CaCl_2$ were obtained from New England Nuclear, Boston, MA. All other compounds were of the highest purity available from commercial sources.

Results

Determination of Na^+-Ca^{2+} exchange found in retinal nerve fibers

In order to establish the sodium dependency of ^{45}Ca uptake, vesicles prepared from axolemma of retinal nerve were pre-equilibrated in various solutions as detailed in Table II. The level of ^{45}Ca

TABLE II

EFFECTS OF PRE-EQUILIBRATION TREATMENTS ON ^{45}Ca UPTAKE

Membrane vesicles prepared from squid retinal nerve fibers were resuspended into pre-equilibration solutions altered and with the additions as listed below. The amount of ^{45}Ca uptake into NaCl pre-equilibrated vesicles was set to be equal to 100% load. In these experiments, the mean ^{45}Ca load was 521 ± 16 (S.E.) pmol Ca^{2+}/mg protein. The percentages which are reported represent the means of at least three experiments of two replicates each $\pm$S.E.

Pre-equilibration solution	Percent of control ^{45}Ca load
425 mM NaCl	100
425 mM NaCl + 0.01 mM tetrodotoxin	112 ± 5.0
425 mM NaCl + 0.1 mM monensin	0.0
425 mM NaCl + 0.1 mM vanadate	98 ± 3.0
425 mM KCl	16 ± 7.0
425 mM choline chloride	9 ± 5.0

uptake was highly dependent on NaCl pre-equilibration. Vesicles pre-equilibrated in either KCl or choline chloride solutions did not take up $^{45}Ca^{2+}$ to any appreciable level during a 5 min load period. Addition of 0.1 mM monensin, a sodium ionophore, resulted in a complete loss of ^{45}Ca uptake into NaCl pre-equilibrated vesicles. This indicates that the $^{45}Ca^{2+}$ remaining on the cellulose acetate filters after washing is due to ^{45}Ca uptake into the interior of vesicles and is not due to surface binding to the vesicle or to trapping of calcium ions in the membrane matrix itself. In addition to KCN, ouabain and oligomycin which were included in all standard solution and showed no interference with Na^+-dependent ^{45}Ca uptake at the concentrations employed (data not shown), sodium vanadate (0.1 mM produced no significant effect on Na^+-dependent ^{45}Ca uptake. Vanadate is a well-established inhibitor of $(Na^+ + K^+)$-ATPase [29], sodium pump [30] and Ca^{2+}-stimulated ATPase activity associated with the enzymatic mechanism for the uncoupled calcium pump in red blood cells [31]. Failure of vanadate to produce similar levels of inhibition establishes that the Na^+-dependent ^{45}Ca uptake in squid retinal axolemma is an entity distinct from these ATP-driven pumps. Additionally, Na^+-dependent ^{45}Ca uptake was not inhibited by addition of 0.01 mM tetrodotoxin, a potent sodium channel blocker.

Na^+-Ca^{2+} exchangers characterized in various other excitable tissues show a high degree of specificity in ion replacements [14,27]. Fig. 1 shows the effect on ^{45}Ca uptake into NaCl pre-equilibrated vesicles of various monovalent cations which were added to Ca-loading solution. In the presence of external K^+, ^{45}Ca uptake was rapid, reaching half the equilibrium value in approx. 1.0 min. Addition of Rb^+, NH_4^+ or choline resulted in a slower rate of Ca^{2+} accumulation but still allowed substantial ^{45}Ca uptake to take place. ^{45}Ca uptake in the presence of Li^+ or Na^+ was greatly reduced and external Na^+ resulted in very little, if any, ^{45}Ca uptake. In similar experiments, external divalent cations were evaluated and the results reported in Fig. 2. For Na-dependent ^{45}Ca uptake, the ranking in saturating the uptake process was $Ca^{2+} > Sr^{2+} \geqslant Ba^{2+} > Mn^{2+}$. Mg^{2+} did not interfere with ^{45}Ca uptake at any of the concentrations examined. In fact, at concentrations greater than

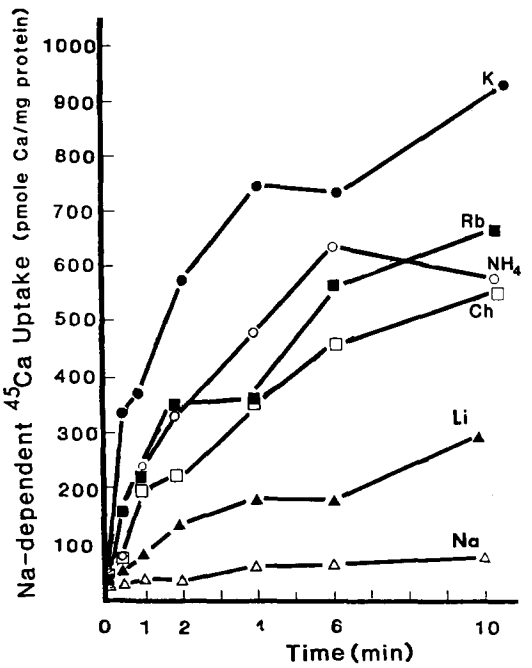

Fig. 1. Effect of external monovalent cations on the rate of ^{45}Ca uptake into NaCl pre-equilibrated vesicles. Ca-loading solution (Table I) was modified by replacing 435 mM KCl (●) with equal molar concentrations of RbCl (■), NH_4Cl (○), choline chloride (□), LiCl (▲) and NaCl (△). The $CaCl_2$ concentration was 0.01 mM for all load experiments.

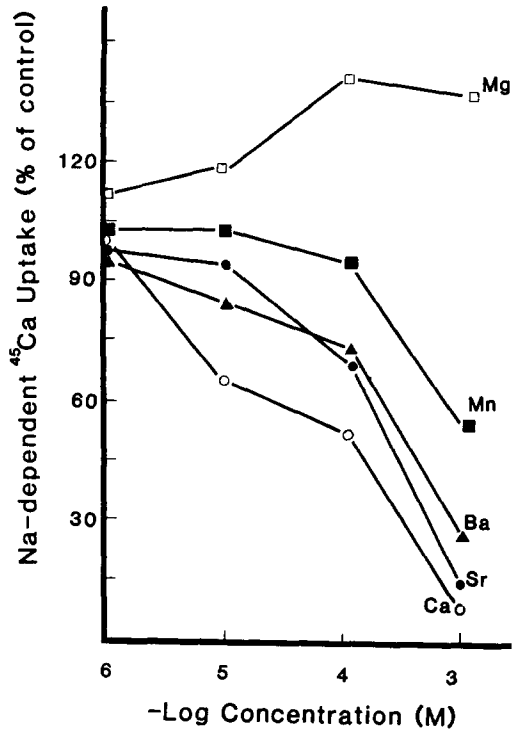

Fig. 2. Effect of external divalent cations on ^{45}Ca uptake into NaCl pre-equilibrates vesicles. Ca-loading solution (Table I) was modified by increasing the concentrations of $MgCl_2$ (□), $MnCl_2$ (■), $BaCl_2$ (▲), $SrCl_2$ (●) and $CaCl_2$ (○) as indicated in the figure. The mean ^{45}Ca load under control conditions (i.e., Ca-loading solution) was 532 ± 8 (S.E.) pmol Ca^{2+}/mg protein.

10^{-5} M, Mg^{2+} produced a slight stimulation of Na^+-dependent ^{45}C uptake.

Another unique feature of Na^+-Ca^{2+} exchange is its reversibility which has been reported both in perfused squid giant axons [32,7,33] and in synaptosomal and sarcolemmal vesicles [27,34]. To determine whether this aspect was evident in retinal axolemmal preparations, NaCl pre-equilibrated vesicles were loaded with $^{45}Ca^{2+}$ and effluxed against various solutions. The results reported in Table III support the contention that Na-dependent ^{45}Ca uptake can be reversed and that $^{45}Ca^{2+}$ is effluxed in a manner dependent on the external Na^+ gradient. Failure of choline to invoke ^{45}Ca efflux shows the importance of having a permeable cation to serve as a counter ion in this efflux mechanism. External Ca^{2+} (10 mM) was also shown to evoke significant ^{45}Ca efflux when compared to an external solution Choline

TABLE III

EFFECT OF EXTERNAL CATIONS ON ^{45}Ca EFFLUX

The percent of total ^{45}Ca load which was lost during a 2 min efflux period was determined as the difference between the amount of radioactivity remaining on the filter after efflux in the presence of various cations compared to the amount of radioactivity remaining after a zero-time efflux period in the presence of 425 mM choline (this value was set equal to 100% ^{45}Ca load). The mean ^{45}Ca load for these experiments was 437 ± 52 (S.E.) pmol Ca^{2+}/mg protein. The reported percentages represent the means ± S.E. of at least three experiments of two replicates each.

External solutions	Percent of ^{45}Ca load lost per 2 min
Choline	4.8 ± 3.0
Na_o-efflux	81.6 ± 9.2
$Li_o + Ca_o$-efflux	59.1 ± 7.4
Li_o-efflux	22.1 ± 5.4
Ca_o-efflux	27.2 ± 4.1

control. Interestingly, the combination of Ca^{2+} and Li^+ stimulated ^{45}Ca efflux above an additive value obtained when vesicles are effluxed against Ca^{2+} and Li^+, individually. These results are consistent with a Ca^{2+}-Ca^{2+} exchange in squid giant axons which is stimulated by Li^+ [10,32,14].

By these procedures, membrane vesicles rapidly accumulated Ca^{2+} only when pre-equilibrated with NaCl. Once loaded with Ca^{2+}, vesicles effluxed it in a Na^+-dependent manner. Thus only when a concentration gradient of Na^+ existed did Ca^{2+} flux occur, reflecting a Na^+-Ca^{2+} mediated exchange.

Effect of ATP on Na^+-Ca^{2+} exchange

One of the most interesting aspects of Na^+-Ca^{2+} exchange is its apparent enhancement by ATP [35,36,14,17]. ATP increases the affinity of the exchange for both Ca^{2+} and Na^+ which is suggestive of a catalytic role for ATP rather than as an energy source [14]. In view of this, it is now realized that the Na^+-Ca^{2+} exchanger has a high affinity for Ca^{2+} and is of major functional importance in the regulation of intraneural free Ca^{2+} levels [8,27,37].

As seen in Fig. 3, addition of ATP resulted in enhanced Na^+-dependent ^{45}Ca uptake into retinal nerve vesicles. Although the overall level of ^{45}Ca uptake was greatly increased as free Ca^{2+} con-

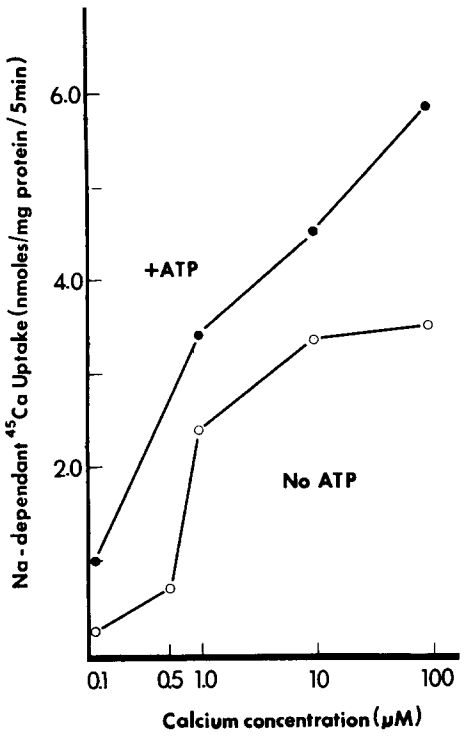

Fig. 3. Effect of calcium concentration on Na^+-dependent ^{45}Ca uptake into axolemma vesicles in the presence (+ATP, ●) and absence (No ATP, ○) of 0.1 mM Tris-ATP. Calcium concentrations were established at nominal values based on a CaEGTA stability constant value of $7.6 \cdot 10^6$ M^{-1} [46]. Approx. 7 μCi of $^{45}CaCl_2$ was added to each buffered sample giving a specific activity range for $^{45}Ca^{2+}$ of 0.2–2.0 Ci/mmol Ca^{2+}.

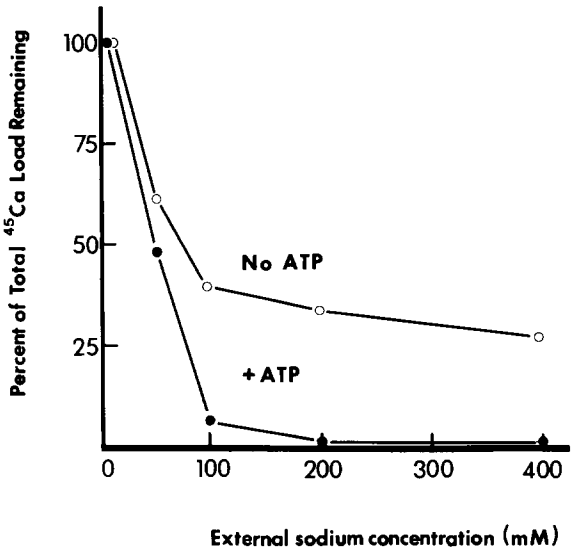

Fig. 4. Effect of external sodium concentration (Na_o) on ^{45}Ca efflux from vesicle pre-loaded with calcium (10^{-5} M CaEGTA) in the presence (+ATP, ●) and absence (No ATP, ○) of 0.1 mM Tris-ATP. The amount of $^{45}Ca^{2+}$ remaining on filters in samples effluxed against choline-containing solution (i.e., choline solution) was set equal to 100% ^{45}Ca load. When sodium concentration was reduced below 425 mM, it was replaced by choline on a equimolar basis.

centration increased, the ATP-promoted aspect of ^{45}Ca uptake was proportionally more pronounced at lower Ca^{2+} concentrations (e.g., less than 1.0 μM). Similarly, vesicles previously loaded with $^{45}Ca^{2+}$ in the presence of ATP were found to efflux their Ca^{2+} load against Na^+ concentrations much more efficiently than vesicles loaded in the absence of ATP (Fig. 4). These results correspond well with observations of Blaustein [14] in squid giant axons and supports the contention that Na^+-Ca^{2+} exchange is enhanced by ATP.

It has been previously reported by us [23–25] that vesicles from retinal nerve have a high level of ATP hydrolysing activity as judged by $[^{32}P]P_i$ liberation from [γ-^{32}P]ATP. A portion of this activity, termed Na^+-Ca^{2+} ATP hydrolysis, was characterized as ouabain-insensitive and selectively stimulated by Na^+ (Li^+), Ca^{2+} and K^+ ions. To study the possibility of ATP hydrolysis in the mechanism of ATP-promoted Na^+-Ca^{2+} exchange, the hydrolysis of [γ-^{32}P]ATP was determined under identical experimental conditions as for Na^+-Ca^{2+} exchange and co-plotted with the ATP-promoted aspect of Na^+-Ca^{2+} exchange (Fig. 5). Interference with ATPases was prevented by the addition of digitoxigenin, oligomycin and vanadate [18]. Under these conditions, there exists a good correlation between the level of ATP-promoted Na^+-Ca^{2+} exchange and Na^+-Ca^{2+} ATP hydrolysis at lower Ca^{2+} concentrations up to 1.0 μM. At elevated Ca^{2+} concentrations, this correlation is lost with Na^+-Ca^{2+} ATP hydrolysis increasing to a higher level of activity than ATP-promoted Na^+-Ca^{2+} exchange.

In experiments which examined the correlation between these two activities during Na^+-dependent Ca^{2+} efflux, both ATP-promoted Na^+-Ca^{2+} exchange and Na^+-Ca^{2+} ATP hydrolysis produced similar saturation curves against external Na^+ concentrations. Under these conditions, both

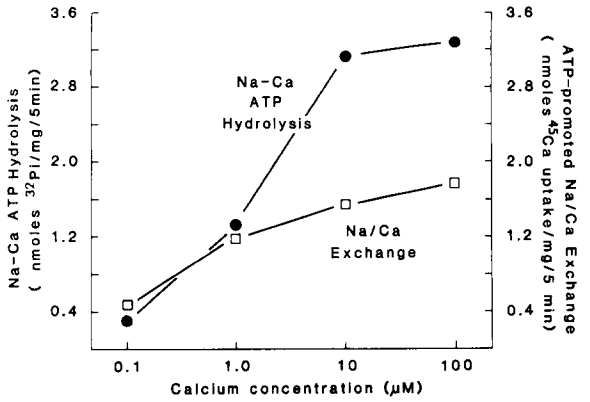

Fig. 5. Comparison of the effect of calcium concentration on ATP-promoted Na^+-Ca^{2+} exchange (□) and Na^+-Ca^{2+} ATP hydrolysis (●). ATP-promoted Na^+-Ca^{2+} exchange is reported as the difference in ^{45}Ca uptake into NaCl pre-equilibrated vesicles in the presence and absence of 0.1 mM ATP. Na^+-Ca^{2+} ATP hydrolysis was calculated as the difference between the inorganic [^{32}P]P_i formed during a 5 min calcium loading period when 0.1 mM [γ-^{32}P]ATP was incubated with NaCl pre-equilibrated vesicles minus the amount of inorganic [^{32}P]P_i formed when [γ-^{32}P]ATP was incubated in the presence of vesicles pre-equilibrated in a solution where NaCl was replaced by choline chloride (425 mM choline chloride). Calcium concentrations were established at nominal values vased on a CaEGTA stability constant value of $7.6 \cdot 10^6$ M^{-1} [46]. In addition to KCN, ouabain and oligomycin, the solutions used in these experiments also contained 10 μM vanadate and 10 μM digitoxigenin.

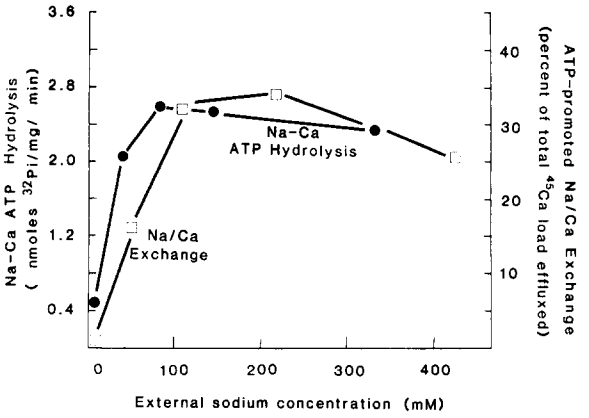

Fig. 6. Comparison of the effect of external sodium concentration on ATP-promoted Na^+-Ca^{2+} exchange (□) and Na^+-Ca^{2+} ATP hydrolysis (●). ATP-promoted Na^+-Ca^{2+} exchange was plotted as the difference between the precent of total ^{45}Ca load effluxed in the presence and absence of 0.1 mM Tris-ATP from axolemma vesicles pre-equilibrated in NaCl and loaded with $^{45}Ca^{2+}$ (10^{-5} M CaEGTA). The amount of $^{45}Ca^{2+}$ remaining on filters in samples effluxed for 2.0 min against choline solution was set equal to 100% ^{45}Ca load. Na^+-Ca^{2+} ATP hydrolysis was determined as the difference between the amount of inorganic [^{32}P]P_i produced from 0.1 mM [γ-^{32}P]ATP when vesicles pre-loaded with Ca^{2+} (10^{-5} M CaEGTA) were effluxed for 2.0 min against Na_o-efflux solution minus the amount of inorganic [^{32}P]P_i former when vesicles were effluxed against choline solution. When sodium concentration was reduced below 425 mM, it was replaced by choline on an equimolar basis. In addition to KCN, ouabain and oligomycin, the solutions used in those experiments also contained 10 μM vanadate and 10 μM digitoxigenin.

reached maximal levels of activity at 100 mM NaCl (Fig. 6).

Further comparisons of ATP-promoted Na^+-Ca^{2+} exchange and Na^+-Ca^{2+} ATP hydrolysis were made using agents which selectively modify various ion fluxes and the results are reported in Table IV. As judged by its I_{50} values, monensin, a Na^+ ionophore which dissipates Na^+ gradients, was particularly inhibitory to both ATP-promoted Na^+-Ca^{2+} exchange and Na^+-Ca^{2+} ATP hydrolysis. Because Na^+-Ca^{2+} exchange is absolutely dependent on the existence of a Na^+ gradient, this parallel sensitivity of both systems to monensin supports the possibility of ATP hydrolysis in the mechanism of ATP-promoted Na^+-Ca^{2+} exchange. The Ca^{2+} ionophore, A23187, was also quite inhibitory to ATP-promoted Na^+-Ca^{2+} exchange but was approximately 100-fold less inhibitory to Na^+-Ca^{2+} ATP hydrolysis. Like monensin, the addition of valinomycin, a K^+ ionophore, resulted in very similar levels of inhibition of both activities.

Neither activity was affected by the addition of either the Na^+ channel blocker, tetrodotoxin, or by the K^+ channel blocker, tetraethylammonium. Tetracaine was inhibitory to both but only at high concentrations and probably reflects its effect on lipid modulation rather than a direct interaction with Ca^{2+} transport systems [38]. Both activities were sensitive to La^{3+} at similar concentrations. La^{3+} is known to replace Ca^{2+} in many transport and binding systems [11,39] and has been reported [27] to be a potent inhibitor of Na^+-Ca^{2+} exchange in synaptosomal membrane vesicles ($K_i = 2$ μM). The decrease in sensitivity to La^{3+} in our preparation either indicates a vast difference in our biological materials or indicates that the ATP-promoted aspect of Na^+-Ca^{2+} exchange in-

TABLE IV

DRUG CONCENTRATION REQUIRED FOR HALF MAXIMUM INHIBITION (I_{50}) OF ATP-PROMOTED Na^+-Ca^{2+} EXCHANGE AND Na^+-Ca^{2+} ATP HYDROLYSIS

The I_{50} values were obtained from log dose-probit activity curves and the values reported represent mean estimated values from three experiments of two replicates each. Concentration values ranged from 10^{-8} to 10^{-3} M. ATP-promoted Na^+-Ca^{2+} exchange activity and Na^+-Ca^{2+} ATP hydrolysis were measured as described in Fig. 5. A23187, calcium ionophore (calimycin); $LaCl_3$, lanthanum chloride.

Drug	I_{50} (M)	
	ATP-promoted Na^+-Ca^{2+} exchange	Na^+-Ca^{2+} ATP hydrolysis
Monensin	$7.5 \cdot 10^{-6}$	$0.8 \cdot 10^{-6}$
A23187	$2.5 \cdot 10^{-6}$	$3.0 \cdot 10^{-4}$
Valinomycin	$7.5 \cdot 10^{-5}$	$5.0 \cdot 10^{-5}$
Tetrodotoxin	$> 10^{-5}$	$> 10^{-5}$
Tetraethylammonium	$> 10^{-3}$	$> 10^{-3}$
Tetracaine	$6.0 \cdot 10^{-4}$	$8.5 \cdot 10^{-4}$
$LaCl_3$	$2.0 \cdot 10^{-3}$	$7.5 \cdot 10^{-4}$
Pentylenetetrazole	$> 10^{-3}$	$> 10^{-3}$
Vanadate	$> 10^{-3}$	$> 10^{-4}$
Ouabain	$> 10^{-3}$	$> 10^{-3}$
Digitoxigenin	$> 10^{-4}$	$> 10^{-4}$
Oligomycin	$> 10^{-4}$	$> 10^{-4}$
Cyanide	$> 10^{-3}$	$> 10^{-3}$

teracts with La^{3+} quite differently than the non-promoted form. Pentylenetetrazole, a CNS stimulant/convulsant which causes Ca^{2+} dependent electrical bursting activity in ganglionic nerve cells [40] had no effect on either systems.

Several agents reported to be inhibitors of adenosine triphosphatases or other ATP-dependent processes [17] were not found to be specific for either ATP-promoted Na^+-Ca^{2+} exchange or Na^+-Ca^{2+} ATP hydrolysis. Vanadate, ouabain and digitoxigenin were all without effect on either activity at concentrations far in excess of that necessary to produce their inhibitory effect on ATP-dependent processes. Similarly, high concentrations of the mitochondrial inhibitor, oligomycin and cyanide were without effect on either activity examined.

Conclusion

There is no doubt as to the fundamental importance of Ca^{2+} homeostasis in excitable tissues such as nerve and muscle. Entry of external Ca^{2+} through plasma membranes of stimulated cells underlies the importance of intracellular free Ca^{2+} concentration on biological processes such as the secretion of neural transmitters and hormones, muscle contractions, permeability of membranes to ions, and conduction of electrical impulses [41,8,9]. The biological ramifications which occur upon Ca^{2+} entry during a single nerve discharge impose at least two necessary restriction on Ca^{2+} regulation in excitable cells. First, intracellular resting Ca^{2+} concentrations must be maintained at exceedingly low levels for the cell to detect a Ca^{2+} signal produced during bioelectrical activity. Secondly, Ca^{2+} entry must be highly interfaced with Ca^{2+} extrusion or sequestration systems in order to maintain Ca^{2+} homeostasis [8,9].

At least four mechanisms which extrude or sequester Ca^{2+} in nerve cells have been implicated in the control of intracellular Ca^{2+} concentration; plasma membrane, endoplasmic reticulum, intrasynaptic vesicles and mitochondria [37]. Besides the removal of Ca^{2+} from cells via the plasma membrane, the remaining three mechanisms represent sequestration processes which are temporary and of finite capacity. Thus, the ultimate removal of cytosolic Ca^{2+} and maintenance of Ca^{2+} homeostasis remains a functions of the plasma membrane.

There is ample evidence supporting the coexistence of two Ca^{2+} flux mechanisms (i.e., (ATP + Mg^{2+})-dependent Ca^{2+} pump and reversible Na^+-Ca^{2+} exchange) operating in neural plasma membranes [42,27,37] and there is no question as to the importance of Na^+-Ca^{2+} exchange in maintaining the Ca^{2+} gradient in squid giant axons [11,43,12,44,19,45,14]. The justification that Na^+-Ca^{2+} exchange was the major Ca^{2+} extruding mechanism of squid giant axons came from Mullins [13] and Baker [9] and is supported by two related findings. First, the Na^+-Ca^{2+} exchanger in synaptolemma is now known to have a distinct high-affinity component for Ca^{2+} with a K_m of approx. 0.5 μM which is only slightly less than the value for the coexisting ATP-dependent Ca^{2+} pump [37]. Because of this, it was suggested that the Ca^{2+} pump primarily functions in the maintenance of low resting levels of cytosolic Ca^{2+} rather than in the removal of larger levels of Ca^{2+} which

result during bioelectrical activity, a function much more suited for the Na^+-Ca^{2+} exchanger with its higher capacity and slightly reduced affinity for Ca^{2+}. Second, Na^+-Ca^{2+} exchange appears to be promoted by ATP. As suggested by Blaustein [14], ATP apparently increased the affinity of the exchange for both Na^+ and Ca^{2+}, thereby producing a more efficient countertransport of these ions in squid giant axons. Using a related system, Reinlib et al. [17] reported a similar enhancement with ATP of Na^+-Ca^{2+} exchange in sarcolemmal vesicles from dog heart. Overall enhancement was approx. 4-fold at 1.0 μM ATP and did not increase at higher ATP concentrations indicating a high ATP affinity for this process. Recently, they have detailed this phenomenon and found the regulation of Na^+-Ca^{2+} exchange to be a cyclic process in which a protein kinase activates the exchanger in a phosphorylation step and a phosphatase deactivates it in a dephosphorylation step. The regulation of the two enzymes is made possible by their different affinity for Ca^{2+}. In the presence of calmodulin, the K_m of Ca^{2+} in the activation process was 0.8 μM and in the deactivation process was about 1.5 μM [18]. As a result of these studies in mammalian muscle, much of the basic mechanism for Na^+-Ca^{2+} exchange as been elucidated and can serve as a model for other systems.

In the present work using retinal axolemmal vesicles, we report an in vitro Na^+-Ca^{2+} exchange activity that is similar in many respects to the Na^+-Ca^{2+} exchange reported in perfused squid giant axons. This exchange is absolutely dependent on the establishment of a Na^+ gradient, shows monovalent and divalent cation specificity, and is highly sensitive to monensin, A23187 and valinomycin but not to ouabain, digitoxigenin, vanadate, pentylenetetrazole, tetrodotoxin or tetraethylammonium. Furthermore, the exchange is promoted by ATP.

The establishment of an in vitro Na^+-Ca^{2+} exchange using cellular fragments of the squid nervous system is important in several ways. First, the question of the role of ATP may be addressed in more detail in such a preparation. Second, the precise concentration of key ions (e.g., Ca^{2+}) can be established and their effect on binding proteins such as calmodulin examined. Third, this preparation is highly suitable for future investigations including the isolation, purification, and reconstitution of exchange components.

As for the relationship between the ATP-promoted aspect of Na^+-Ca^{2+} exchange and Na^+Ca^{2+} ATP hydrolysis measured in this preparation, we were able to point out a number of similarities existing between them, particularly their parallel sensitivity to monensin and similar affinities for Na^+. It is also of interest that the apparent K_m of Ca^{2+} calculated for the ATP-promoted aspect of Na^+-Ca^{2+} exchange is approx. 0.5–0.8 μM. This value is comparable to the half-maximal value of 0.8 μM Ca^{2+} calculated for the activation process of the Na^+-Ca^{2+} exchange in heart sarcolemma which is mediated by a protein kinase and deactivated by a phosphatase [18]. Much work is necessary to answer the questions whether these two activities in squid retinal axons are indeed identical and whether a protein kinase-phosphatase interaction plays a part in the ATP hydrolysis reaction. Notwithstanding, the current work does establish the existence of an ATP-promoted Na^+-Ca^{2+} exchange which exhibits many characteristics that are identical to the exchange in the squid giant axon. Knowing that nature seldom wastes in this manner, such a system is likely to have some function in regulating the ionic environment in these axons, if not via Na^+-Ca^{2+} exchanging.

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