Biochimica et Biophysica Acta, 771 (1984) 53–58
Elsevier

BBA 72038

Na^+-Ca^{2+} EXCHANGE IN THE AXOLEMMA-RICH MEMBRANE VESICLE PREPARATIONS FROM THE WALKING-LEG NERVES OF THE AMERICAN LOBSTER

ARNOLD A. PETERSON [a,b], FUMIO MATSUMURA [a,*] and ESTELLE J. McGROARTY [b,c]

[a] *Pesticide Research Center,* [b] *Department of Biophysics, and* [c] *Department of Biochemistry, Michigan State University, East Lansing, MI (U.S.A.)*

(Received August 9th, 1983)
(Revised manuscript received December 12th, 1983)

Key words: Ca^{2+} transport; Na^+-Ca^{2+} exchange; Nerve membrane; (Lobster)

An axolemma-rich membrane vesicle fraction was prepared from the leg nerve of the lobster, *Homerus americanus*. In this preparation Ca^{2+} transport across the membrane was shown to require a Na^+ gradient (Na^+-Ca^{2+} exchange), and external K^+ was found to facilitate this Na^+-Ca^{2+} exchange activity. In addition, at high Ca^{2+} concentrations (20 mM) a Ca^{2+}-Ca^{2+} exchange system was shown to operate, which is stimulated by Li^+. The Na^+-Ca^{2+} exchange system is capable of operating in the reverse direction, with Ca^{2+} uptake coupled with Na^+ efflux. Such a vesicular preparation has the potential for providing useful experimental approaches to study the mechanism of this important Ca^{2+} extrusion system in the nervous system.

Introduction

The Ca^{2+} concentration within cells is important for many processes, such as secretion of hormones [1], muscle contraction [2], as well as numerous enzyme activities [3]. In neurons it is especially important in the propagation of action potentials [4,5], axonal transport [6] and in the secretion of neurotransmitters [7,8]. Measurements of axoplasmic calcium levels are hampered by the fact that most intracellular calcium is either bound to proteins or sequestered in organelles such as mitochondria and endoplasmic reticulum [9]; hence the resulting free concentration (approx. 10^{-8} to 10^{-10} M) is difficult to determine. Considering the steep gradient of calcium ions across the membrane, it is apparent that an active transport system is necessary to maintain the low internal free Ca^{2+} levels.

The Na^+-Ca^{2+} exchange system transports Ca^{2+} across a membrane against its electrochemical gradient by the flow of Na^+ down its gradient [10]. This process has been demonstrated in squid giant axons [9,11], cardiac muscle vesicles [12,13], brain synaptosomes [14,15], and synaptic membrane vesicles [16].

Despite the wealth of information on the Na^+-Ca^{2+} exchange system in these tissues no established method is presently available to study the biochemical aspects of this phenomenon in axolemmal vesicles in vitro. This is particularly unfortunate since the original studies on Na^+-Ca^{2+} exchange were conducted with squid giant axons in situ and much valuable physiological information is available [9–11]. The giant squid axon itself, however, is unsuitable for biochemical studies because of its low surface area to weight ratio and contamination by nonaxolemmal material. In 1972 Denburg [17] developed a centrifugation

* To whom correspondence should be addressed.
Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

method to obtain a relatively pure axolemmal fraction from the walking leg nerve of the American lobster, *Homerus americanus*. By using this preparation, Baumgold [18] studied the binding of $^{45}Ca^{2+}$ to the membrane material in the presence of various monovalent cations. He found that $^{45}Ca^{2+}$ binding is stimulated by the presence of K^+ and reduced by Na^+. Such an effect is consistent with the expected function of a Na^+-Ca^{2+} exchange system in which Na^+ and Ca^{2+} may compete for the same binding site. On the other hand, his approach does not prove the existence of the Na^+-Ca^{2+} exchange on two accounts: first, cations were added only externally to axolemma vesicles prepared in sucrose and not on opposite sides of the membrane; and second, the role of the (Ca^{2+} + Mg^{2+})-ATPase was not assessed.

In the present study we report that the uptake of Ca^{2+} by lobster axoplasmic vesicles is due to a Na^+-Ca^{2+} exchange system. This preparation, unlike intact synaptosomes or cells, offers greater control of chemical concentrations on either side of the membrane without potential interference from other ions and organelles.

Materials and Methods

Preparation of vesicles. Vesicles enriched with axolemmal membranes were prepared from the walking legs and claws of live lobsters (*Homerus americanus*), following Denburg's isolation method with slight modifications [17]. Nerves were removed, chopped to 0.2 mm pieces using a tissue chopper (McIllwain type) and homogenized in 0.32 M sucrose/20 mM Tris-HCl (pH 7.4). Vesicles were isolated by a series of differential centrifugation steps and the final pellet was resuspended and stored in an 'internal' solution of 420 mM specified salt, 20 mM Tris-HCl, pH 7.4 until used (2 h to 16 h later). All steps were carried out at 4°C. Typically, 1 g (wet weight) of nerve tissue from one lobster yielded 2.2 mg membrane-bound protein as determined by the method of Lowry. Experiments by Marquis et al. [19] have suggested that these vesicles are predominantly right-side-out.

Ca^{2+} influx experiment. 10 μl aliquots of vesicles containing 13.5 μg protein in 'internal' solution were preincubated with 0.1 mM ouabain for 15 min at room temperature. A 0.5 ml aliquot of a 'loading' solution, consisting of 420 mM specified salt, 0.1 mM ouabain, 40 μM $^{45}CaCl_2$ (65 cpm/pmol) (New England Nuclear) in 20 mM Tris-HCl (pH 7.4) was added and the system incubated at room temperature for 5 min (except where specified). Aliquots of 0.2 ml were removed, filtered through Millipore filters (0.45 μm), and washed twice with 8 ml of chilled buffer (420 mM choline chloride, 20 mM Tris-HCl, pH 7.4). The filter was air dried, dissolved in a scintillation solution and counted.

Ca^{2+} efflux experiment. Calcium efflux was measured by the decrease in $^{45}Ca^{2+}$ from preloaded vesicles. Vesicles were loaded with $^{45}Ca^{2+}$ as described above. A 50 μl aliquot was removed, and transferred to 5 ml of 'efflux' buffer, containing Na^+, Li^+ or choline with or without calcium (Table IV), incubated for 2 min, and filtered as described above to estimate $^{45}Ca^{2+}$ remaining in the vesicles. A 2 min incubation time was chosen since by this time the fast efflux was completed.

Other assays. Mitochondrial and lysosomal contamination was estimated by assaying succinate dehydrogenase [20], acid phosphatase [21], and NADH-cytochrome *c* reductase [22] activities. No activities were detected under these assay conditions, suggesting that membranes from these organelles, if present, were of negligible quantities, while the assay for the plasma membrane marker acetylcholinesterase, [23] showed high activity. Inductively coupled plasma-emission spectroscopy of membrane isolates was carried out as described previously [24] to quantitate levels of phosphorus and membrane bound metals.

Results

The composition of the vesicle preparation was determined to be approx. 42% protein and 58% phospholipid, as calculated from phosphorous and protein concentrations. Membrane-bound Ca^{2+} varied between 18 and 54 nmol/mg protein regardless of whether or not the homogenation buffer contained 1 mM EDTA, indicating that this Ca^{2+} is tightly bound. Ca^{2+} uptake results reported in this study assume that this calcium remains bound, and does not affect the Ca^{2+} concentration added. In addition, the preparation also contained an average of 72 nmol iron/mg protein and 22 nmol

TABLE I

INFLUENCE OF THE PRESENCE AND DIRECTION OF Na^+ GRADIENT ON $^{45}Ca^{2+}$ UPTAKE BY THE AXOLEMMA VESICLES

Ionic concn. (mM)	Internal solution	Loading solution	Ca^{2+} uptake (nmol/mg protein)
160 [a]	NaCl	KCl	43.7
	NaCl	NaCl	3.0
	KCl	KCl	4.4
	KCl	NaCl	4.3
450 [b]	NaCl	KCl	65.1
	NaCl	NaCl	2.0

[a] Buffered with 20 mM Hepes, pH 7.3.
[b] Buffered with 20 mM Mops, pH 7.4.

aluminum/mg protein. It is known that aluminum ions alter membrane fluidity [25], and are a possible inhibitor of ATPases [26]. However, since intact nerve tissue from live lobsters also contains similar levels of these ions, they presumably are irreversibly bound.

The results of $^{45}Ca^{2+}$ influx experiments indicate that isolated membrane vesicles have a high Na^+-Ca^{2+} exchange activity which is sensitive to the Na^+ gradient (Table I). Influx in this study refers to Ca^{2+} uptake into the presumably right-side-out vesicles (the reverse of the naturally occurring direction). Plots of Ca^{2+} uptake versus time (Fig. 1) show that the amount of $^{45}Ca^{2+}$ in the vesicles reached a steady-state level after 5 min; hence this incubation time was used for all subsequent experiments. This steady state level represents an accumulation of approx. 3% of the available Ca^{2+}, and thus is not due to a significant decrease in the external concentration of Ca^{2+}.

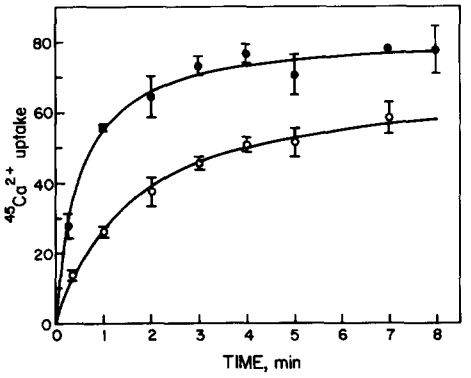

Fig. 1. Time-course of $^{45}Ca^{2+}$ uptake (nmol/mg protein) by the axolemma vesicle preparations. Upper curve (●) is Na^+-Ca^{2+} exchange in the presence of external K^+. In the lower curve (○), K^+ was replaced with Li^+. Internal solution contained 450 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ouabain and 10 mM Mops. The external solution contained 450 mM KCl (or LiCl for the lower curve), 10 mM $MgCl_2$, 40 μM $CaCl_2$, 0.1 mM M ouabain and 10 mM Mops, pH 7.4.

TABLE II

EFFECTS OF THE REPLACEMENT OF INTERNAL Na^+ WITH CATIONS AND SUCROSE ON Ca^{2+} UPTAKE BY AXOLEMMA VESICLES

Solutions contained 10 mM KCl, 10 mM $MgCl_2$, (except 420 mM choline which contained no KCl). Sucrose contained 20 mM $MgSO_4$, 10 mM Mops, pH 7.4. Loading solution contained 410 mM KCl, 10 mM $MgCl_2$, 10 mM NaCl. Values are presented as means ± S.E., the number of determinations given in parentheses.

Internal ionic compositions	Ca^{2+} uptake (nmol/mg protein)
410 mM NaCl	60.4 ± 2.3 (15)
160 mM NaCl, 250 mM choline chloride	26.0 ± 1.6 (8)
410 mM LiCl, 0.1 mM $CaCl_2$	1.0 ± 0.7 (6)
420 mM choline chloride	1.4 ± 0.5 (2)
320 mM sucrose	2.1 ± 0.4 (8)
320 mM sucrose, 0.5 mM EGTA	1.4 ± 0.4 (2)

To test the specificity for sodium in the exchange mechanism, the internal Na^+ was replaced

TABLE III

EFFECTS OF THE REPLACEMENT OF K^+ IN LOADING SOLUTION WITH SOME MONOVALENT CATIONS AND SUCROSE ON Ca^{2+} UPTAKE BY AXOLEMMA VESICLES

Values presented as means ± S.E., the number of determinations in parentheses.

External ionic compositions	Ca^{2+} uptake (nmol/mg protein)
410 mM KCl	42.8 ± 2.3 (18)
410 nM NaCl, 10 mM KCl [a]	1.2 ± 1.1 (10)
160 mM NaCl, 250 mM choline chloride, 10 mM KCl [a]	5.5 ± 3.4 (7)
410 mM LiCl, 10 mM KCl [a]	30.2 ± 2.0 (7)
420 mM choline chloride, 10 mM KCl	21.3 ± 0.9 (4)
420 mM sucrose, 10 mM KCl	11.0 ± 1.6 (4)

[a] Solutions also contain 10 mM $MgCl_2$.

TABLE IV

PERCENTAGES OF $^{45}Ca^{2+}$ EFFLUXED FROM PRE-LOADED VESICLES

Solutions contain 10 mM KCl, 10 mM $MgCl_2$, (420 mM choline chloride contained no KCl)

Ion composition of efflux buffer	% Ca^{2+} effluxed [a]	Statistical difference from control
410 mM NaCl (control)	92.5 ± 1.1 (12)	–
410 mM NaCl, 20 mM $CaCl_2$	95.3 ± 0.6 (5)	*P*, 0.02
410 mM LiCl	70.8 ± 6.4 (6)	*P*, 0.01
410 mM LiCl, 20 mM $CaCl_2$	88.2 ± 1.0 (6)	*P*, 0.025
420 mM choline chloride	62.8 ± 4.7 (9)	*P*, 0.0005
420 mM choline chloride, 20 mM $CaCl_2$	80.7 ± 0.6 (4)	*P*, 0.0005

[a] $\frac{\text{Loaded value} - \text{remaining value}}{\text{Loaded value}} \times 100$. Values are presented as means ± S.E., the number of determinations given in parentheses.

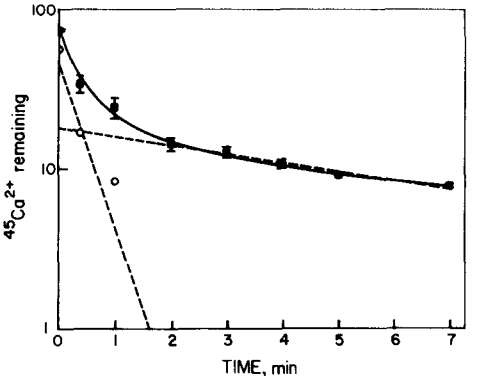

Fig. 2. Time-course of $^{45}Ca^{2+}$ efflux (nmol/mg protein) from preloaded vesicles. The upper curve (■) indicates the actual data. The dotted lines are: --○-- fast efflux (actual values minus line of slow efflux), and ------, slow efflux which was obtained by extending the last five data points (2, 3, 4, 5 and 7 min). Preloading was done as described in Fig. 1 with 450 mM KCl in the external solution. Efflux buffer contained 410 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM ouabain, and 20 mM Tris, pH 7.4.

with a variety of monovalent cations or sucrose (Table II). It was found that only a sodium gradient across the membrane promoted Ca^{2+} uptake, and that decreasing the gradient by replacing internal sodium with choline reduced the level of Ca^{2+} uptake. Lithium, in the presence of a low amount of Ca^{2+} (0.1 mM), was found to be as ineffective as sucrose or choline, showing that a high specificity for Na^+ exists in this system.

To optimize the external ion requirements, the K^+ in the 'loading' solution was replaced by various ions and sucrose (Table III). It was found that Ca^{2+} uptake was reduced though not eliminated, when K^+ was replaced by any of the substitutes tested, implying a specificity for K^+.

The specificity of ion requirements was also assessed in Ca^{2+} efflux assays (Table IV). A greater level of Ca^{2+} efflux was observed when Na^+ was added externally than when Li^+, choline or sucrose was added. High concentrations of external Ca^{2+} increased the efflux of Ca^{2+}, particularly when added along with Li^+.

When the time-course of $^{45}Ca^{2+}$ efflux was studied (Fig. 2), it was found that there are two phases of efflux. The first phase had a $t_{1/2}$ of approx. 0.12 min and the slow phase a $t_{1/2}$ of 6 min. It is likely that the former represents efflux of unbound $^{45}Ca^{2+}$ from extra vesicular spaces or other readily dissociable or accessible sites, and that the latter represents Na^+-Ca^{2+} exchange. This assumption agrees well with the observations from Table IV, that even in the experiment where 420 mM choline was the only cation present approx. 63% of $^{45}Ca^{2+}$ had effluxed during the 2-min incubation period.

Discussion

The results of the current study indicate that axolemma-rich membrane vesicles from lobster walking leg nerves have high Na^+-Ca^{2+} exchange activity. These vesicles are easily and quickly prepared, and have been shown to be relatively free of contamination by other membrane materials. Thus the system may be ideally suited for analysis of the mechanism of axonal Na^+-Ca^{2+} exchange. The ion transport characteristics of this Na^+-Ca^{2+} exchange are similar to those reported in other systems [9,11]. Our results indicate that in the absence of a sodium gradient there is little Na^+-Ca^{2+} exchange activity, though with high levels of Ca^{2+} (20 mM) added to the opposite side of the membrane as $^{45}Ca^{2+}$, a Ca^{2+}-Ca^{2+} exchange is observed. Ca^{2+} efflux with a Na^+ gradient is not enhanced significantly by the addition of external Ca^{2+} (Table IV), suggesting that these are not two

separate mechanisms but the same system operating under different conditions [27]. The slight increase in measured Ca^{2+} efflux with added Ca^{2+} could be due to displacement of surface bound Ca^{2+}. Baumgold [18] previously found that the binding of $^{45}Ca^{2+}$ to axolemmal vesicles from the lobster leg nerve was dependent on the monovalent cation used in the incubating solution. In his experimental design vesicles suspended in sucrose were equilibrated in a salt solution (e.g., 450 mM NaCl or 450 mM KCl) and then incubated with $^{45}CaCl_2$. The accumulation of $^{45}Ca^{2+}$ by the vesicle was assessed through filtration and washing as in this study. Under such experimental conditions the influence of various ions on Ca^{2+} binding on only one side of the membrane was studied. Baumgold observed that greater amounts of Ca^{2+} were bound when K^+ was in the external medium than when Na^+ was present. We have made a similar observation when the external ion composition was varied (Table III). It is interesting to note that Baumgold also found the $^{45}Ca^{2+}$ uptake process to be biphasic [18]. The fast phase was judged to be due to general binding since it was not influenced by ionic changes. This interpretation supports our conclusion that the fast phase reaction observed in both uptake (Fig. 1) and efflux (Fig. 2) is due to nonspecific, monovalent cation insensitive events. The slow phase is believed to represent the actual transport of ions across the membrane.

As for the possibility of involvement of other calcium regulating systems in this assay system, one could eliminate $(Ca^{2+} + Mg^{2+})$-ATPase, as this enzyme under the condition used here is rather labile, and is inactivated after 24 h at $-15°C$. Also $(Ca^{2+} + Mg^{2+})$-ATPase is not activated by Na^+, therefore, the portion of the $^{45}Ca^{2+}$ uptake or efflux which was stimulated by Na^+ cannot be ascribed to the $(Ca^{2+} + Mg^{2+})$-ATPase activity. By the same token, participation of mitochondrial and other Mg^{2+}-ATPases may be ruled out since these ATPases are not differentially stimulated by Na^+.

The specificity for a Na^+ gradient in both the influx and efflux experiments suggests that the same exchange system can operate in both forward and reverse directions, in agreement with previous observations in squid axon [28]. Thus experiments on systems operating in the reverse directions may be used as measures of Na^+-Ca^{2+} exchange system. This conclusion is similar to that reached by other workers [12,13,16] in different tissue preparations.

Acknowledgements

Supported by Michigan Agricultural Experiment Station, Michigan State University (Journal Article No. 11156) and by research grant ES01963 from the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina and by Grant No. 2-50 RR07049-15 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

References

1 Douglas, W.W. and Rubin, J.R.P. (1961) J. Physiol. 159, 40–57
2 Ebashi, S. and Endo, M. (1968) Progr. Biophys. Mol. Biol. 18, 123–183
3 DiPolo, R., Requena, J., Brinley, F.J., Jr., Mullins, L.J., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433–467
4 Tasaki, I., Singer, I. and Takenaka, T. (1965) J. Gen. Physiol. 48, 1095–1123
5 Frankenhaeuser, B. and Hodgkin, A.L. (1957) J. Physiol. 137, 218–244
6 Shecket, G.N. and Lasek, R.J. (1978) in The Cytoskeletal and Contractile Networks of Nonmuscle Cells (Goldman, R., ed.), p. 98, Cold Spring Harbor Laboratory, New York
7 Meech, R.W. (1974) J. Physiol. 237, 259–277
8 Katz, B. and Miledi, R. (1965) Proc. Roy. Soc. (London) Ser. B161, 496–503
9 Requena, J., DiPolo, R., Brinley, F.J., Jr. and Mullins, L.V. (1977) J. Gen. Physiol. 70, 329–353
10 Blaustein, M.P. (1974) Rev. Physiol. Biochem. Pharmacol. 70, 33–82
11 Blaustein, M.P. and Hodgkin, A.L. (1969) J. Membrane Biol. 22, 285–312
12 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235
13 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 590–594
14 Gill, D.L., Grollman, E.F. and Kohn, L.D. (1981) J. Biol. Chem. 256, 184–192
15 Blaustein, M.P. and Ector, A.C. (1976) Biochim. Biophys. Acta 419, 295–308
16 Gill, D.L. (1982) J. Biol. Chem. 257, 10896–10990
17 Denburg, J.L. (1972) Biochim. Biophys. Acta 282, 453–458
18 Baumgold, J. (1979) Biochim. Biophys. Acta 558, 149–165
19 Marquis, J.K., Hilt, D.C., Papadeas, V.A. and Mautner, H.C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2278–2282

20 Osborn, M.H., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biochem. 247, 3962–3972
21 Cotman, C.W. and Matthews, D.A. (1971) Biochim. Biophys. Acta 249, 380–391
22 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438
23 Ellman, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
24 Coughlin, R.T., Tonsager, S. and McGroarty, E.J. (1983) Biochemistry 22, 220–2007
25 Vierstra, R. and Haug, A. (1978) Biochem. Biophys. Res. Commun. 84, 138–143
26 Caldwell, C.R. and Haug, A. (1982) Physiol. Plant. 54, 112–118
27 Blaustein, M.P. (1977) Biophys. J. 20, 79–111
28 Baker, P.F., Blaustein, M.P., Manil, J. and Steinhardt, R.A. (1967) J. Physiol. 191, 100