Biochimica et Biophysica Acta, 986 (1989) 250–256
Elsevier

BBAMEM 74637

Currents related to the sodium-calcium exchange in squid giant axon

Carlo Caputo, Francisco Bezanilla and Reinaldo DiPolo

Centro de Biofisica y Bioquimica, IVIC, Caracas (Venezuela), Department of Physiology, Ahmanson Laboratory of Neurobiology and Jerry Lewis Neuromuscular Research Center, University of California, Los Angeles, CA and The Marine Biological Laboratory, Woods Hole, MA (U.S.A.)

(Received 8 June 1989)

Key words: Sodium-calcium ion exchange; Membrane current; Stoichiometry; (Squid axon)

We report the measurement of a Ca_i-activated membrane current in dialyzed squid axon under membrane potential control with a low-noise voltage clamp. Two additional voltage clamp systems were used to clamp the external guard plates to a value that prevented the establishment of potential differences between the central and lateral compartments of the experimental chamber. This reduced to a minimum the contribution of membrane currents generated at the axon ends to the current measured in the central pool. This latter current was reduced by using internal and external solutions designed to diminish at a maximum membrane currents, while maintaining the conditions for optimal operation of the Na^+-Ca^{2+} exchange. Thus TTX was used to block Na^+ channels and prolonged exposure to K^+-free media was used to eliminate K^+ conductance. The maximum concentration of external sodium was 200 mM. The addition of fixed amounts of free ionic calcium to the internal solution, activated a current whose direction and magnitude depended on the thermodynamic driving forces for calcium and sodium. When the experimental conditions determined an inwardly directed current, this depended on the presence of external sodium, and lithium could not substitute for it. The Ca_i-activated current, was blocked by external lanthanum and showed a high temperature dependence. In experiments in which the reversal potential was measured for the Ca_i- activated current, it was found to be strikingly similar to the value calculated according to $E_r = 3E_{Na} - 2E_{Ca}$, suggesting that the current is the electrical manifestation of the Na^+-Ca^{2+} exchange operating with an stoichiometry of $3Na^+ : 1Ca^{2+}$.

Introduction

In all animal cells, the existence of a steep electrochemical gradient favoring the inward movement of calcium ions requires compensatory mechanisms to maintain the long term calcium balance. In addition many cells require sudden and transient increases in the intracellular ionized calcium concentration for their physiological functions [1]. There is substantial evidence indicating that the Na^+-Ca^{2+} exchange mechanism may contribute to both these homeostatic and loading requirements for calcium ions [2].

Since the work of Blaustein and Hodgkin [3], it has been recognized that the Na^+-Ca^{2+} exchange mechanism in squid giant axons must operate moving more than two sodium ions for each Ca^{2+} transported across the membrane in order to maintain the intracellular calcium concentration at levels below 1 μM. However the precise exchange stoichiometry in squid axons is still lacking; for instance Mullins [2], based on theoretical calculation has proposed a $4Na^+ : 1Ca^{2+}$ stoichiometry, while Blaustein [4] determination of Hill's coefficient is more compatible with a $3Na^+ : 1Ca^{2+}$ scheme. In any case, assuming a stoichiometry greater than $2Na^+ : 1Ca^{2+}$ each transport cycle moving one or more positive charge across the membrane, should generate an electric current whose magnitude and direction should be determined by the sodium and calcium electrochemical gradients. Eisner and Lederer [5] have clearly pointed out the theoretical difficulties for making reasonable predictions about the magnitude of this current without precise information about the factors involved in the exchange mechanism or without direct measurements. The experimental identification and precise measurements of such a current have been hindered by the presence of other ionic currents, including in some preparations a calcium-activated non specific current [6] and by the lack of a specific inhibitor of the

Correspondence: Dr. F. Bezanilla, Department of Physiology, University of California. Los Angeles, CA 90024, U.S.A.

sodium-calcium exchange mechanism. One preparation that has yielded a great amount of quantitative information about the Na^+-Ca^{+2+} exchange, including its voltage sensitivity, is the giant axon of the squid [4,7,8,9]. However, no direct measurements of membrane currents associated with the exchange mechanism in this preparation had been carried out. In this work we describe a membrane current in the squid axon, that is activated by intracellular calcium and is dependent on the presence of sodium ions. We also report the potential dependence of this current and its reversal value. The data strongly suggest that in squid axon the Na^+-Ca^{2+} exchange operates with a stoichiometry of $3Na^+ : 1Ca^{2+}$ confirming the results obtained with other preparations [10–12]. Preliminary reports of this work have been presented elsewhere [13–15].

Methods

The experiments were carried out with giant axons from *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. Immediately after decapitation of the squid, the giant axons were dissected from the mantle in running seawater, and further cleaned in chilled artificial seawater (ASW).

Experimental chamber

The experimental chamber was a modification of one previously used [9]. It contained the essential features for voltage clamping axons segments under internal dialysis conditions (see Fig. 1). The length of the current measuring segment was reduced in order to improve clamping conditions. In addition, the guard cur-

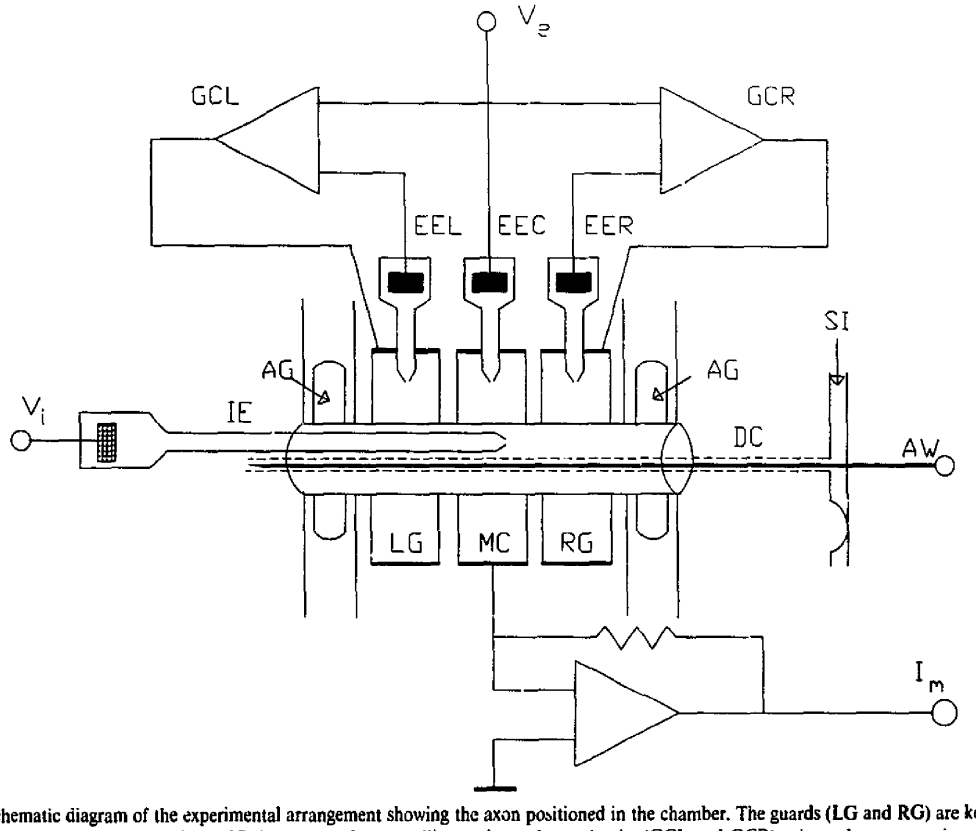

Fig. 1. Schematic diagram of the experimental arrangement showing the axon positioned in the chamber. The guards (LG and RG) are kept at the same potential of the central region (MC) by means of two auxiliary voltage clamp circuits (GCL and GCR) using voltage measuring electrodes EER, EEC and EEL and injecting currents through the external plates. The axial wire (AW) is positioned inside the dialysis capillary (DC) which is inserted in the axon. The dialysis solution (SI) in inserted into the dialysis capillary through a two-way valve that allows the change of solution. The internal electrode (IE) is positioned with its tip in the the central region to measure the internal potential (V_i). The regular voltage clamp to keep control the potential of the center region (not pictured) is done comparing the difference between the internal potential (V_i) and the external potential (V_e) with the command potential and feeding back the current through the axial wire (AW) Membrane current (I_m) is measured with a current to voltage converter. AG are the air gaps.

rent compartments were designed to allow clamping of the current plates to maintain uniformity in the potential and prevent longitudinal current flow. This is especially important in these experiments because long periods of sustained current passing tends to polarize the axial wire and decrease space clamp effectiveness. In fact, without the use of the guard clamp the apparent membrane resistance is very small because the ends contaminate the measurement of the current in the center region. The guard clamp was implemented by measuring the potential with external electrodes (EEL, EER, EEC) which consisted of Ag-AgCl pellets connected to agar bridges filled with 3 M KCl and the difference between the electrodes was maintained close to zero by injecting current through the platinized platinum plates of the chamber. A similar system has been implemented and described in detail by Rakowski [16].

The axon was threaded through holes in the chamber which contained two ends walls, two air gaps (AG in Fig. 1) and two insulated partitions to separate the guards (LG and RG) from the central region (MC). The walls of the partitions were sealed with white petroleum jelly. The dialysis capillary was directly mounted on a valve that allowed the solution change and it contained a black platinized platinum wire to pass current (AW). The internal electrode was made of a 50 to 60 μm glass capillary filled with 0.6 M KCl and the electrical contact was made with a Ag-AgCl pellet. The internal electrode did not contain a floating wire to improve stability. This forced us to slow down the feedback loop to maintain stability of the voltage clamp.

Solutions

The solutions were designed to minimize all the ionic currents except the currents produced by the sodium-calcium exchanger.

The external medium contained (in mN): Na^+, 200 or 0; *N*-methylglucamine (NMG), 220 or 420; Ca^{2+}, 10; Mg^{2+}, 50; Tris-Mops, 10; cyanide, 1; tetrodotoxin (TTX) 0.6 μM (pH 7.8). In some experiments 20 mM of NMG was substituted for tetraethylammonium ion (TEA). The anion was chloride in most of the experiments, and in the early experiments it was nitrate. We found no significant difference in the results. The internal medium contained (in mM): Na^+, 200, 10 or 0; TEA, 100; Mg^{2+}, 2; EGTA, 1; Tris-Mops, 20 (pH 7.4) and glycine variable to adjust osmolality to 1000 mosmol/kg water. The anion was aspartate.

Junction potentials were measured and found to be smaller than 6 mV, consequently records are not corrected for junction potentials. Experiments were done between 18 and 20 °C.

Results

Membrane currents activated by internal calcium (Ca_i)

In order to demonstrate the presence of a membrane current flowing as a consequence of the operation of the sodium-calcium exchange, we chose to add ionized calcium (50 to 500 μM) as the most suitable alternative. It has been shown that intracellular calcium is required not only for the forward mode of the exchange, but also acts as essential activator for the reverse mode [10,17]. The possibility of maneuvering the extracellular concentration of sodium was explored in some experiments, using lithium as a substitute. However, we observed large partially transitory current changes when these ionic substitution were made in the absence of intracellular calcium. Changes in external calcium concentration were not even considered, for possible deleterious effects on the axon membrane under voltage clamp conditions.

Thus the first set of experiments was carried out to demonstrate the existence of a Ca_i-activated membrane current under different experimental conditions. Fig. 2 shows the membrane current of an axon predialyzed with an internal solution containing 20 mM sodium, no potassium and containing no added calcium and 1 mM EGTA to have a free ionic calcium concentration smaller than 10^{-9} M, while being exposed to an external solution containing 200 mM sodium and 10 mM calcium. The current trace in the figure started about 40 min after the beginning of the dialysis, and the membrane

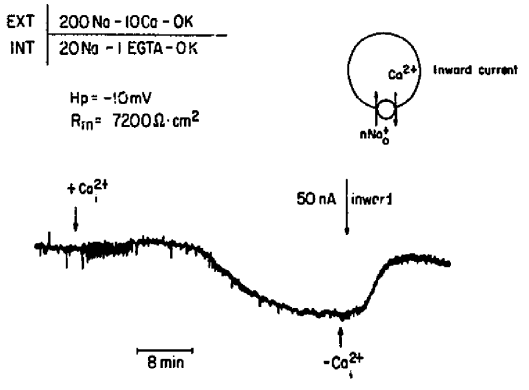

Fig. 2. Effect of raising the intracellular free ionized calcium on the membrane current of a dialyzed squid axon, voltage clamped at −10 mV. Before adding calcium, the axon had been predialyzed with a solution containing 20 mM Na^+, no K^+ and 1-mM EGTA, and exposed to a solution containing 200 mM Na^+, 10 Ca^{2+}, and no K^+. Under these conditions the membrane potential reached a value of about −10 mV after 40 min of dialysis, then the axon was clamped at this same value and the membrane current trace was started. Addition of 200 μM of free calcium activates an inwardly directed current. The insert shows the expected direction of the current resulting from the operation of the Na^+-Ca^{2+} exchange under these experimental conditions.

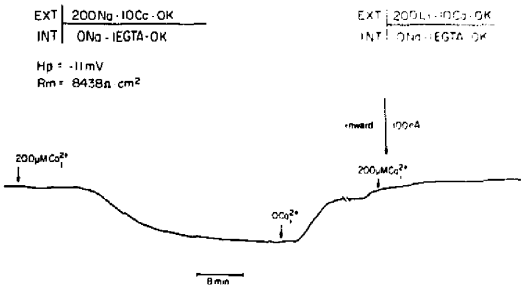

Fig. 3. Effect of sodium on the Ca_i-activated membrane current. The experiment was similar to that of Fig. 2, except that the internal dialysis medium contained no Na^+. In the first part of the experiment, addition of 200 μM calcium in the presence of 200 mM external sodium, induced current activation. After abolishing this effect by internal calcium removal, the external solution was substituted by one containing no Na^+ and 200 mM Li^+. This caused large current fluctuations, not shown in the figure. Under the new conditions, addition of internal calcium fails to activate any current.

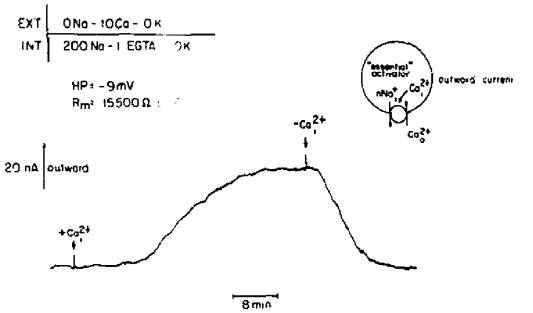

Fig. 4. Effect of reversed sodium gradients on the Ca_i-activated membrane current. In this experiment, the external solution contained no sodium, while the dialysis medium contained 200 mM Na^+. Under these conditions, as it is shown in the insert, the sodium calcium operation should produce an outwardly directed current, that is observed after adding calcium to the dialysis medium; in this case calcium acts as 'essential activator' of the exchange.

potential had decayed to −10 mV, which was the same value at which the axon membrane was held. Under these conditions, addition to the dialysis medium of 1.2 mM calcium to obtain 200 μM free ionic calcium causes an inward current to develop after a delay of approxim. 13 min. This period is compatible with the diffusion time for calcium from the dialysis capillary to the membrane, obtained in experiments in which calcium efflux was measured. The figure also shows that the Ca_i-activated current decays promptly after elimination of calcium from the internal solution, since in this case calcium disappearance from the axoplasm is aided by EGTA chelation.

The experiment of Fig. 3 was designed to explore the sodium dependence of the calcium-activated membrane current. In the first part of the experiment the internal solution contained no sodium, and after internal calcium addition an inwardly directed current developed, indicating that internal sodium is not required for such an effect. After reversing the calcium-activated current with Ca^{2+}-free internal solution, the external solution was changed for one containing 200 mM lithium and no sodium. After the large current changes caused by this substitution had subsided addition of internal calcium caused no effect, demonstrating that the flow of the Ca_i-activated inward current is dependent on the presence of external sodium. An important point of this experiment is the absence of non-specific ionic conductances activated by the addition of intracellular calcium, in contrast to the results obtained in other preparations [6]. Experiments similar to those shown in Figs. 2 and 3 showed that in the presence of external (1 or 2 mM) lanthanum ions addition of intracellular calcium failed to activate the current. In other experiments yet, it was found that 200 μM barium could not substitute for calcium in the activation or maintenance of this current.

Finally in other experiments it was found that the Ca_i-activated current was highly temperature dependent, since decreasing the temperature from 20 to 16 °C reduced the current by 50 and 40% in two different axons.

In the previous experiments the sodium gradient favoured the entry of sodium in exchange for intracellular calcium. Other experiments were carried out inverting or abolishing the sodium gradient. Fig. 4 shows an experiment in which the external and internal solution contained 0 and 200 mM sodium, respectively. Upon addition of internal ionized calcium, an outwardly directed current developed, with the same time

TABLE I

Ca_i-activated membrane currents

Sodium gradient dependence. M.P., membrane potential.

Axon	Diam. (μm)	M.P. (mV)	R_m (kΩ·cm^2)	$[Na]_o$ (mM)	$[Na]_i$ (mM)	$[Ca]_i$ (mM)	I_m (nA/cm^2)
Jn096A	480	−10	7.2	200	20	0.20	−813
Jn106A	480	−11	23	200	20	0.20	−1033
Jn126A	560	−10	16.8	200	20	0.20	−728
Jn126B	600	−18	8.2	0	200	0.20	+235
Jn196A	460	−15	5.8	0	200	0.20	+500
Jn246D	460	−25	2.8	0	200	0.20	+216
Jn256A	520	−10	17	0	0	0.60	0
Jn256B	640	−11	9	0	200	0.60	+350
Jn286A	450	−18	15.7	200	200	0.25	−357
My227B	–	−5	8.3	200	20	0.20	−1206
My247A	500	−7	28.5	200	20	0.20	+68
My267A	500	−8	4.0	200	20	0.20	−660
My277A	550	−11	8.3	200	20	0.20	−330
My287A	550	−9	27.5	200	20	0.13	−448
My307A	600	−12	9.0	200	0	0.13	+461
Jn027A	500	−17	50.0	20	200	0.20	+240
Jn037A	600	−6	41.3	200	20	0.40	−570

course as observed in the previous described experiments. In the absence of a sodium gradient addition of internal calcium caused very small or no currents at all. The results of different experiments in which sodium gradients were varied are summarized in Table I.

Reversal potential of the Ca_i-activated current

A set of experiments was carried out to determine the reversal potential of the Ca_i-activated membrane current in the presence of different sodium gradients. The experiments were similar to those shown in the previous section, except that voltage clamp pulses were applied before, during and immediately after the current activation by intracellular calcium. Fig. 5 shows some results obtained in one such experiment. In each set of records superimposed traces of membrane currents obtained in the presence of internal calcium and in its absence, after the Ca_i-activated current had disappeared, and their subtraction are shown together with the magnitude of the respective voltage clamp pulse. The subtracted traces, giving the current difference are shown at higher amplification. Fig. 6 shows plots of the current-voltage relationship obtained in the same experiment of Fig. 5. In the graph, the triangles represent the currents obtained in the presence and the circles those obtained in the absence of intracellular calcium. In this experiment the internal and external sodium concentrations were 60 and 200 mM, respectively, and assuming a $3Na^+$:$1Ca^{2+}$ stoichiometry a reversal potential $E_r = 3E_{Na} - 2E_{Ca}$ of -6 mV can be calculated. The two lines in the figure appear to cross each other at about -13 mV, which is strikingly near the value calculated for a $3Na^+ : 1Ca^{2+}$ stoichiometry. It is important to note that the experimental value obtained for the reversal potential of this current is substantially different from the value of the sodium equilibrium potential for the conditions of this experiment, +31 mV. Fig. 7 shows the results of another similar experi-

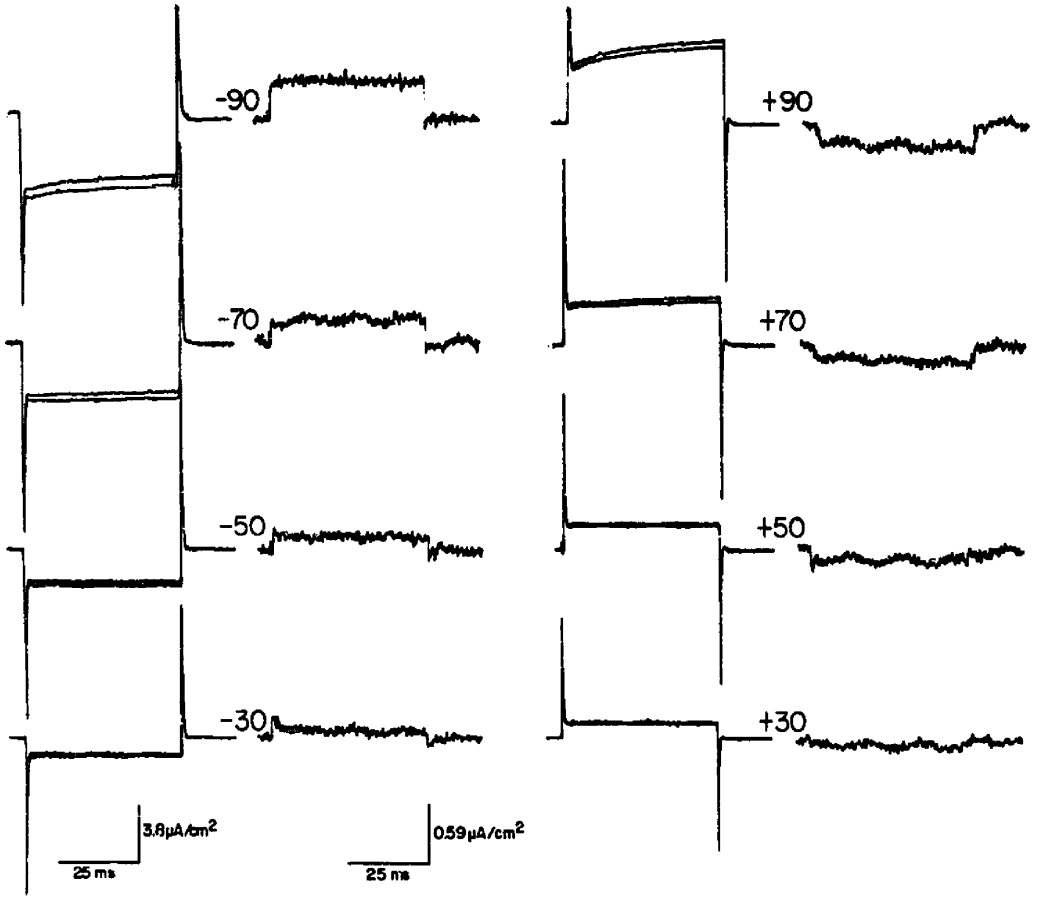

Fig. 5. Membrane current records obtained with voltage clamp pulses in the presence and absence of internal calcium, and the Ca_i-activated current obtained by subtracting them. The raw current records appear at the left of each set, and are shown at less amplification. The numbers indicate the magnitude and polarity of the voltage clamp pulse.

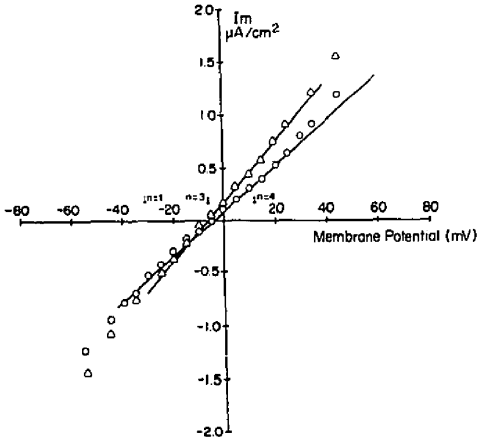

Fig. 6. Current-voltage relationships obtained in the presence (triangles) and in the absence (circles) of internal calcium, in an experiment similar to that shown in Fig. 5. The current values used in the graph were obtained at the trailing edge of the pulse. The arrows point to the theoretical reversal potentials for stoichiometries of $n=1$, $n=3$ and $n=4$, where n is the number of sodium ions exchanging for one calcium ion.

ment, in which the internal and external sodium concentrations were 20 and 200 mM, respectively. In the graph the difference current has been plotted against the membrane potential, after correction for the basal Ca_i-activated current. The reversal value for the current in this case is +69 mV, while the theoretical value calculated according to $3=E_{Na}-2E_{Ca}$ was +65 mV. The results of these and other similar experiments are shown in Table II. The table indicates that in all the experiments performed the experimental values obtained for the reversal potential of the current are rather

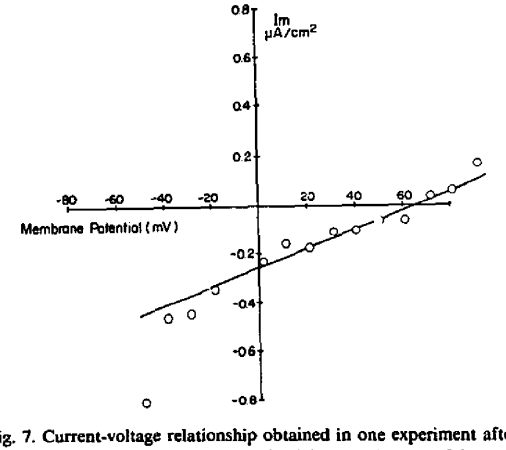

Fig. 7. Current-voltage relationship obtained in one experiment after subtracting the current record obtained in the absence of internal calcium from the corresponding ones obtained in its presence. The current in the graph corresponds to the Ca_i-activated current, representing the result of the operation of the Na^+-Ca^{2+} exchange.

TABLE II

Reversal potential of the Ca_i-activated currents

Axon	Experimental (mV)	Calculated	
		$3E_{Na}-2E_{Ca}$ (mV)	E_{Na} (mV)
NCMY247A	−12	−6	+31
NCMY267B	+74	+76	+59
NCMY277A	+55	+76	+59
NCMY287A	+67	+65	+59
NCMY307A	> +80	> +100	
NCJN027A	< −90	−273	
NCJN037A	+78	+93	+59

similar to the values calculated assuming a 3 Na^+ : 1Ca^{2+} stoichiometry, except for two cases in which the predicted potential values were beyond our experimental testing possibilities. For comparative purposes the values of the sodium equilibrium potential for each experiment are also shown in Table II.

Discussion

Theoretical predictions about the direction and magnitude of calcium fluxes due to the operation of the sodium-calcium ion exchange system have been hindered by the lack of detailed knowledge of the stoichiometry of the system and by its complexity [5]. In fact, although the direction of calcium fluxes depends on the electrochemical gradients for sodium and calcium, their magnitude depends also on the modulatory and, or catalytic function of several extracellular and intracellular ligands including intracellular calcium [4, 7]. Thus, although theoretical considerations and experimental measurements have allowed different authors to reach the conclusion that the sodium-calcium ion exchange should work with a stoichiometry at least greater than two sodium ions for each calcium ion [2,3], a more accurate prediction of the stoichiometry of the system cannot be made unless precise experimental information is available [5]. Recently, some works have appeared reporting more accurate measurements of Na^+-Ca^{2+} exchange currents and stoichiometry for retinal rods [12] and cardiac myocytes [10]. In this work we report the measurements of a calcium-activated, sodium dependent membrane current and although the lack of a specific inhibitor for this current is still an important drawback, the characteristics of the current are such that it can be considered the expression of the electrogenic operation of sodium-calcium ion exchange. In fact (a) the direction and magnitude of the current are determined by the thermodynamic driving forces for sodium and calcium; (b) the inward current activated by calcium, requires external sodium, and lithium cannot substitute for it; (c) the reversal potential of the current is strikingly close to the value calculated accord-

ing to $E_r = 3_{Na} - 2E_{ca}$, and substantially different from the calculated values for a stoichiometry of 4 sodium to 1 calcium, or for the plain sodium equilibrium potential. These three points strongly indicate that this current is due to the operation of the sodium-calcium ion exchange in both its modes of operation: the forward one, by which intracellular calcium is exchanged for extracellular sodium, that produces an inwardly directed current, and the reverse mode, by which intracellular sodium is exchanged for extracellular calcium, and that is associated with an outwardly directed current. Considering the experimental conditions under which these results have been obtained, it is unlikely that a non-specific current, activated by intracellular calcium would behave in a similar way. It is then a fortunate circumstance that no such non-specific current is evident in squid axons under these conditions. Other important characteristics of the current, that are in agreement with independent observations related with Na^+-Ca^{2+} exchange are that (1) it requires internal calcium as a catalytic factor for the reverse mode of operation of the sodium-calcium ion exchange [10,17]; (2) during a voltage clamp pulse it is activated very rapidly, in agreement with the results obtained in experiments in which the forward mode of operation of the Na^+-Ca^{2+} exchange was studied under voltage clamp conditions [9]; (3) the current is blocked by external lanthanum; (4) the current is not activated by barium, and finally (5) the current shows a high temperature dependence.

In conclusion, the Ca_i-activated current reported in this work, can be interpreted as the electrical manifestation of the sodium-calcium ion exchange operating with a stoichiometry of $3Na^+ : 1Ca^{2+}$.

Acknowledgements

This work was supported by the Muscular Dystrophy Association, USPHS grant GM30376, NSF-CONICIT joint program and by a travel grant from the Fundacion Polar to C.C. and R.D.

References

1 Campbell, A.K. (1983) Intracellular Calcium, its Universal Role as Regulator, John Wiley and Sons, New York.
2 Mullins, L.J. (1979) Am. J. Physiol. 236, C103–C110.
3 Blaustein, M.P. and Hodgkin, A.L. (1969) J. Physiol. 200, 497–527.
4 Blaustein, M.P. (1977) Biophys. J. 20, 79–111.
5 Eisner, D.A. and Lederer, W.J. (1985) Am. J. Physiol. 248, C189–202.
6 Colquhoun, D., Neher, E., Reuter, H. and Stevens, C.F. (1981) Nature Lond. 294, 752–754.
7 DiPolo, R. and Beaugé, L. (1984) J. Gen. Physiol. 84, 895–914.
8 DiPolo, R. and Beaugé, L. (1987) J. Gen. Physiol. 90, 505–525.
9 DiPolo, R., Bezanilla, F., Caputo, C. and Rojas, H. (1985) J. Gen. Physiol. 86, 457–478.
10 Kimura, J., Miyamae, S. and Noma, A. (1987) J. Physiol. 384, 199–222.
11 Rasgado-Flores, H. and Blaustein, M.P. (1986) Am. J. Physiol. 21, C499–C505.
12 Yau, K. and Nakatani, K. (1984) Nature 311, 661–663.
13 Caputo, C., DiPolo, R. and Bezanilla, F. (1986) Biophys. J. 49, 232a.
14 DiPolo, R., Caputo, C. and Bezanilla, F. (1987) Biophys. J. 51, 386a.
15 Caputo, C., Bezanilla, F. and DiPolo, R. (1988) Biophys. J. 53, 224a.
16 Rakowski, R.F. (1988) Biophys. J. 55, 663–671.
17 DiPolo, R. and Beaugé, L. (1986) Biochim. Biophys. Acta 54, 298–306.