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In dialyzed squid axons Ca_i^{2+} activates Ca_o^{2+}–Na_i^+ and Na_o^+–Na_i^+ exchanges in the absence of Ca chelating agents

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We used internally dialyzed squid axons to explore whether the reported activatory effect of Ca_i^{2+} on the partial reactions of the Na^+–Ca^{2+} exchange (essential activator) is secondary to the presence of Ca^{2+} chelating agents in the internal medium. The effect of Ca_i^{2+} pulses on both the reverse (Ca_o^{2+}-dependent Na^+ efflux) and Na^+–Na^+ exchange (Na_o^+-dependent Na^+ efflux) modes of the Na^+–Ca^{2+} exchange was studied in axons dialyzed without EGTA. For these experiments a substantial inhibition of the Ca^{2+} buffer capacity of the axoplasm was achieved by the use of Ruthenium red (10–20 μM), cyanide (1 mM) and vanadate (1 mM) in the dialysis solution. Our results indicate that the Ca_i^{2+} requirement of the reverse and Na^+–Na^+ exchange can not be explained by a direct inhibition of the Na^+–Ca^{2+} exchanger by EGTA. In fact, both modes of operation of the exchanger can be activated by internal Ca^{2+} ions in the complete absence of Ca^{2+} chelating agents thus indicating that the 'catalytic' effect of Ca_i^{2+} on the Na^+–Ca^{2+} exchanger is a real phenomenon.

An unexpected finding related to the operation of the Na^+–Ca^{2+} exchange mechanism in squid axons is that intracellular Ca^{2+} is an essential activator of all modes of operation of the exchanger. The effect of Ca_i^{2+} on the turnover of the reverse and Na^+–Na^+ exchange has several common features with that of the forward and Ca^{2+}–Ca^{2+} exchange: (i) activation by Ca_i^{2+} occurs in the micromolar range [1,2], (ii) ATP increases the affinity for Ca_i^{2+} [1,2] and (iii) internal Mg^{2+} inhibits [3–5]. This results suggest the presence of a single internal calcium activatory site for all modes of operation of the exchanger.

The fact that Ca_i^{2+} must bind to the exchanger (or to another molecule(s) that regulates it) adequately explains the inhibition of Ca_o^{2+}-dependent Na^+ efflux observed in intact axons injected with EGTA or the Ca^{2+} indicator quin2 [6,7]. The effect of Ca_i^{2+} (essential activator) in activating all the partial reactions of the Na^+–Ca^{2+} exchange mechanism is not peculiar to squid axons since it has been recently demonstrated in barnacle and cardiac muscle fibers. In the former, Ca_o^{2+}-dependent Na^+ efflux is inhibited at low Ca_i^{2+} [8], in the latter no outward current is generated by the electrogenic reverse exchange (Na_i^+–Ca_o^{2+}) in the absence of internal Ca^{2+} [9]. In all living cell preparations in which the effect of Ca_i^{2+} on the Na^+–Ca^{2+} exchange has been investigated, an exogenous Ca^{2+} chelating agent such as EGTA has always been used to buffer cytoplasmic free calcium [4]. Recently Allen and Baker, working in intact squid axons, have reported that injection of Ca^{2+} buffers such as Ca-EGTA or Ca-BAPTA to give final ionized Ca^{2+} concentrations above 1 μM inhibit Ca_o^{2+}-dependent Na^+ efflux when compared to the magnitude of the Ca_o^{2+}-dependent Na^+ efflux in the absence of chelating agents [10]. This finding opens the question of whether Ca_i^{2+} ions per se have a direct 'catalytic' effect on the Na^+–Ca^{2+} exchanger, or their action may be secondary to an alteration of the exchanger induced by the Ca^{2+} chelating agent.

Since Ca_i^{2+} activation of the reverse Na^+–Ca^{2+} and Na^+–Na^+ exchange was first reported in dialyzed squid axons in which internal Ca^{2+} was stabilized with EGTA [11,12], we have examined whether a rise in Ca_i^{2+} may activate the Ca_o^{2+}- and Na_o^+-dependent Na^+ efflux in axons dialyzed without exogenous Ca^{2+} buffers. In this type of experiments two important requirements must be met: first, in order to increase the ionized Ca_i^{2+} at the inner aspect of the membrane following a $CaCl_2$ pulse at the center of the axon (dialysis capillary), a substantial inhibition of the endogenous axoplasmic

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Ca^{2+} buffer systems must be achieved. Second, the basal level of Ca_i^{2+} prior to the Ca_i^{2+} pulse must be close to or lower than the apparent Ca_i^{2+} affinity of the Ca_o^{2+}- and Na_o^+-dependent Na^+ efflux components ($K_{1/2} = 1-3$ μM in the presence of ATP [3]).

In squid axons, the energy-dependent Ruthenium red-sensitive component accounts for most of the Ca^{2+} buffer capacity of the axoplasm [13]. In fact, injection of $CaCl_2$ in order to get a final concentration of 0.8 mM Ca^{2+} into isolated axoplasm causes only a transient rise in Ca^{2+}. However, in the presence of Ruthenium red (6.4 μM) a similar $CaCl_2$ injection causes the level of Ca^{2+} to remain high and close to 100 μM [13]. In the experiments presented here we have included Ruthenium red (10 μM), cyanide (1 mM) and vanadate (1 mM) in the internal dialysis medium to inhibit energy-dependent components of cytoplasmic Ca^{2+} uptake. In addition, highly porous dialysis capillaries (see Methods) have been used to effectively introduce Ca^{2+} into the axons.

The experiments were performed in two live squid specimens: *Loligo pealei* (Marine Biological Laboratory, Woods Hole, MA, U.S.A.) and *Loligo plei* (IVIC, Caracas, Venezuela). After dissecting and cleaning the axons, they were mounted in a modified dialysis chamber [14]. The standard dialysis solution had the following composition (mM): K-Mops, 310 (pH 7.4); NaCl, 100; $MgCl_2$, 4. Glycine was used to adjust the osmolarity to 1000 mosM. When present, ATP was added as MgATP salt at a final concentration of 2 mM. No EGTA was added to the internal medium. All dialysis solutions containing 10 μM Ruthenium red and 1 mM vanadate. Ultrapure water (Milli-Q; Millipore Corporation) was used to prepare both internal and external solutions. Total Ca^{2+} content present in the dialysis medium from atomic absorption determinations ranged from 2 to 4 μM. The composition of the artificial sea water was as follows (mM): NaCl, 440; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Tris-HCl (pH 7.7), 10. The replacement of Na^+ or Ca^{2+} ions was accomplished using isosmolar amounts of Li^+ and Mg^{2+}, respectively. The osmolarity of all solutions was 1000 mosM. All external solutions contained 200 nM tetrodotoxin (TTX), 10^{-4} M ouabain, and 10 μM bumetanide (a kind gift from Hoffman La Roche Inc., Nutley, NJ, U.S.A.). ATP-Tris (Ca^{2+} free; Sigma) was prepared as a 200 mM stock solution. Mops, Tris and glycine were purchased from Sigma Co. All other salts were from Merck. Experiments were performed at 16–18°C.

The dialysis capillaries used in these experiments were of hollow regenerated cellulose acetate fibers with a nominal molecular weight cut off of 9000 (150 μm o.d., 141 μm i.d., Spectrum, Los Angeles, CA, U.S.A.). Their permeability to Ca^{2+} ions was measured using the same experimental dialysis chamber. The mean permeability coefficient from three different experiments was $9.2 \cdot 10^{-5}$ cm/s For an axon of 500 μM diameter and 1 cm length, Ca^{2+} efflux from these dialysis capillaries dumped Ca^{2+} into the axoplasm at an initial rate of 135 μmol/min per mmol of Ca^{2+} when internally perfused at 1 μl/min. Data from the work of Baker and Umbach [13] show that in the presence of Ruthenium red the Ca^{2+} buffer capacity of the axoplasm is lost after a Ca^{2+} load of 40 μM as compared to a succesfull Ca^{2+} buffering of more than 4000 μM in the absence of the inhibitor. The above considerations indicates that a Ca^{2+} test pulse of 0.2–0.5 mM during 10–15 min should be long enough to initiate a significant rise in the Ca^{2+} at the membrane level.

In order to explore the effect of Ca_i^{2+} on the magnitude of the Ca_o^{2+}-dependent Na^+ efflux (reverse exchange) in the absence of added exogenous Ca^{2+} buffers, axons were externally superfused with standard sea water free of Ca^{2+} and internally dialyzed with a medium free of Ca^{2+} for at least 60 min prior to the addition of the radioactive dialysis medium. Although the exact level of Ca_i^{2+} at the plasma membrane is unknown, a reasonable assumption is that it cannot be greater than that of the experimental solutions (2–4 μM). Fig. 1 shows that under the above conditions the efflux of Na^+ reached a steady level of about 4 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ being totally dependent on Na_o^+ since replacing Na_o^+ for Li_o^+ drops the efflux to less than 1 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$. A Na^+–Na^+ exchange component of less than 5 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ in the presence of ATP indicates that Ca_i^{2+} at the inner membrane must be far below saturation since it has been shown that in the presence of saturating Ca_i^{2+} the Na_o^+-dependent Na^+ efflux is about 25–30 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ [3,12]. In order to establish a control value of the Ca_o^{2+}-dependent Na^+ efflux in the absence of added Ca_i^{2+} the net increment in Na^+ efflux was measured upon addition of 1 mM Ca_o^{2+}. In this particular experiment it amounts to 2.5 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$. In the second part of the experiment the axon was again bathed in an external Li^+ medium causing the efflux of Na^+ to drop to very low values. Under these conditions 200 μM of total calcium was added to the standard dialysis medium. After about 20 min, addition of 1 mM Ca_o^{2+} causes a net increase of 5.5 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ in Na^+ efflux or twice the value obtained without added Ca_i^{2+}. At the end of the experiment the internal Ca^{2+} was removed and 3 mM EGTA were added to the dialysis medium. Under this virtually zero Ca_i^{2+} condition no activation of the Na^+ efflux by Ca_o^{2+} was observed. This type of experiment clearly indicates that even in the absence of an exogenous Ca^{2+} buffer the magnitude of the Ca_o^{2+}-dependent Na efflux (reverse Na^+–Ca^{2+} exchange) is still activated by a rise in the internal ionized Ca_i^{2+}.

Two observations are worth commenting from these experiments: first, axons tend to survive much less than those dialyzed with millimolar concentrations of EGTA.

In fact, an increase in the leak of Na^+ ions was very common after 1 or 2 h of internal dialysis. Although we have no explanation for this phenomenon, it could be due to the presence of toxic metal ions in the experimental solutions, which are normally chelated by EGTA in a standard dialysis experiment. A second interesting point is that when the Ca_o^{2+}-dependent Na^+ efflux (in the absence of added Ca_i^{2+}) was activated by Ca_o^{2+} concentrations greater than 5 mM, Na^+ efflux initially increases rapidly to a steady state but then it slowly increases to higher values. It is feasible that in the presence of an impaired axoplasmic Ca^{2+} buffer (use of metabolic inhibitors) Ca^{2+} entry through the reverse exchange increases the ionized Ca_i^{2+} at the inner membrane thus further activating the exchanger.

In squid axons it has been recently shown that the Ca_i^{2+}-activated Na_o^+-dependent Na^+ efflux component has many features in common with the Na^+/Ca^{2+} exchange mechanism, including: (i) activation by Ca_i^{2+}, ATPγS, and internal alkalinization, (ii) similar activation curves for Ca_i^{2+} and Na_o^+, (iii) inhibition by Mg_i^{2+} and (iv) competitive interaction with Ca_o^{2+} [3,11]. These findings demonstrate that in squid axons the Ca_i^{2+}-activated Na^+–Na^+ exchange component is one of the partial reactions of the Na^+–Ca^{2+} exchange. A similar mechanism has been postulated in cardiac membrane vesicles [15]. We have taken advantage of this observation to explore whether Ca_i^{2+} also activates this mode of operation in axons dialyzed without EGTA. In these experiments no calcium ions are required in the external medium since only Na_o^+-dependent Na^+ efflux is being measured. This has the advantage that no Ca^{2+} entry occurs through the reverse Na^+/Ca^{2+} exchange eliminating any increase in Ca_i^{2+} different from that of the Ca_i^{2+} experimental pulse.

Fig. 2 shows an experiment in which an axon was dialyzed from the beginning with a standard dialysis medium containing 2 mM ATP and no added Ca^{2+} or EGTA. With the axon bathed in artificial sea water containing no Ca^{2+} ions, Na^+ efflux rised to a level of about 20 pmol · cm^{-2} · s^{-1} and then fell progressively to a steady value of 10 pmol · cm^{-2} · s^{-1}. Substitution of Na^+ by Tris causes a marked fall in Na^+ efflux indicating that practically all the efflux of Na^+ under these conditions is Na_o^+-dependent. Addition of 500 μM $CaCl_2$ to the dialysis medium causes a large increase in the Na^+ efflux which is completely abolished upon replacing external sodium for Tris ions. Readmission of Na^+ to the external medium reactivates a large Na^+/Na^+ efflux component which amounts in this experiment to about 30 pmol · cm^{-2} · s^{-1}. At the end of the experiment 3 mM EGTA was added to the dialysis medium causing a rapid fall in the efflux of Na^+ to a steady level of about 6 pmol · cm^{-2} · s^{-1}. The fact that the level of Na^+ efflux in the presence EGTA (virtually zero Ca_i^{2+}) stabilizes to a value higher than the Na^+

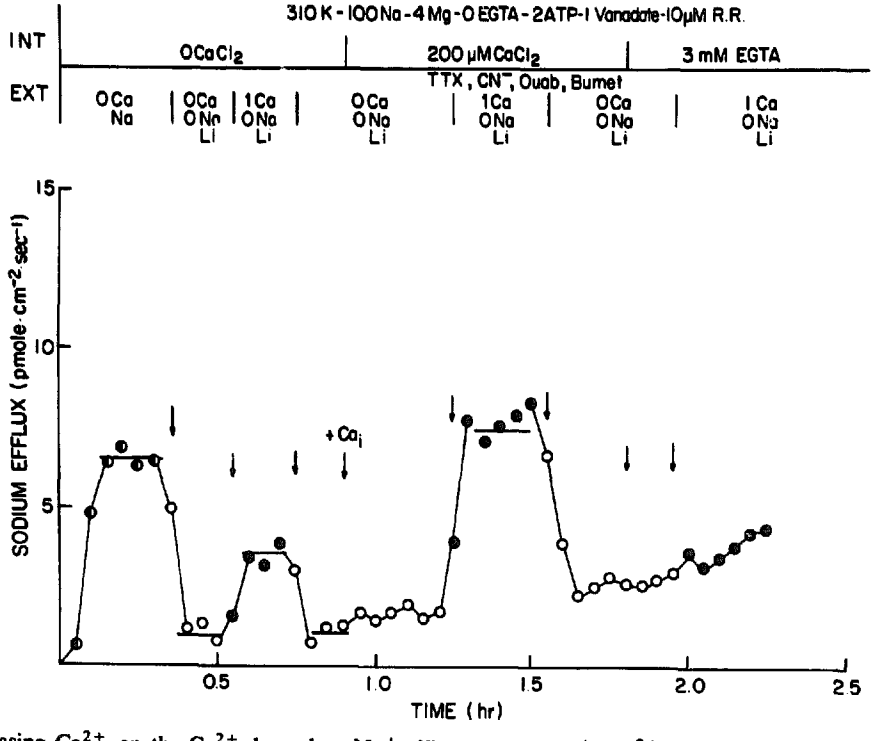

Fig. 1. The effect of increasing Ca_i^{2+} on the Ca_o^{2+}-dependent Na^+ efflux (reverse Na^+–Ca^{2+} exchange) in an axon dialyzed without EGTA. Ordinate Na^+ efflux in pmol·cm^{-2}·s^{-1}. Abscissa, time in hours. Arrows indicate changes in internal and external solutions. ○, Na^+ efflux into Ca^{2+}-free artificial sea water. ○, Na^+ efflux into Ca^{2+}-free lithium-sea water. ●, Na^+ efflux into lithium-sea water containing 1 mM Ca^{2+}. Unless otherwise stated all concentrations are in mmol/l. Axon diameter 495 μm. Temperature, 17°C. RR, Ruthenium red; TTX, tetrodotoxin; Ouab, ouabain; Bumet, bumetamide.

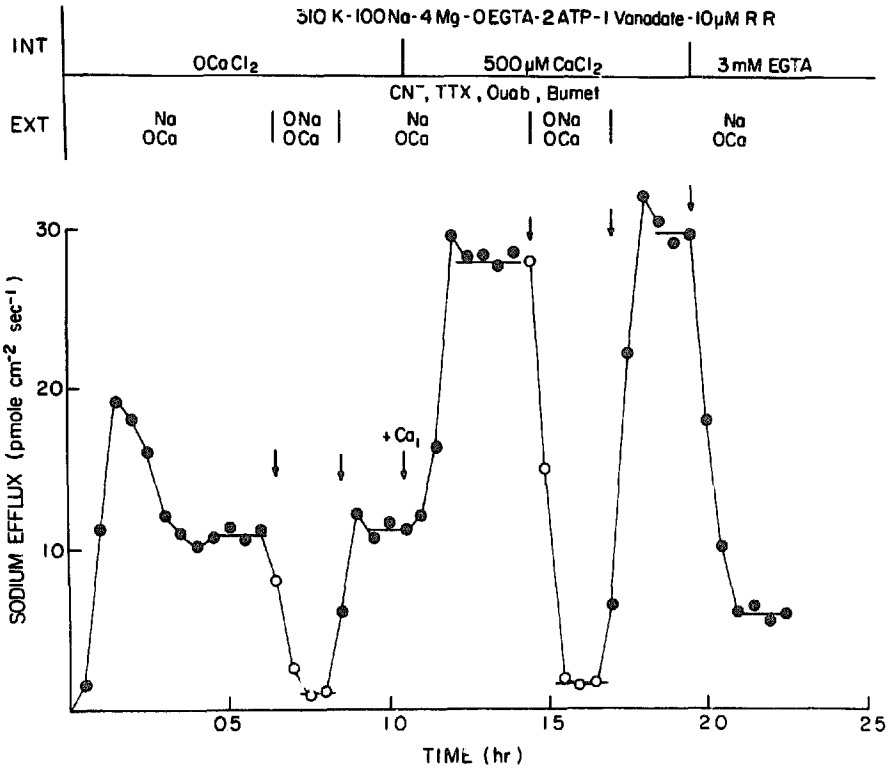

Fig. 2. The effect of increasing Ca_i^{2+} on the Na_o^+-dependent Na^+ efflux (Na^+–Na^+ exchange) in an axon dialyzed without EGTA. Arrows indicate changes in internal and external solutions. ●, Na^+ efflux into Ca^{2+}-free artificial sea water. ○, Na^+ efflux into Na^+- and Ca^{2+}-free sea water (Na^+ ions were substituted by Li^+). Unless otherwise stated all concentrations are in mmol/l. Axon diameter 400 μm. Temperature 17°C. Abbreviations, see legend to Fig. 1.

'leak' is in agreement with the existence in squid axons of an ATP-activated, Ca_i^{2+}-independent, Na_o^+-dependent Na^+ efflux component [12]. The main conclusion drawn from this type of experiments is that internal ionized Ca^{2+} activates the Na_o^+–Na_i^+ exchange in the absence of an exogenous Ca^{2+} buffer. The experiments reported in this paper are at variance with those recently reported by Allen and Baker in intact squid axons which show that the magnitude of the Ca_o^{2+}-dependent Na^+ efflux measured in 10 mM Ca_o^{2+}-Li^+ sea water is greater than that measured after the injection of Ca^{2+} buffers even when the nominal Ca_i^{2+} is in the micromolar range. One possibility is that in injected Na^+ loaded axons during activation of the Ca_o^{2+}-dependent Na^+ efflux (10 mM Ca_o^{2+} Li^+-sea water) the inner face of the carrier may not be at the resting submicromolar state but in the micromolar range due to massive Ca^{2+} entry through the Li^+-activated Na_i^+-dependent Ca^{2+} influx [6]. Although difficult to demonstrate since no values for the ionized Ca^{2+} at the inner membrane have been measured under this conditions, experiments performed with calcium electrodes in intact squid axons demonstrate that Ca^{2+} entry is more readily measured close to the membrane than in the center of the axon [16].

Although we have not carried out a systematic study of the magnitude of the Na_o^+–Na_i^+ and Ca_o^{2+}–Na_i^+ exchanges in the presence and in the absence of EGTA, in four different experiments including that of Fig. 2, the level of the Na^+ efflux in the presence of saturating concentrations of Ca_i^{2+} was only slightly greater (10–20%) in the absence of EGTA than in its presence (see also Refs. 3, 12). Whether this effect represents an alteration of the Na^+/Ca^{2+} exchanger by EGTA remains to be explore. However, against this is the fact that in dialyzed axons a similar activation of the Na_i^+-dependent Ca^{2+} influx by Ca_i^{2+} is observed at two different EGTA concentrations (at constant $[Ca^{2+}]_i$ [2]). The present results clearly show that the reported role of intracellular ionized Ca^{2+} in regulating the exchange ('essential' activator) is a real phenomenon and not the consequence of the presence of Ca^{2+} chelating agents in the internal solutions. These experiments do not discard the possibility that Ca^{2+} chelating agents such as EGTA, EDTA, BABTA or quin-2 may have secondary effects on the Na^+/Ca^{2+} exchanger including modifications of the kinetics of activation by Na^+, Ca^{2+} and/or ATP. Experiments of this type may become difficult due to the uncertainty in the levels of ionized Ca^{2+} at the inner face of the carrier in the absence of exogenous Ca^{2+} buffers in the dialysis medium.

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