Biochimica et Biophysica Acta, 946 (1988) 424–428
Elsevier

BBA 70586

BBA Report

An ATP-dependent Na^+/Mg^{2+} countertransport is the only mechanism for Mg extrusion in squid axons

Reinaldo DiPolo [a] and Luis Beaugé [b]

[a] *Centro de Biofísica y Bioquímica, IVIC, Caracas (Venezuela) and* [b] *División de Biofísica Instituto M. y M. Ferreyra, Córdoba (Argentina)*

(Received 10 June 1988)

Key words: Sodium–magnesium ion exchange; Phosphorylation; (Squid axon)

The components of magnesium efflux in squid axons have been studied under internal dialysis and voltage clamp conditions. The present report rules out the existence of an ATP-dependent, Na_o- and Mg_o-independent Mg^{2+} efflux (ATP-dependent Mg^{2+} pump) leaving the Mg^{2+}–Na^+ exchange system as the only mechanism for Mg^{2+} extrusion. The main features of the Mg^{2+} efflux are: (1) The efflux is completely dependent on ATP. (2) The efflux can be activated either by external Na^+ (forward Mg^{2+}–Na^+ exchange) or external Mg^{2+} (Mg^{2+}–Mg^{2+} exchange). (3) The mobility of the Mg^{2+} exchanger in the Na_o^+-loaded form is greater than that in the Mg^{2+}-loaded one. (4) In variance with the Na^+–Ca^{2+} exchange mechanism, Mg^{2+}–Mg^{2+} exchange is not activated by external monovalent cations. (5) ATPγS replaces ATP in activating Mg^{2+}–Na^+ exchange suggesting that a phosphorylation/dephosphorylation process regulates this transport mechanism.

Magnesium ions are essential for the proper function of several intracellular enzymes, in special those involved in the metabolism of high-energy phosphate compounds. In squid axons, the Mg^{2+} ion distribution across the axolemma is far from equilibrium; its cytoplasmic concentration is much lower (3–4 mM) than that present in the extracellular fluid (44 mM) [1,2]. In fact, if the distribution of Mg^{2+} ions were purely passive, for an internal resting membrane potential of −60 mV and an hemolimph Mg^{2+} concentration of 44 mM [2], the predicted Mg_i^{2+} should be close to 5 M. Previous studies on Mg^{2+} transport in injected and dialyzed squid axons have shown that Mg^{2+} efflux is: (i) largely dependent on external sodium ions [1–3]; (ii) drastically reduced by metabolic poisoning (cyanide or dinitrophenol) [1,2] or apyrase injection [2]; (iii) highly dependent on temperature [2], and (iv) unaffected by changes in membrane potential [1,2]. These results can be best explained by the presence of a plasma membrane Mg^{2+}/Na^+ countertransport system working in an electroneutral fashion in which the Na^+ gradient provides the energy for net Mg^{2+} extrusion, and ATP may serve as a modifier of the carrier. Although the proposed Na^+–Mg^{2+} exchange might be the only mechanism responsible for cell Mg^{2+} homeostasis, at present, an ATP-driven Mg^{2+} pump working in parallel with the Na^+–Mg^{2+} exchange has not been rule out. In fact, two indirect evidences favor the presence of an 'uncoupled' Mg^{2+} pump (ATP dependent, Na_o^+-, Mg_o^{2+}- and Ca_o^{2+}-independent Mg^{2+} extrusion): (i) a significant ATP-dependent Mg^{2+} efflux has been reported in the absence of external Na^+ ions [2,3] and (ii) microsomes prepared from

Correspondence: R. DiPolo, Centro de Biofísica y Bioquímica, IVIC, Apartado 21287, Caracas 1020-A, Venezuela.

membrane fragments of squid nerves fibers show a large Ca^{2+}-independent, ouabain-insensitive ATPase activity which requires Mg^{2+} [4,5].

In this report, we provide conclusive evidence against the existence of an uncoupled Mg^{2+} pump in squid axons, as we have been unable to find any residual Mg^{2+} efflux in the absence of external Na^+, and Mg^{2+} ions in axons dialyzed with millimolar concentrations of ATP. The fact that all measurable Mg^{2+} efflux in this preparation is independent on ATP and stimulated by either external Na^+ or Mg^{2+}, indicates that the Na^+/Mg^{2+} countertransport is the only system responsible for Mg^{2+} homeostasis. A further evidence against the existence of an ATP-dependent Mg^{2+} pump is the finding that the ATP analog ATPγS a substrate for kinases but not for ATPases can also support Na^+–Mg^{2+} exchange.

The experiments were carried out in giant axons from the squid *Loligo pealei* at the Marine Biological Laboratory in Woods Hole, MA, U.S.A. After dissecting and cleaning the axons, they were mounted in a modified dialysis chamber that allows the simultaneous control of both the intracellular medium (internal dialysis) and the membrane potential (voltage clamp). The modified chamber as well as the general experimental techniques have been described in detail elsewhere [6,7]. The standard dyalisis solution had the following composition (mM): NaCl, 40; Mops-K, 300 (pH 7.4); $MgCl_2$, 4; KCl, 100. Glycine was used to adjust the osmolarity to 1000 mosM. When present, ATP was added as MgATP salt at a final concentration of 1 mM. In order to check that the main internal anion Mops (4-morpholinepropanesulfonic acid) does not bind Mg^{2+} ions, experiments were carried out using the dye Coomassie blue following the protocol outlined by DeWeer [2]. A similar dissociation constant for the Mg^{2+}-dye complex was obtained in solutions of: K-Hepes (400 mM), K-Mops (400 mM) or a mixture of Tris-Hepes (25 mM) and KCl (375 mM), thus indicating that no significant binding of Mg^{2+} to Mops occurs in the dialysis solution. The composition of the artificial sea water (ASW) 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 Mg^{2+} ions was done using isoosmolar amounts of Tris, Li^+ and Ca^{2+}, respectively. In all solutions the osmolarity was 1000 mosM. ^{28}Mg was purchased from Oak Ridge National Laboratories. Counting was performed in a liquid scintillation counter mixing 4 ml of the sea water with 5 ml of Aquasol II. When possible, counting was long enough to allow a standard error of counting of less than 1 per cent.

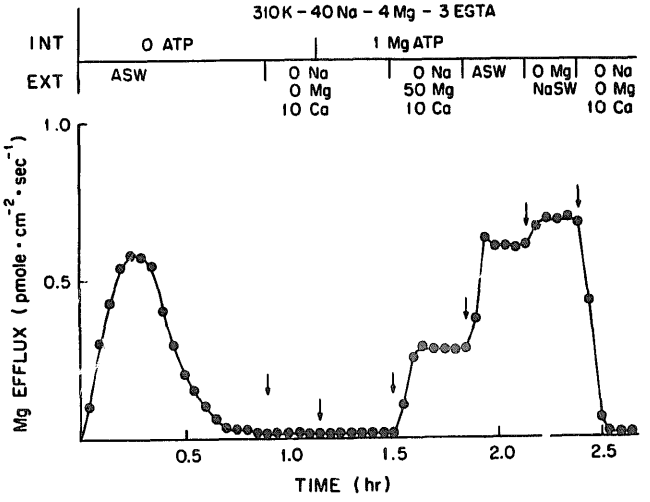

Fig. 1. Components of the Mg^+ efflux in an axon dialyzed without and with ATP. Ordinate: Mg^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscisa: time in hours. The arrows indicate changes in the external and internal solutions. All concentrations are in millimolar. Axon diameter 560 μm. Temp.: 15°C. $V_m = -58$ mV.

The possibility of the existence of an uncoupled (Na_o^+- and Mg_o^{2+}-independent) Mg^{2+} pump was explored in experiments like that illustrated in Fig. 1. An axon was dialyzed from the start with the standard dialysis solution containing radioactive Mg^{2+} and no ATP. With the axon bathed in artificial seawater, Mg^{2+} efflux rised to a maximal level of about 600 fmol·cm^{-2}·s^{-1} falling progressively to less than 10 fmol·cm^{-2}·s^{-1}. This transitory rise in Mg^{2+} efflux, most probably represents the washout of ATP during dialysis. In the virtual absence of internal ATP, removal of Na_o^+ plus Mg_o^{2+}, and later Ca_o^{2+}, produced no effects on the Mg^{2+} efflux levels indicating that under these conditions, the remaining efflux (4–10 fmol·cm^{-2}·s^{-1} from six different axons) is likely to be passive 'leak'. On these basis, the estimated P_{Mg} from the constant field equation amounts to about 10^{-13} cm/s. In the absence of external Na^+ and Mg^{2+}, the addition of 1 mM MgATP to the dialysis medium failed to induce any uncoupled Mg^{2+} efflux. However, when the extracellular

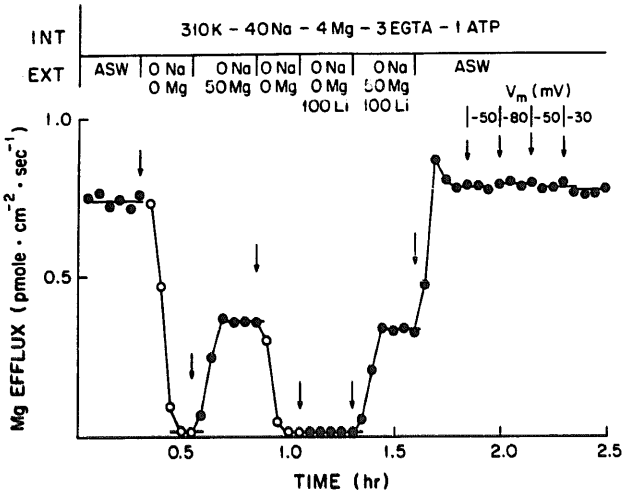

Fig. 2. Lack of an effect of Li^+ ions on the Mg_o^{2+}-dependent Mg^{2+} efflux. Ordinate: Mg^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Notice the absence of an effect of membrane potential on the magnitude of the Mg^{2+} efflux. All concentrations are in millimolar. Temperature: 15°C. V_m = −60 mV. Open circles: Mg^{2+} efflux into Na^+ and Mg^{2+} free sea water (Na^+ ions were substituted by Tris).

Mg^{2+} was restored (in the presence of ATP and in the absence of Na_o^+), an increase in Mg^{2+} efflux to a steady value of around 250 fmol · cm^{-2} · s^{-1} was observed. A further increase in Mg^{2+} efflux to about 600 fmol · cm^{-2} · s^{-1} took place when Na_o^+ and Mg_o^{2+} were simultaneously present. Interestingly, Fig. 1 shows that the removal of Mg_o^{2+} in the presence of full Na_o^+ caused a small but significant increment (see also Fig. 3) in the Mg^{2+} efflux. At the end of the experiment, the removal of both Na_o^+ and Mg_o^{2+} brought the efflux to 'leak' values thus indicating that the external sodium and magnesium stimulation of the Mg^{2+} efflux observed in the presence of ATP are completely reversible.

It is well known that two of the operational modes of the Na^+/Ca^{2+} counter transport system: Ca_o–Na_i (reverse) and Ca_o–Ca_i exchanges, are activated by external monovalent cations ($Li^+ > K^+ > Rb^+$). Whether this is also the case for the Mg^{2+}/Na^+ countertransport system, remained to be explored. The experiment described in Fig. 2 aimed to investigate this point using the Mg_o^{2+}-dependent Mg^{2+} efflux component on the assumption that it represents an homologous exchange mode (Mg_o^{2+}–Mg_i^{2+} exchange) of the Mg^{2+}/Na^+ countertransport system. In this experiment, the axon was predialyzed for 45 min with a standard dialysis medium containing radioactive Mg^{2+} and 1 mM ATP. Notice the absence of a transitory effect on Mg^{2+} efflux due to the presence of ATP from the start of the experiment. From a steady-state value of 750 fmol · cm^{-2} · s^{-1}, the removal of both Na_o^+ and Mg_o^{2+} drastically reduces the Mg^{2+} efflux to a very low value. In the absence of Na_o^+, the Mg_o^{2+}-dependent component (50 mM Mg_o^{2+}) was close to 375 fmol · cm^{-2} · s^{-1}. The effect of 100 mM Li_o^+ on Mg efflux was tested in the absence of both external Na^+ and Mg^{2+} and in the presence of 50 mM Mg_o^{2+}. Two points are worth stressing: first, external Li^+ can not replace Na^+ ions in activating the Mg^{2+}–Na^+ exchange, and second, in varience with the Na^+–Ca^{2+} exchange system [8], the Mg^{2+}–Na^+ exchange mechanism does not seems to have an activatory external monovalent cation site. Clearly a similar Mg_o^{2+}-dependent Mg^{2+} efflux is found with or without Li^+ ions in the external medium. In the last part of the experiment (see Fig. 2) we explored the efflux of Mg^{2+} as a function of the membrane potential. With the axon bathed in artificial sea water and voltage clamped at −50 mV, neither a steady hyperpolarization up to −80 mV or a depolarization to −30 mV affects the level of Mg^{2+} efflux. This confirms previous findings showing that the Mg^{2+}–Na^+ exchange is an electroneutral process [1,2].

The ATP analog ATPγS has recently become a valuable tool for studying ion transport processes across plasma membrane [9,10]. This compound which can act as a subtrate for kinases but not for ATPases [11–13] allows to investigate the effect of ATP on transport process involving Na^+ and Ca^{2+} movements without the interference of pump-mediated fluxes (Na^+/K^+ and Ca^{2+} pump; see Ref. 9). Although previously we have found no detectable Mg_o^{2+}-dependent Na^+ efflux (reverse Mg^{2+}–Na^+ exchange) activated by ATPγS [9], we decided to reinvestigate this matter directly on the Mg^{2+} efflux (forward and Mg_o^{2+}–Mg_i^{2+} exchange modes). The main reason is that the expected magnitude of the Mg_o^{2+}-dependent Na^+ efflux under our present conditions is less than 1 pmol · cm^{-2} · s^{-1} and therefore difficult to detect on the basis of Na^+ flux measurements.

An experiment of the sort used to determine the effect of ATPγS on Mg^{2+} efflux is shown in

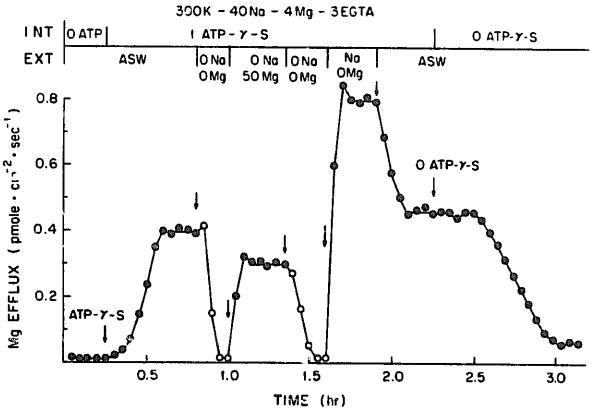

Fig. 3. The effect of ATPγS on the Mg^{2+} efflux. In order to remove all the ATP, the axon was predialyzed for 50 min with a standard free ATP dialysis solution. Arrows indicate changes in internal and external solutions. Ordinate: Mg^{2+} efflux in $fmol \cdot cm^{-2} \cdot s^{-1}$. Abscissa: time in hours. Notice the reversibility of the ATPγS activation upon its removal from the dialysis medium. All concentrations are in millimolar. Axon diameter: 600 μm. Temperature: 16 °C. $V_m = -55$ mV. Open circles: Mg^{2+} efflux into Na^+- and Mg^{2+}-free sea water (Na^+ ions were substituted by Tris).

Fig. 3. Before radioactive Mg^{2+} was added to the internal solution, the axon was predialyzed (50 min) with an ATP-free solution in sea water containing cyanide in order to remove all of the ATP. Therefore, the transient rise in Mg^{2+} efflux observed in the experiment of Fig. 1 was not seen. Addition of 1 mM of ATPγS to the dialysis medium, causes a large increment in Mg^{2+} efflux to a value of about 400 $fmol \cdot cm^{-2} \cdot s^{-1}$. Removal of both Na_o^+ and Mg_o^{2+} brings the efflux back to 'leak' values thus indicating that the stimulation of Mg^{2+} efflux is not an unspecific Mg^{2+} leak. In the second part of the experiment we measured the magnitude of the Mg^{2+} efflux in solutions containing: Mg_o^{2+} but no Na_o^+, Na_o^+ but no Mg_o^{2+}, and both Mg_o^{2+} and Na_o^+. In the presence of Mg_o^{2+} alone, the magnitude of the Mg^{2+} efflux was about 300 $fmol \cdot cm^2 \cdot s^{-1}$ as compared to 800 $fmol \cdot cm^{-2} \cdot s^{-1}$ in the presence of Na_o^+. Interestingly, in the presence of both Mg_o^{2+} and Na_o^+ ions the Mg^{2+} efflux stabilizes at a value between the Mg^{2+} and the Na^+ components. Three points of interest should be notice: (a) ATPγS is able to activate both the Mg^{2+}–Mg^{2+} and the forward Mg^{2+}–Na^+ exchanges. (b) The magnitude of the Na_o^+-dependent component is much larger than that of the Mg_o^{2+} component suggesting a higher mobility of the exchanger in the Na^+ than in the Mg^{2+}-loaded form. (c) In the presence of both Mg_o^{2+} and Na_o^+ ions, total Mg^{2+} efflux is not the addition of the Mg_o^{2+}- and Na_o^+-dependent components but has a much smaller value. This is best explained by the presence of a fraction of the exchanger performing Mg^{2+}–Mg^{2+} exchange in artificial sea water. This last point is in agreement with data obtained in injected squid axons which show that the activation of Mg^{2+} efflux by Na_o^+ is affected by the $[Mg^{2+}]_o$: reducing $[Mg^{2+}]_o$, lowers the apparent K_m for Na_o^+ [2]. In the last part of the experiment of Fig. 3 we tested the reversibility of the activation of Mg^{2+} efflux by ATPγS. Removal of the ATP analog from the dialysis solution, reduces the efflux of Mg^{2+} to almost its ATP free value (in about 48 min of dialysis). As is the case for the Na^+–Ca^{2+} exchange in squid axons [9], the reversibility of the ATPγS effect could indicate that the thiophosphoester form is not stable, or that a phosphatase system(s) present in the axoplasm is able to dephosphorylate the Mg^{2+}–Na^+ exchanger.

In conclusion, our data constitute evidence against the existence of an ATP-dependent, Mg_o^{2+}- and Na_o^+-independent Mg^{2+} efflux in squid axons, thus ruling out the presence of an uncoupled Mg^{2+} pump in this preparation [14]. This leaves the Mg^{2+}/Na^+ countertransport system as the major plasma membrane mechanism responsible for the long term homeostasis of Mg_i^{2+} ions. Moreover, similar to the Na^+/Ca^{2+} countertransport system, the activation of the Mg^{2+}–Na^+ exchange by ATPγS strongly suggest that regulation of Mg^{2+} transport in vivo may occurs by a process of phosphorylation (protein kinases) and dephosphorylation (phosphatases) of the Na^+–Mg^{2+} exchanger.

We wish to thank the director and staff of the Marine Biological Laboratory in Woods Hole, Ma for the facilities put at our disposal. This work was supported by: National Science Foundation Gran BNS-8500595, NIH-1RO1HL39243, CONICIT (S1-1937) Venezuela, Fundación Polar (Venezuela) and CONICET (0155) Argentina.

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