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IN SQUID AXONS INTRACELLULAR Mg^{2+} IS ESSENTIAL FOR ATP-DEPENDENT Na^+ EFFLUX IN THE ABSENCE AND PRESENCE OF STROPHANTHIDIN

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The effect on Na^+ efflux of removal of intracellular Mg^{2+} was studied in squid giant axons dialyzed without internal Ca^{2+}. In the absence of Mg_i^{2+}, ATP was unable to stimulate any efflux of Na^+ above the baseline of about 1 pmol·cm^{-2}·s^{-1}. This behavior was observed in otherwise normal axons and in axons poisoned with 50 μM strophanthidin in the sea water. Reinstatement of 4 mM $MgCl_2$ in excess to ATP in the dialysis solution brought about the usual response of Na^+ efflux to ATP, external K^+ and strophanthidin. The present experiments show that, regardless of the mechanism for the ATP-dependent Na^+ efflux in strophanthidin-poisoned axons, this type of flux shares with the active Na^+ extrusion the need for the stimultaneous presence of intracellular ATP and Mg^{2+}.

In squid giant axons subjected to internal dialysis (60–70 mM Na_i^+/310 mM K_i^+/4 mM Mg_i^{2+} in excess to ATP, 5–10 mM phosphoarginine) and bathed in artificial seawater supplemented with 10 mM K^+, more than 97% of the Na^+ efflux is abolished by removal of ATP [1,2]. The residual Na^+ efflux under 'ATP-free' conditions is about 1 pmol · cm^{-2} · s^{-1}, and many times one-half that value. On the other hand, in axons dialyzed under the same experimental conditions with ATP in the mM range the maximal inhibition of Na^+ efflux attained with strophanthidin or ouabain is no more than 70% of the total Na^+ extrusion [1,3]. The inability of cardiotonic steroids to fully block Na^+ efflux has also been observed in injected nerves [4–7]. This ATP-dependent Na^+ efflux in nerves poisoned with digitalis is corresponded by an influx of Na^+ of the same magnitude, probably representing an Na^+-Na^+ exchange working on a 1 : 1 stoichiometry [3]. These exchange fluxes are insensitive to changes in K_o^+, K_i^+, Mg_o^{2+}, Ca_o^{2+} or Ca_i^{2+} and are not affected by 1 mM intracellular vanadate [3]. There are two important unanswered questions regarding the Na^+ fluxes in the presence of srophanthidin or ouabain. One is whether they do represent true 'digitalis resistant' fluxes (they also exist in unpoisoned nerves), or are new modes of translocation induced by the inhibitors (see Ref. 3). The second question has to do with the role played by ATP: regulatory, phosphorylating, or both. One way to attack the problem is to study the Mg_i^{2+} requirements of Na^+ efflux in axons dialyzed with and without ATP in the absence and presence of strophanthidin. It is known that the (Na^+ + K^+)-ATPase enzyme and the active Na^+, K^+-transport system have an absolute requirement for Mg_i^{2+} [8–10]; in both systems there is an optimal Mg_i^{2+} concentration above and below which activity decreases. In squid axons the effects of Mg_i^{2+} concentrations have been explored by means of the microinjection technique [11]; the optimal Mg_i^{2+} concentrations were found around 10 mM, but the lowest values investigated were never below 0.4 mM. The work described below

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid.

constitutes the first attempt to follow the effects of total Mg_i^{2+} removal on Na^+ efflux in squid axons as they are influenced by ATP and digitalis poisoning.

The experiments were performed on giant axons taken from live specimens of *Loligo pealei,* at the Marine Biological Laboratory at Woods Hole, MA, U.S.A., or of *Doryteutis plei*, at the Instituto Venezolano de Investigaciones Científicas, in Caracas, Venezuela. The general dialysis technique for efflux is described in detail in Refs. 1, 2 and 12. The artificial sea water and dialysis solution compositions are given in the legend to Fig. 1. Other relevant information can be found in the corresponding figure legends. The only point worth stressing here is that in all cases the axons were dialyzed without added calcium and with 1 mM EGTA; in that way we made sure that no Na^+ efflux associated to the Na^+-Ca^{2+} exchange mechanism was present [13].

The experiment described in Fig. 1 shows that the efflux of Na^+ in the presence of 4 mM Mg_i^{2+} and no ATP (about 1 pmol · cm^{-2} · s^{-1}) is not different from that obtained in the absence of both ATP and Mg_i^{2+} or with 3 mM ATP and no Mg_i^{2+}. On the other hand, the total Na^+ efflux regained its usual magnitude and its sensitivity to external K^+ when ATP and Mg^{2+} were simultaneously present in the dialysis solution. The total efflux obtained upon reinstatement of ATP and Mg_i^{2+} has the typical sensitivity to strophanthidin as well (Fig. 2) (about 60% inhibition); in addition, Fig. 2 shows that in the presence of ATP and strophanthidin the efflux of Na^+ is brought to the 'ATP-free' levels when intracellular Mg^{2+} is removed. The results just mentioned are not influenced by the sequence of addition or removal of the ligands. This is also shown in Fig. 3 where the efflux of Na^+ in the absence of Mg_i^{2+} and ATP (about 0.5 pmol · cm^{-2} · s^{-1} in this case) is not modified by the addition of ATP alone or ATP plus 50 μM strophanthidin. In the presence of the nucleotide and the digitalis the inclusion of Mg_i^{2+} produced an increase in Na^+ efflux to the values expected for an otherwise normal axon. Results similar to those described above were obtained when CDTA was used instead of EDTA (not shown) in the Mg^{2+}-free dialysis solutions.

The results presented in this work clearly indicate that, under conditions where the Na^+-Ca^{2+} exchange mechanism is not at work, practically all Na^+ efflux from squid axons depends on the simultaneous presence of intracellular ATP and Mg^{2+}. However, the simplistic explanation of ascribing all the (ATP + Mg_i^{2+})-dependent Na^+

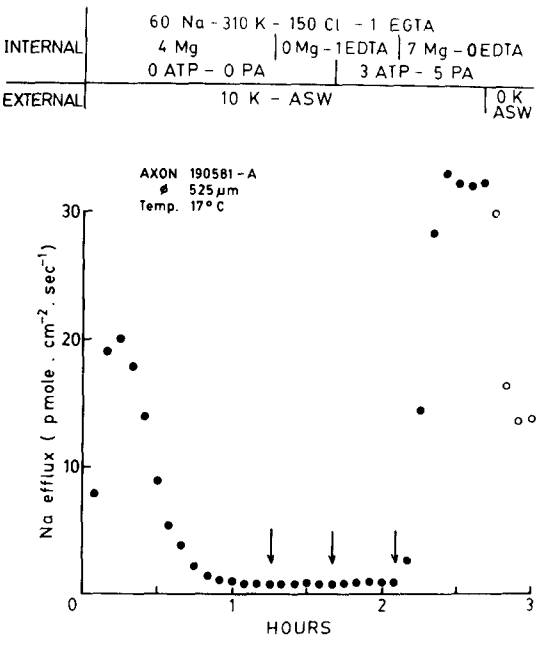

Fig. 1. The effects of intracellular ATP and Mg^{2+}, alone and in combination, on the Na^+ efflux in a dialyzed axon of the squid *Loligo pealei* (Axon 190581-A, Ø 525 μm, temp. 17°C). At zero time the dialysis began with the indicated solutions plus radioactive Na^+. The apparent rise in Na^+ efflux between 0 and 15 min does not represent real flux increase but the time taken for the isotope to reach steady-state distribution. The reduction after 15 min is a consequence of the ATP washout. The composition of solutions was as follows (mM). Artificial sea water (ASW): Na^+, 440; K^+, 10; Ca^{2+}, 10; Mg^{2+}, 50; $Tris^+$, 10; Cl^-, 580; EDTA, 0.1. When K^+ was removed it was replaced by equal amounts of Na^+. The osmolarity was 1000 mosM and the pH (18°C) 7.6. Dialysis solutions: Na^+, 60; K^+, 310; Mg^{2+}, 4 in excess to ATP or none plus 1 mM EDTA (or CDTA); $Tris^+$, 30; Cl^-, 150; aspartate, 260; EGTA, 1; glycine, 330. Total osmolarity was 980 mosM and pH (18°C) 7.3. ATP (vanadium-free) was obtained from Sigma as Tris salt, neutralized with Tris hydroxide and stored at −20°C as a 250 mM solution. Phosphoarginine was also from Sigma as Na^+ salt; it was neutralized with HCl and stored at −20°C as a 400 mM solution. All chemicals used were reagent grade. Counting was performed in a liquid scintillation counter after mixing the sea water samples with 5 ml of scintillator. When possible, counting was long enough to give standard errors of counting of 1%. Unless otherwise stated all concentrations are given in mM.

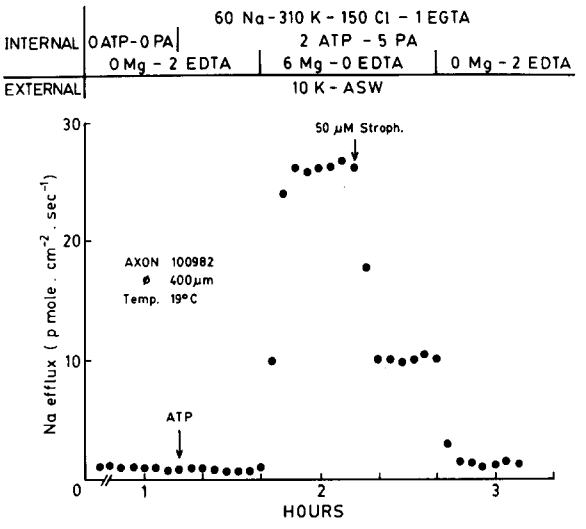

Fig. 2. The effects of intracellular Mg^{2+} on the Na^+ efflux in an axon of the squid *Doryteutis plei* dialyzed without and with ATP in the absence and presence of 50 μM strophanthidin in the sea water. (Axon 100982, ø 400 μm, temp. 19°C). The composition of the solutions can be found in the legend to Fig. 1. Strophanthidin (from Sigma, Co.) was added to the sea water from a 1000-times more concentrated stock solution in pure ethanol. Unless otherwise stated all concentrations are given in mM.

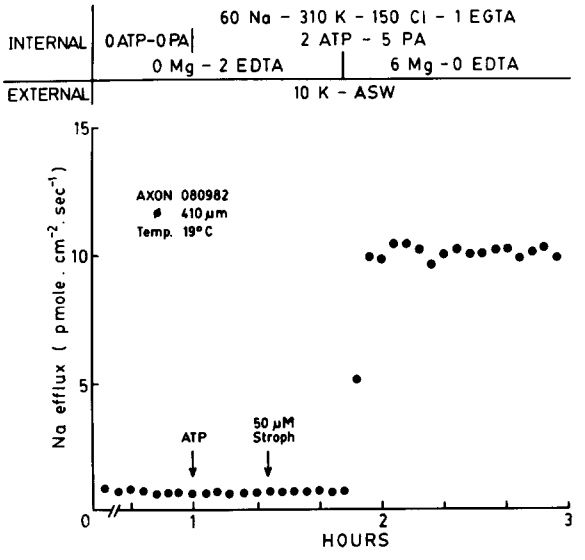

Fig. 3. The need for intracellular Mg^{2+} in order to obtain an ATP-dependent Na^+ efflux in a dialyzed axon of the squid *Doryteutis plei* externally perfused with strophanthidin-containing sea water (Axon 080981, ø 410 μm, temp. 19°C). For details see text and the legend to Fig. 1. Unless otherwise stated, all concentrations are given in mM.

efflux to the normal operation of the Na^+, K^+ pump is challenged by the fact that similar dependence is observed in nerves fully poisoned with strophanthidin. This finding means that the intention of using Mg_i^{2+} as a tool to evaluate the ATP dependent efflux of Na^+ in the absence and presence of digitalis has failed, and the nature of the Na^+ fluxes in digitalis-poisoned axons remains obscure. Nevertheless, it is very important that squid nerves appear different from human red cells, the other preparation where the effects of Mg_i^{2+} on Na^+ fluxes has been investigated; in human red cells, although the Na^+ pump (defined as the ouabain-sensitive Na^+ efflux) is, as expected, affected by the levels of Mg_i^{2+}, the efflux of Na^+ in ouabain-treated cells is completely insensitive to the Mg_i^{2+} concentrations [10]. This suggests that besides Na^+-Ca^{2+} exchange, squid axons have an extra component of Na^+ efflux not present in red blood cells. Whether this is a unique property of nerves or of exitable cells cannot be answered at present, nor there is more information on the controversy about the possibility that cardiotonic steroids can induce Na^+ fluxes in some cells. Induced or not, it is exceedingly interesting and intriguing that ATP is unable to promote any efflux of Na^+ in the presence of digitalis unless there is Mg_i^{2+} present as well. This is another evidence against a regulatory role of ATP of the type seen in the ouabain-sensitive K^+-K^+ exchange [14] for the nucleotide-stimulated disocluding step does not require Mg^{2+} [15]. A regulatory effect where the agent is not ATP but the MgATP complex is not inconceivable; on the other hand, one is always tempted to associate Mg^{2+} plus ATP to some kind of phosphorylating process.

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