In squid nerves intracellular Mg$^{2+}$ promotes deactivation of the ATP-upregulated Na$^{+}$/Ca$^{2+}$ exchanger

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DiPolo, Reinaldo, Graciela Berberián, and Luis Beaugé. In squid nerves intracellular Mg$^{2+}$ promotes deactivation of the ATP-upregulated Na$^{+}$/Ca$^{2+}$ exchanger. Am J Physiol Cell Physiol 279: C1631–C1639, 2000.—We investigated the role of intracellular Mg$^{2+}$ (Mgi) on the ATP regulation of Na$^{+}$/Ca$^{2+}$ exchanger in squid axons and bovine heart. In squid axons and nerve vesicles, the ATP-upregulated exchanger remains activated after removal of cytoplasmic Mg$^{2+}$, even in the absence of ATP. Rapid and complete deactivation of the ATP-stimulated exchange occurs upon readmission of Mgi$^{2+}$. At constant ATP concentration, the effect of intracellular Mg$^{2+}$ concentration ([Mgi$^{2+}$]) on the ATP regulation of exchanger is biphasic: activation at low [Mgi$^{2+}$], followed by deactivation as [Mgi$^{2+}$] is increased. No correlation was found between the above results and the levels of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P$_2$] measured in nerve membrane vesicles. Incoporation of PtdIns(4,5)P$_2$ into membrane vesicles activates Na$^{+}$/Ca$^{2+}$ exchange in mammalian heart but not in squid nerve. Moreover, an exogenous phosphatase prevents MgATP activation in squid nerves but not in mammalian heart. It is concluded that 1) Mg$^{2+}$ is an essential cofactor for the deactivation part of ATP regulation of the exchanger and 2) the metabolic pathway of ATP up-regulation of the Na$^{+}$/Ca$^{2+}$ exchanger is different in mammalian heart and squid nerves.

sodium ion/calcium ion exchange; adenosine 5'-triphosphate; intracellular magnesium ion; phosphorylation-dephosphorylation; phosphoinositides

THE ELECTROGENIC Na$^{+}$/Ca$^{2+}$ exchange mediates the coupled movement of Na$^{+}$ and Ca$^{2+}$ across the plasma membrane of most animal cells, thus playing an important role in Ca$^{2+}$ clearance in excitable tissues (23). One of the main features of this transporter is its modulation by different intra- and extracellular physiological ligands, including Ca$^{2+}$, H$^+$, Na$^+$, K$^+$, ATP, and phosphagens (7, 9, 17, 23). In squid axons, as well as in other preparations, including cardiac and smooth muscle cells, the activity of this transporter is upregulated by MgATP (7, 15, 21).

Although there is general consensus that a phosphorylation-dephosphorylation process controls the state of activation of the exchanger, the mechanism(s) of this upregulation seem(s) to vary not only among different species but also within tissues of the same species (7, 15, 19, 21, 25). In squid nerves, ATP modulation of the Na$^{+}$/Ca$^{2+}$ exchanger (“on effect”) not only requires intracellular Mg$^{2+}$ (Mgi$^{2+}$) but also a soluble cystolic regulatory protein (SCRP) of low molecular weight (4, 16). Conversely, in the mammalian cardiac Na$^{+}$/Ca$^{2+}$ exchanger, ATP stimulation needs Mg$^{2+}$, but, at least in sarcolemmal vesicles (5, 22), it takes place without a soluble regulatory protein (however, see Ref. 24). In the heart exchanger, MgATP stimulation is linked to the phosphatidylinositol metabolism (5, 22) and, when expressed in fibroblasts, to protein kinase C activity (25).

Most studies on ATP stimulation of Na$^{+}$/Ca$^{2+}$ exchange have focused on the “on-activating” effect (2, 6, 9, 18), whereas little is known about the process responsible for the “off-deactivation” part. Reversal phosphorylation is widely accepted as a key mechanism for regulation of several intracellular events that occur in eukariotes in which the dephosphorylation reactions are carried out mostly by phosphatases that require Mg$^{2+}$ (26, 29). In this study, we have used in vivo and in vitro squid nerve preparations to explore whether Mg$^{2+}$ influences the rate at which the ATP-stimulated fraction of the Na$^{+}$/Ca$^{2+}$ exchanges deactivates (“off effect”). We found that, after activation by MgATP, rapid removal of Mg$^{2+}$ locked the exchanger in a highly activated state both in the absence and presence of ATP. Readmission of Mg$^{2+}$ in the absence of the nucleotide rapidly and fully deactivates the stimulated exchanger. The effect of intracellular Mg$^{2+}$ concentration ([Mgi$^{2+}$]) on the ATP upregulated Na$^{+}$/Ca$^{2+}$ exchanger may have important implications under physiopathological conditions such as ischemia and reoxygenation in which ATP concentration ([ATP]) and [Mgi$^{2+}$], suffer

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drastic changes. Part of this work has been previously reported in abstract form (3, 13).

METHODS

Intracellular dialysis of squid giant axons. As described previously (12), squid axons from the Marine Biological Laboratory (Woods Hole, MA; Loligo pealei) and the Instituto Venezolano de Investigaciones Científicas (L. plei) were dialyzed with highly permeable capillaries of regenerated cellulose fibers (210-μm OD; 200-μm ID; molecular weight cutoff of 18 kDa; Spectra/Por 132226; Spectrum, Houston, TX). The standard dialysis medium had the following composition (in mM): 385 Tris-MOPS, 40 NaCl, 5 MgCl₂, 285 glycine, and 1 Tris-EGTA, pH 7.3 and temperature between 17 and 18°C.

The runs were performed with the same buffer at a flow rate of 0.4 ml/min, collecting fractions of 0.25 ml.

The standard external solution had the following composition (in mM): 440 NaCl, 0.3 CaCl₂, 60 MgCl₂, and 10 Tris-Cl, pH 7.6. The osmolarity of all solutions was adjusted to 940 mosmol/l. Removal of external sodium was compensated with lithium. To stop any endogenous production of ATP and to effectively control the intracellular ATP concentration, 1 mM NaCl was always present in the external media. The Ca²⁺ pump component of Ca²⁺ efflux was eliminated by adding 0.1 mM vanadate to all dialysis solutions.

Preparation of squid nerve and bovine heart membrane vesicles. Membrane vesicles from squid optic nerve (Sepioteuthis sepioidea and L. pealei) and bovine heart were prepared by differential centrifugation as described elsewhere (5, 16) and loaded with 300 mM NaCl (nerve) or 160 mM NaCl (heart), 0.1 mM EDTA, and 30 mM MOPS-Tris (pH 7.3 at 20°C for nerve and pH 7.4 at 37°C for heart). Membrane vesicles were ~35% inside out in squid nerve (16) and 40% in mammalian heart (5). Because the ATP regulatory site of the exchanger is located intracellularly (7, 12), all MgATP-stimulated Na⁺ gradient-dependent Ca²⁺ uptake takes place in inside out vesicles only. This population of vesicles also has a powerful MgATP-dependent Ca²⁺ pump that was inhibited by adding vanadate to the incubation solutions.

Partial purification of the SCRP. Squid optic ganglia (brain) were homogenized (in a 1:1 vol/vol ratio) in 20 mM MOPS-Tris (pH 7.3 at 20°C), 0.1 mM EDTA, and an antiprotease cocktail (0.5 mM phenylmethylsulfonyl fluoride plus 10 μg/ml of aprotinin, leupeptin, and pepstatin A), followed by centrifugation at 12,000 g for 10 min. This supernatant was centrifuged further at 100,000 g for 30 min (postmicrosomal fraction). The 100,000-g supernatant fraction went through 100- and 30-kDa cut-off filters (Amicon Centricron). Aliquots of 200 μl (~1.2 mg of total protein) of the 30-kDa fraction suspended in 30 mM Tris-Cl, pH 7.3 at 20°C were passed through an HPLC system using a Superdex-75 column (Pharmacia). The runs were performed with the same buffer at a flow rate of 0.4 ml/min, collecting fractions of 0.25 ml. Fraction 65, which corresponded to a molecular weight of ~15 kDa contained the SCRP (16).

Na⁺ gradient-dependent ⁴⁰Ca uptake in membrane vesicles. ⁴⁰Ca uptake in squid membrane vesicles (16) was measured at 20–22°C by incubating the vesicles (50–60 μg protein) for 10 or 20 s in media with high (300 mM) or low (30 mM) Na⁺ (200 μl total volume). In addition, all extravesicular solutions contained 0.2 mM vanadate and 20 mM MOPS-Tris (pH 7.3 at 20°C) and the Mg²⁺ concentration ([Mg²⁺]) and [ATP] indicated in Figs. 1–10. In low-Na⁺ medium, the osmolarity was compensated with N-methyl-d-glucamine (NMG)-Cl. The reaction was stopped with 0.8 ml of an ice-cold solution containing 20 mM MOPS-Tris, 300 mM KCl, and 1 mM EGTA and was filtered through Whatman GF/F glass filters. The filters were washed with 5 ml of the same solution, immersed in 5 ml of scintillation fluid, and counted in a liquid scintillation counter. To obtain steady counts after addition of the scintillator, the filters were left standing for 4 h and then were counted. The effects of Mg²⁺ on Ca²⁺ transport and phosphoinositide synthesis (see below) were studied in chase experiments. ⁴⁰Ca uptake in bovine heart membrane vesicles was measured at 37°C or at 20°C as indicated (5). Aliquots of 2 μl (5.5 mg/ml) were diluted in 100 μl of solutions of the following composition: 160 or 10 mM NaCl, 20 mM MOPS-Tris (pH 7.4), 0.1 mM EGTA, 0.1 mM digoxigenin, 0.2 mM vanadate, 0.8 μM Ca²⁺, and the concentrations of ATP and Mg²⁺ indicated in Figs. 1–10. Low-Na⁺ solutions contained osmotically equivalent amounts of NMG-Cl. Incorporation of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] into vesicles was done by incubating concentrated vesicles with PtdIns(4,5)P₂ liposomes (0.25 mg/ml vesicle protein) for 5 min at 0°C and then for 1 min at 20°C (1, 15). ⁴⁰Ca uptake measurements were carried out at 20°C in both preparations. In the experiments with alkaline phosphatase (type VII-Nla from bovine intestinal mucosa) before starting Ca²⁺ uptake, concentrated vesicles were preincubated for 2 min at room temperature in the absence of Na⁺ gradient with no Ca²⁺ but with ATP, Mg²⁺, and 0, 50, or 200 U/ml of alkaline phosphatase.

Extraction and TLC separation of phospholipids. Aliquots of 60 μg membrane vesicles were incubated for the indicated times, and solutions are specified in RESULTS. The reactions were stopped with ice-cold chloroform-methanol-HCl, and the lipids were extracted in the organic phase. The TLC plates were developed in a mobile phase of chloroform-methanol-water-concentrated NH₄OH (40:48:10:5 vol/vol/vol/vol) using 20 × 10 cm HP-TLC (Merck). The positions of phosphatidylinositol 4-phosphate [PtdIns(4)P] and PtdIns(4,5)P₂ were detected using commercial standards submitting the plates to saturated iodine vapor (5). The ³²P-labeled phospholipids were visualized in an phosphor screen autoradiography of the plates and were analyzed quantitatively using Imagequant software of the Storm System (Storm-840).

Solutions. All solutions were made with deionized ultrapure (18 MQ) water, (Milli-Q, Millipore). NaCl, KCl, MgCl₂, and CaCl₂ were from Baker. Ouabain, digitoxigenin, Tris-OH, NMG, MOPS, alkaline phosphatase (type VII-Nla from bovine intestinal mucosa), phospholipase C (PLC)-specific phosphatidylinositol (PtdIns; PLC-PtdIns from bovine intestinal mucosa), phospholipase C (PLC)-specific phosphatidylinositol (PtdIns; PLC-PtdIns from Bacillus cereus), PtdIns(4)P, and PtdIns(4,5)P₂ were obtained from Sigma. Tris-ATP and β,γ-methylene-ATP (AMP-PCP) were from Boehringer Mannheim; ⁴⁰Ca as chloride salt and [γ-³²P]ATP were purchased from New England Nuclear. Monoclonal PtdIns(4,5)P₂ antibody was obtained from Perceptive Biosystems (Framingham, MA). The estimations of free Ca²⁺ concentration ([Ca²⁺]) were carried out with the Maxchelator program (Chris Patton Hopkins Marine Station). The dissociation constants for the MgAMP-PCP complex were estimated with Arsenazo III in the same solutions in which the experiments were performed; the values obtained were 0.159 mM at low and 0.65 mM at high ionic strength.

RESULTS

Reversibility and Mg²⁺ dependence of ATP stimulation of Na⁺/Ca²⁺ exchange in internally dialyzed squid axons. Figure 1A shows that, in the presence of 1 mM Mg²⁺, 3 mM ATP stimulates the efflux of Ca²⁺ solely through the extracellular Na⁺ (Na⁺,)-dependent (forward Na⁺/Ca²⁺ exchange) component. At a constant [Mg²⁺], of 1 mM, this effect is reversible upon removal...
of ATP. For a phosphorylation-dephosphorylation requiring system, one would expect that removal of either ATP or Mg\(^{2+}\) would deactivate the exchanger. This is indeed the case in the experiment of Fig. 1A where removal of ATP in the presence of Mg\(^{2+}\) completely deactivates the ATP-stimulated exchanger. On the other hand, as shown in Fig. 1B, if the removal of ATP takes place after Mg\(^{2+}\) has been taken away, no deactivation of the ATP-stimulated exchanger occurs. Figure 1B also shows that removal of Mg\(^{2+}\) in the presence of ATP produces a small but significant increase in Na\(^{+}\)-dependent Ca\(^{2+}\) efflux. When compared with the ATP-independent Na\(^{+}\)-dependent Ca\(^{2+}\) fluxes (~25 fmol cm\(^{-2}\) s\(^{-1}\)), that increase is of the order expected for the noncompetitive partial inhibition of Mg\(^{2+}\) reported earlier in this preparation (11). These results indicate that the exchanger can be maintained in an upregulated state provided Mg\(^{2+}\) is taken away before the removal of ATP. In other experiments (data not shown), as long as the [Mg\(^{2+}\)]\(_i\) was virtually zero, the exchanger could be maintained activated for long periods of time (up to 2 h).

From the reported time constant for MgATP washout in dialyzed squid axons (14), fast and complete removal of intracellular Mg\(^{2+}\) can only be achieved by the use of Mg\(^{2+}\)-chelating agents. We have taken advantage of the Mg\(^{2+}\)-binding capacity of AMP-PCP, a nonhydrolyzable ATP analog that does not interact with the Na\(^{+}/Ca^{2+}\) exchanger (12), to rapidly and entirely deplete Mg\(^{2+}\). Figure 1C shows that the simultaneous withdrawal of Mg\(^{2+}\) and ATP together with the addition of 5 mM AMP-PCP keeps the ATP-stimulated exchanger fully active. That this is indeed the forward mode of Na\(^{+}/Ca^{2+}\) exchange is demonstrated by its total sensitivity to external Na\(^{+}\). An important result shown in Fig. 1D is that the exchangers that remained upregulated after the removal of Mg\(^{2+}\) can be fully deactivated by the readdition of Mg\(^{2+}\). This deactivation is not a result of competition between Mg\(^{2+}\) and intracellular Ca\(^{2+}\) at intracellular sites since removal of Mg\(^{2+}\), as shown at the end of the experiment, produces only a small increment in Ca\(^{2+}\) efflux. However, that increment amounts to double the ATP-independent Ca\(^{2+}\) efflux, which is to be expected from the release of the reported noncompetitive partial inhibition of the exchanger (11). The complete reversibility of the Mg\(^{2+}\)-deactivation of the ATP upregulation of the exchanger is shown in Fig. 2 where, after Mg\(^{2+}\)-induced deactivation at constant [ATP], the upregulated fluxes return to the original levels when the initial low [Mg\(^{2+}\)]\(_i\) is restored.

To evaluate the Mg\(^{2+}\) dependence of the ATP stimulation of Na\(^{+}/Ca^{2+}\) exchange, we performed a series of experiments in which the magnitude of the ATP stimulation was followed at constant [ATP] (4 mM), whereas the concentration of Mg\(^{2+}\) was varied between 0.5 and 10 mM. As shown in Fig. 3, there is a biphasic response: stimulation up to ~1 mM Mg\(^{2+}\) followed by a progressive decline which at 10 mM Mg\(^{2+}\) reaches the values of the unstimulated Na\(^{+}\)-dependent Ca\(^{2+}\) efflux.

Ca\(^{2+}\) exchange fluxes and biochemical studies in squid nerve and bovine heart plasma membrane vesicles: ATP, Mg\(^{2+}\), and phosphoinositides. The use of these vesicles from squid nerve and bovine heart allowed us to perform parallel Ca\(^{2+}\) trans-
Fig. 2. Reversibility of Mg\(^{2+}\) deactivation of the ATP-dependent upregulation of \(\text{Na}^+\)/Ca\(^{2+}\) efflux in dialyzed squid axons. \(\text{Na}_a\) -dependent Ca\(^{2+}\) efflux in the presence of 1 mM intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)) was stimulated by 4 mM ATP. Notice the complete inhibition of the ATP-upregulated exchange fluxes by 10 mM Mg\(^{2+}\) at constant ATP concentration ([ATP]). Return to the initial 1 mM [Mg\(^{2+}\)]\(_i\), produced a reactivation of the ATP-dependent fluxes to their original levels. Temperature: 17.5°C. For more details see RESULTS.

Fig. 3. Dose-response relationship between the ATP-stimulated \(\text{Na}_a\) -dependent Ca\(^{2+}\) efflux and [Mg\(^{2+}\)]\(_i\), at constant [ATP]. \(\text{Na}_a\) -dependent Ca\(^{2+}\) efflux in the presence of 4 mM ATP was measured in 5 different dialyzed squid axons in which Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)) in the dialysis was varied between 0.2 and 10 mM. Points correspond to the mean ± SE normalized to the Ca\(^{2+}\) efflux values obtained with 1 mM [Mg\(^{2+}\)]\(_i\)]. Nos. in parentheses refer to different axons. Notice the bell shape of the ATP activation curve. Temperature: 17.5°C.

Fig. 4. ATP activation curve of \(\text{Na}^+\) gradient-dependent Ca\(^{2+}\) uptake in squid optic nerve plasma membrane vesicles in the presence (●) and absence (○) of soluble cytosolic regulatory protein (SCRP). Broken line shows the ATP activation curve for \(\text{Na}_a\) -dependent Ca\(^{2+}\) efflux in dialyzed squid axons. The Michaelis constant value for ATP in vesicles with SCRP (0.17 mM) is very close to that determined in dialyzed axons [0.2 mM (15)]. Notice that in the absence of SCRP no ATP activation of \(\text{Na}^+\)/Ca\(^{2+}\) exchange is observed. Each point is the mean ± SE of triplicate determinations. Experiments were carried out at 20°C. See METHODS for details.

From the above results, we then pursued to look into the effects of Mg\(^{2+}\) on both the \(\text{Na}^+\)/Ca\(^{2+}\) exchange fluxes and the PtdIns(4,5)\(_P_2\) synthesis in squid nerve membranes. The membrane vesicles in the presence of SCRP were subjected to 1 min of preincubation in the absence of \(\text{Na}^+\) gradient (100 mM NaCl, 200 mM Na-MOPS, 0.15 mM EGTA, 0.2 mM vanadate, and enough CaCl\(_2\) to attain 0.8 \(\mu\)M Ca\(^{2+}\)) with 0.25 mM nonradio-

stimulation of \(\text{Na}^+\) gradient-dependent Ca\(^{2+}\) uptake (see also Refs. 4 and 16).

In the next group of experiments, we followed the phosphoinositide synthesis in both squid nerve and bovine heart under different experimental conditions. Figure 5, A and B, shows that both preparations contain the metabolic machinery to synthesize PtdIns(4)\(_P\) and PtdIns(4,5)\(_P_2\) from PtdIns and MgATP. Moreover, pretreatment of the vesicles with PtdIns-PLC completely abolished PtdIns(4,5)\(_P_2\) production in both preparations (Fig. 5A, lanes 3 and 6). Interestingly, as shown in Fig. 5A, lanes 1 and 2, in squid nerve vesicles, the SCRP does not modify the patterns of phosphatidylinositol synthesis and does not, by itself, lead to the production of either PtdIns(4)\(_P\) or PtdIns(4,5)\(_P_2\). The absolute values of \(^{32}\)P incorporation in PtdIns(4,5)\(_P_2\) under different conditions are shown in Fig. 5B. The data indicate that the PtdIns(4,5)\(_P_2\) production in both squid and bovine heart is quite comparable. We have previously reported that, in the bovine heart, PtdIns(4)\(_P\) production is independent of Ca\(^{2+}\), whereas that of PtdIns(4,5)\(_P_2\) is strongly dependent on this divalent cation (5). Figure 6, A and B, demonstrates that the Ca\(^{2+}\) dependence of PtdIns(4,5)\(_P_2\) production in squid nerve is similar to that seen in bovine heart, thus suggesting that a comparable metabolic polyphosphoinositide machinery is present in both preparations.
active or radioactive (1\textsuperscript{32}P)ATP, 10 \textmu l of the SCRP, and either 0.5 or 10 mM Mg\textsuperscript{2+}. Afterward, one aliquot of vesicles incubated with (32P)ATP was used to estimate the levels of net PtdIns(4,5)P\textsubscript{2} synthesis (Fig. 7A). The other aliquot was used to determine the PtdIns(4,5)P\textsubscript{2} levels after 20 s of incubation in solutions identical to those where Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was assayed. To that aim, vesicles preincubated with radioactive [PtdIns(4,5)P\textsubscript{2} synthesis] and cold (Ca\textsuperscript{2+} uptake) ATP were diluted 15 times in the presence of Na\textsuperscript{+} gradient (Na\textsuperscript{+} substituted for NMG) in solutions free of ATP with final concentrations of either 0.5 or 10 mM Mg\textsuperscript{2+}. There are two points to be stressed in these experimental protocols. First, 0.5 mM Mg\textsuperscript{2+} instead of free Mg\textsuperscript{2+} solutions were used since the vesicles become leaky in the absence of this divalent cation. Nevertheless, as shown in Fig. 3, 0.5 mM Mg\textsuperscript{2+} has no inhibitory effect on the ATP-stimulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Second, the final [ATP] attained during the chase period was only 16 \mu M, a concentration unable to promote either PtdIns(4,5)P\textsubscript{2} synthesis (data not shown) or stimulation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (see Fig. 4). The results in Fig. 7A demonstrate that 10 mM [Mg\textsuperscript{2+}] does not interfere with the synthesis of PtdIns(4,5)P\textsubscript{2}. On the other hand (Fig. 7B), that concentration of Mg\textsuperscript{2+} completely blocks the ATP-stimulated Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake. Therefore, the deactivation of the ATP-stimulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger by high [Mg\textsuperscript{2+}] in squid membrane vesicles is
similar to that reported here in dialyzed squid axons (see Fig. 3) and apparently is unrelated to the levels of PtdIns(4,5)P$_2$.

Figure 8 shows the differential effect of PtdIns(4,5)P$_2$ incorporation in membrane vesicles (27) on the Na$^+$ gradient-dependent Ca$^{2+}$ uptake in squid and heart under similar experimental conditions. As shown, the typical activation by MgATP is observed in both preparations (1, 5, 16). On the other hand, although PtdIns(4,5)P$_2$ causes a large stimulation of the Na$^+$/Ca$^{2+}$ exchanger in the heart, it is completely ineffective in activating the squid exchanger.

Effects of anti-PtdIns(4,5)P$_2$ antibody and PtdIns-PLC in dialyzed squid axons. Experiments in cardiac excised patches demonstrated that the PtdIns(4,5)P$_2$ antibody and treatment with PtdIns-PLC completely block ATP stimulation of the Na$^+$/Ca$^{2+}$ exchanger (22). Although the PtdIns-PLC effectively reduces the PtdIns(4,5)P$_2$ synthesis both in bovine heart and squid nerve (Fig. 5, A and B), this treatment renders squid nerve membrane vesicles highly leaky; this makes it difficult to assess the effect of Na$^+$/Ca$^{2+}$ exchange on in vitro ATP stimulation. We have therefore performed experiments of this kind using dialyzed squid giant axons in which these compounds were intracellularly injected. As shown in Fig. 9, A and B, injection of the PtdIns(4,5)P$_2$ antibody at a final concentration 200 times that required to inhibit the ATP-stimulated exchanger in cardiac giant patches (22) fails to block the ATP-stimulated Na$^+$-dependent Ca$^{2+}$ efflux. No inhibition was observed whether the antibody was injected after (Fig. 9A) or before (Fig. 9B) the addition of ATP. The injected PtdIns-PLC was expected to reach a final concentration in the axon 10 times higher (200 units) than that required to completely inhibit the ATP stimulation of the exchanger in the heart (22). In examining the effect of PtdIns-PLC (see Fig. 9C), we found that, in the absence of ATP, this enzyme causes a small but significant increase in the unspecific Ca$^{2+}$ leak; nevertheless, addition of ATP
still causes stimulation of the Na\textsuperscript{o}+-dependent Ca\textsuperscript{2+} efflux that is indistinguishable from that seen in control axons. Although in experiments of this kind there are no positive controls, the induction of an unspecified Ca\textsuperscript{2+} leak may be taken as evidence that the injected PLC has reached the membrane. Indirect positive controls are the experiments in which the injection of a large protein such as alkaline phosphatase completely blocks ATP and phosphoarginine regulation of the squid Na\textsuperscript{o}/Ca\textsuperscript{2+} exchanger (14).

**DISCUSSION**

This work demonstrates the key role played by Mg\textsuperscript{2+} in the deactivation of the ATP upregulated squid Na\textsuperscript{o}/Ca\textsuperscript{2+} exchanger. The complete reversibility of these Mg\textsuperscript{2+} effects rules out any unspecified deleterious action.

**Fig. 9.** Lack of effect of PtdIns(4,5)\textsubscript{P} \textsubscript{2} antibody [Ab-PtdIns(4,5)\textsubscript{P} \textsubscript{2}] and PtdIns-PLC on MgATP-activated Na\textsuperscript{o}/Ca\textsuperscript{2+} exchange in dialyzed squid axons. •, Ca\textsuperscript{2+} efflux in the presence of external Na\textsuperscript{o}; ○, Ca\textsuperscript{2+} efflux in the absence of external Na\textsuperscript{o}. A: intracellular injection of Ab-PtdIns(4,5)\textsubscript{P} \textsubscript{2} during internal dialysis after addition of ATP; B: intracellular injection of Ab-PtdIns(4,5)\textsubscript{P} \textsubscript{2} during internal dialysis before addition of ATP; C: intracellular injection of PtdIns-PLC (final concentration in the axon of ~200 units) before ATP addition. Notice the lack of effect of these compounds on the ATP stimulation of the Na\textsuperscript{o}-dependent Ca\textsuperscript{2+} efflux.

**Fig. 10.** Effect of alkaline phosphatase on ATP stimulation of Na\textsuperscript{o}/Ca\textsuperscript{2+} exchange in bovine heart sarcolemmal and squid optic nerve vesicles. A: squid nerve membranes. Uptake of \textsuperscript{45}Ca was estimated without or with 50 and 200 U/ml of alkaline phosphatase. B: bovine heart sarcolemmal membranes. Uptake of \textsuperscript{45}Ca was estimated without or with 200 U/ml of alkaline phosphatase. Before starting \textsuperscript{45}Ca uptake, concentrated membrane vesicles were incubated for 2 min at 20°C in the absence of Na\textsuperscript{o} gradient with no Ca\textsuperscript{2+} but with Mg\textsuperscript{2+} and ATP, with and without alkaline phosphatase. In the squid vesicles, all experiments were done in the presence of SCRP. Values are means ± SE of triplicate determinations. See METHODS for details.
of this divalent cation. If phosphorylation by ATP is responsible for activation of the exchanger, the most economical conclusion is that an Mg\textsuperscript{2+}-stimulated dephosphorylation accounts for its deactivation. The biphasic response of the Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux to Mg\textsuperscript{2+} concentrations at constant [ATP] concur with the general notion that a phosphorylation-dephosphorylation process is involved in ATP modulation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. The [Mg\textsuperscript{2+}]\textsubscript{i} required to prevent ATP stimulation indicates that it acts with low apparent affinity (Fig. 3). Furthermore, this Mg\textsuperscript{2+} deactivation cannot be accounted for by the reported noncompetitive partial inhibition already described in this preparation (11). This is evident in Fig. ID where removal of Mg\textsuperscript{2+} in the absence of ATP does not restore the levels of the ATP upregulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange fluxes but only causes the expected release of the inhibition observed in the absence of ATP (11).

Our experiments also show that the influence of [Mg\textsuperscript{2+}]\textsubscript{i} on the ATP regulation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is similar in dialyzed squid axons and in inside out membrane vesicles from squid nerves. This has allowed us to investigate possible relationships between Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange fluxes in the activated (low Mg\textsuperscript{2+}) and deactivated (high Mg\textsuperscript{2+}) states and the synthesis of polyphosphoinositides. The results suggest that phosphoinositides, key in the ATP regulation of the exchanger in mammalian heart, do not seem to be responsible for ATP regulation of the squid. The evidence, although indirect, is given by the lack of correlation between maneuvers leading to ATP activation and deactivation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange and the levels of membrane PtdIns(4,5)P\textsubscript{2}. This lack of correlation occurs despite the fact that bovine heart and squid nerve membranes behave very similarly with respect to their phosphoinositide metabolism. On the one hand, the [Ca\textsuperscript{2+}] dependence of PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} production is the same. On the other hand, a specific phospholipase (PtdIns-PLC) blocks phosphoinositide synthesis in both species (see Fig. 5). Furthermore, the low-molecular-weight cytosolic regulatory protein required for the MgATP stimulation of the squid exchanger does not modify the phosphatidylinositol production in nerve vesicles. Interestingly, as is the case in dialyzed squid axons (14), we found that MgATP modulation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in squid nerve vesicles is completely abolished by an alkaline phosphatase in contrast to the lack of effect in the mammalian heart. One possible explanation is that regulation of the exchanger by phosphorylation/dephosphorylation processes involves mainly proteins in the squid and lipids in the heart.

Recently, it has been shown that the squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange clone, when expressed in frog oocytes, manifests an MgATP stimulation that depends on PtdIns(4,5)P\textsubscript{2} (20). If indeed PtdIns(4,5)P\textsubscript{2} participates in the regulation of the exchanger in the squid, we would have expected, at least in part, modifications of the PtdIns(4,5)P\textsubscript{2} levels in parallel with those of the ATP-modulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange fluxes, and this did not occur. One possibility is that the phosphoinositide pathway is amplified in the oocyte-expressed clone at 32°C (20). Another possibility is that the clone, inserted in an alien cell, behaves differently and becomes sensitive to other membrane environments. In favor of this is the lack of phosphoarginine regulation of the squid clone when expressed in frog oocytes (20). More compelling evidence for the existence of different pathways for MgATP stimulation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in squid nerve and mammalian heart are the results described in Fig. 8. Under similar experimental conditions, incorporation of PtdIns(4,5)P\textsubscript{2} in membrane vesicles does not modify the exchange fluxes in the squid while inducing the reported activation of the exchanger in the heart (1, 5, 27).

The intimate mechanism by which Mg\textsuperscript{2+} deactivate the ATP-stimulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in the squid remains unknown. However, two possibilities are attractive. 1) The first possibility is that the endogenous SCR P is, or behaves as, part of a two-component signal transduction system (30). Therefore, as in most response regulators, the regulatory protein, in addition to being phosphorylated, would have an Mg\textsuperscript{2+}-dependent [acting with low affinity (28)] autophosphatase activity. 2) The second possibility is that an Mg\textsuperscript{2+}-dependent (also with low affinity) phosphatase, perhaps of the protein phosphatase-2C type (8), mediates the dephosphorylation step. Unfortunately, inhibitors of these phosphatases are not known yet (29).

Finally, it must be pointed out that under physiological conditions one would not expect large fluctuations in the cytosolic concentrations of Mg\textsuperscript{2+} and/or ATP. Therefore, in a normal cell, this divalent cation, although essential as a cofactor for ATP regulation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, is not likely to play a regulatory role on the exchanger. However, Mg\textsuperscript{2+} could become crucial in certain pathological conditions leading to changes in its concentration. In fact, alterations in the function of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger have been implicated in the cytosolic Ca\textsuperscript{2+} changes that occur during tissue hypoxia and reoxygenation (see Refs. 31 and 32 and references therein). Besides acidification, a main feature of prolonged ischemia is the dramatic drop in [ATP] (31, 32). Because in most tissues ATP is the major Mg\textsuperscript{2+} buffer (10), an increase in free [Mg\textsuperscript{2+}] is expected under these circumstances. This will create the conditions for an Mg\textsuperscript{2+} inhibition of the ATP-stimulated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, thus contributing to cytosolic Ca\textsuperscript{2+} load.

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