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In squid axons, ATP modulates Na^+-Ca^{2+} exchange by a Ca_i^{2+}-dependent phosphorylation

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In squid axons ATP stimulates both the forward and reverse modes of the Na^+-Ca^{2+} exchange by changing the affinity of the carrier towards Na^+ and Ca^{2+} ions. Whether ATP activates the Na^+-Ca^{2+} antiporter allosterically or is hydrolyzed during activation is still debated. The hypothesis that ATP modulates the Na^+-Ca^{2+} exchange through phosphorylation has been tested by means of [γ-S]ATP, an ATP analog that can act as a substrate for kinases but not for ATPases. Steady-state Ca^{2+} efflux was measured in squid axons dialyzed without ATP and containing either 0.7 or 100 μM Ca_i^{2+}. Addition of 1 mM [γ-S]ATP markedly increases the Na_o^+-dependent component of the Ca^{2+} efflux. The activation by [γ-S]ATP: (1) requires the presence of Mg_i^{2+}, (2) is partially reversible upon removing the analog, (3) is greater than that caused by ATP and (4) only operates on the exchange system since no activation of the ATP-dependent uncoupled Ca^{2+} efflux (Ca^{2+} pump) can be detected. $^{22}Na^+$ experiments were used to monitor the Ca_o-dependent Na^+ efflux (reverse Na^+-Ca^{2+} exchange). Without Ca_i^{2+} and ATP, Na^+ efflux is very small ('leak'). [γ-S]ATP does not activate the efflux of Na^+ in the absence of Ca_i^{2+}. In the presence of Ca_i^{2+} the ATP analog stimulates both the Ca_o- and Na_o-dependent Na^+ efflux components. Interestingly, neither the Na^+ pump, Ca_i^{2+}-independent Na^+-Na^+ exchange, Na_i^+-Mg_i^{2+} exchange or $Na^+/K^+/Cl^-$ cotransport are affected by [γ-S]ATP. The experiments indicate that a Ca_i^{2+}-dependent phosphorylation occurs during the activation of the Na^+-Ca^{2+} exchange by ATP.

Introduction

Phosphorylation is an important cellular regulatory mechanism which has been implicated in the modulation of Ca^{2+} movements across cell membranes including Ca^{2+} channels [1] and Ca^{2+}-pumping ATPases [2]. It is thought that many actions of Ca^{2+} result from activation of protein kinases followed by phosphorylation of specific target proteins [3,4]. Recent experimental evidences indicate that the Na^+-Ca^{2+} exchange mechanism is a complex process which shows transmembrane asymmetry [5] and may be subject to regulation by intracellular Ca^{2+} and ATP [5,6]. In nerve cells, ATP has been shown to stimulate the Na_o^+-dependent Ca^{2+} efflux (forward Na^+-Ca^{2+} exchange) [7,8] as well as the Na_i^+-dependent Ca^{2+} influx (reverse Na^+-Ca^{2+} exchange) [9]. The nucleotide increases the affinities of the external and internal exchange sites towards Na^+ and Ca^{2+} ions [7,8], however, there is no general agreement on the regulatory mechanism that con-

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trols these interactions. Whether ATP activates the exchanger allosterically or it is hydrolyzed during activation, is still something of a puzzle. Nevertheless, the fact that: (a) only hydrolyzable ATP analogs are able to active the exchange [10] and (b) Mg_i^{2+} ions are strictly required [6] suggest that a phosphorylation step occurs during activation of Ca^{2+} transport. In this article the hypothesis that ATP modulates the Na^+-Ca^{2+} exchange through phosphorylation has been tested by means of [γ-S]ATP, an analog of ATP that can act as a substrate for kinases but not for ATPases [11–13]. We now present evidences showing that: (1) in squid axons the ATP analog [γ-S]ATP activates both the forward (Na_o^+-Ca_i^{2+}), reverse (Na_i^+-Ca_o^{2+}), Na_o^+-Na_i^+ and Ca_o^{2+}-Ca_i^{2+} exchange reactions of the countertransport system; the activation is greater than that induced by ATP alone; (2) activation requires Ca_i^{2+} ions and (3) activation is highly specific for the Na^+-Ca^{2+} exchange mechanism since neither the Na^+/K^+ pump, Ca^{2+} pump, ATP-dependent Ca_i^{2+}-independent Na-Na^+ exchange [5,14], Na_i^+-Mg_o^{2+} exchange [15] nor $Na^+/K^+/Cl^-$ cotransport [16] are affected. These results bring evidence that the effects of ATP on Na^+-Ca^{2+} exchange are mediated by a Ca^{2+}-dependent protein kinase system responsible for the phosphorylation of the exchanger.

Methods

The experiments were carried out in giant axons from the squid *Loligo plei* at the Instituto Venezolano de Investigaciones Cientificas in Caracas, Venezuela and *Loligo pealei* at the Marine Biological Laboratory in Woods Hole, MA, U.S.A., using the internal dialysis technique to control the intracellular concentration of low molecular weight solutes and the voltage-clamp technique to control the membrane potential [17]. Washout of internal ATP was achieved using plastic dialysis capillaries with nominal molecular weight cutoff of 9000 (Spectrum LA, CA, U.S.A.).

Solutions. The artificial sea-water had the following composition (mM): K^+, 10, Na^+, 440; Mg^{2+}, 50; Ca^{2+}, 10; $Tris^+$, 10; Cl^-, 580; EDTA, 0.1. The osmolarity was 1000 mosM and the pH (17–18°C) 7.7. The removal of Na^+ and Ca^{2+} was compensated with equiosmolar amounts of Tris and Mg^{2+}, respectively. Ca^{2+}-free sea-water contained 0.5 mM free EGTA. In most of these experiments the external solutions had potassium cyanide (1 mM), tetrodotoxin (300 nM), ouabain (100 μM) and bumetanide (10 μM). The standard dialysis solution had the following composition (mM): K^+, 310; Na^+, 100; Mg^{2+}, 4; $Tris^+$, 30; Cl^-, 78; aspartate, 310; EGTA, 1–3. The osmolarity was adjusted to 990 mosM with glycine and the pH (17°C) to 7.3. The nominal ionized Ca^{2+} was calculated with a multiple-ligand computer program based on a dissociation constant of 0.15 μM for CaEGTA [18] and 1.4 mM for CaATP (De Weer, personal communication). Addition of [γ-S]ATP or ATP was always carried out at constnat Ca^{2+} and Mg^{2+}. ATP (vanadium-free) was obtained from Sigma as Tris salt, neutralized with Tris base and stored at −20°C as 250 mM solution. Adenosine 5′-*O*-(3-thiotriphosphate) was from Boehringer. All reagents were analytical grade. Radioactive solutions were made by adding solid [^{45}Ca]$CaCl_2$ (15–30 mCi/mg; New England Nuclear, Boston, MA) or solid [^{22}Na]NaCl (150 μCi/ml) directly to the internal dialysis medium. Radioactive samples were mixed with 5 ml scintillation solution and counted in a liquid scintillation counter.

Results

The effect of [γ-S]ATP on the components of the Ca^{2+} efflux

Fig. 1 shows an experiment in which the effect of [γ-S]ATP on Ca^{2+} efflux was explored in an axon containing 0.7 μM intracellular Ca^{2+}. To remove all nucleotides from the axoplasm prior to the addition of the ATP analog, the axon was predialyzed for 50 min with an standard dialysis medium containing no ATP. Under these conditions, the efflux of Ca^{2+} reached a steady value of 80 fmol · cm^{-2} · s^{-1}. Subsequent removal of Na_o^+ and Ca_o^{2+} brings the Ca^{2+} efflux to extremely low values. It should be pointed out that under the present experimental conditions more than 85% of the total Ca^{2+} efflux is dependent on external Na^+ [6]. Addition of 1 mM [γ-S]ATP to the dialysis medium causes a 4-fold increase in the level of the Ca^{2+} efflux. Again, the removal of Na_o^+ and Ca_o^{2+} brings the efflux of Ca^{2+} to very

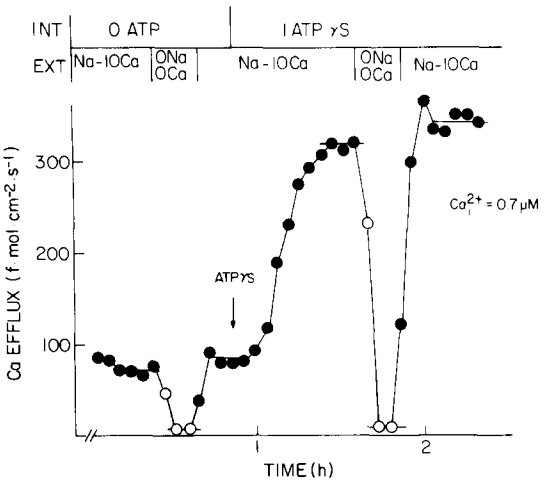

Fig. 1. Effect of [γ-S]ATP on the Na_o^+-dependent Ca^{2+} efflux from an axon of *Loligo plei*. Ordinate: Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. All external solutions contained 1 mM cyanide and 300 nM tetrodotoxin. The axon was voltage-clamped at its own resting potential. The arrow indicates the addition of 1 mM [γ-S]ATP. All concentrations are in millimolar unless otherwise stated. Closed circles refer to Ca^{2+} efflux into artificial seawater. Open circles refer to Ca^{2+} efflux into Na^+- and Ca^{2+}-free seawater. Note that the activation of the Ca^{2+} efflux by the ATP analog is completely dependent on the presence of Na_o^+ and Ca_o^{2+}. No activation of the 'uncoupled' Ca^{2+} efflux component by the analog is observed. Axon diameter 400 μm.

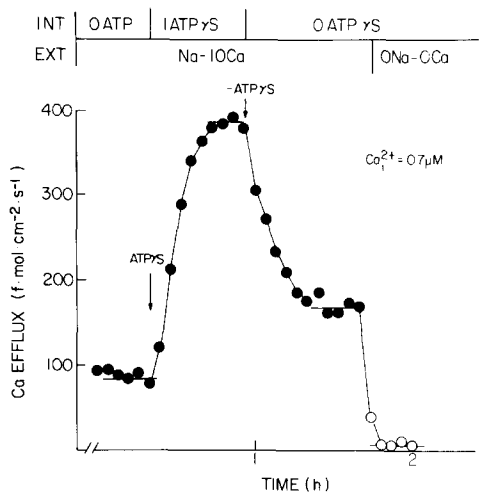

Fig. 2. Partial reversibility of the activating effect of [γ-S]ATP on the Ca^{2+} efflux. In this experiment the axon was dialyzed for 1 h without ATP prior to the addition of the ATP analog. Ordinate: Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Notice that [γ-S]ATP increases the Ca^{2+} efflux by almost 400%. Removal of the analog from the internal dialysis medium partially eliminates the activation of the Na_o^+-dependent Ca^{2+} efflux. Closed circles refer to Ca^{2+} efflux into artificial seawater. Open circles refer to Ca^{2+} efflux into Na^+- and Ca^{2+}-free seawater. Axon diameter 420 μm.

low values. The fact that the level of Ca^{2+} efflux in the absence of Na_o^+ and Ca_o^{2+} is not different from the 'leak' of Ca^{2+} present under these conditions [6], indicates that [γ-S]ATP is unable to activate the ATP-dependent uncoupled Ca^{2+} efflux component (Ca^{2+} pump). Similar to the Mg_i^{2+} requirement for the stimulation of the Na^+-Ca^{2+} exchange by ATP [6,10], no activation by this ATP analog was observed in the absence of Mg_i^{2+} ions (data not shown).

It has been shown that thiophosphorylation of myosin light chain by [γ-S]ATP is resistant to dephosphorylation by the phosphatase system [13]. This contrasts with the activation of the Na^+-Ca^{2+} exchange by [γ-S]ATP as is shown in Fig. 2. After activation of Ca^{2+} efflux by the ATP analog, its removal from the internal medium reverses the stimulating effect by 70%. In four other similar experiments approximately 60% of the activation of the Na^+-Ca^{2+} exchange by [γ-S]ATP could be eliminated upon the removal of the ATP analog from the dialysis medium. This partial reversibility might indicate that the thiophosphorylated Na^+/Ca^{2+} exchanger is not completely stable and/or that the phosphatase system present in the axon is capable of partially dephosphorylating the exchanger.

Since the thiophosphoester formed upon phosphorylation with [γ-S]ATP is hydroyzed slowly by phosphatases [11–13], then if the Na^+-Ca^{2+} exchange mechanism is modulated by a phosphorylation/dephosphorylation process, [γ-S]ATP should to be more effective in activating Na^+-Ca^{2+} exchange than ATP. Fig. 3a shows that this is the case since at saturating concentrations of Ca_i^{2+} (100 μM) and ATP (2 mM), the ATP analog is still capable of further activate the Na_o^+-dependent Ca^{2+} efflux. Similar to the result obtained in the experiment of Fig. 1, the [γ-S]ATP is rather specific in activating the Na_o^+-dependent Ca^{2+} efflux since no Ca^{2+} efflux component related to the Ca^{2+} pump can be detected upon removal of external Na^+ and Ca^{2+}. Fig. 3a also shows that [γ-S]ATP can activate the Ca^{2+}-Ca^{2+} exchange

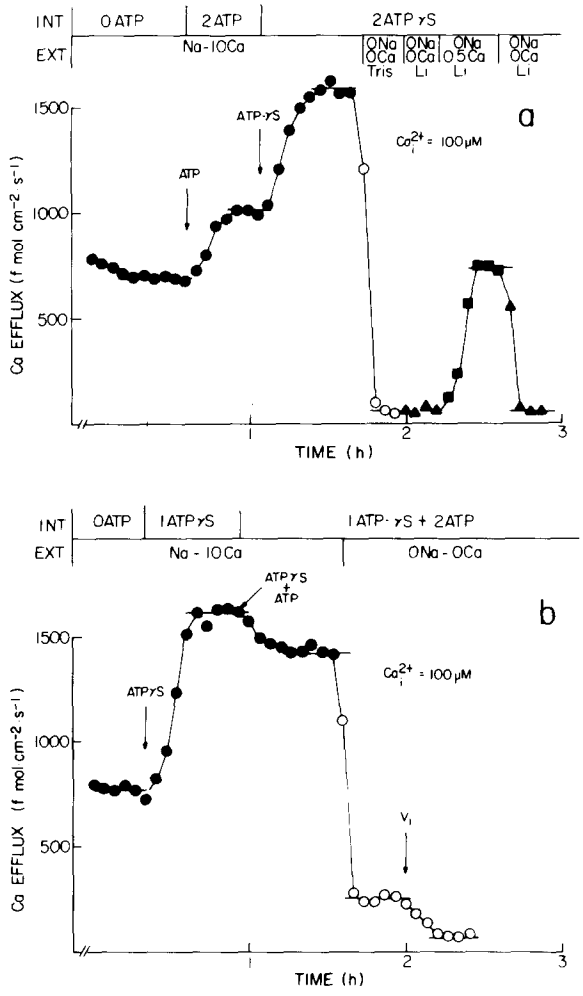

Fig. 3. (a) The effect of ATP and [γ-S]ATP on the Na_o^+- and Ca_o^{2+}-dependent Ca^{2+} efflux at a saturating Ca_i^{2+} concentration. Ordinate: Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. ATP at a saturating concentration (2 mM) causes a moderate activation in the Ca^{2+} efflux. Substitution of ATP by [γ-S]ATP causes a further increase in the Ca^{2+} efflux. Closed circles, efflux into artificial seawater. Open circles, efflux into Na^+ and Ca^{2+} free medium (Na^+ substituted by $Tris^+$). Triangles, efflux into Li^+- and Ca^{2+}-free medium. Squares, efflux into Li^+ and 0.5 mM Ca^{2+}. Note the absence of an uncoupled component of the Ca^{2+} efflux and the large activation of the Ca_o^{2+}-dependent Ca^{2+} efflux. All concentrations are in millimolar. Axon diameter 540 μm. (b) In this experiment the [γ-S]ATP analog was added to the dialysis medium before ATP, causing a large increment in Ca^{2+} efflux. Subsequent addition of ATP in the presence of the analog decreases the [γ-S]ATP-stimulated fraction by 27%. Note the 'uncoupled' Ca^{2+} efflux component in the presence of ATP + [γ-S]ATP and its complete inhibition by 1 mM internal vanadate. Axon diameter 600 μm.

mode of the Na^+/Ca^{2+} exchanger since in the presence of external Li, a procedure which is known to promote Ca^{2+}-Ca^{2+} exchange [19], as much as 0.5 mM Ca_o^{2+} is capable of inducing a large increase in the Ca_o^{2+}-dependent Ca^{2+} efflux. The magnitude of the Ca_o^{2+}-dependent Ca^{2+} efflux component under the above experimental conditions is significantly greater in the presence of [γ-S]ATP than in its absence (data not shown). Fig. 3b shows that if ATP is added to an axon in which the efflux of Ca^{2+} has already been stimulated by [γ-S]ATP, no further activation occurs, but instead a decrease in the steady-state Ca^{2+} efflux level is observed. This behavior, which was consistently seen in other axons, indicates that the Na^+-Ca^{2+} exchange activity is greater in the presence of ATP. The experiment of Fig. 3b also shows that in the presence of both ATP and [γ-S]ATP in the dialysis medium, removal of external Na^+ and Ca^{2+} brings the Ca^{2+} efflux to about 250 fmol·cm^{-2}·s^{-1}, a value within the normal range for the ATP-dependent uncoupled Ca^{2+} efflux at saturating $[Ca_i^{2+}]$ [6]. At the end of the experiment, 1 mM vanadate was added to the internal dialysis medium causing a complete inhibition of the uncoupled Ca^{2+} efflux.

The effect of [γ-S]ATP on the components of the Na^+ efflux

Reversal of the Na^+-Ca^{2+} exchange in dialyzed squid axons is best studied by measuring the Ca_o^{2+}-dependent Na^+ efflux. This procedure has the advantage that any catalytic effect of Ca_i^{2+} can be unambiguously ascribed to Na_i^+-Ca_o^{2+} exchange and not to Ca_i^{2+}-Ca_o^{2+} exchange [5]. If the 'reversal' of the Na^+-Ca^{2+} exchange (Na_i^+-Ca_o^{2+} exchange) is part of the same countertransport system, then in principle it should also be stimulated by [γ-S]ATP. Fig. 4 shows an experiment in which $^{22}Na^+$ efflux was used to monitor Ca_o^{2+}-dependent Na^+ efflux. In order to eliminate the Na^+ efflux components due to the Na^+/K^+ pump [14] and $Na^+/K^+/Cl^-$ cotransport [16], ouabain (100 μM) and bumetanide (10 μM) were added to all external solutions. In the absence of Ca_i^{2+} and ATP, the level of the Na^+ efflux is very small (less than 1 pmol·cm^{-2}·s^{-1}; 'leak' value [14]) even in the absence of Na_o^+ and in the presence of 10 mM Ca_o^{2+}, thus confirming previous findings that the

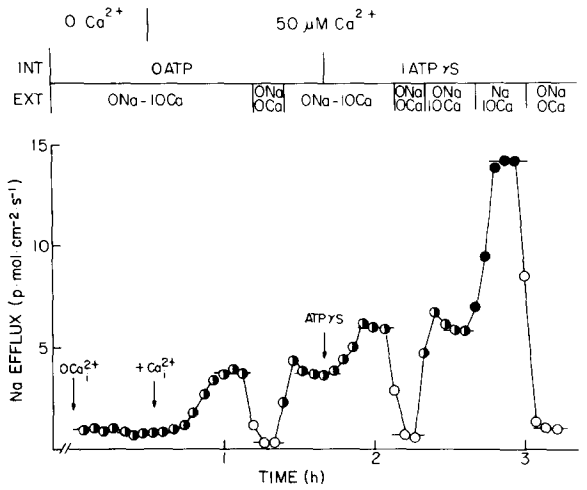

Fig. 4. The effect of [γ-S]ATP on the Ca_o^{2+}-dependent ('reversal') and Na_o^+-dependent Na^+ efflux in the presence of internal Ca^{2+}. Ordinate: Na^+ efflux in pmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Closed circles, Na^+ efflux into artificial seawater. Half filled circles, Na^+ efflux into Na^+-free Ca^{2+}-containing medium. Open circles, Na^+ efflux into Na^+- and Ca^{2+}-free medium. In the absence of ATP and Ca^{2+}, Na^+ efflux into Na^+-free seawater containing 10 mM Ca^{2+} is less than 1 pmol·cm^{-2}·s^{-1}. Ca_i^{2+} activates a Ca_o^{2+}-dependent Na^+ efflux which is further activated by [γ-S]ATP. Notice the large activation of the Na_o^+-dependent Na^+ efflux component by the [γ-S]ATP in the presence of Ca_i^{2+}. All concentrations are in millimolar except Ca^{2+} which is in micromolar. Axon diameter 550 μm.

reverse mode of the Na^+-Ca^{2+} exchange requires micromolar amounts of Ca_i^{2+} for activation [6]. Changing to an internal medium containing 50 μM Ca_i^{2+} causes an increase in the Na^+ efflux which is totally dependent on the presence of external Ca^{2+} since its removal brings the Na^+ efflux again to less than 1 pmol · cm^{-2} · s^{-1}. After reactivating the Ca_o^{2+}-dependent Na^+ efflux component by the addition of 10 mM Ca_o^{2+} (in the absence of Na_o^+), 1 mM internal [γ-S]ATP causes an increase in the steady-state level of the Na^+ efflux. This stimulation by [γ-S]ATP is entirely Ca_o^{2+}-dependent since the removal of Ca_o^{2+} reduces the efflux of Na^+ to very low levels. Furthermore, Fig. 4 shows that [γ-S]ATP also promotes a large Na_o^+-dependent Na^+efflux since addition of Na_o^+ causes the efflux of Na^+ to increase from 6 pmol · cm^{-2} · s^{-1} to about 15 pmol · cm^{-2} · s^{-1}. This Na_o^+-dependent Na^+ efflux observed in the presence of Ca_i^{2+} has recently been suggested in squid axons to be a mode of operation of the Na^+-Ca^{2+} exchange [5] (Na_i^+-Na_o^+ exchange mechanism).

From the experiments of Figs. 1 and 2 it can be concluded that [γ-S]ATP activates the component of the Ca^{2+} efflux that occurs through the Na^+-Ca^{2+} exchange mechanism and not through the ATP-dependent Ca^{2+} pump. An interesting question is how selective is this ATP analog with respect to other known components of the Na^+ efflux (Na^+/K^+ pump, Na^+/Mg^{2+} exchange, $Na^+/Cl^-/K^+$ cotransport, and ATP-dependent Ca_i^{2+}-independent Na^+-Na^+ exchange). Fig. 5 shows that in an axon dialyzed with a standard internal solution containing no Ca_i^{2+} and bathed in artificial seawater containing no ouabain or bumetanide, addition of 1 mM [γ-S]ATP to the dialysis medium causes no change in the level of the Na^+ efflux. However, when internal Ca^{2+} (50 μM) is added to the dialysis medium, [γ-S]ATP is able to activate both a Ca_o^{2+}- and Na_o^+-dependent Na^+ efflux components. Interestingly, the activa-

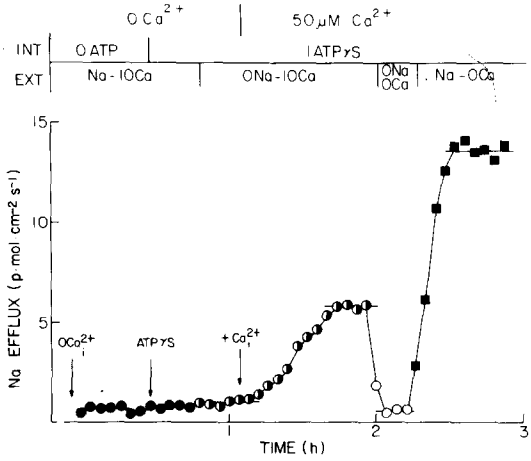

Fig. 5. The effect of [γ-S]ATP on the Na^+ efflux in the absence of internal Ca^{2+}. Ordinate: Na efflux in pmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Closed circles, Na^+ efflux into artificial seawater. Half filled circles, Na^+ efflux into Na^+-free Ca^{2+}-containing medium. Open circles, Na^+ efflux into Na^+- and Ca^{2+}-free medium. Squares, Na^+ efflux into Ca^{2+}-free Na^+-containing medium. In this experiment, no ouabain or bumetanide were included in the external solution. Observe the lack of effect of the [γ-S]ATP on the Na^+ efflux in the absence of Ca_i^{2+} and the activation of the Ca_o^{2+} and Na_o^+-dependent components upon addition of Ca_i^{2+}. Unless otherwise stated, all concentrations are in millimolar. Axon diameter 625 μm.

tion of the Na_o^+-Na_i^+ exchange by the analog was always greater than that of the Ca_o^{2+}-Na_i^+ exchange component.

Discussion

The apparent reversibility of the Na^+-Ca^{2+} exchange reported in intact cells as well as in membrane vesicle preparations has lead to the notion that Ca^{2+} and Na^+ movements in either direction are the manifestation of a simple symmetrical process. However, recent work in squid axons indicates that the Na^+-Ca^{2+} exchange mechanism is far from a simple symmetrical carrier system being a complex mechanism subject to modulation by intracellular ligands [5,6]. In squid axons, two such ligands are known to induce profound changes in the kinetics of the Na^+-Ca^{2+} exchange mechanism: (a) Ca_i^{2+} is known to be required for the activation of Ca^{2+} entry in exchange for internal Na^+ (reverse Na^+-Ca^{2+} exchange; catalytic effect) and for the activation of the Na_o^+-dependent Na^+ efflux component [6,20], and (b) intracellular ATP is known to activate the Na_o^+-dependent Ca^{2+} efflux [7,8], Na_i^+-dependent Ca^{2+} influx [9], Ca_o^+-dependent Na^+ efflux [5] and the Na_o^+-dependent Na^+ efflux [5]. The kinetic effect of ATP seems to be a change in the affinities of the external and internal transport sites of the carrier system to Na^+ and Ca^{2+} ions [7,8,19]. Although important information has been obtained in isolated membrane vesicles [21–23] it appears that these preparations do not exhibit some of the regulatory mechanisms found in intact preparations. Nevertheless, Caroni and Carafoli [24] have recently reported in isolated sarcolemma vesicles that the exchange mechanism can be modified by ATP by a calmodulin-dependent kinase-mediated phosphorylation step. The authors also suggest the presence of a phosphatase system responsible for the dephosphorylation of the Na^+-Ca^{2+} exchange.

In squid axons, the experimental evidence on the effect of ATP suggest that the nucleotide might be hydrolyzed during activation of the Na^+-Ca^{2+} exchange. The evidences are based on two facts: (1) Only hydrolyzable ATP analogs can sustain an activation of the Na^+-Ca^{2+} exchange. Nonhydrolyzable ATP analogs displace ATP from its activatory site causing an inhibition in the ATP stimulated Na^+-Ca^{2+} exchange [10]. (2) No effect of ATP is observed in the absence of internal Mg^{2+} ions [6,10], thus suggesting that MgATP is the substrate for the activation of the Na^+-Ca^{2+} exchange as is the case of several enzymatic reactions in which a phosphorylating step is involved.

We have used the ATP analog [γ-S]ATP which can be used to thiophosphorylate proteins by protein kinases but not by ATPases [11,13], to test the hypothesis that in squid axons the effect of ATP is controlled through a Ca_i^{2+}-sensitive phosphorylation. The data presented in Figs. 1, 2 and 3 show the effect of [γ-S]ATP on the components of the Ca^{2+} efflux and demonstrate that the analog induces a marked increase in the Ca^{2+} efflux which is completley dependent on Na_o^+. Several interesting points emerge from the effect of [γ-S]ATP on Ca^{2+} efflux: (a) The stimulation of the Na_o^+-dependent Ca^{2+} efflux by the ATP analog is much greater than that produced by a similar concentration of ATP. In fact, in axons dialyzed with 0.7 μM Ca_i^{2+} and 2 mM ATP, Na_o^+-dependent Ca^{2+} efflux amounts to about 150 $fmol \cdot cm^{-2}s^{-1}$ (see Ref. 6, Fig. 3) as compared to 300 $fmol \cdot cm^{-2} \cdot s^{-1}$ in the presence of [γ-S]ATP (see Fig. 1). A possible explanation for this difference is that since the thiophosphoester formed upon phosphorylation with [γ-S]ATP is hydrolyzed slowly by phosphatases [12,13], then at a given time more carrier molecules will be phosphorylated by [γ-S]ATP than by ATP. In line with this idea is the finding that addition of [γ-S]ATP to an axon which has been already activated by a saturating concentration of ATP causes a further increase in the Na_o^+-dependent Ca^{2+} efflux (see Fig. 3a). (b) The [γ-S]ATP is not a substrate for the Ca^{2+} pump since no 'uncoupled' Ca^{2+} efflux component could be detected even in the presence of millimolar amounts of the ATP analog (Fig. 1). The unability of [γ-S]ATP to activate the ATP-dependent uncoupled Ca^{2+} efflux can not be ascribed to an inhibition of the Ca^{2+} pump by the analog since as seen in Fig. 3 an uncoupled Ca^{2+} efflux of normal magnitude is observe in the simultaneous presence of [γ-S]ATP and ATP. This fnding is in line with the idea that [γ-S]ATP is substrate for kinases but not for ATPases. (c) An interesting result is the fact that in squid axons the activation

of the Na^+-Ca^{2+} exchanged by [γ-S]ATP is partially reversible (60%) upon removal of the analog from the dialysis medium (see (Fig. 2). This finding contrasts with the irreversible thiophosphorylation of smooth muscle myosin light chain [13] and the irreversible activation of the sarcolemma Na^+/Ca^{2+} exchanger by [γ-S]ATP [24]. The partial reversibility observed in the present experiments could indicate that the thiophosphoester form is less stable or that the phosphatase system present in squid axons is able to dephosphorylate the thioester. (d) Finally, the present experiments show that [γ-S]ATP not only activates the Na_o^+-dependent Ca^{2+} efflux component but also the Ca_i^{2+}-dependent one, an indication that the Ca^{2+}-Ca^{2+} exchange constitutes one of the partial reactions of the Na^+-Ca^{2+} exchange mechanism.

One interesting aspect of the present experiments is the activation of the reverse mode of the Na^+-Ca^{2+} exchange by the ATP analog [γ-S]ATP. The experiment of Fig. 4 confirms previous observations that Ca^{2+} ions from the cytoplasmic side of the membrane are required for the activation of the reverse mode of the Na^+-Ca^{2+} exchange. The ATP analog further stimulates the Ca_i^{2+}-activated Ca_o^{2+}-dependent Na^+ efflux component, thus demonstrating that phosphorylation of the exchange system also affects the reverse reaction of the Na^+-Ca^{2+} exchange mechanism. We have recently postulated that the Ca_i^{2+}-activated Na_i^+-dependent Na^+ efflux is a mode of operation of the Na^+-Ca^{2+} exchange (Na^+-Na^+ exchange) [5]. The activation of this component by [γ-S]ATP is a strong indication that Na^+-Na^+ exchange is one of the partial reactions of the Na^+-Ca^{2+} exchange mechanism. As is the case for the ATP activation of the Na^+ efflux components through the Na^+-Ca^{2+} exchange mechanism, the activation by the ATP analog was found to be always greater for the Na_i^+-Na_o^+ exchange than for the Na_i^+-Ca_o^{2+} exchange component (see Figs. 4 and 5) [5].

Finally, two important points emerge from the data of Fig. 5. First, the absolute requirement of internal ionized calcium for the activation of the reverse Na^+-Ca^{2+} exchange by [γ-S]ATP. This finding is a demonstration that phosphorylation of the Na^+-Ca^{2+} exchange mechanism is mediated by a Ca_i^{2+}-dependent system, most probably a Ca_i^{2+}-activated protein kinase; and second, the high specificity of this ATP analog in activating only those Na^+ fluxes which occurs through the Na^+-Ca^{2+} exchange mechanism. In fact, in the experiemnt of Fig. 5 in which neither ouabain nor bumetanide were added to the external solutions, the analog does not activate the Na^+/K^+ pump nor the $Na^+/K^+/Cl^-$ cotransport. Moreover, the [γ-S]ATP is also unable to activate the reported Ca_i^{2+}-independent, ATP-stimulated, Na_o^+-dependent Na^+ efflux [14], strongly suggesting that the Ca_i^{2+}-independent ATP dependent Na^+-Na^+ exchange and the Na^+-Ca^{2+} exchange are different systems.

In conclusion, our data constitute evidence that a phosphorylation step is involved in the stimulation of the Na^+-Ca^{2+} exchange by ATP. Moreover, the requirement of internal ionized calcium for the analog effect, is indicative that a Ca_i^{2+}-dependent protein kinase is involved in the thiophosphorylation of the exchange system. Our experiments do not show that a phosphatase system is affecting the exchange mechanism. However, the greater effectiveness of [γ-S]ATP over ATP in activating the exchange is consistent with a phosphatase system modulating the Na^+-Ca^{2+} exchange. Finally, the specificity of [γ-S]ATP in activating all modes of operation of the Na^+-Ca^{2+} exchange without apparently affecting other known Ca^{2+} and Na^+ transport, may be of use in the isolation and characterization of the Na^+-Ca^+ exchange mechanism.

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