ATP stimulation of Na\(^+\)/Ca\(^{2+}\) exchange in cardiac sarclemmal vesicles

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Berberián, Graciela, Cecilia Hidalgo, Reinaldo Dípolo, and Luis Beauge´. ATP stimulation of Na\(^+\)/Ca\(^{2+}\) exchange in cardioc sarclemmal vesicles. Am. J. Physiol. 274 (Cell Physiol. 43): C724–C733, 1998.—In cardiac sarclemmal vesicles, MgATP stimulates Na\(^+\)/Ca\(^{2+}\) exchange with the following characteristics: 1) increases 10-fold the apparent affinity for cytosolic Ca\(^{2+}\); 2) a Michaelis constant for ATP of \(\sim 500 \mu\text{M}\); 3) requires micromolar vanadate while millimolar concentrations are inhibitory; 4) not observed in the presence of 20 \(\mu\text{M}\) eosin alone but reinstated when vanadate is added; 5) mimicked by adenosine 5’-O-(3-thiotriphosphate), without the need for vanadate, but not by \(\beta,\gamma\)-methyleneadenosine 5’-triphosphate; and 6) not affected by unspecific protein alkaline phosphatase but abolished by a phosphatidylinositol-specific phospholipase C (PI-PLC). The PI-PLC effect is counteracted by phosphatidylinositol. In addition, in the absence of ATP, \(\Lambda\alpha\)-phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) was able to stimulate the exchanger activity in vesicles pretreated with PI-PLC. This MgATP stimulation is not related to phosphorylation of the carrier, whereas phosphorylation appeared in the phosphoinositides, mainly PIP\(_2\), that communoprecipitate with the exchanger. Vesicles incubated with MgATP and no Ca\(^{2+}\) show a marked synthesis of \(\Lambda\alpha\)-phosphatidylinositol 4-monophosphate (PIP) with little production of PIP\(_2\); in the presence of 1 \(\mu\text{M}\) Ca\(^{2+}\), the net synthesis of PIP\(_2\) is smaller, whereas that of PIP\(_2\) increases ninefold. These results indicate that PIP\(_2\) is involved in the MgATP stimulation of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger through a fast phosphorylation chain: a Ca\(^{2+}\)-dependent PIP formation followed by a Ca\(^{2+}\)-dependent synthesis of PIP\(_2\).

phosphorylation; phosphoinositides; membrane transport

In intact cells, the Na\(^+\)/Ca\(^{2+}\) exchanger is modulated by MgATP (2, 6, 13, 24). The nucleotide stimulates all partial reactions of the carrier (Na\(^+\)/Ca\(^{2+}\), Na\(^+\)/Ca\(^{2+}\), Ca\(^{2+}\)/Na\(^+\), and Ca\(^{2+}\)/Ca\(^{2+}\) exchanges, where subscripts 0 and i refer to extracellular and intracellular, respectively). Among the kinetics effects, there is an increase in the apparent affinity of the transporting sites for Na\(^+\) and Ca\(^{2+}\) (6) as well as that of the regulatory site for intracellular Ca\(^{2+}\) (14). Most of the kinetic data on the MgATP stimulation of Na\(^+\)/Ca\(^{2+}\) exchange by ATP comes from work in injected and dialyzed squid axons. In fact, until recently, this effect was difficult to demonstrate in isolated membrane vesicles. Thus, in cardiac sarclemmal vesicles, an early reported MgATP activation (9) was not seen by others (see Refs. 35, 37), perhaps because some component that regulates the exchanger is modified or lost during membrane isolation (35). However, recent work (11) has shown that in giant membrane patches excised from cardiac myocytes MgATP does activate the Na\(^+\)/Ca\(^{2+}\) exchange current. That finding encouraged us to reinvestigate the MgATP actions in cardiac sarclemmal vesicles. In addition, and with the consideration of all preparations being used, the mechanism(s) of this MgATP regulation is still under debate. Enzyme-catalyzed reactions at phosphoryl centers are numerous, in particular those involving ATP (active transport, muscle contraction, oxidative phosphorylation, and photosynthesis). Phosphorylation reactions are also involved in important modulations by protein kinases and phosphatases. Experiments in dialyzed axons using metal(III)ATP complexes (17), nonhydrolyzable ATP analogs (13), and vanadate (19) favor a phosphorylation-dephosphorylation mechanism (such as a kinase-phosphatase system; see Ref. 15). On the other hand, initial experiments on giant excised patches from cardiac myocytes suggested that changes in membrane phospholipid distribution, caused by a MgATP-dependent amino phospholipid translocase (flippase), could be responsible for the MgATP effect (11). A basic unresolved question is whether the exchanger is phosphorylated. Several attempts to find phosphorylation of the carrier by MgATP have been unsuccessful (1), including the dog cardiac antiporter expressed in COS cells (12). On the other hand, while this work was in preparation, a series of experiments indicated at least two possible ways to account for the MgATP stimulation. On the one hand, a direct phosphorylation of the exchanger, through a direct or indirect action of the protein kinase C (PKC), that correlates with the transport rate was shown in cultured smooth muscle (27). On the other hand, the observed stimulation seen in giant excised patches from cardiac myocytes disappeared after pretreatment with a phospholipase C specific for phosphatidylinositol (PI-PLC), while \(\Lambda\alpha\)-phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) mimicked the MgATP stimulation (25). The results presented here indicate that, in cardiac sarclemmal vesicles, MgATP stimulation of Na\(^+\)/Ca\(^{2+}\) exchange involves the synthesis of PIP\(_2\) from \(\Lambda\alpha\)-phosphatidylinositol (PI) through a fast phosphorylation chain: a Ca\(^{2+}\)-independent formation of \(\Lambda\alpha\)-phosphatidylinositol 4-monophosphate (PIP) followed by a Ca\(^{2+}\)-dependent synthesis of PIP\(_2\). Conversely, phosphorylation of the carrier itself was not detected. An additional important conclusion is that all structures and enzymes responsible for MgATP stimulation are membrane bound and copurify with the exchange activity. Finally, although the actual mechanism is not known, PIP\(_2\) stimulation seems to require
an intimate association with the carrier, since the newly synthesized phosphoinositides and the exchanger communoprecipitate. Parts of this work have already been presented in abstract form (3, 5).

METHODS

Preparation of cardiac membrane vesicles. Cardiac membrane vesicles were prepared by differential centrifugation from beef heart obtained immediately after killing the animal (36). The vesicles were loaded in a solution of 160 mM NaCl, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), and 0.1 mM EDTA; aliquots were stored in the same solution (2–3 mg protein/ml) at −80°C. The orientation of the vesicles was estimated using the asymmetric properties of the Na+/K+ ATPase. Na+/K+ ATPase activity was measured after 30-min preincubation of membranes (0.5 mg/ml) at 20°C in 20 mM MOPS-Tris, pH 7.1, with or without sodium dodecyl sulfate (SDS) (0.3 mg/ml). The basic composition of the assay medium was (in mM) 130 NaCl, 20 KCl, 30 MOPS-Tris (pH 7.4), 3 MgCl2, and 3 ATP ([γ-32P]ATP). The vesicles (data not shown) were usually 40% inside out, 35% right-side out, and 25% leaky (see also Ref. 9). Total protein was measured by the methods of Lowry et al. (32) with modifications (34) using bovine serum albumin as standard.

Na+ gradient-dependent Ca2+ uptake. The Ca2+ uptake was estimated at 37°C by using [35]CaCl2. In routine assays, 2 µl of membranes were diluted in 100 µl of the uptake solutions. These solutions had either low (10 mM) or high (160 mM) Na+. Low Na+ had the equivalent to 260 mosmol N-methylglucamine chloride (NMG-Cl) or bis-tris-propane-Cl (BTP). The concentration of external ionized Mg2+ was kept constant at 5 mM. In addition, the usual extravesicular solutions contained 0.5 mM vanadate and a final Ca2+ concentration (Ca2+2) of 0.8–1.0 µM. In some cases, vanadate was absent, and in others, 20 µM eosin, a Ca2+ pump inhibitor (22), was added. The composition of other solutions used is given in the table and figure legends. The reaction was terminated after 10 s (except in the time-dependent experiments) by adding 1 ml of ice-cold quenching medium [200 mM KCl, 1 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetate acid (EGTA), and 5 mM MOPS-Tris (pH 7.4)]. The vesicles were then harvested by filtration on GF/F (Whatman) glass-fiber filters. To correct for unspecific [35]Ca2+ bound to vesicles and filters, blanks were run in which vesicles were diluted into 160 mM NaCl-20 mM MOPS-Tris, pH 7.4, containing the same concentration of Ca2+ as the assay medium. Experimental points are the means of duplicate or triplicate determinations, and each experiment was repeated at least twice. Because most of the experiments consumed a large amount of vesicles, in many cases different designs correspond to different vesicle preparations; this may well explain the observed variability in the Na+/Ca2+ exchange flux values (see Ref. 36). More details are given in the corresponding figure and table legends.

Phosphorylation of cardiac membrane vesicles. Vesicles (80–100 µg) were incubated for 10 s at 37°C in a media with composition similar to the uptake solutions [20 mM MOPS-Tris (pH 7.4), 20 mM NaCl (osmolarity was matched with NMG-Cl), 0.1 mM EGTA, 0.3 mM vanadate, 0.25 or 0.5 mM [γ-32P]ATP (500 counts·min⁻¹·pmol⁻¹), 3 mM Mg2+, and 0.8 µM Ca2+]. The methods of stopping the reaction depended on the final assay that was programmed: 1) for immunoprecipitation studies, it was done by dilution (1:100, vol/vol) in an ice-cold solution containing 160 mM NaCl, 20 mM MOPS-Tris (pH 7.4), 10 mM EDTA, 1 mM EGTA, 0.5 mM vanadate plus 0.25% Nonidet P-40, 0.5% Triton X-100, and antiprotease cocktail [1 µg/ml leupeptin, pepstatin, and aprotinin and 0.2 mM phenylmethylsulfonyl fluoride (PMSEF)]; and 2) for lipids, extraction by adding 1 ml ice-cold HCl and then 2 ml of an ice-cold mixture of 1:1 (vol/vol) chloroform-methanol.

Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and immunoblotting. Samples of cardiac membrane vesicles (80–100 µg protein) were diluted 1:100 (vol/vol) in an ice-cold solution containing 160 mM NaCl, 20 mM MOPS-Tris (pH 7.4), 10 mM EDTA, 1 mM EGTA, 0.5 mM vanadate plus 0.25% Nonidet P-40, 0.5% Triton X-100, and antiprotease cocktail (1 µg/ml leupeptin, pepstatin, and aprotinin and 0.2 mM PMSEF). After 10 min at 0°C, the suspension was sonicated for 40 s and centrifuged in an Airfuge centrifuge for 5 min. After centrifugation, the supernatant was incubated for 3 h at room temperature with a 300-fold dilution of the guinea pig serum (negative controls were run in parallel with nonimmune guinea pig serum). Protein A-agarose (50 µl/ml, from ICN Biomedicals) was then added, and after an additional 1-h incubation at room temperature, the suspension was centrifuged and the pellet was rinsed three times by repeated centrifugation and resuspension. The immunoprecipitated proteins were eluted by adding 5 µl of five times concentrated SDS sample buffer, were heated for 30 min at 37°C, and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) or to organic extraction for thin-layer chromatography (TLC) separation of the lipids that coprecipitated. Discontinuous SDS-PAGE was carried out according to the method described by Laemmli (29). The proteins in the gel were visualized by Coomassie blue R-250 staining and/or autoradiograph (radiolabeled samples) or electrottransferred to polyvinylidene difluoride (PVDF) transfer membranes for Western blots. Guinea pig antibodies against the NH2-terminal portion of the bovine cardiac Na+/Ca2+ exchange (anti-NCX antiserum) (1) and anti-guinea pig immunoglobulin G-alkaline phosphatase conjugate (Sigma A.7686) were used. For Western blots, the antibody was first adsorbed with nonimmune guinea pig serum immobilized in PVDF membranes. The molecular mass standards were Gibco prestained high molecular weight range.

Extraction and TLC separation of phospholipids. Phospholipids were extracted from cardiac membrane vesicles that had been incubated with [γ-32P]ATP and the uptake solution without or with Ca2+. Once the reaction was stopped with 1 ml of ice-cold 1 N HCl, the lipids were extracted by adding 2 ml of an ice-cold chloroform-methanol mixture (1:1, vol/vol). The suspension was vortexed for 30 s, and the tubes were placed in ice for ~30 min. The organic phase containing the lipids was carefully removed, poured into conical tubes, and dried under a stream of N2 gas while the tubes remained in water at room temperature. A second extraction was not used because initial results showed that it added minimal amount of additional material. The dried lipids were redissolved in 10 µl of chloroform-methanol (1:1, vol/vol) that were then applied in spots of ~3 mm diameter on high-efficiency TLC plates (Analtech, Newark, DE). This procedure was repeated with another 10 µl of the same solution. The plates (20 cm × 10 cm) were preactivated at 140°C for 1 h. The composition of solvent system was methanol-chloroform-water-concentrated NH4OH (48:40:10:5, vol/vol) (21). The 32P-labeled phospholipids were visualized in an autoradiograph of the plates. The identification of phosphatidylinositol monol- and bisphospho- phate was done by comigrating commercial standards and submitting the plates to an atmosphere of saturated iodine vapor. The places where the labeled spots were localized were removed and counted in a liquid scintillation counter.
Solutions. All solutions were made with deionized ultra-pure water (18Ω water, Milli-Q, Millipore). NaCl and KCl were from Baker Uiltrex; all other chemicals were reagent grade. ATP, adenosine 5′-O-(3-thiotriphosphate) (ATPγS), and β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), from Boehringer Mannheim, were transformed into Tris salts by passing them through an Amberlite IR-120-P column. Ouabain, digitoxigenin, Tris-OH, NMG, MOPS, BTP, alkaline phosphatase (type VII-NLA from bovine intestinal mucus), PI-PLC (from Bacillus cereus), PI, and PIP2 were obtained from Sigma Chemical. 45Ca, as chloride salt, and [γ-32P]ATP were purchased from New England Nuclear. Calculations of free [Mg2+] were done taking a dissociation constant (Kd) of 0.091 mM for MgATP (7) and 0.159 mM for MgAMP-PCP (38). The Kd for MgATPγS was assumed to be equal to that of ATP. Free [Ca2+] were estimated by means of the MaxChela-tor program from Chris Patton taking the same Kd values for ATP and ATPγS.

RESULTS

Time dependence, Ca2+ ionophore, and exchange inhibitory peptide effects on the Na+ gradient-dependent Ca2+ uptake in the absence and presence of ATP. Two points had to be first established without ambiguity: 1) that the method for measuring Ca2+ uptake indeed estimated the 45Ca2+ that went into the vesicles, and 2) that our definition of Ca2+ fluxes through the Na+/Ca2+ exchanger, as the Na+ gradient-dependent Ca2+ uptake, was correct. The first point is answered in the experiments illustrated in Fig. 1, where the Na+ gradient-dependent influx of Ca2+ was measured as a function of time in the absence (A) and presence (B) of 1 mM ATP. In both cases, the other components of the incubation solutions were identical, among them 1 µM free Ca2+ and 5 mM free Mg2+. The uptake of Ca2+ can be considered linear at 10 s in both conditions; therefore, that time was used in most assays. In addition, it is clear that the uptake in the presence of ATP doubles that observed in its absence, i.e., there is an ATP stimulation of Ca2+ influx in these vesicles. That the observed fluxes are indeed such and not merely Ca2+ absorbed to some extravascular matrix (9, 36) is shown by the fact that the addition of 30 µM (final concentration) of the Ca2+ ionophore A-23187 produces a release of all radioactivity present in the vesicles, and that is seen without and with ATP. Notice that to inhibit the cardiac Ca2+ pump vanadate was always present (300-500 µM) in the extravascular solution. We also performed similar experiments but adding 20 µg oligomycin/mg total protein to the incubation solutions. This oligomycin concentration was more than enough to completely inhibit mitochondrial Ca2+-ATPase (8). The results (not shown) were identical to those of Fig. 1, A and B, thus ruling out artifacts due to contamination with mitochondrial fragments.

To test for the specificity of these fluxes as expression of Na+/Ca2+ exchange activity, we used the exchange inhibitory peptide (XIP). This peptide inhibits the exchanger by interacting with a small domain in the inner large “regulatory” loop of the protein (31). In Table 1, it can be seen that at 20 µM concentration XIP inhibits the Na+ gradient-dependent Ca2+ uptake. There are some characteristics of that inhibition that are interesting: the inhibition is not total, it increases when the vesicles are preincubated with XIP, and it is more pronounced in the absence of ATP. A partial inhibition of the Na+/Ca2+ exchanger by XIP has already been reported for cardiac vesicles (28) and for dialyzed squid axons (18), as a difference with our preparation, in the squid axons the inhibition was the same without and with ATP (18). In that preparation, XIP had to be injected; therefore, the actual concentration inside the axon was not known. Given the fact that in cardiac vesicles there was an increment in inhibition after preincubation, it is possible that the dose used here was submaximal. At any rate, and taken together, the results of this section indicate that the Na+ gradient-dependent Ca2+ uptake indeed represents Ca2+ flux through the Na+/Ca2+ countertransport system.
Table 1. Effect of XIP on a Na⁺ gradient-dependent Ca²⁺ uptake in bovine heart sarcolemmal vesicles in the absence and presence of ATP

<table>
<thead>
<tr>
<th>XIP, µM</th>
<th>ATP, mM</th>
<th>Na⁺ Gradient-Dependent Ca²⁺ Uptake, nmol mg⁻¹ s⁻¹</th>
<th>Total</th>
<th>ATP dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.40 ± 0.06</td>
<td>1.77 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>None</td>
<td>3.17 ± 0.09</td>
<td>0.54 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1.56 ± 0.10</td>
<td>1.02 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>10*</td>
<td>None</td>
<td>0.86 ± 0.09</td>
<td>0.65 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. ⁴⁵Ca²⁺ uptake was followed at 37°C in media with high (160 mM) or low (10 mM) NaCl, 20 mM MOPS-Tris (pH 7.4), 5 mM free Mg²⁺, 0.5 mM vanadate, 0.1 mM digitoxigenin, 0.1 mM EGTA, 1 µM Ca²⁺ without and with 10 µM exchange inhibitory peptide (XIP) and 1 mM ATP. Low Na⁺ solutions also had the equivalent of 260 mosmol N-methylglucamine chloride (NMG-Cl). *Vesicles were preincubated for 1 min with XIP in absence of ATP and Ca²⁺. For details see text.

Extravascular Ca²⁺ concentration and ATP effect on the Na⁺ gradient-dependent Ca²⁺ uptake. In intact cells, MgATP increases the affinity of the transporting and regulatory sites for intracellular Ca²⁺ (6). To investigate if this is the case in our preparation, we studied the variation of Na⁺ gradient-dependent ⁴⁵Ca²⁺ uptake as a function of the extravascular [Ca²⁺] in the absence and presence of ATP. Figure 2 summarizes the results of three different experiments showing that all ATP stimulation can be explained by an increase in the apparent affinity of intracellular sites for Ca²⁺. Actually, with the Michaelis constant for Ca²⁺ values curve fitted, they changed from 1.03 ± 0.08 µM in the absence of ATP to 0.12 ± 0.01 µM in its presence, i.e., ~10-fold decrease. In the experiments described above, we used NMG as the Na⁺ replacement. If BTP was used instead, the same results were obtained (data not shown), ruling out artifacts due to salt-dependent MgATP stimulation. On the other hand, the results shown in Fig. 2 may account for the variable results found by different authors in the sense that if [Ca²⁺] were nonlimiting, stimulation by MgATP would not be detected. In fact, lithium salts, commonly used as Na⁺ replacement, usually have Ca²⁺ as contaminant (for references, see Ref. 36).

Apparent affinity for ATP, reversibility, and nucleotide specificity for stimulation of Na⁺/Ca²⁺ exchange. Figure 3 summarizes three experiments showing that when ATP is added at the beginning of the uptake period (10 s), together with 1 µM Ca²⁺, it stimulates the exchanger following a hyperbolic curve with a Michaelis constant for ATP of 490 ± 34 µM (n = 3). However, in one case, the curve was not Michaelian, reaching a plateau at 1–2 mM ATP. In following up with the matter, we decided to investigate what was the response when the vesicles were preincubated for 10 s with ATP, in the absence and presence of Ca²⁺, before the uptake period. The results of these experiments are presented in Table 2. When the vesicles were preincubated with 1 µM ATP in the absence of Ca²⁺ and the nucleotide concentration is drastically reduced (to 26 µM) by dilution during the Ca²⁺ uptake period, there is no stimulation of the exchanger, i.e., the Na⁺ gradient-dependent influx of Ca²⁺ is not different from that observed without or with 26 µM ATP. However, a very interesting result is that preincubation with ATP and
ATP STIMULATION OF THE Na⁺/Ca²⁺ EXCHANGER

Table 2. Effects of preincubation with MgATP, in the absence and presence of 1 µM Ca²⁺, on ATP stimulation of a Na⁺ gradient-dependent Ca²⁺ uptake in bovine heart sarcolemmal vesicles

<table>
<thead>
<tr>
<th>[Ca²⁺] During Preincubation, µM</th>
<th>[ATP], mM</th>
<th>Na⁺ Gradient-Dependent Ca²⁺ Uptake, nmol·mg⁻¹·s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>Uptake</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>2.36 ± 0.33</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>2.57 ± 0.30</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.60 ± 0.20</td>
</tr>
<tr>
<td>0.01</td>
<td>0.1</td>
<td>0.77 ± 0.17</td>
</tr>
<tr>
<td>0.026</td>
<td>0.1</td>
<td>2.29 ± 0.29</td>
</tr>
<tr>
<td>0.003</td>
<td>0.1</td>
<td>2.04 ± 0.14</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>1.54 ± 0.08</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1.66 ± 0.18</td>
</tr>
<tr>
<td>0.5</td>
<td>0.015</td>
<td>2.29 ± 0.15</td>
</tr>
<tr>
<td>0.5</td>
<td>0.015</td>
<td>2.36 ± 0.28</td>
</tr>
<tr>
<td>0.5</td>
<td>0.015</td>
<td>0.45 ± 0.17</td>
</tr>
<tr>
<td>1</td>
<td>0.015</td>
<td>2.23 ± 0.21</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. Preincubations were done at 37°C in solutions containing 160 mM Na⁺ (no Na⁺ gradient), 5 mM free Mg²⁺, 0.3 mM vanadate, 20 mM MOPS-Tris (pH 7.4), 0.1 mM digoxigenin, 0.1 mM EGTA, and indicated concentrations of ATP and Ca²⁺. After 10 s, vesicles were diluted (25:100) in uptake solution at same temperature containing none or 150 mM NMG-Cl. ATP stimulation described above could have more than one explanation. For instance, it could adhere to a “regulatory nonphosphorylating” role of the nucleotide or, alternatively, require the phosphorylation of a key structure, not necessarily the carrier itself. To distinguish between these possibilities, we followed the actions of two ATP analogs. One was the nonphosphorylating AMP-PCP, which in other transport systems can sustain the nonphosphorylating regulatory role of ATP (39); the other was ATP₇S, which can act as substrate for kinases but not for ATPases (10, 23) and in squid axons stimulates Na⁺/Ca²⁺ exchange. The results are illustrated in Table 3. AMP-PCP, at 2 mM concentration, failed to stimulate the exchange. On the other hand, ATP₇S behaved exactly as ATP; this similarity included the inability to sustain stimulation if it is removed after a preincubation period in the absence of Ca²⁺.

Table 3. Comparative effects of ATP, AMP-PCP, and ATP₇S on a Na⁺ gradient-dependent Ca²⁺ uptake in bovine heart sarcolemmal vesicles

<table>
<thead>
<tr>
<th>Nucleotide, mM</th>
<th>Nucleotide-Stimulated Na⁺ Gradient-Dependent Ca²⁺ Uptake, nmol·mg⁻¹·s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>Uptake</td>
</tr>
<tr>
<td>None</td>
<td>1 ATP</td>
</tr>
<tr>
<td>None</td>
<td>2 AMP-PCP</td>
</tr>
<tr>
<td>None</td>
<td>1 ATP₇S</td>
</tr>
<tr>
<td>None</td>
<td>0.062 ATP₇S</td>
</tr>
<tr>
<td>1 ATP₇S</td>
<td>1 ATP₇S</td>
</tr>
<tr>
<td>1 ATP₇S</td>
<td>0.062 ATP₇S</td>
</tr>
<tr>
<td>1 ATP₇S</td>
<td>2.04 ± 0.23</td>
</tr>
<tr>
<td>1 ATP₇S</td>
<td>0.19 ± 0.17</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. Both preincubation and uptake times lasted 10 s. Preincubations were done at 37°C in media with high (160 mM) NaCl, 20 mM MOPS-Tris (pH 7.4 at 37°C), 5 mM free Mg²⁺, 0.5 mM vanadate, 0.1 mM digoxigenin, 0.1 mM EGTA, and no Ca²⁺. ⁴Ca²⁺ uptake were followed at 37°C in media with high (160 mM) or low (10 mM) NaCl, 20 mM MOPS-Tris (pH 7.4), 5 mM free Mg²⁺, 0.5 mM vanadate, 0.1 mM digoxigenin, 0.1 mM EGTA, and 1 µM Ca²⁺. Low Na⁺ solutions also had the equivalent of 260 mosmol NMG-Cl. ATP₇S, adenosine 5'-O-(3-thiotriphosphate); AMP-PCP, β,γ-methyleneadenosine 5'-triphosphate. For details see text.

Need for and effects of vanadate. To inhibit the Ca²⁺ pump, the extravesicular solutions contained 200–500 µM vanadate. However, vanadate, depending on the conditions (pH, ionic strength, etc.), can acquire different states (vanadate, metavanadate, vanadyl, etc.); in addition, we cannot be sure that a mixture of forms is present in our case. This raises the possibility that some artifacts, including some derived from complexing Ca²⁺, could be introduced in the estimation of the fluxes. To remove vanadate, we took advantage of a recent observation showing that eosin, at 20 µM concentration, can completely block the Ca²⁺ pump in this preparation (22). Accordingly, we explored the effects of ATP on Na⁺/Ca²⁺ exchange in the presence of 20 µM eosin. Table 4 summarizes these experiments. The first row shows experiments done in the presence of high extravesicular Na⁺, where Ca²⁺ uptake through the Na⁺/Ca²⁺ exchanger is inhibited, so the main route for Ca²⁺...
Ca\(^{2+}\) entry is expected to be the Ca\(^{2+}\) pump. That this is actually the case is indicated by the entries in the second row, left column, of Table 4 where addition of 20 µM eosi inhibits most of this Ca\(^{2+}\) influx; however, to our surprise, under these conditions, ATP also failed to stimulate Na\(^{+}/\)Ca\(^{2+}\) exchange (second row, right column with low extrasynical [Na\(^{+}\)]. As before, in the presence of 200–500 µM vanadate, an ATP stimulation of the exchanger was observed (third row). Comparison of the entries in rows 3 and 4 of Table 4 indicate that eosi by itself does not inhibit the ATP stimulation because that stimulation in the presence of vanadate is roughly the same with and without eosi. At this stage, we must point out that in the absence of ATP the Na\(^{+}\) gradient-dependent Ca\(^{2+}\) fluxes were the same in eosiin alone, vanadate alone, and eosiin plus vanadate (not shown). Finally, the last row describes extremely important results: ATP\(\gamma S\), which as expected, is not a substrate for the Ca\(^{2+}\) pump (left column), stimulates Na\(^{+}/\)Ca\(^{2+}\) exchange even in the absence of vanadate. Therefore, whatever the reason for the need of vanadate to see ATP stimulation of the exchanger (see Discussion), it has nothing to do with artifacts in the estimation of the Ca\(^{2+}\) uptake.

There is another important aspect regarding vanadate effects on this countertransport system. In squid axons, and in the absence of ATP, the response of Na\(^{+}/\)Ca\(^{2+}\) exchange to vanadate is biphasic: a marked stimulation at low concentrations (mean affinity constant of ∼5–10 µM) followed by inhibition at higher concentrations (inhibition constant of ∼1.5 mM) (16). Therefore, we explored whether a similar response was seen in cardiac sarcolemmal vesicles. The results of one of these experiments, described in Table 5, fully reproduce those observed in squid axons: 1) in the absence of ATP, vanadate does not affect Na\(^{+}/\)Ca\(^{2+}\) exchange, and 2) in the presence of 1 mM ATP, there is a marked stimulation of the exchanger at 300 µM vanadate, which is considerably reduced when the vanadate concentration is 3 mM.

Table 5. Effect of low (0.3 mM) and high (3 mM) vanadate concentrations on a Na\(^{+}\) gradient-dependent Ca\(^{2+}\) uptake in bovine heart sarcolemmal vesicles in absence and presence of ATP

<table>
<thead>
<tr>
<th>[Vanadate], mM</th>
<th>[ATP], mM</th>
<th>Na(^{+}) Gradient-Dependent Ca(^{2+}) Uptake, nmol·mg(^{-1})·s(^{-1})</th>
<th>Total ATP dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>None</td>
<td>1.43 ± 0.09</td>
<td>3.22 ± 0.18</td>
</tr>
<tr>
<td>0.3</td>
<td>1</td>
<td>4.65 ± 0.11</td>
<td>0.52 ± 0.22</td>
</tr>
<tr>
<td>3.0</td>
<td>None</td>
<td>1.41 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>1</td>
<td>1.93 ± 0.18</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. Uptake of \(^{45}\)Ca\(^{2+}\) was followed for 10 s at 37°C in solutions of the following composition: 160 or 10 mM NaCl, 5 mM freeMg\(^{2+}\), 20 mM MOPS-Tris (pH 7.4), 0.1 mM EGTA, 0.1 mM digitoxigenin, 0.8 µM Ca\(^{2+}\), and concentrations of ATP and vanadate indicated. Low Na\(^{+}\) solutions also had the equivalent of 260 nmol NMG-Cl. For details, see METHODS.

Absence of carrier phosphorylation and lack of effect of alkaline phosphatase. So far we found that in cardiac sarcolemmal vesicles MgATP stimulation of the Na\(^{+}/\)Ca\(^{2+}\) exchanger can be mimicked by MgATP\(\gamma S\), which is substrate for kinases, whereas AMP-PCP, which is a nonhydrolyzable nucleotide, does not. The kinase phosphorylation hypothesis is strong: the point is what structures become phosphorylated. In searching for the target molecules, we initially investigated the exchanger. To that end, vesicles were incubated under identical conditions where Ca\(^{2+}\) uptake was measured but in the presence of 0.5 mM of [\(^{32}\)P]ATP. The exchanger was immunoprecipitated with a specific anti-Na\(^{+}/\)Ca\(^{2+}\) exchanger antibody, subjected to SDS-PAGE, and blotted. Despite the fact that the Na\(^{+}/\)Ca\(^{2+}\) exchange carrier was detected in the transferred membrane by using the same specific antibody, a 10-day exposure to an X-ray film showed no \(^{32}\)P labeling. These results (not shown) indicated that, under our experimental conditions, the carrier does not become phosphorylated.

In dialyzed squid giant axons, the microinjection of an unspecified alkaline phosphatase reverts all MgATP stimulation of Na\(^{+}/\)Ca\(^{2+}\) exchange (DiPolo and Beauguay, unpublished data). We therefore decided to explore that possibility in our preparation. To that aim, the vesicles were preincubated for 2 min at 37°C with a concentration of alkaline phosphatase similar to that used in axons (200 U/ml), and then the system was assayed for MgATP stimulation of a Na\(^{+}\) gradient-dependent Ca\(^{2+}\) uptake. As indicated in Table 6, both basal and MgATP-stimulated Na\(^{+}/\)Ca\(^{2+}\) exchange were insensitive to the presence of alkaline phosphatase. In control experiments, with the same solutions used in the transport assays, we confirmed the effectiveness of the phosphatase using p-nitrophenyl phosphate as substrate (data not shown). Note that the vanadate concentrations were 0.2 mM in these cases. Although the inhibition constant for vanadate inhibition of this particular phosphatase is >1 mM (data not shown), we decided to reduce vanadate concentration a little.

Involvement of phosphoinositides in the MgATP stimulation of the Na\(^{+}/\)Ca\(^{2+}\) exchanger. At the time these studies were done, a report indicating that in cardiac sarcolemmal vesicles enriched with phosphoinositides there was an increase in Na\(^{+}/\)Ca\(^{2+}\) exchange was published (33). The authors suggested that phosphoinositides, particularly PIP2, could mediate the MgATP effects. If that hypothesis was correct, it could be that newly synthesized \(^{32}\)P labeled phosphoinositides co-precipitated with the exchanger. To that end, vesicles were incubated with radioactive ATP. The actual experimental conditions were 10 s at 37°C in the presence of 500 µM [\(^{32}\)P]ATP, 5 mM Mg\(^{2+}\), 0.3 mM vanadate, 1 µM Ca\(^{2+}\), and low Na\(^{+}\). Figure 4 illustrates one of the autoradiographs and indicates that our expectations were borne out. In Fig. 4, lane 3 (organic extraction of the immunoprecipitated exchanger), we can clearly see a spot corresponding to PIP2 and...
Immediately thereafter, 45Ca2\(^{+}\) was followed for 10 s at 37°C in solutions of the following composition: 160 or 10 mM NaCl, 5 mM free Mg2\(^{+}\), 20 mM MOPS-Tris (pH 7.4), 0.1 mM EGTA, 0.1 mM digitoxigenin, 0.2 mM vanadate, 0.8 µM Ca2\(^{+}\), and concentrations of ATP and alkaline phosphatase indicated. Low Na\(^{+}\) solutions also had the equivalent of 260 mosmol NMG-Cl. For details, see METHODS. In all cases, before starting 45Ca2\(^{+}\) uptake, concentrated vesicles were preincubated for 2 min at 37°C in the absence of Na\(^{+}\) gradient and Ca2\(^{+}\), with or without or with 200 U/ml alkaline phosphatase.

Table 6. Effect of an unspecific alkaline phosphatase on the Na\(^{+}\)/gradient-dependent Ca2\(^{+}\) uptake in bovine heart sarcolemmal vesicles in the absence and presence of ATP

<table>
<thead>
<tr>
<th>ATP, mM</th>
<th>Alkaline Phosphatase, U/ml</th>
<th>Na(^{+})/Gradient-Dependent Ca2(^{+}) Uptake, nmol · mg(^{-1}) · s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.49 ± 0.19</td>
</tr>
<tr>
<td>None</td>
<td>200</td>
<td>1.37 ± 0.18</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>3.36 ± 0.17</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>3.35 ± 0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. Uptake of 45Ca2\(^{+}\) was followed for 10 s at 37°C in solutions of the following composition: 160 or 10 mM NaCl, 5 mM free Mg2\(^{+}\), 20 mM MOPS-Tris (pH 7.4), 0.1 mM EGTA, 0.1 mM digitoxigenin, 0.2 mM vanadate, 0.8 µM Ca2\(^{+}\), and concentrations of ATP and alkaline phosphatase indicated. Low Na\(^{+}\) solutions also had the equivalent of 260 mosmol NMG-Cl. For details, see METHODS. In all cases, before starting 45Ca2\(^{+}\) uptake, concentrated vesicles were preincubated for 2 min at 37°C in the absence of Na\(^{+}\) gradient and Ca2\(^{+}\), with or without or with 200 U/ml alkaline phosphatase.

The immediate next step, and following a recent publication from Hilgemann and Ball (25), was to deplete and reinsert these compounds in the membrane vesicles. The initial experiment was to follow the MgATP effects in vesicles without and with previous exposure to PI-PLC. The vesicles were preincubated for 4 min at 37°C in the absence of Na\(^{+}\) gradient and of extrasolvential Ca2\(^{+}\) (160 mM NaCl, 20 mM MOPS-Tris, pH 7.4) without or with 2 U/ml PI-PLC. After that time, the Na\(^{+}\) gradient-dependent Ca2\(^{+}\) uptake was measured in the absence and presence of 1 mM ATP or 1 mM ATP2S under the usual incubation conditions. Three experiments, each one done in triplicate, are summarized in Table 7. They unmistakably show that pretreatment with PI-PLC completely blocks the stimulation of Na\(^{+}\)/Ca2\(^{+}\) exchange by 1 mM of both ATP and ATP2S. The values for Ca2\(^{+}\) uptake, which were the same in the treated vesicles under the three conditions, are higher than those observed in control vesicles in the absence of nucleotides; although not statistically significant, this was consistently found. In complementary experiments, vesicles initially subjected to the action of PI-PLC (also 4 min at 37°C) were incubated, after PLC removal, for 5 min at 0°C and for an additional minute at 37°C, with no ligands, 200 µM PI, or 50 µM PIP2. Immediately thereafter, 45Ca2\(^{+}\) uptake was assayed in the same incubation solutions with and without 1 mM ATP. One of these experiments, listed in Table 8, shows additional important points: PI by itself does not induce stimulation of Na\(^{+}\)/Ca2\(^{+}\) exchange, but it is able to restore the MgATP stimulation that had been lost by treatment of the vesicles with PI-PLC. In addition, in the absence of ATP, PIP2 produces a stimulation of the exchanger similar to that obtained with 1 mM of the nucleotide plus 200 µM PI.

Relevant to these last findings are the following observations described above: 1) if the vesicles are preincubated with MgATP, stimulation of the exchanger persists upon its removal only if there is Ca2\(^{+}\) in the preincubation solution, and 2) preincubation with MgATP and without Ca2\(^{+}\) increases the apparent affinity for the nucleotide effect. All together, these results suggest that MgATP stimulation occurs via phosphatidylinol phosphorylation that is Ca2\(^{+}\) dependent and very fast. On that basis, we decided to investigate the [32P]JATP of phosphoinositides in membrane vesicles incubated for 10 s at 37°C with 0.5 mM [γ-32P]JATP in the usual Ca2\(^{+}\) uptake solutions in the absence and presence of 1 µM Ca2\(^{+}\). A typical result of one of the experiments performed is illustrated in Fig. 5. In the absence of Ca2\(^{+}\), there is a marked synthesis of PIP with a minute

![Fig. 4. One-dimensional thin-layer chromatography (TLC) separation of lipid extracts from immunoprecipitated protein of Na\(^{+}\)/Ca2\(^{+}\) cardiac sarcolemmal membranes. Shown is an autoradiograph of 32P-labeled phospholipids from the following samples: lane 1, heated (5 min at 80°C) denatured vesicles reacted with phosphorylating media and exposed to NH2-terminal portion of the bovine cardiac Na\(^{+}\)/Ca2\(^{+}\) exchanger (anti-NCX antibody); lane 2, native vesicles exposed to phosphorylating media and then to nonimmune guinea pig serum; lane 3, native vesicles exposed to phosphorylating media and then to anti-NCX antibody. Cardiac sarcolemmal vesicles (100 µg) were incubated for 10 s at 37°C in 20 mM MOPS-Tris (pH 7.4), 150 mM NMG-Cl, 20 mM NaCl, 0.1 mM EGTA, 0.3 mM vanadate, 0.5 mM [γ-32P]JATP, 3 mM Mg2\(^{+}\), equivalent of 260 mosmol NMG-Cl, and 0.8 µM Ca2\(^{+}\). Samples were subjected to immunoprecipitation and then to chloroform extraction and TLC separation as indicated in METHODS. Note the following: 1) on right are indicated positions of respective standards for phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2); 2) TLC were run in a mobile phase of chloroform-methanol-H2O-concentrated NH4OH (40:48:10:5, vol/vol) using 20 cm × 10 cm plates (high-efficiency TLC); and 3) only in lane 3 is phosphorylated PIP2 detected.}
production of PIP₂ in the presence of Ca²⁺, the net synthesis of PIP is reduced and that of PIP₂ increases ninefold. These results are quite satisfying for they can explain what happened on the experiment on fluxes. When there was ATP without Ca²⁺ during the preincubation, a lot of PIP was formed, but, because this compound is not effective in stimulating the exchanger, there was no stimulation upon removal of the nucleotide during the Ca²⁺ uptake period. On the other hand, it produced enough PIP to serve as substrate for PIP₂ production during the uptake period with ATP; that can account for the increase in the apparent affinity for the nucleotide under those circumstances. In addition, to concur with the notion that PIP₂ is responsible for the MgATP stimulation of Na⁺/Ca²⁺ exchange, these results show that the production of PIP₂ is a very fast reaction. Actually, it is so fast that with only a 5-s incubation we obtained ~80% of the levels of synthesis found at 10 s (6.4 ± 0.1 pmol PIP₂/mg protein at 5 s compared with 8.3 ± 0.7 pmol PIP₂/mg protein at 10 s).

Table 7. Effect of preincubation with PI-PLC on Na⁺ gradient-dependent Ca²⁺ uptake in bovine heart sarcoplasmic vesicles in the absence and presence of ATP and ATP₇S

<table>
<thead>
<tr>
<th>Nucleotide, mM</th>
<th>Control</th>
<th>PI-PLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.19 ± 0.14</td>
<td>1.57 ± 0.27</td>
</tr>
<tr>
<td>1 ATP</td>
<td>2.66 ± 0.17</td>
<td>1.55 ± 0.15</td>
</tr>
<tr>
<td>1 ATP₇S</td>
<td>2.52 ± 0.11</td>
<td>1.49 ± 0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 different experiments done in triplicate. In all cases, vesicles (2 mg protein/ml) were preincubated at 37°C in a solution containing 160 mM Na⁺, 20 mM MOPS-Tris (pH 7.4) (no Na⁺ gradient), with or without 2 µM of phosphatidylinositol-specific phospholipase C (PI-PLC). After 4 min, vesicles were diluted (6:150) in uptake solution containing concentrations of ATP and ATP₇S indicated. Uptake time lasted 10 s. See METHODS for details.

Table 8. Effects of PI, without and with ATP, and of PIP₂ on Na⁺ gradient-dependent Ca²⁺ uptake in bovine heart sarcoplasmic vesicles pretreated with PI-PLC

<table>
<thead>
<tr>
<th>Phosphatidylinositol, µM</th>
<th>ATP, mM</th>
<th>Na⁺ Gradient-Dependent Ca²⁺ Uptake, nmol·mg⁻¹·s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 PI</td>
<td>1</td>
<td>1.58 ± 0.15</td>
</tr>
<tr>
<td>200 PI</td>
<td>1</td>
<td>1.47 ± 0.19</td>
</tr>
<tr>
<td>200 PI</td>
<td>1</td>
<td>1.65 ± 0.25</td>
</tr>
<tr>
<td>200 PI</td>
<td>1</td>
<td>2.68 ± 0.18</td>
</tr>
<tr>
<td>50 PIP₂</td>
<td></td>
<td>2.48 ± 0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate determinations. Vesicles (2 mg protein/ml) were preincubated at 37°C in a solution containing 160 mM Na⁺, 20 mM MOPS-Tris (pH 7.4) (no Na⁺ gradient), with or without 2 µM PI-PLC. After 4 min, vesicles were washed with same solution without PLC but with addition of 100 µM PI and centrifuged in a Beckman airfuge at 20 psi for 5 min at 4°C. Vesicles in pellet were resuspended at 2.5–3 mg/ml in same solution without PI and immediately preincubated with or without PI or 1,4-PI of ATP and MgATP at concentrations indicated. This incubation was for 5 min at 0°C and then for 1 min at 37°C. After this treatment, vesicles were diluted (6:150) in uptake solution containing concentrations of ATP and phosphoinositides indicated. Low Na⁺ solutions also had the equivalent of 260 mosmol NMG-Cl. 45Ca²⁺ uptake time lasted 10 s. See METHODS for details.

DISCUSSION

There are three pieces of evidence indicating that the 45Ca²⁺ fluxes considered in this work in fact go through the Na⁺/Ca²⁺ exchanger: 1) the radioactivity measured
is indeed inside the vesicles for it is rapidly lost when the A-23187 Ca\(^{2+}\) ionophore is added to the medium; 2) the uptake, both in the absence and presence of ATP, is inhibited on the cytosolic (extravesicular) side by XIP, a specific inhibitor of the Na\(^{+}/Ca\(^{2+}\)\) exchanger; and 3) we are dealing with a Na\(^{+}\) gradient-dependent (or inhibitable by Na\(^{+}\) on the cis-side) flux. With these considerations, there are two main questions to answer, Why was MgATP stimulation not always detected in this preparation (see Ref. 37) or was seen with different characteristics (9)? What are the mechanisms underlying this regulation?

With regard to the first question, it must also be noted that our results do not coincide completely with others who found an ATP effect in heart preparations. For instance, the Ca\(^{2+}\) concentration needed to see the effect is much smaller than the 50 \(\mu\)M reported for these vesicles (9); actually, had we used that concentration, our system would have been saturated and the stimulation by the nucleotide missed. In addition, in our hands, stimulation by MgATP is insensitive to nonspecific alkaline phosphatases. Also, compared with initial excised patch data (11), we observed 1) a much lower Michaelis constant for ATP, 2) ATP\(_{50}\) stimulation, and 3) an unexpected requirement for vanadate. Regarding later patch-clamp data (25), our results are in much closer agreement (see below). Most likely, the disagreements are related to the possibility that preparations like vesicles and membrane patches are structurally and biochemically incomplete, lacking some soluble, or loosely membrane attached, component required for the metabolic regulation of the exchanger. Relevant to this point is the observation that in squid optic nerve, even in the presence of vanadate, MgATP stimulates the exchanger only when a soluble nerve cytosolic factor is added (4, 20). Even more, if the axons are subjected to prolonged dialysis, the ATP stimulation is lost (4) and can be recovered when the cytosolic factor is injected into the axons (20). This line of reasoning can also apply to exchangers expressed in alien cells that may lack the biochemical machinery present in the natural host cells.

With regard to the mechanism of stimulation, the first thing to notice is that MgATP produces a 10-fold increase in the apparent affinity for cytosolic Ca\(^{2+}\), i.e., stimulation does not happen at saturating Ca\(^{2+}\) concentrations. An increment in the apparent affinity for cytosolic Ca\(^{2+}\), both for regulatory and transport sites, due to MgATP has also been seen in dialyzed squid axons (16). Needless to say, the molecular mechanisms underlying those affinity changes remain unknown; however, in our case, they seem related to the production of PIP\(_2\). In this regard, our results concur with recent work of Hilgeman and Ball (25) with the additional information that the effect of MgATP involves a cascade of at least two reactions: 1) synthesis of PIP from PI that does not require Ca\(^{2+}\) and 2) synthesis of PIP\(_2\) from PIP for which the presence of Ca\(^{2+}\) is essential. Notice that the need of a Ca\(^{2+}\)-dependent phosphorylation mechanism to account for MgATP stimulation of the Na\(^{+}/Ca\(^{2+}\)\) exchanger was proposed as early as in 1986 for squid giant axons (14). The fact that PIP\(_2\) coinmunoprecipitates with the exchanger may speak of a close association between these two molecules; however, and this must also be stressed, the way(s) PIP\(_2\) interacts with the countertransport system remains unknown. However, this may not necessarily be the only way for MgATP stimulation. On the one hand, phosphorylation of the carrier molecule, induced by growth factor (27) and PKC (26) and accompanied by stimulation of the exchanger, has been reported in whole cells. On the other, when rat heart exchanger is expressed in COS cells, the inhibition of Na\(^{+}/Ca\(^{2+}\) exchange after ATP depletion is similar to that observed when there is a disruption of the cytoskeleton (12). All these results open the possibility to multiple regulatory paths in this system.

Finally, an extremely interesting aspect of this work, and for which we have no explanation, has to do with vanadate. This compound is needed for MgATP and not for MgATP\(_{50}\) stimulation and produces a biphasic response, stimulation followed by a decline. The original idea put forward to account for similar results in squid axons was a protein kinase-protein phosphatase system (2, 24). That idea was sustained by some properties of vanadate: 1) it stimulates tyrosine kinases (39), 2) it is a powerful inhibitor of phosphotyrosil and less potent inhibitor of serine/threonine phosphatases (40), and 3) it inhibits, with an inhibition constant in the millimolar range, other protein kinases (unpublished data). In our vesicle preparation, we did not detect phosphorylation of the carrier but other protein bands did incorporate \(^{32}\)P; the same happens in squid axon membranes, and that phosphorylation pattern is altered by vanadate. Therefore, can we ensure that none of these proteins is related to the metabolic regulation of the exchanger? No doubt in isolated cardiac vesicles the PIP\(_2\) formation is essential MgATP stimulation of the exchanger, but is it the only molecule involved in that process? Micromolar vanadate concentrations also interact with the metabolism of phosphoinositides, stimulating PI phosphorylation (42), but nothing is known about the effects of millimolar vanadate concentration on phosphoinositides metabolism. Similar lack of information is found when considering the interactions of ATP\(_{50}\) in these metabolic paths. Actually, the efforts of our laboratories are presently oriented in that direction.

We thank Myriam Siravegna for skillful technical assistance. We also thank the generosity of Dr. Kenneth Phillipson who provided us with XIP and of Dr. John P. Reeves who gave us the antibody against the NH\(_2\)-terminal portion of the bovine cardiac Na\(^{+}/Ca\(^{2+}\) exchanger. We are thankful to Frigorifico Bustos and Beltran (Cordoba, Argentina) for providing beef hearts.

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