Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4433–4437, July 1985
Cell Biology

Inositol trisphosphate modification of ion transport in rough endoplasmic reticulum

(Ca^{2+} pump/KCl cotransport/K^+ conductance/Ca^{2+} conductance/stimulus secretion coupling)

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Communicated by Jared M. Diamond, April 12, 1985

ABSTRACT **The ion transport properties of the rough endoplasmic reticulum (RER) from liver have been defined by using measurements of active and potential gradient-driven transport. The Ca^{2+} pump is shown to be electrogenic, and both ATP and potential difference is able to drive vanadate-inhibitable Ca^{2+} uptake into the RER. ATP-dependent Ca^{2+} transport into the RER depends on the presence of tetraethylammonium-sensitive cation conductance and a furosemide-inhibited cation/chloride cotransport pathway. Inositol trisphosphate does not affect either of the monovalent ion translocation systems but activates a Ca^{2+} conductance in the RER, allowing efflux of RER Ca^{2+} stores into the cytosol in exchange for K^+ uptake.**

The elevation of free intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) is recognized as a central event in stimulus–secretion coupling in many tissues (1). It has been shown that release of Ca^{2+} from intracellular stores provides at least part of the Ca^{2+} necessary for this increment, and these stores have been identified with a membrane component fractionating with the rough endoplasmic reticulum (RER) (2). Inositol 1,4,5-trisphosphate (Ins-P_3), which is released into the cytosol from phosphatidylinositol bisphosphate in plasma membrane, has been identified as the intermediary messenger between agonist binding and release of Ca^{2+} (3) by experiments in which this compound has been added to permeabilized hepatocytes or pancreatic acinar cells (4, 5). In the pancreas at least, whereas there is a steady-state elevation of Ins-P_3, there is a transient elevation of $[Ca^{2+}]_{in}$ (6). Accumulation of Ca^{2+} in a cellular compartment and its release requires cooperation between a primary or secondary active transport system and cognate ion pathways within the same membrane. This paper attempts to define the transport properties of the endoplasmic reticulum fraction associated with Ca^{2+} signaling in hepatocytes.

METHODS

A fraction enriched in RER was prepared by differential and sucrose step-gradient centrifugation from rat liver homogenate as described (7). The vesicles of a density greater than 44.5% sucrose were washed and suspended in a medium containing 250 mM sucrose, 5 mM Hepes/Tris (pH 7.0), 0.02% NaN_3, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (solution A). The ion transport properties of the relevant membrane vesicles were determined by defining the conditions required for the uptake or efflux of $^{45}Ca^{2+}$ and $^{86}Rb^+$. The radioactivity of the vesicles was determined by passing 50 μl of the vesicle suspension through a Dowex 50X8 cation-exchange column with two 0.75-ml aliquots of 250 mM sucrose as eluent (8).

The ionic requirements for ATP-dependent Ca^{2+} uptake were determined by measuring $^{45}Ca^{2+}$ trapped after the addition of vesicles to a medium containing 5 mM Hepes/Tris (pH 7.0), 3 mM $MgSO_4$, 1 mM ATP/Tris, 10 μM Ca^{2+} acetate, 0.1 μCi (1 Ci = 37 GBq) of $^{45}Ca^{2+}$, and, selectively, the following at 100 mM: (solution B), NaCl, tetramethylammonium chloride (Me_4NCl), or K gluconate at 37°C. Samples of the vesicle suspension were taken every 20 sec. The conductance requirements for ATP-dependent Ca^{2+} uptake were determined in vesicles preloaded with solution B containing K gluconate after the addition of valinomycin or tetrachlorosalicylanilide (TCS) prior to the addition of ATP or in vesicles preloaded with solution B containing 250 mM sucrose instead of K gluconate with or without TCS (5 μM).

The potential generated by ATP-dependent Ca^{2+} uptake was assessed by measuring ATP and Ca^{2+}-dependent efflux of $^{86}Rb^+$ from vesicles equilibrated with a solution containing 250 mM sucrose, 3 mM $MgSO_4$, 0.2 mM EGTA, 10 mM KCl, and 1.0 μCi of $^{86}Rb^+$ in the absence and presence of 5 μM TCS.

The presence of a monovalent cation conductance was monitored by preloading vesicles with a K salt of an impermeant anion, removing the extravesicular salt by passing the suspension over a cation-exchange column and adding the K gluconate-containing vesicles to a solution containing 250 mM sucrose, 5 mM Hepes/Tris (pH 7.0), and 0.4 μCi of $^{86}Rb^+$. As necessary, Ins-P_3 or tetraethylammonium chloride (Et_4NCl) were added, and the uptake of radioactivity was followed over a period of 30 min. Since the addition of a proton conductance in the form of 5 μM TCS blocked $^{86}Rb^+$ uptake, this method defines the presence of a K^+ conductance (9).

The net uptake of $^{86}Rb^+$ was followed after addition of sucrose-equilibrated vesicles to a medium containing 250 mM sucrose, 5 mM Hepes/Tris, 0.2 mM EGTA, 10 mM KCl or K gluconate, and 0.4 μCi of $^{86}Rb^+$ in the absence or presence of bumetanide or furosemide at 1.5 mM or of Et_4N^+ at 5 mM. The relationship of KCl uptake to ATP-dependent Ca^{2+} uptake was determined by the effect of furosemide or bumetanide on ATP-dependent $^{45}Ca^{2+}$ uptake when vesicles were placed in KCl-containing solution B.

The effect of Ins-P_3 on ATP-dependent Ca^{2+} uptake was measured after a steady-state Ca^{2+} gradient was obtained after addition of ATP to vesicles suspended in KCl. In other experiments, ATP and extravesicular Ca^{2+} were removed by passing the suspension over a collapsed Sephadex column (8)

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Abbreviations: RER, rough endoplasmic reticulum; Et_4NCl, tetraethylammonium chloride; Me_4NCl, tetramethylammonium chloride; Ins-P_3, inositol trisphosphate; $[Ca^{2+}]_{in}$, free intracellular Ca^{2+} concentration; TCS, tetrachlorosalicylanilide.

after loading with $^{45}Ca^{2+}$ as above, and the vesicles were suspended in sucrose or KCl with or without Ins-P_3.

The effect of membrane potential on Ca^{2+} uptake into the vesicles was measured by adding vesicles preloaded with K gluconate to K^+-free $^{45}Ca^{2+}$-containing solutions and variously adding vanadate, Et_4N^+, valinomycin, and Ins-P_3.

Protein was determined by the Lowry procedure (10), and all reagents were of the highest purity available. Ins-P_3 was kindly provided by R. F. Irvine, Babraham, Cambridge University, England.

RESULTS

In preliminary experiments (see Fig. 5), Ins-P_3 was found to release Ca^{2+} from the RER fraction after loading in the presence of ATP. The experiments discussed below were then designed to determine the nature of the Ca^{2+} pump, associated ion pathways, and the membrane modification carried out by Ins-P_3.

Ca^{2+} Uptake in RER. Uptake of Ca^{2+} into the vesicles required the addition of ATP. Furthermore, it varied according to the ionic composition of the medium to which the vesicles were added (Fig. 1*a*). The fastest and largest uptake was in the presence of KCl. Substitution of K^+ by Me_4N^+ or of Cl^- by gluconate$^-$ reduced uptake to less than a third of that in KCl. It should be noted, however, that ATP still enhanced uptake even in the presence of K gluconate. The addition of valinomycin and TCS in the presence of K gluconate increased ATP-dependent Ca^{2+} uptake to the level of that found in the presence of KCl. Substitution of K^+ by Na^+ reduced uptake by 40%. When vesicles were suspended in sucrose medium, the addition of ATP resulted in a rate and level of uptake similar to that found in K gluconate, and the addition of 5 μM TCS resulted in a large stimulation of the rate and magnitude of Ca^{2+} uptake (Fig. 1*b*).

Pump Potential. Given the presence of a K^+ conductance (see below), transport of Ca^{2+} into the vesicles, if electrogenic, would result in K^+ or Rb^+ efflux. As shown in Fig. 2, the loading of KCl into vesicles was fairly rapid with a $t_{1/2}$ of about 2 min. The addition of ATP alone in the absence of Ca^{2+} resulted in no measured efflux of counts from the preequilibrated vesicles. However, the addition of Ca^{2+} resulted in a transient loss of counts to about one-third of the equilibrium value. This efflux was completely prevented by the addition of a large proton conductance in the form of TCS.

K^+ Conductance. K^+ or Rb^+ gradient-driven $^{86}Rb^+$ uptake that is sensitive to TCS has been shown to be due to the presence of electrical coupling between K^+ gradient-dependent diffusion potential and a K^+ (or cation) conductance (9). When vesicles were preloaded with 100 mM K gluconate, dilution into K^+-free medium containing tracer quantities of $^{86}Rb^+$ resulted in a time-dependent uptake of isotope (Fig. 3). This was blocked by Et_4N^+, a K^+-conductance blocker, and was not influenced by the presence of Ins-P_3, showing that K^+ conductance is present, as already indicated in Fig. 2, and that Ins-P_3 does not alter this conductance since uptake rate was unchanged and uptake was still entirely sensitive to Et_4N^+.

Net K^+ Uptake. The data above suggest the presence of an electrogenic Ca^{2+} pump with a K^+ conductance compensating charge flow to allow for net Ca^{2+} uptake. This requires an uptake pathway for KCl to provide the necessary internal K^+ for pump potential-mediated Ca^{2+} for K^+ exchange. When net $^{86}Rb^+$ uptake was monitored, as in Fig. 4, removal of Cl^- sharply reduced uptake, and the net uptake was insensitive to Et_4N^+. Furosemide, but not bumetanide, reduced KCl flux to below the gluconate level. The relevance of this KCl uptake system to ATP-dependent Ca^{2+} uptake was shown by the finding that ATP-induced Ca^{2+} uptake was blocked by furosemide (ED_{50} = 0.5 mM) but not by bumetanide up to 2 mM (data not shown).

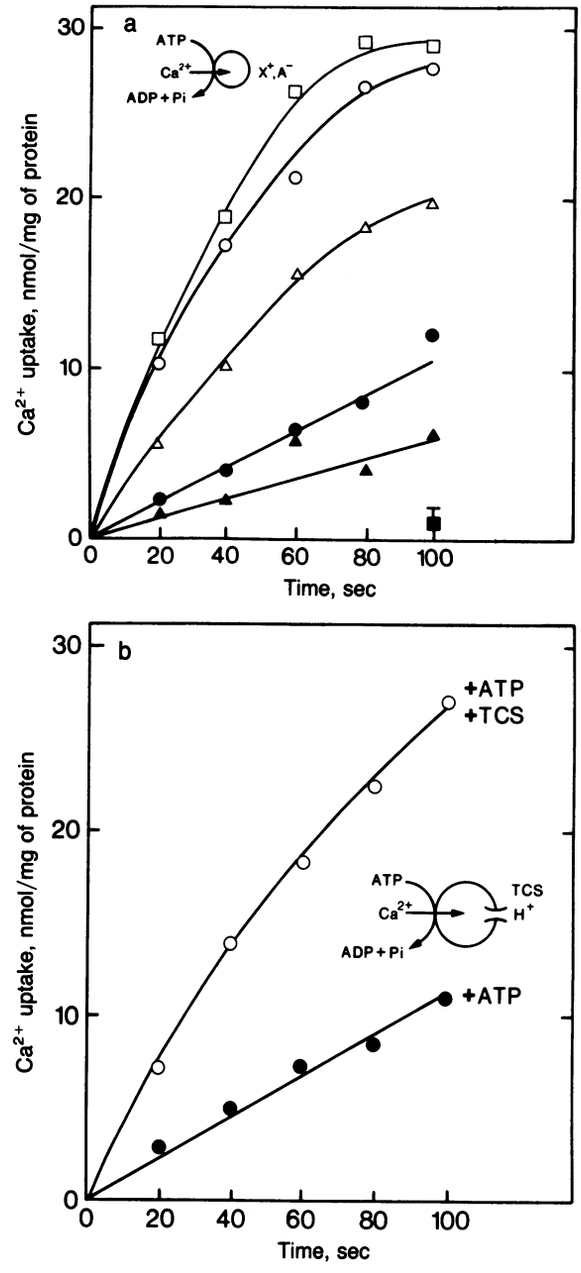

FIG. 1. Ionic requirement of ATP-dependent Ca^{2+} uptake into RER vesicles. Ca^{2+} uptake at 37°C was initiated by the addition of 30 μg of vesicles into reaction medium of the indicated composition also containing 10 μM Ca^{2+} and 2.5 × 10^5 cpm of ^{45}Ca. (*a*) The reaction medium was solution B (KCl, 1 mM ATP) (○) or similar solution in which 100 mM NaCl (△), 100 mM K gluconate (▲), 100 mM Me_4NCl (●), or 100 mM K gluconate/5 μM TCS/5 μM valinomycin (□) replaced the KCl. ■, Solution B without ATP. (*b*) The reaction medium was solution B in which 250 mM sucrose replaced KCl, and TCS when present was at 5 μM. At the indicated incubation periods, vesicles were separated from the medium by elution through Dowex columns as described.

In general, Cs^+ substitutes poorly for K^+ in biological conductances but is equally well transported by valinomycin (11). Thus, uptake is reduced when CsCl replaced KCl. If KCl entry is due to coupling between a K^+ and Cl^- conductance, then the addition of valinomycin will result in rapid CsCl entry and extrusion of the valinomycin-Cs complex due to Ca^{2+} entry through the pump. If no Cl^- conductance is present, then the addition of valinomycin will result in development of a positive intravesicular potential, which will restrict pump-dependent Ca^{2+} uptake. In this case, the

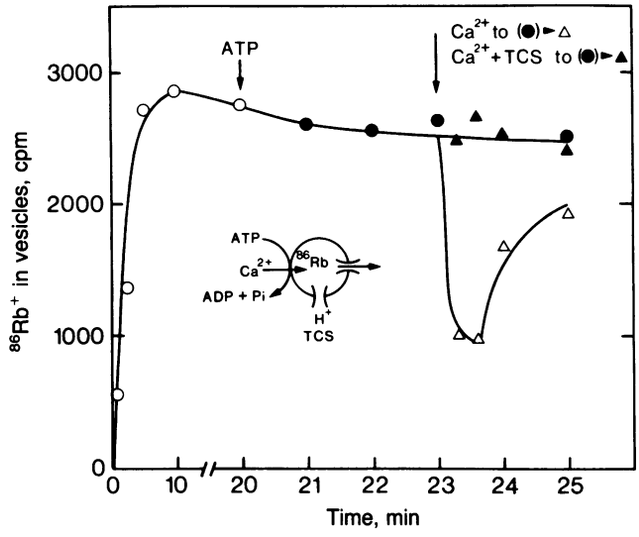

FIG. 2. ATP- and Ca^{2+}-induced K^+ efflux from RER vesicles. RER vesicles were added to medium containing 250 mM sucrose, 3 mM $MgSO_4$, 0.2 mM EGTA, 10 mM KCl, and 10^6 cpm of $^{86}Rb^+$. Where indicated, ATP was added to a final concentration of 1 mM. Then Ca^{2+} with or without TCS was added (final concentrations: 10 μM and 5 μM, respectively). The $^{86}Rb^+$ in vesicles was measured after removal of extravesicular medium on Dowex columns.

further addition of TCS by providing a proton shunt conductance for net valinomycin-Cs entry will stimulate the pump as effectively as KCl. The data of Table 1 show that uptake of Ca^{2+} in the presence of CsCl was about two-thirds of that obtained in the presence of valinomycin and TCS. Valinomycin alone inhibited Ca^{2+} uptake, showing that the Cl^- conductance was low in this preparation. Therefore, the data in this section argue for direct rather than electrical

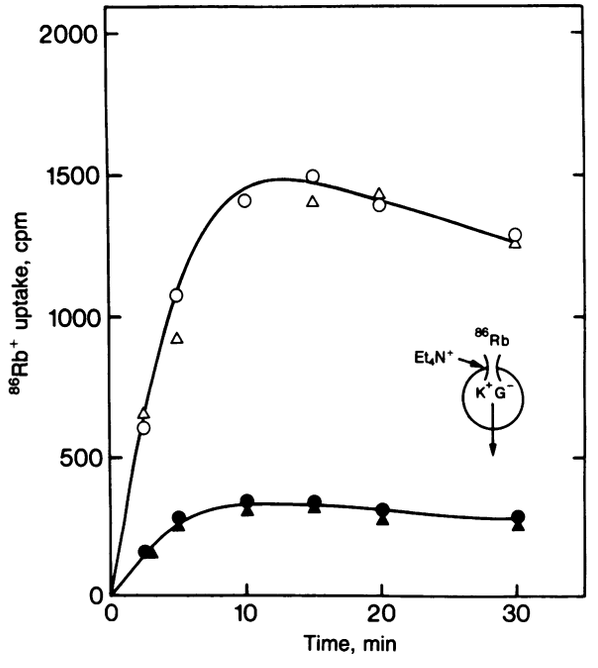

FIG. 3. K^+ gradient-dependent $^{86}Rb^+$ uptake into RER vesicles. K gluconate (K^+G^-)-equilibrated vesicles were eluted through a Dowex column to remove extravesicular K^+ and then diluted into a medium containing 250 mM sucrose, 5 mM Hepes/Tris, and 4×10^5 cpm of $^{86}Rb^+$. ○, Control; ●, control with 5 mM Et_4N^+; △, control with 5 μM Ins-P_3; ▲, control with 5 μM Ins-P_3 and 5 mM Et_4N^+. All incubations were at 0°C.

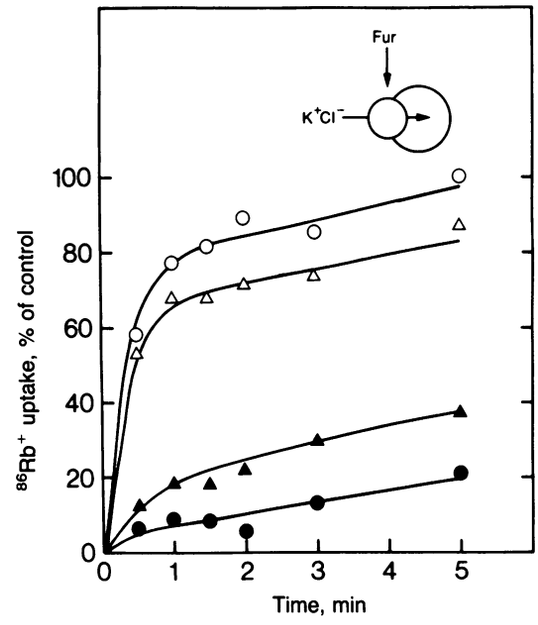

FIG. 4. KCl uptake into RER vesicles. Sucrose-equilibrated RER vesicles were added to medium containing 250 mM sucrose, 5 mM Hepes/Tris, 0.2 mM EGTA, 4×10^5 cpm of $^{86}Rb^+$, and 10 mM KCl with (△) or without (○) 5 mM Et_4N^+ or 10 mM K gluconate with (●) or without (▲) 1.5 mM furosemide (Fur). The amount of $^{86}Rb^+$ in vesicles was determined after Dowex column elution as before.

coupling between K^+ and Cl^- for a net KCl uptake by the vesicles containing the Ca^{2+} pump.

Effects of Ins-P_3. As reasoned in the discussion, the RER preparation from the data above contains an ATP-dependent Ca^{2+} uniport pump, a K^+ conductance, and a K^+/Cl^- cotransport pathway. In the intact cell, Ins-P_3 releases Ca^{2+} from intracellular stores, and in this section, the Ins-P_3-mediated changes in the membrane surrounding the putative Ca^{2+} store are examined.

When vesicles were allowed to accumulate Ca^{2+} in the presence of ATP and KCl, the addition of Ins-P_3 resulted in a transient efflux of Ca^{2+}, reducing vesicle Ca^{2+} levels by about 30–50%. A second addition of Ins-P_3 also released Ca^{2+}; but a slightly lower level of release was obtained (Fig. 5*a*). Further, when vesicles were preloaded with ^{45}Ca in the presence of ATP and KCl and the extracellular medium was removed by passage over a Sephadex column, there was a slow release of Ca^{2+} when vesicles were diluted into sucrose or KCl medium. The addition of Ins-P_3 resulted in a rapid release of counts only when dilution was into a KCl medium and not into the sucrose medium (Fig. 5*b*). Thus, this preparation retains Ins-P_3 sensitivity and demonstrates the conditions necessary for Ins-P_3-mediated Ca^{2+} release.

If Ins-P_3 activates a Ca^{2+} conductance in the RER preparation, it should be possible to demonstrate the presence of this

Table 1. Effect of the membrane potential on ATP-dependent Ca^{2+} uptake into the RER

Addition	ATP-dependent Ca^{2+} uptake, % of control
Sucrose/10 mM CsCl	100
+ 5 μM TCS	118
+ 5 μM Val	60
+ 5 μM TCS/5 μM Val	175

ATP-dependent Ca^{2+} uptake was measured as described in Fig. 1*b* except that the incubation medium also contained 10 mM CsCl and the indicated concentrations of TCS and valinomycin (Val). Control Ca^{2+} uptake was 25 nmol/mg of protein per min.

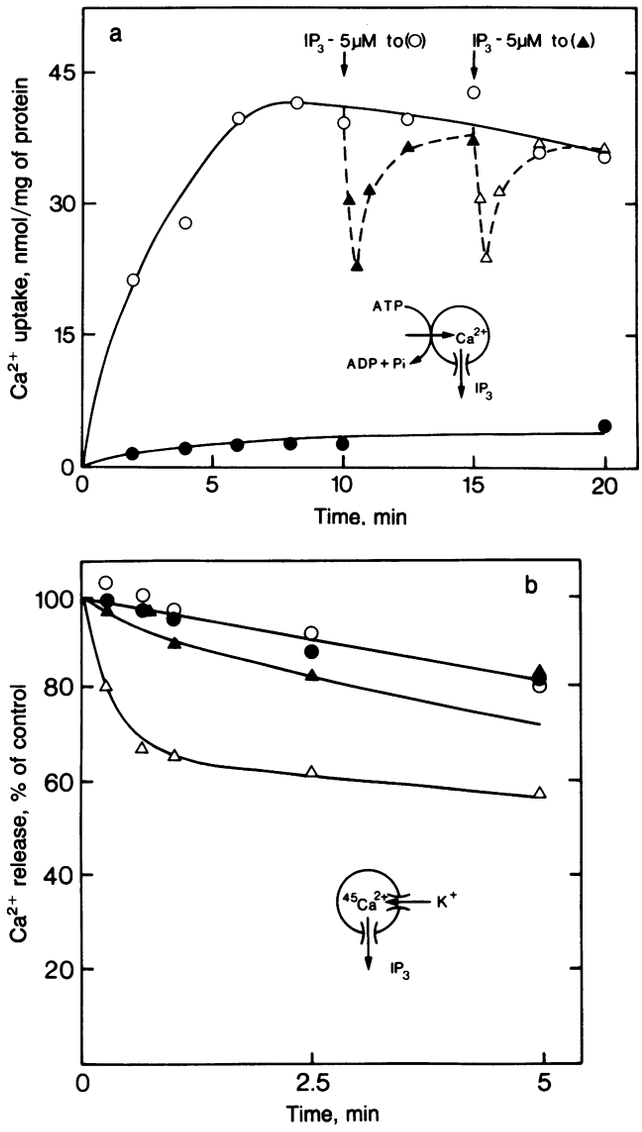

FIG. 5. Ins-P_3 (IP_3)-induced Ca^{2+} release from RER vesicles. (*a*) Ca^{2+} uptake at 37°C, in the presence (○) or absence (●) of ATP was measured as in Fig. 1. ▲ and △, Successive additions (arrows) of 5 μM Ins-P_3 to the same suspension of ATP-containing vesicles (○). (*b*) RER vesicles were loaded with $^{45}Ca^{2+}$ as in *a*, after which KCl, ATP, and Ca^{2+} were removed from the medium by centrifugation through a Sephadex column. The vesicles were added to medium containing 5 mM Hepes/Tris and 250 mM sucrose with (○) or without (●) 5 μM Ins-P_3 or 100 mM KCl with (△) or without (▲) 5 μM Ins-P_3. All experimental incubations were at 0°C.

by using the technique of Ca^{2+} gradient-driven ^{45}Ca uptake. Thus, when vesicles were preloaded with nonradioactive Ca^{2+} and then diluted into sucrose containing ^{45}Ca, Ins-P_3 addition resulted in an enhanced ^{45}Ca uptake that was reversed by the addition of 100 mM K gluconate to the external medium (data not shown).

Site of Ins-P_3 Action. A uniport pump should be reversible in terms of either ion gradient or potential (12); thus, it is possible that Ins-P_3 acts directly on the pump or elsewhere in the membrane to induce Ca^{2+} efflux. The data of Fig. 6 address this question. The vesicles were preloaded with K gluconate and diluted into K^+-free medium containing $^{45}Ca^{2+}$. Under these conditions, the K^+ diffusion potential induced Ca^{2+} uptake. This Ca^{2+} uptake was sensitive to Et_4N^+, showing that a K^+ conductance was necessary for this phenomenon and, further, was sensitive to vanadate, an ATPase inhibitor. The addition of valinomycin did not relieve the vanadate inhibition, showing that vanadate effect was not on the K^+ conductance (Fig. 6 *left*). In the presence of Ins-P_3, similar experiments gave different results. First, there was a more rapid uptake of Ca^{2+}, but vanadate only partially blocked this uptake. A combination of vanadate and Et_4N^+ was required to reduce uptake to background levels, and the addition of valinomycin to restore the K^+ conductance brought the Ca^{2+} uptake back to the vanadate-alone levels. These data show that the addition of Ins-P_3 adds an additional Ca^{2+} pathway to the vesicle membrane, separate from the catalytic subunit of the ATPase.

DISCUSSION

Ca^{2+} fulfills its role as a second messenger by alteration of $[Ca^{2+}]_{in}$. This is achieved either by changes in the Ca^{2+} permeability of the plasma membrane or by release of Ca^{2+} from cellular stores (13). For the latter process to be effective, it must be reversible, such as by combination of an uptake and release system. In noncontractile tissue, it has been suggested that the intracellular compartment regulating $[Ca^{2+}]_{in}$ is the RER (13). Thus, a subcellular fraction from rat liver enriched in RER has been shown to contain an active Ca^{2+} uptake system, the necessary accompanying ion pathways for ATP-mediated Ca^{2+} uptake, and Ins-P_3-sensitive Ca^{2+} release pathways.

ATP-induced Ca^{2+} uptake depends on the ionic composition of the suspension medium. KCl was the most effective salt, but the inhibition of uptake due to the absence of a permeant anion could be overcome by a combination of ionophores such as valinomycin and TCS, in the presence of K^+. This finding indicates that the Ca^{2+} uptake is electrogenic but, since K^+ was present, does not exclude the possibility of an electrogenic Ca^{2+}-for-K^+ exchange. However, since a proton conductance in the membrane, in the absence of salt, allows essentially normal Ca^{2+} uptake, it can be concluded that the Ca^{2+} pump in the RER fraction is a uniport type (14), requiring only a shunt conductance for net Ca^{2+} pumping to occur. The low level of Ca^{2+} uptake seen in the absence of salts and TCS must be accounted for by an inherent proton conductance of the RER membrane. This may be an artefact of the vesicle preparation, as has been shown for basal-lateral vesicles of the parietal cell (8). A further expectation for a uniport mechanism is its reversibility by potential. A K^+ diffusion potential was able to move Ca^{2+} into the RER by a vanadate-sensitive pathway, further confirming the uniport nature of the pump.

An electrogenic pump must result in generation of a potential difference across the membrane. This was established by demonstrating a TCS-sensitive transient efflux of $^{86}Rb^+$ from KCl-loaded vesicles due to the addition of ATP and then Ca^{2+}. The transient nature of the potential is expected in the situation where net ion accumulation occurs, since, initially, pump activity is expressed entirely as a potential and finally as a Ca^{2+} gradient (15). A 3-fold K^+ gradient would reflect a maximal transmembrane potential of 28 mV, if a homogeneous population of vesicles and insignificant Cl^- conductance are assumed.

For net uptake to occur, both a shunting conductance and an entry pathway for the shunting ion or ions must be present. In the RER preparation, the presence of a K^+ conductance that is sensitive to Et_4N^+ was established by demonstration of a Et_4N^+-sensitive K^+ countertransport. This would allow Ca^{2+} accumulation by the pump, provided K^+ was present inside the vesicles. Provision of this K^+ was by a furosemide-sensitive, Et_4N^+-insensitive, Cl^--dependent pathway. Valinomycin in the presence of CsCl, rather than KCl, inhibited, rather than stimulated, ATP-dependent Ca^{2+} uptake. Thus, a K^+/Cl^- cotransport system similar to that

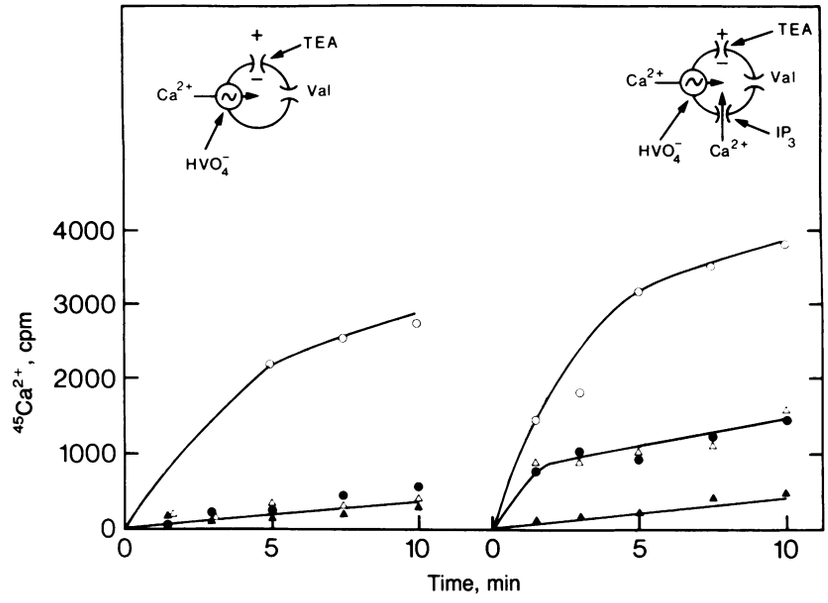

FIG. 6. K^+ gradient-driven Ca^{2+} uptake. K gluconate-equilibrated RER vesicles were eluted through Dowex column to remove external K^+. The vesicles were added to medium containing 250 mM sucrose, 5 mM Hepes/Tris, 3 mM $MgSO_4$, 10 μM Ca acetate, and $^{45}Ca^{2+}$. ○, Control; ●, control with 1 mM HVO_4^-; ▲, control with 1 mM HVO_4^- and 5 mM Et_4N^+; △, control with 1 mM HVO_4^-, 5 mM Et_4N^+, and 5 μM valinomycin (Val). (*Left*) Experiments in the absence of Ins-P_3. (*Right*) Experiments in the presence of 5 μM Ins-P_3. IP_3, Ins-P_3; TEA, Et_4N^+.

recently described in erythrocytes may be present in the membrane of interest (16). The necessary role of this pathway in Ca^{2+} uptake was shown by the furosemide inhibition of Ca^{2+} uptake.

Ins-P_3 was able to release Ca^{2+} from this subcellular fraction as expected of Ins-P_3 as an intermediary messenger in the Ca^{2+} pathway of stimulus secretion coupling (4, 5). Ins-P_3 does not modify either the K^+ conductance or K^+/Cl^- cotransport pathway but activates a Ca^{2+} conductance (channel) in the RER membrane. This pathway is active enough so that Ca^{2+} release can occur in spite of continuing pump activity and is active as long as Ins-P_3 is present. The conductive nature of the pathway was established by showing Ins-P_3-sensitive Ca^{2+} gradient-driven ^{45}Ca uptake, a K^+ conductance requirement for Ca^{2+} release, and the appearance of a vanadate-insensitive potential gradient-driven Ca^{2+}-uptake pathway. Since some of these Ins-P_3 effects were demonstrated after ATP removal, the continuing presence of ATP is not essential for the Ins-P_3 effect to be manifest. Only about one-third of the accumulated Ca^{2+} was released, showing that the RER preparation was heterogeneous with respect to its Ins-P_3 sensitivity. Thus, only a fraction of the RER *in situ* may be concerned with Ins-P_3-determined regulation of $[Ca^{2+}]_{in}$.

The data presented here can be summarized in the model of Fig. 7. The relevant Ca^{2+} pump is presented as an ATP-driven uniport pump. In parallel to the pump electromotive force, there is a Et_4N^+-sensitive and relatively K^+-selective conductance, a furosemide-sensitive and bumetanide-insensitive K^+/Cl^- symport pathway, and an Ins-P_3-sensitive Ca^{2+} conductance or channel. This model can readily account for loading of the Ca^{2+} stores in the absence of agonist and for their unloading upon the release of Ins-P_3 after activation of phospholipase C by agonist binding to a receptor.

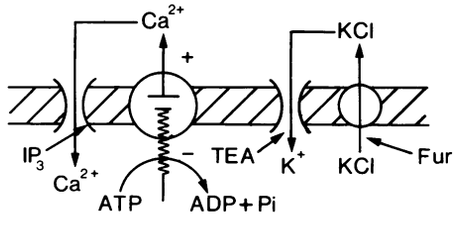

FIG. 7. Model of the ion pathways found in the RER membrane. IP_3, Ins-P_3; TEA, Et_4N^+; Fur, furosemide.

This study was supported by funds from the Research Services of the Veterans Administration and by National Institutes of Health Grants AM 17328 and AM 33010.

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