J. Membrane Biol. 109, 173–186 (1989)

The Journal of
Membrane Biology
© Springer-Verlag New York Inc. 1989

Characterization of Inositol 1,4,5-Trisphosphate-Sensitive (IsCaP) and -Insensitive (IisCaP) Nonmitochondrial Ca^{2+} Pools in Rat Pancreatic Acinar Cells

Frank Thévenod*, Martine Dehlinger-Kremer, Thomas P. Kemmer**, Anna-Luise Christian, Barry V.L. Potter†, and Irene Schulz

Max-Planck-Institut für Biophysik, D-6000 Frankfurt am Main 70, Federal Republic of Germany, and †Department of Chemistry, University of Leicester, Leicester LE1 7RH, United Kingdom

Summary. We have measured Ca^{2+} uptake and Ca^{2+} release in isolated permeabilized pancreatic acinar cells and in isolated membrane vesicles of endoplasmic reticulum prepared from these cells. Ca^{2+} uptake into cells was monitored with a Ca^{2+} electrode, whereas Ca^{2+} uptake into membrane vesicles was measured with $^{45}Ca^{2+}$. Using inhibitors of known action, such as the H^+ ATPase inhibitors NBD-Cl and NEM, the Ca^{2+} ATPase inhibitor vanadate as well as the second messenger inositol 1,4,5-trisphosphate (IP_3) and its analog inositol 1,4,5-trisphosphorothioate (IPS_3), we could functionally differentiate two nonmitochondrial Ca^{2+} pools. Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} pool (IsCaP) occurs by a MgATP-dependent Ca^{2+} uptake mechanism that exchanges Ca^{2+} for H^+ ions. In the absence of ATP Ca^{2+} uptake can occur to some extent at the expense of an H^+ gradient that is established by a vacuolar-type MgATP-dependent H^+ pump present in the same organelle. The other Ca^{2+} pool takes up Ca^{2+} by a vanadate-sensitive Ca^{2+} ATPase and is insensitive to IP_3 (IisCaP). The IsCaP is filled at "higher" Ca^{2+} concentrations ($\sim 10^{-6}$ mol/liter) which may occur during stimulation. The low steady-state [Ca^{2+}] of $\sim 10^{-7}$ mol/liter is adjusted by the IisCaP.

It is speculated that both Ca^{2+} pools can communicate with each other, the possible mechanism of which, however, is at present unknown.

Key Words H^+ pump · H^+ ATPase inhibitors · vanadate · inositol 1,4,5-trisphosphorothioate · Ca^{2+} electrode · acridine orange

Introduction

The intracellular messenger for hormone-induced Ca^{2+} release is inositol 1,4,5-trisphosphate (IP_3), a product of the receptor-mediated breakdown of plasma membrane phosphatidylinositol 4,5-bisphosphate [5, 26]. The IP_3-sensitive Ca^{2+} pool has not yet been localized. In the exocrine pancreas, evidence suggests that it is part of the endoplasmic reticulum (ER) [25]. Recently it has been proposed that a novel calsequestrin-containing organelle referred to as "calciosome" is the IP_3-sensitive Ca^{2+} pool [32]. It is generally believed that Ca^{2+} uptake into intracellular nonmitochondrial Ca^{2+} pools is mediated by Ca^{2+} ATPases present in endoplasmic [13, 15] and sarcoplasmic reticulum [12]. Following IP_3-induced Ca^{2+} release, Ca^{2+} is also actively taken back into the IP_3-sensitive Ca^{2+} pool and this Ca^{2+} uptake is dependent on ATP [8]. In vacuolar membrane vesicles of oat roots two Ca^{2+} pools with different Ca^{2+} uptake mechanisms have been described: A vanadate-sensitive Ca^{2+} ATPase associated with the endoplasmic reticulum and a vanadate-insensitive Ca^{2+}/H^+ antiport system located in the tonoplast [23]. It was assumed that the pH gradient established by the tonoplast H^+ ATPase is used to drive Ca^{2+} accumulation via the Ca^{2+}/H^+ exchanger. Furthermore, IP_3 has been shown to release Ca^{2+} from the vacuole of the oat root cell [24]. Since the Ca^{2+} ATPase activity is very low in the ER of oat root cells, it could not be decided if IP_3 also induced Ca^{2+} release from the ER.

In a recent study on Ca^{2+} transport in isolated permeabilized parotid cells and isolated ER, we have described both a vanadate-sensitive and a vanadate-insensitive MgATP-dependent Ca^{2+} uptake mechanism [31]. The latter was assumed to be located in an IP_3-insensitive Ca^{2+} pool. Both protonophores and H^+ ATPase inhibitors decreased vanadate-insensitive Ca^{2+} uptake which appeared to operate in exchange for H^+ ions. In the absence of ATP, Ca^{2+} uptake could occur to some extent at the expense of the H^+ gradient established by an H^+ pump located in the same IP_3-sensitive Ca^{2+} pool [31]. In the present study we have investigated the

* *Present address:* Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106.

** *Present address:* Klinikum der Universität Ulm, Medizinische Klinik und Poliklinik, Postfach 3880, D-7900 Ulm, FRG.

mechanism of Ca^{2+} uptake into an IP_3-sensitive Ca^{2+} pool (IsCaP) from isolated pancreatic acinar cells and pancreatic ER. Using different ATPase inhibitors we could functionally characterize and distinguish the IsCaP from other IP_3-insensitive Ca^{2+} pools (IisCaP). The data indicate that at least two nonmitochondrial Ca^{2+} pools act together to regulate cytosolic free Ca^{2+} concentration. The IsCaP is filled at "higher" Ca^{2+} concentrations (apparent $K_m \sim 2 \times 10^{-5}$ mol/liter [Ca^{2+}]), which may occur during stimulation, when cytosolic Ca^{2+} rises to 10^{-6} mol/liter or higher [19]. Furthermore, our data suggest that Ca^{2+} uptake into the IsCaP is mediated by a MgATP-dependent Ca^{2+}/H^+ exchange mechanism that can also operate in the absence of ATP at the expense of an H^+ gradient. We assume that acidification of the IsCaP necessary for Ca^{2+}/H^+ exchange is provided by a vacuolar type H^+ ATPase. The other Ca^{2+} uptake mechanism that is present in the IisCaP adjusts the low cytosolic free Ca^{2+} concentration at $\sim 10^{-7}$ mol/liter found at rest. It is a vanadate-sensitive Ca^{2+} ATPase which operates at lower Ca^{2+} concentrations (apparent $K_m \sim 7 \times 10^{-7}$ mol/liter) than the Ca^{2+} uptake mechanism of the IsCaP. Although it appears from our data that both the IsCaP and the IisCaP are separate pools, we do not exclude that both pools could communicate under some circumstances. The nature of this "communication" is unknown and could involve fusion of both Ca^{2+} pools [18], or Ca^{2+}-induced Ca^{2+} release [7], as described in other cell types.

Abbreviations

AO:	acridine orange
CCCP:	carbonylcyanide-*m*-chlorophenylhydrazone
CK:	creatine kinase
CP(Na):	phosphocreatine (sodium salt)
EDTA:	(ethylenedinitrilo)tetraacetic acid
EGTA:	(ethylene-bis-(oxyethylenenitrilo))tetraacetic acid
ER:	endoplasmic reticulum
HEPES:	N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid
IP_3:	myo-inositol 1,4,5-trisphosphate
IPS_3:	myo-inositol 1,4,5-trisphosphorothioate
NaN_3:	sodium azide
Na_3VO_4:	sodium vanadate
NBD-Cl:	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NEM:	N-ethylmaleimide
NTA:	nitrilotriacetic acid

Materials and Methods

Materials

Reagents were obtained from the following sources: CP (sodium salt), ATP (tris- and dipotassium salt), the protease inhibitor benzamidine, EDTA, EGTA, Na_3VO_4, bovine serum albumin (lyophylized), NTA, and NBD-Cl from Sigma (Deisenhofen, FRG); antimycin A, oligomycin, NEM, HEPES, the dye Serva Blue G, and Triton X-100 from Serva (Heidelberg, FRG). NaN_3, glucose, active charcoal, Tris, and D-mannitol were purchased from Merck (Darmstadt, FRG). CK (350 U/mg at 25°C), trypsin inhibitor (from hen egg white), hexokinase (from yeast, 140 U/mg at 25°C), and CCCP were from Boehringer (Mannheim, FRG). Nigericin was from Calbiochem (Giessen, FRG). Lanthanum chloride was from Fluka (Buchs, Switzerland). Collagenase, type III (from *Clostridium histolyticum*, 150 U/mg at 37°C) was purchased from Worthington (Freehold, NJ). IP_3 and IPS_3 were synthesized as described earlier [10]. $^{45}CaCl_2$ (4–30 Ci/g) and (γ-^{32}P) adenosine 5′-triphosphate, tetra(triethylammonium) salt (1000–3000 Ci/mmol) were from New England Nuclear Chemicals (Dreieich, FRG). Acridine orange (AO) was from Eastman Kodak (Rochester, NY). Ca^{2+}-selective electrode membranes containing the neutral carrier N,N′-di-((11-ethoxycarbonyl)undecyl)-N,N′,4,5,-tetramethyl-3,6-dioxaoctane amide (ETH 1001) were purchased from Glasbläserei W. Möller (Zurich, Switzerland). All other reagents were of analytical grade.

Methods

Preparation of Cells and Endoplasmic Reticulum

Rat pancreatic acinar cells were prepared as described previously [2, 27], with slight modifications. Briefly, pancreatic tissue from six male Wistar rats (200–250 g), which were fasted overnight, was digested with collagenase (120 U/ml) in 30 ml of a standard Krebs-Ringer-HEPES (KRH) medium. The KRH contained (in mmol/liter): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES adjusted with NaOH to pH 7.4, 10 glucose, 0.01% trypsin inhibitor, and 0.2% albumin, gassed with 100% O_2 and shaken in a water bath at 190 oscillations at 37°C for 15 min. Single cells were obtained by incubating the tissue with a 2 mmol/liter EDTA-containing KRH solution for 10 min and subsequent digestion with collagenase (180 U/ml) for a further 60 min in the Ca^{2+}-containing standard medium. To permeabilize the plasma membrane, isolated cells were washed three times with a nominally Ca^{2+}-free solution, as described previously [27] and stored as a concentrated suspension on ice up to 4 hr. For isolation of endoplasmic reticulum, cells were washed twice in an ice-cold homogenization buffer containing (in mmol/liter): mannitol 280, HEPES 5, KCl 10, $MgCl_2$ 1, benzamidine 1, adjusted with Tris/HCl to pH 7.0. Homogenization and subsequent fractionation steps were performed in the cold. Cells were homogenized in 36 ml of the same buffer by 50 strokes at 900 rpm in a tight-fitting Teflon-gas Potter-Elvehjem homogenizer. To remove remaining intact cells, the resulting homogenate was centrifuged for 4 min at 100 × *g* and the pellet was rehomogenized. The combined homogenate was centrifuged at 1000 × *g* for 13 min, and the supernatant was further centrifuged at 11,000 × *g* for 15 min in a Beckman 60 Ti type rotor. The 11,000 × *g* pellet was composed of a whitish fluffy layer and a yellowish bottom layer, which were separated. The fluffy layer is enriched by about twofold in ER [25]. It was resuspended in standard homogenization buffer at a protein concentration of 20 mg/ml, which was adjusted using the protein determination method of Bradford [6]. Vesicles were stored in liquid nitrogen until use for a maximum of 14 days.

Ca^{2+} Electrode Measurements

Ca^{2+}-specific macroelectrodes were built using the neutral carrier ETH 1001 in PVC and calibrated in EDTA-buffered, NTA-buffered and in unbuffered solutions as previously described

[27]. To determine Ca^{2+} uptake, permeabilized cells (5–8 mg protein/ml) were added to 3 ml of a solution containing, in mmol/liter: 120 KCl, 25 HEPES, 6 $MgCl_2$, 5 K_2ATP, 10 CP, 10 U/ml CK, 0.01 antimycin A, 0.005 oligomycin, 10 NaN_3, pH 7.4 with KOH, and test substance where indicated. The decrease in medium-free Ca^{2+} concentration due to cellular Ca^{2+} uptake was measured at 25°C with the Ca^{2+}-specific electrode as previously described [27].

Measurements of $^{45}Ca^{2+}$ Uptake

One milligram of membrane vesicle protein was preincubated for 15 min in a standard incubation medium of a total volume of 1 ml, containing basically (in mmol/liter): KCl 155, HEPES 5, $CaCl_2$ 0.034 (corresponding to 0.002 free Ca^{2+} concentration), EDTA 0.2, $MgCl_2$ 2.4 (corresponding to 1.3 free Mg^{2+} concentration), oligomycin 0.005, antimycin A 0.01, sodium azide 10, and 1 μCi/ml of $^{45}CaCl_2$, pH 7.0 adjusted with Tris/HCl. Test substances or the solvent used as a control were added from stock solutions at volumes not exceeding 1% (vol/vol). $^{45}Ca^{2+}$ uptake was initiated by the addition of MgATP to a final concentration of 1 mmol/liter and was measured with a rapid filtration method, as described previously [4]. MgATP-dependent $^{45}Ca^{2+}$ transport into vesicles was calculated as the difference between Ca^{2+} content in the presence and absence of MgATP. Variations in the composition of buffers and in the experimental procedures are described in details in the legends to figures and the Table.

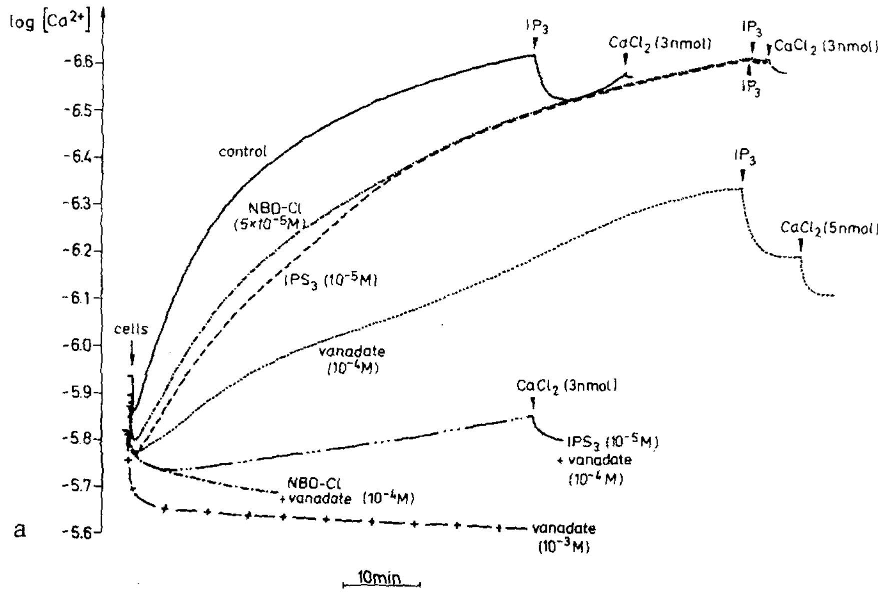
Assay for Determination of (Ca^{2+} + Mg^{2+})-ATPase Activity

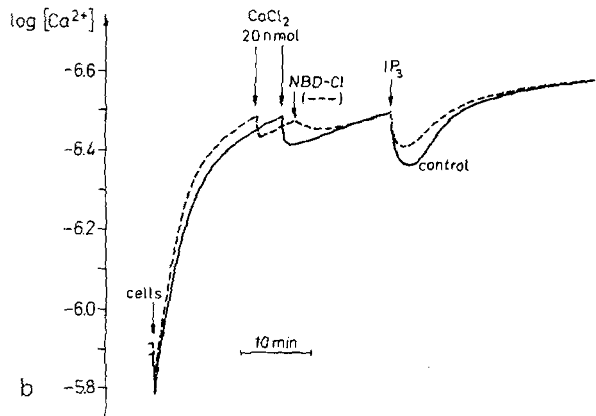
(Ca^{2+} + Mg^{2+})-ATPase activity was determined by measuring $^{32}P_i$ liberated from (γ-^{32}P) ATP during the reaction according to the method of Bais [3] as described previously [13, 15]. 100 μg of 11,000 × *g* fluffy layer protein were preincubated for 15 min in 200 μl of an incubation medium containing (in mmol/liter): KCl 130, antimycin A 0.01, oligomycin 0.01, sodium azide 5, benzamidine 1, HEPES 18 adjusted with Tris to pH 7.0, EDTA 3, $MgSO_4$ 3.7 (corresponding to 1.0 free Mg^{2+}), $CaSO_4$ 0.3 (corresponding to 0.001 free Ca^{2+}). In "Ca^{2+}-free" media, 3 mmol/liter EGTA without added Ca^{2+} was used. Total Mg^{2+} concentration was 1.1 mmol/liter (corresponding to 1.0 free Mg^{2+}) under these conditions. Free Ca^{2+}- and Mg^{2+} concentrations of the media used for Ca^{2+} uptake and (Ca^{2+} + Mg^{2+})-ATPase studies were calculated with a computer program using the true proton-, Ca^{2+}- and Mg^{2+} stability constants for ATP, EDTA and EGTA as described previously [13, 27]. Stock solutions of test substances or the solvent used as a control were added at 1% (vol/vol) to the medium. The phosphorylation reaction was started by addition of ATP solution containing 0.1 mmol/liter Tris-ATP and (γ-^{32}P) ATP (10 μCi, 5 μmol/liter final concentration) to the incubation medium and was carried out for 20 sec at room temperature (22–25°C) as described previously [13]. The reaction was terminated by addition of ice-cold stop solution containing 10% trichloroacetic acid, 10 mmol/liter KH_2PO_4, and 1 mmol/liter Tris-ATP. The samples were kept on ice for 10 min and were then centrifuged for 5 min at 2,250 × *g* at 4°C. After centrifugation, a 20-μl aliquot of the supernatant was mixed with 600 μl of active charcoal solution (125 mg/ml 1 N HCl). The sample was centrifuged at 2,500 × *g* for 10 min at 4°C and 100-μl aliquots of the resulting supernatant were mixed with 4 ml of scintillator Rotiszint 22X, and $^{32}P_i$ liberated was counted in a LKB 1214 Rackbeta liquid scintillation counter. The radioactivity of a control sample obtained in the absence of membrane protein was subtracted from each sample. The specific Ca^{2+} ATPase activity was determined by subtracting the ATPase activity in the absence of Ca^{2+} from that in the presence of Ca^{2+}.

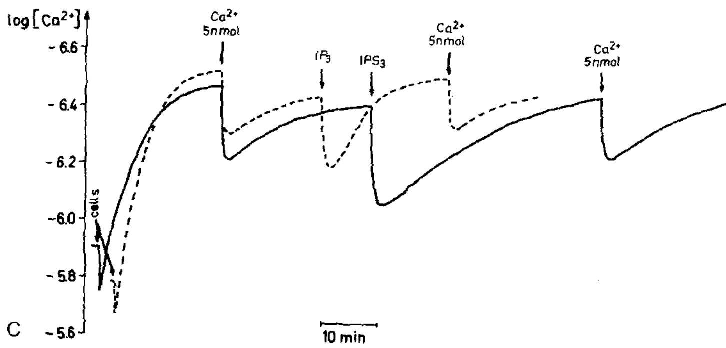
Results

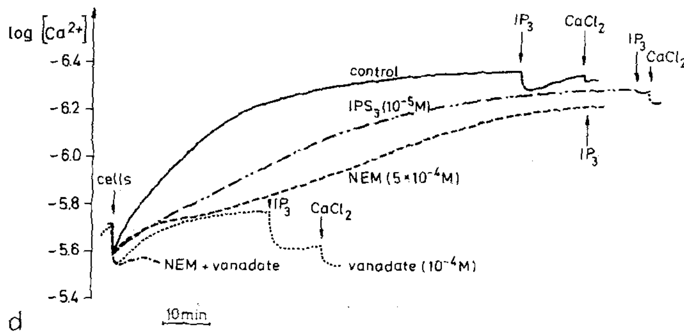
Effect of ATPase Inhibitors, of Inositol 1,4,5-Trisphosphate (IP_3) and of Inositol 1,4,5-Trisphosphorothioate (IPS_3) on Nonmitochondrial MgATP-Dependent Ca^{2+} Uptake into Permeabilized Pancreatic Acinar Cells

In a recent study on Ca^{2+} transport in isolated permeabilized parotid cells and ER we had found both a vanadate-sensitive and a vanadate-insensitive Ca^{2+} uptake. The latter was decreased in the presence of protonophores or H^+ ATPase inhibitors [31]. In order to investigate if an H^+ gradient-dependent nonmitochondrial Ca^{2+} uptake mechanism is present in pancreatic acinar cells, we have studied MgATP-dependent Ca^{2+} uptake into permeabilized pancreatic acinar cells in the presence of the H^+ ATPase inhibitors NBD-Cl and NEM. As shown in Fig. 1*a–d,* in the presence of MgATP isolated, permeabilized pancreatic acinar cells took up Ca^{2+} from a medium with a Ca^{2+} concentration of ~2 × 10^{-6} mol/liter until a steady-state of ~2 × 10^{-7} mol/liter Ca^{2+} was reached. Upon addition of IP_3 (5 × 10^{-6} mol/liter) at steady-state, Ca^{2+} was released from nonmitochondrial intracellular Ca^{2+} stores which was followed by Ca^{2+} reuptake. If cells were incubated in the presence of an H^+ ATPase inhibitor such as NBD-Cl (5 × 10^{-5} mol/liter; Fig. 1*a*), at a concentration that completely abolished nonmitochondrial MgATP-driven H^+ transport in isolated pancreatic membrane vesicles from endoplasmic reticulum [30], Ca^{2+} uptake rate was reduced (Fig. 1*a*). In three experiments Ca^{2+} uptake rate in the presence of NBD-Cl (5 × 10^{-5} mol/liter) as measured at 10^{-6} mol/liter Ca^{2+} was 42% ± 7 SE of the control Ca^{2+} uptake rate. However, the low steady-state free Ca^{2+} of ~2 × 10^{-7} mol/liter was still reached in the presence of NBD-Cl within ~80 min. Inhibition of the H^+ pump obviously prevented the filling of the IsCaP with Ca^{2+}, since addition of IP_3 at steady state to cells, which had been incubated with NBD-Cl, did not induce any release of Ca^{2+} (Fig. 1*a*). This effect of NBD-Cl was not due to inhibition of the IP_3-sensitive Ca^{2+} release mechanism, since subsequent to NBD-Cl addition at steady state, IP_3 did induce Ca^{2+} release (Fig. 1*b*). Longer incubation periods up to 50 min following NBD-Cl addition at steady state were also tested and showed no inhibitory effect of IP_3 on Ca^{2+} release. A further indication that in the presence of NBD-Cl only an IP_3-insensitive Ca^{2+} pool had been filled is the Ca^{2+} uptake observed in the presence of the nonhydrolysable IP_3 analog IPS_3 (*see* Fig. 1*a*).


log [Ca2+]
IP3
CaCl2 (3nmol)
IP3
CaCl2 (3nmol)
IP3
control
NBD-Cl (5×10-5M)
IPS3 (10-5M)
IP3
CaCl2 (5nmol)
cells
vanadate (10-4M)
CaCl2 (3nmol)
IPS3 (10-5M) + vanadate (10-4M)
NBD-Cl + vanadate (10-4M)
vanadate (10-3M)
10min
a


log [Ca2+]
CaCl2 20 nmol
NBD-Cl (---)
IP3
control
cells
10 min
b


log [Ca2+]
Ca2+ 5nmol
IP3
IPS3
Ca2+ 5nmol
Ca2+ 5nmol
cells
10 min
c


log [Ca2+]
IP3
CaCl2
IP3
CaCl2
control
IPS3 (10-5M)
IP3
cells
NEM (5×10-4M)
IP3
CaCl2
NEM + vanadate
vanadate (10-4M)
10min
d

IPS_3 released Ca^{2+} as effectively as IP_3 [28], (Fig. 1*c*). Since IPS_3 is not metabolized to IP_2 or to IP_4 [29, 33] and is also a potent inhibitor of the 5 phosphatase [11] one can assume that it is not broken down. Presumably the IsCaP remains empty as long as IPS_3 is present and Ca^{2+} reuptake should occur into the IisCaP. Consequent addition of IP_3 to IPS_3-pretreated cells did not release any Ca^{2+} (*see* Fig. 1*a*). It is striking that both Ca^{2+} uptake curves of cells pretreated with either NBD-Cl or IPS_3 are very similar. Ca^{2+} uptake in the presence of NBD-Cl or of IPS_3 was reduced by a similar amount that could be released by IP_3 when added to control cells (*see* Fig. 1*a*). In five experiments Ca^{2+} uptake rate in the presence of 10^{-5} mol/liter IPS_3 as measured at 10^{-6} mol/liter [Ca^{2+}] was 44% ± 6 SE of the control Ca^{2+} uptake rate. This similarity of Ca^{2+} uptake curves further indicates that in the presence of NBD-Cl the IsCaP had not been filled. Similarly, when another H^+ ATPase inhibitor, NEM, at concentrations that abolished H^+ uptake [30], was added to cells, Ca^{2+} uptake was also reduced, following closely the Ca^{2+} uptake curve in the presence of IPS_3 (Fig. 1*d*). In three experiments Ca^{2+} uptake rate in the presence of NEM (5×10^{-4} mol/liter) as measured at 10^{-6} mol/liter [Ca^{2+}] was 41% ± 19 SE of the control Ca^{2+} uptake rate. The steady-state free Ca^{2+} concentration was similar as in the control; however, steady state was reached with a delay of about 30–50 min. Again as with NBD-Cl, Ca^{2+} release was not observed when IP_3 was added at steady state (Fig. 1*d*). However, if NEM was added to control cells at steady-state [Ca^{2+}], subsequent addition of IP_3 induced Ca^{2+} release, indicating that NEM did not inhibit the IP_3-sensitive Ca^{2+} release mechanism (*data not shown*). These experiments indicate that a Ca^{2+} pool which was not sensitive to IP_3 could adjust the low free Ca^{2+} concentration of $\sim 2 \times 10^{-7}$ mol/liter observed in nonstimulated pancreatic cells [19, 27].

When incubation was performed in the presence of the inhibitor of the (Ca^{2+} + Mg^{2+})-ATPase vanadate at a concentration that nearly completely inhibits the (Ca^{2+} + Mg^{2+})-ATPase of pancreatic ER [13], (Table), permeabilized pancreatic acinar cells still took up Ca^{2+}. However, the Ca^{2+} uptake rate was markedly reduced. In seven experiments Ca^{2+} uptake rate was 38% ± 10 in the presence of 10^{-4} mol/liter vanadate as compared to the control Ca^{2+} uptake rate as measured at 10^{-6} mol/liter [Ca^{2+}]. The steady state of 2×10^{-7} mol/liter, which was adjusted by control cells, was not reached by vanadate-treated cells within the time of observation (*see* Fig. 1*a* and *d*). Nevertheless, the IP_3-sensitive Ca^{2+} pool had taken up Ca^{2+} in the presence of vanadate, since Ca^{2+} could be released upon addition of IP_3 (Fig. 1*a* and *d*), Ca^{2+} reuptake, however, was markedly inhibited due to inhibition of the Ca^{2+} pump in the IisCaP, which appeared to be stronger than the Ca^{2+} uptake mechanism in the IsCaP.

When vanadate was added to cells together with IPS_3 (Fig. 1*a*) Ca^{2+} uptake was nearly completely abolished. In the presence of both vanadate (10^{-4} mol/liter) and NBD-Cl or with vanadate and NEM (Fig. 1*a* and *d*), Ca^{2+} uptake was completely abolished, indicating that the effects of vanadate plus H^+ ATPase inhibitors are additive. Since IPS_3 + vanadate did not abolish Ca^{2+} uptake completely, it appears that H^+-dependent Ca^{2+} uptake also takes place into IP_3-insensitive Ca^{2+} pools. Thus Ca^{2+} uptake mainly occurs into three nonmitochondrial Ca^{2+} pools: into the IsCaP (sensitive to IP_3, to H^+ ATPase inhibitors and largely insensitive to vanadate at 10^{-4} mol/liter), into the same type of Ca^{2+} pool but lacking an IP_3 receptor, and into the

Fig. 1. (*a*) Effect of NBD-Cl (5×10^{-5} mol/liter) of vanadate (10^{-4} and 10^{-3} mol/liter), of inositol 1,4,5-trisphosphate (IP_3, 5×10^{-6} mol/liter and its analog inositol 1,4,5-phosphorothioate (IPS_3, 10^{-5} mol/liter) on nonmitochondrial Ca^{2+} uptake into isolated permeabilized pancreatic acinar cells. A concentrated suspension of cells (80 mg protein/ml) was preincubated for 20 min in standard incubation medium with or without NBD-Cl and vanadate at indicated concentrations. Cells were then 10-fold diluted into the same buffer with indicated substances for Ca^{2+} uptake measurement with a Ca^{2+} electrode as described previously [27]. IP_3 (5×10^{-6} mol/liter) and $CaCl_2$ (3 or 5 nmol) at a final concentration of 10^{-6} mol/liter and 1.7×10^{-6} mol/liter, respectively, were added for calibration where indicated. Typical for 3–6 experiments. (*b*) Effect of NBD-Cl on IP_3-induced Ca^{2+} release. Cells were added to standard incubation buffer and Ca^{2+} uptake was monitored with a Ca^{2+} electrode until steady-state [Ca^{2+}] was reached. $CaCl_2$ (final concentration 6.7×10^{-6} mol/liter), NBD-Cl (5×10^{-5} mol/liter) and IP_3 (5×10^{-6} mol/liter) were added where indicated. Typical for two experiments. (*c*) Effects of IP_3 (----, 5×10^{-7} mol/liter) and IPS_3 (—, 5×10^{-6} mol/liter) on Ca^{2+} release. Cells were added to standard incubation buffer and Ca^{2+} uptake was monitored with a Ca^{2+} electrode. IP_3 and IPS_3 were added where indicated. Ca^{2+} calibration pulses were given at a final concentration of 1.7×10^{-6} mol/liter. Typical for five experiments. (*d*) Effects of the H^+ ATPase inhibitor NEM (5×10^{-4} mol/liter), of the Ca^{2+} ATPase inhibitor vanadate (10^{-4} mol/liter), of IP_3 (5×10^{-6} mol/liter) and its analog IPS_3 (10^{-5} mol/liter), on Ca^{2+} uptake and Ca^{2+} release in isolated permeabilized pancreatic acinar cells. Cells were preincubated for 20 min in standard incubation buffer with or without NEM (5×10^{-4} mol/liter), vanadate (10^{-4} mol/liter) or with both substances. Cells that had been preincubated with NEM or vanadate were added to incubation buffer with the same substances as in the preincubation period. Cells without inhibitors in the preincubation period were added to standard incubation buffer with IPS_3 (10^{-5} mol/liter) or without additions (control). $CaCl_2$ (3 nmol, 10^{-6} mol/liter) and IP_3 (5×10^{-6} mol/liter) were added where indicated. Typical for three experiments

Table. Effect of ionophores and inhibitors on MgATP-dependent $^{45}Ca^{2+}$ uptake and ATPase activity into isolated vesicles from pancreatic endoplasmic reticulum

Compound	Conc. (mol/liter)	$^{45}Ca^{2+}$ uptake % of control	n	ATPase activity			% of control	n
				With Ca^{2+}	Without Ca^{2+}	Ca^{2+}-dependent		
Without (control)		100.0 (8.05 ± 0.78 nmol/mg protein × 20 min)	29	1.52 ± 0.11[c]	0.73 ± 0.08	0.78 ± 0.06	100.0	14
Ethanol 0.27% (control)		100.0 (9.76 ± 1.25 nmol/mg protein × 20 min)	21	1.50 ± 0.10[c]	0.68 ± 0.08	0.83 ± 0.04	100.0	13
Vanadate	2×10^{-3}	15.8 ± 2.1[c]	30	0.66 ± 0.08	0.58 ± 0.07	0.08 ± 0.03[f]	10.3	5
Vanadate	1×10^{-4}	51.2 ± 10.5[a]	6	1.06 ± 0.17	0.89 ± 0.19	0.16 ± 0.10[f]	20.5	6
NEM	5×10^{-4}	54.7 ± 16.6	3	0.77 ± 0.10[b]	0.49 ± 0.07	0.28 ± 0.05[f]	35.9	4
NEM	1×10^{-4}	80.4 ± 6.1	5	1.30 ± 0.24[b]	0.79 ± 0.17	0.51 ± 0.12[d]	65.4	6
NBD-Cl	1×10^{-4}	22.6 ± 1.5[c]	5	1.23 ± 0.19[c]	0.69 ± 0.13	0.57 ± 0.08[e]	63.9	7
NBD-Cl	5×10^{-5}	47.8 ± 11.6[a]	5	1.38 ± 0.21[a]	0.77 ± 0.13	0.61 ± 0.17	73.5	7
NBD-Cl	1×10^{-5}	64.3 ± 8.2[a]	7	1.46 ± 0.12[c]	0.68 ± 0.09	0.78 ± 0.08	94.0	4
Vanadate	2×10^{-3}							
+ NEM	5×10^{-4}	0.0 ± 0.0[c]	3	0.68	0.58	0.10	13.0	2
+ NBD-Cl	1×10^{-5}	1.0	2	0.70 ± 0.03	0.62 ± 0.04	0.08 ± 0.02[f]	10.0	5
CCCP	1×10^{-5}	80.1 ± 4.5[c]	8	1.80 ± 0.18[c]	0.82 ± 0.08	0.97 ± 0.10	117.0	8
Nigericin	1×10^{-5}	76.5 ± 5.2[c]	8	1.96 ± 0.22[c]	0.97 ± 0.13	0.99 ± 0.11	119.0	4
A23187	2×10^{-5}	5.0 ± 2.0[c]	3	1.90 ± 0.69[c]	0.86 ± 0.39	1.04 ± 0.31	125.0	3

Membrane vesicles (0.5 mg/ml) were preincubated for 15 min at 25°C in the presence of mitochondrial inhibitors and test compounds or their carriers. $^{45}Ca^{2+}$ uptake or ATPase activity assays were started by addition of ATP to the medium, as described in Materials and Methods. Values are means ± SE of separate experiments (n). Significance for $^{45}Ca^{2+}$ uptake in the presence and absence of test compounds and for MgATPase activity with and without Ca^{2+} was calculated by student's t-test for paired values. ATPase activity is expressed in nmol P_i liberated/mg protein × 20 sec.

[a] ($P < 0.02$), [b] ($P < 0.01$), [c] ($P < 0.005$). The difference for (Ca^{2+} + Mg^{2+})-ATPase activity with and without test compounds was calculated by the unpaired t-test. [d] ($P < 0.05$), [e] ($P < 0.01$), [f] ($P < 0.005$).

IisCaP (sensitive to vanadate and insensitive to IP_3 and to H^+ ATPase inhibitors). At 10^{-3} mol/liter vanadate Ca^{2+} uptake into the IsCaP was also inhibited since no Ca^2 uptake could be detected (*see* Fig. 1*a*). The IisCaP can adjust a low cytosolic free Ca^{2+} concentration of about 2×10^{-7} mol/liter. Filling of this pool is not prevented by IPS_3. The IsCaP does not adjust a low steady-state free Ca^{2+} concentration within a period comparable to the adjustment by the IisCaP at the experimental conditions used.

Effect of Protonophores and ATPase Inhibitors on MgATP-Dependent $^{45}Ca^{2+}$ Uptake and (Ca^{2+} + Mg^{2+})-ATPase Activity in Pancreatic ER Vesicles

In order to further characterize Ca^{2+} uptake as well as (Ca^{2+} + Mg^{2+})-ATPase activity in both Ca^{2+} pools we have performed experiments with isolated pancreatic membrane vesicles enriched in endoplasmic reticulum (ER) using $^{45}Ca^{2+}$ and (γ-^{32}P) ATP, respectively. Similar to that in parotid ER [31], in pancreatic ER a decrease of Ca^{2+} uptake in the presence of protonophores or H^+ ATPase inhibitors was observed. As shown in the Table in the presence of protonophores such as CCCP or the electroneutral K^+/H^+ exchanger nigericin, Ca^{2+} uptake was reduced to 80.1 ± 4.5% and 76.5 ± 5.2% of the control, respectively (mean ± SEM of eight experiments). H^+ ATPase inhibitors (NEM and NBD-Cl), which abolish ATP-driven H^+ uptake into vesicles from endoplasmic reticulum [30] reduced Ca^{2+} uptake by 25–80%, depending on the concentration. Vanadate at 2×10^{-3} mol/liter, which had no effect on ATP-driven H^+ uptake [30], drastically reduced both $^{45}Ca^{2+}$ uptake and (Ca^{2+} + Mg^{2+})-ATPase activity, whereas at 10^{-4} mol/liter vanadate the concentration mostly used in the present studies 51.2 ± 10.5% of total Ca^{2+} uptake remained although ~80% of (Ca^{2+} + Mg^{2+})-ATPase activity was inhibited. Since NBD-Cl (5×10^{-5} mol/liter), which abolishes MgATP-driven H^+ uptake completely [30], had only a small inhibitory effect on (Ca^{2+} + Mg^{2+})-ATPase as compared to the control (Table), we conclude that the decrease of MgATP-dependent Ca^{2+} uptake observed with NBD-Cl or of NEM was mainly due to a Ca^{2+} uptake mechanism other than

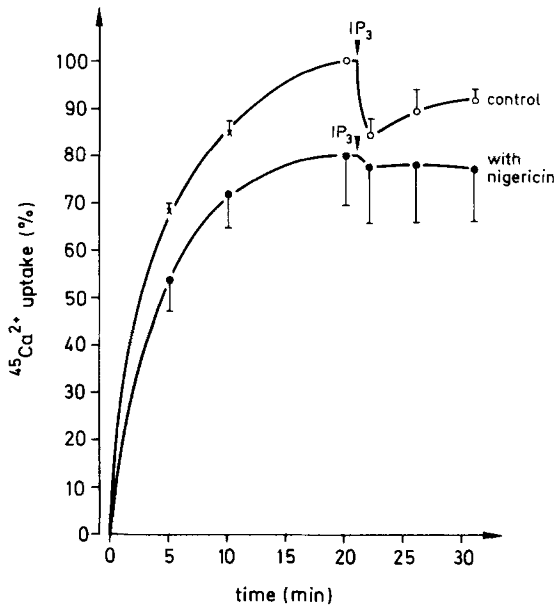


Fig. 2. Measurement of $^{45}Ca^{2+}$ uptake into isolated membrane vesicles from pancreatic endoplasmic reticulum (ER). ER was isolated from isolated pancreatic acinar cells and MgATP-dependent $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. Nigericin (10^{-5} mol/liter) was present in the incubation medium and IP_3 (5×10^{-6} mol/liter) was added where indicated. $^{45}Ca^{2+}$ uptake in the absence of ATP is subtracted from the $^{45}Ca^{2+}$ uptake curves. The values show means ± SE from four separate experiments

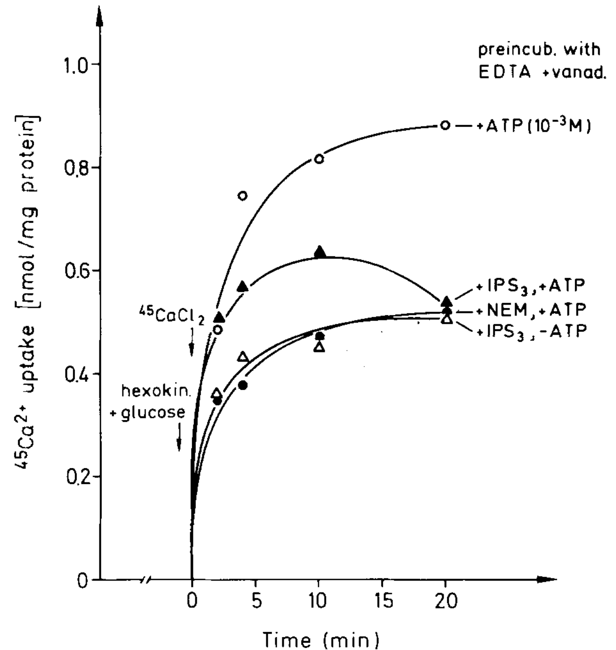


Fig. 3. $^{45}Ca^{2+}$ uptake into membrane vesicles from isolated pancreatic endoplasmic reticulum. Isolated membrane vesicles were preincubated for 15 min in the presence of EDTA (0.2 mmol/liter) at a free Mg^{2+} concentration of 1.3 mmol/liter and vanadate (2×10^{-3} mol/liter) with or without ATP (10^{-3} mol/liter), IPS_3 (10^{-5} mol/liter) and NEM (5×10^{-4} mol/liter) as indicated. Following preincubation, hexokinase (5 U/ml) + glucose (10 mmol/liter) and $^{45}CaCl_2$ (final concentration 2×10^{-6} mol/liter) were added as indicated and $^{45}Ca^{2+}$ uptake was determined as described in Materials and Methods. One experiment out of three similar ones

the vanadate-sensitive (Ca^{2+} + Mg^{2+})-ATPase. If both vanadate and H^+ ATPase inhibitors were added together $^{45}Ca^{2+}$ uptake was abolished, although 10% of (Ca^{2+} + Mg^{2+})-ATPase activity remained. In the presence of protonophores (Ca^{2+} + Mg^{2+})-ATPase was not inhibited. Figure 2 and the Table show that MgATP-dependent $^{45}Ca^{2+}$ uptake into an IsCaP was diminished in the presence of nigericin (10^{-5} mol/liter). Since in the presence of nigericin IP_3 did not significantly release any Ca^{2+}, we conclude that the IP_3-sensitive Ca^{2+} pool had not been filled in the presence of this protonophore. In four experiments MgATP-dependent $^{45}Ca^{2+}$ uptake after 20 min of incubation was reduced from 12.4 ± 4.2 SE nmol/mg protein in the controls to 8.6 ± 1.6 nmol/mg protein in the presence of nigericin; maximal release of Ca^{2+} by IP_3 was diminished from 2.5 ± 1.1 nmol/mg protein in the controls to 0.6 ± 0.3 nmol/mg protein in the nigericin experiments (means ± SEM). Similar data were also obtained with CCCP. In two experiments MgATP-dependent $^{45}Ca^{2+}$ uptake was decreased in the mean from 5.6 nmol/mg protein to 4.9 nmol/mg protein after 20 min. IP_3-induced Ca^{2+} release was reduced from 0.6 nmol/mg protein to 0.2 nmol/mg protein in the presence of CCCP. If the reason for diminished Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} pool (IsCaP) was due to dissipation of an H^+ gradient by the protonophores, these data suggest that the H^+ gradient is important for Ca^{2+} uptake into the IsCaP.

Evidence for the Presence of Ca^{2+}/H^+ Exchange in the IsCaP

If Ca^{2+} uptake is coupled to an H^+ gradient it could occur via a MgATP-driven Ca^{2+}/H^+ countertransport or via a MgATP-independent Ca^{2+}/H^+ exchanger. In the latter case Ca^{2+} uptake should occur at the expense of an H^+ gradient and should be possible in the absence of ATP. In order to probe for this possibility we have performed the experiment shown in Fig. 3. ER vesicles were preincubated in the presence of vanadate and EDTA to inhibit all Ca^{2+} uptake, but with MgATP to allow H^+ uptake by a vanadate-insensitive "V"-type H^+ pump present in the same membrane fraction [30].

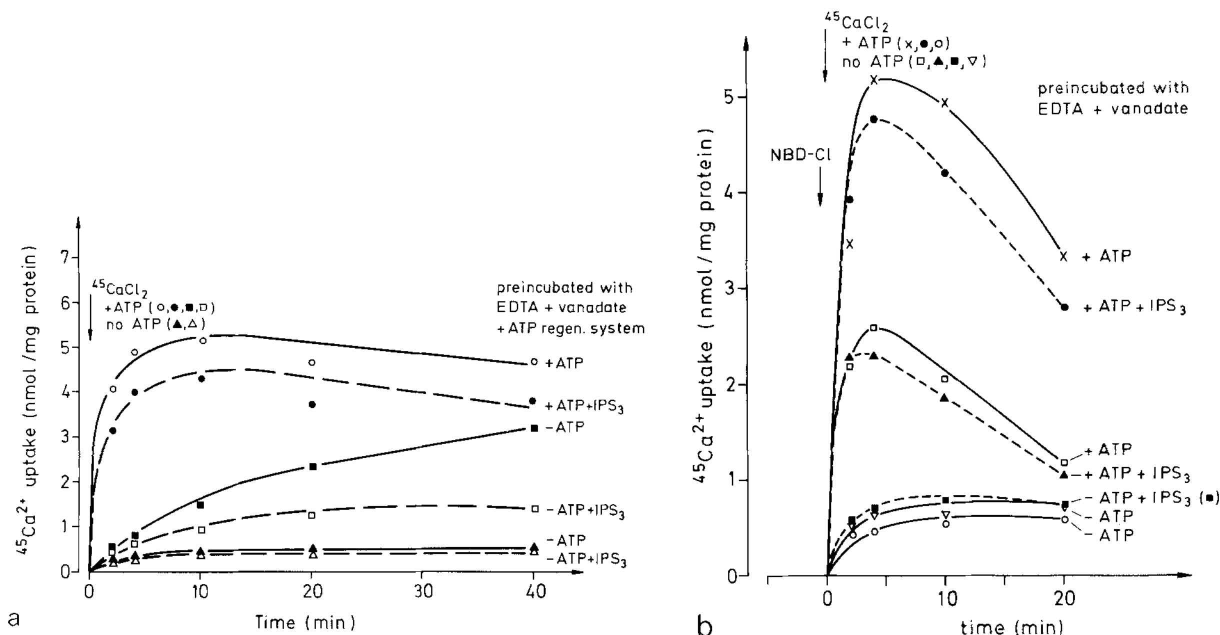


Fig. 4. (*a*) Effect of acute addition of ATP on $^{45}Ca^{2+}$ uptake into ER vesicles at different preincubation conditions. ER vesicles were preincubated for 15 min in the presence of EDTA (0.2 mol/liter) of vanadate (10^{-4} mol/liter) with an ATP-regenerating system (8 units CK + 10 mmol/liter CP) and with or without ATP (10^{-3} mol/liter) and IPS_3 (10^{-5} mol/liter) as indicted at the right side of the figure. Following preincubation $^{45}Ca^{2+}$ (final concentration 2×10^{-6} mol/liter) and ATP (10^{-3} mol/liter) was added as indicated by the arrow at time 0. $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. One out of three similar experiments. (*b*) Effect of ATP on $^{45}Ca^{2+}$ uptake in the presence of a preformed H^+ gradient. ER vesicles were preincubated at conditions similar to those in *a* as indicated on the right. At time 0, $^{45}Ca^{2+}$ (2×10^{-6} mol/liter) and NBD-Cl (10^{-4} mol/liter) to inhibit the H^+ pump were added to all assays and ATP was added where indicated. $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. One out of two similar experiments

Subsequent addition of $^{45}Ca^{2+}$ resulted in Ca^{2+} uptake from a medium in which all ATP had been hydrolyzed by addition of glucose and hexokinase. Complete hydrolysis of ATP in the incubation medium was verified by demonstrating that simultaneous addition of both ATP and the regenerating system followed by addition of $^{45}Ca^{2+}$ within 2 min did not cause any $^{45}Ca^{2+}$ uptake (*data not shown*). If vesicles had not been preincubated with ATP or if the preincubation medium contained ATP and NEM (Fig. 3) or ATP and NBD-Cl (*not shown*) to inhibit H^+ uptake, $^{45}Ca^{2+}$ uptake into membrane vesicles was reduced to the values of the control without ATP preincubation (Fig. 3). MgATP-independent $^{45}Ca^{2+}$ uptake was also reduced as compared to the control if the medium contained IPS_3 during both preincubation and incubation periods (*see* Fig. 3). In three different experiments $^{45}Ca^{2+}$ uptake was significantly reduced from 0.76 ± 0.03 nmol/mg protein to 0.60 ± 0.03 mol/mg protein at 4 min ($P < 0.01$, paired *t*-test) and from 0.89 ± 0.08 nmol/mg protein to 0.71 ± 0.07 nmol/mg protein ($P < 0.02$, paired *t*-test), when control $^{45}Ca^{2+}$ uptake was compared to $^{45}Ca^{2+}$ uptake in the presence of NEM or of IPS_3, respectively. These data show that Ca^{2+} uptake into an IsCaP can be achieved in the absence of ATP at the expense of an H^+ gradient generated by a "V"-type H^+ pump and most likely occurs via a Ca^{2+}/H^+ exchanger. It was now interesting to find out if this Ca^{2+}/H^+ exchange can also operate as a Ca^{2+} ATPase, i.e., if it is a MgATP-driven Ca^{2+} pump that exchanges Ca^{2+} for H^+ ions. In order to study this question the following points were investigated: Does the Ca^{2+}/H^+ exchanger that fills an IsCaP in the presence of an H^+ gradient operate at a higher rate in the presence of MgATP? If it uses MgATP, is the H^+ gradient then necessary at all?

When vesicles were preincubated with ATP in the presence of an ATP-regenerating system to allow H^+ uptake and with vanadate and EDTA to inhibit Ca^{2+} uptake, addition of $^{45}Ca^{2+}$ and additional ATP caused a quick $^{45}Ca^{2+}$ uptake that could be diminished by IPS_3. (In three experiments $^{45}Ca^{2+}$ uptake was decreased from 5.8 ± 0.7 to 5.1 ± 0.8 nmol Ca^{2+}/mg protein after 10 min) (*see* Fig. 4*a*).) If vesicles were preincubated with vanadate, EDTA, but without ATP, so that neither active Ca^{2+} transport nor H^+ uptake could take place, addition of $^{45}Ca^{2+}$ and ATP caused slower Ca^{2+} uptake as compared to vesicles that had been preincubated with MgATP. Again in the presence of IPS_3, $^{45}Ca^{2+}$ uptake was decreased in three experiments from 3.3 ± 0.9 to 2.7 ± 0.9 nmol Ca^{2+}/mg protein after 10 min (*see* Fig. 4*a*). If the same experimental conditions were tested, but NBD-Cl (1×10^{-4} mol/liter) was

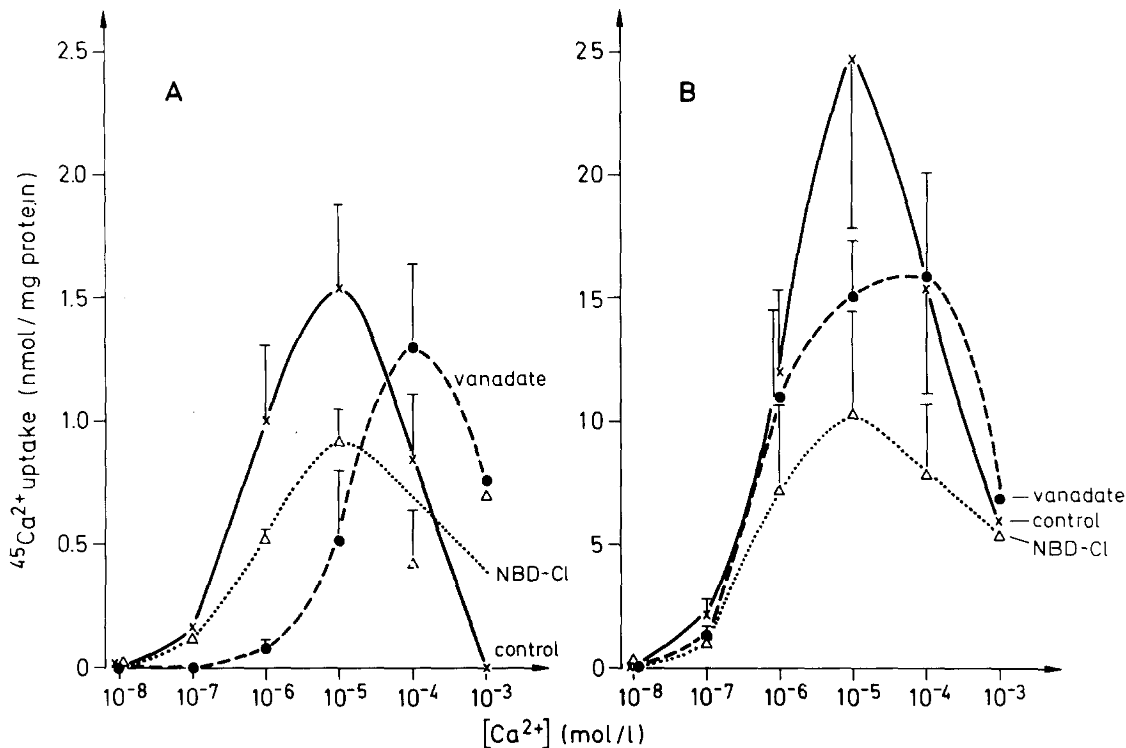


Fig. 5. Effect of ATPase inhibitors (NBD-Cl, 5×10^{-5} mol/liter) and vanadate (10^{-4} mol/liter) on $^{45}Ca^{2+}$ uptake at different free Ca^{2+} concentrations in the incubation medium. ER vesicles were preincubated for 15 min with or without inhibitors and at indicated free $^{45}Ca^{2+}$ concentrations adjusted with EDTA (1 mmol/liter) as described in Materials and Methods. $[Ca^{2+}]$ was checked back with the Ca^{2+} electrode. $^{45}Ca^{2+}$ uptake was started by addition of ATP (10^{-3} mol/liter) and determined after 10 sec (*A*) and after 20 min (*B*) of incubation. The data show mean values ± SE from 4–5 experiments. $^{45}Ca^{2+}$ uptake in the absence of ATP had been subtracted

added to the medium just before addition of $^{45}Ca^{2+}$, to block the H^+ pump, $^{45}Ca^{2+}$ uptake was observed only in vesicles that had been preincubated with MgATP, i.e., in which an H^+ gradient had been previously built up (Fig. 4*b*). This $^{45}Ca^{2+}$ uptake at inhibited H^+ pump with and without acute addition of ATP but at preformed H^+ gradient was overshooting, indicating dissipation of the H^+ gradient at inhibited H^+ pump. It was also diminished in the presence of IPS_3 (Fig. 4*b*). If ATP was acutely added to the medium, Ca^{2+} uptake was higher than without acute addition of ATP and the IPS_3 effect appeared to be higher (0.6 nmol $^{45}Ca^{2+}$/mg protein at 10 min) as compared to the experiment without acute addition of ATP (0.2 nmol $^{45}Ca^{2+}$/mg protein at 10 min). This indicates that the Ca^{2+}/H^+ exchanger can also use ATP for Ca^{2+} uptake. The observation that IPS_3 did not abolish $^{45}Ca^{2+}$ uptake following acute addition of ATP could mean that only part of Ca^{2+}/H^+ exchanging Ca^{2+} pools are sensitive to IP_3. It could also mean that only part of Ca^{2+} was released from an IP_3-sensitive Ca^{2+} pool. If vesicles had not been preincubated with ATP and ATP was acutely added subsequently to NBD-Cl, Ca^{2+} uptake did not occur. The data in Figs. 3 and 4 indicate that in the presence of vanadate no Ca^{2+} uptake takes place if formation of an H^+ gradient is prevented either by inhibition of the H^+ pump with NBD-Cl or by omission of MgATP. A preformed H^+ gradient stimulates Ca^{2+} uptake into the IsCaP most likely via a Ca^{2+}/H^+ exchange mechanism in the absence of ATP (Fig. 3) or in the presence of ATP at continuous H^+ uptake. Ca^{2+} uptake is slower if an H^+ gradient had not been preformed but was allowed to be formed by acute addition of ATP to the $^{45}Ca^{2+}$ uptake medium (Fig. 4). However, if an H^+ gradient had not been preformed or was not allowed to be formed due to addition of NBD-Cl (Fig. 4*b*) ATP did not stimulate Ca^{2+} uptake.

Effect of Different Free Ca^{2+} Concentrations on MgATP-Dependent $^{45}Ca^{2+}$ Uptake into Membrane Vesicles from ER

For further differentiation of the Ca^{2+} uptake mechanisms present in the vanadate-insensitive IsCaP and in the vanadate-sensitive IisCaP, we have studied Ca^{2+} dependence of MgATP-dependent $^{45}Ca^{2+}$ uptake into the IsCaP ($^{45}Ca^{2+}$ uptake in the presence of vanadate) as well as of MgATP-dependent $^{45}Ca^{2+}$ uptake into the vanadate-sensitive IisCaP ($^{45}Ca^{2+}$ uptake in the presence of NBD-Cl). As shown in Fig. 5*A*, within the initial 10 sec the IsCaP (in the

presence of vanadate, 1×10^{-4} mol/liter) took up $^{45}Ca^{2+}$ at higher Ca^{2+} concentrations, starting at $\sim 10^{-6}$ mol/liter as compared to the IisCaP (in the presence of NBD-Cl). Maximal Ca^{2+} uptake into the IsCaP during the initial 10 sec was observed at a Ca^{2+} concentration of 10^{-4} mol/liter free Ca^{2+}. Half-maximal $^{45}Ca^{2+}$ uptake was found at $\sim 2 \times 10^{-5}$ mol/liter free Ca^{2+} concentration. The IisCaP (in the presence of NBD-Cl; 5×10^{-5} mol/liter) started to take up Ca^{2+} at a low Ca^{2+} concentration of about 10^{-7} mol/liter with highest uptake at 10^{-5} mol/liter and was reduced at 10^{-4} mol/liter Ca^{2+}. Half-maximal $^{45}Ca^{2+}$ uptake was found at $\sim 7 \times 10^{-7}$ mol/liter. After 20 min of incubation with MgATP (Fig. 5*B*) the Ca^{2+} uptake curve for the IsCaP (in the presence of vanadate) had shifted to the left, indicating that after that time Ca^{2+} had also been taken up by the vanadate-insensitive Ca^{2+} pool (IsCaP) at lower Ca^{2+} concentrations between 10^{-8} and 10^{-6} mol/liter Ca^{2+}. Highest uptake into this Ca^{2+} pool was obtained at 10^{-5}–10^{-4} mol/liter. The other Ca^{2+} pool (vanadate-sensitive IisCaP as studied in the presence of NBD-Cl) still had its optimum at a Ca^{2+} concentration of 10^{-5} mol/liter after 20 min.

Discussion

The aim of the present study was to separate functionally different nonmitochondrial Ca^{2+} pools and to characterize their Ca^{2+} uptake mechanisms. Our data indicate that at least two different nonmitochondrial Ca^{2+} pools adjust free Ca^{2+} concentration in pancreatic acinar cells.

The IP_3-Sensitive Ca^{2+} Pool (IsCaP)

Part of total Ca^{2+} uptake is sensitive to IP_3 and to IPS_3. As shown in Fig. 1*a* and *d*, in the presence of IPS_3 less Ca^{2+} is taken up as compared to the control but the steady-state free $[Ca^{2+}]$ is reached in the presence of IPS_3. Ca^{2+} uptake into this IP_3-sensitive Ca^{2+} pool is inhibited by H^+-ATPase inhibitors such as NBD-Cl or NEM, since IP_3 did not release any Ca^{2+} from NBD-Cl- or NEM-pretreated cells. Furthermore, Ca^{2+} uptake curves in the presence of these inhibitors closely follow the Ca^{2+} uptake curve in the presence of IPS_3 (*see* Fig. 1*a* and *d*), indicating that a similar amount of Ca^{2+} had not been taken up. Since NBD-Cl did not inhibit IP_3-induced Ca^{2+} release when added at steady state (*see* Fig. 1*b*) this indicates that NBD-Cl did not inhibit the IP_3-sensitive Ca^{2+} release mechanism (*see* Fig. 1*a*). Similar data as for NBD-Cl added at steady state (*see* Fig. 1*b*) were also obtained for NEM (*not shown*). We have recently demonstrated the presence of an H^+ ATPase in pancreatic vesicles from endoplasmic reticulum (ER) [30]. This H^+ ATPase belongs to the vacuolar type of H^+ ATPases that has no phosphorylated intermediate and is not inhibited by vanadate. However, NBD-CL and NEM, typical blockers of the vacuolar type of H^+ ATPases, present in ER, Golgi, endosomes, clathrin-coated vesicles and lysosomes [1, 16, 20] inhibited H^+ uptake into pancreatic ER vesicles [30]. Since protonophores and H^+ ATPase inhibitors decreased Ca^{2+} uptake into an IP_3-releasable Ca^{2+} pool (*see* Figs. 1*a* and *d*, 2, 3, and the Table) we assume that Ca^{2+} uptake into the IsCaP is dependent on an H^+ gradient. This H^+ gradient could be established by the H^+ ATPase which we had found in the same preparation of ER membrane vesicles that was also used for the present Ca^{2+} uptake studies [30]. It should be noted, however, that it cannot be concluded from the present study that the H^+ pump found in an ER fraction of pancreatic acinar cells [30] is only located in IP_3-sensitive Ca^{2+} pools. As shown in Fig. 4, only part of H^+-dependent Ca^{2+} uptake is sensitive for IP_3. This could indicate the presence of Ca^{2+} pools with an H^+ ATPase and a Ca^{2+}/H^+ exchanger but without IP_3 receptors. In addition, it is quite possible that vacuolar type H^+ ATPases are located in organelles without any Ca^{2+} uptake mechanisms.

In order to explore if Ca^{2+} could be taken up into an IP_3-sensitive Ca^{2+} pool in the absence of ATP by coupling to an H^+ gradient, we have performed experiments in which vesicles had been preincubated in the presence of vanadate and EDTA to inhibit any Ca^{2+} uptake, but in the presence of ATP to allow H^+ uptake (*see* Fig. 3). When glucose and hexokinase were added to hydrolyse all ATP in the medium, added $^{45}Ca^{2+}$ was taken up at a higher amount as compared to $^{45}Ca^{2+}$ uptake into vesicles which had not been preincubated with ATP or had been preincubated with ATP and NEM or NBD-Cl. In the presence of IPS_3, $^{45}Ca^{2+}$ uptake was decreased, indicating that the Ca^{2+} pool, which takes up Ca^{2+} in the absence of ATP at the expense of an H^+ gradient, is sensitive to IP_3. The ATP-independent, H^+-gradient-dependent Ca^{2+} uptake was only $\sim$25% of average ATP-dependent Ca^{2+} uptake in the presence of vanadate (Table, Fig. 4). The reason for this discrepancy could be due to quick dissipation of the H^+ gradient in the absence of ATP [30]. It could also be due to the possibility that a Ca^{2+}/H^+ exchanger could operate as a Ca^{2+}/H^+ ATPase in the presence of ATP. The latter point was investigated in detail in the experiments shown in Fig. 4*a* and *b*. If membrane vesicles had not been preincubated with ATP, and ATP together with $^{45}Ca^{2+}$ was then added to the medium, $^{45}Ca^{2+}$ uptake was

slower than in vesicles which had been preincubated with ATP (Fig. 4*a*). Since it takes ~20 min until H^+ uptake reaches a steady state [30], this experiment could indicate that $^{45}Ca^{2+}$ uptake is slower as long as an H^+ gradient has not been built up as compared to $^{45}Ca^{2+}$ uptake in the presence of a preformed H^+ gradient. A similar slow Ca^{2+} uptake was also seen in cells in the presence of vanadate (*see* Fig. 1*a*). The rate-limiting step for slow Ca^{2+} uptake in the presence of vanadate is probably formation of an H^+ gradient and not the Ca^{2+}/H^+ exchanger itself. If the H^+ gradient is present in vivo all the time and not dissipated by IP_3 (for which we have no evidence) Ca^{2+} uptake via Ca^{2+}/H^+ exchanger should be faster and similar to the conditions shown in Fig. 4*b* in which ATP was present in the preincubation periods. $^{45}Ca^{2+}$ uptake could be enhanced further if ATP was acutely added to vesicles with preformed H^+ gradient and an inhibited H^+ pump (*see* Fig. 4*b*). We therefore assume that the Ca^{2+}/H^+ exchanger can operate as an ATPase. This conclusion, although indirect, seems to be supported by the data showing some 10% remaining Ca^{2+} ATPase activity in the presence of vanadate plus NEM or NBD-Cl (Table). Other possibilities, such as an allosteric effect of ATP or an activation of the Ca^{2+}/H^+ exchanger due to protein phosphorylation, should also be considered.

The IP_3-Insensitive Ca^{2+} Pool (IisCaP)

In previous studies we had shown that ATP-driven Ca^{2+} uptake into nonmitochondrial Ca^{2+} pools is inhibited by vanadate [27]. This vanadate-inhibitable Ca^{2+} uptake is promoted by a Mg-dependent Ca^{2+} ATPase that shows all properties of a Ca^{2+} transport ATPase and which is also inhibited by vanadate [13].

In the presence of vanadate (10^{-4} mol/liter) Ca^{2+} uptake was markedly reduced (Figs. 1*a* and *d*, Table); however, in contrast to Ca^{2+} uptake in the presence of NBD-Cl or NEM, IP_3 was still able to release Ca^{2+}, indicating that Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} was still operating in the presence of 10^{-4} mol/liter vanadate. At 10^{-3} mol/liter vanadate, however, Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} pool was further reduced and could not be detected any more by use of the Ca^{2+} electrode (Fig. 1*a*). If both vanadate (10^{-4} mol/liter) plus NBD-Cl (*see* Fig. 1*a*, Table), or vanadate plus NEM (*see* Fig. 1*d*, Table) were added, Ca^{2+} uptake was abolished. In the presence of both vanadate and IPS_3 a small NBD-Cl and NEM-inhibitable Ca^{2+} uptake remained, indicating Ca^{2+} uptake into a third Ca^{2+} pool with an H^+-dependent Ca^{2+} uptake but lacking an IP_3 receptor. It could also mean that IPS_3 or IP_3, respectively, do not release all Ca^{2+} from an IP_3-sensitive Ca^{2+} pool. Since Ca^{2+} uptake in the presence of both vanadate and an H^+ ATPase inhibitor was abolished, but some 10% of Ca^{2+} ATPase activity remained (*see* Table), we conclude that this 10% of Ca^{2+} ATPase activity is either no transport Ca^{2+} ATPase or present in broken membrane vesicles, or that 10% of remaining Ca^{2+} ATPase activity is not sufficient to promote any measurable Ca^{2+} uptake with the Ca^{2+} electrode. This remaining 10% of Ca^{2+} ATPase activity in the presence of both vanadate and NEM or NBD-Cl could also be due to ATPase activity of the Ca^{2+}/H^+ exchanger (*see* Fig. 4*b*).

Different Ca^{2+} Uptake Characteristics in Both IsCaP and IisCaP

In order to further characterize Ca^{2+} uptake into both IsCaP and IisCaP we have studied Ca^{2+} uptake at different Ca^{2+} concentrations in the presence of NBD-Cl to inhibit Ca^{2+} uptake into the IsCaP and in the presence of vanadate, to inhibit Ca^{2+} uptake into the IisCaP. As shown in Fig. 5, in the presence of vanadate Ca^{2+} is only taken up at relatively high Ca^{2+} concentrations, starting at 10^{-6} mol/liter. This observation could indicate that the IsCaP is only filled at Ca^{2+} concentrations that occur during hormonal stimulation in the cytosol of the cell, i.e., at ~10^{-6} mol/liter or higher [19]. Filling of the IsCaP at $[Ca^{2+}]$ around 10^{-6} mol/liter could be demonstrated by addition of IP_3 which caused Ca^{2+} release (*see* Fig. 1*a* and *d*). From Fig. 1*a* it appears that the IsCaP can also adjust steady-state Ca^{2+}, but this takes longer time than for the IisCaP. This observation is also expressed in the Ca^{2+} uptake curve in the presence of vanadate, which had shifted to the left after 20 min of incubation (*see* Fig. 5*B*). When Ca^{2+} uptake into the IsCaP is inhibited by NBD-Cl (*see* Fig. 5*A*) the remaining Ca^{2+} uptake occurs at low concentrations between 10^{-8} and 10^{-6} mol/liter $[Ca^{2+}]$. Consequently this Ca^{2+} pool (IisCaP) must be the one that rapidly adjusts the low $[Ca^{2+}]$ steady state that occurs in the cell during rest. It remains open, if this interpretation is applicable to in vivo conditions, since the difference in the time courses of Ca^{2+} uptake into the IsCaP and IisCaP is mainly due to the absence or presence of an H^+ gradient.

Are Both IsCaP and IisCaP Connected?

From the data presented it appears that both IsCaP and IisCaP are separate Ca^{2+} pools, e.g. in the presence of NBD-Cl, Ca^{2+} uptake takes place, but the

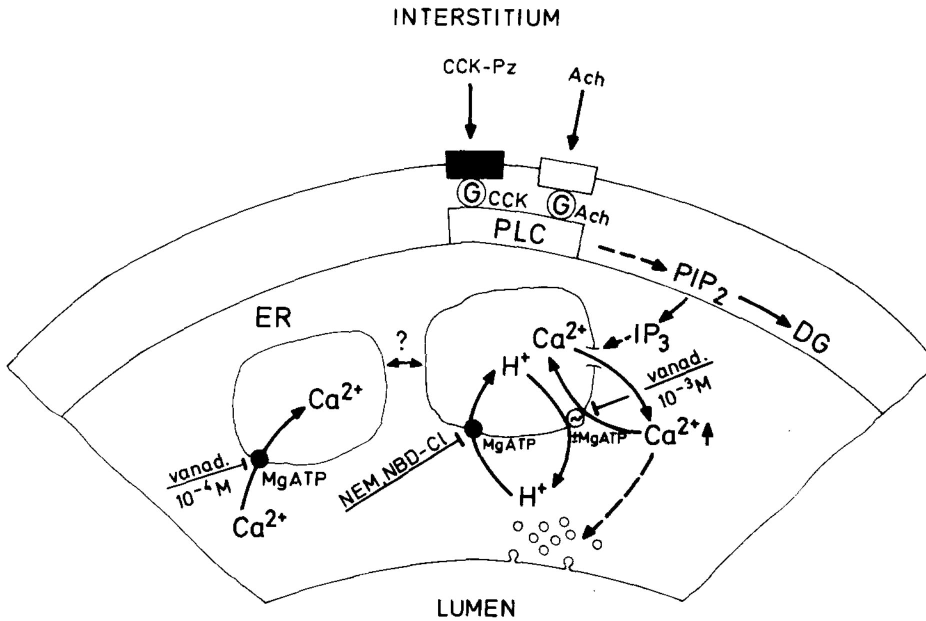


Fig. 6. Model for Ca^{2+} release and Ca^{2+} uptake into nonmitochondrial Ca^{2+} stores (probably belonging to the endoplasmic reticulum, *ER*). Phospholipase C (*PLC*) activation by hormones such as cholecystokinin-pancreozymin (*CCK-Pz*) or acetylcholine (*Ach*) is mediated by GTP-binding proteins (G_{CCK} and G_{Ach} [21, 22]). Phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed to diacylglycerol (*DG*) and inositol 1,4,5 trisphosphate (IP_3). IP_3 releases Ca^{2+} from a Ca^{2+} pool that leads to increase of cytosolic [Ca^{2+}] and activation of further processes involved in enzyme secretion. Ca^{2+} reuptake occurs into the same Ca^{2+} pool by a Ca^{2+}/H^+ exchanging ATPase. Ca^{2+} uptake can also occur in the absence of ATP at the expense of an H^+ gradient which is established by a NBD-Cl- and NEM-inhibitable H^+ pump present in the same organelle. Ca^{2+} uptake also occurs into another Ca^{2+} pool by a vanadate-sensitive Ca^{2+} ATPase. It is likely that both Ca^{2+} pools communicate, but the mechanism for this communication is not yet clear

IsCaP is not filled (*see* Fig. 1*a*), whereas in the presence of vanadate the IsCaP is filled. In the presence of both NBD-Cl plus vanadate, of NEM plus vanadate or of IPS_3 plus vanadate, Ca^{2+} uptake into both Ca^{2+} pools is nearly completely abolished. If total Ca^{2+} uptake would occur into one Ca^{2+} pool only, which is also sensitive for IP_3, one should expect that in the presence of NBD-Cl or of NEM the vanadate-sensitive Ca^{2+} pump should be able to fill this IP_3-sensitive Ca^{2+} pool and it should be possible to release Ca^{2+} with IP_3. On the other hand, it is difficult to imagine that a Ca^{2+} pool is filled but not emptied. It could be possible that there are other messengers which release Ca^{2+} from the IisCaP, or that both pools somehow communicate with each other. For the previous possibility metabolic products of IP_3, cAMP, diacylglycerol or GTP [18] would come into question. So far we have not found any compound of physiological relevance that releases Ca^{2+} from the IisCaP. Another possibility is that both Ca^{2+} pools are connected and that Ca^{2+} uptake by a vanadate-sensitive Ca^{2+} ATPase occurs at one end, whereas Ca^{2+} release at the other end of this Ca^{2+} pool, similar to that known from the sarcoplasmic reticulum with its terminal cisternae where Ca^{2+} is released, and the longitudinal tubules where the Ca^{2+} pumps are located [9]. The possibility that the different Ca^{2+} pools are permanently connected is unlikely since in this case one would expect an IP_3 effect following Ca^{2+} uptake in the presence of H^+ ATPase inhibitors or protonophores (*See* Figs. 1*a*, *d* and 2). Cross talk between both IsCaP and IisCaP could involve IP_4 and/or GTP-controlled conveyance of Ca^{2+} [14, 17, 18]. In a previous paper [32] we have suggested that in parotid acinar cells only one Ca^{2+} pool is involved and that the vanadate-sensitive Ca^{2+} ATPase transports Ca^{2+} in exchange for H^+ and that in the absence of ATP or in the presence of vanadate this Ca^{2+} uptake mechanism can operate to some extent at the expense of an H^+ gradient. This conclusion was drawn from the observation that the inhibitory effects of both vanadate and protonophores were more than additive. In the presence of either compound Ca^{2+} uptake was reduced by ~70% following 20 min of incubation. We did not, however, exclude two separate mechanisms for Ca^{2+} uptake into nonmitochondrial Ca^{2+} pools of parotid acinar cells. In vacuolar membrane vesicles of oat roots also two separate Ca^{2+} pools have been postulated [23]. In the present study we have used lower vanadate concentrations to inhibit Ca^{2+} uptake and are inclined

to assume different Ca^{2+} pools (IsCaP and IisCaP). However, at higher vanadate concentrations (10^{-3} or 2×10^{-3} mol/liter) at which Ca^{2+} uptake was still present in parotid cells, Ca^{2+} uptake is completely inhibited in pancreatic acinar cells ([15], Fig. 1*a*). It therefore appears that in the pancrease 10^{-3} mol/liter vanadate also decreases Ca^{2+} uptake into the IsCaP. Since vanadate does not inhibit the H^+ ATPase [30], it is possible that the Ca^{2+}/H^+ exchanger is an ATPase (for which evidence is given in Fig. 4*b*), and that this Ca^{2+} ATPase is inhibited by vanadate at high concentration (i.e., $>10^{-3}$ mol/liter). With this assumption it would still be possible to assume two different Ca^{2+} pools (an IsCaP and an IisCaP) as shown in the model of Fig. 6. Since at present we are not able to isolate and to separate different Ca^{2+} pools from pancreatic acinar cells by fractionation methods, we cannot directly demonstrate different Ca^{2+} uptake mechanisms and different sensitivities of inhibitors in different Ca^{2+} pools. However, we think that these functional studies as reported here might help to further characterize intracellular Ca^{2+} pools and their Ca^{2+} uptake mechanisms.

We wish to thank Prof. Dr. K.J. Ullrich for helpful discussions. We gratefully acknowledge financial support from SERC and the Research Corporation Trust. B.V.L.P. is a Lister Institute Research Fellow.

F.T. was supported by the "Jung-Stiftung für Wissenschaft und Forschung" and "Deutsche Forschungsgemeinschaft" (Th 345/1-1). T.P.K. was supported by "Deutsche Forschungsgemeinschaft" (Ke 354/1-1).

References

1. Al-Awqati, Q. 1986. Proton-translocating ATPases. *Annu. Rev. Cell Biol.* **2:**179–199
2. Amsterdam, A., Jamieson, J.D. 1972. Structural and functional characterization of isolated pancreatic exocrine cells. *Proc. Natl. Acad. Sci. USA* **69:**3028–3032
3. Bais, R. 1975. A rapid and sensitive radiometric assay for adenosine triphosphatase activity using cerenkov radiation. *Anal. Biochem.* **63:**271–273
4. Bayerdörffer, E., Streb, H., Eckhardt, L., Haase, W., Schulz, I. 1984. Characterization of calcium uptake into rough endoplasmic reticulum of rat pancreas. *J. Membrane Biol.* **81:**69–82
5. Berridge, M.J., Irvine, R.F. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (London)* **312:**315–321
6. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254
7. Breemen, C., van, Cauvin, C., Johns, A., Leijten, P., Yamamoto, H. 1986. Ca^{2+} regulation of vascular smooth muscle. *Fed. Proc.* **45:**2746–2751
8. Burgess, G.M., Irvine, R.F., Berridge, M.J., McKinney, J.S., Putney, J.W., Jr. 1984. Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes. *Biochem. J.* **224:**41–746
9. Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56:**395–433
10. Cooke, A.M., Gigg, R., Potter, B.V.L. 1987. Myo-inositol 1,4,5-tris-phosphorothioate: A novel analogue of a biological second messenger. *J. Chem. Soc. Commun.* **698:**1525–1526
11. Cooke, A.M., Nahorski, S.R., Potter, B.V.L. 1989. *FEBS Lett. (in press)*
12. Haynes, D.H. 1983. Mechanism of Ca^{2+} transport by Ca^{2+}-Mg^{2+}-ATPase pump: Analysis of major states and pathways. *Am. J. Physiol.* **244:**G3–G12
13. Imamura, K., Schulz, I. 1985. Phosphorylated intermediate of (Ca^{2+} + K^+)-stimulated Mg^{2+}-dependent transport ATPase in endoplasmic reticulum from rat pancreatic acinar cells. *J. Biol. Chem.* **260:**11339–11347
14. Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.M., Wreggett, K.A. 1988. Inositol phosphates: Proliferation, metabolism and function. *Phil. Trans. R. Soc. (London) B* **320:**281–298
15. Kemmer, T.P., Bayerdörffer, E., Will, H., Schulz, I. 1987. Anion dependence of Ca^{2+} transport and (Ca^{2+} + K^+)-stimulated Mg^{2+}-dependent transport ATPase in rat pancreatic endoplasmic reticulum. *J. Biol. Chem.* **262:**13758–13764
16. Mellman, I., Fuchs, R., Helenius, A. 1986. Acidification of the endocytotic and exocytotic pathways. *Annu. Rev. Biochem.* **55:**663–700
17. Mullaney, J.M., Chueh, S.-H., Ghosh, T.K., Gill, D.L. 1987. Intracellular calcium uptake activated by GTP. *J. Biol. Chem.* **262:**13865–13872
18. Mullaney, J.M., Yu, M., Ghosh, T.K., Gill, D.L. 1988. Calcium entry into the inositol 1,4,5-trisphosphate-releasable calcium pool is mediated by a GTP-regulatory mechanism. *Proc. Natl. Acad. Sci. USA* **85:**2499–2503
19. Ochs, D.L., Korenbrot, J.I., Williams, J.A. 1983. Intracellular free calcium concentrations in isolated pancreatic acini: Effects of secretagogues. *Biochem. Biophys. Res. Commun.* **117:**122–128
20. Rudnick, G. 1986. ATP-driven H^+ pumping into intracellular organelles. *Annu. Rev. Physiol.* **48:**403–413
21. Schäfer, R., Christian, A.-L., Schulz, I. 1988. Photoaffinity labeling with GTP-γ-azidoanilide of a cholera toxin-sensitive 40 kDa protein from pancreatic acinar cells. *Biochem. Biophys. Res. Commun.* **155:**1051–1059
22. Schnefel, S., Banfić, H., Eckhardt, L., Schultz, G., Schulz, I. 1988. Acetylcholine and cholecystokinin receptors functionally couple by different G-proteins to phospholipase C in pancreatic acinar cells. *FEBS Lett.* **230:**125–130
23. Schumaker, K.S., Sze, H. 1985. A Ca^{2+}/H^+ antiport system driven by the proton electrochemical gradient of a tonoplast H^+-ATPase from oat roots. *Plant Physiol.* 1111–1117
24. Schumaker, K.S., Sze, H. 1987. Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. *J. Biol. Chem.* **262:**3944–3946
25. Streb, H., Bayerdörffer, E., Haase, W., Irvine, R.F., Schulz, I. 1984. Effect of inositol-1,4,5-trisphosphate on isolated subcellular fractions of rat pancreas. *J. Membrane Biol.* **81:**241–253
26. Streb, H., Irvine, R.F., Berridge, M.J., Schulz, I. 1983. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature (London)* **306:**67–69
27. Streb, H., Schulz, I. 1983. Regulation of cytosolic free Ca^{2+} concentration in acinar cells of rat pancreas. *Am. J. Physiol.* **245:**G347–G357

28. Taylor, C.W., Berridge, M.J., Brown, K.D., Cooke, A.M., Potter, B.V.L. 1988. *Myo*-inositol 1,4,5-trisphosphorothioate mobilizes intracellular calcium in Swiss 3T3 cells and *Xenopus* oocytes. *Biochem. Biophys. Res. Commun.* **150**:626–632
29. Taylor, C.W., Berridge, M.J., Cooke, A.M., Potter, B.V.L. 1989. Inositol 1,4,5-trisphosphorothioate: A stable analogue of inositol 1,4,5-trisphosphate which mobilizes intracellular calcium. *Biochem. J. (in press)*
30. Thévenod, F., Kemmer, T.P., Christian, A.L., Schulz, I. 1989. Characterization of MgATP-driven H^+ uptake into a microsomal vesicle fraction from rat pancreatic acinar cells. *J. Membrane Biol.* **107**:263–275
31. Thévenod, F., Schulz, I. 1988. H^+-ion dependent calcium uptake into an inositol 1,4,5-trisphosphate sensitive calcium pool from rat parotid gland. *Am. J. Physiol.* **255**:G429–G440
32. Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., Lew, D.P. 1988. "Calciosome," a cytoplasmic organelle: The inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? *Proc. Natl. Acad. Sci. USA* **85**:1091–1095
33. Willcocks, A.L., Potter, B.V.L., Cooke, A.M., Nahorski, S.R. 1988. *Myo*-inositol 1,4,5-trisphosphorothioate binds to specific ^3H-inositol 1,4,5-trisphosphate sites in rat cerebellum and is resistant to 5-phosphatase. *Eur. J. Pharmacol.* **155**:181–183

Received 8 December 1988