J. Membrane Biol. 144, 111–120 (1995)

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 1995

Characterization of Two Different Ca^{2+} Uptake and IP_3-sensitive Ca^{2+} Release Mechanisms in Microsomal Ca^{2+} Pools of Rat Pancreatic Acinar Cells

T. Ozawa,* F. Thévenod, I. Schulz

II. Physiologisches Institut der Universität des Saarlandes 66421 Homburg (Saar), Federal Republic of Germany

Received: 29 July 1994/Revised: 11 November 1994

Abstract. We have examined the effect of the Ca^{2+} (Mg^{2+})-ATPase inhibitors thapsigargin (TG) and vanadate on ATP-dependent $^{45}Ca^{2+}$ uptake into IP_3-sensitive Ca^{2+} pools in isolated microsomes from rat pancreatic acinar cells. The inhibitory effect of TG was biphasic. About 40–50% of total Ca^{2+} uptake was inhibited by TG up to 10 nM (apparent $K_i \approx 4.2$ nM, Ca^{2+} pool I). An additional increase of inhibition up to 85–90% of total Ca^{2+} uptake could be achieved at 15 to 20 nM of TG (apparent $K_i \approx 12.1$ nM, Ca^{2+} pool II). The rest was due to TG-insensitive contaminating plasma membranes and could be inhibited by vanadate (apparent $K_i \approx 10$ μM). In the absence of TG, increasing concentrations of vanadate also showed two phases of inhibition of microsomal Ca^{2+} uptake. About 30–40% of total Ca^{2+} uptake was inhibited by 100 μM of vanadate (apparent $K_i \approx 18$ μM, Ca^{2+} pool II). The remaining 60–70% could be inhibited either by vanadate at concentrations up to 1 mM (apparent $K_i \approx 300$ μM) or by TG up to 10 nM (Ca^{2+} pool I). The amount of IP_3-induced Ca^{2+} release was constant at ≈25% over a wide range of Ca^{2+} filling. About 10–20% remained unreleasable by IP_3. Reduction of IP_3-releasable Ca^{2+} in the presence of inhibitors showed similar dose-response curves as Ca^{2+} uptake (apparent $K_i \approx$ 3.0 nM for IP_3-induced Ca^{2+} release as compared to ≈4.2 nM for Ca^{2+} uptake at TG up to 10 nM) indicating that the highly TG-sensitive Ca^{2+} pump fills the IP_3-sensitive Ca^{2+} pool I. At TG concentrations >10 nM which blocked Ca^{2+} pool II the apparent K_i values were ≈11.3 and ≈12.1 nM, respectively. For inhibition by vanadate up to 100 μM the apparent K_i values were ≈18 μM for Ca^{2+} uptake and ≈7 μM for Ca^{2+} release (Ca^{2+} pool II). At vanadate concentrations up to 1 mM the apparent K_i values were ≈300 and ≈200 μM, respectively (Ca^{2+} pool I). Both Ca^{2+} pools I and II also showed different sensitivities to IP_3. Dose-response curves for IP_3 in the absence of inhibitors (control) showed an apparent K_m value for IP_3 at 0.6 μM. In the presence of TG (inhibition of Ca^{2+} pool I) the curve was shifted to the left with an apparent K_m for IP_3 at 0.08 μM. In the presence of vanadate (inhibition of Ca^{2+} pool II), the apparent K_m for IP_3 was 2.1 μM. These data allow the conclusion that there are at least three different Ca^{2+} uptake mechanisms present in pancreatic acinar cells: TG- and IP_3-insensitive but highly vanadate-sensitive Ca^{2+} uptake occurs into membrane vesicles derived from plasma membranes. Two Ca^{2+} pools with different TG-, vanadate- and IP_3-sensitivities are most likely located in the endoplasmic reticulum at different cell sites, which could have functional implications for hormonal stimulation of pancreatic acinar cells.

Key words: Thapsigargin — Vanadate — Ca^{2+} pump — Ca^{2+} ATPase — SERCA — Endoplasmic reticulum

Introduction

We have previously shown the presence of three different Ca^{2+} pools in pancreatic acinar cells [9]: (i) an IP_3-sensitive Ca^{2+} pool; (ii) a caffeine-sensitive Ca^{2+} pool, both of which take up Ca^{2+} via a Ca^{2+} uptake mechanism which is suggested to be a Ca^{2+}/H^+ exchanger and largely insensitive to vanadate at 100 μM; (iii) a Ca^{2+} pool which is insensitive to both IP_3 and caffeine and which takes up Ca^{2+} via a vanadate (100 μM) inhibitable Ca^{2+} ATPase [13]. Thapsigargin (TG), a sequiterpene lactone inhibits ATP-dependent Ca^{2+} uptake into intracellular calcium pools in various cell types [19, 22, 24,

* *Present address:* Department of Physiology, Tohoku University, School of Medicine, Seiryocho 2, 980 Sendai, Japan

Correspondence to: I. Schulz

29] including pancreatic acinar cells [17]. In rat parotid acinar cells [12, 22] and in bovine adrenal chromaffin cells [8], TG at a high concentration of 2 μM appeared to act only on the IP_3-sensitive Ca^{2+} pool, an IP_3-insensitive Ca^{2+} pool remained unaffected. In other cells, such as neuronal [23, 28] and smooth muscle cells [4], TG also affected IP_3-insensitive Ca^{2+} pools. The TG concentrations used to obtain maximal inhibition of Ca^{2+} uptake were higher than 100 nM [4, 11, 22]. However, in rat brain microsomes total Ca^{2+} uptake was inhibited about 90% by TG at nanomolar concentrations, and only 40% of the TG-sensitive Ca^{2+} uptake was due to Ca^{2+} accumulation into an IP_3-sensitive Ca^{2+} pool [28]. The origin of the TG-resistant Ca^{2+} uptake and how IP_3-sensitive Ca^{2+} pools relate to TG-sensitive and insensitive Ca^{2+} pumps remained unclear. In the present study, we have examined the Ca^{2+} uptake and Ca^{2+} release properties of IP_3-sensitive Ca^{2+} pools using two inhibitors of Ca^{2+} ATPases: thapsigargin and vanadate.

Our findings indicate the presence of at least three different Ca^{2+} transport mechanisms in pancreatic acinar cells. Ca^{2+} pool I: (≈50% of total IP_3-sensitive Ca^{2+} pools) has a low sensitivity to IP_3 and contains an ATP driven Ca^{2+} pump with high sensitivity to TG and a low sensitivity to vanadate. Ca^{2+} pool II (≈40% of IP_3-sensitive Ca^{2+} pools) has a higher sensitivity to IP_3 and contains an ATP driven Ca^{2+} pump with lower TG and higher vanadate sensitivity than Ca^{2+} pool I. Ten to 15 percent of the total Ca^{2+} uptake is neither sensitive to TG up to 100 nM nor to IP_3, largely due to Ca^{2+} uptake into plasma membrane vesicles. This Ca^{2+} uptake could be blocked, however, by vanadate at a lower concentration (estimated $K_i \approx 10$ μM) than Ca^{2+} uptake into IP_3-sensitive Ca^{2+} pools. Half maximal inhibitory concentrations of vanadate in this range have been described for the plasma membrane Ca^{2+} ATPase of hepatocytes [1], cardiac sarcolemma and erythrocyte membranes [7], whereas those of thapsigargin and vanadate for Ca^{2+} uptake into Ca^{2+} pools I and II are similar to the half maximal inhibitory concentrations found for the sarcoendoplasmic reticulum Ca^{2+} ATPases SERCA 2 and SERCA 3, respectively [15, 16, 27].

Abbreviations

BSA:	bovine serum albumin
EDTA:	(ethylenediamine)tetraacetic acid
ER:	endoplasmic reticulum
HEPES:	N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid
IP_3:	D-myo-inositol 1,4,5-trisphosphate
Na_3VO_4:	sodium vanadate
TG:	thapsigargin

Materials and Methods

Creatine kinase was obtained from Boehringer (Mannheim, FRG). Adenosine trisphosphate dipotassium salt (K_2ATP), sodium orthovanadate (Na_3VO_4), EDTA, bovine serum albumin (BSA), creatine phosphate (disodium salt), D-myo-inositol trisphosphate (potassium salt) and thapsigargin were purchased from Sigma (Munich, FRG). Collagenase of *Clostridium histolyticum* was from Worthington (Freehold, NJ). Oligomycin was from Serva (Heidelberg, FRG). $^{45}CaCl_2$ (11–30 Ci/g) was purchased from New England Nuclear Chemicals (Dreieich, FRG).

Preparation of Pancreatic Microsomes

Pancreatic microsomal vesicles were prepared from isolated rat pancreatic acinar cells as described previously [3, 9]. Briefly, after centrifugation of cell homogenate in a "mannitol buffer" (pH 7.0) at 11,000 × *g*, the "fluffy layer" on top of the pellet was collected. This fraction is enriched about twofold in endoplasmic reticulum [20]. This microsomal fraction represents a heterogeneous population of vesicles unrelated to the polarity of the cell.

Preparation of Pancreatic Plasma Membranes

Purified "plasma membrane" vesicles were prepared using a $MgCl_2$ precipitation method [2]. Briefly, cell homogenate was suspended in a "mannitol buffer" containing 11 mM $MgCl_2$. Following precipitation of membranes in the presence of $MgCl_2$ and low-speed centrifugation (400 × *g* and 3,000 × *g*) purified plasma membranes were obtained by high-speed (25,000 × *g*) centrifugations of the supernatant. These procedures were repeated three times. Finally, the pellet of the third high-speed centrifugation at 25,000 × *g* was collected as purified "plasma membranes" and used for Ca^{2+} uptake. In some cases, a "fluffy layer" fraction was prepared from the 400 × *g* pellet of the $MgCl_2$ precipitate, washed in "mannitol buffer" and centrifuged at 11,000 × *g* to obtain a microsomal fraction with less contamination by plasma membranes than in the method described above [3]. The microsomal vesicles and "plasma membrane" vesicles were used immediately or kept frozen in liquid nitrogen until use. Protein concentration was determined by the method of Bradford [5] using BSA as a standard.

Measurement of $^{45}Ca^{2+}$ Uptake and $^{45}Ca^{2+}$ Release

Microsomal vesicles (1 mg protein) were preincubated for 15 min at 25°C in 1 ml of a buffer containing (mM): KCl 155, HEPES 5, $CaCl_2$ 0.0327 (corresponding to 0.002 free Ca^{2+} concentration), EDTA 0.2, $MgCl_2$ 2.90, (corresponding to 1.0 free Mg^{2+} concentration), oligomycin 0.01, creatine phosphate 10, creatine kinase 8 U/ml, and 1μCi/ml of $^{45}CaCl_2$ adjusted to pH 7.0 with Tris/HCl. Test substances or the solvents ($DMSO/H_2O$) used as controls were added from stock solutions in volumes not exceeding 0.4% vol/vol. $^{45}Ca^{2+}$ uptake was initiated by the addition of ATP (potassium salt) at a final concentration of 2 mM.

"Plasma membrane" vesicles (70 μg protein) were preincubated in 500 μl of a buffer containing 20 μCi/ml $^{45}CaCl_2$. To obtain maximal $^{45}Ca^{2+}$ uptake, the concentration of free calcium in the medium and the final concentration of ATP were increased to 0.01 and 5 mM, respectively [2]. Otherwise, the composition of the medium was the same as for "microsomal" $^{45}Ca^{2+}$ uptake. At indicated times, aliquots were removed from the incubation medium and vesicles were separated from the incubation medium by a rapid filtration technique [2, 3]. MgATP dependent $^{45}Ca^{2+}$ uptake into vesicles was calculated as the difference between $^{45}Ca^{2+}$ content in the presence and absence of ATP. IP_3 was added from stock solutions in a volume of 0.5% (vol/vol). To calculate IP_3-induced $^{45}Ca^{2+}$ release, $^{45}Ca^{2+}$ content of membrane vesicles was determined 10–20 min after addition of IP_3 and subtracted from steady

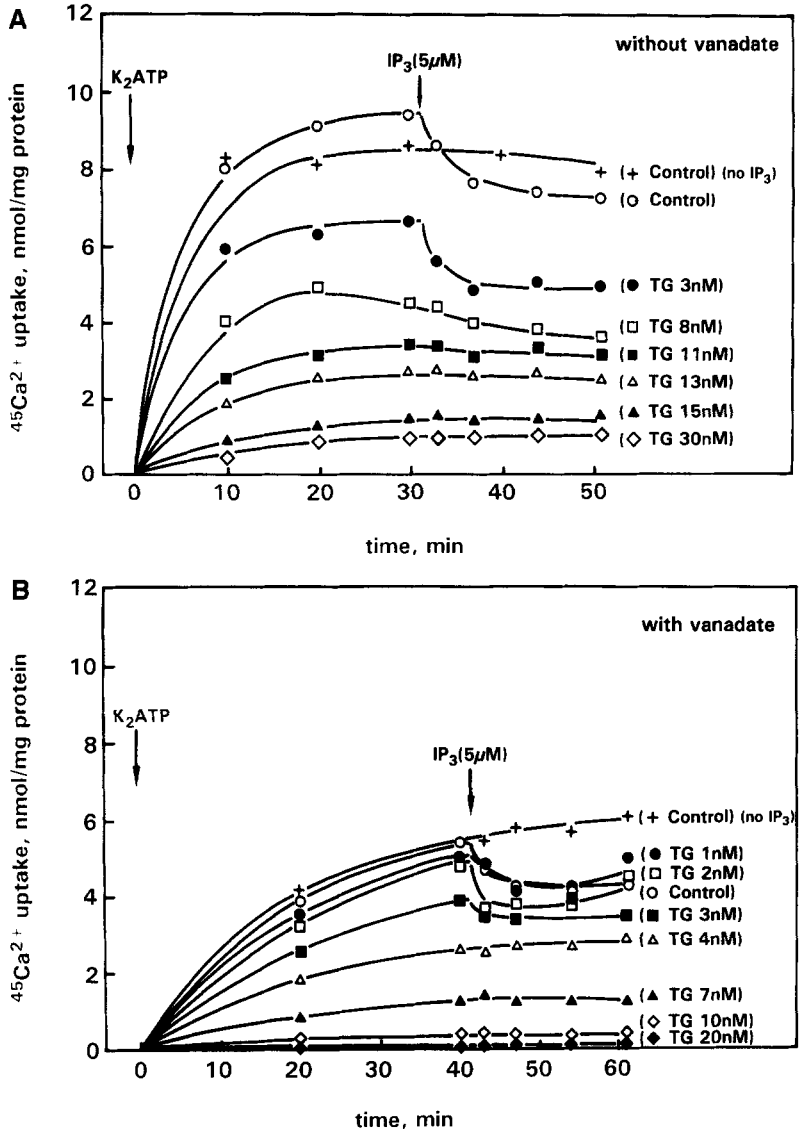


Fig. 1. Effect of thapsigargin (TG) on MgATP-induced $^{45}Ca^{2+}$ uptake and IP_3-induced $^{45}Ca^{2+}$ release in pancreatic microsomes. Vesicles (1 mg protein) were preincubated for 15 min in 1 ml of a KCl-HEPES buffer in the absence (*A*) or presence (*B*) of vanadate (100 μM) and in the absence or presence of indicated TG concentrations. $^{45}Ca^{2+}$ uptake was initiated by the addition of 2 mM $K_2ATP \cdot IP_3$ (5 μM) in 4 μl of incubation buffer that was added as indicated. In the control the same volume of buffer without IP_3 was added. $^{45}Ca^{2+}$ uptake in the absence of ATP is subtracted from $^{45}Ca^{2+}$ uptake in the presence of ATP. The experiments shown are representative of 3 to 17 similar experiments.

state $^{45}Ca^{2+}$ content before addition of IP_3. To account for nonspecific leakage of $^{45}Ca^{2+}$ from the vesicles control experiments were performed with addition of buffer instead of IP_3. The radioactivity was counted in a LKB 1214 Rackbeta liquid scintillation counter.

Results

Effects of Thapsigargin on $^{45}Ca^{2+}$ Uptake and the Size of IP_3-releasable $^{45}Ca^{2+}$ Pools in the Presence and Absence of Vanadate

Figure 1 shows that the rate of $^{45}Ca^{2+}$ uptake into microsomal vesicles is lower in the presence of vanadate (100 μM) (Fig. 1*B*) as compared to the control without vanadate (Fig. 1*A*). Furthermore, in microsomes steady state $^{45}Ca^{2+}$ uptake at 30–40 min is reduced to ≈70% of the control in the presence of vanadate (100 μM) (Fig. 1 and the Table). Thapsigargin inhibits $^{45}Ca^{2+}$ uptake in a

Table. Effect of vanadate on steady state level of $^{45}Ca^{2+}$ uptake in "microsomal" and "plasma membrane" vesicles

Vanadate concentration (μM)	$^{45}Ca^{2+}$ uptake into vesicles of			
	Microsomes (%)		Plasma membranes (%)	
0 (control)	100		100	
1		(ND)	79	(n = 2)
10	86	(n = 2)	53	(n = 2)
100	73 ± 8.7	(n = 4)	17	(n = 2)
200	39 ± 3.2	(n = 3)		(ND)
500	17	(n = 2)	9.7	(n = 1)
1000	7.5	(n = 1)		(ND)

Conditions for measurements of $^{45}Ca^{2+}$ uptake into microsomes and plasma membrane vesicles are described in Materials and Methods. $^{45}Ca^{2+}$ uptake is calculated as percent of control uptake. 100% is 10.6 ± 0.9 nmol/mg protein for microsomes ($n = 4$) and 9.9 nmol/mg protein for plasma membranes ($n = 2$). ND = not determined

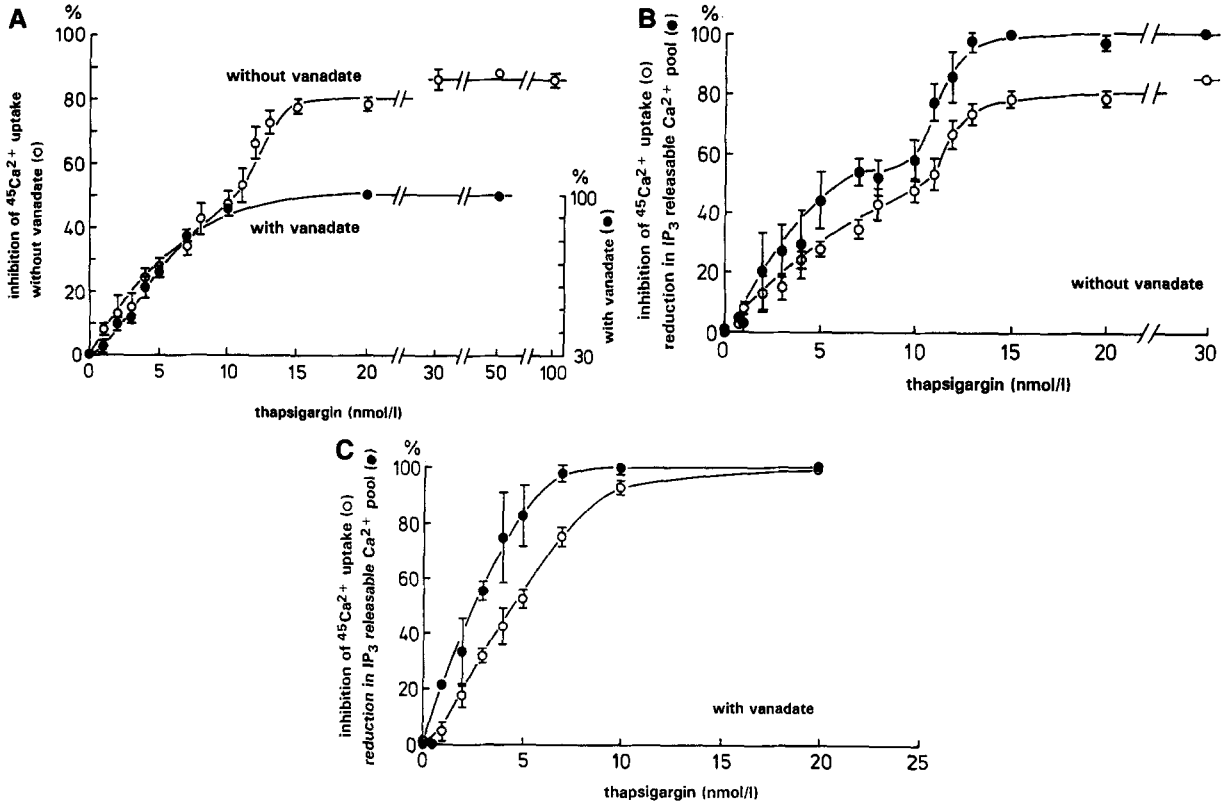


Fig. 2. (*A*) Effect of thapsigargin on $^{45}Ca^{2+}$ uptake in the presence (●) and absence (○) of vanadate (100 μM). Each point shows the mean value ± SE expressed as percent inhibition of steady state $^{45}Ca^{2+}$ uptake in controls (100%) measured at 30 min in 3 to 17 experiments of the type shown in Fig. 1. Values without SE are from 1 to 2 experiments. In the absence of both vanadate and thapsigargin the mean value of $^{45}Ca^{2+}$ uptake at 30 min in 17 experiments was 9.5 ± 0.7 nmol/mg protein (0% inhibition) and in the presence of 10 nM thapsigargin 5.7 ± 0.8 nmol/mg protein (n = 7). In the presence of vanadate about 30% of control $^{45}Ca^{2+}$ uptake was inhibited. The remaining mean $^{45}Ca^{2+}$ uptake at 30 min was 6.7 ± 0.6 nmol/mg protein in 14 experiments. This remaining Ca^{2+} uptake was inhibited by thapsigargin concentrations between 10 and 20 nM. (*B*) Comparison of the thapsigargin (TG) effect on $^{45}Ca^{2+}$ uptake (○) and on the size of the IP_3-releasable Ca^{2+} pool (●). Each point shows the mean value ± SE as percent inhibition of steady state $^{45}Ca^{2+}$ uptake in controls, i.e., without TG (○) at 30 min of IP_3-induced Ca^{2+} release in controls 30 to 50 min after addition of ATP in 3 to 17 experiments of the type shown in Fig. 1. Values without SE are from 1 to 2 experiments. The $^{45}Ca^{2+}$ uptake curve is the same as that shown in (*A*). IP_3-releasable $^{45}Ca^{2+}$ in control conditions (value for 0% inhibition) was 2.3 ± 0.2 nmol/mg protein in 17 experiments. At 10 nM of thapsigargin it was 1.1 ± 0.2 nmol/mg protein (n = 7) and at 13 nM of thapsigargin it was 0.1 ± 0.1 nmol/mg protein, (n = 4). (*C*) Effect of thapsigargin on $^{45}Ca^{2+}$ uptake (○) and on the size of the IP_3-releasable $^{45}Ca^{2+}$ pool (●) in the presence of vanadate (100 μM). Experiments were performed as described in the legend for Fig. 1*B*. Each value is the mean ± SE from 3 to 14 experiments. Values without SE are from 1 experiment. Values for Ca^{2+} uptake are taken from Fig. 2*A*. The mean value for IP_3-releasable Ca^{2+} in the presence of vanadate and without thapsigargin (0% inhibition) was 1.7 ± 0.2 nmol/mg protein, n = 14.

dose dependent manner both in the presence (Fig. 1*B*) and in the absence (Fig. 1*A*) of vanadate. In the presence of vanadate, nanomolar concentrations of TG strongly inhibit $^{45}Ca^{2+}$ uptake and complete inhibition is seen at 10 nM of TG (Fig. 1*B*). However, in the absence of vanadate, some 50% of $^{45}Ca^{2+}$ uptake still remains at 10 nM of TG (Figs. 1 and 2*A*). As shown in Fig. 2*A*, the TG effect is biphasic, inhibition further increases at TG concentrations higher than 10 nM and maximal inhibition of ≈85% of control $^{45}Ca^{2+}$ uptake is seen between 15 to 20 nM TG. Some 15% of Ca^{2+} uptake is TG-insensitive even at 100 nM of TG (Fig. 2*A*). This part of Ca^{2+} uptake can be released, however, in the presence of the Ca^{2+} ionophore A23187 (*data not shown*). We assume that this small amount of TG-insensitive Ca^{2+} uptake is due to Ca^{2+} uptake into plasma membrane vesicles (*see below*) contaminating the "fluffy layer" fraction [20], which is known to be TG-insensitive in human erythrocytes [24]. When inhibition of Ca^{2+} uptake at different TG concentrations is compared to the size of the IP_3-releasable Ca^{2+} pool (Fig. 2*B*), the first phase of ≈50–60% of total IP_3-releasable Ca^{2+} is abolished in the presence of 10 nM TG indicating that Ca^{2+} uptake with higher sensitivity to TG is responsible for the filling of ≈50–60% of IP_3-sensitive Ca^{2+} pools (Fig. 2*B*). Hill plot evaluation of this first phase shows an apparent $K_i \approx 4.3$ nM TG for Ca^{2+} uptake and of 3.2 nM for IP_3-releasable Ca^{2+} pool (*Hill plot not shown*). The second phases for

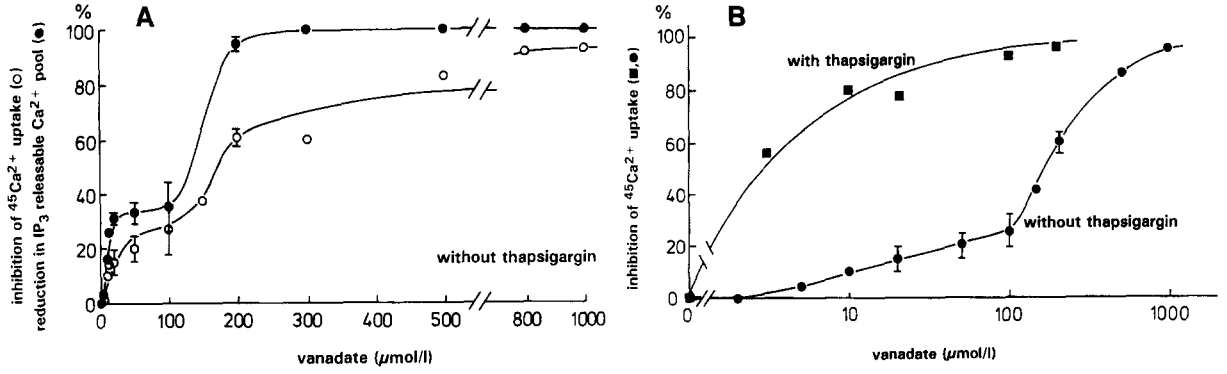


Fig. 3. (*A*) Comparison of the effect of vanadate on the size of the IP_3-releasable $^{45}Ca^{2+}$ pool (●) and $^{45}Ca^{2+}$ uptake (○) in the absence of thapsigargin. Zero percent inhibition was 10.6 ± 0.9 nmol/mg protein (n = 4) for $^{45}Ca^{2+}$ uptake and 2.6 ± 0.4 nmol/mg protein (n = 4) for $^{45}Ca^{2+}$ release. Data are mean values ± SE from 2 to 4 experiments. (*B*) Effect of vanadate on $^{45}Ca^{2+}$ uptake in the presence (■) and absence (●) of thapsigargin (TG 10 nM). The data in the presence of thapsigargin are from one experiment. Zero percent inhibition was 6.6 nmol/mg protein for $^{45}Ca^{2+}$ uptake with thapsigargin and 10.6 ± 0.9 nmol/mg protein (n = 4) for $^{45}Ca^{2+}$ uptake without thapsigargin. Data in the absence of thapsigargin are mean values ± SE from 2 to 4 experiments.

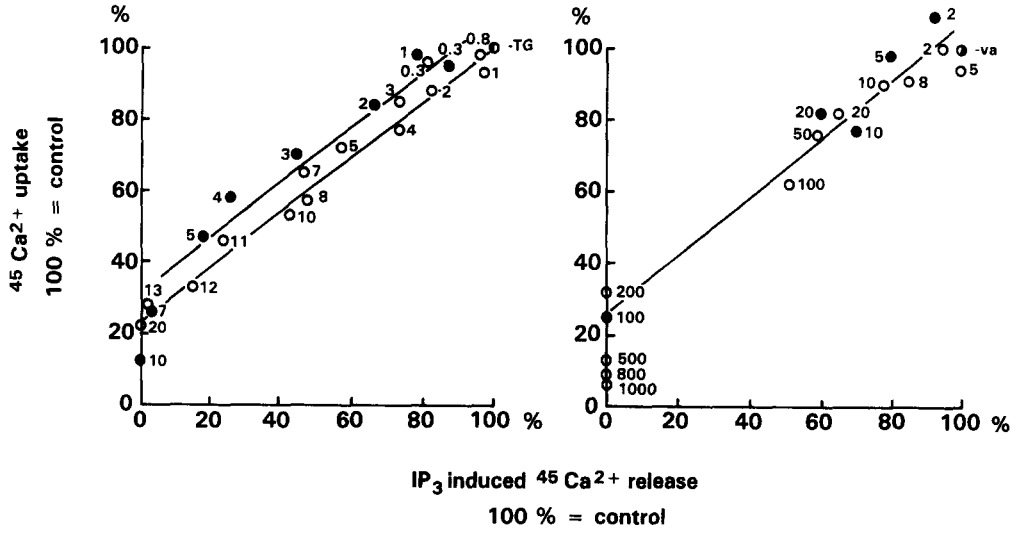


Fig. 4. Relationship between $^{45}Ca^{2+}$ uptake and the size of the IP_3-releasable $^{45}Ca^{2+}$ pool in the presence of different concentrations of thapsigargin (TG) or vanadate (Va). Left panel shows the effect of different *TG* concentrations (numbers represent inhibitor concentrations in nM) in the absence (○) or presence (●) of 100 μM vanadate. 100% = control $^{45}Ca^{2+}$ uptake or IP_3-releasable $^{45}Ca^{2+}$ without thapsigargin in the absence or presence of 100 μM vanadate. Right panel shows the effect of different vanadate concentrations (numbers in μM) in the absence (○) or presence (●) of 10 nM thapsigargin. 100% = control $^{45}Ca^{2+}$ uptake or IP_3-releasable $^{45}Ca^{2+}$ without vanadate in the absence or presence of 10 nM thapsigargin.

both inhibition of Ca^{2+} uptake and reduction in IP_3-induced Ca^{2+} release also show comparable apparent K_i values for TG (≈12.1 nM and ≈11.3 nM, respectively).

When Ca^{2+} uptake of the first phase of inhibition by TG in the absence of vanadate is compared to Ca^{2+} uptake in the presence of 100 μM vanadate, TG dose dependencies for both Ca^{2+} uptake curves are the same (*see* Fig. 2*A*, apparent K_i ≈ 4.3 and 4.2 nM TG in the absence and presence of vanadate, respectively). As shown in Fig. 2*C*, in the presence of 100 μM vanadate IP_3-induced Ca^{2+} release is abolished at 10 nM TG indicating that the IP_3-releasable Ca^{2+} pools had not been filled at 10 nM TG. Apparent K_i values of ≈4.2 nM TG for Ca^{2+} uptake and ≈3.0 nM for IP_3-induced Ca^{2+} release are similar to apparent K_i values for the first phases in the absence of vanadate (*see* Fig. 2*B*). This indicates that Ca^{2+} uptake into the IP_3-releasable Ca^{2+} pools with high sensitivity to TG is largely insensitive to vanadate at a concentration of 100 μM.

Effects of Vanadate on $^{45}Ca^{2+}$ Uptake and the Size of IP_3-releasable $^{45}Ca^{2+}$ Pools in the Presence and Absence of Thapsigargin

In the absence of TG, increasing concentrations of vanadate also show two phases of inhibition for both Ca^{2+}

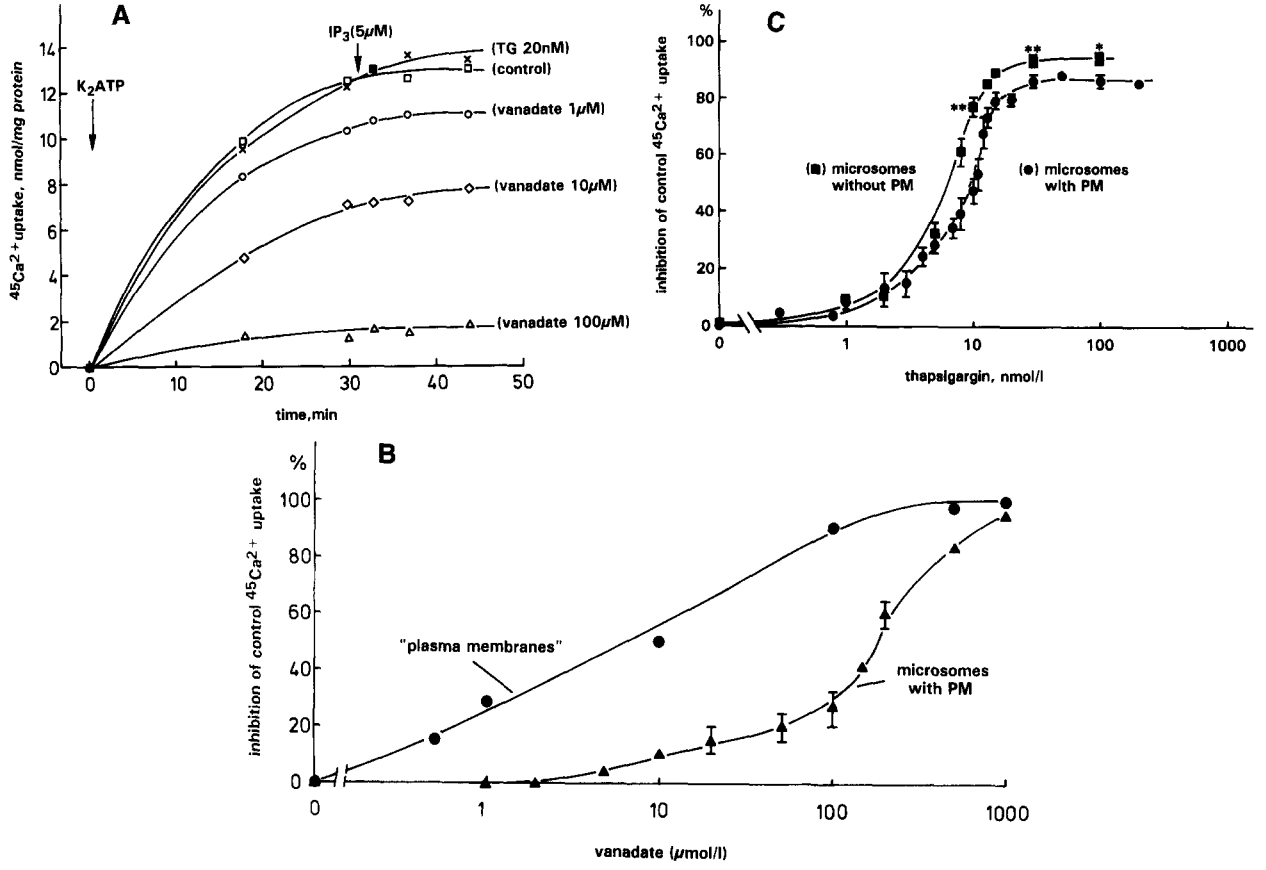


Fig. 5. (*A*) $^{45}Ca^{2+}$ uptake into vesicles from a plasma membrane fraction purified as described in Materials and Methods in the presence of 20 nM thapsigargin or of vanadate at indicated concentrations. IP_3 (5 μM) was added to vesicles, where indicated. One representative experiment is shown out of three similar ones. (*B*) Effect of vanadate on $^{45}Ca^{2+}$ uptake in pancreatic microsomes with contaminating plasma membranes PM (▲) and in isolated "plasma membrane" vesicles (●). Plasma membranes were purified as described in Materials and Methods. Data for $^{45}Ca^{2+}$ uptake are mean values from two separate experiments of the type shown in Fig. 5*A*. The Ca^{2+} uptake curve for microsomes is the same as that shown in Fig. 3*A*. (*C*) Effect of thapsigargin on $^{45}Ca^{2+}$ uptake into microsomes ("fluffy layer") and into the same fraction depleted in "plasma membranes." "Plasma membranes" and microsomes without "plasma membranes" were prepared as described in Materials and Methods. $^{45}Ca^{2+}$ uptake at 30 min was 9.5 ± 0.7 nmol/mg protein in microsomes with "plasma membranes" and 7.8 ± 1.1 nmol/mg protein without "plasma membranes" (0% inhibition). Mean values ± SE from 3 to 14 experiments; significant differences of $^{45}Ca^{2+}$ uptake between microsomes with and without "plasma membranes" at the same thapsigargin concentration were calculated using Student's *t*-test for unpaired values ($*P < 0.05$ $**P < 0.01$).

uptake and of reduction in the size of IP_3-releasable Ca^{2+} pools. A plateau is indicated at ≈100 μM vanadate (Fig. 3*A*) at which concentration about 30% of both Ca^{2+} uptake and IP_3-induced Ca^{2+} release are reduced (first phase with apparent K_i values of ≈18 μM and ≈7 μM, respectively; Fig. 3*A*). Ca^{2+} uptake and the IP_3-releasable Ca^{2+} pool can be abolished by vanadate concentrations >200 μM (second phase, *see* Fig. 3*A*). Apparent K_i values of vanadate for the second phases are ≈300 μM for Ca^{2+} uptake and ≈200 μM for IP_3-releasable Ca^{2+}. Both Ca^{2+} uptake and IP_3-induced Ca^{2+} release with low sensitivity to vanadate are highly sensitive to TG (*see* Fig. 2*C*). In the presence of 10 nM TG, a shift of the Ca^{2+} uptake curve to the left is seen (Fig. 3*B*). This indicates that in the presence of 10 nM TG, the remaining Ca^{2+} uptake with low sensitivity to TG has a higher sensitivity to vanadate than the highly TG-sensitive part of Ca^{2+} uptake (*see* Fig. 2*A* and *B*). Under these conditions, complete inhibition of Ca^{2+} uptake can be obtained by vanadate at concentrations between 100 and 200 μM and the apparent K_i value of ≈3 μM is similar to that of the first, highly vanadate-sensitive phase of Ca^{2+} uptake without TG (*see* Fig. 3*A* and *B*).

Filling of Ca^{2+} Pools is Linearly Correlated to IP_3-releasable Ca^{2+}

A replot of the data of Figs. 2 and 3 shows that IP_3-induced $^{45}Ca^{2+}$ release is linearly correlated to the filling of the Ca^{2+} pools, whether or not TG or vanadate are used to inhibit Ca^{2+} uptake (Fig. 4). About 20% of Ca^{2+} is taken up but not released by IP_3. This indicates that the size of the Ca^{2+} pools determines the amount of

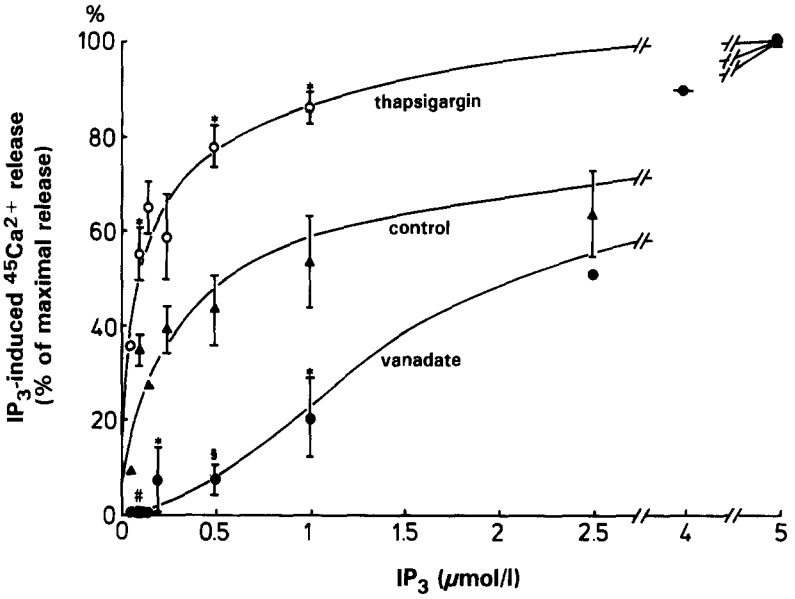


Fig. 6. Dose-response curves for IP_3-induced $^{45}Ca^{2+}$ release in the absence of inhibitors (control ▲) and presence of 10 nM thapsigargin (○) or 100 μM vanadate (●). IP_3-induced Ca^{2+} release was calculated from the difference between mean $^{45}Ca^{2+}$ uptake at steady state before addition of IP_3 (collected in 1 min intervals at 27–30 min after addition of ATP) and mean $^{45}Ca^{2+}$ content 2–6 min (five values determined in 1-min intervals) following addition of IP_3. Points are mean values ± SEM from 3–5 and without SEM from two separate experiments. #$P < 0.001$, §$P < 0.025$, *$P < 0.05$ using students t-test for paired comparison of controls versus thapsigargin or of controls versus vanadate at the same IP_3-concentration.

IP_3-releasable Ca^{2+} and that ≈20% of Ca^{2+} uptake either remains in the IP_3-sensitive Ca^{2+} pools or belongs to IP_3-insensitive Ca^{2+} uptake, part of which could be represented by plasma membranes [1, 7].

$^{45}Ca^{2+}$ Uptake into Plasma Membrane Vesicles

As shown in Fig. 2*A* and *B*, ≈15% of Ca^{2+} uptake is not inhibitable by thapsigargin even at the high concentration of 100 nM. It can be inhibited, however, by 100 μM vanadate (*see* Fig. 2*A*, *C*). The Ca^{2+} which had been taken up in the presence of a high thapsigargin concentration (100 nM) can be released by the Ca^{2+} ionophore A23187, but not by IP_3 (*not shown*). It is therefore likely that this small part of Ca^{2+} uptake is due to contaminating plasma membrane vesicles. Ca^{2+} uptake into purified "plasma membrane" vesicles is shown in Fig. 5*A*. Neither IP_3 nor TG have any effect on Ca^{2+} uptake into the "plasma membrane" fraction. However, vanadate is effective. As can be seen from Fig. 5*A*, 100 μM vanadate inhibits Ca^{2+} uptake by more than 80% indicating a higher vanadate sensitivity of Ca^{2+} uptake into plasma membrane vesicles than of microsomal Ca^{2+} uptake (Table). Replot of the data demonstrates a shift of the Ca^{2+} uptake curve to lower vanadate concentrations for purified "plasma membranes" as compared to microsomes containing plasma membranes (Fig. 5*B*). When plasma membranes are removed from the "fluffy layer" fraction, we observe stronger inhibition of Ca^{2+} uptake at the same TG concentration as compared to Ca^{2+} uptake into "fluffy layer" vesicles containing plasma membranes (Fig. 5*C*). In the presence of 30 nM of TG, the TG-insensitive component of $^{45}Ca^{2+}$ uptake into microsomes containing plasma membranes is significantly reduced from 14 ± 0.6% ($n = 3$) to 6.9 ± 1.2% ($n = 3$, $P < 0.01$) in microsomes without plasma membranes (*see* Fig. 5*C*). These results indicate that at least part of the TG-insensitive but vanadate-sensitive Ca^{2+} uptake into microsomal vesicles of the "fluffy layer" is due to Ca^{2+} uptake into plasma membrane vesicles.

Ca^{2+} Pools with Different Thapsigargin- and Vanadate Sensitivities have Different IP_3-sensitivities

In order to characterize further Ca^{2+} uptake into Ca^{2+} pools with different thapsigargin- and vanadate sensitivities, IP_3-induced $^{45}Ca^{2+}$ release was measured from membrane vesicles ("fluffy layer") at different IP_3-concentrations in the absence or presence of thapsigargin (10 nM) or of vanadate (100 μM). When maximal IP_3-induced $^{45}Ca^{2+}$ release is set to 100% for each condition tested (3.7 ± 0.9 nmol/mg protein for controls, 3.2 ± 1 nmol/mg protein in the presence of thapsigargin and 0.6 ± 0.1 nmol/mg protein in the presence of vanadate, $n = 3$), apparent K_m values for IP_3-induced $^{45}Ca^{2+}$ release are estimated to 0.6 μM for control, to 0.08 μM in the presence of thapsigargin and to 2.1 μM in the presence of vanadate (Fig. 6).

Discussion

Our previous studies on isolated pancreatic permeabilized acinar cells have shown the presence of IP_3-sensitive and IP_3-insensitive Ca^{2+} pools [9, 25]. We have now further characterized IP_3-sensitive Ca^{2+} pools in a microsomal fraction ("fluffy layer" of an 11,000 × g pellet) which has been previously shown to contain the

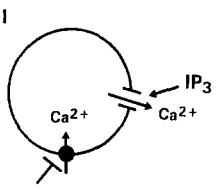
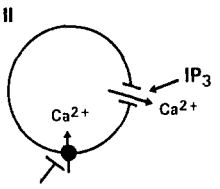
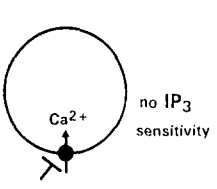
	microsomes (ER)		plasma membranes
	I 	II 	
sensitivity:	low vanadate high TG low IP_3	high vanadate low TG high IP_3	highest vanadate no TG no IP_3
% of total Ca^{2+} uptake	40 - 50 %	30 - 40 %	10 - 20 %
K_i TG for Ca^{2+} uptake IP_3 induced Ca^{2+} release	 4.2 nM 3.0 nM	 12.1 nM 11.3 nM	 - -
K_i vanadate for Ca^{2+} uptake IP_3 induced Ca^{2+} release	 300 μM 200 μM	 18 μM 7 μM	 10 μM -
K_m IP_3 for Ca^{2+} release	 2.1 μM	 80 nM	 -

Fig. 7. Model for Ca^{2+} pools in pancreatic membrane vesicles with different sensitivities to thapsigargin, vanadate and IP_3. For further explanations see text.

highest enrichment in IP_3-induced Ca^{2+} release [20]. In addition to vanadate, thapsigargin, an inhibitor of Ca^{2+} uptake into IP_3-sensitive Ca^{2+} pools [22, 24] and of Ca^{2+} (Mg^{2+}) ATPases in different cell types [19] has been used. The main finding of this study provides evidence for two different Ca^{2+} pools characterized by different sensitivities to the inhibitors of Ca^{2+} uptake, vanadate and thapsigargin, and different sensitivities of Ca^{2+} release to IP_3.

Two Different IP_3-sensitive Ca^{2+} Pools with Different Ca^{2+} Uptake and Ca^{2+} Release Mechanisms

As shown in Fig. 4, there is a linear relationship between Ca^{2+} uptake and IP_3-induced Ca^{2+} release, whether or not Ca^{2+} uptake is inhibited by thapsigargin or vanadate. This indicates that the Ca^{2+} that has been taken up into all IP_3-sensitive Ca^{2+} pools is released by IP_3 at the maximally effective concentration of 5 μM [21]. Since separation of microsomal membranes into different fractions representing distinct Ca^{2+} pools with different Ca^{2+} pumps could not be achieved, K_i values for Ca^{2+} uptake and Ca^{2+} release are only rough estimates that characterize these Ca^{2+} pools functionally. Emphasis is put more on evidence for differences in Ca^{2+} uptake than on correct determination of apparent K_i values. Furthermore, we have considered the possibility that the two distinct Ca^{2+} uptake mechanisms are located in separate Ca^{2+} pools with different sensitivities to IP_3. Figure 6 shows that in the presence of 10 nM thapsigargin, the remaining Ca^{2+} release is highly IP_3-sensitive with an apparent K_m value for IP_3 of 0.08 μM as compared to the control apparent K_m value of 0.6 μM. However, if the Ca^{2+} pool with high IP_3-sensitivity is blocked by vanadate (100 μM), Ca^{2+} release with low sensitivity to IP_3 is still present (K_m for IP_3 2.1 μM).

These results are interesting with respect to the recent observations that following hormonal stimulation of pancreatic acinar cells, initial Ca^{2+} release occurs at the luminal cell pole, which is followed by subsequent Ca^{2+} release from basal cell sites [14]. Thorn et al. have provided evidence for functional IP_3-receptors with high affinity to IP_3 in the secretory pole region and IP_3-receptors with a lower IP_3-affinity in the basal pole region [26]. The type 3 IP_3 receptor has been localized to the apex of pancreatic acinar cells by immunocytochemical studies, whereas the distribution of other IP_3-receptor subtypes in pancreas has not been established [18]. Figures 7 and 8 show models that combine recent findings [14, 18, 26] with our present data. We assume two IP_3-sensitive Ca^{2+} pools: a larger Ca^{2+} pool I (≈40–50% of total Ca^{2+} uptake) located at the basolateral cell side contains a Ca^{2+} pump with high sensitivity to thapsigargin (apparent $K_i \approx$ 3–4 nM), and low sensitivity to vanadate (apparent $K_i \approx$ 200–300 μM). The Ca^{2+} release mechanism of this Ca^{2+} pool has a low sensitivity to IP_3 (apparent $K_m \approx$ 2.1 μM). A smaller IP_3-sensitive Ca^{2+} pool II (30–40% of total Ca^{2+} uptake) is located at the

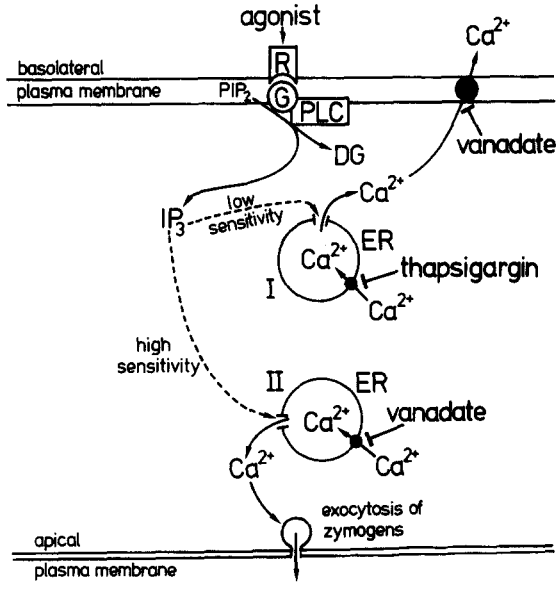


Fig. 8. Speculative model for apically and basolaterally located Ca^{2+} pools with different Ca^{2+} uptake and Ca^{2+} release mechanisms which combines recent findings with the present data. The apically located Ca^{2+} pool has a Ca^{2+} pump with a high vanadate and a low thapsigargin sensitivity and a Ca^{2+} release mechanism with a high sensitivity to IP_3. The basolaterally located Ca^{2+} pool has a Ca^{2+} pump with a high thapsigargin and low vanadate sensitivity and a low sensitivity to IP_3. For further details see text.

apical cell side. It has a Ca^{2+} pump with opposite sensitivities to inhibitors, i.e., high sensitivity to vanadate (apparent $K_i \approx 10$–20 μM) and low sensitivity to thapsigargin (apparent $K_i \approx 11$–12 nM). The Ca^{2+} release mechanism of the Ca^{2+} pool II has a high sensitivity to IP_3 (apparent $K_m \approx 0.08$ μM).

Is Ca^{2+} Uptake into Two Different Ca^{2+} Pools Brought about by Different Ca^{2+} ATPases?

We do not have yet any evidence for the types of Ca^{2+} ATPases, which could underly these Ca^{2+} uptake mechanisms. The half maximal inhibitory concentrations of vanadate for Ca^{2+} uptake are similar to those determined for sarcoendoplasmic reticulum Ca^{2+} ATPases SERCA 2 and SERCA 3 [15, 16, 27]. Thapsigargin specificity has been tested on all of the known intracellular-type calcium pumps of the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase family (SERCA) following expression of full-length cDNA clones encoding SERCA 1, SERCA 2a, SERCA 2b and SERCA 3 enzymes in COS cells [16]. Thapsigargin inhibited all of the SERCA isozymes with equal potency. Complete inhibition was obtained at 25 nM. In the studies by Lytton et al. [16] and Thastrup et al. [24], high concentrations of thapsigargin (up to 100 nM) were used without paying attention to smaller thapsigargin concentrations in the nanomolar range up to 10 nM. However, a study on Ca^{2+} stores of endoplasmic reticulum from rat brain, as in the present study, revealed maximal inhibitory concentrations of 10 nM with an IC_{50} value of 2 nM TG [28]. As shown in Fig. 2*A* an apparent K_i value of $\approx$ 3–4 nM can be evaluated from the first phase of Ca^{2+} uptake. This K_i value is further substantiated, when Ca^{2+} uptake is measured in the presence of 100 μM of vanadate which abolishes the second phase of TG-induced inhibition of Ca^{2+} uptake and leaves a Ca^{2+} uptake curve identical to the first phase of Ca^{2+} uptake. Recently, two isoforms of the SERCA-2b type Ca^{2+}, (Mg^{2+})-ATPase have been described in pancreatic endoplasmic reticulum [10]. However, no attempts have been made to determine whether they possess different functional properties and cellular localization [10]. Northern blotting of pancreatic tissue has demonstrated mRNA for SERCA types 2b and 3 [6]. It therefore appears likely that these Ca^{2+} ATPases are involved in Ca^{2+} uptake into IP_3-sensitive Ca^{2+} pools.

In addition to the two Ca^{2+} uptake mechanisms in intracellular microsomal membranes, we found a third Ca^{2+} pump with high sensitivity to vanadate ($K_i \approx 10$ μM) and no sensitivity to thapsigargin and IP_3. This pump is most likely located in the plasma membrane. Half maximal inhibitory concentration of vanadate for Ca^{2+} uptake at $\approx$10 μM (*see* Fig. 5*B*) has been described for plasma membrane Ca^{2+} ATPases of hepatocytes [1], cardiac sarcolemma and erythrocyte membrane [7]. Further studies will have to determine whether or not both Ca^{2+} uptake mechanisms in intracellular membranes as described in the present study can be assigned to different types of SERCA-type Ca^{2+} ATPases located in Ca^{2+} pools with different sensitivities to IP_3 and distinct locations in pancreatic acinar cells.

This work was supported by the "Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 246." The authors wish to thank Dr. Klaus-Dieter Preuß for valuable discussions and Mrs. Gabriele Mörschbächer for excellent secretarial help.

References

1. Bachs, O., Famulski, K.S., Mirabelli, F., Carafoli, E. 1985. ATP-dependent Ca^{2+} transport in vesicles isolated from the bile canalicular region of the hepatocyte plasma membrane. *Eur. J. Biochem.* **147**:1–7
2. Bayerdörffer, E., Eckhardt, L., Haase, W., Schulz, I. 1985. Electrogenic calcium transport in plasma membrane of rat pancreatic acinar cells. *J. Membrane Biol.* **84**:45–60
3. 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
4. Bian, J., Ghosh, T.K., Wang, J.-C., Gill, D.L. 1991. Identification of intracellular calcium pools: selective modification by thapsigargin. *J. Biol. Chem.* **266**:8801–8806
5. 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
6. Burk, S.E., Lytton, J., MacLennan, D.H., Shull, G.E. 1989. cDNA

cloning, functional expression, and mRNA tissue distribution of a third organellar Ca^{2+} pump. *J. Biol. Chem.* **264:**18561–18568

7. Carafoli, E., Zurini, M. 1982. The Ca^{2+}-pumping ATPase of plasma membranes: purification, reconstitution and properties. *Biochim. Biophys. Acta* **683:**279–301
8. Cheek, T.R., Thastrup, O. 1989. Internal Ca^{2+} mobilization and secretion in bovine adrenal chromaffin cells. *Cell Calcium* **10:**213–221
9. Dehlinger-Kremer, M., Zeuzem, S., Schulz, I. 1991. Interaction of caffeine-, IP_3- and vanadate-sensitive Ca^{2+} pools in acinar cells of the exocrine pancreas. *J. Membrane Biol.* **119:**85–100
10. Dormer, R.L., Capurro, D.E., Morris, R., Webb, R. 1993. Demonstration of two isoforms of the SERCA-2b type Ca^{2+}, Mg^{2+}-ATPase in pancreatic endoplasmic reticulum. *Biochim. Biophys. Acta* **1152:**225–230
11. Ely, J.A., Ambroz, C., Baukal, A.J., Christensen, S.B., Balla, T., Catt, K.J. 1991. Relationship between agonist- and thapsigargin-sensitive calcium pools in adrenal glomerulosa cells: thapsigargin-induced Ca^{2+} mobilization and entry. *J. Biol. Chem.* **266:**18635–18641
12. Foskett, J.K., Roifman, C.M., Wong, D. 1991. Activation of calcium oscillations by thapsigargin in parotid acinar cells. *J. Biol. Chem.* **266:**2778–2782
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. Kasai, H., Augustine, G.J. 1990. Cytosolic Ca^{2+} gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature* **348:**735–738
15. Lytton, J., Westlin, M., S.E., Shull, G.E., MacLennan, D.H. 1992. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J. Biol. Chem.* **267:**14483–14489
16. Lytton, J., Westlin, M., Hanley, M.R. 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase Family of calcium pumps. *J. Biol. Chem.* **266:**17067–17071
17. Metz, D.C., Patto, R.J., Mrozinski, J.E., Jr., Jensen, R.T., Turner, R.J., Gardner, J.D. 1992. Thapsigargin defines the roles of cellular calcium in secretagogue-stimulated enzyme secretion from pancreatic acini. *J. Biol. Chem.* **267:**20620–20629
18. Nathanson, M.H., Fallon, M.B., Padfield, P.J., Maranto, A.R. 1994. Localization of the type 3 inositol 1,4,5-trisphosphate receptor in the Ca^{2+} wave trigger zone of pancreatic acinar cells. *J. Biol. Chem.* **269:**4693–4696
19. Sagara, Y., Inesi, G. 1991. Inhibition of the sarcoplasmic reticulum Ca^{2+} transport ATPase by thapsigargin at subnanomolar concentrations. *J. Biol. Chem.* **266:**13503–13506
20. 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
21. 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* **306:**67–69
22. Takemura, H., Hughes, A.R., Thastrup, O., Putney, J.W., Jr. 1989. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* **264:**12266–12271
23. Takemura, H., Ohshika, H., Yokosawa, N., Oguma, K., Thastrup, O. 1991. The thapsigargin-sensitive intracellular Ca^{2+} pool is more important in plasma membrane Ca^{2+} entry than the IP_3-sensitive intracellular Ca^{2+} pool in neuronal cell lines. *Biochem. Biophys. Res. Commun.* **180:**1518–1526
24. Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. USA* **87:**2466–2470
25. Thévenod, F., Dehlinger-Kremer, M., Kemmer, T.P., Christian, A.-L., Potter, B.V.L., Schulz, I. 1989. Characterization of inositol 1,4,5-trisphosphate-sensitive (IsCaP) and -insensitive (IisCaP) nonmitochondrial Ca^{2+} pools in rat pancreatic acinar cells. *J. Membrane Biol.* **109:**173–186
26. Thorn, P., Lawrie, A.M., Smith, P.M., Gallacher, D.V., Petersen, O.H. 1993. Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonists and inositol trisphosphate. *Cell* **74:**661–668
27. Verboomen, H., Wuytack, F., De Smedt, H., Himpens, B., Casteels, R. 1992. Functional difference between SERCA2a and SERCA2b Ca^{2+} pumps and their modulation by phospholamban. *Biochem. J.* **286:**591–596
28. Verma, A., Hirsch, D.J., Hanley, M.R., Thastrup, O., Christensen, S.B., Snyder, S.H. 1990. Inositol trisphosphate and thapsigargin discriminate endoplasmic reticulum stores of calcium in rat brain. *Biochem. Biophys. Res. Commun.* **172:**811–816
29. Xuan, Y.-T., Wang, O.-L., Whorton, A.R. 1992. Thapsigargin stimulates Ca^{2+} entry in vascular smooth muscle cells: nicardipine-sensitive and -insensitive pathways. *Am. J. Physiol.* **262:**C1258–C1265