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Interaction of Caffeine-, IP_3- and Vanadate-Sensitive Ca^{2+} Pools in Acinar Cells of the Exocrine Pancreas

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Summary. Previous studies have shown the existence of functionally distinguishable inositol 1,4,5-trisphosphate- (IP_3) sensitive and IP_3-insensitive nonmitochondrial intracellular Ca^{2+} pools in acinar cells of the exocrine pancreas. For further characterization of Ca^{2+} pools, endoplasmic reticulum (ER) membrane vesicles were separated by Percoll gradient centrifugation which allowed us to distinguish five discrete fractions designated P_1 to P_5 from the top to the bottom of the gradient. Measuring Ca^{2+} uptake and Ca^{2+} release with a Ca^{2+} electrode, we could differentiate three nonmitochondrial intracellular Ca^{2+} pools: (*i*) an IP_3-sensitive Ca^{2+} pool (IsCaP), vanadate- and caffeine-insensitive, (*ii*) a caffeine-sensitive Ca^{2+} pool (CasCaP), vanadate- and IP_3-insensitive, and (*iii*) a vanadate-sensitive Ca^{2+} pool (VasCaP), neither IP_3- nor caffeine-sensitive, into which Ca^{2+} uptake is mediated via a Ca^{2+} ATPase sensitive to vanadate at 10^{-4} mol/liter. A fourth Ca^{2+} pool is neither IP_3- nor caffeine- or vanadate-sensitive. Percoll fraction P_1 contained essentially the IsCaP, CasCaP and VasCaP and was mainly used for studies on Ca^{2+} uptake and Ca^{2+} release.

When membrane vesicles were incubated in the presence of caffeine (2×10^{-2} mol/liter), Ca^{2+} uptake up to the steady state [Ca^{2+}] did not appear to be altered as compared to the control Ca^{2+} uptake. However, in control vesicles spontaneous Ca^{2+} release occurred after the steady state had been reached, whereas caffeine-pretreated vesicles did not spontaneously release Ca^{2+}. Addition of IP_3 at steady state [Ca^{2+}] induced similar Ca^{2+} release followed by Ca^{2+} reuptake in both caffeine-pretreated and control vesicles. However, when caffeine was acutely added at steady state, Ca^{2+} was released from all Ca^{2+} pools including the IsCaP. Following Ca^{2+} reuptake after IP_3 had been added, a second addition of IP_3 to control vesicles induced further but smaller Ca^{2+} release, and a third addition resulted in a steady Ca^{2+} efflux by which all Ca^{2+} that had been taken up was released. This steady Ca^{2+} release started at a Ca^{2+} concentration between 5.5–8 $\times 10^{-7}$ mol/liter and could also be induced by the IP_3 analogue inositol 1,4,5-trisphosphorothioate (IPS_3) or by addition of Ca^{2+} itself. Ruthenium red (10^{-5} mol/liter) inhibited both caffeine-induced as well as Ca^{2+}-induced but not IP_3-induced Ca^{2+} release. Heparin (100 μg/ml) inhibited IP_3- but not caffeine-induced Ca^{2+} release. The data indicate the presence of at least three separate Ca^{2+} pools in pancreatic acinar cells: the IsCaP, CasCaP and VasCaP. During Ca^{2+} uptake these Ca^{2+} pools appear to be separate. However, when steady state is reached, we assume that these Ca^{2+} pools come into contact and total Ca^{2+} release from all three pools can occur. The mechanism of this "contact" of Ca^{2+} pools is not clear but seems to be different from that induced by GTP in the presence of polyethylene glycol, which probably involves fusion of membranes.

Key Words fusion · ruthenium red · heparin · GTP

Introduction

In many different cell types hormones and neurotransmitters act by releasing Ca^{2+} from intracellular stores. Whereas in nonexcitable cells inositol 1,4,5-trisphosphate (IP_3) is an important intracellular messenger for Ca^{2+} release (Streb et al., 1983; Berridge & Irvine, 1984) in excitable tissues, the main process that leads to elevation of cytosolic free-Ca^{2+} concentration is Ca^{2+} entry from the extracellular space through voltage-gated Ca^{2+} channels (Hosey & Lazdunsky, 1988). In special tissues, such as the smooth muscle, Ca^{2+} entry into the cell induces Ca^{2+} release from a Ca^{2+} store that is also sensitive to caffeine. In this cell type the Ca^{2+}-induced Ca^{2+} release mechanism seems to be present in addition to the IP_3-sensitive Ca^{2+} release mechanism (Leijten & van Breemen, 1984; Kanaide, Shogakiuchi & Nakamura, 1987; Kanmura, Raeymaekers & Casteels, 1989). In skeletal muscle, the initial signal for Ca^{2+} release is an electrical depolarization of the transverse T-tubule membrane which is detected by the voltage-dependent dihydropyridine-sensitive receptor (DHPR). Ca^{2+} release from the sarcoplasmic reticulum (SR) occurs through a channel in close contact to the DHPR, which is generally referred to as ryanodine receptor, since it binds the plant alkaloid ryanodine (Lai et al., 1988; Takeshima et al., 1989; Valdivia & Coronado, 1989). Ca^{2+} release can be induced by the pharmacological actions of ryanodine or of caffeine or by increase in the concentration of free intracellular [Ca^{2+}] (Endo, 1977; Martonosi, 1984). The coexistence of both IP_3-sensitive and caffeine-sensitive Ca^{2+} stores has been described for

the dorsal root ganglion neurons (Thayer, Perney & Miller, 1988) and for smooth muscles (Kanaide et al., 1987).

We have recently characterized two different — an IP_3-sensitive (IsCaP) and an IP_3-insensitive (IisCaP) — Ca^{2+} stores in pancreatic acinar cells. Whereas the IsCaP takes up Ca^{2+} via a Ca^{2+}/H^+ exchanger that is stimulated in the presence of ATP, the IisCaP is filled from the cytosol by an ATP-dependent vanadate-inhibitable Ca^{2+} pump (Thévenod et al., 1989*a*). Any Ca^{2+} release mechanism for the latter Ca^{2+} store has not been found so far. Evidence suggests that in pancreatic acinar cells receptor-mediated stimulation of cytoplasmic Ca^{2+} spikes is generated by IP_3-evoked Ca^{2+} release causing pulses of Ca^{2+}-induced Ca^{2+} release from Ca^{2+} pools close to the plasma membrane. Caffeine at low concentrations (1 mM) was shown to broaden these Ca^{2+} spikes (Osipchuk et al., 1990). We have recently reported that a Ca^{2+} channel is present in the endoplasmic reticulum of pancreatic acinar cells, that can be activated by caffeine and inhibited by ruthenium red (Schmid et al., 1990). This Ca^{2+} channel, however, appears to be different from the classical ryanodine receptor present in skeletal, cardiac and smooth muscle cells (Rousseau, Smith & Meissner, 1987; Fleischer et al., 1985; Kanmura et al., 1988) since it is voltage dependent, not sensitive to ryanodine and calcium at the conditions applied had no activating effect (Schmid et al., 1990). In the present study we report on IP_3- and caffeine- (or Ca^{2+})-induced Ca^{2+} release from pancreatic endoplasmic reticulum. If caffeine, IP_3 or vanadate are present in the incubation medium, Ca^{2+} uptake does not appear to be altered as compared to the control and the same steady state $[Ca^{2+}]$ is reached. The effects of IP_3 (in the presence of caffeine) and of caffeine (in the presence of IP_3, IPS_3) are the same as in the control. We therefore think that both IP_3- and caffeine-induced Ca^{2+} release mechanisms are located in two different Ca^{2+} pools. However, when caffeine or the nonhydrolyzable analogue of IP_3, IPS_3, is added at steady state all Ca^{2+} that previously had been taken up is released. It therefore appears that during Ca^{2+} uptake these pools are separate and come into connection at steady state by an unknown mechanism, which could involve the intravesicular Ca^{2+} or H^+ concentration or both. At conditions, at which connection of Ca^{2+} pools is induced like in the presence of polyethylene glycol and GTP (Ghosh et al., 1989), Ca^{2+} can be also released from all three Ca^{2+} stores by either IP_3, by IPS_3, by caffeine or by calcium. However, the GTP-induced "connection" probably involves fusion of Ca^{2+} pools and appears to be different with respect to the kinetics of IP_3- and caffeine-induced Ca^{2+} release and Ca^{2+} reuptake as compared to interaction of Ca^{2+} pools in the absence of GTP.

Abbreviations

CasCaP: caffeine-sensitive Ca^{2+} pool
CK: creatine kinase
CP: phosphocreatine (sodium salt)
EDTA: ethylenediaminetetraacetic acid
ER: endoplasmic reticulum
GTP: guanosine-5′-triphosphate
GTPγS: guanosine-5′-o-(3-thiotriphosphate)
HEPES: N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid
IP_3: D-myo-inositol 1,4,5-trisphosphate
IPS_3: DL-myo-inositol 1,4,5-trisphosphorothioate
IisCaP: IP_3-insensitive Ca^{2+} pool
IsCaP: IP_3-sensitive Ca^{2+} pool
NaN_3: sodium azide
NBD-Cl: 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole
PEG: polyethylene glycol
PMSF: phenylmethylsulfonyl fluoride
VasCaP: vanadate-sensitive Ca^{2+} pool

Materials and Methods

Materials

Reagents were obtained from the following sources: K_2ATP, benzamidine, bovine serum albumin (lyophilized), EDTA, GTP, Na_3VO_4, NBD-Cl, PMSF, CP (sodium salt), pyruvic acid and succinic acid from Sigma Chemical (St Louis, MO); CK (350 U/mg at 25°C) and trypsin inhibitor (from soybean) from Boehringer (Mannheim, FRG); NaN_3, D-mannitol, ruthenium red, caffeine and tris(hydroxymethyl)aminomethan from Merck (Darmstadt, FGR); antimycin A, oligomycin, HEPES, heparin and leupeptin from Serva (Heidelberg, FRG); PEG (M_r 6000) from Fluka (Buchs, Switzerland); Percoll (density: 1.129 g/ml, osmolality: 17 mOsmol/kg H_2O) from Deutsche Pharmacia (Freiburg, FRG); and collagenase from *Clostridium histolyticum* type III (132 U/mg at 37°C) from Worthington (Freehold, NJ). All other reagents were of analytical grade.

Ca^{2+}-selective electrode membranes containing the neutral carrier N,N-di(11-ethoxycarbonyl)undecyl)-N,N-4,5-tetramethyl-3,6-dioxaoctane amide were purchased from Glasbläserei W. Möller (Zürich, Switzerland).

D-myo-inositol 1,4,5-trisphosphate (IP_3) was a gift from Dr. R. Irvine and DL-myo-inositol 1,4,5-trisphosphorothioate (IPS_3) was kindly provided by Dr. B.V.L. Potter.

Methods

Preparation of Subcellular Fractions Enriched in Endoplasmic Reticulum

Pancreatic vesicles enriched in endoplasmic reticulum were prepared from 12 male Wistar rats (200–250 g each), which had been fasted overnight. At first acinar cells were prepared by collagenase digestion in a standard Krebs-Ringer-HEPES (KRH)

solution containing (in mmol/liter): NaCl 120, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 0.1, HEPES 10, glucose 15, trypsin inhibitor 0.1 mg/ml and albumin 0.2% (wt/vol) (pH 7.4); as described previously (Streb & Schulz, 1983). Following 60-min digestion and mechanical dissociation by sequential passage through polypropylene pipet tips of decreasing tip diameter, cells were centrifuged through 20 ml of the above solution containing 4% albumin. Isolated cells were then washed twice in an ice-cold "mannitol buffer" containing (in mmol/liter): mannitol 280, HEPES 5, KCl 1.0, $MgCl_2$ 1, benzamidine 1, PMSF 0.2, leupeptin 0.001 and 20 μg/ml trypsin inhibitor, adjusted with Tris to pH 7.0.

Subsequent homogenization and fractionation steps were performed in the cold. Cells were homogenized in the "mannitol buffer" at a protein concentration of about 10 mg/ml by 50 strokes with a tightly fitting Potter-Elvejhem glass/Teflon homogenizer which was motor-driven at 900 rpm.

The resulting homogenate was centrifuged for 5 min at 100 × *g* and the pellet was rehomogenized. The combined homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was further centrifuged at 11,000 × *g* for 15 min in a Beckmann Model J-21C centrifuge using a Beckmann JA20 rotor. The resulting pellet was composed of an upper whitish fluffy layer and a yellowish bottom layer, which were separated.

The fluffy layer which was enriched by about twofold in ER (as shown by the distribution of the marker enzymes, RNA and NADPH-cytochrome-*c*-reductase), (Streb et al., 1984) was further fractionated by centrifugation on a Percoll gradient (Thévenod et al., 1989*b*). For that purpose 1 ml of vesicle suspension was layered over 9 ml of a solution composed of "mannitol buffer" containing 11% (wt/vol) Percoll, pH 7.0 adjusted with 1 N HCl. The resulting density was 1.035 g/cm^3. A gradient of densities from 1.020 to 1.141 g/cm^3 was generated by spinning the tubes at 41,000 × *g* for 40 min in a Beckman Model L350 ultracentrifuge using a Beckman Ti 60 rotor.

In the upper half of the Percoll gradient a thick white layer is present. Five 2-ml fractions (P_1 to P_5) were collected from the top to the bottom of the gradient. For collection of P_1 it is critical to include the very upper part of the white layer. To separate vesicles from Percoll, each fraction was diluted 20 times with "mannitol buffer" and spun down at 200,000 × *g* for 60 min in a Beckman Ti 60 rotor. The pellets were resuspended in the "mannitol buffer" at a protein concentration of about 20 mg/ml. Vesicles were either used immediately or kept frozen in liquid nitrogen until use.

Measurement of Ca^{2+} Uptake with the Ca^{2+} Electrode

Isolated membrane vesicles were incubated at a protein concentration of 1 mg/ml in 2 ml of "incubation buffer" containing (in mmol/liter): KCl 120, HEPES 25, $MgCl_2$ 6, K_2ATP 5, KH_2PO_4 1.2, CP (sodium salt) 10, K succinate 5, K pyruvate 5, oligomycin 0.005, antimycin A 0.01, sodium azide 10 and 10 U/ml CK (pH 7.0) at 25°C. The solution was continuously stirred and the free-calcium concentration of the medium was recorded with a Ca^{2+}-specific macro-electrode (neutral carrier ETH 1001) as described previously (Streb & Schulz, 1983).

In order to convert the change in free-Ca^{2+} concentration to total amount of calcium, the system was calibrated for each individual determination by additions of known amounts of calcium.

Protein Determination

Proteins were measured by the method of Bradford (1976) using bovine serum albumin as standard.

Results

In previous studies we have shown that two distinct Ca^{2+} uptake mechanisms are present in two nonmitochondrial intracellular Ca^{2+} pools of acinar cells from the exocrine pancreas. A Ca^{2+}/H^+ exchanger mediates Ca^{2+} uptake into an IP_3-sensitive Ca^{2+} pool (IsCaP) and a vanadate-sensitive Ca^{2+} ATPase mediates Ca^{2+} uptake into an IP_3-insensitive Ca^{2+} pool (IisCaP) (Thévenod et al., 1989*a*). In order to separate the IP_3-sensitive Ca^{2+} pool from other Ca^{2+} pools, we have examined in subcellular fractions of the ER separated by Percoll gradient centrifugation the distribution (*i*) of the IP_3 effect, (*ii*) of the caffeine effect, (*iii*) the effect of the Ca^{2+} ATPase inhibitor vanadate and (*iv*) the effect of the H^+ ATPase inhibitor NBD-Cl on Ca^{2+} uptake.

Distribution of the IP_3 Effect

Subfractionation of endoplasmic reticulum on the Percoll gradient allowed us to distinguish five discrete fractions, from P_1 (the lightest fraction) to P_5 (the heaviest fraction). Ca^{2+} uptake into these fractions as measured with a Ca^{2+} electrode is shown in Fig. 1. A significant Ca^{2+} uptake could only be measured in the three lightest fractions P_1, P_2, and P_3. The vesicles from these fractions took up Ca^{2+} from a medium with a free-Ca^{2+} concentration of about 10^{-6} mol/liter until a steady state of free-Ca^{2+} concentration of about 3.5×10^{-7} mol/liter was reached. In the Percoll fraction P_4 very little Ca^{2+} uptake was observed and in fraction P_5 there was even less Ca^{2+} uptake.

Upon addition of IP_3 (5×10^{-6} mol/liter) to the Percoll fractions P_1, P_2 and P_3 Ca^{2+} was released. The IP_3 effect was highest in the Percoll fraction P_1 and decreased from P_1 to the Percoll fraction P_3. In Percoll fractions P_4 and P_5 no IP_3 effect could be observed (Fig. 1 and Table 1). In the Percoll fraction P_1 Ca^{2+} released by IP_3 was taken up again. This Ca^{2+} reuptake occurred via a vanadate-sensitive Ca^{2+} ATPase, since in the presence of 10^{-4} mol/liter of this (Ca^{2+} + Mg^{2+})-ATPase inhibitor reuptake was abolished (Figs. 1 and 2*a*).

These data suggest that the IP_3-sensitive Ca^{2+} pool, as well as the Ca^{2+} pool into which Ca^{2+} is taken up again via a vanadate-sensitive Ca^{2+} ATPase, are both localized in the light Percoll fraction P_1 (*see also* Table 2).

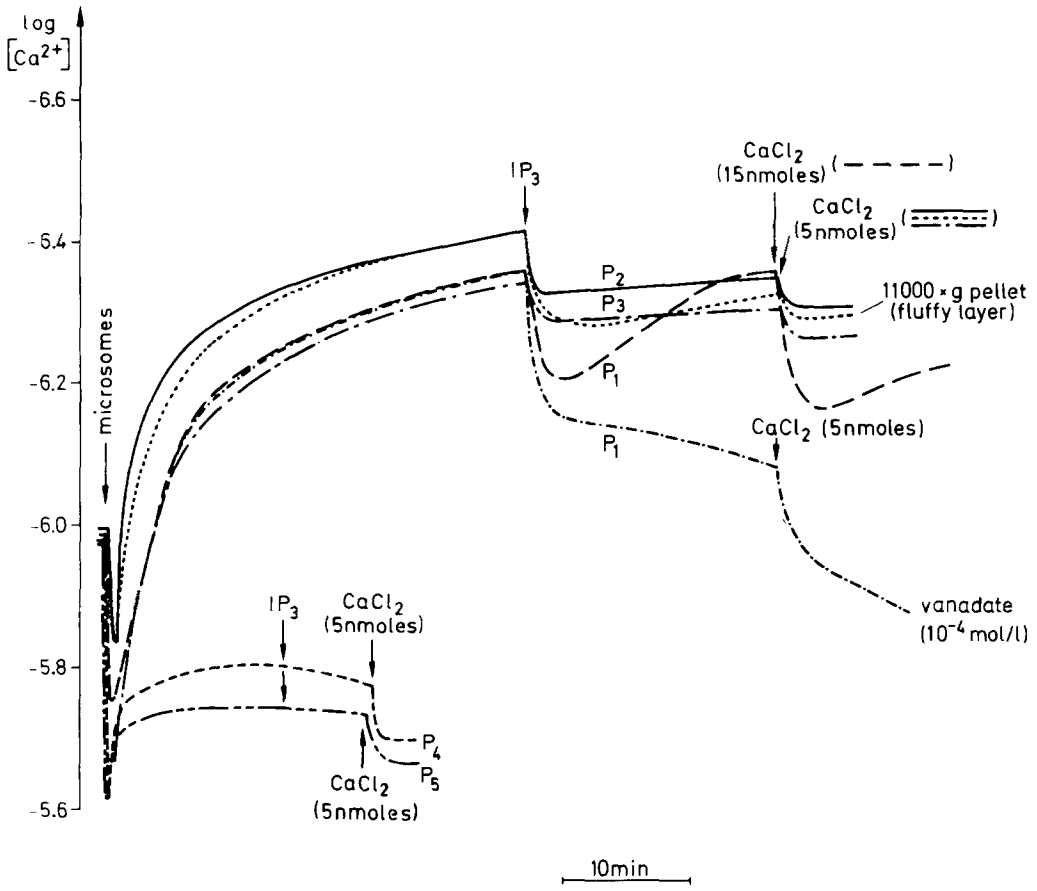


Fig. 1. Ca^{2+} uptake and Ca^{2+} release from membrane vesicles (P_1–P_5) of the Percoll gradient and from the "fluffy layer." A concentrated suspension of membrane vesicles were added to the standard incubation medium in the absence or presence of vanadate as described in Methods. Ca^{2+} uptake was monitored with a Ca^{2+} electrode until steady-state free-Ca^{2+} concentration was reached. Where indicated IP_3 at a final concentration of 5×10^{-6} mol/liter was added. For calibration 5 and 15 nmol $CaCl_2$ was added at final concentrations of 2.5×10^{-6} mol/liter and 7.5×10^{-6} mol/liter calcium, respectively. Typical for six similar experiments

Effect of Vanadate and of NBD-Cl on Ca^{2+} Uptake

As shown in Fig. 2*a–c*, preincubation and incubation of membrane vesicles of the Percoll fractions P_1, P_2 and P_3 with 10^{-4} mol/liter vanadate, apparently did not affect Ca^{2+} uptake and the low steady-state free-Ca^{2+} concentration of about 3.5×10^{-7} mol/liter was reached within the same time as in control conditions. However, in the Percoll fractions P_4 and P_5 vanadate abolished Ca^{2+} uptake (Fig. 2*d*). It thus appears that in the three lightest fractions P_1, P_2 and P_3 the vanadate-sensitive Ca^{2+} uptake mechanism is not critically involved in the adjustment of the low steady-state free-calcium concentration. However, when IP_3 was added at steady state to vanadate-preincubated vesicles, Ca^{2+} reuptake was inhibited in Percoll fractions P_1, P_2 and P_3. From the degree of vanadate-inhibited Ca^{2+} reuptake it appears that the vanadate-sensitive Ca^{2+} pool (VasCaP) is highest in fraction P_1 and decreases in the Percoll fractions P_2 and P_3 (Fig. 2*a–c* and Table 2). This is also supported by the absence of apparent Ca^{2+} reuptake in fractions P_2 and P_3 which in P_1 follows the Ca^{2+} release induced by IP_3 in the absence of vanadate (Fig. 2).

Ca^{2+} uptake mediated by the vanadate-sensitive Ca^{2+} ATPase occurs into an IP_3-insensitive Ca^{2+} pool, whereas Ca^{2+} uptake into the IP_3-sensitive Ca^{2+} pool (IsCaP) is mediated by a Ca^{2+}/H^+ exchanger (Thévenod et al., 1989*a*). In the Percoll fractions P_4 and P_5 no Ca^{2+} uptake remained in the presence of 10^{-4} mol/liter vanadate (Fig. 2*d*). NBD-Cl did not significantly affect Ca^{2+} uptake in these fractions (*data not shown*). In these fractions also no Ca^{2+} release could be induced by IP_3. This suggests that the VasCaP is the main calcium pool present in these heavy fractions P_4 and P_5 (Fig. 2*d* and Table 2).

Incubation of vesicles with 10^{-5} mol/liter NBD-

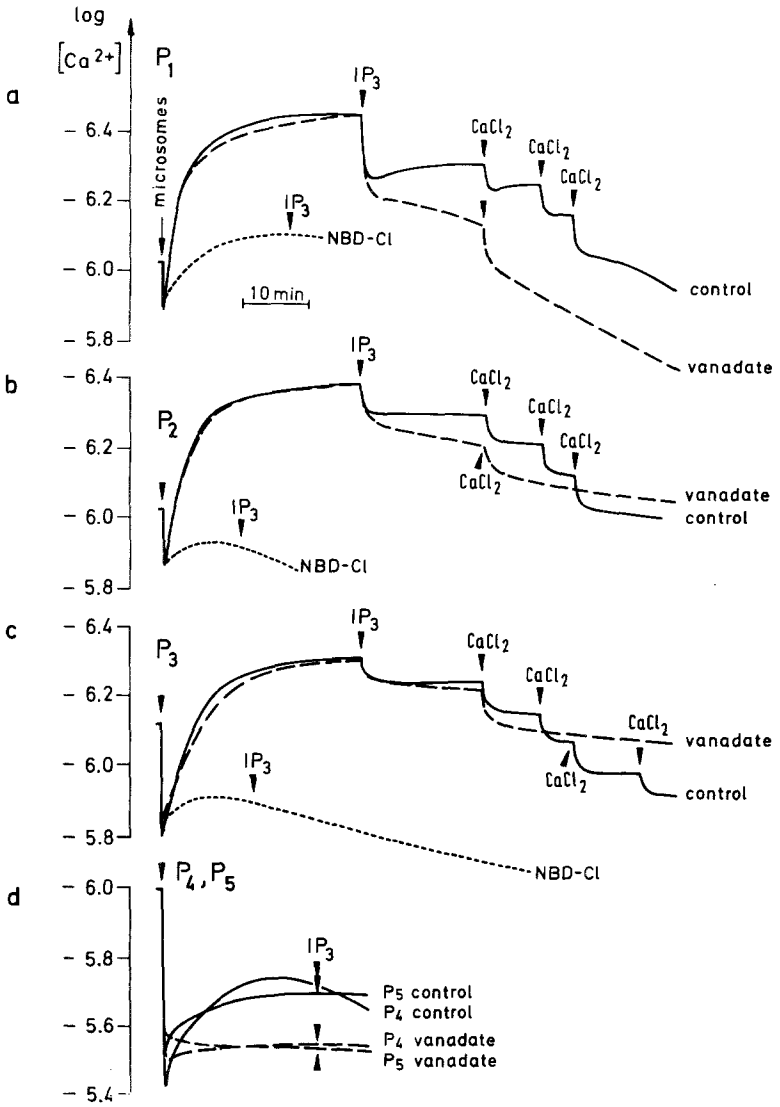


Fig. 2. Effect of vanadate (10^{-4} mol/liter) and of NBD-Cl (10^{-5} mol/liter) on nonmitochondrial Ca^{2+} uptake and Ca^{2+} release in isolated membrane vesicles of Percoll fractions P_1 (*a*) of P_2 (*b*) of P_3 (*c*) and of P_4 and P_5 (*d*). Membrane vesicles (at a final concentration of 1 mg protein/ml) preincubated for 15 min in standard incubation medium without or with 10^{-4} mol/liter vanadate or with 10^{-5} mol/liter NBD-Cl (as indicated) were incubated in the same buffer in the presence of the same substances as during preincubation. Ca^{2+} uptake was monitored with a Ca^{2+} electrode as described in Methods. Where indicated IP_3 at a final concentration of 5×10^{-6} mol/liter or $CaCl_2$ (5 nmol) at a final concentration of 2.5×10^{-6} mol/liter calcium were added. Typical for three similar experiments

Table 1 Distribution of the IP_3 effect in subcellular vesicle fractions from exocrine pancreas separated by Percoll gradient centrifugation

Subcellular fractions	IP_3 effect (Ca^{2+} release) nmol/mg protein	*n*
Fluffy layer	4.65 ± 1.01	10
P_1	6.72 ± 1.59	9
P_2	3.68 ± 0.40	8
P_3	1.86 ± 0.16	8
P_4	0	8
P_5	0	8

Values are means ± SEM of separate experiments (*n*).

Table 2. Distribution of the functionally differentiable nonmitochondrial intracellular Ca^{2+} pools in the Percoll fractions P_1–P_5

Intracellular Ca^{2+} pools	Percoll fractions				
	P_1	P_2	P_3	P_4	P_5
IsCaP IP_3 sensitive Vanadate insensitive	+ + +	+ +	+	−	−
CasCaP Caffeine sensitive Vanadate insensitive	+ + +	+	−	−	−
VasCaP Vanadate sensitive	+ + +	+ +	+	+ + +	+ + +

Symbols (+ and −) represent the relative enrichment in the respective Ca^{2+} pools.

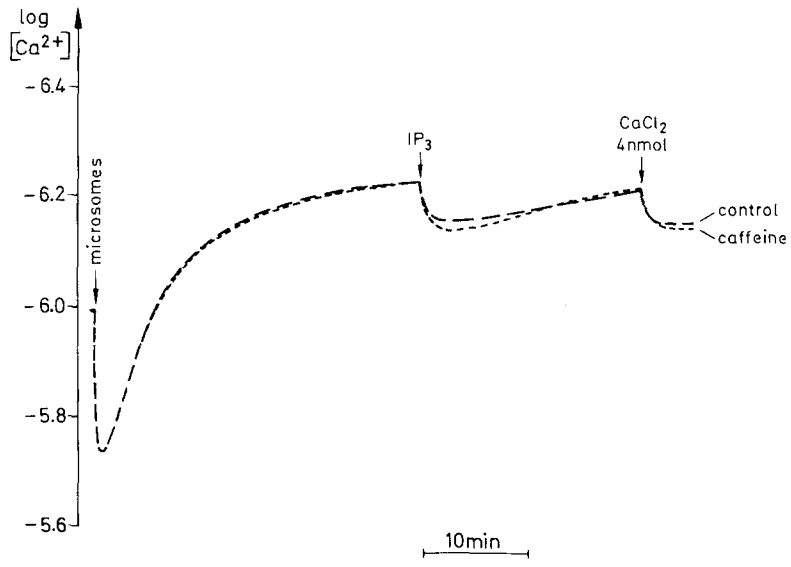


Fig. 3. Effect of caffeine (2×10^{-2} mol/liter) on Ca^{2+} uptake and Ca^{2+} release from membrane vesicles of the Percoll fraction P_1. Membrane vesicles of the Percoll fraction P_1 (1 mg protein/ml) were incubated in standard incubation medium with or without 2×10^{-2} mol/liter caffeine as indicated. Ca^{2+} uptake was monitored with a Ca^{2+} electrode as described in Methods. IP_3, at a final concentration of 5×10^{-6} mol/liter and $CaCl_2$ (4 nmol) at a final concentration of 2×10^{-6} mol/liter, were added where indicated. Typical for three similar experiments

Cl, a concentration that completely abolished non-mitochondrial MgATP-driven H^+ transport in isolated pancreatic membrane vesicles from ER and Ca^{2+} uptake into the IsCaP (Thévenod et al., 1989*a*,*b*), produced a partial inhibition of Ca^{2+} uptake in membrane vesicles of the Percoll fraction P_1 and a total inhibition in membrane vesicles of the Percoll fractions P_2 and P_3 (Fig. 2*a–c*). This indicates that Ca^{2+} uptake into the Percoll fractions P_2 and P_3 occurs via the Ca^{2+}/H^+ exchanger identified in the IsCaP and also in similar Ca^{2+} pools lacking the IP_3 receptor of isolated permeabilized pancreatic acinar cells (Thévenod et al., 1989*a*). Since in fractions P_2 and P_3, only a small IP_3 effect was detected the main Ca^{2+} pool in these fractions appears to be the IP_3- and vanadate-insensitive Ca^{2+} pool with the Ca^{2+}/H^+ exchanger.

Caffeine- and Ca^{2+}-Induced Ca^{2+} Release: Interaction of Both Caffeine- and IP_3-Sensitive Ca^{2+} Pools

The methylxanthine caffeine has been shown to induce Ca^{2+} release from the sarcoplasmic reticulum of smooth muscles, where it activates the Ca^{2+} channel responsible for Ca^{2+}-induced Ca^{2+} release (Endo, 1977; Rousseau & Meissner, 1989). When Ca^{2+} loading of isolated pancreatic membrane vesicles (fraction P_1) was performed in the presence of 2×10^{-2} mol/liter caffeine in the incubation medium, neither Ca^{2+} uptake nor the IP_3 effect were apparently affected (Fig. 3). However, when caffeine was acutely added at steady state, Ca^{2+} release was observed in the Percoll fraction P_1 (Figs. 4*a* and 5*a*). In 10 experiments with the Percoll fraction P_1, Ca^{2+} was released by caffeine with a rate of 2.88 ± 0.01 SE nmol/mg protein·min. In P_2 and in the "fluffy layer," the rates of caffeine-induced Ca^{2+} release were small and only seen at inhibited Ca^{2+} reuptake in the presence of vanadate. Under these conditions the rates were only 0.31 ± 0.05 SEM nmol/mg protein · min in the P_2 fraction (Fig. 5*b*) and 0.86 ± 0.01 SEM nmol/mg protein · min in the "fluffy layer" (*data not shown*). When Ca^{2+} release was induced by caffeine in fraction P_1 all Ca^{2+} that had been previously taken up was released and subsequent addition of IP_3 did not further induce any significant Ca^{2+} release (Fig. 4*a*). However, when IP_3 or its metabolic stable analog IPS_3 (Cooke, Gigg & Potter, 1987; Willcock, Potter & Cooke, 1988) was added first, subsequent addition of caffeine induced further Ca^{2+} release (Figs. 4*a* and 6). Assuming that the IsCaP was empty under these conditions, this indicates that in addition to an IsCaP also a CasCaP exists which can be functionally discriminated from the IsCaP. Similarly under conditions at which the VasCaP is not filled, a caffeine effect still can be induced (Fig. 6). The data shown in Figs. 4*a* and 6 together with the data shown in Fig. 3 indicate that in fraction P_1 IsCaP and caffeine-sensitive Ca^{2+} pools (CasCaP) are separate intracellular Ca^{2+} pools which come into connection when they are filled and when steady state $[Ca^{2+}]$ had been reached. The experiment presented in Fig. 7 suggests that this "connection" is initiated by the CasCaP. Incubation of membrane vesicles from the Percoll fraction P_1 in the presence of 2×10^{-2} mol/liter caffeine abolished the "spontaneous" Ca^{2+} release observed in control vesicles (Fig. 7). This "spontaneous" Ca^{2+} release from control vesicles incubated with ATP and an ATP-regenerating system (*see* Methods) could not be due to consumption of ATP, since additional administra-

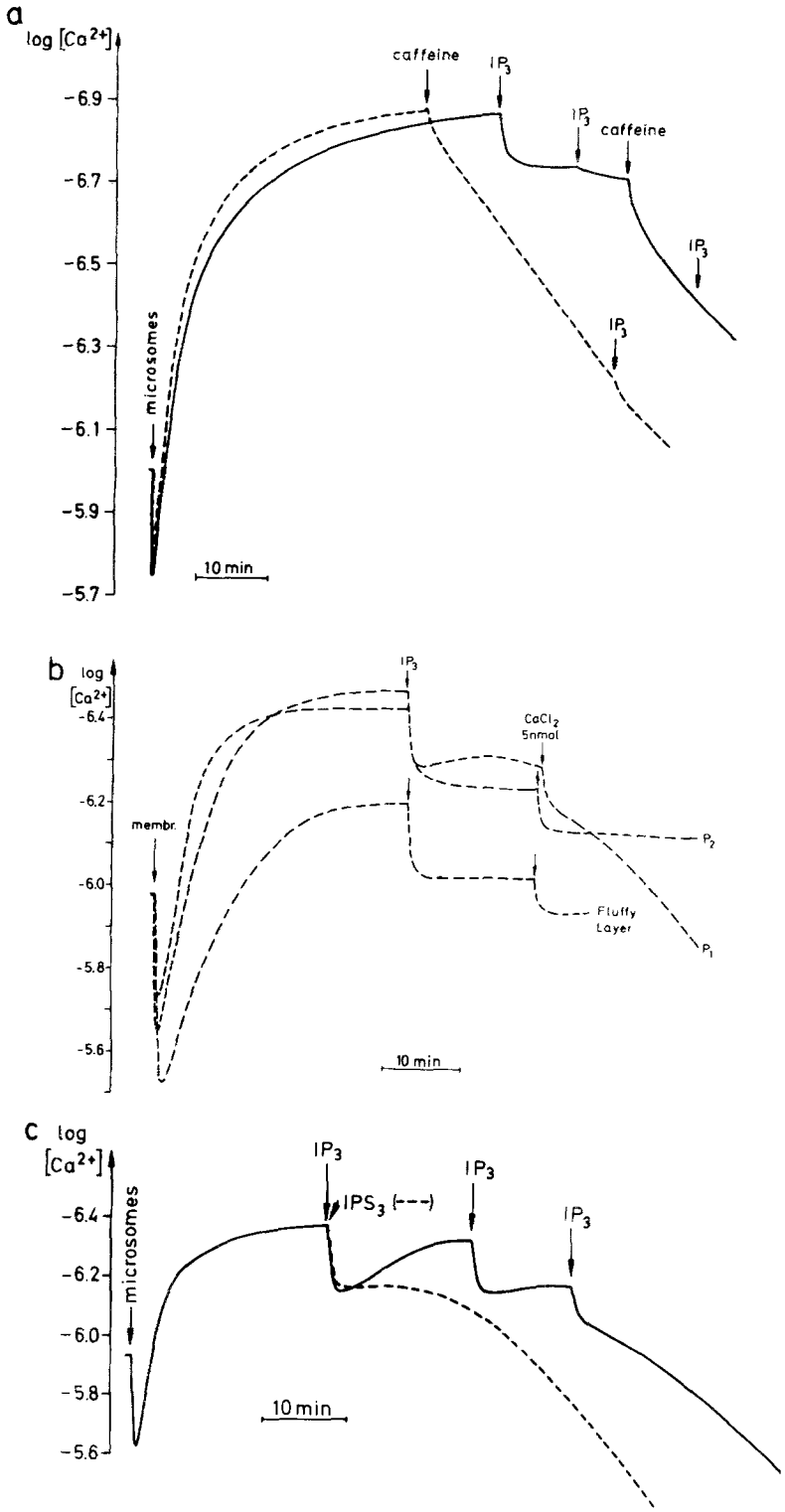
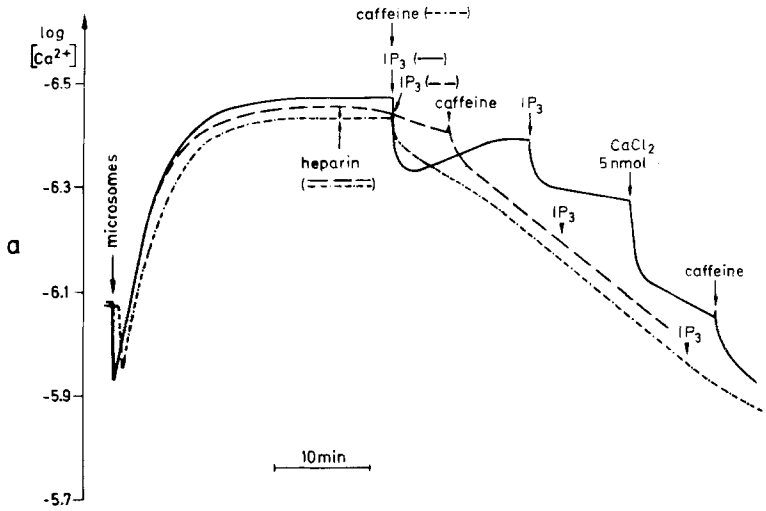
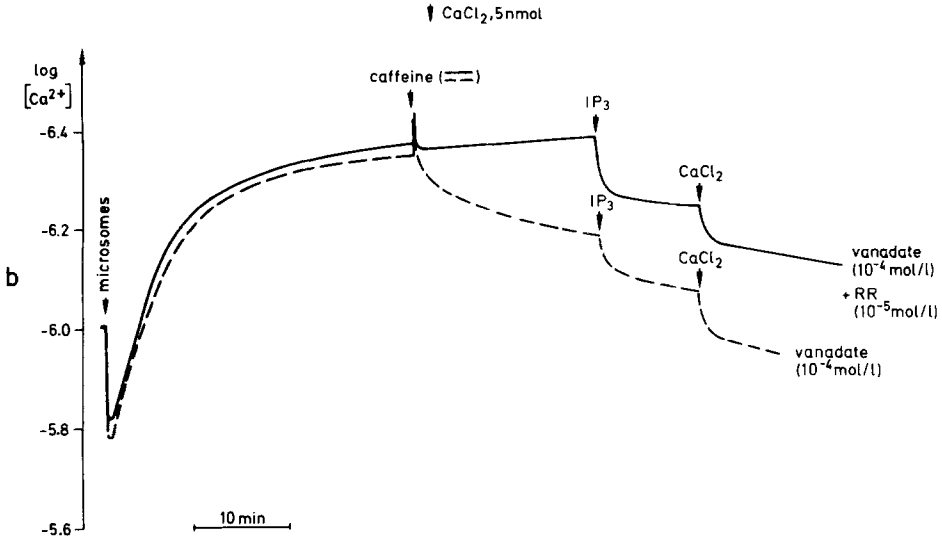
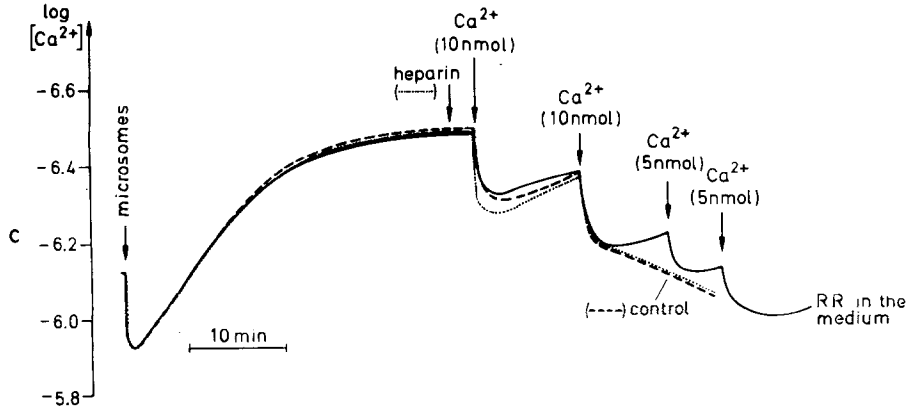


Fig. 4. (*a*) Effects of IP_3 and caffeine on Ca^{2+} release from pancreatic membrane vesicles (fraction P_1 of the Percoll gradient). Membrane vesicles (1 mg protein/ml) were incubated under standard conditions as described in Methods. Where indicated IP_3 (5×10^{-6} mol/liter) and caffeine (2×10^{-2} mol/liter) were added. Typical for three similar experiments. (*b*) Effects of IP_3 and $CaCl_2$ on Ca^{2+} release from pancreatic membrane vesicles of the "fluffy layer" and the Percoll fractions P_1 and P_2. Membrane vesicles (1 mg protein/ml) were incubated in standard incubation medium. Where indicated, IP_3 at a final concentration of 5×10^{-6} mol/liter and $CaCl_2$ (5 nmol) at a final concentration of 2.5×10^{-6} mol/liter calcium were added. Typical for three similar experiments. (*c*) Effect of IP_3 and IPS_3 on Ca^{2+} release from isolated membrane vesicles of the Percoll fraction P_1. Membrane vesicles (1 mg protein/ml) were incubated under control conditions as described in Methods. Where indicated IP_3, at a final concentration of 5×10^{-6} mol/liter and IPS_3 at a final concentration of 3×10^{-5} mol/liter, were added. One out of two similar experiments

tion of 2×10^{-3} mol/liter ATP did not influence Ca^{2+} efflux (*not shown*). Similarly in vanadate-treated vesicles where the extent of Ca^{2+} release is enhanced as compared to control by preventing the competing Ca^{2+} reuptake process, the presence of caffeine decreased the "spontaneous" Ca^{2+} release (Fig. 7). On the other hand, when Ca^{2+} uptake into the IsCaP was avoided by incubating the vesicles with 5×10^{-5} mol/liter IPS_3, the rate of "spontaneous" Ca^{2+} release was similar as in control vesicles


a
log [Ca2+]
-6.5
-6.3
-6.1
-5.9
-5.7
microsomes
heparin
caffeine
IP3
CaCl2 5nmol
10min


CaCl2,5nmol
b
log [Ca2+]
-6.4
-6.2
-6.0
-5.8
-5.6
microsomes
caffeine
IP3
CaCl2
vanadate (10-4 mol/l)
+RR (10-5mol/l)
vanadate (10-4mol/l)
10 min


c
log [Ca2+]
-6.6
-6.4
-6.2
-6.0
-5.8
microsomes
heparin
Ca2+ (10nmol)
Ca2+ (10nmol)
Ca2+ (5nmol)
Ca2+ (5nmol)
control
RR in the medium
10 min

(Fig. 7). When vesicles from the Percoll fraction P_1 were preincubated at the same experimental conditions but without ATP for 1 hr before Ca^{2+} uptake was initiated by addition of ATP, Ca^{2+} uptake and "spontaneous" Ca^{2+} release rates were the same as those shown in Fig. 7 (*data not shown*). We therefore do not think that "spontaneous" Ca^{2+} release could be explained by a decay of membrane vesicles. The outside calcium concentration is also important for Ca^{2+} release after steady state [Ca^{2+}] had been reached. In 10 experiments with fraction P_1 further Ca^{2+} was released when a critical free-Ca^{2+} concentration of 6.75 ± 0.36 (SEM) × 10^{-7} mol/liter had been reached. This is demonstrated in Figs. 1, 4*b*, and 5*a* and *c* when Ca^{2+} was added or in Fig. 4*c* where the increase of [Ca^{2+}] was induced by the nonhydrolyzable IP_3 analogue IPS_3. This Ca^{2+}-induced Ca^{2+} release was not observed in P_2 or the "fluffy layer" (Fig. 4*b*).

Caffeine and IP_3 Act on Two Different Ca^{2+} Channels

Single-channel recordings from microsomal fraction of smooth muscles incorporated into lipid bilayer have shown that heparin inhibits the IP_3-induced opening of sarcoplasmic reticulum Ca^{2+} channels without affecting the caffeine-induced Ca^{2+} release (Ehrlich & Watras, 1988). When heparin (100 μg/ml) was added to vesicles of the Percoll fraction P_1 at steady state prior to IP_3 addition, the IP_3 effect was abolished but the subsequent caffeine effect remained (Fig. 5*a*). Heparin did not affect the caffeine effect neither did it inhibit the Ca^{2+} release induced by successive additions of Ca^{2+} pulses (Fig. 5*c*).

When ruthenium red (RR), an inhibitor of the Ca^{2+}-sensitive Ca^{2+} release channel of cardiac and skeletal sarcoplasmic reticulum (Miyamoto & Racker, 1982; Rousseau et al., 1986), was present in the incubation medium at a concentration of 10^{-5} mol/liter, the caffeine effect was abolished but the IP_3 effect remained unaffected (Fig. 5*b*). The Ca^{2+} release induced by two successive calcium pulses was also inhibited in the presence of RR (Fig. 5*c*).

This suggests that in acinar cells of the exocrine pancreas IP_3 and caffeine and/or calcium act on two different Ca^{2+} release channels. Ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum (Rousseau et al., 1987), had no effect on Ca^{2+} release as induced by caffeine, by Ca^{2+} or by IP_3 at tested concentration of 10^{-5} and 3 × 10^{-4} mol/liter (*data not shown*).

Action of GTP on the Effect of Caffeine and IP_3

In order to examine if "connection" of Ca^{2+} pools described above (*see* Figs. 3, 4 and 6) could be "Ca^{2+} conveyance" between Ca^{2+} pools, as had been postulated to occur in the presence of GTP (Mullaney et al., 1988; Ghosh et al., 1989) with or without PEG we have studied the action of GTP on both IP_3- and caffeine-induced Ca^{2+} release in isolated membrane vesicles.

As shown in Fig. 8*a* when membrane vesicles of the Percoll fraction P_1 were incubated in the absence of PEG, addition of 10^{-5} mol/liter GTP at steady state neither did induce any Ca^{2+} release nor did it influence the Ca^{2+} release induced by caffeine. However, when incubation was performed in the presence of the membrane fusogen PEG (3%) (Fig. 8*b* and *c*), addition of 10^{-5} mol/liter GTP caused Ca^{2+} release followed by Ca^{2+} reuptake. The rate of GTP-induced Ca^{2+} release as well as the rate of Ca^{2+} reuptake were slow when compared to the rates after addition of IP_3 (Fig. 8*c*). In the presence of GTP reuptake of Ca^{2+} did not occur into the IsCaP, since subsequent addition of IP_3 at steady state did not result in further Ca^{2+} release (Fig. 8*b*). Without GTP and PEG, however, Ca^{2+} reuptake following IP_3-induced Ca^{2+} release partially filled an IsCaP since a second addition of IP_3 caused a second Ca^{2+} release (*see* Fig. 5*a*). The effect of GTP could not be observed with its nonhydrolyzable analogue GTPγS (Fig. 8*c*). When GTP, in the presence of PEG, was added together with IP_3 an enhancement of the latter

Fig. 5. (*a*) Effect of heparin on IP_3 and caffeine-induced Ca^{2+} release from membrane vesicles (fraction P_1 of the Percoll gradient). Membrane vesicles (1 mg protein/ml) were incubated in standard incubation medium. Heparin (100 μg/ml), IP_3 (5 × 10^{-6} mol/liter), caffeine (2 × 10^{-2} mol/liter) and $CaCl_2$ 5 nmol (final concentration of 2.5 × 10^{-6} mol/liter) were added where indicated. One out of two similar experiments. (*b*) Effect of caffeine and IP_3 on Ca^{2+} release from vanadate- and ruthenium red (RR)-pretreated membrane vesicles (fraction P_2 of the Percoll gradient). Microsomes preincubated for 15 min in standard incubation with vanadate (10^{-4} mol/liter) and with or without RR (10^{-5} mol/liter) (as indicated), were incubated in the same buffer in the presence of the same substances as during preincubation. Caffeine (10^{-2} mol/liter), IP_3 (5 × 10^{-6} mol/liter) and $CaCl_2$ 5 nmol (final concentration of 2.5 × 10^{-6} mol/liter) were added where indicated. One out of two similar experiments. (*c*) Effect of heparin and RR on Ca^{2+}-induced Ca^{2+} release from membrane vesicles of the Percoll fraction P_1. Membrane vesicles, at a final concentration of 1 mg protein/ml, were incubated in standard incubation medium in the absence or presence of 10^{-5} mol/liter RR. Heparin (100 μg/ml) and $CaCl_2$ pulses of 5 or 10 nmol (final concentration of 2.5 × 10^{-6} mol/liter and 5 × 10^{-6} mol/liter, respectively) were added where indicated

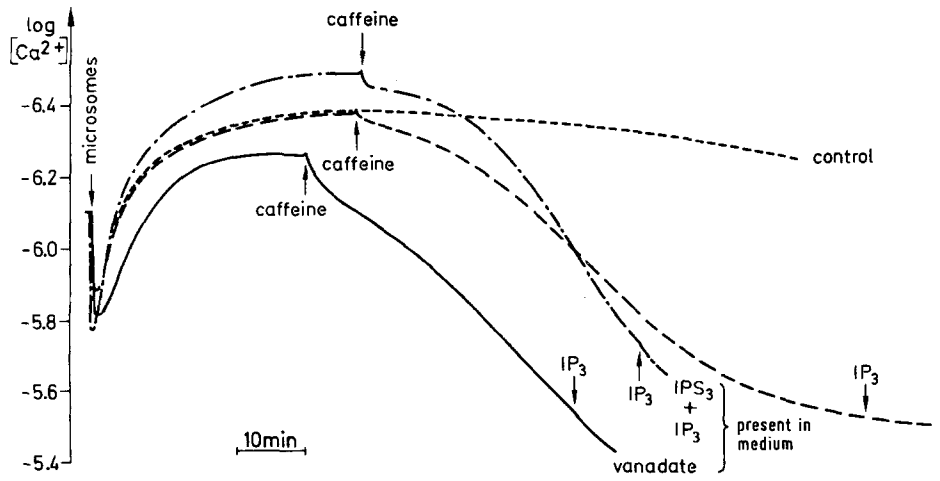


Fig. 6. Caffeine-induced calcium release from pancreatic membrane vesicles (Percoll fraction P_1) in the presence of vanadate (10^{-4} mol/liter) or of IPS_3 + IP_3 (3×10^{-5} mol/liter and 5×10^{-6} mol/liter, respectively). Following 15-min preincubation of membrane vesicles in a standard incubation medium with or without 10^{-4} mol/liter vanadate, membrane vesicles with vanadate preincubation were added to incubation buffer with vanadate (10^{-4} mol/liter) and vesicles without vanadate preincubation were added to IP_3 (5×10^{-6} mol/liter) + IPS_3 (3×10^{-5} mol/liter) containing incubation buffer as indicated. Caffeine at a final concentration of 2×10^{-2} mol/liter was added where indicated. One out of two similar experiments

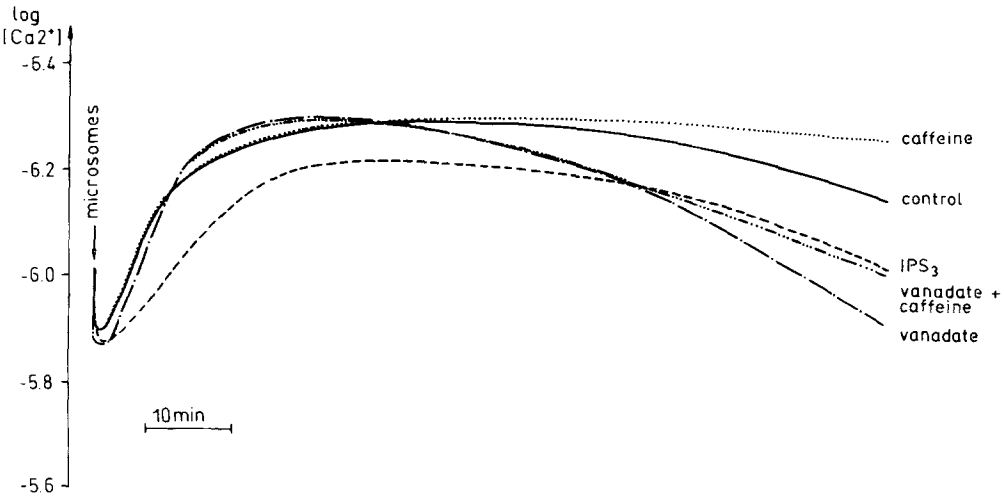


Fig. 7. Effects of caffeine (2×10^{-2} mol/liter), vanadate (10^{-4} mol/liter), IPS_3 (5×10^{-5} mol/liter), caffeine + vanadate, and IPS_3 + vanadate on Ca^{2+} uptake and subsequent Ca^{2+} release from membrane vesicles of the Percoll fraction P_1. Membrane vesicles (1 mg protein/ml) preincubated for 15 min without or with vanadate were incubated in standard incubation medium in the absence or presence of either caffeine, vanadate, IPS_3, caffeine + vanadate, or IPS_3 + vanadate as indicated. Ca^{2+} uptake was monitored with a Ca^{2+} electrode as described in Methods. One out of three similar experiments

effect was observed (Fig. 8*c*). When GTP, in the presence of PEG, was added together with caffeine, the amount of Ca^{2+} released was also higher than with GTP alone (Fig. 8*b*), and in contrast to effects of caffeine alone (Fig. 4*a*) Ca^{2+} was taken up again.

Discussion

We have previously described the presence of two nonmitochondrial Ca^{2+} pools in pancreatic acinar cells: an IP_3-sensitive Ca^{2+} pool (IsCaP), which takes up Ca^{2+} via a Ca^{2+}/H^+ exchanger, and an IP_3-insensitive Ca^{2+} pool, into which Ca^{2+} uptake is mediated by a vanadate-sensitive Ca^{2+} transport ATPase. While IP_3 releases about 30% of all Ca^{2+} that had been taken up by cells, any mechanism by which Ca^{2+} is released from the IP_3-insensitive Ca^{2+} stores has not been found so far. Ghosh et al. (1989) gave evidence that in the presence of GTP Ca^{2+} conveyance between IP_3-sensitive and IP_3-insensi-

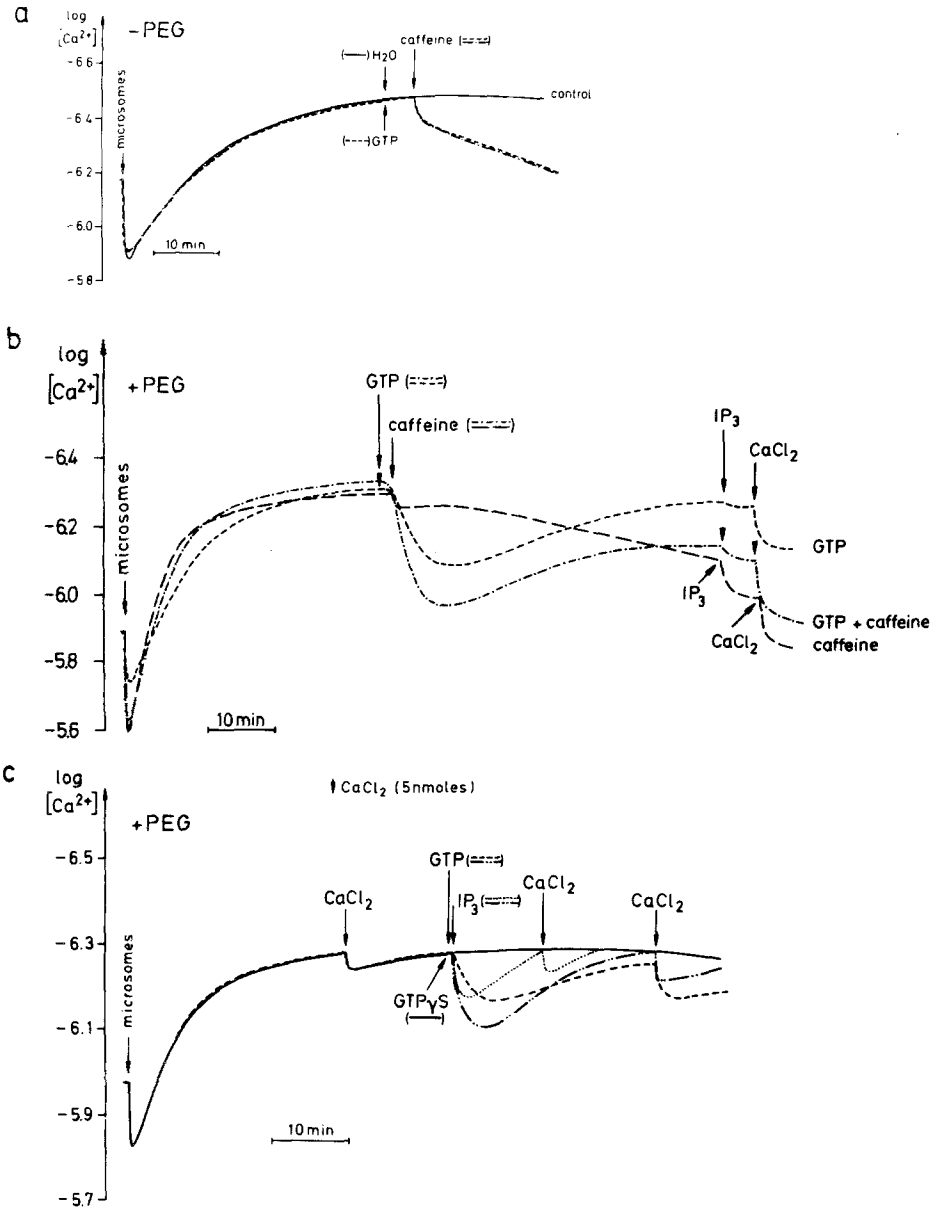


Fig. 8. (*a*) Effects of GTP and caffeine on Ca^{2+} release from membrane vesicles of the Percoll fraction P_1. Membrane vesicles at a final concentration of 1 mg protein/ml were incubated under standard conditions. Where indicated GTP (final concentration of 10^{-5} mol/liter) and caffeine (final concentration of 2×10^{-2} mol/liter) were added. (*b*) Effect of GTP, of caffeine, of GTP + caffeine, and of IP_3 on Ca^{2+} release from membrane vesicles of the Percoll fraction P_1 in the presence of PEG (3%). Membrane vesicles at a final concentration of 1 mg protein/ml were incubated in standard incubation medium supplemented with 3% (wt/vol) PEG. Where indicated GTP (final concentration of 10^{-5} mol/liter), caffeine (final concentration of 2×10^{-2} mol/liter), IP_3 (final concentration of 5×10^{-6} mol/liter) and $CaCl_2$ 5 nmol (final concentration of 2.5×10^{-6} mol/liter) were added. Typical for five similar experiments. (*c*) Effects of GTP, of GTPγS, of IP_3 and of GTP + IP_3 on Ca^{2+} release from membrane vesicles (fraction P_1 of the Percoll gradient) in the presence of PEG (3%). Membrane vesicles (1 mg protein/ml) were incubated in standard incubation medium supplemented with 3% (wt/vol) PEG. Where indicated GTP (final concentration of 10^{-5} mol/liter), GTPγS (final concentration of 10^{-5} mol/liter), IP_3 (final concentration of 5×10^{-6} mol/liter) and $CaCl_2$ 5 nmol (final concentration of 2.5×10^{-6} mol/liter calcium) were added. Typical for four similar experiments

tive Ca^{2+} stores occurs. The physiological significance of these observations, however, is not clear.

The present study investigates the question if "communication" between IsCaPs and IisCaPs could be produced by IP_3-induced Ca^{2+} release from the IsCaP causing Ca^{2+}-induced Ca^{2+} release from the IisCaP or alternatively by "connection" of Ca^{2+} pools similar to that reported for GTP-induced Ca^{2+} conveyance between Ca^{2+} pools (Ghosh et al., 1989). The former point was examined by using caf-

feine, an activator of Ca^{2+}-induced Ca^{2+} release channels in the sarcoplasmic reticulum (Endo, 1977; Rousseau & Meissner, 1989). In pancreatic acinar cells Osipchuk et al. (1990) have shown that caffeine alone had no effect on the cytoplasmic Ca^{2+} concentration, but potentiated the Ca^{2+}-releasing action of acetylcholine, IP_3 and GTPγS, an activator of G-protein coupling phospholipase C. We were also unable to find any caffeine effect in isolated permeabilized acinar cells or in a crude fraction of endoplasmic reticulum (fluffy layer) (*data not shown*). A small caffeine-induced Ca^{2+} release could be observed only in the presence of vanadate, which inhibits Ca^{2+} reuptake of released Ca^{2+}, so that a small effect became visible (*data not shown*). However, in the lightest fraction of the Percoll gradient (P_1), in which the IP_3 effect is higher than in the "fluffy layer," a caffeine-sensitive Ca^{2+} pool (CasCaP) is present (Figs. 4*a*, 5*a*, 6 and 8*a*). Percoll fraction P_1 also contains a vanadate-sensitive Ca^{2+} pool (VasCaP) since, Ca^{2+} reuptake following IP_3 addition was inhibited in the presence of vanadate (Figs. 1 and 2*a*).

Fractions P_2 and P_3 contain less IP_3-sensitive Ca^{2+} pools, less vanadate-sensitive Ca^{2+} pools and no or only small amounts of caffeine-sensitive Ca^{2+} pools. It appears that the IP_3-, caffeine- and vanadate-insensitive Ca^{2+} pools in fractions P_2 and P_3 are mainly of the type described previously (Thévenod et al., 1989*a*) which have the Ca^{2+}/H^+ exchanger but no IP_3 receptors and could be inhibited by NBD-Cl (Thévenod et al., 1989*a*; Fig. 2*b* and *c*). In fractions P_4 and P_5 mainly the VasCaP and neither IsCaP nor CasCaP are present (Table 2). Since fraction P_1 contains all types of Ca^{2+} pools: the IsCaP (IP_3-sensitive), an IisCaP (IP_3-insensitive, vanadate-insensitive), the VasCaP (vanadate-sensitive, IP_3-insensitive), and the CasCaP (caffeine-sensitive), it appeared the most suitable fraction to study the interplay between these Ca^{2+} pools.

Caffeine- and Ca^{2+}-Induced Ca^{2+} Release

The caffeine- and Ca^{2+}-induced Ca^{2+} release mechanism as described here appears to be different from that described for skeletal, cardiac or smooth muscle (Fleischer et al., 1985; Rousseau et al., 1987; Kanmura et al., 1988). The main difference is its insensitivity to ryanodine in the present study and in the study on the caffeine-sensitive Ca^{2+} channel present in the pancreatic endoplasmic reticulum (Schmid et al., 1990), whereas ruthenium red inhibited caffeine and Ca^{2+}-induced Ca^{2+} release (Fig. 5*b* and *c*) similar to that in the sarcoplasmic reticulum (Ohnishi, 1979; Miyamoto & Racker, 1981). Caffeine- and calcium-induced Ca^{2+} release were not inhibited by heparin, an inhibitor of the IP_3 effect (Fig. 5*a* and *c*). Therefore the IP_3-induced Ca^{2+} release mechanism is different from that of Ca^{2+}-induced Ca^{2+} release.

Are the Caffeine- (Ca^{2+}-) Sensitive and IP_3-Sensitive Ca^{2+} Pools Separate Stores?

The following discusses the question if the IsCaP and CasCaP are separate, partly overlapping or come into contact under some circumstances. If membrane vesicles were preincubated in the presence of caffeine, no apparent inhibition of Ca^{2+} uptake occurred and the IP_3 effect was the same in both caffeine-pretreated and -untreated vesicles, indicating that the IsCaP is not identical or overlapping with the CasCaP (Fig. 3). Ca^{2+} uptake remains even more stable in the presence of caffeine as compared to the control, which shows "spontaneous" Ca^{2+} release (Fig. 7).

However, when caffeine was added acutely at steady state, complete Ca^{2+} release occurred from IP_3-pretreated vesicles (Fig. 6) as well as from control vesicles which include the IsCaP (Figs. 6 and 4*a*). One possibility to explain these different effects of caffeine when present during Ca^{2+} uptake and when added after steady state [Ca^{2+}] had been reached is the assumption that Ca^{2+} pools come into contact at steady state and that this "contact" is initiated by the CasCaP. If caffeine was in the medium, the CasCaP is supposed not to be filled with Ca^{2+} and to remain uncoupled from other Ca^{2+} pools which do not show "spontaneous" Ca^{2+} release (Fig. 7). However, if Ca^{2+} uptake occurs into the CasCaP in the absence of caffeine "spontaneous" Ca^{2+} release occurs from all Ca^{2+} pools which had taken up Ca^{2+} previously indicating connection of Ca^{2+} pools. Opening of the Ca^{2+} release channel could be favored by the intravesicular Ca^{2+} concentration when the CasCaP had been filled. In the presence of caffeine added at steady state this effect would be enhanced. In a previous study we have reported about the presence of a caffeine-sensitive Ca^{2+} channel in membranes from endoplasmic reticulum (ER) as measured with the patch-clamp method. This Ca^{2+} channel is voltage sensitive and opens at inside-positive potential differences (Schmid et al., 1988). In the absence of a Cl^- conductance small Ca^{2+} efflux from the Ca^{2+} pool would generate an inside-negative potential difference and closure of the Ca^{2+} channel. We had previously described electrogenic Ca^{2+} uptake into membrane vesicles from pancreatic ER that could be inhibited by vanadate (Kemmer et al., 1987). A Cl^- channel

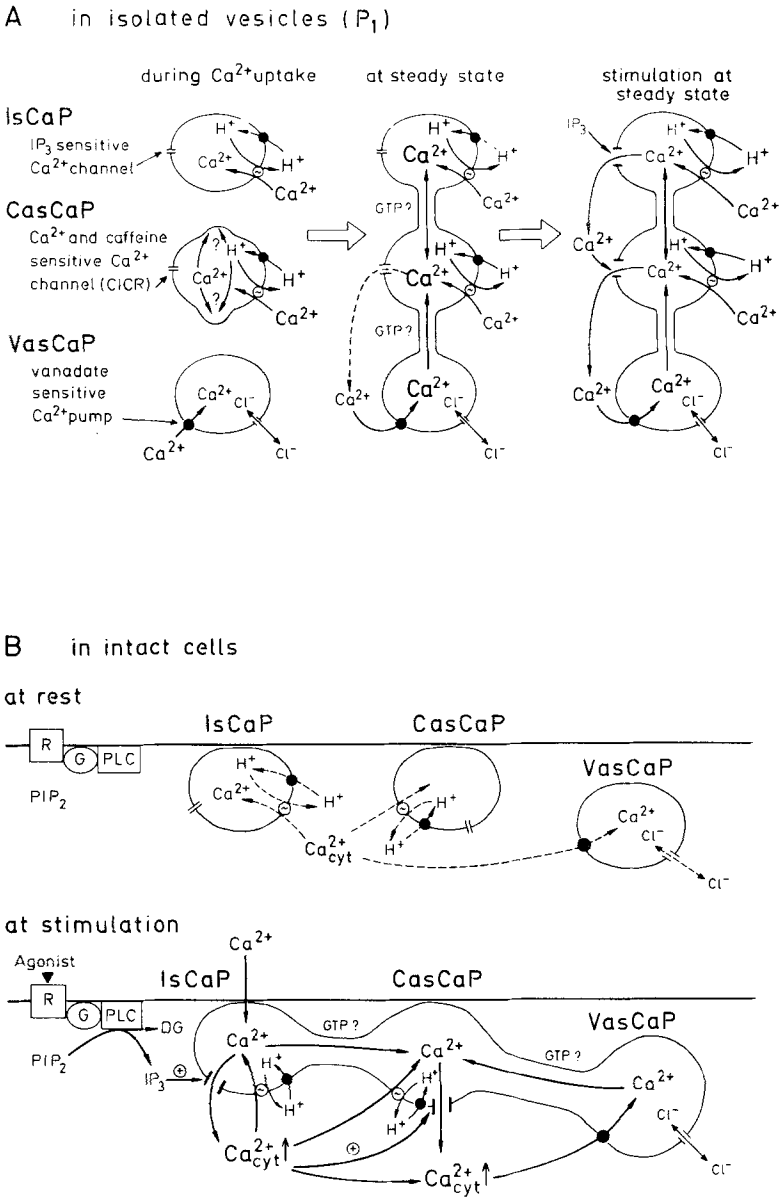


Fig. 9. Model for Ca^{2+} uptake and IP_3- and Ca^{2+}-induced Ca^{2+} release from different Ca^{2+} pools in endoplasmic reticulum of pancreatic acinar cells. We assume the presence of three separate Ca^{2+} pools in pancreatic acinar cells. An IP_3-sensitive Ca^{2+} pool (IsCaP) takes up Ca^{2+} via a Ca^{2+}/H^+ exchanger (Thévenod et al., 1989*a*). A Ca^{2+} and caffeine-sensitive Ca^{2+} pool (CasCaP) with the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism probably contains the same Ca^{2+} uptake mechanism as the IsCaP but no IP_3 receptors. (In addition similar Ca^{2+} pools with the Ca^{2+}/H^+ exchanger but without IP_3 receptors and without CICR or with both types of Ca^{2+} release mechanisms are also possible). The third Ca^{2+} pool, VasCaP, contains an electrogenic vanadate-inhibitable Ca^{2+} pump (Kemmer et al., 1987) a Cl^- channel (Schmid et al., 1988) and neither IP_3-receptors nor the CICR mechanism. During Ca^{2+} uptake these Ca^{2+} pools stay separate. However, at steady state connection of Ca^{2+} pools proceeds from the CasCaP by a mechanism that could involve GTP (Ghosh et al., 1989) and/or signals such as the intravesicular $[Ca^{2+}]$ or a low pH reached at steady state. It is assumed that under the experimental conditions in isolated vesicles (*A*) Ca^{2+} uptake into the CasCaP and connection of Ca^{2+} pools occurs without previous IP_3-induced Ca^{2+} release from the IsCaP. However, in intact cells there may be only little Ca^{2+} in the CasCaP at rest. During stimulation cytosolic $[Ca^{2+}]$ is increased by IP_3-induced Ca^{2+} release and Ca^{2+} may be taken up into the CasCaP (*B*). This leads to connection of Ca^{2+} pools and coupling to the VasCaP containing Cl^- channels which allows Ca^{2+} efflux from the CasCaP and other Ca^{2+} pools connected to the CasCaP. Ca^{2+} release will lead to disconnection of the Ca^{2+} pools due to decreased intravesicular $[Ca^{2+}]$ or increased pH as a consequence of increased Ca^{2+}/H^+ exchange during Ca^{2+} efflux from the CasCaP. The idea that progression of the Ca^{2+} signal occurs from the surface cell membrane to the cell interior through the IsCaP is based on models by Berridge and Irvine (1989), Goldbeter et al. (1990) and Putney (1986) as well as on data from Osipchuk et al. (1990)

present in the ER (Schmid et al., 1988) could compensate Ca^{2+} uptake by charge equilibration. Connection of the CasCaP containing the voltage-sensitive Ca^{2+} channel with Cl^--containing vesicles at steady state $[Ca^{2+}]$ would allow Ca^{2+} efflux from the CasCaP.

The mechanism, by which the connection of Ca^{2+} pools at steady state occurs, is not clear. As will be discussed below it seems to be different from that which can be activated by GTP.

Ca^{2+} Release in the Presence of GTP

As shown in Fig. 8*b* Ca^{2+} release could be induced by addition of GTP in the presence of PEG. In the presence of caffeine GTP-induced Ca^{2+} release was higher than with GTP alone (Fig. 8*b*). Similarly, the IP_3 effect was higher in the presence of GTP and PEG (Fig. 8*c*). However, total Ca^{2+} release was not seen in the absence of vanadate and Ca^{2+} reuptake into a VasCaP occurred. Following addition of GTP, subsequent addition of IP_3 did not result in further Ca^{2+} release, indicating that the IsCaP had been emptied in the presence of GTP.

The experiments with GTP also show that a vesicle-outside Ca^{2+} concentration higher than 8×10^{-7} mol/liter was reached without following Ca^{2+} release (Fig. 8*b*). Ca^{2+} reuptake occurring at concentrations higher than 10^{-6} mol/liter $[Ca^{2+}]$ was *not* due to an effect of GTP itself but was rather a consequence of fusion in the presence of PEG (Comerford & Dawson, 1988). This is shown in Fig. 8*a*, demonstrating that without PEG in the medium the caffeine effect was not enhanced in the presence of GTP and Ca^{2+} reuptake did not occur.

The differences of caffeine effects, seen in GTP + PEG-treated (Fig. 8*b*) and -untreated vesicles (Figs. 4, 5*a* and 8*a*), allow the conclusion that the mechanisms of GTP-induced Ca^{2+} conveyance between Ca^{2+} pools (Mullaney et al., 1987), which likely involves fusion of Ca^{2+} pools, is different from that occurring in GTP-untreated membranes as described here.

Summarizing, Fig. 9 shows our model, which assumes at least three different Ca^{2+} pools, which are probably separate during Ca^{2+} uptake, but come into connection at steady state, so that Ca^{2+} conveyance between these pools can occur. In addition, a Ca^{2+} pool with the Ca^{2+}/H^+ exchanger but lacking IP_3 receptors and Ca^{2+}-induced Ca^{2+} release mechanism, such as present in fractions P_2 and P_3, might also occur in pancreatic cells but is not included in the model.

The vanadate- and IP_3-sensitive Ca^{2+} pools had been already described previously (Thévenod et al., 1989*a*). In addition, we postulate a Ca^{2+} pool with a Ca^{2+}-induced Ca^{2+} release mechanism that is also sensitive to caffeine. At a vesicle-outside (cytosolic) Ca^{2+} concentration of $5.5–8 \times 10^{-7}$ mol/liter this channel opens, probably favored by a high intravesicular Ca^{2+} concentration that is reached in this pool at steady state. Since in the presence of caffeine in the medium the steady state $[Ca^{2+}]$ remains more stable than in the control (Fig. 7), it appears that connection of Ca^{2+} pools at steady state proceeds from the caffeine-sensitive Ca^{2+} pool indicated by "budding off" of the membrane in the CasCaP. If the CasCaP remains depleted of Ca^{2+} in the presence of caffeine it remains uncoupled from other Ca^{2+} pools and significant Ca^{2+} release does not occur.

Connection of Ca^{2+} pools could occur as a consequence of the intravesicular Ca^{2+} concentration or pH in the CasCaP reached at steady state. Recently evidence has been obtained that vesicular traffic between the ER and the Golgi apparatus requires GTP and Ca^{2+} ions (Sambrook, 1990). A role of pH in fusion was obtained in experiments with liposomes demonstrating that clathrin enhanced fusion at acidic pH in the phosphatidylserine-containing vesicles (Hong, Yoshimura & Papahadjopoulos, 1985). We assume that in experiments with isolated vesicles (Percoll fraction P_1) (Fig. 9*A*) at a buffer concentration of $\approx 2 \times 10^{-6}$ mol/liter Ca^{2+} uptake into the CasCaP leads to filling of this pool with Ca^{2+}. However, since any caffeine effect was not seen in resting intact pancreatic acinar cells, there may be only little Ca^{2+} in the CasCaP at rest. During stimulation cytosolic Ca^{2+} is increased by IP_3-induced Ca^{2+} release and Ca^{2+} may be taken up into the CasCaP (Fig. 9*B*; Goldbeter, Dupont & Berridge, 1990). As had been shown by Osipchuk et al. (1990) caffeine evoked regular Ca^{2+} spikes when applied in the presence of a subthreshold dose of acetylcholine or in the presence of a subthreshold intracellular Ca^{2+} infusion (Wakui, Potter & Petersen, 1989), whereas in the absence of stimulation caffeine did not evoke any effect (Osipchuk et al., 1990). This indicates that Ca^{2+} stored in the CasCaP is released into the cytosol in a process activated by increased cytosolic $[Ca^{2+}]$. According to our model (Fig. 9) Ca^{2+} release from the CasCaP would be possible only if an intravesicular signal (such as the intravesicular $[Ca^{2+}]$ or pH) allows connections to be established to the VasCaP containing Cl^- channels.

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