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Differences in uptake, storage and release properties between inositol trisphosphate-sensitive and -insensitive Ca\textsuperscript{2+} stores in permeabilized pancreatic acinar cells

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Abstract — Rabbit pancreatic acinar cells, permeabilized by saponin treatment, were used to study the kinetics of ATP-dependent Ca\textsuperscript{2+} uptake and release in inositol 1,4,5-trisphosphate (Ins-1,4,5-P\textsubscript{3})-sensitive and -insensitive stores. Permeabilized acinar cells rapidly accumulated Ca\textsuperscript{2+} to steady-state. At steady state, approximately 60% of actively stored Ca\textsuperscript{2+} resided in the Ins-1,4,5-P\textsubscript{3}-sensitive store. Kinetic analysis of the Ca\textsuperscript{2+} uptake process revealed that the initial Ca\textsuperscript{2+} uptake rate was 1.7 times higher in the Ins-1,4,5-P\textsubscript{3}-sensitive store as compared to the Ins-1,4,5-P\textsubscript{3}-insensitive store. On the other hand, the Ca\textsuperscript{2+} uptake capacity was 1.6 times higher in the Ins-1,4,5-P\textsubscript{3}-sensitive store as compared to the Ins-1,4,5-P\textsubscript{3}-insensitive store. The Ca\textsuperscript{2+} uptake rate in the Ins-1,4,5-P\textsubscript{3}-sensitive store remained virtually constant for at least 4 min, whereas in the Ins-1,4,5-P\textsubscript{3}-insensitive Ca\textsuperscript{2+} store this rate progressively declined with time. These observations are compatible with: (I) an Ins-1,4,5-P\textsubscript{3}-sensitive store containing relatively few Ca\textsuperscript{2+} pumps but possessing a relatively high Ca\textsuperscript{2+} uptake capacity, which may reflect the presence of a substantial amount of Ca\textsuperscript{2+} binding protein; and (ii) an Ins-1,4,5-P\textsubscript{3}-insensitive Ca\textsuperscript{2+} store containing relatively many Ca\textsuperscript{2+} pumps but possessing a relatively low Ca\textsuperscript{2+} uptake capacity, which may reflect the presence of little if any Ca\textsuperscript{2+} binding protein. The data presented are consistent with the idea of a heterogeneous distribution of Ca\textsuperscript{2+} pumps, Ca\textsuperscript{2+} binding proteins and Ca\textsuperscript{2+} release channels between intracellular Ca\textsuperscript{2+} storage organelles.

Intracellular Ca\textsuperscript{2+} stores play an important role in cellular Ca\textsuperscript{2+} homeostasis and signal transduction [1]. Many extracellular signals, including hormones, neurotransmitters and physical stimuli, evoke the release of Ca\textsuperscript{2+} from Ca\textsuperscript{2+} storage organelles through the intermediation of inositol 1,4,5-trisphosphate, produced upon the receptor activated hydrolysis of phosphatidyl inositol 4,5-bisphosphate.
The development of Ca$^{2+}$ imaging techniques has enabled to study the spatial and temporal distribution of such receptor evoked cytoplasmic Ca$^{2+}$ signals in intact cells. Using these techniques, pancreatic acinar cells were demonstrated to respond to receptor activation with a rapidly spreading Ca$^{2+}$ signal, initiated at the secretory pole [2-6]. This initial rise in free cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) was shown to be independent of external Ca$^{2+}$ [2,3,7], indicating that it involves the Ins-1,4,5-P$_3$-stimulated release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores. Infusion of a minimal effective concentration of Ins-1,4,5-P$_3$ at the basolateral side of the acinar cell evoked a Ca$^{2+}$ rise restricted to the apical side of the cell, whereas infusion of a high concentration of Ins-1,4,5-P$_3$ evoked a global [Ca$^{2+}$]$_i$ rise [3,5]. In addition, infusion of Ca$^{2+}$ revealed the presence of Ca$^{2+}$-sensitive Ca$^{2+}$ stores in the apical region of the acinar cell [3]. These observations demonstrate that pancreatic acinar cells possess Ca$^{2+}$ stores with different Ca$^{2+}$ release mechanisms, namely Ca$^{2+}$ stores with Ins-1,4,5-P$_3$-operated Ca$^{2+}$ release channels and Ca$^{2+}$ stores with Ca$^{2+}$-activated Ca$^{2+}$ release channels, and that these different Ca$^{2+}$ stores are situated in different areas of the cell. In addition, they demonstrate that Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores are heterogeneous in their response to Ins-1,4,5-P$_3$.

Thus far, the mechanism underlying the heterogeneity between Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores with respect to their releasability by Ins-1,4,5-P$_3$ is unclear. Basically, there are two explanations for these differences in releasability between internal Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores. Firstly, Ins-1,4,5-P$_3$-operated Ca$^{2+}$ release channels may display different sensitivities towards activation by Ins-1,4,5-P$_3$. It has been demonstrated that multiple Ins-1,4,5-P$_3$ receptor subunits can be obtained from separate genes [8,9] and/or the process of alternative splicing [10] and it has been suggested that this may give rise to multiple affinity binding sites. Thus far, immunological studies have revealed the presence of the type 3, rather than the type 1, subtype of the Ins-1,4,5-P$_3$ receptor in the pancreatic acinar cell [11]. In addition, the potency of Ins-1,4,5-P$_3$ in releasing Ca$^{2+}$ has been shown to be altered by phosphorylation of the receptor protein [12-14]. Furthermore, physiological changes in free cytosolic Ca$^{2+}$ concentration [15-18] and cellular pH [19,20] have been demonstrated to control Ins-1,4,5-P$_3$ binding. Moreover, the intravesicular Ca$^{2+}$ content has been implicated in regulation of the sensitivity of the Ins-1,4,5-P$_3$-operated Ca$^{2+}$ release channel for activation by Ins-1,4,5-P$_3$ [21-24]. Secondly, heterogeneity between Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores can be explained by differences in the relative amount of Ca$^{2+}$ pumps as compared to the amount of Ins-1,4,5-P$_3$-operated Ca$^{2+}$ release channels [25]. In the case of a relatively high amount of Ca$^{2+}$ pumps, Ins-1,4,5-P$_3$-evoked Ca$^{2+}$ release at low Ins-1,4,5-P$_3$ concentrations will be largely compensated for by active Ca$^{2+}$ uptake, whereas the same Ins-1,4,5-P$_3$ concentrations will rapidly empty stores possessing relatively few pumps.

We have previously shown that permeabilized pancreatic acinar cells contain at least three distinct subpopulations of nonmitochondrial Ca$^{2+}$ storage organelles: (i) stores sensitive to Ins-1,4,5-P$_3$; (ii) stores sensitive to GTP; and (iii) stores insensitive to Ins-1,4,5-P$_3$ and GTP [26]. In subsequent studies, we demonstrated that Ins-1,4,5-P$_3$-sensitive and insensitive stores could also be distinguished by virtue of differences in releasability by ruthenium red [27] and the aminosteroid U73122 [28]. The Ins-1,4,5-P$_3$-sensitive stores being more sensitive to both compounds than the Ins-1,4,5-P$_3$-insensitive stores.

In order to gain more insight into possible mechanisms underlying the heterogeneity between intracellular Ca$^{2+}$ stores, permeabilized pancreatic acinar cells were used to study the kinetics of Ca$^{2+}$ uptake and release in both the Ins-1,4,5-P$_3$-sensitive store, previously shown to consist of a heterogeneous population of Ca$^{2+}$ storage organelles with respect to their releasability by Ins-1,4,5-P$_3$ [25], and the Ins-1,4,5-P$_3$-insensitive store. The data obtained are consistent with the idea of a heterogeneous distribution of Ca$^{2+}$ pumps, Ca$^{2+}$ binding proteins and Ca$^{2+}$ release channels between Ins-1,4,5-P$_3$-sensitive and -insensitive Ca$^{2+}$ storage organelles.

Materials and methods

Permeabilization of pancreatic acinar cells

Rabbit pancreatic acinar cells were isolated by enzymatic digestion using collagenase and hyaluronidase.
as previously described [26]. Acinar cells, permeabilized by saponin treatment, were washed twice and resuspended in a Ca^{2+} uptake medium (4 mg protein/ml) containing 120 mM KCl, 1.0 mM MgCl₂, 1.2 mM KH₂PO₄, 5 mM pyruvate, 5 mM succinate, 0.5 mM EGTA, 0.5 mM nitrilotriacetic acid, 0.5 mM HEEDTA, 0.2 mg/ml soybean trypsin inhibitor and 20 mM HEPES and adjusted to pH 7.1 with KOH [25-28]. Permeabilized acinar cells were stored on ice until use.

**Ca^{2+} uptake and release experiments**

Ca^{2+} uptake was started by adding 10 μl of the permeabilized cell suspension to 90 μl of Ca^{2+} uptake medium which contained in addition: 0 or 1 mM NaATP, 10 mM creatine phosphate, 10 U/ml creatine kinase, 3% (w/v) polyethylene glycol (MW 4000) and 5 μCi ⁴⁵Ca^{2+}/ml. The free Mg^{2+} (0.9 mM) and Ca^{2+} (as indicated) concentrations were adjusted as described by Schoenmakers et al. [29]. At 0.5 and 1 μM ambient free Ca^{2+}, 5 μM ruthenium red was included in the medium to prevent mitochondrial Ca^{2+} uptake. At this concentration, ruthenium red does not interfere with Ca^{2+} uptake in nonmitochondrial Ca^{2+} stores [27]. The incubations, performed at 37°C, were stopped by adding 1 ml of ice-cold stop solution containing 150 mM KCl, 5.0 mM MgCl₂, 1.0 mM EGTA and 20 mM HEPES/KOH (pH 7.1) and the suspension was rapidly filtered (Schleicher and Schuell, GF92, Dassel, Germany). The filters were washed twice and counted for radioactivity. Total Ca^{2+} was calculated and expressed as nmol Ca^{2+} per mg of protein. Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP.

Protein was determined with a commercial Coomassie Blue kit (Bio-Rad) after treatment of the cells with 0.1% Triton X-100. γ-Globulin (Bio-Rad) was used as a standard.

**Ca^{2+} exchange experiments under steady-state conditions**

Permeabilized acinar cells were loaded with Ca^{2+} in the presence of radioactive tracer in Ca^{2+} uptake medium in which the concentration of each of the divalent cation chelators was reduced to 0.15 mM. The ambient free Ca^{2+} concentration was adjusted to 0.19 μM. After 10 min of Ca^{2+} loading, the Ca^{2+} uptake medium was diluted 3-fold with medium in which the concentration of each of the chelators was increased to 2 mM and which did not contain ⁴⁵Ca^{2+}. The ambient free Ca^{2+} concentration was maintained at 0.19 μM. This procedure resulted in a 28-fold reduction in specific activity of ⁴⁵Ca^{2+}, thus allowing the study of unidirectional Ca^{2+} fluxes under steady-state conditions.

In a second type of exchange experiment, permeabilized acinar cells were loaded with Ca^{2+} to steady-state in the absence of radioactive tracer. After 10 min of Ca^{2+} loading, ⁴⁵Ca^{2+} was added and accumulated Ca^{2+} was determined at the indicated times.

**Analysis of the data**

The kinetics with which Ca^{2+} was accumulated in the Ins-1,4,5-P₃-sensitive and -insensitive stores was analysed by means of the nonlinear regression computer program InPlot (GraphPad Software for Science, San Diego, CA, USA).

**Materials**

Collagenase was purchased from Cooper Biomedical Inc., Malvern, PA, USA. Polyethylene glycol (MW 4000), EGTA and ruthenium red were obtained from Merck, Darmstadt, Germany; hyaluronic acid, phosphocreatine and creatine kinase were from Boehringer, Mannheim, Germany; NaATP, bovine serum albumin, Triton X-100, HEEDTA, nitrilotriacetic acid, saponin, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor and trypan blue were from Sigma, St Louis, MO, USA; HEPES was from Research Organics Inc., Cleveland, OH, USA; thapsigargin was from LC Services Corporation, Woburn, MA, USA; ⁴⁵Ca^{2+} (20 mCi/ml) was from New England Nuclear, Dreieich, Germany. Ins(1,4,5)P₃ and heparin were generously supplied by Dr P. Westerduin, Organon Scientific Development Group, Oss, The Netherlands. All other chemicals were of analytical grade.
Results

Time-dependence of ATP-dependent Ca\(^{2+}\) uptake in Ins-1,4,5-P\(_3\)-sensitive and -insensitive Ca\(^{2+}\) stores

Pancreatic acinar cells, permeabilized by saponin treatment, maximally accumulated 2.77 (SE = 0.27, n = 24) nmol Ca\(^{2+}\)/mg of protein in an energy-dependent Ca\(^{2+}\) storage pool when incubated at an ambient free Ca\(^{2+}\) concentration of 190 nM and a temperature of 37°C. Steady-state Ca\(^{2+}\) uptake was reached within 7 min following the addition of ATP (Fig. 1A) and subsequent addition of Ins-1,4,5-P\(_3\) maximally released 65% of actively stored Ca\(^{2+}\). In order to study the kinetics of the Ca\(^{2+}\) uptake process in individual Ca\(^{2+}\) stores, Ca\(^{2+}\) uptake experiments were performed in the presence of a maximally effective concentration of Ins-1,4,5-P\(_3\). Figure 1A shows that under these conditions steady-state Ca\(^{2+}\) uptake reached a maximum of 1.20 nmol Ca\(^{2+}\)/mg of protein (SE = 0.15, n = 4) within 3 min following the addition of ATP. Steady-state Ca\(^{2+}\) uptake remained maximal for another 7 min, indicating that no significant Ins-1,4,5-P\(_3\) degradation occurred. The steady-state level attained in the presence of a maximally effective concentration of

**Fig. 1** Time-dependence of active Ca\(^{2+}\) uptake in Ins-1,4,5-P\(_3\)-sensitive and -insensitive intracellular Ca\(^{2+}\) stores. Permeabilized pancreatic acinar cells were loaded with Ca\(^{2+}\) in the absence (closed circles) or presence (closed triangles) of a maximally effective concentration of Ins-1,4,5-P\(_3\) (30 µM). The ambient free Ca\(^{2+}\) concentration was 0.19 µM and the incubations were performed at 37°C. The reactions were terminated at the indicated times. The open symbols represent the residual Ca\(^{2+}\) content in control cells loaded with Ca\(^{2+}\) for 10 min and subsequently incubated in the presence of either saline (open circle) or 30 µM Ins-1,4,5-P\(_3\) (open triangle) for another 2 min. The residual Ca\(^{2+}\) content was calculated and corrected for uptake in the absence of ATP. The values presented are the mean ± SE of at least 4 independent experiments. (A) Time-dependence of Ca\(^{2+}\) uptake in the absence (closed circles) and presence (closed triangles) of 30 µM Ins-1,4,5-P\(_3\). (B) Time-dependence of Ca\(^{2+}\) uptake in the Ins-1,4,5-P\(_3\)-sensitive store. The dashed line represents a tentative fit of the Ca\(^{2+}\) uptake process to a monoexponential rate equation. The fit was obtained by fixing the maximal Ca\(^{2+}\) uptake value. (C) Time-dependence of Ca\(^{2+}\) uptake in the Ins-1,4,5-P\(_3\)-insensitive store. The dashed line represents the fit of the Ca\(^{2+}\) uptake process to a monoexponential rate equation. The fit was obtained without fixing the maximal Ca\(^{2+}\) uptake value.
Ins-1,4,5-P₃ was virtually similar to that reached 2 min after maximal stimulation of steady-state loaded control cells (Fig. 1C, open triangle).

Non-linear regression analysis revealed that the uptake process in this store occurred according to a monoexponential rate equation with a half-time of 1.1 min⁻¹ (Fig. 1C). The initial Ca²⁺ uptake rate in the Ins-1,4,5-P₃-insensitive store was calculated to be 0.79 nmol Ca²⁺/mg protein.min⁻¹. The time-dependence of the Ca²⁺ uptake in the Ins-1,4,5-P₃-sensitive store was estimated by subtracting the Ca²⁺ uptake values measured in the presence of Ins-1,4,5-P₃ from those measured in the absence of Ins-1,4,5-P₃. Figure 1B shows that Ca²⁺ uptake in the Ins-1,4,5-P₃-sensitive store did not reach steady-state before 7 min following the addition of ATP. Ca²⁺ uptake was virtually linear with time during the first 4 min and could by no means be fitted to a monoexponential rate equation. The dotted line represents a tentative fit to a monoexponential rate equation. Linear regression analysis revealed an initial Ca²⁺ uptake rate of 0.28 nmol Ca²⁺/mg of protein.min⁻¹.

**Ca²⁺-dependence of steady-state ATP-dependent Ca²⁺ uptake in Ins-1,4,5-P₃-sensitive and -insensitive Ca²⁺ stores**

The steady-state Ca²⁺ uptake level dose-dependently increased with increasing of the ambient free Ca²⁺ concentration (Fig. 2). At 1.0 µM free Ca²⁺, steady-state Ca²⁺ uptake reached a value 1.3-fold higher (3.57 nmol Ca²⁺/mg of protein (SE = 0.21, n = 3)) than that obtained at an ambient free Ca²⁺ concentration of 0.19 µM. In relative terms, the size of the Ins-1,4,5-P₃-sensitive store, determined by challenging steady-state loaded cells with a maximally effective concentration of Ins-1,4,5-P₃ (20 µM), did not change upon increasing of the ambient free Ca²⁺ concentration (see also [30]). At 0.19 µM, 0.5 µM and 1.0 µM [Ca²⁺]free, 20 µM Ins-1,4,5-P₃ released 63% (1.73 nmol Ca²⁺/mg of protein (SE = 0.05, n = 3)), 64% (2.01 nmol Ca²⁺/mg of protein (SE = 0.06, n = 3)) and 62% (2.21 nmol Ca²⁺/mg of protein (SE = 0.17, n = 3)) of actively stored Ca²⁺, respectively.

**Ca²⁺-dependence of initial ATP-dependent Ca²⁺ uptake rates in Ins-1,4,5-P₃-sensitive and -insensitive Ca²⁺ stores**

In order to assure linearity of ATP-dependent Ca²⁺ uptake with time in experiments aimed to study the Ca²⁺-dependence of ATP-dependent Ca²⁺ uptake, incubations were terminated after 1 min in the case of an ambient free Ca²⁺ concentration of 0.19 µM and after 0.5 min in the case of 0.5 µM and 1.0 µM free Ca²⁺ (see, Fig. 1A, closed circles). At 0.19 µM free Ca²⁺, permeabilized acinar cells accumulated 0.88 nmol Ca²⁺ per mg of protein per min (SE = 0.04, n = 3) (Fig. 3). This value increased with increasing of the ambient free Ca²⁺ concentration to reach a value of 2.02 nmol Ca²⁺ per mg of protein per min (SE = 0.05, n = 3) at 1.0 µM free Ca²⁺. When initial Ca²⁺ uptake was studied in the presence of a maximally effective concentration of Ins-1,4,5-P₃ (20 µM), reflecting ATP-dependent Ca²⁺ uptake in the Ins-1,4,5-P₃-insensitive store, a value of 0.55 nmol Ca²⁺ per mg of protein per min (SE = 0.01, n = 3) was obtained at 0.19 µM free.

![Fig. 2](https://example.com/fig2.png) Dependence of the size of the Ins-1,4,5-P₃-sensitive and -insensitive intracellular Ca²⁺ store on the ambient free Ca²⁺ concentration. Permeabilized pancreatic acinar cells were loaded with Ca²⁺ to steady-state at the indicated ambient free Ca²⁺ concentrations for 10 min. At 10 min, either saline (closed circles) or a maximally effective concentration of 20 µM Ins-1,4,5-P₃ (closed triangles) was added and the incubations were terminated 2 min later. Actively stored Ca²⁺ is expressed in nanomoles of Ca²⁺ per milligram of acinar protein. The results presented are the mean ± SE of at least 3 independent experiments, each of which performed in triplicate.
mally effective concentration of Ins-1,4,5-P₃, resulted in a rapid reuptake of Ca²⁺ (Fig. 4A). This procedure allowed to estimate the Ca²⁺ uptake rate in the Ins-1,4,5-P₃-sensitive store without interference of the Ins-1,4,5-P₃-insensitive store. The Ca²⁺ reuptake rate was calculated to be 0.43 nmol Ca²⁺/mg of protein.min⁻¹. This value correlated well with that observed in the Ca²⁺ uptake experiments (Figs 1B and 3). The heparin-induced reuptake of Ca²⁺ was completely prevented by thapsigargin, demonstrating the exclusive involvement of intracellular Ca²⁺-ATPases.

The effect of decreasing the luminal Ca²⁺ content of the Ins-1,4,5-P₃-sensitive Ca²⁺ pool on the Ca²⁺ pump activity was studied in a ⁴⁵Ca²⁺/⁴⁵Ca²⁺ exchange experiment. Permeabilized acinar cells were loaded with Ca²⁺ to steady-state in the absence of ⁴⁵Ca²⁺. At 9.5 min either saline or Ins-1,4,5-P₃ (10 µM) was added followed at 10 min by a tracer amount of ⁴⁵Ca²⁺. In the presence of 10 µM Ins-1,4,5-P₃, when the Ins-1,4,5-P₃-sensitive Ca²⁺ pool was maximally depleted, the rate of labeling of the remainder of the energy-dependent store was markedly reduced as compared to the control (Fig. 4B). Subsequent addition of heparin (150 U/ml) led to a rapid increase in the rate of labeling, reflecting the increased uptake of Ca by the Ins-1,4,5-P₃-sensitive Ca²⁺ pool. Interestingly, the initial rate of labeling was not different between control and Ins-1,4,5-P₃-treated cells. It should be noted that the Ca²⁺ uptake values presented in Figure 4B are not corrected for those obtained in the absence of ATP.

Exchange of ⁴⁵Ca²⁺ under steady-state conditions

In order to study the rate at which Ca²⁺ was lost from permeabilized acinar cells loaded to steady-state in the presence of a tracer amount of ⁴⁵Ca²⁺, the specific activity of the tracer was reduced 28 times without changing the ambient free Ca²⁺ concentration. Figure 5A shows that under conditions of steady-state Ca²⁺ loading, 30% of actively stored ⁴⁵Ca²⁺ was lost within the first minute after reduction of the specific activity. Thereafter, ⁴⁵Ca²⁺ was lost in a monoexponential manner. Simultaneous addition of a maximally effective concentration of Ins-1,4,5-P₃, which under normal conditions released 62% of the steady-state Ca²⁺ content, resulted in a
loss of 90% of actively stored $^{45}\text{Ca}^{2+}$ within the first minute after reduction of the specific activity. The latter observation suggests that the large decrease of the $^{45}\text{Ca}^{2+}$ content observed during the first minute of the exchange experiments performed in the absence of Ins-1,4,5-P$_3$ is mainly due to a high turnover rate of the Ins-1,4,5-P$_3$-insensitive pool. The $^{45}\text{Ca}^{2+}$ content of the Ins-1,4,5-P$_3$-sensitive store decayed in a monoexponential fashion with a half-time of 6.5 min (time constant = 0.11 min$^{-1}$) (Fig. 5B).

In a previous study, using thapsigargin to study the rate of Ca$^{2+}$ loss, the Ca$^{2+}$ content of the Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ store was found to decay with a half-time of 7.0 min [25]. The passive Ca$^{2+}$ permeability of the Ins-1,4,5-P$_3$-insensitive store was considerably higher since exchange was virtually completed within 1 min (Fig. 5A). If the exchange process had occurred with the same time constant as observed for the Ins-1,4,5-P$_3$-sensitive store, the $^{45}\text{Ca}^{2+}$ content of Ins-1,4,5-P$_3$-insensitive store would have been reduced by 10% rather than 30% per minute.

Discussion

The data presented in this study demonstrate that internal Ca$^{2+}$ stores, normally discriminated by virtue
Fig. 5 Effect of Ins-1,4,5-P<sub>3</sub> on the unidirectional Ca<sup>2+</sup> efflux in the presence of Ca<sup>2+</sup> pumping. (A) Permeabilized cells were loaded with Ca<sup>2+</sup> at an ambient free Ca<sup>2+</sup> concentration of 0.19 µM in a medium containing 0.15 mM of each of the three bivalent cation chelators. After 10 min, the concentration of the radioactive tracer was reduced 28-fold without changing the ambient free Ca<sup>2+</sup> concentration as described in Materials and methods. Saline (closed circles) or Ins-1,4,5-P<sub>3</sub> (closed triangles), at a final concentration of 30 µM, was included in the dilution medium. The reactions were quenched at the indicated times. The residual <sup>45</sup>Ca<sup>2+</sup> content was corrected for the ATP-independent binding, which was determined in parallel incubations performed in the absence of ATP. The values presented are expressed as percentage of the residual amount of radioactive tracer present at 10 min (open circle). The results presented are the mean ± SE of 3 independent experiments. (B) Represents the exchange activity in the Ins-1,4,5-P<sub>3</sub>-sensitive store. The data presented in (B) are calculated from the data presented in (A).

of their releasability by Ins-1,4,5-P<sub>3</sub>, differ also in a number of other aspects.

Firstly, from the observation that the initial Ca<sup>2+</sup> uptake rate measured in the presence of a maximally effective concentration of Ins-1,4,5-P<sub>3</sub>, thus reflecting active Ca<sup>2+</sup> uptake in the Ins-1,4,5-P<sub>3</sub>-insensitive store, was 1.7 times higher than that calculated for the Ins-1,4,5-P<sub>3</sub>-sensitive store it can be deduced that the Ins-1,4,5-P<sub>3</sub>-insensitive store contains 1.7 times more Ca<sup>2+</sup> pumps than the Ins-1,4,5-P<sub>3</sub>-sensitive store. Furthermore, from the observation that increasing of the ambient free Ca<sup>2+</sup> concentration from 0.19 µM to 1.0 µM led to an increase in Ca<sup>2+</sup> uptake rate which was not significantly different between both stores it can be concluded that both subclasses of Ca<sup>2+</sup> pumps are equally sensitive to activation by ambient free Ca<sup>2+</sup>. However, it can not be ruled out that the differences in initial Ca<sup>2+</sup> uptake rate, observed in the present study, reflect differences in turn-over rate among the two subclasses of Ca<sup>2+</sup> pumps. Such differences may be intrinsic to the pumping protein itself or the result of a kinase-dependent phosphorylation reaction. Another possibility is the development of a membrane potential opposing ATP-dependent Ca<sup>2+</sup> uptake, which may be different between both stores.

Secondly, the uptake capacity of the Ins-1,4,5-P<sub>3</sub>-sensitive store was 1.6 times higher than that of the Ins-1,4,5-P<sub>3</sub>-insensitive store. With increasing of the ambient free Ca<sup>2+</sup> concentration, the steady-state
Ca\(^{2+}\) content of both stores increased to the same extent as was revealed by the observation that a maximally effective concentration of Ins-1,4,5-P\(_3\) invariably released 60\% of actively stored Ca\(^{2+}\).

Thirdly, Ca\(^{2+}\) uptake in the Ins-1,4,5-P\(_3\)-sensitive store was virtually linear with time during the first 4 min following its initiation. By contrast, the rate at which Ca\(^{2+}\) was actively accumulated in the Ins-1,4,5-P\(_3\)-insensitive store decreased progressively with time. This observation may be explained by the presence of substantial amounts of a Ca\(^{2+}\) binding protein in the Ins-1,4,5-P\(_3\)-sensitive store. This conclusion is supported by recent immunolocalization studies [31]. Using antibodies directed against calciopestrin, a Ca\(^{2+}\) binding protein present in the terminal cisternae of the sarcoplasmic reticulum, a calciopestrin-like protein was demonstrated to be present in discrete organelles, referred to as calciosomes. Recently, this protein has been identified as calcreticulin [32]. Evidence in support of the colocalization of this Ca\(^{2+}\) binding protein and the Ca\(^{2+}\) pump was obtained in studies using antibodies raised against the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase [33]. Both antibodies were demonstrated to recognize proteins present in small cytosolic structures distributed throughout the acinar cell. Whether these particular structures also contain the Ins-1,4,5-P\(_3\) receptor is still unclear. Interestingly, however, subcellular fractionation studies using canine pancreatic homogenates revealed that the distribution pattern of the Ins-1,4,5-P\(_3\) receptors was significantly different from that of Ca\(^{2+}\) pumps and Ca\(^{2+}\) binding proteins [34,35]. This observation is consistent with the data obtained in the present study, suggesting a heterogeneous distribution of Ca\(^{2+}\) pumps and Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels between intracellular Ca\(^{2+}\) storage organelles. However, it can not be ruled out that the greater extent of linearity of Ca\(^{2+}\) uptake, observed for the Ins-1,4,5-P\(_3\)-sensitive store in the present study, reflects a larger mean vesicle size, a slower development of an inhibitory membrane potential, or a lower sensitivity of pump to inhibition by intravesicular Ca\(^{2+}\).

Fourthly, the turn-over rate of Ca\(^{2+}\) in the Ins-1,4,5-P\(_3\)-insensitive store was found to be extremely high. This was concluded from the observation that under steady-state conditions the Ins-1,4,5-P\(_3\)-insensitive store lost 80\% of its actively stored \(^{45}\)Ca\(^{2+}\) within the first minute following a 28-fold decrease in specific activity of the radioactive tracer in the medium. Similarly, permeabilized acinar cells, loaded with Ca\(^{2+}\) to steady-state in the absence of \(^{45}\)Ca\(^{2+}\) and stimulated with a maximally effective concentration of Ins-1,4,5-P\(_3\) 30 s before the addition of \(^{45}\)Ca\(^{2+}\) to the medium, accumulated \(^{45}\)Ca\(^{2+}\) at a rate equal to that obtained with unstimulated cells during the first minute of incubation in the presence of the radioactive tracer. These observations suggest that Ca\(^{2+}\), actively taken up in this store, may largely be present in the unbound form.

In contrast to the Ins-1,4,5-P\(_3\)-insensitive store, the Ins-1,4,5-P\(_3\)-sensitive store is exchanging more slowly. This is in agreement with the idea that the passive Ca\(^{2+}\) permeability of the Ins-1,4,5-P\(_3\)-sensitive store is considerably less than that of the Ins-1,4,5-P\(_3\)-insensitive store. From the observation that the thapsigargin-evoked loss of Ca\(^{2+}\) from the Ins-1,4,5-P\(_3\)-sensitive store is a first-order process it can be concluded that the free Ca\(^{2+}\) concentration in this store is gradually decreasing during passive Ca\(^{2+}\) efflux. This is consistent with the idea that virtually all Ca\(^{2+}\), actively taken up in the Ins-1,4,5-P\(_3\)-sensitive store, is loosely bound to Ca\(^{2+}\) binding proteins and rapidly exchangeable with Ca\(^{2+}\) present in the unbound Ca\(^{2+}\) state. In the case of Ca\(^{2+}\) being tightly bound, pump inhibition would have led to a considerably slower and linear rather than monoexponential efflux of Ca\(^{2+}\), as has been observed in stores loaded with Ca\(^{2+}\) precipitating anions [36].

The use of Ca\(^{2+}\) precipitating anions such as oxalate, with a dissociation constant for Ca\(^{2+}\) of about 1 mM [37], demonstrates that the intravesicular free Ca\(^{2+}\) concentration is in the millimolar range. Recently, millimolar Ca\(^{2+}\) concentrations have been measured in intracellular Ca\(^{2+}\) stores of permeabilized hepatocytes using the fluorescent Ca\(^{2+}\) selective probe chlortetracycline [38]. This is consistent with a functional role for Ca\(^ {2+}\) sequestering proteins, such as calcreticulin, which have been shown to bind Ca\(^{2+}\) with affinities in the millimolar range [32].

From the experimental data obtained with the permeabilized cell system, the picture emerges that the pancreatic acinar cell contains essentially two distinct intracellular Ca\(^{2+}\) stores of mitochondrial origin. One store, containing relatively few
Ca\(^{2+}\) pumps but displaying a high Ca\(^{2+}\) uptake capacity, possibly due to the presence of a Ca\(^{2+}\) binding protein, and released by the action of Ins-1,4,5-P\(_3\), and a second store containing relatively many Ca\(^{2+}\) pumps but displaying a low Ca\(^{2+}\) uptake capacity and not released by the action of Ins-1,4,5-P\(_3\).

Putney and co-workers have postulated that both Ca\(^{2+}\) pumps and Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels reside in the same Ca\(^{2+}\) storage organelle but are spatially separated. Permeabilization of the cells could then lead to the formation of smaller organelles containing either Ca\(^{2+}\) pumps and little if any Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels or Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels and little if no Ca\(^{2+}\) pumps [39], whereas subcellular fractionation could lead to the formation of microsomal vesicles containing either Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels and no Ca\(^{2+}\) pumps or Ca\(^{2+}\) pumps and no Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels [40]. Evidence in support of this idea has recently been provided by Thomas and coworkers demonstrating that treatment with digitonin of hepatocytes in suspension resulted in a considerable fragmentation of the endoplasmic reticulum [38]. On the other hand, no fragmentation was observed upon permeabilization of hepatocytes attached to cover-slips. Interestingly, all effective Ins-1,4,5-P\(_3\) concentrations released virtually all Ca\(^{2+}\) storage organelles in permeabilized attached hepatocytes, whereas in permeabilized suspended hepatocytes Ins-1,4,5-P\(_3\) released only a fraction of actively stored Ca\(^{2+}\) in a quantal manner. These observations suggest that in the intact hepatocyte Ins-1,4,5-P\(_3\)-sensitive and Ins-1,4,5-P\(_3\)-insensitive stores are functionally coupled. The observation of Thomas and coworkers [41] that the extent of fragmentation of the endoplasmic reticulum was significantly reduced in the presence of GTP might be indicative for the dynamic nature of such a coupling process. We have recently shown that addition of thapsigargin to intact pancreatic acinar cells, prestimulated with a maximal concentration of cholecystokinin-octapeptide, resulted in a significant increase in free cytosolic Ca\(^{2+}\) concentration [28]. Although this observation appears to be in agreement with the presence of a substantial agonist-insensitive Ca\(^{2+}\) store next to the agonist-sensitive Ca\(^{2+}\) store, some reservation should be made since the rising phase of the thapsigargin-evoked Ca\(^{2+}\) transient was significantly faster in the presence than in the absence of the agonist, suggesting the presence of Ca\(^{2+}\) storage organelles which are not completely released by the agonist as a result of compensatory Ca\(^{2+}\) pumping. On the other hand, it should be noted that Schulz and coworkers failed to demonstrate vesiculization of the endoplasmic reticulum upon treatment with saponin of pancreatic acinar cells in suspension [42]. Moreover, Short et al. [43] reported that Ins-1,4,5-P\(_3\) released only a fraction of actively stored Ca\(^{2+}\) in a quantal manner in permeabilized attached DDTMF-2 smooth muscle cells, suggesting that this cell type, in contrast to the hepatocyte [38], contains both Ins-1,4,5-P\(_3\)-sensitive and -insensitive Ca\(^{2+}\) storage organelles. It is our current working hypothesis that the intact pancreatic acinar cell contains separate Ins-1,4,5-P\(_3\)-sensitive and -insensitive Ca\(^{2+}\) storage organelles which are dynamically coupled depending on the physiological state of the cell [26]. In this context, it is worthwhile to study the characteristics of these separate stores in the permeabilized preparation.

In a previous study [25], we have postulated that the Ins-1,4,5-P\(_3\)-sensitive store consists of a heterogeneous population of discrete Ca\(^{2+}\) accumulating organelles containing different amounts of Ca\(^{2+}\) pumps relative to Ins-1,4,5-P\(_3\)-operated Ca\(^{2+}\) release channels. Ins-1,4,5-P\(_3\)-sensitive stores displaying relatively few Ca\(^{2+}\) pumps are more sensitive to Ins-1,4,5-P\(_3\) due to a low compensatory Ca\(^{2+}\) pumping activity and their presence may therefore be restricted to that part of the apical pole of the acinar cell where the initial Ca\(^{2+}\) rise occurs and which has been referred to as 'trigger zone' [3]. On the other hand, Ins-1,4,5-P\(_3\)-sensitive stores containing relatively many Ca\(^{2+}\) pumps are less sensitive to Ins-1,4,5-P\(_3\) due to a high compensatory Ca\(^{2+}\) pumping activity and may therefore be situated in the basolateral area of the acinar cell. Thus far, it is unclear which store contains the Ca\(^{2+}\)-activated Ca\(^{2+}\) release channel. However, it is tempting to speculate that it is the Ins-1,4,5-P\(_3\)-insensitive store, which may then be situated in the apical area of the cell, where Ca\(^{2+}\) infusion has been demonstrated to initiate a heparin-insensitive rise in free cytosolic Ca\(^{2+}\) concentration [3]. However, up to the present we were unable to demonstrate the existence of a caffeine- and/or
rynnodine-sensitive Ca\textsuperscript{2+} release mechanism in permeabilized pancreatic acinar cells [27,28].

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