Ca²⁺-sensitivity of inositol 1,4,5-trisphosphate-mediated Ca²⁺ release in permeabilized pancreatic acinar cells

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Hormonal and phorbol ester pretreatment of pancreatic acinar cells markedly decreases the Ins(1,4,5)P₃-induced release of actively stored Ca²⁺ [Willems, Van Den Broek, Van Os & De Pont (1989) *J. Biol. Chem.* **264**, 9762–9767]. Inhibition occurred at an ambient free Ca²⁺ concentration of 0.1 μM, suggesting a receptor-mediated increase in Ca²⁺-sensitivity of the Ins(1,4,5)P₃-operated Ca²⁺ channel. To test this hypothesis, the Ca²⁺-dependence of Ins(1,4,5)P₃-induced Ca²⁺ release was investigated. In the presence of 0.2 μM free Ca²⁺, permeabilized cells accumulated 0.9 nmol of Ca²⁺/mg of acinar protein in an energy-dependent pool. Uptake into this pool increased 2.2- and 3.3-fold with 1.0 and 2.0 μM free Ca²⁺ respectively. At 0.2, 1.0 and 2.0 μM free Ca²⁺, Ins(1,4,5)P₃ maximally released 0.53 (56%), 0.90 (44%) and 0.62 (20%) nmol of Ca²⁺/mg of acinar protein respectively. Corresponding half-maximal stimulatory Ins(1,4,5)P₃ concentrations were calculated to be 0.5, 0.6 and 1.4 μM, suggesting that the affinity of Ins(1,4,5)P₃ for its receptor decreases beyond 1.0 μM free Ca²⁺. The possibility that an inhibitory effect of sub-micromolar Ca²⁺ is being masked by the concomitant increase in size of the releasable store is excluded, since Ca²⁺ release from cells loaded in the presence of 0.1 or 0.2 μM free Ca²⁺ and stimulated at higher ambient free Ca²⁺ was not inhibited below 1.0 μM free Ca²⁺. At 2.0 and 10.0 μM free Ca²⁺, Ca²⁺ release was inhibited by approx. 30% and 75% respectively. The results presented show that hormonal pretreatment does not lead to an increase in Ca²⁺-sensitivity of the release mechanism. Such an increase in Ca²⁺-sensitivity to sub-micromolar Ca²⁺ is required to explain sub-micromolar oscillatory changes in cytosolic free Ca²⁺ by a Ca²⁺-dependent negative-feedback mechanism.

INTRODUCTION

One of the earliest events in receptor-mediated Ca²⁺ mobilization by pancreatic secretagogues is the activation of a phosphodiesterase with specificity for phosphatidylinositol 4,5-bisphosphate, which results in rapid formation of the intracellular messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] [1,2]. Streb *et al.* [3], using permeabilized pancreatic acinar cells, were the first to demonstrate that Ins(1,4,5)P₃ releases Ca²⁺ from intracellular stores characterized previously as vesicular, ATP-dependent and non-mitochondrial, and more recently referred to as calciosomes [4]. The role of Ins(1,4,5)P₃ in mobilizing intracellular Ca²⁺ has been established in a wide variety of tissues [5]. Analysis of secretagogue-induced increases in fluorescence from pancreatic acinar cells, loaded with Ca²⁺-selective fluorescent indicators, revealed that during the initial phase of secretagogue-induced Ca²⁺ mobilization increases in free cytosolic Ca²⁺ concentration ([Ca²⁺]_i) are independent of extracellular Ca²⁺ and thus reflect Ins(1,4,5)P₃-induced Ca²⁺ release from internal stores [6–9]. Close examination of the time-dependence of secretagogue-induced increases in [Ca²⁺]_i revealed that [Ca²⁺]_i increases within seconds, reaches a peak at about 5 s (500–700 nM) and returns to near pre-stimulatory levels (100–150 nM) within 1.5 min after stimulation [9–11]. Recently, however, sinusoidal oscillations in [Ca²⁺]_i have been shown to be superimposed on the rising Ca²⁺ signal in single pancreatic

acinar cells stimulated by acetylcholine [12]. In two other studies the initial increase in [Ca²⁺]_i was shown to be followed by secondary oscillations of smaller amplitude [11,13]. Ca²⁺ oscillations have been described in several cell types (see [14–16]), and it has been speculated that feedback inhibition of Ins(1,4,5)P₃-induced Ca²⁺ release by ambient free Ca²⁺ could provide a mechanism to generate cyclic changes in [Ca²⁺]_i [17]. In pancreatic acinar cells oscillatory changes are observed at sub-micromolar [Ca²⁺]_i [11–13], which means that Ins(1,4,5)P₃-induced Ca²⁺ release has to be inhibited by sub-micromolar [Ca²⁺]_i. However, in rat cerebellum microsome Ca²⁺ did not inhibit Ins(1,4,5)P₃-induced Ca²⁺ release at concentrations below 1.0 μM, whereas at concentrations above 1.0 μM Ins(1,4,5)P₃-induced Ca²⁺ release was progressively inhibited [17]. This may suggest that in the microsomal preparation some cytosolic factor is lacking which enhances the Ca²⁺-sensitivity of the Ins(1,4,5)P₃ receptor. In addition, we have recently shown that hormonal pretreatment of pancreatic acinar cells significantly inhibits subsequent Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized cells [18]. Interestingly, this inhibitory effect was observed at a free Ca²⁺ concentration of 0.1 μM, which may suggest that in hormone-treated cells the Ca²⁺-sensitivity of the Ins(1,4,5)P₃ receptor is increased.

In order to test these possibilities, we studied the Ca²⁺-dependence of Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized pancreatic acinar cells preincubated in the

Abbreviations used: CCK-8, C-terminal octapeptide of cholecystokinin; [Ca²⁺]_i, free cytosolic calcium concentration; [Ca²⁺]_{free}, free calcium concentration; HEEDTA, N-(2-hydroxyethyl)ethylenediamine-NN'-triacetic acid.

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absence and presence of the C-terminal octapeptide of cholecystokinin (CCK-8). The results obtained with permeabilized pancreatic acinar cells confirm previous observations with other cell types (for a review, see ref. [19]) that $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release is inhibitable by high ambient Ca^{2+} . However, no inhibition occurred at sub-micromolar Ca^{2+} concentrations. Moreover, the inhibitory action of CCK-8 pretreatment was independent of the free Ca^{2+} concentration, indicating that the inhibitory effect of hormonal treatment does not depend on stimulus-induced increases in Ca^{2+} -sensitivity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

EXPERIMENTAL

Rabbit pancreatic acinar cells were prepared essentially as described by Amsterdam & Jamieson [20]. The method is based on two successive incubations with collagenase and hyaluronidase in a Krebs-Ringer bicarbonate medium (pH 7.4) containing 119 mM-NaCl, 3.5 mM-KCl, 1.2 mM- KH_2PO_4 , 25 mM- NaHCO_3 , 0.1 mM- CaCl_2 , 1.2 mM- MgCl_2 , 5.8 mM-glucose, 0.2 mg of soya-bean trypsin inhibitor/ml and an amino acid mixture (Eagle [21]), with, in between, a bivalent-cation-chelating step. After digestion, the tissue was dispersed by pipetting, filtered through nylon gauze and purified by centrifugation through a 4% (w/v) albumin layer. The intactness of the isolated acinar cells was demonstrated by Trypan Blue exclusion. Pretreatment of acinar cells with 0.1 μM -CCK-8 was carried out as described previously [18].

Isolated pancreatic acinar cells were washed twice, resuspended in a high- K^+ medium (1 mg of protein/ml) containing 135 mM-KCl, 1.0 mM- MgCl_2 , 1.2 mM- KH_2PO_4 , 0.1 mM-phenylmethanesulphonyl fluoride, 0.2 mg of soya-bean trypsin inhibitor/ml and 10 mM-Hepes (pH 7.4) and permeabilized with saponin (30 $\mu\text{g}/\text{ml}$, 10 min, 25 °C). After saponin treatment less than 25% of the cells excluded Trypan Blue.

Permeabilized acinar cells were washed twice and resuspended in a Ca^{2+} -uptake medium (5–8 mg of protein/ml) containing 120 mM-KCl, 1.0 mM- MgCl_2 , 1.2 mM- KH_2PO_4 , 5 mM-pyruvate, 5 mM-succinate, 0.5 mM-EGTA, 0.5 mM-nitrilotriacetic acid, 0.5 mM-HEEDTA and 0.2 mg of soya-bean trypsin inhibitor/ml and adjusted to pH 7.1 with KOH. The mitochondrial Ca^{2+} -uptake inhibitor Ruthenium Red (10 μM) was added, and the permeabilized cells were kept at 0 °C. Ca^{2+} uptake was started by adding 10 μl of permeabilized cells to 90 μl of Ca^{2+} -uptake medium (pH 7.1), which contained in addition: 10 mM-phosphocreatine, 10 units of creatine kinase, 5 μCi of $^{45}\text{Ca}^{2+}/\text{ml}$, 10 μM -Ruthenium Red and 0 or 10 mM-MgATP. The free Mg^{2+} (1.45 mM) and Ca^{2+} (as indicated) concentrations were adjusted as described by Van Heeswijk *et al.* [22]. At 10 min 1.0 ml of ice-cold stop solution containing 150 mM-KCl, 5.0 mM- MgCl_2 , 1.0 mM-EGTA and 20 mM-Hepes/KOH (pH 7.1) was added, and the suspension was rapidly filtered (Schleicher and Schüll, GF 92). The filters were washed with 2 $\times$ 2.0 ml of ice-cold stop solution, dissolved in scintillation fluid and counted for radioactivity. Total Ca^{2+} was calculated and expressed as nmol/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, Richmond, CA, U.S.A.) after treatment of the cells with 0.1% Triton X-100. The Ca^{2+} -

uptake and -release experiments described in this paper were performed at 37 °C.

Permeabilized pancreatic acinar cells were loaded with Ca^{2+} in the above Ca^{2+} -uptake medium at the free Ca^{2+} concentrations indicated. At 10 min $\text{Ins}(1,4,5)\text{P}_3$ was added, and after 1 min the incubation was stopped. Each concentration of $\text{Ins}(1,4,5)\text{P}_3$ was tested in triplicate. In a second series of experiments permeabilized acinar cells were loaded with Ca^{2+} at 0.1 or 0.2 μM free Ca^{2+} . At 10 min the free Ca^{2+} concentration of the medium was adjusted to the indicated values by adding 100 μl of Ca^{2+} -uptake medium (pH 7.1), from which MgATP, creatine kinase, phosphocreatine and $^{45}\text{Ca}^{2+}$ were absent, but which contained in addition Ruthenium Red (10 μM), vanadate (final concn. 0.5 mM), calculated amounts of MgCl_2 to maintain the free Mg^{2+} concentration at 1.45 mM and calculated amounts of CaCl_2 to generate the indicated free Ca^{2+} concentrations. At 30 s $\text{Ins}(1,4,5)\text{P}_3$ was added, and the reaction was quenched with ice-cold stop solution 1 min after addition of $\text{Ins}(1,4,5)\text{P}_3$.

Collagenase was purchased from Cooper Biomedical, Malvern, PA, U.S.A. Ruthenium Red was obtained from Merck, Darmstadt, Germany; CCK-8 was synthesized by Dr. H. M. Rajh in the Department of Organic Chemistry, University of Nijmegen, The Netherlands; MgATP, bovine serum albumin, $\text{Ins}(1,4,5)\text{P}_3$, Triton X-100, HEEDTA, nitrilotriacetic acid, EGTA, saponin, Hepes, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor and Trypan Blue were from Sigma, St. Louis, MO, U.S.A.; hyaluronidase, phosphocreatine and creatine kinase were from Boehringer, Mannheim, Germany. $^{45}\text{CaCl}_2$ (20 mCi/ml) was purchased from New England Nuclear, Dreieich, Germany. All other reagents were of reagent grade.

RESULTS

At a free Ca^{2+} concentration of 0.1 μM and in the presence of Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake, ATP-dependent Ca^{2+} uptake by permeabilized pancreatic acinar cells reaches a steady state within 10 min when incubated at 37 °C [18]. In the present study, steady-state Ca^{2+} uptake by permeabilized acinar cells, incubated at 0.2 μM free Ca^{2+} , was calculated to be 0.94 (S.E.M. = 0.17, $n = 7$) nmol of Ca^{2+}/mg of protein. Steady-state Ca^{2+} uptake by energy-dependent internal stores increased with increasing $[\text{Ca}^{2+}]_{\text{free}}$ (Fig. 1). Compared with the maximal uptake value reached in the presence of 0.2 μM free Ca^{2+} , a 2.2- and 3.3-fold increase in steady-state Ca^{2+} uptake was observed with 1.0 and 2.0 μM free Ca^{2+} respectively.

Maximal $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized pancreatic acinar cells loaded in the presence of 0.1 μM free Ca^{2+} occurs within 30 s after addition of $\text{Ins}(1,4,5)\text{P}_3$, and no re-uptake of Ca^{2+} takes place for at least 90 s [18]. In the present study, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was measured 60 s after addition of $\text{Ins}(1,4,5)\text{P}_3$. Fig. 2(a) shows that $\text{Ins}(1,4,5)\text{P}_3$ released Ca^{2+} dose-dependently at all three $[\text{Ca}^{2+}]_{\text{free}}$ tested. In absolute terms, however, the amounts of Ca^{2+} released at 1.0 μM free Ca^{2+} were significantly higher than those released at 0.2 μM as well as at 2.0 μM free Ca^{2+} . A more detailed presentation of the release values obtained is given in a Lineweaver-Burk plot (Fig. 2b). At 0.2, 1.0 and 2.0 μM free Ca^{2+} , maximal values for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release were calculated to be 0.53, 0.90 and

Fig. 1. Ca²⁺-dependence of Ca²⁺ uptake by permeabilized rabbit pancreatic acinar cells

Isolated rabbit pancreatic acinar cells, permeabilized by saponin treatment, were resuspended (5–8 mg of protein/ml) in a Ca²⁺-uptake medium (pH 7.1) to which 10 μM-Ruthenium Red was added, and were kept on ice before use. Ca²⁺ uptake was started by adding 10 μl of permeabilized cells to 90 μl of Ca²⁺-uptake medium to which 0 or 10 mM-MgATP was added. Incubations were carried out at 37 °C with the indicated free Ca²⁺ concentrations, including 5 μCi of ⁴⁵Ca²⁺/ml. At 10 min, the reaction mixture was quenched in 1.0 ml of ice-cold stop solution and the suspension was rapidly filtered. ⁴⁵Ca²⁺ retained on the filter after rinsing was determined by liquid-scintillation counting. Total Ca²⁺ retained on the filter was calculated and expressed in nmol/mg of acinar protein. Actively stored Ca²⁺ is defined as the difference in total Ca²⁺ retained on the filter after incubation in the presence and absence of ATP. The values presented are means ± S.E.M. of six to seven experiments.

0.62 nmol of Ca²⁺/mg of protein, with half-maximal stimulatory concentrations of Ins(1,4,5)P₃ of 0.5, 0.6 and 1.4 μM respectively. In relative terms, Ins(1,4,5)P₃ maximally released 56 %, 44 % and 20 % of actively stored Ca²⁺. Fig. 2(c) shows that the [Ca²⁺]_{free}-dependent increase in the amount of non-releasable Ca²⁺ virtually parallels the [Ca²⁺]_{free}-dependent increase in actively stored Ca²⁺.

In order to test the possibility that an inhibitory effect of free Ca²⁺ on Ins(1,4,5)P₃-induced Ca²⁺ release is being

Fig. 2. Ca²⁺-dependence of Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized pancreatic acinar cells

Isolated rabbit pancreatic acinar cells, permeabilized by saponin treatment, were loaded with Ca²⁺ in the presence and absence of MgATP as described in the legend of Fig. 1. At 10 min Ins(1,4,5)P₃ was added at the indicated concentrations, and after 1 min the reaction was stopped. Each Ins(1,4,5)P₃ concentration was tested in triplicate. Actively stored Ca²⁺ was calculated as described in the legend of Fig. 1. At each [Ca²⁺]_{free} the amount of actively stored Ca²⁺ in permeabilized cells not stimulated with Ins(1,4,5)P₃ is set at 100 %, to which the release values are related. The release values obtained with 0.2 μM (●) and 2.0 μM (◆) free Ca²⁺ were compared with those at 1.0 μM (■) free Ca²⁺ by Student's *t* test (***P* < 0.005; ***P* < 0.01; **P* < 0.05). The values presented are

means ± S.E.M. of six to seven experiments. The percentage of actively stored Ca²⁺ released by Ins(1,4,5)P₃ is given as a function of the Ins(1,4,5)P₃ concentration in a Line-weaver-Burk plot (b). From this plot half-maximal stimulatory concentrations of Ins(1,4,5)P₃ of 0.5, 0.6 and 1.4 μM and maximal release values of 0.53, 0.90 and 0.62 nmol of Ca²⁺/mg of protein were calculated for 0.2 (●), 1.0 (■) and 2.0 (◆) μM free Ca²⁺ respectively. The maximal release values (●) were used to calculate the size of the non-releasable pool (◆) (c).

Fig. 3. Ca^{2+} -dependence of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized rabbit pancreatic acinar cells loaded with Ca^{2+} at sub-micromolar $[\text{Ca}^{2+}]_{\text{free}}$

Isolated pancreatic acinar cells, permeabilized by saponin treatment, were loaded with Ca^{2+} at 0.1 (■) and 0.2 (●) μM free Ca^{2+} as described in the legend of Fig. 1. At 10 min, free Ca^{2+} was reset to the indicated values by adding 100 μl of Ca^{2+} -uptake medium, from which MgATP , creatine kinase, phosphocreatine and $^{45}\text{Ca}^{2+}$ were absent, but which contained in addition vanadate (final concn. 0.5 mM). Vanadate effectively blocked Ca^{2+} uptake by internal stores not sensitive to Ruthenium Red (result not shown), and was added to maintain the specific radioactivity of internally stored Ca^{2+} . At 30 s $\text{Ins}(1,4,5)\text{P}_3$ was added at final concentrations of 1.5 (●) and 0.3 (■) μM , and the reaction was stopped 1 min later. Each $\text{Ins}(1,4,5)\text{P}_3$ concentration was tested in triplicate. Actively stored Ca^{2+} was calculated as described in the legend of Fig. 1. At each free Ca^{2+} concentration the amount of actively stored Ca^{2+} in permeabilized cells not stimulated with $\text{Ins}(1,4,5)\text{P}_3$ is set at 100%, to which the release values are related. The values given are means $\pm$ S.E.M. of three (■) or five (●) experiments.

masked by a concomitant $[\text{Ca}^{2+}]_{\text{free}}$ -dependent increase in the amount of $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} , permeabilized pancreatic acinar cells were loaded at 0.1 or 0.2 μM free Ca^{2+} and stimulated with $\text{Ins}(1,4,5)\text{P}_3$ at different $[\text{Ca}^{2+}]_{\text{free}}$ (Fig. 3). At 10 min, $[\text{Ca}^{2+}]_{\text{free}}$ was reset by adding 100 μl of Ca^{2+} -uptake medium from which MgATP , creatine kinase, phosphocreatine and $^{45}\text{Ca}^{2+}$ were absent, but which contained vanadate. The final concentration of vanadate was 0.5 mM. Vanadate effectively inhibits ATP-dependent Ca^{2+} uptake by internal stores and was added to maintain the specific radioactivity of internally stored $^{45}\text{Ca}^{2+}$. $\text{Ins}(1,4,5)\text{P}_3$ was added 30 s after changing $[\text{Ca}^{2+}]_{\text{free}}$. At concentrations below 1.0 μM , Ca^{2+} had no effect on the amount of actively stored Ca^{2+} released by 0.3 μM - or 1.5 μM - $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 3). At 2.0 μM free Ca^{2+} , however, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was inhibited by approx. 30%. No Ca^{2+} re-uptake was observed for at least 5 min after addition of 1.0 μM - $\text{Ins}(1,4,5)\text{P}_3$.

Specific binding of $\text{Ins}(1,4,5)\text{P}_3$ to adrenocortical microsomes has been shown to be inhibited by ATP [23]. To evaluate the effect of ATP on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, permeabilized cells were loaded at 0.1 μM free Ca^{2+} and stimulated with 0.3 μM - $\text{Ins}(1,4,5)\text{P}_3$ in the presence of either 0.5 mM- or 5 mM-ATP. Table 1 shows that the amount of Ca^{2+} released in the presence of the

Table 1. ATP-dependence of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized pancreatic acinar cells

Isolated rabbit pancreatic acinar cells, permeabilized by saponin treatment, were loaded with Ca^{2+} at 0.1 μM free Ca^{2+} and in the absence and presence of 1 mM-ATP. At 10 min, free Ca^{2+} was reset to the indicated values as described in the legend of Fig. 3. The final ATP concentration was adjusted to 0.5 mM or 5 mM, and vanadate (0.5 mM) was present in all the incubations. At 30 s, 0.3 μM - $\text{Ins}(1,4,5)\text{P}_3$ was added and the reaction was stopped 1 min later. Actively stored Ca^{2+} was calculated as described in the legend of Fig. 1. At each free Ca^{2+} concentration, the amount of actively stored Ca^{2+} in permeabilized cells not stimulated with $\text{Ins}(1,4,5)\text{P}_3$ is set at 100%, to which the release values are related. The values given are means $\pm$ S.E.M. of three experiments.

$[\text{Ca}^{2+}]_{\text{free}}$ (μM) ... [ATP] (mM)	Ca^{2+} release (%)		
	0.1	1.0	10.0
0.5	20.5 $\pm$ 3.8	31.1 $\pm$ 7.9	7.3 $\pm$ 3.5
5.0	23.9 $\pm$ 6.1	27.0 $\pm$ 6.3	6.2 $\pm$ 8.5

Fig. 4. Ca^{2+} -dependence of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized pancreatic acinar cells pretreated with CCK-8

Isolated pancreatic acinar cells, preincubated with saline (○, □) or 0.1 μM -CCK-8 (●, ■) for 30 min at 37 °C, and permeabilized by saponin treatment, were loaded with Ca^{2+} at 0.1 μM free Ca^{2+} . At 10 min, free Ca^{2+} was reset to the indicated values as described in the legend of Fig. 3. At 30 s, $\text{Ins}(1,4,5)\text{P}_3$ was added at final concentrations of 1.5 μM (○, ●) and 0.3 μM (□, ■), and the reaction was stopped 1 min later. Each $\text{Ins}(1,4,5)\text{P}_3$ concentration was tested in triplicate. Actively stored Ca^{2+} was calculated as described in the legend of Fig. 1. At each free Ca^{2+} concentration the amount of actively stored Ca^{2+} in permeabilized cells not stimulated with $\text{Ins}(1,4,5)\text{P}_3$ is set at 100%, to which the release values are related. The values given are means $\pm$ S.E.M. of four to five experiments.

Table 2. Effect of free Ca²⁺ on Ca²⁺-loaded permeabilized pancreatic acinar cells incubated in the presence of vanadate

Isolated rabbit pancreatic acinar cells, preincubated with saline or 0.1 μ M-CCK-8 and permeabilized by saponin treatment, were loaded with Ca²⁺ at 0.1 μ M free Ca²⁺ as described in the legend of Fig. 1. At 10 min, free Ca²⁺ was reset to the indicated values as described in the legend of Fig. 3. Vanadate (0.5 mM) was present in all the incubations. At 90 s, the reaction was stopped as described in the legend of Fig. 1. In each experiment the amount of actively stored Ca²⁺ in permeabilized cells incubated at the free Ca²⁺ concentration at which they were loaded (0.1 μ M) is set at 100%, to which all other values are related. The results presented are means $\pm$ S.E.M. of four to five experiments.

[Ca ²⁺] _{free} (μ M)	Pretreatment ...	Actively stored Ca ²⁺ (% of control)	
		Saline	CCK-8
0.1		100	100
0.3		92 $\pm$ 4	109 $\pm$ 15
1.0		87 $\pm$ 6	104 $\pm$ 7
10.0		76 $\pm$ 5	79 $\pm$ 9

rather high concentration of 5 mM-ATP was not significantly different from the release value obtained with the sub-physiological concentration of 0.5 mM-ATP.

Pretreatment of pancreatic acinar cells with 0.1 μ M-CCK-8 significantly inhibits the Ins(1,4,5)P₃-induced release of actively stored Ca²⁺ [18]. This inhibitory action of hormonal pretreatment had been measured in permeabilized cells loaded and stimulated at a free Ca²⁺ concentration of 0.1 μ M. In the present study, pancreatic acinar cells permeabilized after pretreatment with 0.1 μ M-CCK-8 were loaded at 0.1 μ M free Ca²⁺ and stimulated with Ins(1,4,5)P₃ at different [Ca²⁺]_{free} as described above. Fig. 4 shows that, at all three sub-micromolar [Ca²⁺]_{free} tested, the Ins(1,4,5)P₃-induced release of Ca²⁺ from permeabilized CCK-8-treated cells was inhibited to the same extent, indicating that hormonal pretreatment does not lead to an increase in Ca²⁺-sensitivity of the Ins(1,4,5)P₃-operated Ca²⁺ channel. At 10 μ M free Ca²⁺, Ins(1,4,5)P₃-induced Ca²⁺ release was dramatically decreased in both control (approx. 75%) and CCK-8-treated (approx. 80%) acinar cells. Table 2 shows that the internal Ca²⁺ store, loaded at 0.1 μ M free Ca²⁺, released Ca²⁺ in response to increases in [Ca²⁺]_{free}. The effect was more pronounced in control cells, although at 10 μ M free Ca²⁺ both the control and the CCK-8-pretreated cells lost approx. 20% of actively stored Ca²⁺ within 90 s after increasing [Ca²⁺]_{free}.

DISCUSSION

In several cell types, including the pancreatic acinar cell [11–13], oscillatory changes in [Ca²⁺]_i have now been demonstrated during both the initial and the sustained phases of Ca²⁺ mobilization [14–16]. The observation that Ca²⁺ inhibits Ins(1,4,5)P₃ binding to its putative receptor in rat cerebellum membranes [24] has led to the hypothesis that feedback inhibition of Ins(1,4,5)P₃ bind-

ing to its receptor could provide a mechanism to generate such cyclic changes in [Ca²⁺]_i [17]. When testing this hypothesis, it was found that Ca²⁺ did inhibit Ins(1,4,5)P₃-induced Ca²⁺ release from rat cerebellum microsomes, but only at relatively high concentrations (> 1.0 μ M), and not at the sub-micromolar concentrations at which [Ca²⁺]_i oscillates in the intact pancreatic acinar cell [11–13]. Our results with permeabilized cells confirm the data obtained with the microsomal preparation, in that Ins(1,4,5)P₃-induced Ca²⁺ release is inhibited by Ca²⁺ only at concentrations above 1.0 μ M. However, inhibition of Ins(1,4,5)P₃-induced Ca²⁺ release by high ambient Ca²⁺ is not a universal phenomenon. For example, in permeabilized hepatocytes such an inhibition was not observed [25]. Ins(1,4,5)P₃ binding to rat cerebellum microsomes appears to be more sensitive to Ca²⁺ than is Ins(1,4,5)P₃-induced Ca²⁺ release [17]. Differences in experimental conditions may explain part of this discrepancy. For example, Ca²⁺ release was measured at a pH far from optimal for binding. Inhibition of Ins(1,4,5)P₃ binding to its putative receptor by sub-micromolar Ca²⁺ is also not a universal phenomenon. Ins(1,4,5)P₃ binding to bovine adrenocortical microsomes was not inhibited by ambient free Ca²⁺ up to a concentration of 0.5 μ M [23]. One explanation for the lack of Ca²⁺-sensitivity in some systems might be the absence of 'calmedin' (see below).

The fact that Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized pancreatic acinar cells is not inhibited by sub-micromolar free Ca²⁺ seems to rule out the possibility that some cytosolic factor mediating the inhibition of the Ins(1,4,5)P₃ receptor by Ca²⁺ is lost in microsomal preparations. On the other hand, Danoff *et al.* [26] recently described the existence of a membrane-associated protein, referred to as 'calmedin', in cerebellar tissue which increases the Ca²⁺-sensitivity of the Ins(1,4,5)P₃ receptor. Since this membrane-associated protein is expected to be present in permeabilized cells as well as microsomal preparations, an additional mechanism seems to be required to increase the Ca²⁺-sensitivity of the Ins(1,4,5)P₃ receptor to sub-micromolar Ca²⁺ concentrations. In fact, we have recently shown that, at a free Ca²⁺ concentration of 0.1 μ M, Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized pancreatic acinar cells is markedly decreased after hormonal pretreatment (carbamoylcholine, CCK-8) [18]. The present study clearly indicates that in CCK-8-treated cells Ins(1,4,5)P₃-induced Ca²⁺ release is suppressed in a [Ca²⁺]_{free}-independent manner. Therefore, the inhibitory effect of hormonal pretreatment is unlikely to be due to an increased Ca²⁺-sensitivity of the Ins(1,4,5)P₃ receptor. In addition, Wakui *et al.* [27] recently showed that oscillatory changes in [Ca²⁺]_i are also induced by the non-metabolizable InsP₃ analogue inositol trisphosphothioate, suggesting that oscillations in [Ca²⁺]_i can occur at constant InsP₃ concentration and that fluctuating Ins(1,4,5)P₃ levels are not essential for repetitive rises in [Ca²⁺]_i.

Permeabilized pancreatic acinar cells rapidly accumulate Ca²⁺ in an energy-dependent store, and we have shown previously that, at 0.1 μ M free Ca²⁺, ATP-dependent Ca²⁺ uptake reaches a steady state of 0.9–1.5 nmol of Ca²⁺/mg of protein within 10 min at 37 °C [18]. In the present study we show that energy-dependent Ca²⁺ uptake increases with increasing [Ca²⁺]_{free}. A similar Ca²⁺-dependence of ATP-dependent Ca²⁺ accumulation

was found in rat pancreas [28]. In addition to the latter study, we determined the absolute amount of actively stored Ca^{2+} releasable by $\text{Ins}(1,4,5)\text{P}_3$ at three $[\text{Ca}^{2+}]_{\text{free}}$. In absolute terms, the maximal amount of $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} first increases (1.7-fold at $1.0\ \mu\text{M}$ relative to $0.2\ \mu\text{M}$ free Ca^{2+}), but then decreases (0.7-fold at $2.0\ \mu\text{M}$ relative to $1.0\ \mu\text{M}$ free Ca^{2+}). At $2.0\ \mu\text{M}$ free Ca^{2+} the half-maximal concentration for $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was increased 2.5-fold, indicating that with increasing $[\text{Ca}^{2+}]_{\text{free}}$ the affinity of $\text{Ins}(1,4,5)\text{P}_3$ binding to the receptor decreases. In relative terms, $\text{Ins}(1,4,5)\text{P}_3$ maximally releases 56%, 44% and 20% of actively stored Ca^{2+} . The data presented suggest that an additional, non- $\text{Ins}(1,4,5)\text{P}_3$ -releasable, store becomes rapidly filled at higher Ca^{2+} concentrations. This opens the possibility that at $[\text{Ca}^{2+}]_{\text{free}}$ above $1.0\ \mu\text{M}$ this non- InsP_3 -releasable store actively accumulates Ca^{2+} at the expense of the $\text{Ins}(1,4,5)\text{P}_3$ -releasable store. As a consequence, the absolute amount of Ca^{2+} to be released by $\text{Ins}(1,4,5)\text{P}_3$ decreases at higher $[\text{Ca}^{2+}]_{\text{free}}$. Relative to the amount of Ca^{2+} released by $1.0\ \mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ at $0.2\ \mu\text{M}$ free Ca^{2+} , a 1.5-fold increase was observed at $1.0\ \mu\text{M}$ free Ca^{2+} and a 0.7-fold decrease at $2.0\ \mu\text{M}$ free Ca^{2+} (Fig. 2a). A similar decrease (0.7-fold) in the amount of Ca^{2+} released by $1.5\ \mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ was observed for permeabilized cells loaded at $0.2\ \mu\text{M}$ free Ca^{2+} and stimulated at $2.0\ \mu\text{M}$ free Ca^{2+} . These observations suggest that with increasing $[\text{Ca}^{2+}]_{\text{free}}$ the $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} pool becomes smaller and the affinity of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor decreases. Recently, Irvine [29] proposed a model for the regulation of free cytosolic Ca^{2+} in which the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store is refilled from such a non-releasable pool by the action of inositol 1,3,4,5-tetrakisphosphate. We have shown previously that pretreatment with carbamoylcholine or CCK-8 did not lead to significant changes in size of the energy-dependent Ca^{2+} pool. The size of the InsP_3 -releasable Ca^{2+} store, however, seemed to be decreased in hormone-treated permeabilized cells. In the light of the above model, it is interesting to investigate the effect of inositol tetrakisphosphate on the size of the $\text{Ins}(1,4,5)\text{P}_3$ -releasable store in permeabilized cells pretreated with carbamoylcholine or CCK-8. Evidence has been provided that the Ca^{2+} -uptake mechanism of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool is not inhibited by vanadate (for a review, see ref. [30]). However, in our studies the amount of Ca^{2+} bound to permeabilized cells in the absence of ATP was the same as the amount of Ca^{2+} bound in the presence of both ATP and vanadate. In addition, this amount of Ca^{2+} was not releasable by the Ca^{2+} ionophore A23187 [18], indicating that in the presence of vanadate no Ca^{2+} is accumulated in a vesicular store.

In rat cerebellar microsomes steady-state Ca^{2+} uptake also increased with increasing $[\text{Ca}^{2+}]_{\text{free}}$, and $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release progressively decreased at $[\text{Ca}^{2+}]_{\text{free}}$ above $1.0\ \mu\text{M}$ [17]. However, the possibility of an inhibitory effect by sub-micromolar $[\text{Ca}^{2+}]_{\text{free}}$ being masked by an enlargement of the $\text{Ins}(1,4,5)\text{P}_3$ -releasable pool was not considered. To test this possibility, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized pancreatic acinar cells loaded at a particular (sub-micromolar) $[\text{Ca}^{2+}]_{\text{free}}$ was measured at increasing $[\text{Ca}^{2+}]_{\text{free}}$. Also under these conditions we observed no inhibition at sub-micromolar $[\text{Ca}^{2+}]_{\text{free}}$. In these experiments vanadate was included in the medium to maintain the specific radioactivity of internally stored Ca^{2+} . As has been shown for

human neutrophils [31], vanadate had no effect on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized pancreatic acinar cells, indicating that $\text{Ins}(1,4,5)\text{P}_3$ does not act by inhibiting the Ca^{2+} pump of the internal store [32].

Berridge *et al.* [14] recently discussed possible mechanisms by which cells could generate oscillatory changes in $[\text{Ca}^{2+}]_{\text{i}}$. Ca^{2+} -induced Ca^{2+} release was implicated, as well as oscillatory changes in $\text{Ins}(1,4,5)\text{P}_3$ production. In the present study, the amount of actively stored Ca^{2+} , measured 90 s after changing $[\text{Ca}^{2+}]_{\text{free}}$, was found to be decreased with increasing $[\text{Ca}^{2+}]_{\text{free}}$. Therefore, part of the energy-dependent Ca^{2+} pool seems to be released in a $[\text{Ca}^{2+}]_{\text{free}}$ -dependent manner. It is unclear, however, which store, the InsP_3 -releasable or non- InsP_3 -releasable, is sensitive to $[\text{Ca}^{2+}]_{\text{free}}$.

In conclusion, the results presented do not support the idea that feedback inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release by Ca^{2+} plays a role in generating oscillatory changes in $[\text{Ca}^{2+}]_{\text{i}}$. Moreover, the fact that the inhibitory action of CCK-8 pretreatment on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release proved to be independent of the free Ca^{2+} concentration indicates that hormonal pretreatment does not lead to an increase in the Ca^{2+} -sensitivity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Pretreatment of pancreatic acinar cells leads to inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from permeabilized cells by a mechanism not yet resolved. Whether this mechanism is part of the mechanism by which the cell generates oscillatory changes in $[\text{Ca}^{2+}]_{\text{i}}$, deserves further investigation.

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