Cell Calcium (1991) 12, 1–9

Effect of GTP and Ca^{2+} on inositol 1,4,5-trisphosphate induced Ca^{2+} release from permeabilized rat exocrine pancreatic acinar cells

R. ENGLING[1], K.J. FÖHR[1], T.P. KEMMER[2] and M. GRATZL[1]

[1] *Abteilung Anatomie und Zellbiologie der Universität Ulm, Ulm, FRG*
[2] *Abteilung Innere Medizin II der Universität Ulm, Ulm, FRG*

Abstract — The effects of Ca^{2+} and GTP on the release of Ca^{2+} from the inositol 1,4,5-trisphosphate (IP_3) sensitive Ca^{2+} compartment were investigated with digitonin permeabilized rat pancreatic acinar cells.

The amount of Ca^{2+} released due to IP_3 directly correlated with the amount of stored Ca^{2+} and was found to be inversely proportional to the medium free Ca^{2+} concentration. Ca^{2+} release induced by 0.18 μM IP_3 was half maximally inhibited at 0.5 μM free Ca^{2+}, i.e. at concentrations observed in the cytosol of pancreatic acinar cells.

GTP did not cause Ca^{2+} release on its own, but a single addition of GTP (20 μM) abolished the apparent desensitization of the Ca^{2+} release which was observed during repeated IP_3 applications. This effect of GTP was reversible. GTPγS could not replace GTP. Desensitization still occurred when GTPγS was added prior to GTP. The reported data indicate that GTP, stored Ca^{2+} and cytosolic free Ca^{2+} modulate the IP_3 induced Ca^{2+} release.

Rises in intracellular free Ca^{2+} play essential roles in receptor-mediated signal transduction pathways. Both GTP and inositol 1,4,5-trisphosphate (IP_3) are involved in these processes. IP_3 is produced by the hydrolysis of an inositol lipid (phosphatidyl-inositol-4,5-bisphosphate) located at the plasma membrane and released into the cytosol. A GTP binding protein couples the receptor to the hydrolytic enzyme. Within the cell IP_3 mobilizes Ca^{2+} from non-mitochondrial stores, probably the endoplasmic reticulum. Ca^{2+} is taken up again by a cation [1] and anion dependent [2] Ca^{2+} transport ATPase located in the endoplasmic reticulum. The second messenger function of IP_3 was first recognized in exocrine pancreatic acinar cells [3] and subsequently in a wide variety of different cells [4].

In addition to its role at the level of the plasma membrane, GTP appears also to be involved in intracellular signal transduction. This function, in modulating the Ca^{2+}-mobilizing mechanism, is still poorly understood [cf. 5, 6]. In studying intracellular Ca^{2+} fluxes a decreased response to repeated exposures with the same concentration of

Abbreviations used : EGTA, Ethylene-glycol-bis (2-aminoethylether)-N,N,N',N'- tetra acetic acid; GTPγS, Guanosine 5'-O-[3-thio]triphosphate; GDPβS, Guanosine 5'-O-[2-thio]diphosphate; IP_3, Inositol 1,4,5-trisphosphate; IP_2, Inositol 1,4-bisphosphate; IP_4, Inositol 1,3,4,5-tetrakisphosphate; MOPS, Morpholinopropane sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; pHMB, Parahydroxymercuribenzoate

IP_3 has been observed [7, 8]. This is also shown here for rat pancreatic acinar cells. Thus, in some endocrine and exocrine cells the response to IP_3 desensitizes. On the other hand, the response to IP_3 is reproducible for a few hours with no sign of desensitization in permeabilized tumour cells such as rat insulinoma or rat pheochromocytoma cells [9].

Here we report on the apparent desensitization of the IP_3-induced Ca^{2+} release in permeabilized exocrine pancreatic cells. This effect is abolished by GTP, which suggests that GTP modulates the IP_3 induced Ca^{2+} release within the cells. Also Ca^{2+} itself plays an important role in this process. The data indicate that both the amount of Ca^{2+} stored inside the endoplasmic reticulum as well as cytosolic free Ca^{2+} influence the IP_3 induced Ca^{2+} release.

Materials and Methods

Chemicals

Collagenase was purchased from Serva (Heidelberg, FRG). Soybean trypsin inhibitor, IP_2, and IP_3 were from Boehringer (Mannheim, FRG), IP_4 was kindly given by Dr R. Irvine (Cambridge, UK). Ca^{2+} selective membranes containing the neutral carrier ETH 129 were a generous gift of W. Simon (ETH Zürich, Switzerland). KCl suprapur was from Merck (Darmstadt, FRG), and Azur A from Fluka (Neu-Ulm, FRG). All other chemicals were of analytical grade.

Isolation of exocrine pancreatic acinar cells

Pancreatic acinar cells were prepared as described previously by Amsterdam and Jamieson [10] with modifications according to Streb and Schulz [11]. Briefly, pancreatic tissue from four male Wistar rats (200–250 g) fasted overnight was chopped with scissors and incubated in a standard medium containing (in mM): 145 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.4), 15 glucose, 0.2 % BSA, 0.01 % soybean trypsin inhibitor plus collagenase (150 U/ml) for 15 min at 37°C. In order to obtain single cells, a washing step in the above Ca^{2+}-free medium ($CaCl_2$ replaced by 1 mM EDTA, without collagenase) was interposed, followed by a further digestion with collagenase (210 U/ml) for 50–60 min at 37°C. During cell isolation the medium was continuously gassed with oxygen in a water bath at 37°C. Acinar cells were then mechanically dissociated by sequential passages through polypropylene pipettes of decreasing tip diameter (about 5, 3, and 2 mm) after 40 min of the second collagenase digestion step. The cell suspension was diluted with standard medium, and centrifuged for 5 min at 85 *g*. The pellet was resuspended in standard buffer and filtered through a double layer of medical gauze, layered over 30 ml of standard medium containing 8% albumin. The suspension was centrifuged again. Finally the pellet was washed thrice in the standard medium as described above.

Permeabilization of cells with digitonin

The obtained cell suspension was washed thrice in medium A containing (in mM): 150 KCl, 20 MOPS (pH 7.2), 5 NaN_3, and 1 EGTA. Permeabilization with digitonin (10 μM final) was usually carried out in the same medium on ice for 10 min. Digitonin was added from a stock solution containing DMSO. Permeabilization was controlled by counting the Azur A (0.25 %) stained cells. The permeabilized cells were washed thrice in medium B (medium A without EGTA).

Measurement of free Ca^{2+} concentration

Ca^{2+} specific minielectrodes prepared as described [12] were calibrated before and after each experiment. The poly(vinyl chloride) membrane of the electrode contained the recently developed neutral carrier ETH 129, which allows reliable measurements of Ca^{2+} down to the nanomolar range [13]. Experiments were carried out at room temperature and pH 7.2 in 400 μl of medium B containing 3–6 x 10^6 cells. 10^7 cells correspond to 1.5 mg of cell protein. During Ca^{2+} uptake experiments, medium B contained 1 mM Mg^{2+}, and during Ca^{2+} release experiments, medium B contained 2 mM ATP and 2 mM Mg^{2+}. The absolute amount of Ca^{2+} fluxes was calibrated in each experiment by the addition of suitable dilutions

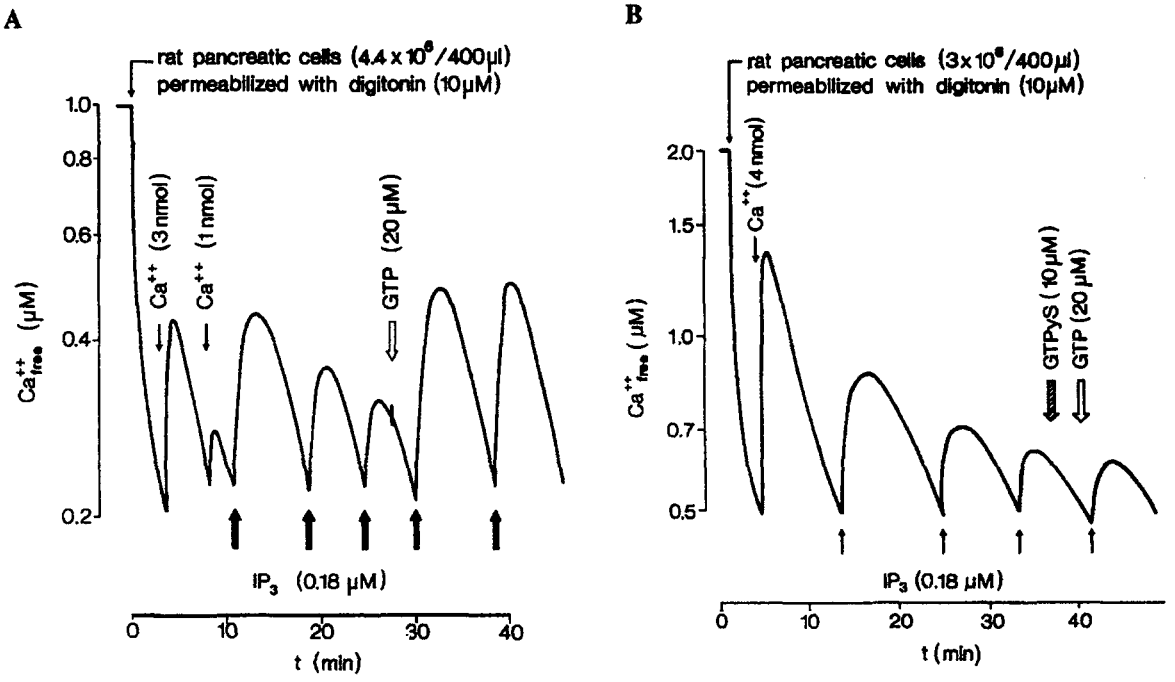


Fig. 1 Effects of GTP or GTPγS on IP_3 induced Ca^{2+} release

(A) Effect of GTP after repeated exposure to IP_3. The cells were loaded with 4 nmol Ca^{2+}. Then, sequential additions of 0.18 µM IP_3 led to a decreased Ca^{2+} release. After addition of a single dose of 20 µM GTP the amounts of Ca^{2+} released by IP_3 were similar in size as observed during the first release.

(B) Effect of GTPγS and GTP after repeated response to IP_3. The cells were first loaded with 4 nmol Ca^{2+}. During successive additions of 0.18 µM IP_3, a diminished Ca^{2+} release was observed. When 10 µM GTPγS was added prior to 20 µM GTP, the IP_3 response could not be restored by the addition of GTP as seen in Figure 1A.

(C) Summary of the results presented in Figure 1A and 1B. Result of 13 experiments (mean ± SD) are shown which were carried out exactly as in Figure 1A and 1B, respectively. The amount of Ca^{2+} released during the first addition of IP_3 (0.18 µM) was defined as 100 %. It corresponds to 2–6 nmol $Ca^{2+}/10^7$ cells. This value depends on the Ca^{2+} accumulated by the stores as well as the free Ca^{2+} concentration in the medium. After the third addition of 0.18 µM IP_3, either 20 µM GTP was added before the fourth addition of IP_3 (open column) or 10 µM GTPγS was added prior to 20 µM GTP followed by the fourth addition of IP_3 (hatched column). For experimental design see Figure 1A and 1B

of a neutral Ca^{2+} standard (Orion, Lorch). Guanosine nucleotides were present as indicated in the figure legends.

Results

The apparent desensitization of the IP_3-induced Ca^{2+} release is abolished by GTP

When permeabilized cells were added to medium B containing 2 mM ATP and 2 mM Mg^{2+}, a rapid decrease of the free Ca^{2+} concentration to about 0.1–0.4 µM occurred (Fig. 1A). In order to avoid

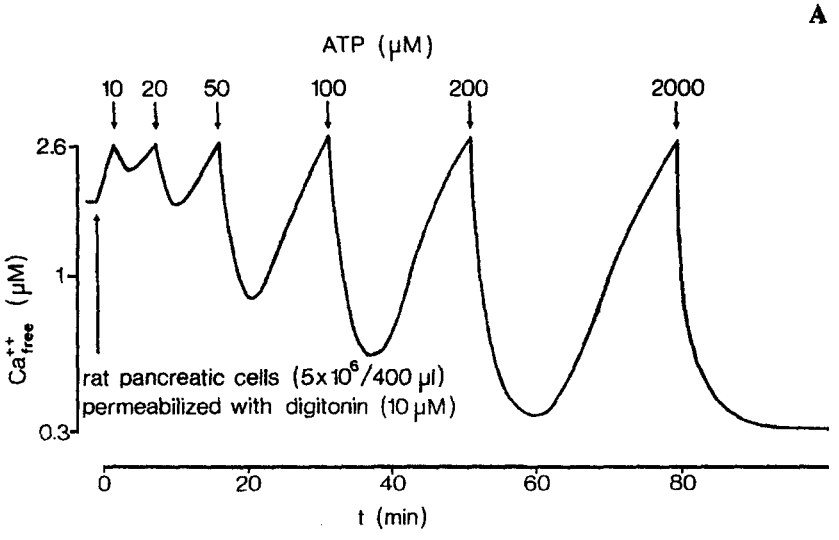
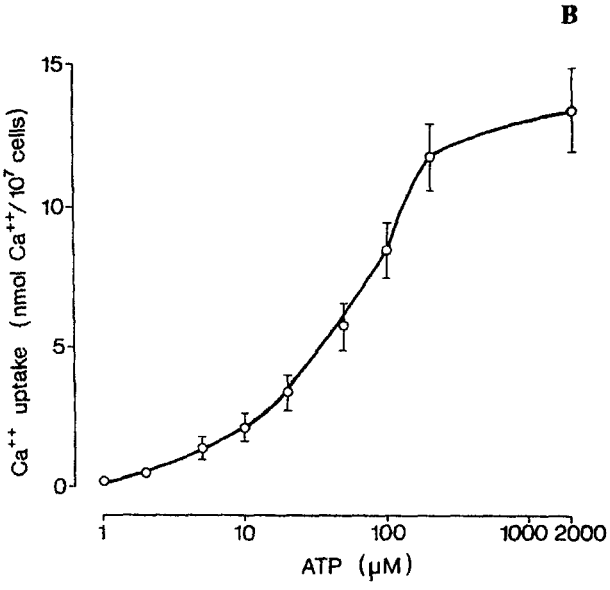


Fig. 2 Ca^{2+} uptake by permeabilized pancreatic acinar cells as a function of the ATP concentration.
(A) Increasing amounts of ATP were added to permeabilized cells in medium B containing 1 mM Mg^{2+} and micromolar free Ca^{2+} concentrations. The free Ca^{2+} concentration is transiently decreased with the addition of low amounts of ATP. Maximal Ca^{2+} uptake is reached at about 200 μM ATP. At higher ATP concentrations the attained Ca^{2+} level was kept constant. (B) Results of 5 experiments (mean ± SD) as described in Figure 2A. The amount of Ca^{2+} uptake was calibrated by adding known amounts of Ca^{2+} from a neutral stock solution to the medium

Ca^{2+} uptake by mitochondria, 5 mM sodium azide (NaN_3) was present throughout the experiments. Addition of further pulses of Ca^{2+} only transiently increased the free Ca^{2+} concentration in the medium followed by immediate Ca^{2+} uptake. Consecutive additions of IP_3 (0.18 μM, final) resulted in a Ca^{2+} release which gradually decreased during further additions of the same amount of IP_3 to permeabilized rat exocrine pancreatic acinar cells (Fig. 1A). This behaviour was also observed with endocrine cells such as bovine adrenal glomerulosa cells and bovine adrenal chromaffin cells and was termed desensitization [7, 8]. This decrease, which was also observed after a single addition of IP_3 followed by long incubation (not shown), was abolished by a single dose of 20 μM GTP (Fig. 1A). Then, IP_3 induced Ca^{2+} release remained constant, and was similar in size to that observed in the first IP_3 induced release (Fig. 1A & 1C).

Though GTPγS could not replace GTP in abolishing desensitization (not shown), it was able to inhibit the effect of GTP (Fig. 1B). Thus, when GTPγS was added prior to GTP, desensitization proceeded (Fig. 1B). Figure 1C summarizes the results of 13 experiments in which GTP or GTPγS plus GTP were added after the third addition of IP_3 (same protocol as Fig. 1A & 1B). These experiments clearly indicate, that the effect of GTP on desensitization is blocked completely by GTPγS.



GDPβS (100 μM) neither acted like GTP nor like GTPγS during IP_3 induced Ca^{2+} release. However, an excess of GDP (100 μM) abolished desensitization. This shows that part of the added GDP might have been phosphorylated to GTP by the cells in the presence of 2 mM ATP. The ineffectiveness of GDPβS, a GDP analogue which does not undergo phosphorylation, supports this interpretation. The effect of GTP was not due to inhibition of the Ca^{2+} uptake system. Ca^{2+} uptake with increasing concentrations of ATP is shown in

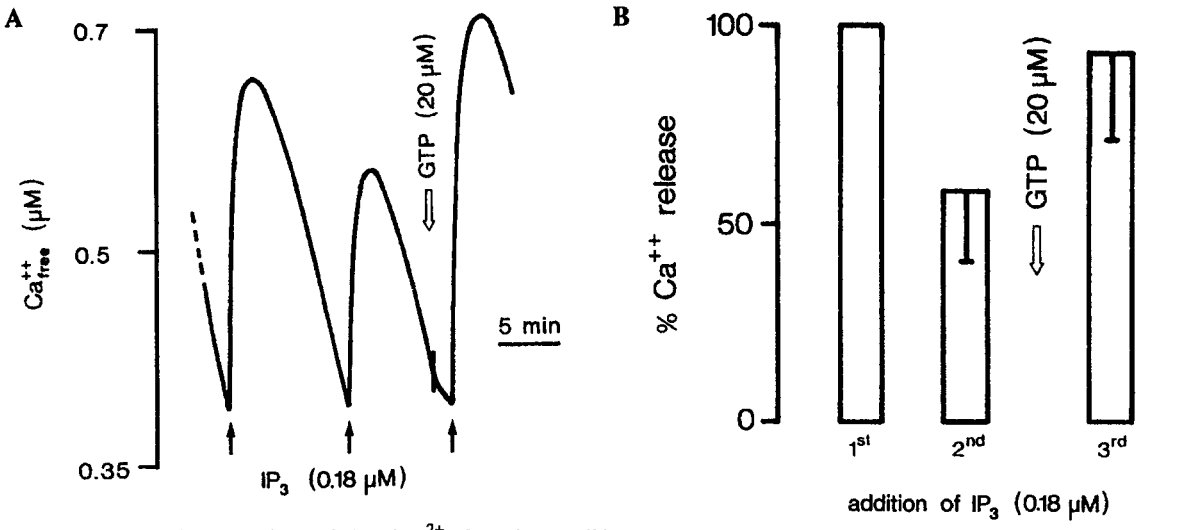


Fig. 3 The effect of GTP on the IP_3 induced Ca^{2+} release is reversible..

(A) When cells, first analyzed as shown in Figure 1A, were washed twice by centrifugation (350 g_{av}, 2 min) and resuspended in medium B the response to 0.18 μM IP_3 again desensitized. However, after addition of 20 μM GTP a full response to the IP_3 occurred. This experiment is to be compared with that shown in Figure 1A.

(B) In 6 independent experiments carried out as in Figure 3A, an almost identical desensitization was observed when compared to the situation described in Figure 1C. The amount of Ca^{2+} released during the first addition of IP_3 (0.18 μM) was defined as 100 % (n = 6, ± SD)

Figure 2A. At low ATP concentrations stored Ca^{2+} is released again upon consumption of ATP (*see* also [9]) while at 2 mM ATP a constant free Ca^{2+} concentration is maintained for about 2 h. With 200 μM ATP, 10.8 ± 1.1 nmol Ca^{2+} are taken up by 10^7 cells in the absence and 11.1 ± 1.2 nmol (n = 3; ±SD) in the presence of 20 μM GTP. This finding indicates that also with 2 mM ATP, the effect of GTP (20 μM) on the IP_3 induced Ca^{2+} release was not due to inhibition of the Ca^{2+} uptake. It is also interesting to note, that, in the absence of IP_3, neither GTP, GDP, GTPγS, nor GDPβS themselves caused Ca^{2+} release.

The effect of GTP was reversible since its removal resulted in the same behaviour as observed before its addition. This was demonstrated by removing GTP by centrifugation (*see* legend to Fig. 3) from cell preparations which had already been treated as shown in Figure 1. After resuspending these cells and repeating the same experiment, desensitization was observed again and added GTP restored full response to IP_3 (Fig. 3). In a similar way the reversibility of the inhibitory action of GTPγS in blocking the GTP effect was established (data not shown).

Effect of stored and medium Ca^{2+} on IP_3 induced Ca^{2+} release

The stable response of the permeabilized cells in the presence of 20 μM GTP allowed the analysis of the Ca^{2+} release as a function of the IP_3 concentration. As shown in Figure 4, Ca^{2+} release depended on the IP_3 concentration. At Ca^{2+} concentrations found in resting acinar cells (0.2 μM), half maximal Ca^{2+} release was achieved with 0.12 μM IP_3. However about 4 times higher concentrations of IP_3 are required at Ca^{2+} concentrations found in stimulated cells (1.2 μM) to give the same amount of Ca^{2+} release. Other inositol compounds like inositol, IP_2 and IP_4 could not replace IP_3.

Ca^{2+} release depends on the medium Ca^{2+} concentration, which in permeabilized cells, corresponds to the cytosolic free Ca^{2+} concentration. Compared to 0.1 μM free Ca^{2+}, Ca^{2+} release due to a constant amount of IP_3 (0.18 μM) decreased with increasing medium free Ca^{2+} concentration (Fig. 5). Half maximal inhibition of Ca^{2+} release occurred at about 0.5 μM free Ca^{2+} (Fig. 5). At 10 μM free Ca^{2+}, IP_3 even at higher concentrations (5 μM) could not cause Ca^{2+} release. However, stored Ca^{2+}

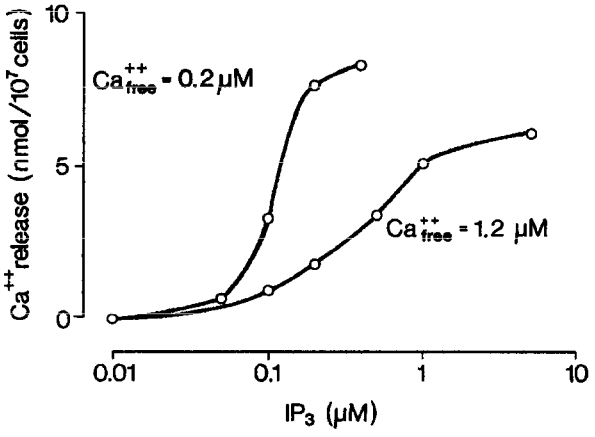


Fig. 4 Ca^{2+} release as a function of the IP_3 concentration at 0.2 and 1.2 μM free Ca^{2+}.
The Ca^{2+} stores were first maximally filled with Ca^{2+} as shown in Figure 6 (several additions of Ca^{2+} until no further Ca^{2+} uptake occurred). Then 20 μM GTP was added and Ca^{2+} release with IP_3 concentrations between 0.01 and 5 μM was started. Each addition of IP_3 resulted in a transient Ca^{2+} release. The higher basal free Ca^{2+} concentration (1.2 μM) was achieved by addition of an appropriate amount of Ca^{2+} from a neutral stock solution prior to the addition of increasing amounts of IP_3 as described above. The free Ca^{2+} concentrations given in the figure indicate the readings of the Ca^{2+} electrode when IP_3 was added. The amount of Ca^{2+} released was calibrated as described in Figure 2B. This figure shows 2 of 4 similar experiments giving the same results

could still be released, indicating that the Ca^{2+} gradient was sufficient to allow Ca^{2+} efflux from the Ca^{2+} compartments investigated. To deplete cellular Ca^{2+} stores we used 50 μM pHMB instead of A23187 since the ionophore caused changes in the response of our Ca^{2+} electrode.

The amount of released Ca^{2+} is not only governed by the free Ca^{2+} in the medium but also by the amount of stored Ca^{2+} (Fig. 6). In the presence of GTP, the amount of released Ca^{2+} during repetitive addition of the same IP_3 concentration was identical. Addition of small amounts of Ca^{2+} resulted in transient increases in medium Ca^{2+} followed by an immediate uptake of Ca^{2+} into the non-mitochondrial storage system (Fig. 6). In this way the amount of Ca^{2+} released by IP_3 was enhanced stepwise (Fig. 6). In the absence of GTP, addition of exogenous Ca^{2+} did not prevent the decreased Ca^{2+} release observed during consecutive IP_3 applications. From the data described in the previous two paragraphs, it can be concluded that Ca^{2+} release by IP_3 is directly correlated to the amount of stored Ca^{2+} but inversely to the medium free Ca^{2+} concentration. It also shows that, due to the dynamic properties of the intracellular Ca^{2+} store, adaptation of the cells to the physiological requirements is possible.

Discussion

Neuronal, endocrine or exocrine secretory cells may use extracellular and/or intracellular Ca^{2+} to control secretion by exocytosis. The intracellular systems

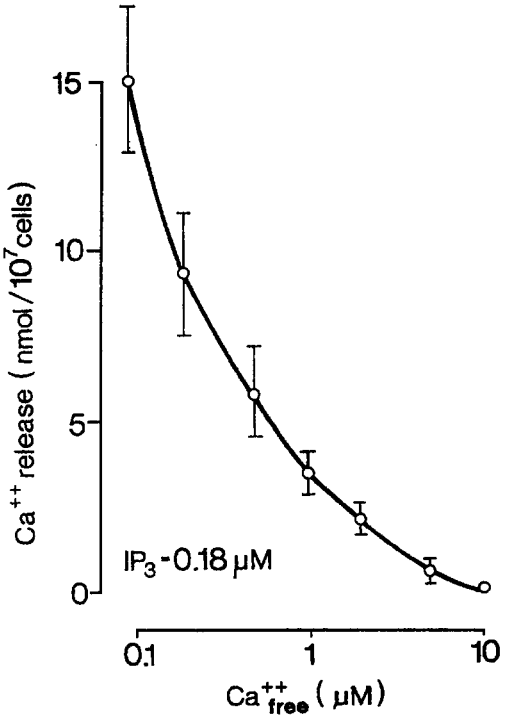


Fig. 5 Ca^{2+} release as a function of the free Ca^{2+} concentration. The Ca^{2+} stores were first loaded with Ca^{2+} (as in Fig. 1A & 1B) and 20 μM GTP was added to avoid desensitization. The results (mean ± SD) of 5 experiments are shown. Release was carried out with 0.18 μM IP_3. The release experiment was started with IP_3 at 0.1 μM free Ca^{2+}. The higher free Ca^{2+} concentrations were obtained by additions of suitable amounts of Ca^{2+} from a stock solution. The Ca^{2+} concentration indicated at the abscissa gives the Ca^{2+} concentration at which IP_3 was added

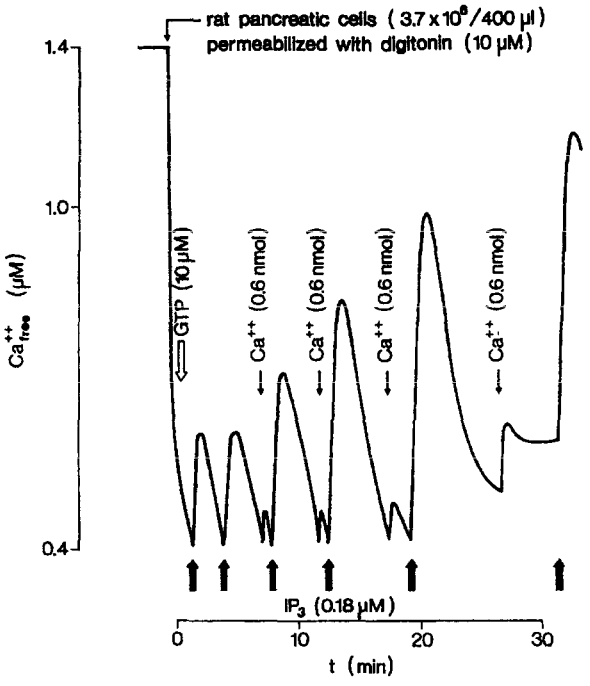


Fig. 6 The amount of Ca^{2+} released depends on the Ca^{2+} preloading.

Permeabilized cells were challenged with pulses of 0.18 µM IP_3. Ca^{2+}, added between IP_3 injections, was rapidly sequestered. It stepwise increased the amount of Ca^{2+} released by IP_3

participating in regulation of the secretory response can be studied with subcellular fractions or permeabilized cell preparations.

IP_3, produced in intact cells as a consequence of receptor activation, is the principal agent causing the release of Ca^{2+} from a membrane bound compartment, probably the endoplasmic reticulum, which can store Ca^{2+} due to the presence of an ATP dependent Ca^{2+} uptake system. Free Ca^{2+} of the medium (corresponding in permeabilized cells to the cytosol) inhibits IP_3-induced Ca^{2+} release in some cell types and not in others. Experiments with platelets [14], indicated that medium free Ca^{2+} up to 100 µM does not influence the response to IP_3. On the other hand saponin-permeabilized neuroblastoma cells have been observed to be unable to release Ca^{2+} in the presence of 10 µM free Ca^{2+} [15]. We observed in this study, that acinar pancreatic cells exhibit a similar behaviour (Figs 4 and 6). Release of Ca^{2+} by the non-mitochondrial system is progressively inhibited with increasing Ca^{2+} concentrations, suggesting an autoregulatory role of cytoplasmic free Ca^{2+}. Indeed half maximal inhibition of IP_3-induced Ca^{2+} release was observed at 0.5 µM free Ca^{2+}, a concentration expected to occur in intact cells. Furthermore, the IP_3 sensitivity of the Ca^{2+} release was greatly altered by a change of the free Ca^{2+} concentration from 0.2 µM to 1.2 µM. These findings may be explained by the recently discovered inhibition of IP_3-binding to its receptor by Ca^{2+} [16–18]. In contrast to our findings, where the Ca^{2+} release continuously decreased with the free Ca^{2+} concentration, a biphasic pattern was reported by other groups with an optimum around 1 µM free Ca^{2+} [19, 20].

It is known from studies of permeabilized rat pancreatic acinar cells or pancreatic subcellular fractions, that the amount of Ca^{2+} stored in the endoplasmic reticulum can be increased [21, 22]. An important finding in this study is that the additional stored Ca^{2+} is directly available for the IP_3 induced Ca^{2+} release, which suggests that the regulation of intracellular Ca^{2+} by the IP_3 sensitive Ca^{2+} store depends not only on the presence of IP_3 but also is a function of the cytosolic free Ca^{2+} as well as the filling state of the corresponding compartments.

Cellular GTP may also play an important role during intracellular Ca^{2+} release. GTP can either release Ca^{2+} independently of IP_3 [23–25] or act together with IP_3 [26, 27]. Both effects of GTP are generally dependent on the presence of polyethyleneglycol, albumin or similar substances. None of these colloids were used in the present study.

We observed that Ca^{2+} release by permeabilized pancreatic acinar cells gradually diminished during several applications of IP_3. A similar behaviour has been reported for adrenal glomerulosa and adrenal medullary cells and termed desensitization [7, 8]. By contrast, rat insulinoma cells or rat pheochromocytoma cells release a constant amount of Ca^{2+} during repeated IP_3 applications [9, 28]. The desensitization exhibited by pancreatic acinar cells was instantaneously abolished by GTP, and sensitivity was fully restored to subsequent IP_3 applications. This effect could not be mimicked by non hydrolysable analogues. Thus GTP may play an

sequestration in permeabilized rat pancreatic acini. Am. J. Physiol., 23, C621-C627.
22. Bayerdörffer E. Streb H. Eckardt L. Haase W. Schulz I. (1984) Characterization of calcium uptake into rough endoplasmic reticulum of rat pancreas. J. Membr. Biol., 81, 69-82.
23. Ueda T. Cheuh SH. Noel MW. Gill DL. (1986) Influence of inositol 1,4,5-trisphosphate and guanine nucleotides on intracellular calcium release within the N1E-115 neuronal cell line. J. Biol. Chem., 261, 3184-3192.
24. Gill DL. Ueda T. Chueh SH. Noel MW. (1986) Ca^{2+} release from endoplasmic reticulum is mediated by a guanine nucleotide regulatory mechanism. Nature, 320, 461-464.
25. Henne V. Söling HD. (1986) Guanosine 5′-triphosphate releases calcium from rat liver and guinea pig parotid gland endoplasmic reticulum independently of inositol 1,4,5-trisphosphate. FEBS Lett., 202, 267-273.
26. Dawson AP. (1985) GTP enhances inositol trisphosphate-stimulated Ca^{2+} release from rat liver microsomes. FEBS Lett., 185, 147-150.
27. Thomas AP. (1988) Enhancement of the inositol 1,4,5-trisphosphate-releasable Ca^{2+} pool by GTP in permeabilized hepatocytes. J. Biol. Chem., 263, 2704-2711.
28. Prentki M. Corkey BE. Matschinsky FM. (1985) Inositol 1,4,5-trisphosphate and the endoplasmic reticulum calcium cycle of a rat insulinoma cell line. J. Biol. Chem., 260, 9185-9190.
29. Kimura Y. Hirata M. Hamachi T. Koga T. (1988) Possible physiological role of guanosine triphosphate and inositol 1,4,5-trisphosphate in Ca^{2+} release in macrophages stimulated with chemotactic peptide. Biochem. J., 249, 531-536.
30. Ghosh TK. Mullaney JM. Tarazi FI. Gill DL. (1989) GTP-activated communication between distinct inositol 1,4,5-trisphosphate-sensitive and -insensitive calcium pools. Nature, 340, 236-239.
31. Dawson AP. Comerford JG. Fulton DV. (1986) The effect of GTP on inositol 1,4,5-trisphosphate-stimulated Ca^{2+} efflux from a rat liver microsomal fraction. Biochem. J., 234, 311-315.

Please send reprint requests to : Prof. Dr. M. Gratzl, Abteilung Anatomie und Zellbiologie, der Universität Ulm, Postfach 4066, D-7900 Ulm, FRG.

Received : 6 August 1990
Revised : 15 November 1990
Accepted : 19 November 1990