J. Membrane Biology 81, 241–253 (1984)

The Journal of
Membrane Biology

Effect of Inositol-1,4,5-Trisphosphate on Isolated Subcellular Fractions of Rat Pancreas

H. Streb, E. Bayerdörffer, W. Haase, R.F. Irvine†, and I. Schulz
Max-Planck-Institut für Biophysik, 6000 Frankfurt (Main) 70, Federal Republic of Germany,
and †Department of Biochemistry, ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England

Summary. We have previously shown that inositol-1,4,5-trisphosphate (IP_3) releases Ca^{2+} from an intracellular calcium store in permeabilized acinar cells of rat pancreas (H. Streb et al., 1983, *Nature (London)* **306**:67–69). This observation suggests that IP_3 might provide the missing link between activation of the muscarinic receptor and Ca^{2+} release from intracellular stores during stimulation. In order to localize the intracellular IP_3-sensitive calcium pool, IP_3-induced Ca^{2+} release was measured in isolated subcellular fractions. A total homogenate was prepared from acinar cells which had been isolated by a collagenase digestion method. Endoplasmic reticulum was separated from mitochondria, zymogen granules and nuclei by differential centrifugation. Plasma membranes and endoplasmic reticulum were separated by centrifugation on a sucrose step gradient or by precipitation with high concentrations of $MgCl_2$. IP_3-induced Ca^{2+} release per mg protein in the total homogenate was the same as in leaky cells and was sufficiently stable to make short separation procedures possible. In fractions obtained by either differential centrifugation at 7000 × *g*, sucrose-density centrifugation, or $MgCl_2$ precipitation there was a close correlation of IP_3-induced Ca^{2+} release with the endoplasmic reticulum markers ribonucleic acid (r = 0.96, 1.00, 0.91, respectively) and NADPH cytochrome *c* reductase (r = 0.63, 0.98, 0.90, respectively). In contrast, there was a clear negative correlation with the mitochondrial markers cytochrome *c* oxidase (r = −0.64) and glutamate dehydrogenase (r = −0.75) and with the plasma membrane markers ($Na^+ + K^+$)-ATPase (r = −0.81) and alkaline phosphatase (r = −0.77) in all fractions analyzed. IP_3-induced Ca^{2+} release was distributed independently of zymogen granule or nuclei content of the fractions as assessed by electron microscopy. The data suggest that inositol-1,4,5-trisphosphate releases Ca^{2+} from endoplasmic reticulum in pancreatic acinar cells.

Key Words stimulus secretion coupling · pancreatic acinar cells · intracellular calcium stores · calcium transport · inositol phosphates

Introduction

Activation of Ca^{2+} mobilizing receptors such as the muscarinic receptor in the exocrine pancreas is generally accompanied by the breakdown of phosphatidylinositol [5, 19]. Recent evidence suggests that the primary target of receptor-coupled hydrolysis may be the polyphosphoinositides, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate rather than phosphatidylinositol itself. In a great number of tissues rapid hydrolysis of the polyphosphoinositides has been demonstrated [2, 6, 8, 10, 11, 16, 18, 21–23, 30, 31, 33]. Furthermore, the primary hydrolysis products were shown to be inositol-1,4-bisphosphate and/or inositol-1,4,5-trisphosphate (IP_3) [2, 6, 7, 11, 18]. However, the relationship between Ca^{2+} release and polyphosphoinositide breakdown remained unclear.

Recently we have shown that in pancreatic acinar cells with permeabilized plasma membrane IP_3 in μmolar concentrations releases Ca^{2+} from an intracellular calcium store [28]. As IP_3-induced Ca^{2+} release was unaltered when cells were incubated in the presence of mitochondrial inhibitors, we suggested that a nonmitochondrial Ca^{2+} store was probably involved. The total amount of Ca^{2+} released by sequential addition of IP_3 or the acetylcholine analogue carbachol (in either order) was constant, indicating that indeed both substances act on the same intracellular calcium pool.

These results suggested that IP_3 functions as a second messenger between the muscarinic receptor and the intracellular calcium store. It therefore seemed important to localize more precisely the target organelle of IP_3.

In the data described below we report on the relationship between IP_3-induced Ca^{2+} release and the distribution of subcellular organelles in a variety of fractions prepared by four separate fractionation procedures based upon three different principles. Our results suggest that IP_3 releases Ca^{2+} from the endoplasmic reticulum, rather than from plasma membranes, mitochondria, or zymogen granules.

Materials and Methods

Materials

Reagents were obtained from the following sources: K_2ATP, phosphocreatine (sodium salt), benzamidine, and 2-oxoglutaric acid from Sigma Chemical (St. Louis, Mo.); cytochrome *c*, lactate dehydrogenase, pyruvate kinase, creatine kinase, phosphoenolpyruvate, NADH, NADPH, and RNA from Boehringer (Mannheim, Germany); and Triton X and bovine serum albumin (lyophilized) from Serva (Heidelberg, Germany). Sodium azide, sodium dithionate, the test kit for alkaline phosphatase (no. 3344), trypan blue, and ouabain were purchased from Merck (Darmstadt, Germany) and collagenase (from clostridium histolyticum) type III from Worthington (Freehold, N.J.). All other reagents were of analytical grade.

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

Inositol-1,4,5-trisphosphate (IP_3) was prepared from ox brain by alkaline hydrolysis of a crude inositide fraction, followed by Dowex column chromatography, and then paper chromatography, essentially as described by Grado and Ballou [13]. After elution from the paper, the 1,4,5 IP_3 was cleaned of any contaminating cations by passage down a Dowex 50 W column and neutralized with KOH. IP_3 therefore is mainly the potassium salt.

Preparation of Cells and Isolated Fractions

a) Preparation of Isolated Acinar Cells and a Total Homogenate

Isolated acinar cells from rat pancreas (male Wistar rats) were prepared by a collagenase digestion method according to Amsterdam and Jamieson [3] with slight modifications [29]. The plasma membranes of the isolated cells were permeabilized by washing the cells twice with a nominally Ca^{2+}-free solution containing (in mmol/liter): KCl 135, HEPES 10, $MgCl_2$ 1, pH 7.4. This treatment resulted in an increase in trypan blue uptake—as an indicator of cell leakiness—from below 10% before washing to 70 to 90% within 30 min after washing [29]. Cells were stored as a concentrated suspension on ice in the same medium until measurement (up to 4 hr).

A total homogenate was generally prepared from acinar cells isolated as above to reduce contamination by endocrine cells, duct cells, fibrocytes and erythrocytes. After isolation, cells were washed twice with a buffer containing (in mmol/liter): mannitol 280, HEPES 10, KCl 10, $MgCl_2$ 1, benzamidine 1, pH 7.4 (from now on referred to as mannitol buffer). Cells were then homogenized in mannitol buffer at a protein concentration of about 10 mg/ml using 50 strokes at 900 rpm with a very tightly fitting motor-driven Potter-Elvejhem glass/Teflon® homogenizer. To remove the small amount (about 10%) of remaining intact cells, the resulting homogenate was centrifuged for 10 min at 100 × *g* and the pellet was either discarded or rehomogenized. Homogenization and all subsequent fractionation procedures were performed in the cold room. Subcellular fractions were generally suspended in, and diluted with, mannitol buffer.

b) Differential Centrifugation

A total homogenate was centrifuged at 11,000 × *g* for 15 min in a Beckman Model L2 65 B ultracentrifuge using a Beckman 60 Ti fixed angle rotor. The supernatant was removed, a loosely attached layer on the pellet ("fluffy layer") was separated from the firm pellet ("pellet") and both fractions were resuspended in mannitol buffer.

For subfractionation of the "pellet" it was diluted to a protein concentration of 4.4 ± 0.2 mg protein/ml and centrifuged at 7000 × *g* for 15 min in a Beckman Model J-21 C centrifuge using a Beckman JA 20 rotor. The resulting pellet ("heavy pellet") and the supernatant ("light pellet") were separated, the heavy pellet resuspended and both fractions spun down at 18,000 × *g* for 15 min using the same rotor.

c) Sucrose-Density Centrifugation

For preparation of a sucrose step gradient, sucrose was dissolved in a solution containing (in mmol/liter): HEPES 10, KCl 10, $MgCl_2$ 1, benzamidine 1, pH 7.4. The "fluffy layer" obtained from six rats by differential centrifugation (*see* Section *b*) was brought to 1.25 M sucrose by addition of 2 M sucrose buffer. The sample (7 ml) was layered over a 1-ml cushion of 2 M sucrose and overlayered with 3 ml of 0.3 M sucrose. The step gradient was then centrifuged at 150,000 × *g* for 90 min in a Beckman Model L2 65 B ultracentrifuge using a Beckman Type 35 rotor. The material banding at the interfaces between 0.3 M/1.25 M sucrose (=S1) and 1.25 M/2 M sucrose (=S2) was collected.

d) $MgCl_2$ Precipitation

A total pancreatic homogenate from six rats was suspended in 80 ml of mannitol buffer, and then $MgCl_2$ was added to a final concentration of 11 mmol/liter and the homogenate allowed to stand for 15 min. It was then diluted 1 : 1 with buffer containing the same (11 mmol/liter) $MgCl_2$ concentration and spun at 400 × *g* for 10 min in a Beckman Model J-21 C centrifuge using a Beckman JS 7.5 rotor. The resulting supernatant and precipitate were separated, the precipitate resuspended, and both fractions were centrifuged at 25,000 × *g* for 15 min using a Beckman SW 27 swing-out rotor (Beckman Model L2 65 B ultracentrifuge). The final pellets were resuspended in mannitol buffer.

For the determination of IP_3-induced Ca^{2+} release, the isolated fractions were stored on ice for up to 3 hr. The content of protein, RNA, and marker enzyme activity was measured in samples that had been stored frozen at −20°C.

Determination of Inositol-1,4,5-trisphosphate-Induced Ca^{2+} Release

Cells, total homogenate, or isolated fractions were incubated at 25°C in 3 ml of a solution containing (in mmol/liter): KCl 120, HEPES 25, $MgCl_2$ 6, K_2ATP 5, creatine phosphate (sodium salt) 10, and 10 U/ml creatine kinase, pH 7.4. The solution was con-

tinuously stirred and the free Ca^{2+} concentration of the medium recorded with a Ca^{2+}-specific macro-electrode (neutral carrier ETH 1001) as described before [1, 29]. The amount of calcium taken up into or released from permeabilized cells or isolated fractions was determined by measuring the decrease or increase in free Ca^{2+} concentration of the incubation medium, respectively. In order to convert the changes in free Ca^{2+} concentration to total amounts of calcium, the system was calibrated for each individual determination by known calcium additions (*compare* Figs. 1 and 2). Ca^{2+} release was always determined using the saturating concentration of IP_3 at 2.5 μmol/liter [28].

Measurement of Protein, RNA and Marker Enzymes

For all assays a Beckman Model 25 spectrophotometer was employed.

For determination of *protein*, samples were precipitated in 10% (wt/vol) trichloroacetic acid (TCA) and dissolved in 1 M NaOH. Protein content was then measured according to Lowry et al. [17] using bovine serum albumin as standard.

For determination of *ribonucleic acid*, samples were precipitated with TCA in the presence of 0.1% bovine serum albumin, washed twice with 5% TCA and hydrolyzed by incubating for 20 min at 90°C in the presence of 5% TCA. RNA content was measured with orcinol according to Hatcher and Goldstein [14].

(Na^+ + K^+)-ATPase was measured according to Scharschmidt et al. [24] with the following modifications: The concentration of ATP was reduced to 2.5 mmol/liter and that of phosphoenolypyruvate to 2 mmol/liter. The ouabain-inhibited fraction of total ATPase activity was determined in the same sample by adding ouabain directly to the cuvette to a final concentration of 1.5 mmol/liter.

Alkaline phosphatase was determined as the rate of hydrolysis of *p*-nitrophenyl phosphate at 37°C using a Merck test kit (no. 3344).

Cytochrome c oxidase was measured in a 30-mmol/liter phosphate buffer at pH 7.4 and 37°C, containing 0.8 mmol/liter cytochrome *c* that had previously been reduced by 20 mmol/liter sodium dithionite. Samples were preincubated with 0.0015% Triton X.

Glutamate dehydrogenase was determined in a NADH-coupled assay at 25°C according to Schmidt [25].

NADPH cytochrome c reductase was measured using the method of Sottocasa et al. [27].

Electron Microscopy

For electron microscopy the suspended fractions were spun at 18,000 × *g* for 15 min in a Beckman SW 50 rotor (Beckman Model L2 65 B ultracentrifuge) and fixed as pellets with 5% (vol/vol) glutaraldehyde in 0.1 mol/liter sodium cacodylate buffer, pH 7.4. Small blocks of the whole pellet were cut, enclosed in agar, postfixed with 1% (wt/vol) osmic acid in cacodylate buffer, dehydrated with alcohol, and embedded in Spurr's resin. Sections parallel to the axis of the pellet were cut, stained with uranyl acetate and lead citrate, and examined in a Phillips 300 electron microscope.

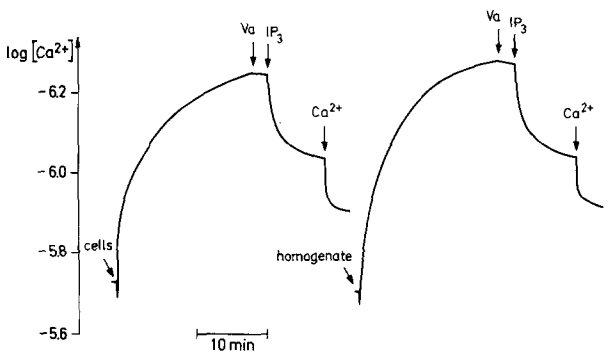

Fig. 1. Comparison of IP_3-induced Ca^{2+} release in leaky cells and total homogenate. Leaky acinar cells and total homogenate of rat pancreas were prepared and incubated as described in Materials and Methods. Ca^{2+} uptake was started by addition of leaky cells or homogenate to the medium to a final concentration of 0.94 mg protein/ml and 0.83 mg protein/ml, respectively. Original traces of medium-free Ca^{2+} concentration as measured with the Ca^{2+}-specific electrode are given. Where indicated, vanadate (*Va*) to a final concentration of 0.2 mmol/liter, inositol-1,4,5-trisphosphate (IP_3) to a final concentration of 2.5 μmol/liter and Ca^{2+} (10 nmol) were added. Typical for 3 experiments

Results

Comparison of Leaky Cells and Total Homogenate

IP_3-induced Ca^{2+} release has thus far only been demonstrated in permeabilized cell preparations [15, 28]. To see if the effect depends upon the presence of whole cells or on some cytosolic or loosely attached factor retained in the permeabilized cells, a total homogenate was tested.

Permeabilized cells and total homogenate prepared from the same cells were compared to avoid scatter between different preparations. No measurable difference between the two preparations could be detected (Fig. 1). In three experiments, the homogenate showed 97 ± 11% of the IP_3-induced Ca^{2+} release per mg protein as measured in leaky cells under the same conditions. In these experiments, vanadate, which abolishes nonmitochondrial Ca^{2+} uptake [29], was added after Ca^{2+} uptake was complete and before IP_3 was added. This was done for the following reason: In the absence of vanadate, IP_3-induced Ca^{2+} release in both permeabilized cells and homogenate is generally followed by Ca^{2+} reuptake [28]. Therefore, in leaky cells, in contrast to the total homogenate, some of the Ca^{2+} released by IP_3 might be taken up again before being released to the incubation medium and before being measured by the Ca^{2+}-sensitive electrode. This would result in

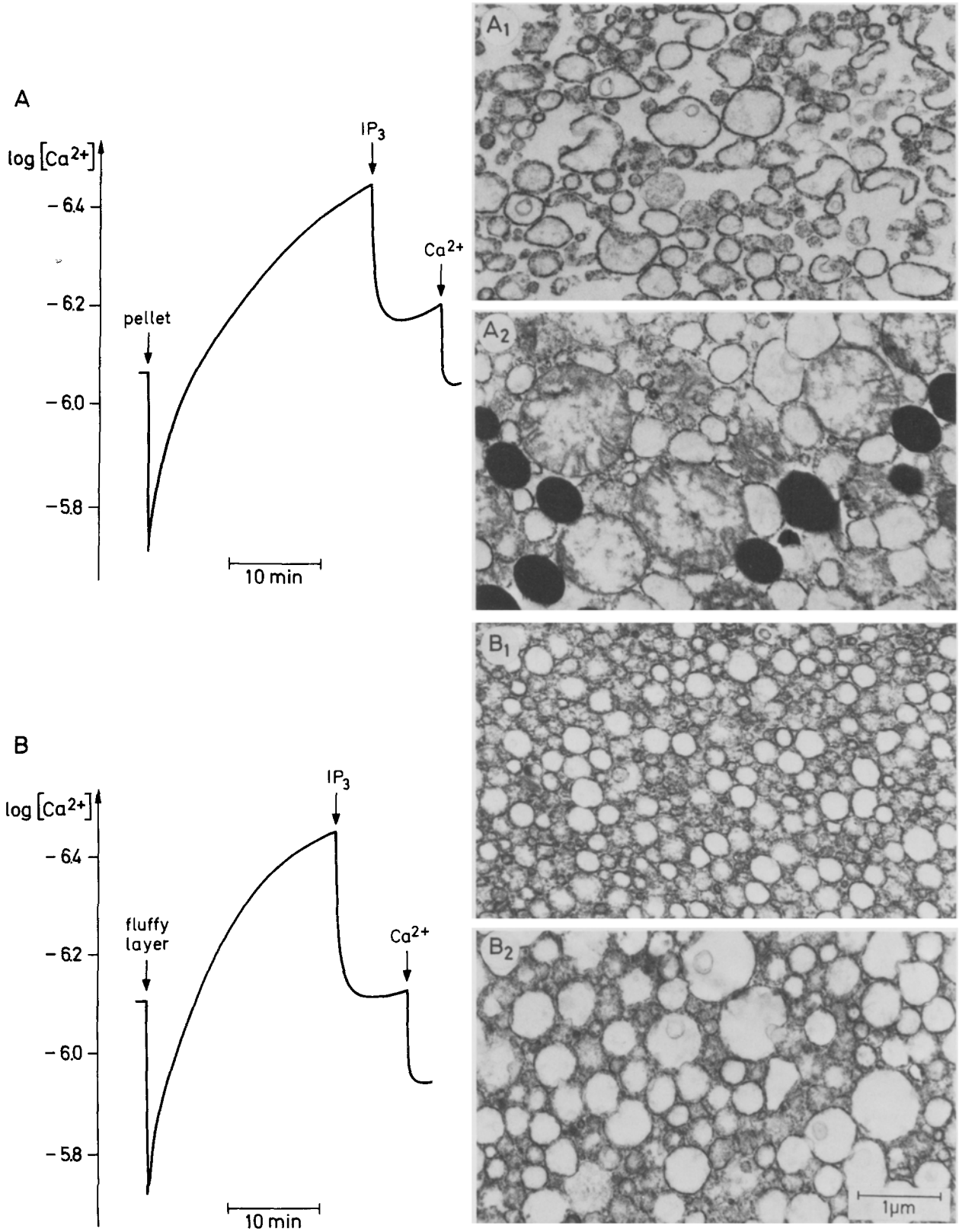
A
log [Ca2+]
-6.4
-6.2
-6.0
-5.8
IP3
Ca2+
pellet
10 min
A1
A2
B
log [Ca2+]
-6.4
-6.2
-6.0
-5.8
IP3
Ca2+
fluffy layer
10 min
B1
B2
1µm

Table 1. Differential centrifugation at 11,000 × *g*

	$(Na^+ + K^+)$-ATPase	Alkaline phosphatase	Cytochrome *c* oxidase	Glutamate dehydrogenase	Ribonucleic acid (RNA)	NADPH-cyt-*c* reductase	IP_3-induced Ca^{2+} release
Homogenate	27 ± 2	28 ± 6	132 ± 24	375 ± 32	152 ± 17	4.2 ± 0.5	2.7 ± 0.5
Pellet	22 ± 3	25 ± 1	279 ± 64	418 ± 32	85 ± 21	2.9 ± 0.3	2.8 ± 0.4
Fluffy layer	43 ± 7	34 ± 13	16 ± 3	34 ± 3	194 ± 25	4.3 ± 0.6	2.8 ± 0.3

Total homogenate of rat pancreas (= homogenate, 10.6 ± 2.4 mg protein/ml) was centrifuged at 11,000 × *g* for 15 min and a loosely attached layer on the pellet (= fluffy layer) separated from the firm pellet (= pellet) as described in Materials and Methods. Enzyme activities are expressed in mU/mg protein, RNA in μg/mg protein and Ca^{2+} release in nmol/mg protein. Results are given as mean ± SE of three different preparations.

an underestimate of the effect of IP_3 on cells. Indeed, in the absence of vanadate, an apparent 30% increase of the IP_3-induced Ca^{2+} release was observed in the homogenate as compared to leaky cells.

The effect in the homogenate, like that in permeabilized cells, proved sufficiently stable during storage on ice and with respect to mechanical alteration to allow short, further fractionation procedures to be carried out. After storage on ice for 4 hr, 82% of the effect was recovered and after 5.5 hr, 70%. When the homogenate was centrifuged at 27,000 × *g* for 15 min and the pellet resuspended in its own total supernatant, IP_3-induced Ca^{2+} release was unaltered. Similarly, vigorous vortexing for 10 min did not alter the IP_3-induced Ca^{2+} release.

Differential Centrifugation at 11,000 × *g*

It was the general strategy of the fractionation studies reported below to produce significant differences for one or more subcellular components by a single and short fractionation step, and to correlate the IP_3-induced Ca^{2+} release with marker enzymes and electron microscopy. This approach had to be chosen instead of the preparation of pure organelle fractions, because lengthy preparations resulted in too pronounced a loss in IP_3 "sensitivity" of the tissue to allow valid conclusions (*see* Discussion). We attempted to treat all fractions that were compared as similarly as possible. If the supernatant of a certain separation step was too dilute to enable measurement of IP_3-induced Ca^{2+} release directly and therefore had to be centrifuged down, the corresponding pellet was also resuspended and spun down in the same rotor. Different principles of separation were used throughout to avoid selective inactivation of IP_3-induced Ca^{2+} release by any particular method.

To obtain a rough separation of mitochondria, zymogen granules and nuclei on the one hand, and microsomes on the other, a total homogenate was centrifuged at 11,000 × *g* for 15 min. A loosely attached layer on the pellet ("fluffy layer") was separated from the firm pellet ("pellet"). IP_3-induced Ca^{2+} release was determined in both fractions as described for cells or homogenate. The distribution of subcellular organelles was assessed by marker enzymes and electron microscopy. Cytochrome *c* oxidase and glutamate dehydrogenase were measured as mitochondrial markers, $(Na^+ + K^+)$-ATPase and alkaline phosphatase as markers for plasma membranes and ribonucleic acid (RNA) and NADPH cytochrome *c* reductase as markers for endoplasmic reticulum. No marker enzyme for zymogen granules or nuclei was used. Both structures can already be pelleted almost quantitatively by differential centrifugation at the much lower centrifugal force of 1000 × *g* under comparable conditions [20], so that electron microscopy seemed sufficient to confirm that indeed no nuclei or zymogen granules are contained in the fluffy layer.

As shown in Fig. 2 and Table 1 differential centrifugation at 11,000 × *g* resulted in the accumulation of nearly all mitochondria, zymogen granules and nuclei in the pellet as opposed to the fluffy layer fraction. In contrast, there were only small differences between the two fractions for the microsomal markers.

Fig. 2 (*facing page*). Comparison of IP_3-induced Ca^{2+} release in "pellet" (*A*) and "fluffy layer" (*B*). Pellet and fluffy layer were prepared from total homogenate by centrifugation at 11,000 × *g*. IP_3-induced Ca^{2+} release was determined in the standard medium as described in Materials and Methods. Where indicated inositol-1,4,5-trisphosphate (IP_3) to a final concentration of 2.5 μmol/liter and Ca^{2+} (10 nmol) were added. For electron microscopy the fractions were pelleted at 18,000 × *g* and fixed as pellets. Representative micrographs of the upper (A_1,B_1) and basal (A_2,B_2) layer of each fraction are shown (×15,000). Typical for 3 experiments

Table 2. Subfractionation of 11,000 × *g* pellet at 7000 × *g*

	$(Na^+ + K^+)$-ATPase	Alkaline phosphatase	Cytochrome *c* oxidase	Glutamate dehydrogenase	Ribonucleic acid (RNA)	NADPH-cyt-*c* reductase	IP_3-induced Ca^{2+} release
Pellet	28 ± 3	25 ± 6	326 ± 47	568 ± 54	132 ± 19	5.1 ± 0.4	3.1 ± 0.8
Heavy pellet	41 ± 4	34 ± 6	386 ± 49	1020 ± 147	94 ± 20	5.6 ± 0.4	1.4 ± 0.4
Light pellet	26 ± 3	19 ± 3	79 ± 7	132 ± 24	363 ± 12	8.6 ± 1.6	3.2 ± 0.4

The firm pellet obtained by centrifugation at 11,000 × *g* (*see* Table 1) was suspended (4.4 ± 0.2 mg protein/ml) and centrifuged for 15 min at 7000 × *g*. Both the supernatant (= light pellet) and the pellet (= heavy pellet) after resuspension were spun down at 18,000 × *g* for 15 min as described in Materials and Methods. Enzyme activities are expressed in mU/mg protein, RNA in μg/mg protein and Ca^{2+} release in nmol/mg protein. Results are given as mean ± SE of five different preparations.

The fluffy layer appeared to be an essentially pure microsomal fraction with a small mitochondrial contamination as judged by both electron microscopy and enzyme markers. The mitochondrial markers cytochrome *c* oxidase and glutamate dehydrogenase were reduced in the fluffy layer by a factor of 8 and 11, respectively, compared to total homogenate and by a factor of 17 and 12, respectively, compared to the pellet fraction (Table 1). No nuclei or zymogen granules could be detected in the fluffy layer by electron microscopy.

The pellet fraction was heterogeneous, containing, in addition to a considerable amount of rough endoplasmic reticulum and plasma membrane, about 95% of the mitochondria and all zymogen granules and nuclei (Fig. 2, Table 1).

Although practically all nuclei, zymogen granules and mitochondria were localized in the pellet fraction, IP_3-induced Ca^{2+} release was the same in pellet and fluffy layer (Fig. 2, Table 1). This result strongly suggests that IP_3 does not release Ca^{2+} from any one of these structures alone. A pronounced and selective damage of IP_3 sensitivity of the pellet as opposed to the fluffy layer can be excluded by the observation that about 70% of the IP_3-induced Ca^{2+} release observed in the homogenate was recovered in the two fractions.

The small differences for the microsomal markers between the two fractions do not allow clear conclusions to be drawn. The specific activities of the plasma membrane marker enzymes, $(Na^+ + K^+)$-ATPase and alkaline phosphatase, and the endoplasmic reticulum markers, RNA and NADPH cytochrome *c* reductase, were between 1.4- and 2.3-fold greater in the fluffy layer compared to the pellet.

It therefore seems clear that the IP_3-induced Ca^{2+} release in the fluffy layer was due to a microsomal structure rather than to mitochondria, zymogen granules or nuclei. In order to determine whether the IP_3-induced Ca^{2+} release observed in the pellet can also be accounted for by the microsomal structures present in this fraction, it is necessary to further separate the bulk of the endoplasmic reticulum from mitochondria, zymogen granules and nuclei by a subfractionation of the pellet.

Subfractionation of 11,000 × *g* Pellet at 7000 × *g*

Differential centrifugation of the diluted pellet at 7000 × *g* was used to separate the endoplasmic reticulum still present in the 11,000 × *g* pellet from mitochondria and zymogen granules. A "light pellet" and a "heavy pellet" were obtained. As for pellet and fluffy layer (Table 1), IP_3-induced Ca^{2+} release and marker enzymes were determined in both fractions and electron microscopy was performed.

A shown in Table 2 and Fig. 3, the light pellet was considerably enriched in endoplasmic reticulum, while the bulk of mitochondria were in the heavy pellet. The endoplasmic reticulum markers RNA and NADPH cytochrome *c* reductase were enriched 3.9- and 1.5-fold, respectively, in the light pellet while the mitochondrial markers cytochrome *c* oxidase and glutamate dehydrogenase were enriched 4.9-fold and 7.7-fold, respectively, in the heavy pellet (Table 2). No zymogen granules or nuclei could be detected in the light pellet by electron microscopy (Fig. 3). Only small differences between light and heavy pellets for the plasma membrane markers were obtained.

IP_3-induced Ca^{2+} release was enriched 2.3-fold in the light pellet parallel to the endoplasmic reticulum markers and in opposition to the mitochondria (Table 2) and zymogen granules (Fig. 3). When the individual data for heavy and light pellets of all five separate preparations were correlated, a clear positive correlation of IP_3-induced Ca^{2+} release to RNA ($r = 0.82$) and NADPH cytochrome *c* reductase ($r = 0.71$) was found. There was a negative correlation with the mitochondrial markers cytochrome *c*

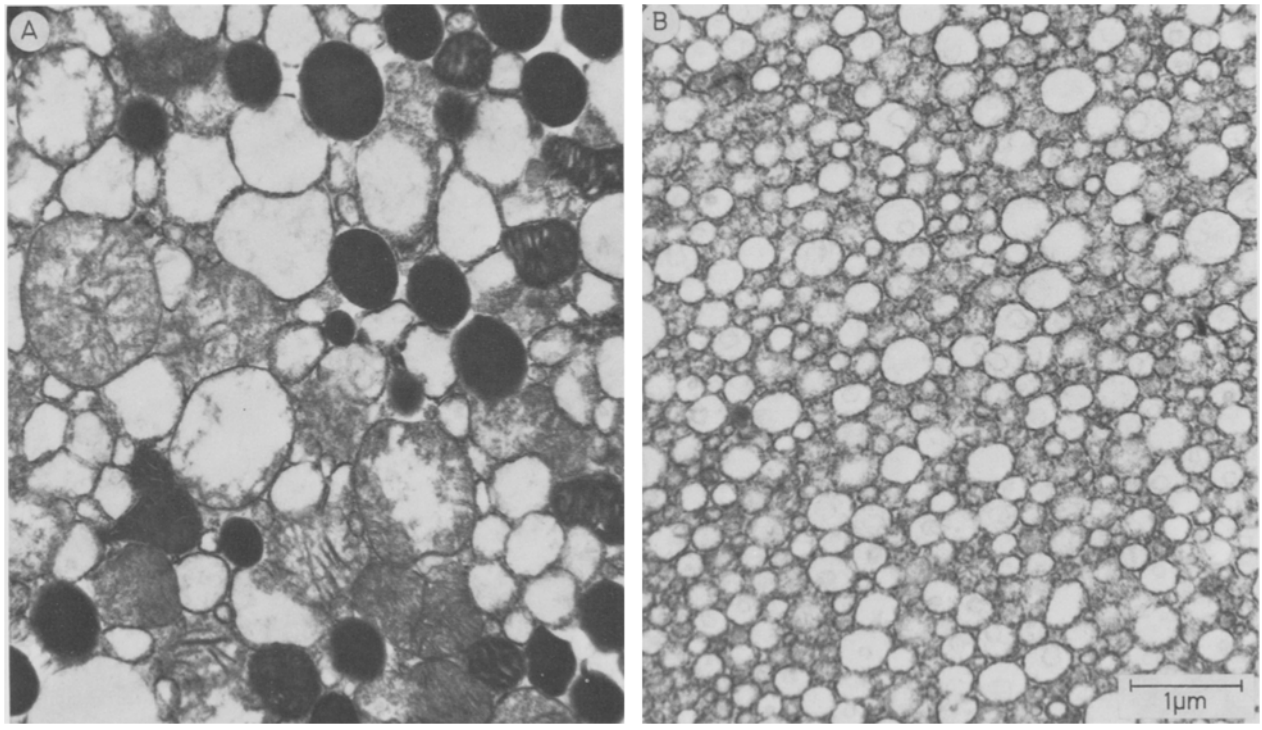

Fig. 3. Electron micrographs of "heavy pellet" (*A*) and "light pellet" (*B*). Heavy pellet and light pellet were obtained by differential centrifugation at 7000 × *g* of "pellet." For electron microscopy fractions were spun at 18,000 × *g* and fixed as pellets. Representative micrographs of the basal layer of both fractions are shown (×15,000)

oxidase ($r = -0.76$) and glutamate dehydrogenase ($r = -0.84$) (Table 5). When the sum of both IP_3-induced Ca^{2+} release and marker enzyme activity measured in heavy and light pellets for each experiment was standardized to 1, an excellent correlation with RNA ($r = 0.96$), a moderate positive correlation with NADPH cytochrome *c* reductase ($r = 0.63$), and a clear negative correlation to mitochondrial markers was calculated ($r = -0.92$, Table 5). This result suggests that the IP_3-induced Ca^{2+} release measured in the 11,000 × *g* pellet is, as in the fluffy layer of the 11,000 × *g* centrifugation (Table 1), due to endoplasmic reticulum.

In order to further establish or disprove the positive correlation of IP_3-induced Ca^{2+} release and endoplasmic reticulum markers, we attempted in the following data to prepare fractions with varying endoplasmic reticulum content using separation methods based upon different principles. In addition, we tried to separate endoplasmic reticulum from plasma membranes, as thus far the differences in the plasma membrane content of the fractions analyzed were too small to exclude definitely plasma membranes as an IP_3-sensitive store. Two methods based on two different principles were used: sucrose-density centrifugation and precipitation with high $MgCl_2$ concentrations.

Sucrose-Density Centrifugation

The fluffy layer obtained by differential centrifugation at 11,000 × *g* of a total homogenate was brought to 1.25 M sucrose, layered on top of a cushion of 2 M sucrose, and overlayered with 0.3 M sucrose. After centrifugation at 150,000 × *g* for 90 min, the membranes banding at the interfaces 0.3 M/1.25 M sucrose (=S1) and 1.25 M/2 M sucrose (=S2) were recovered and analyzed. IP_3-induced Ca^{2+} release and plasma membrane and endoplasmic reticulum markers were determined as before.

As shown in Table 3, the plasma membrane markers ($Na^+ + K^+$)-ATPase and alkaline phosphatase were enriched 12- and 15-fold, respectively, in S1 compared to S2 while the endoplasmic reticulum markers RNA and NADPH cytochrome *c* reductase were enriched 5.7- and 4.7-fold respectively, in S2 compared to S1.

IP_3-induced Ca^{2+} release was enriched 5.9-fold in S2 compared to S1 parallel to the endoplasmic reticulum markers and against the marked differences of plasma membrane markers. The following correlation coefficients with IP_3-induced Ca^{2+} release were calculated: RNA 0.79, NADPH cytochrome *c* reductase 0.68, ($Na^+ + K^+$)-ATPase −0.72, alkaline phosphatase −0.53 (Table 5). When the sum of IP_3-

Table 3. Separation of plasma membranes and endoplasmic reticulum by centrifugation on a discontinuous sucrose density gradient

	$(Na^+ + K^+)$-ATPase	Alkaline phosphatase	Ribonucleic acid (RNA)	NADPH-cyt-*c* reductase	IP_3-induced Ca^{2+} release
Fluffy layer	38 ± 1	36 ± 6	309 ± 31	6.1 ± 0.5	3.7 ± 1.0
S1	181 ± 21	161 ± 44	77 ± 15	2.3 ± 0.7	0.7 ± 0.4
S2	15 ± 6	11 ± 1	436 ± 23	10.9 ± 0.9	4.1 ± 1.6

The fluffy layer, obtained by differential centrifugation at 11,000 × *g* of total homogenate (*see* Table 1), was placed on a discontinuous sucrose gradient as described in Materials and Methods. The bands at the interfaces 0.3 M/1.25 M sucrose (=S1) and 1.25 M/3 M sucrose (=S2) were analyzed. Enzyme activities are expressed in mU/mg protein, RNA in μg/mg protein and Ca^{2+} release in nmol/mg protein. Results are given as mean ± SE of three different preparations.

Table 4. Separation of plasma membranes and endoplasmic reticulum by Mg^{2+} precipitation

	$(Na^+ + K^+)$-ATPase	Alkaline phosphatase	Ribonucleic acid (RNA)	NADPH-cyt-*c* reductase	IP_3-induced Ca^{2+} release
Homogenate	30 ± 1	28 ± 2	188 ± 9	4.4 ± 0.5	4.0 ± 1.0
Supernatant	90 ± 16	45 ± 13	132 ± 20	2.1 ± 0.3	1.0 ± 0.2
Precipitate	22 ± 1	33 ± 2	263 ± 9	6.4 ± 0.2	3.1 ± 0.7

Total homogenate from rat pancreas (3.4 ± 0.4 mg protein/ml) was treated with 10 mmol/liter $MgCl_2$ for 15 min to form a precipitate enriched in endoplasmic reticulum (= precipitate) and a supernatant enriched in plasma membranes (= supernatant) as described in Materials and Methods. Enzyme activities are expressed in mU/mg protein, RNA in μg/mg protein and Ca^{2+} release in nmol/mg protein. Results are given as mean ± SE of three different preparations.

Table 5. Correlation of marker enzymes and IP_3-induced Ca^{2+} release

	$(Na^+ + K^+)$-ATPase	Alkaline phosphatase	Cytochrome *c* oxidase	Glutamate dehydrogenase	Ribonucleic acid (RNA)	NADPH-cyt-*c* reductase
Differential centrifugation	0.48	0.27	−0.30	−0.26	0.27	0.78
at 11,000 × *g*	(−0.30)	(−0.50)	(0.21)	(−0.23)	(−0.45)	(−0.38)
Differential centrifugation	−0.53	−0.55	−0.92	−0.92	0.96	0.63
at 7000 × *g*	(−0.49)	(−0.27)	(−0.76)	(−0.84)	(0.82)	(0.71)
Sucrose density	−0.98	−0.99	—	—	1.00	0.98
centrifugation	(−0.72)	(−0.53)	—	—	(0.79)	(0.68)
Mg^{2+} precipitation	−0.97	−0.71	—	—	0.91	0.90
	(−0.68)	(−0.26)	—	—	(0.87)	(0.84)

Marker enzyme activity and RNA content were correlated with IP_3-induced Ca^{2+} release for the individual data obtained for "pellet" and "fluffy layer" (11,000 × *g* centrifugation), "light pellet" and "heavy pellet" (7000 × *g* centrifugation), "S1" and "S2" (sucrose density centrifugation) and "supernatant" and "precipitate" ($MgCl_2$ precipitation). Results were standardized by setting the sum of the activity of marker enzymes, RNA content and IP_3-induced Ca^{2+} release for each pair of fractions to 1 for each experiment. The correlation coefficients for the nonstandardized data are given in brackets.

induced Ca^{2+} release and marker enzyme activity in S1 and S2 for each preparation was standardized to 1, correlation was considerably improved: for the endoplasmic reticulum markers the positive correlation was increased to 1.00 (RNA) and 0.98 (NADPH cytochrome *c* reductase), for the plasma membrane markers a negative correlation of −0.98 ($Na^+ + K^+$-ATPase) and −0.99 (alkaline phosphatase) were calculated (Table 5).

$MgCl_2$ Precipitation

A method originally described for the purification of microvilli in kidney [9] was used to separate endoplasmic reticulum from plasma membranes. Although smaller enrichments for plasma membranes and endoplasmic reticulum were obtained with this separation procedure compared to sucrose density centrifugation, it seemed important to confirm the

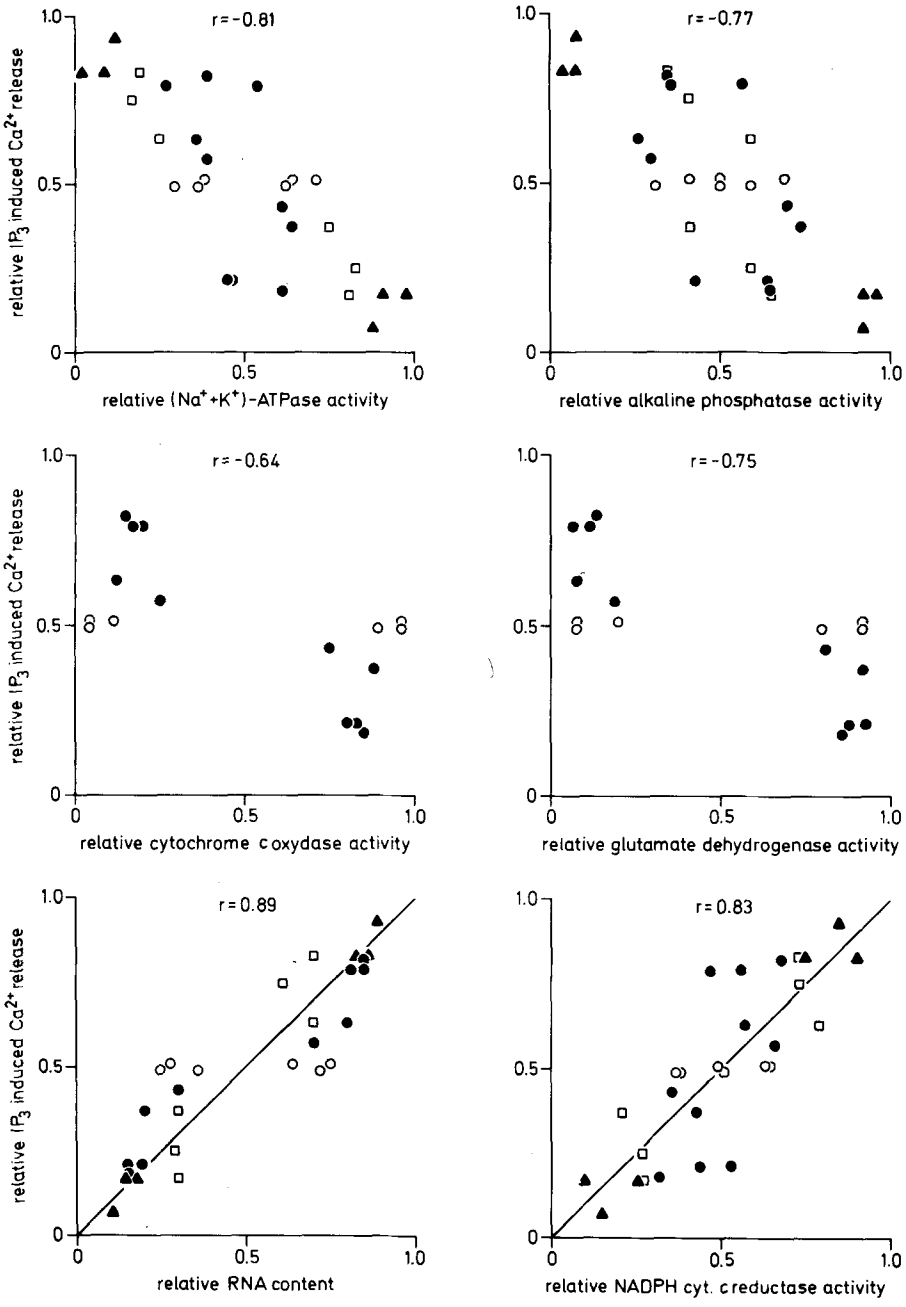

Fig. 4. Correlation of marker enzymes and IP_3-induced Ca^{2+} release. Individual data for IP_3-induced Ca^{2+} release are plotted against marker enzyme activity and RNA content of the same fraction for: "pellet" and "fluffy layer" (○), "heavy pellet" and "light pellet" (●), "S1" and "S2" (▲) and "supernatant" and "precipitate" (□). Data were standardized as described in the legend to Table 5. r = correlation coefficient

sucrose-data by a method based on a completely different principle. Total homogenate was treated with 11 mmol/liter $MgCl_2$ for 15 min and the precipitate formed was separated from the supernatant by low-speed centrifugation.

As shown in Table 4, the plasma membrane markers ($Na^+ + K^+$)-ATPase and alkaline phosphatase were enriched 4.1- and 1.4-fold, respectively, in the supernatant, and the endoplasmic reticulum markers RNA and NADPH cytochrome *c* reductase 2.0- and 3.0-fold, respectively, in the precipitate.

IP_3-induced Ca^{2+} release was enriched 3.1-fold in the precipitate, again parallel to the endoplasmic reticulum markers. A high positive correlation of 0.87 with RNA and 0.84 with NADPH cytochrome *c* reductase was calculated (Table 5).

In Fig. 4 the individual data for all four different separation methods are summarized. An overall positive correlation of IP_3-induced Ca^{2+} release and the endoplasmic reticulum markers RNA ($r = 0.89$) and NADPH cytochrome *c* reductase ($r = 0.83$) was calculated. In contrast, there was a negative correlation with the plasma membrane markers ($Na^+ + K^+$)-ATPase ($r = -0.81$) and alkaline phosphatase ($r = -0.77$) and the mitochondrial markers cytochrome *c* oxidase ($r = -0.64$) and glutamate dehydrogenase ($r = -0.75$) when all data were considered.

Discussion

Discussion of Methodological Approach

In order to localize the intracellular calcium store sensitive to IP_3, isolated subcellular fractions were prepared and IP_3-induced Ca^{2+} release was correlated with their content of subcellular organelles. To obtain reliable results with this approach two conditions are essential. 1) The content of the different organelles in the fractions analyzed must be assessed unambiguously with specific marker enzymes or electron microscopy. 2) The sensitivity to IP_3 must remain unchanged during the isolation procedures, or at least be reduced to a similar extent in fractions that are compared. The term "sensitivity" in this context is chosen to denote anything that might change the amount of Ca^{2+} released by IP_3 from the same amount of organelle present, e.g. decreases in affinity of some kind of "IP_3 receptor" due to storage or mechanical alteration, or increased leakiness of vesicles, which will result in smaller Ca^{2+} accumulation and subsequent release. Considerable experimental effort was applied to meet these conditions. Marker enzymes and other markers for plasma membrane, mitochondria and endoplasmic reticulum were generally determined in pairs with different localization on the same organelle. For mitochondria the matrix enzyme glutamate dehydrogenase and the membrane-bound enzyme cytochrome *c* oxidase were measured. A parallel distribution of the two markers indicates that intact mitochondria are present rather than isolated mitochondrial membranes or released marker enzymes. For endoplasmic reticulum RNA was determined as a marker of rough endoplasmic reticulum (which constitutes more than 90% of the endoplasmic reticulum present in exocrine pancreas). To include also the smooth endoplasmic reticulum, NADPH cytochrome *c* reductase, generally assumed to be a specific endoplasmic reticulum marker, was also measured. $(Na^+ + K^+)$-ATPase and alkaline phosphatase were determined as markers for plasma membranes. Furthermore, the distribution of mitochondria and rough endoplasmic reticulum was determined by electron microscopy. Electron microscopy is of little value to assess the distribution of plasma membranes as even in enriched fraction there is only a small percentage of plasma membranes which cannot be distinguished unambiguously from smooth endoplasmic reticulum or isolated mitochondrial membranes. The distribution of zymogen granules and nuclei was determined by electron microscopy only. This seemed sufficient for the following reasons: For the scope of this study and the conclusions reached, it was only necessary to show that the pellets obtained by differential centrifugation at $11{,}000 \times g$ and $7000 \times g$ contained about 90% or more of zymogen granules and nuclei. Both structures can be pelleted almost quantitatively at the much lower centrifugal force of $1000 \times g$ under similar conditions [20]. Moreover, zymogen granules and nuclei are considerably heavier than mitochondria and will be even more enriched in the heavier fractions. Since the mitochondria were already enriched sufficiently to be excluded as an IP_3-sensitive pool, zymogen granules and nuclei appear even less likely from the fractionation data. Finally, although electron microscopy can scarcely distinguish small differences in organelle content, it can quite clearly establish the absence of a given organelle in a certain fraction. Fractions were generally fixed as pellets. Thus zymogen granules and nuclei were always concentrated in the basal regions and could be searched for selectively.

In general, closely comparable results for the different markers of the same structure and electron-microscopical analysis were obtained (Tables 1–4, Figs. 2, 3). The parallelism of the results obtained for each pair of markers is also reflected by the very similar correlation coefficients to IP_3-induced Ca^{2+} release (Table 5). The only exception was some discrepancy for the plasma membrane markers, $(Na^+ + K^+)$-ATPase and alkaline phosphatase, in the $MgCl_2$ precipitation: although both markers were enriched in the supernatant, the enrichment of the $(Na^+ + K^+)$-ATPase was much higher, probably due to a selective action of high $MgCl_2$ concentrations or luminal and basolateral plasma membranes [9]. There was some tendency of RNA to be enriched to a greater extent in the lighter fractions than NADPH cytochrome *c* reductase (Tables 1–3), which might be explained by the presence of free ribosomes.

Only a small, if any, reduction of IP_3 "sensitivity" was observed due to homogenization or a single differential centrifugation step (*see* Results, Table 1). Subsequent centrifugations, however, led to some reduction of IP_3 sensitivity: IP_3-induced Ca^{2+} release was generally enriched to a smaller degree than the endoplasmic reticulum markers when the isolated fractions were compared to their respective starting material (Tables 2–4). As no sufficient separation of endoplasmic reticulum from both plasma membranes and mitochondria could be obtained by a single differential centrifugation step, this reduction of sensitivity had to be accepted. Great care was therefore taken to treat all fractions to be compared as similar as possible regarding centrifugation steps, time and conditions of storage and analysis of IP_3-induced Ca^{2+} release (*see* Results). Further-

more, only large differences of organelle content in the compared fractions (10 : 1 or above (Tables 1, 3)) were accepted to exclude a certain organelle. Thus, even if some selective damage had occurred in one fraction, the general conclusions would remain unaltered. Finally, a variety of different separation procedures based upon three different principles were used which gave consistent results (Fig. 4, Table 5).

Localization of IP_3-Sensitive Calcium Store

It seems valid to conclude from our data that IP_3 releases Ca^{2+} from the endoplasmic reticulum. In three different separation procedures (7000 × *g* differential centrifugation, sucrose density centrifugation, $MgCl_2$ precipitation) high positive correlations of IP_3-induced Ca^{2+} release with the endoplasmic reticulum markers RNA and NADPH cytochrome *c* reductase were obtained (Table 5). Correlation of IP_3-induced Ca^{2+} release and the endoplasmic reticulum markers was rather low for the differential centrifugation at 11,000 × *g* (Table 1). It therefore is important to consider that differential centrifugation separates vesicles of the same density according to size, the bigger vesicles being concentrated in the heavier fractions (Fig. 2). Assuming that the same inside/outside concentration gradient of Ca^{2+} can be maintained in vesicles of different size, the bigger vesicles can accumulate—and probably release—more Ca^{2+} per unit membrane area and amount of membrane marker than the smaller vesicles. This might lead to an apparent enrichment of IP_3-induced Ca^{2+} release relative to markers in the heavier fraction (Table 1). When a starting material of more homogeneously sized vesicles was used (7000 × *g* centrifugation) or a separation principle not dependent on vesicle size employed (sucrose density centrifugation, $MgCl_2$ precipitation) good correlation with the endoplasmic reticulum markers was obtained (Table 5). For all individual data obtained in 14 completely separate fractionations using four different procedures an overall correlation of $r = 0.89$ with RNA and $r = 0.83$ with NADPH cytochrome *c* reductase was calculated (Fig. 4).

In contrast, there was either no or even a negative correlation of IP_3-induced Ca^{2+} release with the mitochondrial markers cytochrome *c* oxidase and glutamate dehydrogenase (Table 5, Fig. 4), suggesting that IP_3 does not release Ca^{2+} from mitochondria. This confirms our previous results obtained with inhibitors in leaky isolated cells [28]: when the mitochondrial calcium pool was depleted by incubation of the cells in the presence of the mitochondrial inhibitors antimycin A and oligomycin, IP_3-induced Ca^{2+} release was unaltered. In contrast, vanadate, which does not interfere with mitochondrial Ca^{2+} uptake but abolishes all nonmitochondrial uptake [29], produced a clear inhibition [28].

There was also either no or a negative correlation with the plasma membrane markers (Na^+ + K^+)-ATPase and alkaline phosphatase (Table 5, Fig. 4). In sucrose-density centrifugation the plasma membrane markers were enriched 12- to 15-fold in the lighter fraction (S1) while IP_3-induced Ca^{2+} release was enriched about sixfold in the heavier fraction (S2) (Table 3). This result, which was confirmed by $MgCl_2$ precipitation, seems to exclude the plasma membrane as an IP_3-sensitive store.

No correlation coefficients for zymogen granules or nuclei could be calculated as their distribution was assessed qualitatively by electron microscopy. However, as IP_3-induced Ca^{2+} release was the same or about threefold enriched in fractions containing no zymogen granules or nuclei compared to fractions that contained all of them (Figs. 2, 3; Tables 1, 2) this issue seems sufficiently clarified.

IP_3 as the Second Messenger of the Muscarinic Receptor

It also seems necessary to interpret our results in the light of the probable second messenger function of IP_3 (*see* Introduction). It certainly fits nicely with the idea of a second messenger, that Ca^{2+} release can be observed in isolated fractions (Fig. 2) and that the full effect can be recovered in total homogenate (Fig. 1). Neither we nor to our knowledge anyone else has ever published Ca^{2+} release with a secretagogue in total homogenate or isolated fractions. This observation should finally rule out the possibility that IP_3 might release Ca^{2+} by acting as an acetylcholine analogue on the muscarinic receptor.

Based on the assumption that IP_3 and muscarinic agonists release Ca^{2+} from the same intracellular calcium store [28], our results suggest that secretagogues release Ca^{2+} from endoplasmic reticulum. In other words, in close analogy to the situation in skeletal muscle [12], the endoplasmic reticulum in exocrine pancreas would not only function to regulate cytosolic Ca^{2+} concentration [4, 29] but would also release Ca^{2+} to trigger enzyme secretion. This conclusion is at variance with the hypothesis previously favored by us, namely that the "trigger pool" is closely associated with the plasma membrane [26]. The main reasons to favor a plasma membrane site were the following:

1. Secretagogue-induced Ca^{2+} release is still observed in permeabilized cells, where a second mes-

senger on stimulation should be released to the incubation medium rather than acting on an intracellular trigger pool [29]. This observation thus indicated that no second messenger at all was involved and the Ca^{2+} pool in direct contact to the plasma membrane receptor. However, it does not exclude a second messenger function of IP_3 for several reasons. Also in permeabilized cells IP_3 will not be released instantaneously to the incubation medium and sufficient messenger concentrations might build up for a short period. No calcium release by secretagogues was ever observed in total homogenate or isolated plasma membrane preparations. To obtain secretagogue-induced Ca^{2+} release in permeabilized cells, high concentrations of the secretagogues had to be used [29]. As there is a close coupling of receptor occupation and phospholipid breakdown, probably much higher amounts of messenger are produced as compared to the *in vivo* situation. Finally, the endoplasmic reticulum in pancreatic acinar cells is in close proximity to the basolateral plasma membrane so that only short distances have to be overcome.

2. Substances that do not enter the cells such as La^{3+} are able to abolish secretagogue-induced Ca^{2+} release [32]. This observation does of course "not exclude the possibility that La^{3+} could act on the plasma membrane to interfere with the generation of a signal that controls a trigger pool located elsewhere" [32].

The data presented above do not exclude that after stimulation some small amount of Ca^{2+} might be released from the plasma membrane by a mechanism not mediated by IP_3, but they clearly suggest that IP_3 releases Ca^{2+} from endoplasmic reticulum.

The technical assistance of Frau B. Moewes and Frau L. Eckhardt is gratefully acknowledged. H.S. was supported by the Deutsche Forschungsgemeinschaft (grant No. Schu 429/2-1).

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Received 5 March 1948; revised 3 May 1984