Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7777–7781, August 1994
Cell Biology

Localization of inositol trisphosphate receptor subtype 3 to insulin and somatostatin secretory granules and regulation of expression in islets and insulinoma cells

(insulin secretion/membrane protein/calcium channel/glucose/diabetes mellitus)

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Contributed by Hewson Swift, May 2, 1994

ABSTRACT **Calcium ions play a central role in stimulus–secretion coupling in pancreatic β cells, and an elevation of cytosolic Ca^{2+} levels is necessary for insulin secretion. Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+} stores in the β cell by binding to specific receptors that are ligand-activated Ca^{2+} channels. The inositol trisphosphate receptors comprise a family of structurally related proteins with distinct but overlapping tissue distributions. Previous studies indicated that the predominant inositol trisphosphate receptor subtype expressed in rat pancreatic islets was the protein designated IP3R-3. We have confirmed the expression of IP3R-3 in pancreatic islets by immunohistocytochemistry and localized this protein to the secretory granules of insulin-secreting β cells and somatostatin-secreting δ cells by immunogold electron microscopy. Secretory granules contain high levels of Ca^{2+}, and the presence of IP3R-3 in the granule provides a mechanism for mobilizing granule Ca^{2+} stores in response to glucose and/or hormones. The release of Ca^{2+} from granule stores would increase the Ca^{2+} concentration in the surrounding cytoplasm and promote rapid exocytosis of granules, especially those granules in close proximity to the plasma membrane. The levels of IP3R-3 were increased in pancreatic islets of diabetic rats and rats that had been refed after a period of fasting. They were also increased in rat insulinoma RINm5F cells cultured in 25 mM glucose compared with cells cultured in 5 mM glucose. The localization of IP3R-3 to secretory granules of insulin-secreting β cells and somatostatin-secreting δ cells suggests that granule Ca^{2+} stores actively participate in the secretory process and that their release is regulated by inositol 1,4,5-trisphosphate. The regulation of IP3R-3 levels by glucose, diabetes, and refeeding may allow the β cell to adjust the insulin secretory response to changing physiological conditions.**

An increase in intracellular Ca^{2+} concentration is required for insulin secretion in pancreatic β cells (1–3), and the oscillatory electrical activity and correlated pulsatile release of insulin observed in β cells in response to glucose appear to depend upon a close interplay between plasma membrane ion channels and intracellular inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores (4). Secretory granules contain high levels of a readily mobilizable pool of Ca^{2+} whose release is promoted by glucose-induced depolarization of the β-cell plasma membrane, implying that secretory granules may actively participate in the regulation of the Ca^{2+} concentration in the surrounding cytoplasm (5–10). However, the mechanisms regulating the release of granular Ca^{2+} stores in β cells are unknown.

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IP_3 plays an important role in the regulation of insulin secretion. The cellular actions of IP_3 are mediated by specific receptors that function as ligand-activated, Ca^{2+}-selective channels (11). They have been identified in the endoplasmic reticulum (12), nucleus (13), plasma membrane (14, 15), plasma membrane-derived vesicles (16, 17), nerve terminals (18), and chromaffin granules (19). Molecular cloning studies have shown that the IP_3 receptors comprise a family of structurally related proteins. cDNAs encoding four different subtypes (IP3R-1 to -4) have been isolated, and each subtype has a distinct tissue distribution (11, 20, 21). IP_3 receptor subtype 3 (IP3R-3) is expressed at high levels in rat insulinoma cells and is the predominant IP_3 receptor subtype expressed in normal adult rat pancreatic islets (22). The present study was undertaken to define the subcellular localization of IP3R-3 in islets and to determine whether its expression is regulated. These studies show that IP3R-3 is present in the secretory granules of insulin-secreting β cells and somatostatin-secreting δ cells. The levels of IP3R-3 are increased in islets in response to diabetes and fasting/refeeding and in cultured insulinoma cells by glucose.

MATERIALS AND METHODS

Antisera. The rabbit anti-rat IP3R-3 polyclonal antibody IPR3AB3 was directed to a peptide corresponding to the carboxyl-terminal 15 amino acids, residues 2656–2670 (RQRLGFVDVQNCMSR), of rat IP3R-3 (22). Antibody AP45 was raised to a peptide corresponding to amino acids 2432–2444 (VSEVSVPEILEED) of rat IP3R-3. Both antibodies were affinity purified by chromatography on a peptide-coupled Sepharose column (CNBr-activated Sepharose 4B, Pharmacia LKB) as described (23). The sequences of rat IP3R-1, -2, and -3 are unique in the regions from which the peptides were selected for antibody production (22).

Isolation of Pancreatic Islets. Pancreatic islets were isolated from adult male Wistar rats and Zucker diabetic fatty male rats (ZDF/Gmi-*fa/fa*) (Genetic Models, Indianapolis), a rat model of non-insulin-dependent (type 2) diabetes mellitus, and lean littermates (+/+ and +/*fa*) by the collagenase method of Lacy and Kostianovsky (24). Collagenase P was purchased from Boehringer Mannheim. Isolated islets were collected by centrifugation through a discontinuous gradient of Ficoll (Sigma). Harvested islets were washed three times with Hanks' balanced salts solution and picked by hand under a dissecting microscope. Approximately 300–400 islets

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP3R-3, IP_3 receptor subtype 3.

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were obtained from each animal, and islets from three rats were combined for immunoblotting experiments.

Cell Culture Studies. RINm5F and COS-7 cells were plated during the afternoon in 100-mm-diameter dishes at a concentration of 2.5×10^5 cells per dish in Dulbecco's modified Eagle's medium with low (5 mM) glucose (GIBCO/BRL) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (50 units/ml), and streptomycin sulfate (50 μg/ml). The following day was counted as day 0 and medium was changed every day with low-glucose (5 mM) or high-glucose (25 mM) DMEM, supplemented as described above, to ensure that cells were exposed to a constant glucose concentration. Exposure to high glucose was started at day 0 (for 8 days of culture in high glucose), day 3 (for 5 days of culture), day 5 (for 3 days of culture), day 6 (for 2 days of culture), and day 7 (for 1 day of culture). In all cases, cells were collected at day 8, at which time they had been confluent for several days.

Immunoblotting. Crude membranes were prepared from rat pancreas, pancreatic islets, and cultured cells by homogenization (25 strokes with a Teflon/glass homogenizer) in ice-cold buffer A (50 mM Tris·HCl, pH 8.0/1 mM EDTA/10% sucrose/1 mM phenylmethylsulfonyl fluoride). The membranes were pelleted by centrifugation at 120,000 × *g* for 40 min at 4°C and resuspended in buffer A. The protein concentration was determined by using a Bio-Rad protein assay kit and a bovine gamma globulin standard (Bio-Rad). Fifty micrograms of membrane protein was separated by SDS/5% PAGE and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in 20 mM Tris·HCl, pH 7.4/500 mM NaCl/0.1% Tween 20 (TTBS) and then incubated with rat antibody IPR3AB3 at 0.4 μg/ml in TTBS. After washing, the bound antibody was localized by chemiluminescence using ECL Western blot detection reagents (Amersham). The magnitude of the changes in IP3R-3 levels in islets and RINm5F cells in response to different metabolic states and glucose, respectively, was determined by densitometric scanning of the Western blots.

Immunohistocytochemistry. Adult male rats were anesthetized by intraperitoneal injection of 7% chloral hydrate (100 mg/100 g of body weight). The pancreas was resected and placed in 4% paraformaldehyde/0.1 M sodium tetraborate, pH 9.0/15% sucrose at 4°C overnight. The tissue was frozen on dry ice, and 12-μm sections were cut on a cryostat and mounted on gelatin- and poly(L-lysine)-coated slides. Sections were incubated with antiserum IPR3AB3 at a dilution of 1:500 in phosphate-buffered saline and primary antibody binding was visualized with a Vectastain ABC Kit (Vector Laboratories) without peroxidase quenching. To confirm the specificity of antibody binding, sections were also incubated with antiserum that had been preabsorbed with the immunizing peptide (10 μg/ml) at 4°C overnight.

Immunogold Electron Microscopy. Pancreatic islets were fixed for 4 hr at room temperature in Karnovsky's fixative containing 4% paraformaldehyde, 5% glutaraldehyde, 3.5 mM $CaCl_2$, and 1% sorbitol at pH 7.4. Samples were then rinsed in 0.1 M cacodylate buffer (pH 7.4) for 12 hr at 4°C, dehydrated, and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkaiburg, Germany) at 60°C according to the manufacturer's directions. Sections were placed on carbon-coated nickel grids and rinsed for 30 min in phosphate-buffered saline containing 3% bovine serum albumin. Grids were floated with the section side down for 2 hr at room temperature on drops of primary antiserum diluted in this buffer, then rinsed dropwise with the buffer. The grids were then treated in the same way with goat anti-rabbit IgG conjugated to 20-nm gold particles (Electron Microscopy Sciences, Fort Washington, PA) diluted 50-fold in the aforementioned buffer. After a water rinse, the grids were counterstained for 15 min with 3% uranyl acetate. Electron microscopy was performed on a Siemens (Iselin, NJ) 101 electron microscope operated at 80 keV.

RESULTS

Expression of IP3R-3 in Pancreatic Islets. Affinity-purified antibody IPR3AB3, raised against the carboxyl-terminal 15 amino acids of IP3R-3, reacted specifically with the 300-kDa IP3R-3 protein (Fig. 1). The abundance of IP3R-3 was much higher in total membranes from pancreatic islets than from pancreas, suggesting that IP3R-3 was expressed primarily in islets, which comprise about 5% of the total pancreas volume (25). The localization of IP3R-3 to islets was confirmed by immunohistocytochemistry (Fig. 2). Immunostaining was present only in the islets of Langerhans. There was no staining of cells in the exocrine pancreas. The presence of staining throughout the islet indicated that IP3R-3 was present at least in the insulin-secreting β cells, which comprise ≈70% of the cells in the islet. No immunostaining was detected when IPR3AB3 was preincubated with the peptide immunogen (Fig. 2*B*).

Localization of IP3R-3 to Secretory Granules of β and δ Cells. Immunogold electron microscopy was used to identify the islet cell types that express IP3R-3 as well as to determine its subcellular distribution. The four major cell types present in the islets of Langerhans (insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, and pancreatic-polypeptide-producing PP cells) can be distinguished with the electron microscope on the basis of the ultrastructural characteristics of their secretory granules and cell bodies (26). Immunogold electron microscopy using IPR3AB3 as the primary antibody showed that IP3R-3 was expressed in β cells (Fig. 3*A*) and δ cells (Fig. 3*C*) with no detectable labeling in α cells (Fig. 3*B*) or PP cells (Fig. 3*E*). In the β and δ cells, immunogold labeling was restricted to the secretory granules. The same pattern of labeling was observed with another antibody, AP45, which was raised against an IP3R-3-specific peptide from the Ca^{2+} channel domain of the receptor (Fig. 3 *D* and *F*). No labeling was observed when IPR3AB3 and AP45 were preabsorbed with their corresponding peptide immunogens.

Most of the secretory granules in the β and δ cells were labeled, including granules adjacent to the plasma membrane and undergoing exocytosis (Fig. 3*D*). No labeling of the

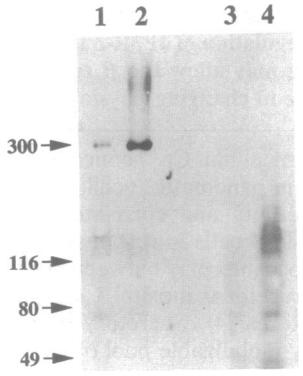

FIG. 1. Immunoblot analysis of IP3R-3. Fifty micrograms of total membrane proteins was separated by SDS/5% PAGE and IP3R-3 was detected by immunoblotting using an affinity-purified antibody (IPR3AB3) against the carboxyl-terminal 15 amino acids of the protein. Lane 1, rat pancreas; lane 2, rat pancreatic islets; lane 3, rat pancreas incubated with antibody preabsorbed with peptide immunogen (10 μg/ml) for 12 hr at 4°C; and lane 4, rat islets incubated with preabsorbed antibody. The sizes (kDa) of IP3R-3 (300 kDa) and three reference proteins (prestained β-galactosidase, bovine serum albumin, and ovalbumin) are noted on the left.

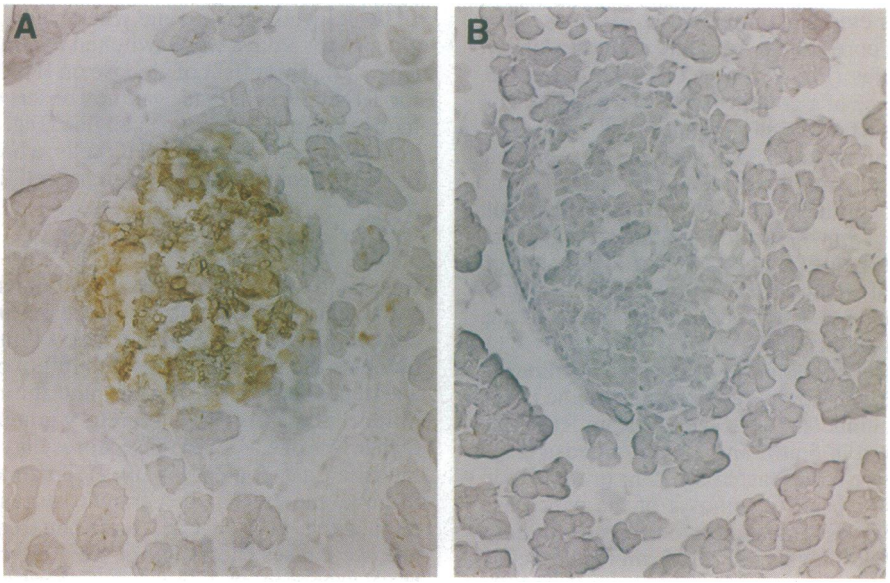

FIG. 2. Immunohistocytochemical localization of IP3R-3 in rat pancreas. (*A*) Section (12 μm) prepared as described in *Materials and Methods* was incubated overnight at 4°C with a 1:500 dilution of rabbit antiserum IPR3AB3 (antibody was not affinity purified). Immunostaining was detected by the avidin–biotin horseradish peroxidase method. (*B*) Reactivity of a rat pancreatic section with antiserum IPR3AB3 that had been preabsorbed with peptide immunogen (10 μg/ml) for 12 hr at 4°C.

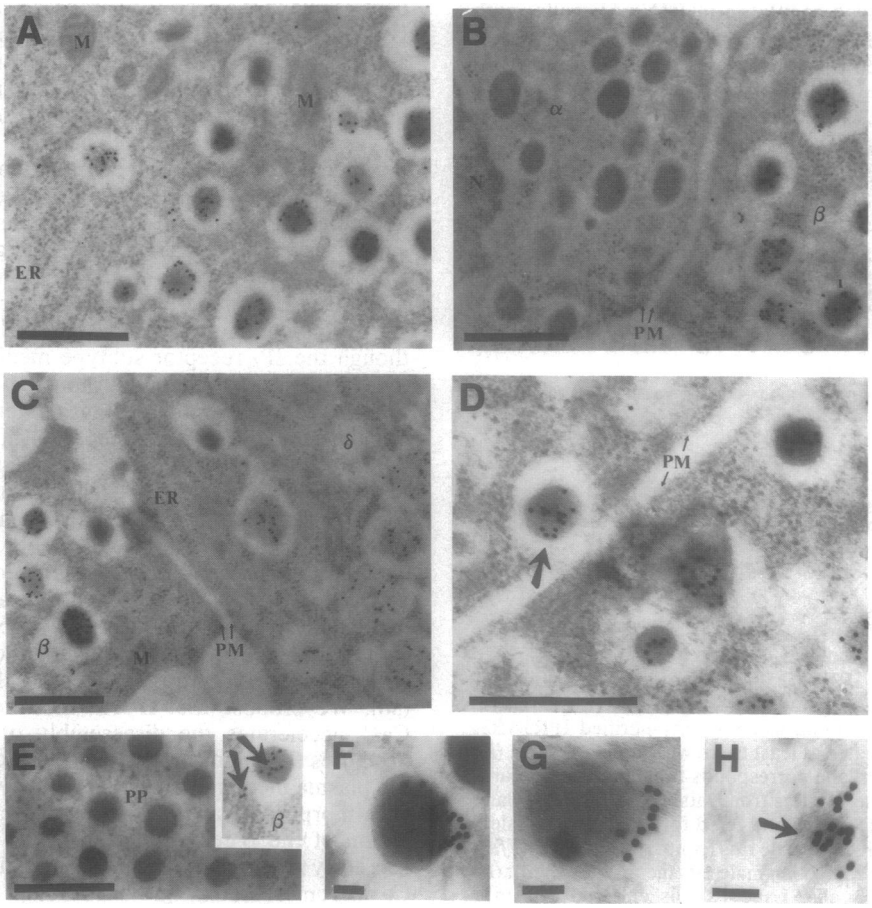

FIG. 3. Localization of IP3R-3 in rat islets by immunogold electron microscopy. Sections were incubated with rat IP3R-3-specific affinity-purified antibody IPR3AB3 (*A–C*, *E*, *G*, and *H*) or AP45 (*D* and *F*). (*A*) Insulin-secreting β cell. Immunostaining is localized to insulin-secretory granules. M, mitochondria; ER, endoplasmic reticulum. (*B*) Glucagon-secreting α cell. No immunostaining is evident. α, α cell; β, β cell; N, nucleus; PM, plasma membrane. (*C*) Somatostatin-secreting δ cell. Immunostaining is localized to somatostatin-secretory granules. δ, δ cell. (*D*) Site of exocytosis in a pancreatic β cell. Note the granule fusing with the plasma membrane (arrow) and the absence of decoration of the plasma membrane. (*E*) Pancreatic polypeptide-secreting PP cell. Note the absence of immunostaining. *Inset* shows a secretory granule of a neighboring β cell with positive labeling in the granule core and membrane (arrows). PP, PP cell. (*F* and *G*) Cluster-like immunostaining at the periphery of β-cell secretory granules. (*H*) Pattern of immunostaining on the surface of a granule in a skimmed section. Note the shading corresponding to the granule core underneath the gold particles (arrow). (*A–E*, bar = 1 μm; *F–H*, bar = 0.1 μm.)

plasma membrane was evident. Immunogold particles were detected in the granule core (Fig. 3 *A–E*) and at the periphery of the secretory granules (Fig. 3 *F* and *G*). In a tangential section across the surface of a granule, there were clusters of gold particles on the surface of the granule (Fig. 3*H*). The labeling seen in the granule cores was specific. It was seen with both antibodies and could be blocked by preincubation of the antibodies with peptide. We believe that the labeling of the granule core results from disruption of the fragile granule ultrastructure during fixation, embedding, or sectioning of the islet preparations. It may also be due to a rapid degradation of the receptor *in vivo*, leading to an elimination of the IP3R-3 with the secretory products.

Regulation of IP3R-3 Expression in Islets and Insulinoma Cells. Total membranes were prepared from pancreatic islets of rats in different metabolic states as well as from RINm5F insulinoma cells cultured in 5 mM and 25 mM glucose (Fig. 4). Adult Wistar rats were either fasted for 72 hr or fasted for 72 hr and refed for 24 hr prior to removal of the pancreas. There was a 3.4-fold increase (mean of two separate studies; 3.0- and 3.9-fold) in IP3R-3 levels in islets from fasted/refed rats compared with fasted controls (Fig. 4*A*, lanes 1 and 2). Islets from diabetic ZDF male rats (10 weeks of age; glycemia, 469 ± 51 mg/dl) had 4.4-fold higher levels (mean of two separate studies; 3.3- and 5.5-fold) of IP3R-3 than islets from nondiabetic lean controls (10 weeks of age; glycemia, 130 ± 0.3 mg/dl) (Fig. 4*A*, lanes 3 and 4).

IP3R-3 levels were also regulated in RINm5F cells, with levels being 3.6-fold greater (mean of three studies; 2.2-, 3.0-, and 5.5-fold) in cells cultured in 25 mM glucose compared with those grown in 5 mM glucose (Fig. 4*B*). The increase in IP3R-3 levels was evident after 2 days in 25 mM glucose and was maintained during the 8-day period that cells were cultured in 25 mM glucose. The increase in IP3R-3 levels in RINm5F cells cultured in 25 mM glucose was reversible, with

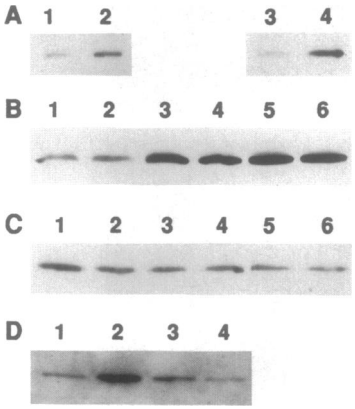

FIG. 4. Regulation of IP3R-3 expression. Fifty micrograms of total membrane protein was separated by SDS/5% PAGE and IP3R-3 was detected by immunoblotting using affinity-purified IPR3AB3. Each experiment was repeated at least twice with similar results. (*A*) Rat pancreatic islets; each lane corresponds to membrane preparations from three rats. Lane 1, islets from rats fasted for 72 hr; lane 2, islets from rats fasted for 72 hr and refed for 24 hr; lane 3, islets from lean nondiabetic ZDF/Gmi male rats (genotype, +/+ or +/*fa*); lane 4, islets from diabetic ZDF/Gmi male rats (genotype, *fa*/*fa*). (*B*) Insulin-secreting RINm5F cells. Lane 1, cells cultured in 5 mM glucose for 8 days; lanes 2–6, cells cultured in 25 mM glucose for 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), 5 days (lane 5), or 8 days (lane 6). (*C*) COS-7 cells. Lane 1, cells cultured in 5 mM glucose for 8 days; lanes 2–6, cells cultured in 25 mM glucose for 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), 5 days (lane 5), or 8 days (lane 6). (*D*) Insulin-secreting RINm5F cells. Lane 1, cells cultured in 5 mM glucose for 3 days; lane 2, cells cultured in 25 mM glucose for 3 days; lane 3, cells cultured in 25 mM glucose for 3 days and then 5 mM glucose for 2 days; lane 4, cells cultured in 25 mM glucose for 3 days and then 5 mM glucose for 5 days.

IP3R-3 levels decreasing when cells cultured in 25 mM glucose for 3 days were switched to 5 mM glucose for 2 days (Fig. 4*D*). This effect of glucose on IP3R-3 levels was specific for RINm5F cells and was not observed in COS-7 cells, a monkey kidney fibroblast cell line (Fig. 4*C*); Hep G2 cells, a human hepatoblastoma; CHO cells, a hamster ovary cell line; or AtT-20 cells, a mouse pituitary cell line (data not shown).

DISCUSSION

Regulated secretion of insulin and other hormones and neurotransmitters requires an increase in intracellular Ca^{2+} concentration (1–3). In the case of insulin secretion, this involves the glucose-stimulated influx of Ca^{2+} through voltage-dependent Ca^{2+} channels and the release of Ca^{2+} from intracellular stores. The secretory granules of β cells contain high levels of Ca^{2+} and these stores may comprise 25–40% of the islet Ca^{2+} content (5–10). Hellman and his colleagues (7, 8) have shown that the granule stores represent a readily mobilizable pool of Ca^{2+} and that treatment of islets with glucose causes the efflux of Ca^{2+} from the granules. They have proposed that the β-cell secretory granules actively participate in the regulation of intracellular Ca^{2+} levels.

The demonstration of IP3R-3 in the secretory granules of β and δ cells suggests that these organelles represent an IP_3-regulated store of Ca^{2+} that is mobilized during the secretory process. Moreover, it implies that IP_3 plays a central role in the secretory process *per se* in these cells. The IP_3 levels in β cells are increased by hormones such as acetylcholine and cholecystokinin acting through receptors coupled to phospholipase C (1, 2). The metabolism of glucose has also been reported to stimulate the accumulation of IP_3 (reviewed in ref. 1), and we speculate that IP_3 generated by this mechanism mobilizes secretory granule Ca^{2+} stores. The effect of glucose on accumulation of IP_3 is specific for glucose and other metabolizable secretagogues, as non-fuel secretagogues have no effect. Such IP_3-regulated granule Ca^{2+} stores are not unique to β and δ cells and have been previously described in chromaffin granules (19), the catecholamine-containing vesicles of the adrenal medulla, although the IP_3 receptor subtype mediating the response in these cells has not been determined. The absence of IP3R-3 in α and PP cells indicates that movement of the secretory granules to the plasma membrane must be regulated by a different mechanism in these cells than in β and δ cells.

In polarized secretory cells, liberation of intracellular Ca^{2+} from IP_3-sensitive stores leads to a transient increase in Ca^{2+} levels in the secretory pole which serves to prime the exocytotic process (27, 28). Regulated exocytosis involves changes in the cytoskeleton, with disassembly of actin filaments following activation of Ca^{2+}-dependent actin-severing proteins (29). The disassembly of the actin network then allows secretory granules to access the plasma membrane (30). We propose that IP3R-3-mediated release of granule Ca^{2+} accelerates the disassembly of actin filaments and promotes the migration of the insulin-containing granules to the plasma membrane (Fig. 5). This hypothesis is consistent with morphological studies showing changes in the organization of the β-cell actin web at the site of fusion of the secretory granule with the plasma membrane (31). Release of Ca^{2+} from secretory granules could also be amplified by Ca^{2+}-induced Ca^{2+} release, a process seen in many secretory cells that involves mobilization of Ca^{2+} stores from the endoplasmic reticulum and results in the generation of Ca^{2+} waves and oscillations (11, 27, 28). Release of Ca^{2+} by granules that are reaching the plasma membrane could also directly activate the Ca^{2+}-binding proteins of the granule membrane that are involved in the formation of the fusion pore (32), thereby directly regulating exocytosis. Finally, if the integrity of IP3R-3 in granules undergoing exocytosis is

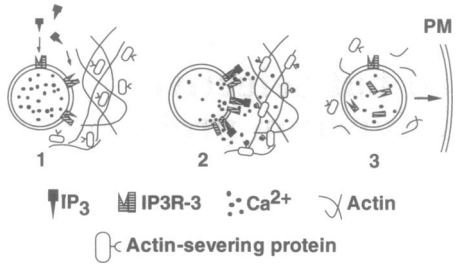

FIG. 5. Model for IP3R-3 function in secretion. IP_3 binding to IP3R-3 in secretory granule membrane (*1*) causes release of granule Ca^{2+} stores, resulting in a local increase in Ca^{2+} levels and activation of actin-severing proteins (*2*). Dissociation of the actin network allows granule migration to the plasma membrane (PM) and exocytosis (*3*).

maintained, IP3R-3 may also mediate the uptake of extracellular Ca^{2+}. Such a mechanism has been proposed to explain the phenomenon of secondary Ca^{2+} entry in polarized secretory cells (28).

In summary, localization of IP3R-3 to the secretory granules of insulin and somatostatin secreting cells suggests that these organelles act as IP_3-sensitive Ca^{2+} stores. We propose that mobilization of Ca^{2+} from these stores initiates and/or augments insulin and somatostatin secretion and that regulation of IP3R-3 levels allows the cell to adapt this secretory response to changing physiological conditions.

We are very grateful to Drs. K. S. Polonsky and J. Tang and Ms. D. Ostrega for providing rat pancreatic islets. The antibody IPR3AB3 was produced in the Radioimmunoassay Core Laboratory of the Diabetes Research and Training Center of the University of Chicago. We thank Drs. D. F. Steiner, K. S. Polonsky, L. Philipson, and M. W. Roe for their helpful comments on this manuscript. This work was supported by the Howard Hughes Medical Institute, U.S. Public Health Service grants (DK20595, DK42086, and DK44840), and the Juvenile Diabetes Foundation International. A.M.D. was supported in part by a Mentor-based fellowship from the American Diabetes Association.

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