**Creation of an Inositol 1,4,5-Trisphosphate-sensitive Ca\(^{2+}\) Store in Secretory Granules of Insulin-producing Cells**

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Olivier Blondel, Graeme I. Bell\(^1\), Maya Moody\(^1\), Richard J. Miller\(^1\), and Simon J. Gibbons\(^1\)

From the \(^1\)Howard Hughes Medical Institute and Departments of Biochemistry and Molecular Biology and Medicine, the \(^2\)Department of Molecular Genetics and Cell Biology, and the \(^3\)Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

A rapid rise in the cytosolic free Ca\(^{2+}\) concentration due to influx of extracellular Ca\(^{2+}\) or mobilization from intracellular stores is the primary trigger for exocytosis from secretory cells. Our understanding as to the precise role of Ca\(^{2+}\) mobilization has been complicated by the presence of several types of Ca\(^{2+}\) stores in most cells. We now demonstrate that overexpression of the type 3 inositol 1,4,5-trisphosphate (IP\(_3\)) receptor in insulin-secreting \(\beta\)TC-3 cells results in the creation of a unique IP\(_3\)-sensitive Ca\(^{2+}\) pool, restricted to the insulin secretory granules of these cells.

The cellular actions of inositol 1,4,5-trisphosphate (IP\(_3\)), are mediated by specific receptors that function as ligand-activated, Ca\(^{2+}\)-selective channels (1). They have been identified in endoplasmic reticulum (2), nucleus (3), plasma membrane (4, 5), nerve terminals (6), and chromaffin granules (7). Molecular cloning studies have shown that the IP\(_3\) receptors comprise a family of structurally related proteins. cDNAs encoding four different subtypes (IP\(_3\)-R 1 to 4) have been isolated, and each subtype has a distinct tissue distribution (1, 8–11). We have previously shown that the IP\(_3\) receptor subtype 3, IP\(_3\)-R, is expressed at high levels in rat insulinoma cells and is the predominant IP\(_3\) receptor expressed in normal adult rat pancreatic islets (10). In this paper we describe studies on the contribution of IP\(_3\)-R3 to the regulation of intracellular Ca\(^{2+}\) signaling. To do this, we transfected \(\beta\)TC-3 cells with a construct encoding IP\(_3\)-R3 and generated clones expressing this protein.

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\$ To whom correspondence and reprint requests should be addressed: Howard Hughes Medical Inst., University of Chicago, 5841 South Maryland Ave., MC1028, Chicago, IL 60637. Tel.: 312-702-9116; Fax: 312-792-0271.

\(1\) The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; IP\(_3\)-R3, type 3 inositol 1,4,5-trisphosphate receptor; CCh, carbamyl choline.

**EXPERIMENTAL PROCEDURES**

**Transfection of \(\beta\)TC-3 Cells and Detection of IP\(_3\)-R 3 by Immunoblotting**—The full-length cDNA sequence coding for rat IP\(_3\)-R3 (nucleotides 1–8806) (10) was cloned into pcCB6-Neo, which contains the human cytomegalovirus promoter and the neo gene resistance gene (a gift from D. Russell, M. Roth, and C. Brewer, University of Texas Southwestern Medical Center, Dallas, TX), to generate pcCB6-IP3R3. \(\beta\)TC-3 cells were transfected by electroporation either with the pcCB6-IP3R3 (IP\(_3\)-R3-\(\beta\)TC cells) or with the pcCB6-neo vector alone (C-\(\beta\)TC cells) as a control. Fifty μg of total membrane proteins were separated by 5% SDS-polyacrylamide gel electrophoresis and IP\(_3\)-R3 detected by immunoblotting using an affinity-purified polyclonal rabbit antibody (IP3R3A3) (380 ng/ml) against the COOH-terminal 15 amino acids (residues 2656–2670, RQRLGFVDVQNCMSR) of the rat protein (10). The sequences of rat IP3-R1-2, and -3 are unique in the region from which the peptide was selected for antibody production (10). Crude membranes were prepared from cultured cells, and Western blots were prepared and visualized by chemiluminescence techniques as described previously (10).

**Measurement of Intracellular Ca\(^{2+}\) Concentrations ([Ca\(^{2+}\)]\(_i\)) by Fura-2-based Digital Imaging**—[Ca\(^{2+}\)]\(_i\) was determined by digital fluorescence imaging in a medium containing (in mM): NaCl, 137; KCl, 5; MgSO\(_4\), 0.41; MgCl\(_2\), 0.49; CaCl\(_2\), 2; KH\(_2\)PO\(_4\)/H\(_2\)O, 0.64; NaHCO\(_3\), 3; glucose, 5.5; Hepes, 20, pH 7.4. Calcium-free medium contained 0.2 μM EGTA. Cells were loaded with 5 μM Fura-2 acetoxy methyl ester (Molecular Probes, Eugene, OR) at room temperature (22–24 °C) for 30 min and then allowed to fully lyse the ester in fresh medium for 30 min. [Ca\(^{2+}\)]\(_i\) was measured as described previously (12). Responses to bath-applied agonists were deemed acceptable for analysis according to the following criteria: 1) a stable [Ca\(^{2+}\)]\(_i\) for 30 s prior to drug application and 2) a rise in [Ca\(^{2+}\)]\(_i\) of at least 15 nM following carbamyl choline (CCh) application, which returned to baseline following washout. The peak Ca\(^{2+}\) was the maximal [Ca\(^{2+}\)]\(_i\) rise in the presence of CCh. The total Ca\(^{2+}\) change was calculated by summing the differences between the [Ca\(^{2+}\)]\(_i\) and the mean base line at each time point during the response. Statistical significance was calculated using a two-tailed Student's t test.

**Subcellular Localization of IP3-R3 by Immunogold Electron Microscopy**—Cultured cells were fixed for 4 h at room temperature in Karnovsky's fixative containing 4% paraformaldehyde, 5% glutaraldehyde, 3.5% CaCl\(_2\), and 1% sorbitol, pH 7.4. Samples were then rinsed in 0.1 μM cacodylate buffer, pH 7.4, for 12 h at 4 °C, dehydrated, and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany) at 65 °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 h at room temperature on drops of affinity-purified IP3R3Ab3 antisera (25 μg/ml) diluted in the buffer and 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. The electron microscopy was done using a Siemens 101 electron microscope operated at 80 kV.

**Materials**—Cyclopiazonic acid and thapsigargin were obtained from RBI (Natick, MA). All other chemicals were of reagent grade.

**RESULTS AND DISCUSSION**

Immunoblotting of total membrane proteins from \(\beta\)TC-3 cells transfected with rat IP3-R3 (IP3-R3-\(\beta\)TC) showed constitutive expression of the IP3-R3 protein (Fig. 1). The level of IP3-R3 expression in IP3-R3-\(\beta\)TC cells at passage 2 post-transfection was comparable with that measured in RINm5F cells, which express high levels of this protein endogenously (10). The expression IP3-R3 in these cells was unstable and declined during repeated passage, despite maintaining the cells in neomycin, and the IP3-R3 protein could not be detected at passage 5 (Fig. 1). Fura-2-based digital imaging was used to compare [Ca\(^{2+}\)]\(_i\) signals following stimulation by CCh of phospholipase...
C-coupled muscarinic receptors (13) in IP3R3-βTC and control cells (C-βTC) at passages 2–3. There was no apparent difference in the base-line [Ca²⁺], between the two cell types, but the baseline did show considerable variation from cell to cell, for example some cells exhibited periodical, large oscillations in [Ca²⁺], in control saline containing 2 mM Ca²⁺. Unless the baseline [Ca²⁺], was stable, the cells were not included in the analysis because it was not possible to separate the base-line oscillatory [Ca²⁺], responses from the effects of CCh. These oscillations were not more or less common in IP3R3-βTC cells. Initial studies examined the changes in [Ca²⁺], in normal physiological saline and showed that IP3R3-βTC cells were more responsive to CCh than C-βTC cells (Fig. 2). The mean time to peak and the duration of the [Ca²⁺], signal following CCh stimulation were similar for IP3R3-3-expressing and control cells. The differences between IP3R3-βTC and C-βTC cells were qualitatively similar when Ca²⁺ was removed from the medium, consistent with the expressed IP3R-3 functioning as an intracellular IP₃-activated Ca²⁺ channel. In both 0 and 2 mM extracellular Ca²⁺, a higher proportion of IP3R3-βTC cells responded to 10 μM CCh, and the changes in [Ca²⁺], were larger in IP3R3-βTC than C-βTC cells (Fig. 2). As previously reported by Prentki et al. (14) for another insulin-secreting cell line (HIT), individual cells had distinct patterns of response to CCh. This diversity was observed in both IP3R3-βTC cells and C-βTC cells. Although the responses were larger and the responding cells more numerous in IP3R3-βTC clones there were no apparent differences in the pattern of [Ca²⁺], changes between these cells and cells transfected with the vector alone.

In the presence of thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca²⁺-ATPase that depletes intracellular Ca²⁺ stores (15), the responses to CCh were almost completely abolished in C-βTC cells (Fig. 3, A and B). In contrast, when the same experiments were performed with IP3R3-βTC cells, nearly 40% of cells still exhibited a robust response to CCh in normal Ca²⁺-containing saline (Fig. 3, B and C). These responses were observed even in the absence of extracellular Ca²⁺, indicating that the response was due to release from intracellular stores. However, a second application of CCh in the presence of thapsigargin failed to produce further Ca²⁺ responses in IP3R3-βTC cells (Fig. 3C). Therefore thapsigargin was unable to “deplete” all CCh-mobilizable Ca²⁺ stores in IP3R3-βTC cells but did block refilling of these stores. It was possible that the absence of a response to CCh after thapsigargin treatment in C-βTC cells was due to the smaller effect of CCh in these cells. Fig. 3A shows that even when CCh elicits an abnormally large [Ca²⁺], change, thapsigargin prevents subsequent [Ca²⁺], responses to CCh. The effect of cyclopiazonic acid, a Ca²⁺-ATPase inhibitor that may be less selective than thapsigargin (16), was essentially the same (Fig. 3D). We also tested whether the increased sensitivity to CCh in the IP3R3-βTC cells was entirely due to a Ca²⁺ store, which was not depleted by thapsigargin by calculating the total [Ca²⁺], change in response to thapsigargin. Curiously, IP3R3-βTC cells had significantly lower thapsigargin responses than C-βTC cells (IP3R3-βTC cells: peak [Ca²⁺], rise = 279 ± 26 nm, total Ca²⁺ change = 15,682 ± 1745, n = 71; C-βTC cells: peak [Ca²⁺], rise = 472 ± 17 nm, total Ca²⁺ change = 33,292 ± 156, n = 59). The above data show that the IP3R-3-regulated Ca²⁺ store is distinct from the major endogenous IP₃-sensitive store and unusual in that it is intrinsically less leaky to Ca²⁺ because blockade of Ca²⁺-ATPases does not deplete the store but does prevent refilling (see Fig. 3C).

These results suggest that IP3R-3-sensitive Ca²⁺ stores may have a unique localization within IP3R3-βTC cells. In order to address this issue, we used Immunogold electron microscopy and an IP3R-3-specific antibody to examine the distribution of IP3R-3 in IP3R3-βTC cells and C-βTC cells (Fig. 4). There was Immunogold labeling of secretory granules in both IP3R3-βTC cells and C-βTC cells (Fig. 4) with the levels being significantly greater in the former. Over 80% of secretory granules were labeled in IP3R3-βTC cells (80.8% ± 3, n = 4 fields) whereas less than 50% of granules were labeled in the C-βTC cells (48.5% ±

**Fig. 1.** Expression of IP3R-3 in control and transfected insulin-secreting cell lines. Lane 1, RINm5F cells; lane 2, non-transfected βTC-3 cells; lanes 3–5, IP3R3-βTC cells at passage 2 (lane 3), 3 (lane 4), and 5 (lane 5) (note that the level of expression of IP3R-3 in the transfected IP3R3-βTC cells decreases with passage number; lane 6, RINm5F cells reacted with antibody preabsorbed with 10 μg/ml peptide antigen for 12 h at 4°C; lanes 7 and 8, IP3R3-βTC cells at passage 2 (lane 7) and passage 3 (lane 8) incubated with preabsorbed antibody. The mobilities of molecular weight standards are shown on the left.

**Fig. 2.** Transfection of βTC-3 cells with the pc86-IP3R3 construct altered the [Ca²⁺], responses following application of CCh. A, 1 and 10 μM CCh caused clear increases in [Ca²⁺], in a larger proportion of IP3R3-βTC cells (IP3R3) than C-βTC cells (Control) in both Ca²⁺-containing and Ca²⁺-free medium (0 [Ca²⁺],). Results are the means ± S.E. of the percent responding cells for each experiment; the total number of cells studied is indicated for information only (***, indicates p < 0.01 versus 10 μM CCh, control). B, CCh responses were larger in IP3R3-βTC than C-βTC cells. C, both peak [Ca²⁺], and total Ca²⁺ changes following the application of 10 μM CCh were larger in IP3R3-βTC cells. This difference was also observed in Ca²⁺-free medium (0 [Ca²⁺],). Results are the means ± S.E. for all the cells indicated; only the response to the first application of CCh was analyzed (*, indicates p < 0.05 versus control; ***, indicates p < 0.005 versus control; ****, indicates p < 0.001 versus control).
FIG. 3. Response of cells to CCh after application of inhibitors of intracellular Ca\textsuperscript{2+}-ATPases to deplete the intracellular Ca\textsuperscript{2+} stores. A, application of 500 nm thapsigargin was sufficient to prevent a response to CCh in C\texttext{-}\beta\text{TC} cells. B, a response to CCh in the presence of thapsigargin was only observed in one C\texttext{-}\beta\text{TC} cell whereas 40% of IP3R3\texttext{-}\beta\text{TC} cells responded. Only cells that clearly responded to CCh immediately before thapsigargin application were included for analysis. Data are the means (± S.E.) of the percent responding cells for each experiment; cell numbers are also given (*, indicates p < 0.05 by unpaired t test). C, in IP3R3\texttext{-}\beta\text{TC} cells, responses were still observed in the presence of thapsigargin in Ca\textsuperscript{2+}-free medium, but further applications of Ca\textsuperscript{2+} had no effect (typical of 10 cells from three clones). D, responses to CCh were also observed in IP3R3\texttext{-}\beta\text{TC} cells following the application of cyclopiazonic acid, another Ca\textsuperscript{2+}-ATPase inhibitor (typical of 17 cells from 3 clones). CCh did not elicit a response in C\texttext{-}\beta\text{TC} cells under the same conditions. Breaks in the trace indicate pauses in data collection when the cells were not illuminated.

FIG. 4. Subcellular localization of IP3R-3 in insulin-producing cell lines. a, \beta\text{TC} cells transfected with vector pCB6-neo. The arrows indicate secretory granules. b, \beta\text{TC} cells transfected with pCB6-IP3R3. The secretory granules are more intensely labeled than in control cells shown in a. Bar = 0.5 \mu m. In all experiments, preincubation of the IP3R3AB3 antibody with its peptide antigen (10 \mu g/ml) gave no labeling.

7.6, n = 4 fields, p < 0.01), and the number of gold particles per labeled granule was much lower in the C\texttext{-}\beta\text{TC} cells (C\texttext{-}\beta\text{TC} = 1.89 ± 0.2 immunogold particles/labeled granule, IP3R3\texttext{-}\beta\text{TC} = 8.35 ± 7.6 particles/labeled granule, n = 4 fields each, p < 0.01). There was no labeling of the endoplasmic reticulum, mitochondria, or other structures in cells overexpressing IP3R-3. The Immunogold labeling was primarily localized to the periphery of the core or even the core itself. We believe that this may be a consequence of the fact that the granule membrane is poorly preserved in these preparations and collapses onto the core during fixation and embedding of the cells. These results are consistent with our previous observations in rat pancreatic islets showing that IP3R-3 is specifically localized to the secretory granules of insulin-secreting \beta cells and somatostatin-secreting \delta cells (17).

The selective localization of IP3R-3 receptors in secretory granules suggests a role for this Ca\textsuperscript{2+} store in the control of insulin secretion. Our imaging data suggest that the secretory granule-associated IP3R-3 is functional and regulates the release of Ca\textsuperscript{2+} from this store. Insulin secretory granules are known to sequester Ca\textsuperscript{2+} following glucose stimulation in isolated pancreatic islets (18), a mechanism that is considered a key event in stimulus-secretion coupling (19). The pumping of Ca\textsuperscript{2+} in secretory granules is believed by some authors to occur through a Ca\textsuperscript{2+}-ATPase (see Ref. 19). Our imaging data suggest that the secretory granule-associated IP3R-3 is functional and can regulate the release of Ca\textsuperscript{2+} from this store. Our results indicate that once CCh has been applied in the presence of thapsigargin and has led to the release of the remaining Ca\textsuperscript{2+} from granule stores, CCh is no longer effective. This observation is consistent with the existence of a thapsigargin-sensitive Ca\textsuperscript{2+}-ATPase in the granule membrane whose activity is needed for a rapid refilling of the granule Ca\textsuperscript{2+} store. The fact that secretory granules are able to retain their Ca\textsuperscript{2+} during continuous application of Ca\textsuperscript{2+}-ATPase inhibitors confirms earlier reports showing that the passive permeability of the insulin secretory granule membrane to Ca\textsuperscript{2+} is much lower than the membrane of other Ca\textsuperscript{2+}-storing organelles (20). Although IP3R-3 has been reported in rat pancreatic \beta cells (17), it is unlikely that IP3R-3 is sine qua non for insulin release as regulated secretion does occur from normal \beta\text{TC} cells (21), which have only low levels of this receptor. Nonetheless, it may be an important element in the modulation of insulin secretion by agents that act as agonists at phospholipase C-linked receptors (22, 23). Synthesis of IP\textsubscript{3} in response to such stimuli may promote local Ca\textsuperscript{2+} release from granules that could potentiate either movement of granules through the cytoskeleton, fusion of granules with the plasma membrane, or both (23). Furthermore, changes in the levels of IP3R-3 may be an important element in the control of insulin release in both normal and pathophysiological circumstances.

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