

## cAMP-dependent Mobilization of Intracellular $\text{Ca}^{2+}$ Stores by Activation of Ryanodine Receptors in Pancreatic $\beta$ -Cells

A  $\text{Ca}^{2+}$  SIGNALING SYSTEM STIMULATED BY THE INSULINOTROPIC HORMONE GLUCAGON-LIKE PEPTIDE-1-(7–37)\*

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Glucagon-like peptide-1 (GLP-1) is an intestinally derived insulinotropic hormone currently under investigation for use as a novel therapeutic agent in the treatment of type 2 diabetes mellitus. *In vitro* studies of pancreatic islets of Langerhans demonstrated that GLP-1 interacts with specific  $\beta$ -cell G protein-coupled receptors, thereby facilitating insulin exocytosis by raising intracellular levels of cAMP and  $\text{Ca}^{2+}$ . Here we report that the stimulatory influence of GLP-1 on  $\text{Ca}^{2+}$  signaling results, in part, from cAMP-dependent mobilization of ryanodine-sensitive  $\text{Ca}^{2+}$  stores. Studies of human, rat, and mouse  $\beta$ -cells demonstrate that the binding of a fluorescent derivative of ryanodine (BODIPY FL-X ryanodine) to its receptors is specific, reversible, and of high affinity. Rat islets and BTC3 insulinoma cells are shown by reverse transcriptase polymerase chain reaction analyses to express mRNA corresponding to the type 2 isoform of ryanodine receptor-intracellular  $\text{Ca}^{2+}$  release channel (RyR2). Single-cell measurements of  $[\text{Ca}^{2+}]_i$  using primary cultures of rat and human  $\beta$ -cells indicate that GLP-1 facilitates  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR), whereby mobilization of  $\text{Ca}^{2+}$  stores is triggered by influx of  $\text{Ca}^{2+}$  through L-type  $\text{Ca}^{2+}$  channels. In these cells, GLP-1 is shown to interact with metabolism of D-glucose to produce a fast transient increase of  $[\text{Ca}^{2+}]_i$ . This effect is reproduced by 8-Br-cAMP, but is blocked by a GLP-1 receptor antagonist (exendin-(9–39)), a cAMP antagonist ((Rp)-cAMPS), an L-type  $\text{Ca}^{2+}$  channel antagonist (nimodipine), an antagonist of the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$  ATPase (thapsigargin), or by ryanodine. Characterization of the CICR mechanism by voltage clamp analysis also demonstrates a stimulation of  $\text{Ca}^{2+}$  release by caffeine. These findings provide new support for a model of  $\beta$ -cell signal transduction whereby GLP-1 promotes CICR by sensitizing intracellular  $\text{Ca}^{2+}$  release channels to the stimulatory influence of cytosolic  $\text{Ca}^{2+}$ .

Glucagon-like peptide-1 (GLP-1)<sup>1</sup> is a potent blood glucose-lowering hormone that stimulates secretion of insulin from pancreatic  $\beta$ -cells, and which is under intensive clinical investigation for use in treatment of type 2 diabetes mellitus (1, 2). The two naturally occurring and biologically active isopeptides of GLP-1 with therapeutic potential are GLP-1-(7–37) and GLP-1-(7–36)amide (3, 4). They are derived by post-translational processing of proglucagon, and are synthesized and secreted by enteroendocrine L-cells of the distal intestine. The timing of the secretion of GLP-1 from these cells is such that circulating levels of the hormone rise coincident with the post-prandial increase of blood glucose concentration. By binding to its receptors located on  $\beta$ -cells, GLP-1 synergizes with glucose to induce insulin secretion, thereby increasing the concentration of circulating insulin to a level above that attributable to glucose alone. This augmentation of glucose-stimulated insulin secretion by the gut hormone GLP-1 is termed the incretin effect and plays a critical role in the maintenance of systemic glucose homeostasis (5).

The GLP-1 receptor located on  $\beta$ -cells is a G protein-coupled receptor that mediates stimulatory effects of the hormone on adenylyl cyclase (6). GLP-1 raises intracellular levels of cyclic cAMP, activates protein kinase A (PKA), and exerts pleiotropic effects on  $\beta$ -cell signal transduction (7–9). PKA-mediated protein phosphorylation interacts with metabolism of D-glucose at a late step in stimulus-secretion coupling to facilitate exocytosis of insulin-containing secretory granules (10–12). GLP-1 also produces cellular depolarization (13–15) and a large increase of  $[\text{Ca}^{2+}]_i$  (14, 16–20), effects reproduced by activators of cAMP signaling (21–23). The best evidence available indicates that the GLP-1-induced rise of  $[\text{Ca}^{2+}]_i$  serves as an important trigger for exocytosis of insulin. The vital role  $\text{Ca}^{2+}$  plays in this process is emphasized by studies demonstrating that the insulin secretagogic effect of GLP-1 is markedly attenuated by treatments that prevent the rise of  $[\text{Ca}^{2+}]_i$  (16, 24).

We have focused on early events in GLP-1 signal transduction in order to ascertain how the rise of  $[\text{Ca}^{2+}]_i$  that serves as a trigger for secretion of insulin is achieved. In general, the alterations of  $[\text{Ca}^{2+}]_i$  observed upon exposure of  $\beta$ -cells to

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<sup>1</sup> The abbreviations used are: GLP-1, glucagon-like peptide-1; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PKA, protein kinase A; BODIPY FL-X, 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino) hexanoic acid; CICR,  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release; RyR, ryanodine receptor; FBS, fetal bovine serum; SERCA, sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase; VDCC, voltage-dependent  $\text{Ca}^{2+}$  channel;  $\text{IP}_3$ , inositol trisphosphate; cADPR, cyclic ADP-ribose; K-ATP, ATP-sensitive  $\text{K}^+$  channel; 8-Br-cAMP, 8-bromo-cAMP; bp, base pair(s); SES, standard extracellular salt solution.

GLP-1 can be described as having transient (seconds) or sustained (minutes) kinetics. The sustained rise of  $[Ca^{2+}]_i$  results from influx of  $Ca^{2+}$  that is a consequence of three distinct mechanisms. First, GLP-1 potentiates glucose-induced closure of ATP-sensitive  $K^+$  channels (K-ATP) (13), thereby generating cellular depolarization, activation of voltage-dependent  $Ca^{2+}$  channels (VDCCs), and influx of  $Ca^{2+}$ . Second, GLP-1 exerts a direct stimulatory influence on the entry of  $Ca^{2+}$  through dihydropyridine-sensitive (L-type) VDCCs (9). Third, GLP-1 stimulates the opening of  $Ca^{2+}$ -activated nonselective cation channels that are permeant to  $Ca^{2+}$  as well as to  $Na^+$  (14, 25). These three processes act in concert to augment oscillatory electrical activity,  $Ca^{2+}$  influx, and pulsatile secretion of insulin characteristic of whole islets of Langerhans.

More poorly understood is the fast transient increase of  $[Ca^{2+}]_i$  observed in response to GLP-1 (18, 19). Here we report that this action of the hormone requires the presence of extracellular  $Ca^{2+}$ , can occur independent of any change of membrane potential, and is reproduced by pharmacological activators of cAMP signaling. Typically, such transient effects of hormones on  $[Ca^{2+}]_i$  are considered to be a consequence of the mobilization of  $Ca^{2+}$  stores. GLP-1 might stimulate release of inositol trisphosphate ( $IP_3$ )-sensitive  $Ca^{2+}$  stores because it increases inositol phosphate production in COS-7 cells transfected with the recombinant GLP-1 receptor (26) or in *Xenopus* oocytes injected with GLP-1 receptor mRNA (27). However, GLP-1 has only a small effect on inositol phosphate production in freshly isolated islets (24, 28) or HIT-T15 insulinoma cells (16). Therefore, an issue of central importance to our understanding of GLP-1 signal transduction concerns exactly how cAMP interacts with  $\beta$ -cell  $Ca^{2+}$  stores to promote their mobilization.

It has been proposed that  $Ca^{2+}$  is mobilized by GLP-1 during the process of  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR), whereby influx of  $Ca^{2+}$  across the plasma membrane triggers release of  $Ca^{2+}$  from  $Ca^{2+}$  stores regulated by ryanodine receptor  $Ca^{2+}$  release channels (20). This hypothesis is controversial due to conflicting findings concerning what role ryanodine receptors play as regulators of  $\beta$ -cell  $Ca^{2+}$  signaling. One objection raised concerns the uncertain effectiveness of cyclic ADP-ribose (cADPR) in this system. cADPR is a candidate second messenger that mobilizes ryanodine-sensitive  $Ca^{2+}$  stores in some cell types (29). It is derived from  $\beta$ -NAD<sup>+</sup> via an enzymatic process catalyzed by an ADP-ribosyl cyclase designated CD38 (30). Okamoto and co-workers reported that an  $IP_3$ -insensitive  $Ca^{2+}$  store was mobilized by cADPR in rat islet microsomes (31–33). However, cADPR failed to act in *ob/ob* mouse  $\beta$ -cells or RINm5F insulinoma cells (34–38).  $Ca^{2+}$  signaling in rat  $\beta$ -cells was also not influenced by an antagonist of cADPR (37, 39), and levels of cADPR in rat islets were insensitive to alterations of glucose concentration (40), findings that contradict the Okamoto hypothesis. Therefore, the significance of ryanodine receptors as mediators of GLP-1 signal transduction remained unclear.

Here we report functional expression of ryanodine receptors in  $\beta$ -cells derived from human, rat, and C57BL/6J mouse islets. Assays of islet mRNA by the reverse transcriptase-polymerase chain reaction (RT-PCR) indicated a preferential expression of the type 2 isoform of ryanodine receptor (RYR2) in these tissues. The subcellular location of ryanodine receptors was visualized in  $\beta$ -cells by fluorescence microscopy using BODIPY FL-X ryanodine as a fluorophore.  $Ca^{2+}$  imaging and patch clamp analyses of  $\beta$ -cells demonstrated that GLP-1 produces a fast transient increase of  $[Ca^{2+}]_i$  as a consequence of CICR initiated by influx of  $Ca^{2+}$  through L-type VDCCs. It is concluded that this process of CICR is mediated by a cAMP-

signaling system that targets a ryanodine-sensitive source of intracellular  $Ca^{2+}$ .

#### EXPERIMENTAL PROCEDURES

**Preparation of Human, Rat, and Mouse Islet Cell Cultures**—Human islets were obtained from Dr. C. Ricordi (Diabetes Research Institute, University of Miami School of Medicine, Miami, FL). Rat islets were isolated from pentobarbital-anesthetized male Sprague-Dawley rats (200–250 g; Charles River Laboratories, Wilmington, MA) according to Lacy and Kostianovsky (41) by digestion with collagenase (Roche Molecular Biochemicals). Mouse islets were isolated by digestion of pancreata obtained from male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Islets were dispersed into single cell suspensions by digestion with trypsin-EDTA, and by trituration through a fire-polished Pasteur pipette. Cell suspensions were plated onto glass coverslips (25CIR-1; Fisher) coated with 1 mg/ml concanavalin A (type V; Sigma), which facilitates adherence of  $\beta$ -cells to glass surfaces. Cell cultures were maintained in a humidified incubator (95% air, 5%  $CO_2$ ) at 37 °C in RPMI 1640 culture media containing 11.1 mM glucose, 2 mM glutamine, 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**Preparation of Insulinoma Cell Cultures**—BTC3 and BTC6 cells (passages 62–78 and 24–34, respectively) were obtained from Dr. S. Efrat (Albert Einstein College of Medicine, New York, NY). MIN6 cells (passages 25–35) were provided by J. Miyazaki (Kyoto University, Japan). HIT-T15 cells (passages 65–70) were obtained from the American Type Culture Collection (Rockville, MD). Cell cultures were maintained on plastic tissue culture dishes (Falcon 3003; Becton Dickinson, Franklin Lakes, NJ) containing media supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. BTC3 and BTC6 cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose, 15% heat-inactivated horse serum, and 2.5% FBS. MIN6 cells were maintained in Dulbecco's modified Eagle's medium composed of 25 mM glucose, 15% FBS, and 74  $\mu$ M 2-mercaptoethanol. HIT-T15 cells were maintained in Ham's F-12K medium containing 10 mM glucose, 10% horse serum, and 2.5% FBS.

**RT-PCR Analyses of Ryanodine Receptor Isoforms**—Total RNA was extracted from rat islets and insulinoma cell cultures using guanidinium thiocyanate. The RNA was fractionated by density gradient centrifugation on a CsCl cushion, and poly(A) mRNA was isolated by chromatographic separation on an oligo(dT) affinity column. First strand cDNA synthesis was initiated using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Copy Kit; Invitrogen Corp., Carlsbad, CA). A cDNA synthesis reaction to which no reverse transcriptase was added served as a negative control. PCR primer pairs corresponding to RYR isoforms were identified by analysis of the published sequences (42, 43) of RYR cDNAs using Oligo version 4.0 primer analysis software (National Biosciences Inc., Plymouth, MN). The design of the PCR primers is listed in Table I. PCR reactions were catalyzed using recombinant *Taq* DNA polymerase, PCR reaction buffer, and dNTPs supplied by TaKaRa Biologicals (Shiga, Japan). The thermal cycling parameters consisted of an initial denaturation step for 5 min at 95 °C, followed by 36 cycles consisting of: 30 s at 95 °C, 60 s at 55 °C, and 90 s at 72 °C. The final extension step was for 5 min at 72 °C. PCR products were resolved by 1% agarose gel electrophoresis, and DNA was stained with ethidium bromide for fluorescence detection. PCR products corresponding to RYR were extracted from agarose gels by solubilization in NaI and by extraction with silica (GeneClean II; Bio 101 Inc., La Jolla, CA). The purified PCR products were ligated into the pCRII cloning vector by the T/A cloning method (Invitrogen Corp.). Ligation products were used to transform INV $\alpha$ F' cells (Invitrogen Corp.), and transformants were isolated by antibiotic resistance selection on agar plates containing ampicillin. Individual clones were selected and amplified by inoculation of Luria broth. Cloned plasmid DNAs were extracted from bacterial cultures using the Rapid Pure Miniprep procedure (Bio 101 Inc.). Determination of plasmid DNA sequences containing RYR PCR products was accomplished by the dideoxynucleotide sequencing method of Sanger in combination with polyacrylamide gel electrophoresis (Sequenase version 2.0 DNA sequencing kit; U. S. Biochemical Corp.).

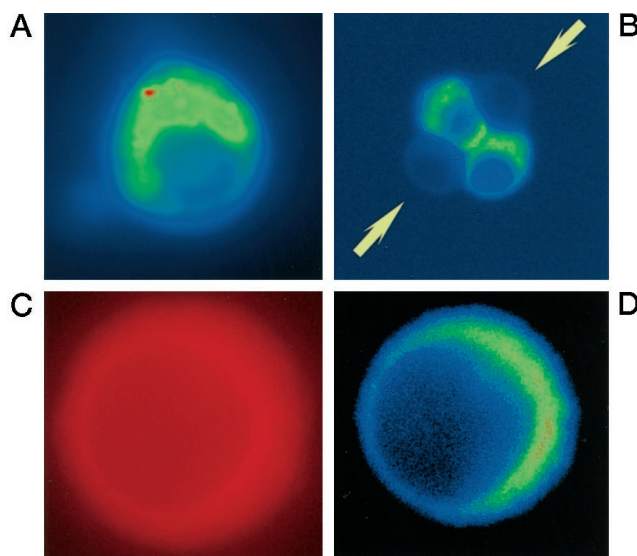
**Fluorescence Microscopy, Immunocytochemistry, and FluorImager Analyses**—The binding of BODIPY FL-X ryanodine (Molecular Probes Inc., Eugene, OR) to its receptor was evaluated by fluorescence microscopy using primary cultures of dispersed islet cells. An Eclipse E600 upright microscope (Nikon, Melville, NY) equipped with 20, 40, and 100 $\times$  Plan Fluor objectives was interfaced with a digital video imaging system comprising a Photometrics Ltd. Sensys cooled CCD camera

(Tucson, AZ) and a MacIntosh G3 computer (Apple Computers, Cupertino, CA) running IP Lab Spectrum software (Scanalytics Inc., Fairfax, VA). A mercury lamp served as the excitation light source, and the emitted light was filtered using standard fluorescein and rhodamine filter sets. Individual islet cells were allowed to adhere to the numbered grid surfaces of CELLocate coverslips (55- $\mu$ m square size; Eppendorf, Hamburg, Germany). The cells were fixed in 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. The fixed and permeabilized cells were incubated at room temperature in Hanks' balanced salt solution containing the specified concentration of BODIPY FL-X ryanodine. Immunocytochemistry for detection of insulin-like immunoreactivity was performed using 5% normal goat antiserum as a blocking agent. The primary antiserum was guinea pig anti-insulin (Dako, Carpinteria, CA) used at a dilution factor of 1:100. The secondary antiserum was rhodamine-conjugated goat anti-guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA). Quantitative analyses of islet cell suspensions were performed using a Molecular Dynamics FluorImager running ImageQuant software (Sunnyvale, CA). An argon laser served as the excitation light source, and fluorescence was imaged on 96-well tissue culture plates (Falcon 3072; ~20,000 islet cells/well) using a fluorescein 530-nm discriminating filter set. Estimates of RYR2 affinity were based on the use of permeabilized cells where access of BODIPY FL-X ryanodine to its binding site was unimpeded. Although the apparent  $K_d$  for fluorophore binding was ~4 nM, higher concentrations of ryanodine were required to block CICR in living cells, where access of ryanodine to its binding site was impeded.

**Measurement of Intracellular  $Ca^{2+}$  Concentration**—Cells were maintained on glass coverslips 24–48 h prior to each experiment. The cell culture media was replaced with a standard extracellular salt solution (SES) containing (in mM): 138 NaCl, 5.6 KCl, 2.6  $CaCl_2$ , 1.2 mM  $MgCl_2$ , 10 mM HEPES (pH 7.4), and 7.5 mM D-glucose. The SES was supplemented with 1  $\mu$ M fura-2 acetoxyethyl ester (fura-2-AM; Molecular Probes Inc.), 2% fetal bovine serum, and 0.02% Pluronic F-127 (w/v; Molecular Probes Inc.). Cells were exposed to the fura-2 AM-containing solution for 15 min at 22 °C. The loading solution was then removed, and the cells were equilibrated in fresh SES for 10 min at 22 °C. Experiments were performed at 32 °C using a Zeiss IM35 inverted microscope (Thornwood, NJ) outfitted with a temperature-controlled stage, a superfusion system, and a 100 $\times$  Nikon UVF objective. Dual wavelength excitation microspectrofluorimetry was performed ratiometrically at 1-s intervals using a digital video imaging system (Ion-Optix Corp., Milton, MA). Calibration of the raw fluorescence values was performed as described previously (22) using fura-2 pentapotassium salt dissolved in calibration buffer solutions from Molecular Probes (Calcium Calibration Kit 1 with Magnesium). Insulin-containing  $\beta$ -cells were positively identified at the end of each experiment by fluorescence microscopy in combination with immunocytochemistry.

**Patch Clamp Electrophysiological Techniques**—The resting potential and holding current were measured under current clamp or voltage clamp using the tight seal, whole cell, perforated patch configuration (13, 14). Pipettes were pulled from borosilicate glass (Kimax-51; Kimble Glass Inc., Vineland, NJ), fire-polished (final tip resistances of 2–3 megohms), and tip-dipped in a standard patch pipette solution containing (in mM): 95  $Cs_2SO_4$ , 7  $MgCl_2$ , and 5 HEPES (pH 7.4). Pipettes were then back-filled with patch pipette solution supplemented with 240  $\mu$ g/ml nystatin. The pipette solutions did not contain fura-2 and were considered to be nominally  $Ca^{2+}$ -free. Patch pipettes were connected to the head stage of an EPC-9 patch clamp amplifier (Heka Electronics, Lambrecht, Germany) interfaced with a personal computer running Pulse version 8.0 acquisition software (Instrutech Corp., Mineola, NY). The series resistance ( $R_s$ ) and cell capacitance ( $C_m$ ) were monitored following seal formation, and experiments were conducted when  $R_s$  declined to 12–25 megohms and  $C_m$  increased to 10–40 picofarads. In voltage clamp experiments,  $R_s$  was compensated for by 60%. Electrical access to the cytosol was confirmed by noting approximately -60-mV resting potential, and by noting an increase of  $[Ca^{2+}]_i$  in response to a voltage step from -70 to 0 mV. Experiments were rejected if such a rise of  $[Ca^{2+}]_i$  was not observed. Measurements of membrane potential and current were digitized using an Instrutech VR-10A digital data recorder and stored on magnetic tape for subsequent analysis using pClamp version 6.0 software (Axon Instruments Corp., Foster City, CA).

**Preparation of Test Solutions**—GLP-1-(7–37), exendin-4, and exendin-(9–39) were from Peninsula Laboratories (Belmont, CA). 8-Br-cAMP, nimodipine, ryanodine, caffeine, and thapsigargin were from Sigma. (Rp)-cAMPS was from BioLog Life Sciences Institute (Bremen, Germany). Test solutions were dissolved in SES prior to each experiment. For studies examining the effects of peptides, the SES also contained 0.05% human serum albumin (fraction V; Sigma) to protect



**FIG. 1. Labeling of human pancreatic islet cells by fluorescent BODIPY FL-X ryanodine.** Primary cultures of human islet cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated at 22 °C for 1 h in Hanks' buffered saline containing 1 nM BODIPY FL-X ryanodine. A pseudocolor image of fluorescence due to binding of the fluorophore demonstrated a perinuclear pattern of labeling (panel A; original magnification,  $\times 40$ ). Imaging of a cluster of islet cells demonstrated that not all cells were labeled (panel B, arrows; original magnification,  $\times 20$ ). A double-labeling technique demonstrated that a single human  $\beta$ -cell labeled by BODIPY FL-X ryanodine (panel D; original magnification,  $\times 100$ ) also contained insulin-like immunoreactivity as visualized using a rhodamine-conjugated secondary antiserum (panel C; original magnification,  $\times 100$ ).

against their absorption to glass or plastic surfaces. Solutions to which no  $Ca^{2+}$  was added were prepared by substituting  $MgCl_2$  for  $CaCl_2$ . Solutions containing 44 mM KCl were prepared by substituting KCl for NaCl on an isosmotic basis. Test solutions were applied to individual cells from micropipettes using a PicoSpritzer II pneumatic pressure ejection system (General Valve Corp., NJ) as described previously (13).

## RESULTS

**BODIPY FL-X Ryanodine as a Fluorescent Probe for Detection of Ryanodine Receptors**—We tested for expression of high affinity ryanodine receptors in pancreatic  $\beta$ -cells by using the fluorophore BODIPY FL-X ryanodine. Cells derived from dispersed human, rat, or mouse islets were allowed to adhere to coverslips, on which was etched a numbered grid system. This allowed positive identification of individual cells by microscopic inspection. Cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and bathed for 1 h at 22 °C in saline containing BODIPY FL-X ryanodine. A microscope-based digital imaging system was used to acquire images of fluorescence attributable to binding of BODIPY FL-X ryanodine to its receptors.

Specific binding of BODIPY FL-X ryanodine to human islet cells was observed using a low concentration (1 nM) of the fluorophore (Fig. 1, panel A). A perinuclear pattern of fluorescence was observed (panel A), consistent with expression of ryanodine receptors in a subcellular compartment that is likely to include the endoplasmic reticulum. The affinity of BODIPY FL-X ryanodine for these receptor sites appeared to be cell type-specific because not all cells in a cluster of human islet cells were labeled (arrows, panel B). BODIPY FL-X, itself, was not effective in this assay (data not shown), thereby confirming that the unconjugated fluorophore has no binding affinity. These human islet cells labeled by BODIPY FL-X ryanodine included authentic  $\beta$ -cells, as demonstrated by their co-expression of insulin-like immunoreactivity (cf. panels C and D). Quantitative analyses of human islet cells using a FluorImager

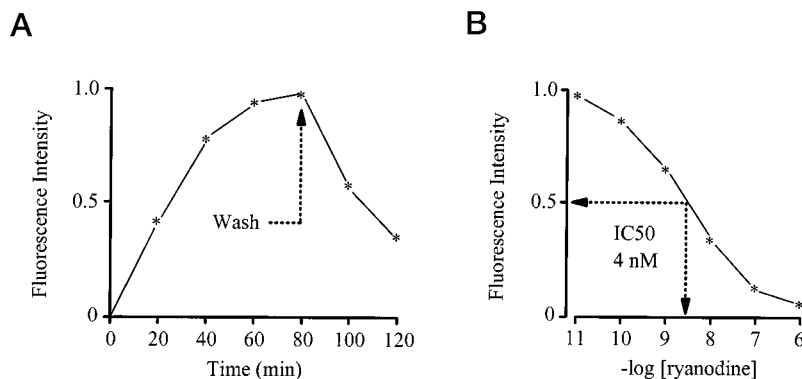


FIG. 2. **The interaction of BODIPY FL-X ryanodine with its receptor was saturable, reversible, and of high affinity.** Human islet cell suspensions maintained on 96-well tissue culture plates were fixed, permeabilized, and incubated with saline containing BODIPY FL-X ryanodine. Labeling of cells by the fluorophore was quantitated using an argon laser-based imaging system. The maximal intensity of fluorescence detected was assigned an arbitrary value of 1.0. Measurements were normalized to this value. *A*, cells were exposed to 1 nM BODIPY FL-X ryanodine for 20, 40, 60, or 80 min at 22 °C. This led to a time-dependent increase of labeling that decreased following removal of the fluorophore from the extracellular solution (as indicated by *Wash*). *B*, high affinity binding of BODIPY FL-X ryanodine to its receptor was diminished by prior treatment of islet cells for 60 min at 22 °C with the indicated concentration of non-fluorescent ryanodine. A concentration of 4 nM ryanodine reduced the binding of 1 nM BODIPY FL-X ryanodine by 50%.

demonstrated that the binding of BODIPY FL-X ryanodine was saturable and reversible (Fig. 2A). Pretreatment of human islet cells with 1  $\mu$ M non-fluorescent ryanodine blocked the subsequent binding of BODIPY FL-X ryanodine to its receptor (Fig. 2B). The  $IC_{50}$  for displacement of binding was determined to be  $\sim$ 4 nM. These experiments conducted with human islet cells were reproduced successfully using islets derived from Sprague-Dawley rats and C57BL/6J mice (data not shown).

**RT-PCR Analyses of Ryanodine Receptor Isoforms**—RT-PCR analyses of the three known isoforms of RYR were performed using PCR primers that match the published partial sequences (42, 43) of cDNAs corresponding to mouse ryanodine receptors (Table I). These primers target regions of RYR cDNA that code for the M1-M4 membrane-spanning and carboxyl terminus portions of the RYR protein (44). The samples of cDNA we tested were derived from poly(A) mRNA extracted from rat islets. Amplification of rat islet cDNA using an RYR2-specific primer set (F04/R04) demonstrated that a product of the expected size (423 bp) was resolved by gel electrophoresis (Fig. 3A). In contrast, PCR products were not obtained when we amplified aliquots of a rat islet cDNA synthesis reaction mixture to which no reverse transcriptase was added (Fig. 3A; negative control). The cloned 423-bp PCR product derived from rat islets was sequenced in both directions and evaluated by a National Center for Biotechnology BLAST analysis using the GenBank data base. Determination of the nucleic acid sequence confirmed that it was homologous (>90% identity) to the RYR2 sequence derived by screening of a mouse cDNA library (42). This sequence corresponds to sequence 13084–13506 (see Ref. 45) of rabbit cardiac RYR2.

It may be argued that our samples of rat islet cDNA are contaminated by cDNAs derived from non-endocrine cell types that express RYR2. Therefore, we sought to determine if RYR2 is detectable by RT-PCR of cDNA derived from insulin-secreting cell lines that serve as model systems for analysis of  $\beta$ -cell function. To this end, poly(A) mRNA was extracted from mouse BTC3 cells for use in synthesis of cDNA. As was the case for rat islet cDNA, the RYR2-specific F04/R04 primer set generated a 423-bp PCR product when using BTC3 cDNA as template (Fig. 3A). When cloned and characterized, this BTC3 PCR product was found to be identical in sequence to that reported for mouse RYR2. Three additional primer sets were chosen to validate the presence of RYR2 mRNA in rat islets or insulinoma cells. RYR2 F01/R01 and F02/R02 primer pairs (Table I) generated PCR products of the expected sizes (303 and 258 bp, respectively)

when used for amplification of cDNA from rat islets (Fig. 3B) and BTC3 cells (Fig. 3C). Sequence analysis confirmed that these PCR products correspond to RYR2. The 303- and 258-bp PCR products are homologous to sequences 14757–15059 and 14802–15059 of rabbit cardiac RYR2 (45). We also observed that the RYR2 F03/R03 primer pair generated a product of the expected size (1179 bp) when amplifying cDNA from rat islets (data not shown). The RYR2 F03/R03 primers target a sequence in mouse and rabbit cardiac RYR2 that encompasses the entire carboxyl terminus starting at position 14805 of rabbit RYR2 and extending into the 3'-untranslated region (42, 45).

We also sought to determine whether type 1 or type 3 isoforms of RYR are detectable by RT-PCR in rat islets or insulinoma cells. Therefore, RT-PCR was performed using RYR1- or RYR3-specific PCR primer sets (Table I). As an internal control, the same batches of cDNA that yielded RYR2 were used in each PCR reaction. Four primer sets corresponding to RYR1 were tested. Of these, two sets (RYR1-F01/R01; F02/R02) yielded faint PCR products of the appropriate sizes (298 and 385 bp, respectively) when amplifying cDNA from BTC3 cells but not rat islets (data not shown). The 298-bp PCR product was sequenced, whereby it was determined to be identical to that previously reported for mouse RYR1 (42, 43). Also tested were four RYR3-specific primer sets. Of these, only one set (RYR3-F01/R01) generated a PCR product of the expected size (344 bp). This abundant PCR product was detected when amplifying cDNA derived from rat islets, BTC3 cells, and mouse brain (data not shown). Sequence analysis determined it not to correspond to any known sequence in GenBank. Instead, it corresponded most closely (93% identity) to mt19d02.rl, an expressed sequence tag (accession no. A178752) derived from a mouse cDNA library.

**A Physiological Role for GLP-1 and Ryanodine Receptors in  $\beta$ -Cell  $Ca^{2+}$  Signaling**—GLP-1 is proposed to influence  $Ca^{2+}$  signaling in  $\beta$ -cells by mobilizing ryanodine-sensitive  $Ca^{2+}$  stores (9, 20). To test this possibility, measurements of  $[Ca^{2+}]_i$  were obtained from single rat  $\beta$ -cells maintained in primary cell culture and equilibrated in saline containing 7.5 mM D-glucose. Application of 10 nM GLP-1-(7–37) produced a significant increase of  $[Ca^{2+}]_i$  in 34 of 50 cells. These responses were tabulated on the basis of kinetic parameters (Table II). A monophasic response was measured in 26 cells, whereas 8 cells exhibited a biphasic response. Monophasic responses consisted of a fast transient increase of  $[Ca^{2+}]_i$  lasting 20–30 s (Fig. 4A)

TABLE I  
PCR primers used for amplification of RYR cDNAs

The design of the PCR primers was based on the published partial sequences of mouse ryanodine receptors (42, 43). Listed are the four sets of forward (F01–F04) and reverse primers (R01–R04) that target the type 1, 2, or 3 ryanodine receptor isoforms. The predicted sizes (in base pairs) of the PCR products are listed on the right.

| Isoform | Forward primer                  | Reverse primer                 | Size  |
|---------|---------------------------------|--------------------------------|-------|
| RYR1    | F01 5'-TCACTCACAATGGAAAGCAG-3'  | R01 5'-AGCAGAATGACGATAACGAA-3' | bp    |
|         | F02 5'-TGGGGCACTACAATAACTTC-3'  | R02 Same as RYR1 R01           | 298   |
|         | F03 5'-GGGTCTGGTGTATGGCTCTGG-3' | R03 5'-CGGGCTGCTCTGTAATGTAG-3' | 385   |
|         | F04 5'-TTGCCATCAACTTCATCTTA-3'  | R04 5'-TCATCGCCCTTACCTCCTC-3'  | 268   |
| RYR2    | F01 5'-GTATGTGGGCGTCCGTGCTG-3'  | R01 5'-TAGTTGGCCAGGTTATGTTC-3' | 303   |
|         | F02 5'-AGACCCAGCAGGAGACGAGT-3'  | R02 Same as RYR2 R01           | 258   |
|         | F03 5'-CCCAGCAGGAGACGAGTATG-3'  | R03 5'-TGGGGAGGGTAAATGTGACG-3' | 1,179 |
|         | F04 5'-CTGAAGAGCCTGAAGAGCA-3'   | R04 5'-GCCGGCATTGGGTTGTGAG-3'  | 423   |
| RYR3    | F01 5'-GGTTTTCACTGACAACCTCCT-3' | R01 5'-TGCTCCACCTGCTCTTACG-3'  | 344   |
|         | F02 5'-TTCTTTGCTGCTCATCTGTT-3'  | R02 5'-AATGACGAAGAAGAAGAGG-3'  | 354   |
|         | F03 5'-AGGTTCTTGCTCTGTTTGT-3'   | R03 5'-TGCTTTGGCCTCTTCTACTG-3' | 537   |
|         | F04 5'-CTATCAACTTCATCTCTGTT-3'  | R04 5'-AATTTCTTTCTCCCTTTTGA-3' | 266   |

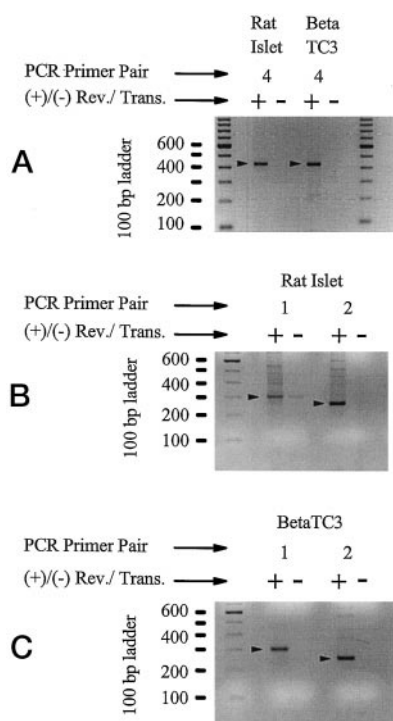


FIG. 3. RT-PCR analysis of RYR2 in rat islets and mouse BetaTC3 insulinoma cells. A, RT-PCR amplification of cDNA derived from rat islets or BetaTC3 cells was achieved using RYR2-specific primer pair 4, designated as RYR2 F04/R04 (Table I). A 423-bp PCR product was resolved by gel electrophoresis, as indicated by arrowheads in lanes designated as (+) *Rev. Trans.* (for reverse transcriptase). No PCR product was detected when amplifying a cDNA synthesis reaction mixture to which no reverse transcriptase was added (lanes designated as (-) *Rev. Trans.*). B and C, RT-PCR amplification of cDNA derived from rat islets (B) or BetaTC3 insulinoma cells (C) was accomplished using RYR2-specific primer pairs 1 and 2, designated as RYR F01/R01 and RYR F02/R02 (Table I). The 303- and 258-bp PCR products were resolved by gel electrophoresis and are indicated by arrowheads in lanes designated as (+) *Rev. Trans.*

or a slowly developing and sustained increase lasting several minutes (Fig. 4B). Biphasic responses consisted of an initial transient increase of  $[Ca^{2+}]_i$  followed by a sustained effect (Fig. 4C).

These actions of GLP-1 resemble those effects known to be produced by pharmacological activators of cAMP signaling. The membrane-permeant cAMP agonist 8-Br-cAMP produced a fast transient increase of  $[Ca^{2+}]_i$  similar in time course and magnitude to that produced by GLP-1 itself (Fig. 4A). A similar effect was also obtained during exposure of  $\beta$ -cells to 10  $\mu$ M

TABLE II  
Kinetic parameters of the GLP-1-induced rise of  $[Ca^{2+}]_i$

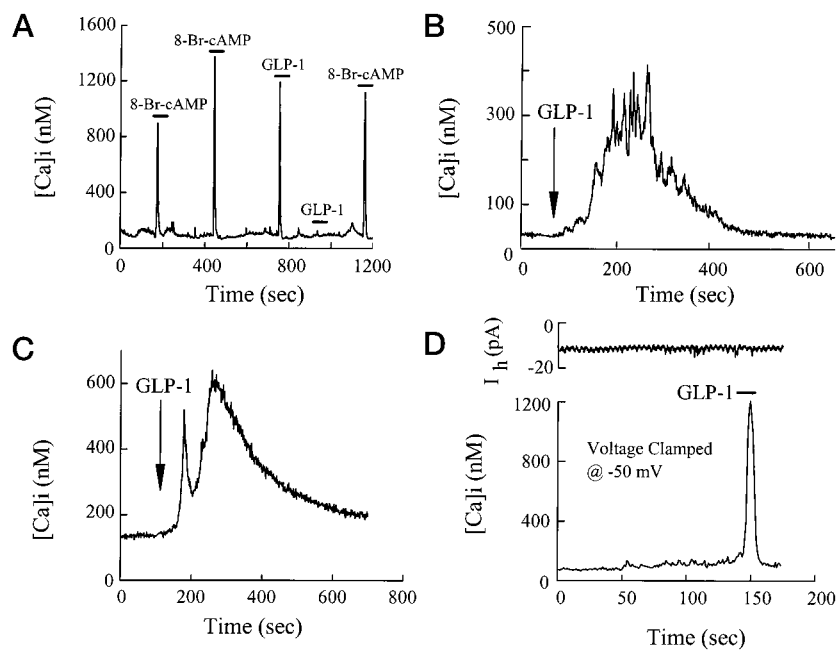
Measurements refer to the mean  $\pm$  standard deviation increase of  $[Ca^{2+}]_i$  (in nM) observed during exposure of single fura-2-loaded rat  $\beta$ -cells to 10 nM GLP-1-(7–37) under conditions in which cells were equilibrated in a saline solution containing 7.5 mM D-glucose. Three types of responses to GLP-1 were noted: transient, sustained, and biphasic.

| Response type | Fraction of cells responding | Increase of $[Ca^{2+}]_i$ (mean $\pm$ S.D.)          |
|---------------|------------------------------|--|
| Transient     | 12/50                        | 490 $\pm$ 65   |
| Sustained     | 14/50                        | 621 $\pm$ 82   |
| Biphasic      | 8/50                         | 443 $\pm$ 30 (transient)<br>587 $\pm$ 78 (sustained) |
| No effect     | 16/50                        |  |

forskolin or 10  $\mu$ M of the cAMP agonist (Sp)-cAMPS (data not shown). As expected for a signal transduction mechanism mediated by cell surface receptors, the action of GLP-1 exhibited desensitization, whereas the effect of 8-Br-cAMP was readily repeatable (Fig. 4A). Voltage clamp analysis using the perforated patch technique demonstrated that a fast transient increase of  $[Ca^{2+}]_i$  could also be measured when GLP-1 was applied to cells in which the membrane potential was maintained at -50 mV but not -80 mV (Fig. 4D). Under these conditions, the increase of  $[Ca^{2+}]_i$  was not associated with a detectable change of membrane current (Fig. 4D; trace labeled  $I_h$ ). Therefore, the fast transient increase of  $[Ca^{2+}]_i$  was unlikely to result from influx of  $Ca^{2+}$  as a consequence of the activation of nonselective cation channels (14, 25) or cyclic nucleotide-gated ion channels.

Detailed pharmacological studies were performed to characterize the signaling system by which GLP-1 stimulates a fast transient increase of  $[Ca^{2+}]_i$ . To achieve this goal, the transient response was studied in isolation using cells for which no sustained increase of  $[Ca^{2+}]_i$  was observed. The action of GLP-1 was characterized with respect to: 1) the requirement for a normoglycemic concentration of extracellular D-glucose, 2) the ligand selectivity of the GLP-1 receptor, 3) the action of (Rp)-cAMPS as a membrane-permeant antagonist of cAMP signaling, 4) the action of nimodipine as a dihydropyridine antagonist of L-type  $Ca^{2+}$  channels, 5) the action of thapsigargin as a blocker of endoplasmic reticular  $Ca^{2+}$  uptake mediated by the SERCA class of  $Ca^{2+}$  ATPases, and 6) the action of ryanodine as an antagonist of CICR mediated by intracellular  $Ca^{2+}$  release channels.

We observed that equilibration of  $\beta$ -cells in a normoglycemic concentration (7.5 mM) of glucose allowed for a fast transient



**FIG. 4. GLP-1-(7-37) and 8-Br-cAMP stimulated a transient and/or sustained increase of  $[Ca^{2+}]_i$  in primary cultures of rat  $\beta$ -cells.** Ratiometric determinations of  $[Ca^{2+}]_i$  were obtained by microspectrofluorimetry of single  $\beta$ -cells loaded with fura-2. *A*, a fast transient increase of  $[Ca^{2+}]_i$  was observed during repeated 30-s applications of 1 mM 8-Br-cAMP or 10 nM GLP-1-(7-37) (application of test solutions indicated by horizontal bars). *B*, a slowly developing and sustained increase of  $[Ca^{2+}]_i$  was measured following a 30-s application of 10 nM GLP-1-(7-37) (arrow indicates the time at which application of GLP-1 commenced). *C*, a biphasic increase of  $[Ca^{2+}]_i$  was observed following a 30 s application of 10 nM GLP-1-(7-37). *D*, 10 nM GLP-1-(7-37) produced a fast transient increase of  $[Ca^{2+}]_i$  when applied to a  $\beta$ -cell in which the membrane potential was held at  $-50$  mV using the voltage clamp technique. The action of GLP-1 was not associated with a change of holding current ( $I_h$ , top trace). Each panel indicates observations obtained from 4 different cells. The  $\beta$ -cell phenotype was established by detection of insulin-like immunoreactivity (data not shown).

increase of  $[Ca^{2+}]_i$  in response to brief application (30 s) of GLP-1 or 8-Br-cAMP (Fig. 5A). Levels of  $[Ca^{2+}]_i$  increased from  $98 \pm 30$  nM to nearly 600 nM, an increase of  $490 \pm 65$  nM for GLP-1 (Fig. 5A; Table II). In marked contrast, the actions of GLP-1 and 8-Br-cAMP were diminished by equilibration of cells in lower concentrations of glucose (Fig. 5A). Responses observed during exposure of cells to 2.0 or 0.8 mM glucose were measured not as a spike-like increase of  $[Ca^{2+}]_i$ , but as a small sustained effect (data not shown). This diminishment in response size was accompanied by a decrease in the fraction of cells exhibiting any response at all (Fig. 5A). Evidently, these fast transient increases of  $[Ca^{2+}]_i$  are all-or-none phenomena generated in a manner strictly dependent on exposure of  $\beta$ -cells to a normoglycemic concentration of glucose. This conclusion is consistent with the established role of glucose metabolism as a positive regulator of  $Ca^{2+}$  uptake, thereby maintaining the filling state of intracellular  $Ca^{2+}$  stores (46, 47).

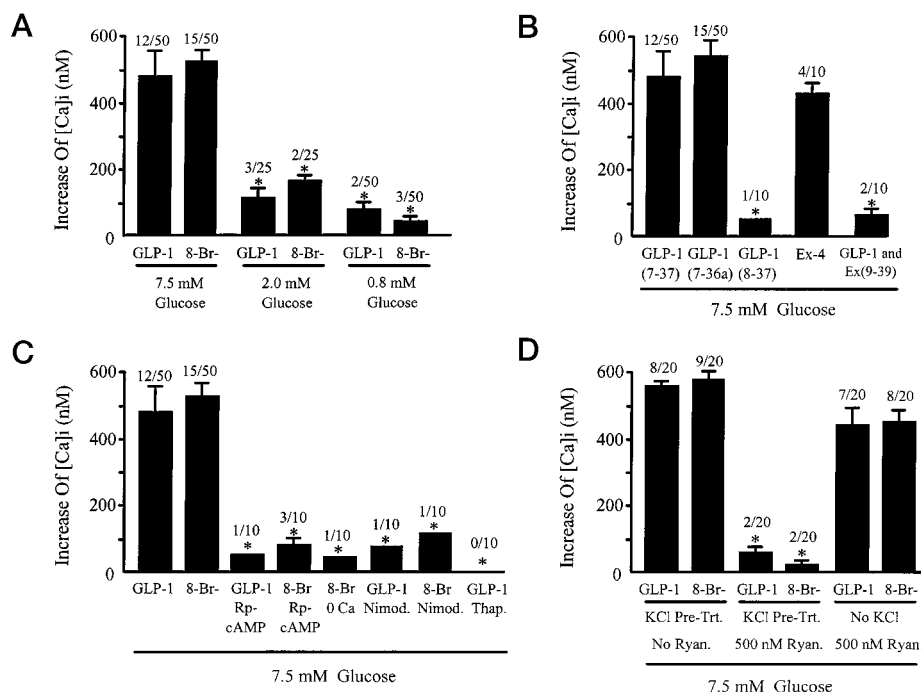
It was also established that the stimulatory action of GLP-1 in this assay exhibited pharmacological specificity characteristic of the cloned GLP-1 receptor isolated from a rat islet cDNA library (3, 6). GLP-1-(7-37) and GLP-1-(7-36)amide isopeptides were effective, whereas GLP-1-(8-37) was ineffective (Fig. 5B). Exendin-4, a peptide isolated from *Heloderma*, and which resembles in structure GLP-1, also exhibited agonist activity (Fig. 5B). In contrast, no effect of GLP-1-(7-37) was observed after treatment of cells with exendin-(9-39) (Fig. 5B), a peptide antagonist of the GLP-1 receptor.

A cAMP signaling system that regulates influx of  $Ca^{2+}$  through L-type  $Ca^{2+}$  channels is likely to be an important determinant of this response. Equilibration of cells for 30 min at  $37^\circ C$  in saline containing the cAMP antagonist (Rp)-cAMPS blocked the fast transient increases of  $[Ca^{2+}]_i$  measured in response to GLP-1 or 8-Br-cAMP (Fig. 5C). The actions of GLP-1 and 8-Br-cAMP were also blocked by brief exposure of cells to a  $Ca^{2+}$ -free solution (Fig. 5C), or by treatment with the

L-type  $Ca^{2+}$  channel antagonist nimodipine (Fig. 5C). It was also observed that this signaling system targets an intracellular source of  $Ca^{2+}$  that is mobilized as a consequence of  $Ca^{2+}$  influx. No effect of GLP-1 was observed after treatment of cells with the SERCA  $Ca^{2+}$  ATPase inhibitor thapsigargin (Fig. 5C).

The stimulatory actions of GLP-1 and 8-Br-cAMP that resulted in a fast transient increase of  $[Ca^{2+}]_i$  were blocked by prior equilibration of islet cells for 5 min at  $37^\circ C$  in saline containing 44 mM KCl and 500 nM ryanodine (Fig. 5D). This concentration of KCl produces cellular depolarization and  $Ca^{2+}$  influx, thereby promoting high affinity binding of ryanodine to  $Ca^{2+}$  release channels that open as a consequence of CICR (44). No such antagonist action of 500 nM ryanodine was observed using islet cells not pretreated with 44 mM KCl (Fig. 5D). Pharmacological concentrations of ryanodine (20–100  $\mu M$ ) were also observed to be effective provided that islet cells were incubated for 30 min at  $37^\circ C$  in saline containing 5.6 mM KCl (data not shown). Our use of 44 mM KCl to “unmask” the potent antagonist action of ryanodine is reminiscent of a previous study of RINm5F cells in which ryanodine was reported to be an antagonist of  $Ca^{2+}$  signaling only under conditions in which cells were pretreated with caffeine (48). Such observations may help explain conflicting reports documenting the effectiveness (48), limited effectiveness (49), or lack of effect (35, 50–53) of ryanodine as a blocker of  $Ca^{2+}$  signaling in  $\beta$ -cells.

**Caffeine as a Sensitizer of  $Ca^{2+}$ -induced  $Ca^{2+}$  Release Mediated by Ryanodine Receptors**—Caffeine is a methylxanthine that acts as a co-agonist with cytosolic  $Ca^{2+}$  to facilitate CICR mediated by ryanodine receptors in cardiac myocytes (54). Whether ryanodine receptors also mediate stimulatory effects of caffeine on  $[Ca^{2+}]_i$  in  $\beta$ -cells remains controversial. We examined what effect caffeine exerts on  $Ca^{2+}$  signaling in HIT-T15 insulinoma cells, a hamster  $\beta$ -cell line that expresses GLP-1 receptors (16), L-type VDCCs (16, 21), and the type 2 isoform of RYR (55). We found that application of caffeine



**FIG. 5. Pharmacology of GLP-1 signal transduction.** Population studies in *A–D* refer to actions of GLP-1 and 8-Br-cAMP to produce a fast transient increase of  $[Ca^{2+}]_i$  in rat or human  $\beta$ -cells. Measurements are the mean  $\pm$  S.D. increase of  $[Ca^{2+}]_i$  above base line. Also indicated is the fraction of cells that exhibited a  $\geq 400$  nM increase of  $[Ca^{2+}]_i$ . Cultures were equilibrated in the indicated concentrations of glucose for 30 min at 37 °C. GLP-1, exendin-4, exendin-(9–39), and 8-Br-cAMP were applied to individual cells for 30 s. All other solutions were administered by a superfusion system. Asterisks (\*) indicate a statistically significant difference from control ( $p \leq 0.001$ ,  $t$  test; control defined as the response to GLP-1(7–37) or 8-Br-cAMP when cells were bathed in saline containing 7.5 mM glucose alone). *A*, three sets of rat islet cell cultures were equilibrated in 7.5, 2.0, or 0.8 mM glucose as indicated. Application of 10 nM GLP-1(7–37) or 1 mM 8-Br-cAMP (8-Br) produced a fast transient increase of  $[Ca^{2+}]_i$  in 24% and 30% of cells equilibrated in 7.5 mM glucose, respectively. Only a small increase of  $[Ca^{2+}]_i$  was observed when the saline contained 2.0 or 0.8 mM glucose. Under these conditions the percentage of cells responding to either test substance was reduced. *B*, GLP-1(7–37), GLP-1(7–36)amide, and exendin-4 (10 nM each) were effective agonists, whereas GLP-1(8–37) was ineffective. The action of 10 nM GLP-1(7–37) was blocked by prior exposure of rat islet cells to 1  $\mu$ M exendin-(9–39). *C*, application of 10 nM GLP-1(7–37) or 1 mM 8-Br-cAMP failed to increase  $[Ca^{2+}]_i$  when rat islet cultures were pretreated for 30 min at 37 °C with 10  $\mu$ M (Rp)-cAMPS (Rp-), 10  $\mu$ M nimodipine (Nimod.), or 10  $\mu$ M thapsigargin (Thap.). 8-Br-cAMP was also ineffective when cells were exposed for 120 s to a solution that contained no added  $Ca^{2+}$  (0 Ca). *D*, application of 10 nM GLP-1(7–37) or 1 mM 8-Br-cAMP to human islet cells superfused with saline containing 5.6 mM KCl failed to increase  $[Ca^{2+}]_i$  when cultures were pretreated for 5 min at 37 °C with 44 mM KCl and 500 nM ryanodine. No effect of ryanodine was observed in cells not pretreated with 44 mM KCl.

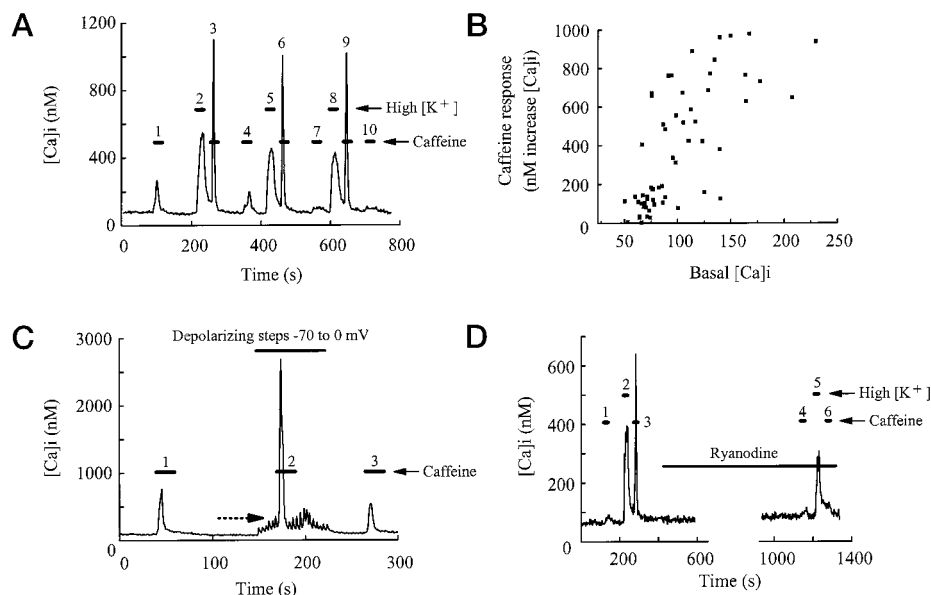
produced a fast transient increase of  $[Ca^{2+}]_i$  in 25 of 30 cells tested (Fig. 6A). This response was similar in magnitude and time course to that produced by GLP-1 or 8-Br-cAMP in rat  $\beta$ -cells (*cf.* Figs. 4A and 6A). As might be expected for a process of CICR, the action of caffeine was critically dependent on the initial  $[Ca^{2+}]_i$ . There existed a positive correlation between the  $[Ca^{2+}]_i$  measured prior to and during application of caffeine (Fig. 6B). This correlation appears to explain our finding that the action of caffeine was potentiated by prior exposure of HIT-T15 cells to a depolarizing concentration (44 mM) of KCl that produced a rise of  $[Ca^{2+}]_i$  on its own (Fig. 6A).

It is important to note that the action of caffeine was unrelated to its previously reported inhibitory effect on K-ATP (51). Caffeine produced an increase of  $[Ca^{2+}]_i$  in HIT-T15 cells voltage clamped at  $-70$  mV (Fig. 6C). This action of caffeine was potentiated by repetitive cellular depolarization to 0 mV (Fig. 6C). Such repetitive stimuli generated influx of  $Ca^{2+}$  through VDCCs, thereby explaining the gradual increase of  $[Ca^{2+}]_i$  observed prior to application of caffeine (Fig. 6C; arrow with dots). From these observations, it is evident that a close functional coupling existed between influx of  $Ca^{2+}$  and the subsequent mobilization of caffeine-sensitive  $Ca^{2+}$  stores in this cell type. Support for such a mechanism of CICR is also provided by our finding that the interaction of 44 mM KCl and caffeine to produce a fast transient increase of  $[Ca^{2+}]_i$  was attenuated by prior exposure of HIT-T15 cells to ryanodine (Fig. 6D).

## DISCUSSION

The precise mechanism by which cAMP-elevating agents such as GLP-1 regulate  $Ca^{2+}$  signaling and insulin secretion in  $\beta$ -cells continues to be a topic of considerable controversy. Here we present findings in support of an important role for the type 2 isoform of ryanodine receptor as a mediator of intracellular  $Ca^{2+}$  signaling activated by GLP-1(7–37). RT-PCR analyses of rodent islet cells and insulinoma cell lines indicated a preferential expression of RYR2 in comparison to RYR1 or RYR3. These receptors were visualized by fluorescence microscopy in fixed, permeabilized human, mouse, and rat  $\beta$ -cells using the BODIPY-FL-X ryanodine fluorophore. Functional studies also demonstrated that application of ryanodine to  $\beta$ -cells blocked the fast transient increase of  $[Ca^{2+}]_i$  observed in response to GLP-1(7–37). Taken together, these findings appear to substantiate an important role for ryanodine receptors as mediators of GLP-1 signal transduction.

*Evidence for Expression of Ryanodine Receptors in Pancreatic  $\beta$ -Cells*—Islam and colleagues have used the RNase protection assay to detect RYR2 mRNA in *ob/ob* mouse islets and BTC3 cells (56). The riboprobes used in that study were derived by *in vitro* transcription of RYR cDNAs equivalent to partial sequences of RYRs 1–3 from mouse (42, 43). These sequences were used in the present study to identify RYR-specific PCR primers for RT-PCR analyses of rat islets. Our findings complement those of Islam and co-workers, whereby we demon-



**FIG. 6. Sensitization of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release by caffeine.** Caffeine (10 mM) and KCl (44 mM) were applied to individual HIT-T15 cells from micropipettes as indicated by bars. The extracellular solutions contained 0.8 mM glucose. **A**, potentiation of the caffeine-induced rise of  $[\text{Ca}^{2+}]_i$  by prior stimulation with KCl. An initial application of caffeine produced a small transient increase of  $[\text{Ca}^{2+}]_i$  (bar labeled 1) that diminished in size during repeated administration of caffeine (bars 4, 7, and 10). Application of KCl produced a rise of  $[\text{Ca}^{2+}]_i$  that was repeatable (bars 2, 5, and 8). A larger increase of  $[\text{Ca}^{2+}]_i$  was observed when caffeine was applied immediately prior to recovery of the response to KCl (bars 3, 6, and 9). This interaction of caffeine and KCl tended not to exhibit tachyphylaxis. **B**, positive correlation of  $[\text{Ca}^{2+}]_i$  measured prior to and during application of caffeine. Elevated basal  $[\text{Ca}^{2+}]_i$  favored a larger increase of  $[\text{Ca}^{2+}]_i$  in response to caffeine ( $n = 12$  cells). **C**, voltage clamp analysis of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. Caffeine produced a transient increase of  $[\text{Ca}^{2+}]_i$  when the membrane potential was maintained at  $-70$  mV (bars 1 and 3). A gradual increase of  $[\text{Ca}^{2+}]_i$  was observed when the membrane potential was shifted from  $-70$  to  $0$  mV for 100 ms at 2-s intervals (arrow with dots). This depolarization-induced rise of  $[\text{Ca}^{2+}]_i$  allowed for a much larger increase of  $[\text{Ca}^{2+}]_i$  in response to caffeine (bar 2). **D**, antagonism by ryanodine. The interaction of KCl and caffeine to produce an increase of  $[\text{Ca}^{2+}]_i$  was demonstrated (bars 1–3). Ryanodine (50  $\mu\text{M}$ ) was then administered via a superfusion system and cells were equilibrated at  $32^\circ\text{C}$  for 15 min. Ryanodine blocked the interaction of KCl and caffeine to produce a fast transient increase of  $[\text{Ca}^{2+}]_i$  (bars 4–6). The trace for  $[\text{Ca}^{2+}]_i$  was interrupted for  $\sim 300$  s during which a light was turned on in order to monitor the superfusion system.

strate expression of RYR2 mRNA corresponding to a region of the receptor that encompasses the transmembrane spanning domains as well as the carboxyl terminus. Takasawa and colleagues have also used RT-PCR to detect RYR2 mRNA in islets obtained from C57BL/6J mice but not *ob/ob* mice or RINm5F insulinoma cells (33). It was proposed that the *ob/ob* model of diabetes/obesity is characterized by a defect of  $\text{Ca}^{2+}$  signaling such that levels of RYR2 protein are reduced (33). Should this prove to be the case, it may provide an explanation for the failure of previous studies to detect functional expression of ryanodine receptors in *ob/ob* mouse islets (35, 37, 51–53) or RINm5F cells (Refs. 34, 36, and 50, but see Ref. 48).

Interpretation of findings obtained by RNase protection or RT-PCR analysis is complicated by uncertainties concerning whether or not there is functional expression of ryanodine receptor protein. Therefore, we tested for expression of ryanodine receptor binding sites by fluorescence microscopy using the BODIPY FL-X ryanodine fluorophore. Specific high affinity binding of BODIPY FL-X ryanodine was observed in human islet, rat islet, and C57BL/6J mouse islet cells. The binding was detected in permeabilized cells, was found to be saturable and reversible, and was blocked by pretreatment of cells with non-fluorescent ryanodine. Not all cells were labeled with BODIPY FL-X ryanodine, but those that did included  $\beta$ -cells that stained positively with anti-insulin antiserum. Therefore, BODIPY FL-X ryanodine might serve as a useful fluorescent probe for future studies examining the subcellular distribution of ryanodine receptors in organellar structures of the  $\beta$ -cell.

**Caffeine as a Probe for  $\text{Ca}^{2+}$  Signaling Mediated by Ryanodine Receptors**—It is well established that  $\text{IP}_3$  receptors play a highly significant role in the process by which hormones and neurotransmitters mobilize  $\text{Ca}^{2+}$  stores in the  $\beta$ -cell (57–60). It is also known that it is the type 3 isoform of the  $\text{IP}_3$  receptor

that is expressed at high levels in this cell type (61). Much less well understood is the functional significance of  $\text{IP}_3$ -insensitive  $\text{Ca}^{2+}$  stores. An  $\text{IP}_3$ -insensitive non-mitochondrial source of  $\text{Ca}^{2+}$  was observed by Islam and co-workers to be mobilized in a synergistic fashion during treatment of RINm5F cells with thimerosal and caffeine (50). Although thimerosal and caffeine will potentiate CICR from ryanodine-sensitive  $\text{Ca}^{2+}$  stores, the mechanism by which caffeine exerts its action in RINm5F cells was called into question. Studies of *ob/ob* mouse  $\beta$ -cells failed to demonstrate a caffeine-sensitive source of intracellular  $\text{Ca}^{2+}$  (35, 51, 52). It was argued instead that caffeine raised  $[\text{Ca}^{2+}]_i$  not by stimulatory effects on ryanodine receptors, but by inhibitory actions on K-ATP, whereby cellular depolarization produced influx of  $\text{Ca}^{2+}$  via VDCCs (51).

The findings of the present study contradict several of these assertions and provide new evidence for an important role of ryanodine receptors in  $\beta$ -cell  $\text{Ca}^{2+}$  signaling. We observed GLP-1 and caffeine to be effective under conditions of voltage clamp, producing a fast transient increase of  $[\text{Ca}^{2+}]_i$  in a manner independent of K-ATP (Figs. 4D and 6C). As expected for a process of CICR, the actions of ryanodine and caffeine were shown to be facilitated by KCl-induced depolarization (Figs. 5D and 6A) or by stepwise changes of membrane potential (Fig. 6C). A positive correlation was also established linking an increase of basal  $[\text{Ca}^{2+}]_i$  to an increased responsiveness of HIT-T15 cells to caffeine (Fig. 6B). Most importantly, the actions of GLP-1 and caffeine were found to be blocked by ryanodine (Figs. 5D and 6D). Such observations provide compelling evidence for a ryanodine- and caffeine-sensitive source of intracellular  $\text{Ca}^{2+}$  that is mobilized as a consequence of CICR, a process activated in a *direct* manner by GLP-1. Notably, these findings differ from those of Islam and co-workers (56), who



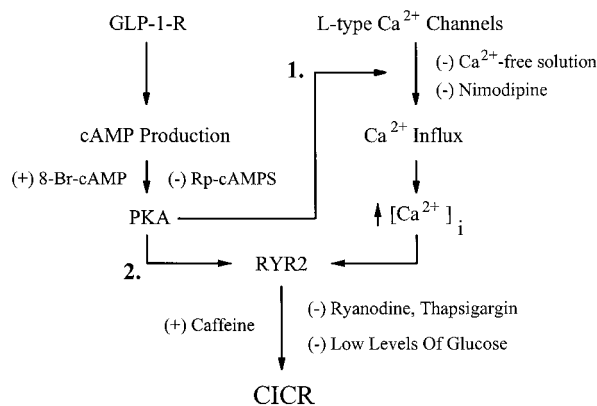


FIG. 7. A model to explain stimulatory actions of GLP-1 on  $\beta$ -cell  $\text{Ca}^{2+}$  signaling. GLP-1 stimulates the production of cAMP and activation of PKA. This effect is mimicked by 8-Br-cAMP and blocked by (Rp)-cAMPS. PKA-mediated phosphorylation promotes the opening of L-type voltage-dependent  $\text{Ca}^{2+}$  channels and influx of  $\text{Ca}^{2+}$  (step 1). This step is blocked by a  $\text{Ca}^{2+}$ -free extracellular solution or by treatment with nimodipine. PKA also sensitizes the type 2 isoform of ryanodine receptor (RYR2) to stimulatory effects of cytosolic  $\text{Ca}^{2+}$  (step 2). Steps 1 and 2 summate to trigger CICR. The process of CICR is initiated by caffeine and is blocked by ryanodine, thapsigargin, or an extracellular solution containing a low concentration of glucose.

observed no such direct stimulatory action of cAMP to release  $\text{Ca}^{2+}$  stores in mouse *ob/ob*  $\beta$ -cells.

**An Interaction of cAMP, L-type VDCCs, and Ryanodine Receptors to Regulate CICR**—We report that GLP-1 stimulates a fast transient increase of  $[\text{Ca}^{2+}]_i$  under conditions in which  $\beta$ -cells are equilibrated in a normoglycemic but not hypoglycemic concentration of glucose. This action of the peptide is mediated by receptors, the pharmacological properties of which match those of the GLP-1 receptor cloned from rat islets. Analyses of the signal transduction mechanism stimulated by GLP-1 demonstrates that its effects on  $[\text{Ca}^{2+}]_i$  are mediated by cAMP. The action of GLP-1 is blocked by the cAMP antagonist (Rp)-cAMPS, and is mimicked by the agonist 8-Br-cAMP. Evidence is also presented implicating two distinct types of  $\text{Ca}^{2+}$  channels as targets of the GLP-1 signaling system. Treatments that targeted L-type  $\text{Ca}^{2+}$  channels (nimodipine) or  $\text{Ca}^{2+}$  release channels (ryanodine) render GLP-1 ineffective. Finally, the source of  $\text{Ca}^{2+}$  mobilized by GLP-1 is shown to be depleted by thapsigargin, thereby implicating the SERCA class of  $\text{Ca}^{2+}$  ATPases in its regulation.

These findings are interpreted in the model illustrated in Fig. 7, which shares important features with previous models (20, 56, 69). GLP-1 stimulates production of cAMP and activation of PKA, thereby exerting a dual effect. The hormone augments influx of  $\text{Ca}^{2+}$  and an increase of  $[\text{Ca}^{2+}]_i$  by promoting the opening of L-type  $\text{Ca}^{2+}$  channels (step 1). It also sensitizes RYR2 to stimulatory influences of cytosolic  $\text{Ca}^{2+}$ , thereby facilitating CICR from a  $\text{Ca}^{2+}$  store, the filling state of which is maintained by metabolism of glucose (step 2). The fast transient increase of  $[\text{Ca}^{2+}]_i$  that results is measured in cells voltage clamped at  $-50$  mV but not  $-80$  mV because GLP-1 promotes partial activation of the L-type  $\text{Ca}^{2+}$  current, thereby allowing the opening of  $\text{Ca}^{2+}$  channels at a membrane potential ( $-50$  mV) slightly less negative than the resting potential. This influx of  $\text{Ca}^{2+}$  is responsible for CICR mediated by RYR2 sensitized to the stimulatory effects of cytosolic  $\text{Ca}^{2+}$ . Such a model is consistent with studies demonstrating that PKA facilitates permeation by  $\text{Ca}^{2+}$  of L-type  $\text{Ca}^{2+}$  channels (62) and ryanodine receptors (63, 64).

It should be emphasized that the model presented in Fig. 7 does not rule out a role for  $\text{IP}_3$  receptors as mediators of GLP-1 signal transduction. GLP-1 has a small stimulatory effect on

inositol phosphate production in rat islets (24, 28), and cAMP enhances  $\text{Ca}^{2+}$  release from  $\text{IP}_3$ -sensitive stores in hepatocytes (65, 66). Furthermore, permeation by  $\text{Ca}^{2+}$  of the type 1  $\text{IP}_3$  receptor is facilitated by PKA (67), although such an effect has yet to be demonstrated for the type 3  $\text{IP}_3$  receptor expressed in  $\beta$ -cells. The model also does not take into account actions of GLP-1 to induce a sustained increase of  $[\text{Ca}^{2+}]_i$ , an effect upon which the transient response may or may not be superimposed (cf. Fig. 4, B and C). We propose that such sustained actions of GLP-1 result from its inhibitory effect on ATP-sensitive  $\text{K}^+$  channels (13) and/or its stimulatory action on nonselective cation channels (14, 25).

**Conclusion**—It is now established that influx of  $\text{Ca}^{2+}$  through L-type  $\text{Ca}^{2+}$  channels produces an increase of  $[\text{Ca}^{2+}]_i$  that serves as a powerful trigger for secretion of insulin. Exocytosis occurs in localized patches of  $\beta$ -cell plasma membrane where secretory granules co-localize with L-type  $\text{Ca}^{2+}$  channels (68). Influx of  $\text{Ca}^{2+}$  through these channels triggers exocytosis by producing a fast transient increase of  $[\text{Ca}^{2+}]_i$  that is spatially restricted (68). The efficacy of  $\text{Ca}^{2+}$  as a trigger for exocytosis is limited by processes ( $\text{Ca}^{2+}$  buffering, extrusion, re-uptake) that maintain  $[\text{Ca}^{2+}]_i$  at a level subthreshold for secretion. By facilitating  $\text{Ca}^{2+}$  influx and CICR, GLP-1 may allow for a localized and explosive increase of  $[\text{Ca}^{2+}]_i$  that triggers secretion in a highly efficient manner. Therefore, the functional significance of CICR initiated by GLP-1 in the  $\beta$ -cell may be that it serves to amplify any increase of  $[\text{Ca}^{2+}]_i$  arising from entry of  $\text{Ca}^{2+}$  via the L-type  $\text{Ca}^{2+}$  channels. In this manner, GLP-1 might stimulate two interrelated and closely coupled processes ( $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  mobilization) that determine whether or not an increase of  $[\text{Ca}^{2+}]_i$  is sufficient to reach threshold for initiation of exocytosis.

## REFERENCES

- Nathan, D. M., Schreiber, E., Mojsos, S., and Habener, J. F. (1992) *Diabetes Care* **15**, 270–276
- Nauck, M. A., Holst, J. J., Willms, B., and Schmiegel, W. (1997) *Exp. Clin. Endocrinol. Diabet.* **105**, 187–195
- Fehmann, H.-C., Goke, R., and Goke, B. (1995) *Endocr. Rev.* **16**, 390–410
- Drucker, D. J. (1998) *Diabetes* **47**, 159–169
- Kreyman, B., Williams, G., Ghatei, M. A., and Bloom S. R. (1987) *Lancet* **2**, 1300–1303
- Thorens, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8641–8645
- Holz, G. G., and Habener, J. F. (1992) *Trends Biochem. Sci.* **17**, 388–393
- Leech, C. A., Holz, G. G., and Habener, J. F. (1996) *Annals N. Y. Acad. Sci.* **805**, 81–93
- Gromada, J., Holst, J. J., and Rorsman, P. (1998) *Pflugers Arch.* **435**, 583–594
- Gromada, J., Ding, W.-G., Barg, S., Renstrom, E., and Rorsman, P. (1997) *Pflugers Arch.* **434**, 515–524
- Gromada, J., Bokvist, K., Ding, W.-G., Holst, J. J., Nielsen, J. H., and Rorsman, P. (1998) *Diabetes* **47**, 57–65
- Komatsu, M., Schermerhorn, T., Aizawa, T., and Sharp, G. W. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10728–10732
- Holz, G. G., Kuhlreiber, W. M., and Habener, J. F. (1993) *Nature* **361**, 362–365
- Holz, G. G., Leech, C. A., and Habener, J. F. (1995) *J. Biol. Chem.* **270**, 17749–17757
- Kato, M., Ma, H.-T., and Tatemoto, K. (1996) *Regul. Pept.* **62**, 23–27
- Lu, M., Wheeler, M. B., Leng, X. H., and Boyd, A. E., III (1993) *Endocrinology* **132**, 94–100
- Fridolf, T., and Ahren, B. (1993) *Mol. Cell. Endocrinol.* **96**, 85–90
- Yada, T., Itoh, K., and Nakata, M. (1993) *Endocrinology* **133**, 1685–1692
- Cullinan, C. A., Brady, E. J., Saperstein, R., and Leibowitz, M. D. (1994) *Cell Calcium* **15**, 391–400
- Gromada, J. S., Dissing, K., Bokvist, K., Renstrom, E., Frokjaer-Jensen, J., Wulff, B. S., and Rorsman, P. (1995) *Diabetes* **44**, 767–774
- Rajan, A. S., Hill, R. S., and Boyd, A. E., III (1989) *Diabetes* **38**, 874–880
- Grapengeter, E., Gylfe, E., and Hellman, B. (1991) *J. Biol. Chem.* **266**, 12207–12210
- Wang, J. L., Corbett, J. A., Marshall, C. A., and McDaniel, M. L. (1993) *J. Biol. Chem.* **268**, 7785–7791
- Fridolf, T., and Ahren, B. (1991) *Diabetes Res.* **16**, 185–191
- Leech, C. A., and Habener, J. F. (1997) *J. Biol. Chem.* **272**, 17987–17993
- Wheeler, M. B., Lu, M., Dillon, J. S., Leng, X. H., Chen, C., and Boyd, A. E. (1993) *Endocrinology* **133**, 57–62
- Gromada, J., Anker, C., Bokvist, K., Knudsen, L. B., and Wahl, P. (1998) *FEBS Lett.* **425**, 277–280
- Zawalich, W. S., Zawalich, K. C., and Rasmussen, H. (1992) *Regul. Pept.* **44**, 277–283
- Gallone, A. (1993) *Science* **259**, 325–326
- Koguma, T., Takasawa, S., Tohgo, A., Karasawa, T., Furuya, Y., Yonekura, H.,

- and Okamoto, H. (1994) *Biochim. Biophys. Acta* **1223**, 160–162
31. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) *Science* **259**, 370–373
  32. Okamoto, H., Takasawa, S., and Nata, K. (1997) *Diabetologia* **40**, 1485–1491
  33. Takasawa, S., Akiyama, T., Nata, K., Kuroki, M., Tohgo, A., Noguchi, N., Kobayashi, S., Kata, I., Katada, T., and Okamoto, H. (1998) *J. Biol. Chem.* **273**, 2497–2500
  34. Islam, M. S., Larsson, O., and Berggren, P. O. (1993) *Science* **262**, 584–585
  35. Tengholm, A., Hagman, C., Gylfe, E., and Hellman, B. (1998) *Diabetes* **47**, 1224–1230
  36. Rutter, G. A., Theler, J. M., Li, G., Wollheim, C. B. (1994) *Cell Calcium* **16**, 71–80
  37. Webb, D. L., Islam, M. S., Efanov, A. M., Brown, G., Kohler, M., Larsson, O., and Berggren, P. O. (1996) *J. Biol. Chem.* **271**, 19074–19079
  38. Islam, M. S., and Berggren, P. O. (1997) *Diabetologia* **40**, 1480–1484
  39. Willmott, N. J., Galione, A., and Smith, P. A. (1995) *Cell Calcium* **18**, 411–419
  40. Malaisse, W. J., Kanda, Y., Inageda, K., Scruel, O., Sener, A., and Katada, T. (1997) *Biochem. Biophys. Res. Commun.* **231**, 546–548
  41. Lacy, P. E., and Kostianovsky, M. (1967) *Diabetes* **16**, 335–337
  42. Giannini, G., Conti, A., Mammarella, S., Scrobogna, M., and Sorrentino, V. (1995) *J. Cell Biol.* **128**, 893–904
  43. Ayabe, T., Kopf, G. S., and Schultz, R. M. (1995) *Development* **121**, 2233–2244
  44. McPherson, P. S., and Campbell, K. P. (1993) *J. Biol. Chem.* **268**, 13765–13768
  45. Otsu, K., Willard, H. F., Khanna, V. K., Zorato, F., Green, N. M., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 13472–13483
  46. Wollheim, C. B., and Sharp, G. W. G. (1981) *Physiol. Rev.* **61**, 914–973
  47. Chow, R. H., Lund, P. E., Loser, S., Panten, U., and Gylfe, E. (1995) *J. Physiol.* **485**, 607–617
  48. Chen, T. H., Lee, B., Yang, C., and Hsu, W. H. (1996) *Life Sci.* **58**, 983–990
  49. Willmott, N. J., Galione, A., and Smith, P. A. (1995) *FEBS Lett.* **371**, 99–104
  50. Islam, M. S., Rorsman, P., and Berggren, P. O. (1992) *FEBS Lett.* **296**, 287–291
  51. Islam, M. S., Larsson, O., Nilsson, T., and Berggren, P. O. (1995) *Biochem. J.* **306**, 679–686
  52. Liu, Y. J., Grapengiesser, E., Gylfe, E., and Hellman, B. (1996) *Arch. Biochem. Biophys.* **334**, 295–302
  53. Islam, M. S., Kindmark, H., Larsson, O., and Berggren, P. O. (1997) *Biochem. J.* **321**, 347–354
  54. Ehrlich, B. E., Kaftan, E., Bezprozvannaya, S., and Bezprozvannaya, I. (1994) *Trends Pharmacol. Sci.* **15**, 145–149
  55. Taniguchi, T., Yamada, Y., Yasuda, K., Kubota, A., Ihara, Y., Kagimoto, S., Kuroe, A., Watanabe, R., Inada, A., and Seino, Y. (1996) *Endocrinol. Metabol.* **3**, 135–138
  56. Islam, M. S., Leibiger, I., Leibiger, B., Rossi, D., Sorrentino, V., Ekstrom, T. J., Westerblad, H., Andrade, F. H., and Berggren, P. O. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6145–6150
  57. Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J., and Wollheim, C. B. (1984) *Nature* **309**, 562–564
  58. Wolf, B. A., Comens, P., Ackermann, K., Sherman, W. R., and McDaniel, M. L. (1985) *Biochem. J.* **227**, 965–969
  59. Ammala, C., Larsson, O., Berggren, P. O., Bokvist, K., Juntti-Berggren, L., Kindmark, H., and Rorsman, P. (1991) *Nature* **353**, 849–852
  60. Barker, C. J., Nilsson, T., Kirk, C. J., Mitchell, R. H., and Berggren, P. O. (1994) *Biochem. J.* **297**, 265–268
  61. Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) *J. Biol. Chem.* **268**, 11356–11363
  62. Trautwein, W., and Hescheler, J. (1990) *Annu. Rev. Physiol.* **52**, 257–274
  63. Hain, J., Onoue, H., Mayrleitner, M., Fleischer, S., and Schindler, H. (1995) *J. Biol. Chem.* **270**, 2074–2081
  64. Ozawa, T. (1998) *Biochem. Biophys. Res. Commun.* **246**, 422–425
  65. Burgess, G. M., Bird, G., Obie, J. F., and Putney, J. W., Jr. (1991) *J. Biol. Chem.* **266**, 4772–4781
  66. Nakada, S., Rhee, S. K., Hamanaka, H., and Mikoshiba, K. (1994) *J. Biol. Chem.* **269**, 6735–6742
  67. Chatton, J. V., Cao, Y., Liu, H., and Stucki, J. W. (1998) *Biochem. J.* **330**, 1411–1416
  68. Bokvist, K., Eliasson, L., Ammala, C., Renstrom, E., and Rorsman, P. (1995) *EMBO J.* **14**, 50–57
  69. Gromada, J., Dissing, S., and Rorsman, P. (1996) *Br. J. Pharmacol.* **118**, 769–775