RyR2 and Calpain-10 Delineate a Novel Apoptosis Pathway in Pancreatic Islets*

Cells are programmed to die when critical signaling and metabolic pathways are disrupted. Inhibiting the type 2 ryanodine receptor (RyR2) in human and mouse pancreatic β-cells markedly increased apoptosis. This mode of programmed cell death was not associated with robust caspase-3 activation prompting a search for an alternative mechanism. Increased calpain activity and calpain gene expression suggested a role for a calpain-dependent death pathway. Using a combination of pharmacological and genetic approaches, we demonstrated that the calpain-10 isofrom mediated ryanodine-induced apoptosis. Apoptosis induced by the fatty acid palmitate and by low glucose also required calpain-10. Ryanodine-induced calpain activation and apoptosis were reversed by glucagon-like peptide or calpain-10. Ryanodine-induced calpain activation and fatty acid palmitate and by low glucose also required specific intracellular Ca²⁺ influx during chronic hyperglycemia, high levels of free fatty acids, hypoxia or hypoglycemia, endoplasmic reticulum (ER) stress, and loss of growth factor signaling (1–12). Whether various inducers of apoptosis employ distinct molecular mechanisms has not been systematically studied. Intracellular Ca²⁺ stores play an important role in the regulation of apoptosis in many cell types (13, 14). The present study was undertaken to test the hypothesis that alterations in specific intracellular Ca²⁺ stores may induce apoptosis in pancreatic β-cells. There are at least three classes of intracellular Ca²⁺ stores in β-cells, and these are sensitive, respectively, to inositol trisphosphate (IP₃)/thapsigargin, nicotinic acid adenine dinucleotide phosphate, and cyclic ADP ribose/ryanodine (15–18). In many cell types, ryanodine receptor Ca²⁺ channels (RyR) transmit Ca²⁺ signals directly to closely associated mitochondria (19). In the MIN6 β-cell line, RyR were shown to regulate ATP production (20). Because of their role in regulating intracellular Ca²⁺ and mitochondrial function, we focused specifically on RyR as likely mediators of β-cell apoptosis. Of the three RyR subtypes, two have been reported to be present in β-cells, RyR1 and RyR2. The latter is more abundant and can be distinguished from the former by its insensitivity to dantrolene (21, 22). Ryanodine, a plant alkaloid, is the most specific probe for all RyR subtypes, and its activity is lost in RyR-deficient cells (23, 24).

In the present study, we examined the role of RyR in the survival of human and mouse pancreatic islets. We uncovered a novel apoptosis pathway that is initiated when Ca²⁺ flux through RyR2 is blocked. The mechanism of ryanodine-induced programmed cell death shares important features with palmitate-induced apoptosis, and both require activation of calpain-10, a type 2 diabetes susceptibility gene with previously unknown function.

The pancreatic β-cell plays a central role in the pathogenesis of diabetes mellitus. A reduction in β-cell mass mediated at least in part by an increase in apoptosis is characteristic of the diabetic state (1–3). It is becoming clear that several pathways can lead to β-cell apoptosis, including cytokine signaling, excessive Ca²⁺ influx during chronic hyperglycemia, high levels of free fatty acids, hypoxia or hypoglycemia, endoplasmic reticulum (ER) stress, and loss of growth factor signaling (1, 3–12). Whether various inducers of apoptosis employ distinct molecular mechanisms has not been systematically studied. Intracellular Ca²⁺ stores play an important role in the regulation of apoptosis in many cell types (13, 14). The present study was undertaken to test the hypothesis that alterations in specific intracellular Ca²⁺ stores may induce apoptosis in pancreatic β-cells. There are at least three classes of intracellular Ca²⁺ stores in β-cells, and these are sensitive, respectively, to inositol trisphosphate (IP₃)/thapsigargin, nicotinic acid adenine dinucleotide phosphate, and cyclic ADP ribose/ryanodine (15–18). In many cell types, ryanodine receptor Ca²⁺ channels (RyR) transmit Ca²⁺ signals directly to closely associated mitochondria (19). In the MIN6 β-cell line, RyR were shown to regulate ATP production (20). Because of their role in regulating intracellular Ca²⁺ and mitochondrial function, we focused specifically on RyR as likely mediators of β-cell apoptosis. Of the three RyR subtypes, two have been reported to be present in β-cells, RyR1 and RyR2. The latter is more abundant and can be distinguished from the former by its insensitivity to dantrolene (21, 22). Ryanodine, a plant alkaloid, is the most specific probe for all RyR subtypes, and its activity is lost in RyR-deficient cells (23, 24).

In the present study, we examined the role of RyR in the survival of human and mouse pancreatic islets. We uncovered a novel apoptosis pathway that is initiated when Ca²⁺ flux through RyR2 is blocked. The mechanism of ryanodine-induced programmed cell death shares important features with palmitate-induced apoptosis, and both require activation of calpain-10, a type 2 diabetes susceptibility gene.

MATERIALS AND METHODS

Reagents—High purity ryanodine, dantrolene, ALLM and DEVD-CHO were purchased from Calbiochem (La Jolla, CA) and kept as 1000× stocks in MeSO. Thapsigargin (Calbiochem) was dissolved in ethanol (ETOH). Palmitate was in dissolved ETOH/NaOH at 55 °C. Human glucagon-like-peptide 1 (GLP-1) was purchased from Peninsula (Belmont, CA). RPMI 1640 medium contained 10 mM glucose unless otherwise indicated. Mouse islets (1), and MIN6 cells (9) using RPMI 1640 media (with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin). RPMI 1640 medium contained 10 mM glucose unless otherwise indicated. Human glucagon-like-peptide 1 (GLP-1) was purchased from Peninsula (Belmont, CA). Fluo 488 or 596 were applied for 2 h. Controls using no primary antibody or no second antibody were negative for each experiment. A Fluoview™ laser scanning confocal microscope (Olympus, Melville, NY) was used for studies of RyR localization.

Cell Culture and Ca²⁺ Imaging—Human islets were obtained from the Washington University Human Islet Isolation Core Lab. Standard culturing methods were employed as described for human islets (16), mouse islets (1), and MIN6 cells (9) using RPMI 1640 media (with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin). RPMI 1640 medium contained 10 mM glucose unless otherwise indicated. Human and mouse islets were gently dispersed, loaded with 1 μM Fura-4F-AM for single-cell Ca²⁺ imaging studies as described (1, 16). Ringer’s solutions contained 3 mM glucose, unless otherwise indicated.

Caspase Expression and Activity—Calpain mRNA was measured semiquantitatively in 50 islets using reverse transcriptase-PCR with GAPDH and β-actin as internal standards, as described (1). Calpain activity was measured in intact mouse islets loaded with the fluorogenic...
calpain substrate, Boc-Leu-3-O-[3H]methyl-D-Met-CMAC [10 mmol/liter], as described previously (25, 26). Calpain activity was quantified by using the initial slope (0–10 min).

Measurement of Apoptosis—We used four independent methods to measure apoptosis in primary islets and MIN6 cells: namely, PCR-enhanced DNA laddering, ApoPercentage dye labelling, cell density/viability, and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). The PCR-enhanced DNA ladder analysis was adapted from the ApoAlert Kit (Clontech, Palo Alto, CA) to measure apoptosis in groups of 8–15 islets. This method is semi-quantitative and extremely sensitive (i.e., requires very little tissue). This approach uses adapter nucleotides and short PCR runs to selectively amplify DNA ladders. Briefly, after lysing islets at 55 °C for 15 min, genomic DNA was isolated by using the DNeasy kit (Qiagen, Valencia, CA), taking great care not to break the large strands of DNA; concentration was quantified by UV spectrophotometry. 200 ng of genomic DNA was ligated to adapters using T4 ligase (New England Biolabs, Beverly, MA) and heat-melted to form a single-stranded DNA ligation mixture. After denaturing PCR using specific oligonucleotides, products were separated on a gel in TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). DNA ladders were quantified as the amount intensity on an imaging plate using the AlphaImager 2000 System (Alpha Innotech, San Leandro, CA) and normalized to the intensity of the untreated/wild-type lane. For each gel, DNA ladder density was expressed as a percentage of untreated/wild-type lane. BTC scaffold density is expressed as a percentage of untreated/wild-type lane. BTC scaffold density is expressed as a percentage of untreated/wild-type lane.

Apoptosis in single isolated human or mouse islet cells was examined qualitatively using the ApoPercentage dye (Biocolor, Belfast, Northern Ireland), as described previously (1). This dye labels live cells bright pink that are undergoing phosphatidylserine translocation to the outer plasma membrane, a characteristic of apoptosis, but not necrosis. Non-apoptotic cells remain clear.

Cell death was measured quantitatively in cultures of MIN6 cells by measuring viability/cell density and by TUNEL analysis using the DeadEnd Colorimetric kit (Promega, Madison, WI) as described (1). The number of cells in each well and the number of TUNEL-positive cells were quantified using MetaMorph™ image analysis software. To further characterize MIN6 cell apoptosis, cells were stained with antibody recognizing the active, cleaved form of caspase-3 (Trevigen, Gaithersburg, MD). The unpaired test was used to test the significance of differences between groups. Differences were considered significant when p < 0.05. Results are presented as mean ± S.E.

RESULTS

Localization of Active RyR in Human and Mouse β-Cells—Immunofluorescence staining demonstrated the presence of RyR in a majority of human and mouse β-cells (Fig. 1A). Concanavalin studies of RyR localization showed a punctate and vesicular pattern of expression (Fig. 1B). Although previous studies have documented the presence of specific ryanodine-binding
sites and mRNA for RyR2 in human and rodent β-cells (17, 22), whether these Ca\(^{2+}\) channels are functional has not been directly tested. Nanomolar concentrations of ryanodine, which bind to the open RyR channel pore and increase the probability that the channel will open (27, 28), evoked an increase in cytosolic Ca\(^{2+}\) in β-cells from mouse islets (Fig. 1C) and human islets (18). This finding suggests that β-cell RyR are active under basal conditions. Micromolar concentrations of ryanodine, which inhibit the RyR, did not elevate cytosolic Ca\(^{2+}\) in unstimulated cells (Fig. 1D). These findings provide strong evidence that functional RyR are present in both human and mouse β-cells.

**Role of RyR in β-Cell Apoptosis**—We employed several approaches to determine whether ryanodine-induced apoptosis in cultured islets, including PCR-enhanced DNA ladder analysis, a sensitive technique for detecting DNA fragments generated during apoptosis but not necrosis (1). DNA ladders, reflecting the organized cleavage of DNA, were detected within 36 h after exposure of human islets (Fig. 2A) or mouse islets (see below) to a blocking concentration (10 μM) of ryanodine. In contrast,

---

**Fig. 2.** Inhibiting RyR2 induces apoptosis in human and mouse islets that is not caspase-3-dependent. A, DNA laddering was significantly greater than control in human islets cultured for 36 h in 10 μM ryanodine (252 ± 33% versus control, p < 0.05), but not 1 nm ryanodine (109 ± 6% versus control) or 10 μM dantrolene (115 ± 16% versus control) (n = 4). The average intensity (±S.E.) of the DNA ladders is indicated as a percentage of control islets. B, cell density analysis of MIN6 cells demonstrated a significant loss of cells in cultures treated with 10 μM ryanodine (71 ± 2% versus control, p < 0.05), 1 μM thapsigargin (44 ± 1% versus control, p < 0.05) or 250 μM palmitate (93 ± 3% versus control, p < 0.05; n = 3). A significant increase in red TUNEL-positive cell nuclei is also seen in these representative images. C, normalized to control, the percentage of active caspase-3-positive MIN6 cells (stained red), relative to the total number of cells in each treatment, was 129 ± 7% for ryanodine (p < 0.05), 167 ± 15% for thapsigargin (p < 0.05), and 232 ± 3% for palmitate (p < 0.05; n = 3). D, in ryanodine-treated mouse islets, the caspase-3 inhibitor DEVD-CHO (10 μM) did not block apoptosis (123 ± 22%; n = 3). DEVD-CHO reduced apoptosis 69 ± 11% (p < 0.05) in mouse islets treated with 1 μM thapsigargin (n = 3).
pancreas sections from wild-type (CastRIP mice was compared after 60-h culture in 10 mM glucose control conditions (95 ± 22% versus 88%), increased calpain activity over control (n = 8). B, effect of the calpain inhibitor ALLM on apoptosis under 10 mM glucose basal conditions (103 ± 22% versus no ALLM) and apoptosis induced by 60-h culture with 1 μM thapsigargin (98 ± 6% versus no ALLM), 10 μM ryanodine (49 ± 12% versus no ALLM, p < 0.05) or 250 μM palmitate (72 ± 7% versus no ALLM, p < 0.05, n = 4–6). C, transgene construct for mice that over-express calpastatin in β-cells (CastRIP). D, representative immunohistochemical staining using an antibody to human calpastatin in paraffin-embedded pancreas sections from wild-type (left panel) and CastRIP mice (right panel). No staining is seen in an islet (left panel, blue outline) from wild-type mice. E, reduced calpain activity in islets isolated from CastRIP mice. Asterisks denote significant difference (p < 0.05) from control. F, apoptosis in islets from CastRIP mice was compared after 60-h culture in 10 mM glucose control conditions (95 ± 11% versus wild type), 1 μM thapsigargin (102 ± 5% versus wild type), 10 μM ryanodine (55 ± 1% versus wild type, p < 0.05) or 250 μM palmitate (65 ± 8% versus wild type, p < 0.05) (n = 8).

activation of RyR with 1 nm ryanodine or inhibition of RyR1 with dantrolene did not induce significant apoptosis. We confirmed that 10 μM ryanodine induced apoptosis by measuring phosphatidylserine translocation in human β-cells (not shown), as well as by measuring a decrease in cell number and an increase in TUNEL-positive cells in the MIN6 cell line (Fig. 2B). Together, these results indicate that inhibition of basal Ca2⁺ release by thapsigargin or dantrolene-induced apoptosis but had no effect on thapsigargin-induced apoptosis or basal apoptosis (Fig. 3B). ALLM also blocked apoptosis induced by palmitate. A genetic approach was used to confirm the role of calpain in β-cell apoptosis. We generated transgenic mice that express calpastatin, an endogenous calpain inhibitor protein (30), exclusively in β-cells using the rat insulin promoter (CastRIP mice; Fig. 3C). Calpain activity was reduced more than 50% in islets from these mice (Fig. 3E), and this was associated with reductions in apoptosis induced by ryanodine or palmitate (Fig. 3F). The reduction in β-cell apoptosis with ALLM and calpastatin, both of which inhibit multiple isoforms of calpain, establishes an important role for a calpain-mediated pathway in this process.

Role of the Calpain System in β-Cell Apoptosis—Next, we sought to determine the specific isoform of calpain that is involved in ryanodine- and palmitate-induced apoptosis. Ry-
anodine increased calpain-10 mRNA in mouse islets by ~2.5-fold (Fig. 4, confirmed by real-time PCR, not shown), but had no significant effect upon calpain 1 and calpain 2 mRNA (not shown). Importantly, the ryanothine-induced increase in mRNA was an early event, evident by 12 h and lasting at least 5 days. To define the role of calpain-10 in β-cell apoptosis, we examined Capn10−/− islets from mice in which the calpain-10 gene had been deleted (Fig. 5A). Ryanothine failed to increase calpain activity in Capn10−/− islets (Fig. 5B). Ryanothine-induced apoptosis and palmitate-induced apoptosis were also prevented in Capn10−/− islets (Fig. 5C). Conversely, ryanothine-induced apoptosis was enhanced in islets from transgenic mice with β-cell-specific overexpression of human calpain-10 (Fig. 6, A–C, Capn10RIP). Capn10RIP islets showed enhanced calpain activity (Fig. 6D), suggesting that our assay measures the activity of calpain-10, in addition to other calpains. Together, the results strongly suggest that calpain-10 mediates apoptosis induced by inhibition of RyR2. The observation that apoptosis resulting from exposure to palmitate was inhibited in Capn10−/− islets and enhanced in Capn10RIP islets suggests that cell death induced by free fatty acids shares common steps with the RyR2 pathway.

Effects of GLP-1 on Calpain Activity and Apoptosis—We next tested whether agents known to promote β-cell survival may act by modulating the RyR2/calpain-10 pathway. GLP-1, a potent inhibitor of β-cell apoptosis (31), mobilizes intracellular Ca2+ and activates mitochondria via RyR (17, 20). GLP-1 decreased basal calpain activity and abolished ryanothine-induced calpain activation (Fig. 7A). GLP-1 also prevented apoptosis and cell loss in MIN6 cells treated with ryanothine (Fig. 7B). Similar results were seen with the GLP-1 receptor agonist, exendin-4 (not shown). Thus, the anti-apoptotic effects of GLP-1 may, in part, be due to activation of RyR2 and subsequent inhibition of calpain activity.

Interaction between Glucose and RyR2—The glucose level to which pancreatic islets are exposed is another important determinant of apoptosis (1, 4–6, 32). First, we wanted to determine the relationship between glucose signaling and RyR2. Surprisingly, ryanothine had significant effects on Ca2+ homeostasis during stimulation with glucose or KCl, in contrast to the situation in the basal conditions (3 mM glucose) described in Fig. 1. Concentrations of ryanothine that inhibit RyR potentiated the Ca2+ response to elevated glucose in a subpopulation of human or mouse β-cells (Fig. 8, A and B) and Ca2+ responses to 30 mM KCl in all cells (Fig. 8C), suggesting that ryanothine-sensitive
Ca²⁺ stores are involved in Ca²⁺ uptake under these conditions. In contrast, dantrolene evoked an immediate decrease in Ca²⁺ in stimulated β-cells, consistent with a role for RyR1 in Ca²⁺-induced Ca²⁺ release (CICR). Neither stimulating RyR with 1 mM ryanodine nor blocking IP₃R with xestospongin C had any effect upon depolarization-induced Ca²⁺ responses (18). These findings suggest that RyR2 play a novel non-CICR role during glucose-induced Ca²⁺-influx, whereas RyR1 mediates CICR in β-cells. Having established the link between glucose signaling and RyR2, we examined whether high glucose modulates calpain activity and protects islets from ryanodine-induced apoptosis, because short-term exposure (i.e., 2 days) to high glucose (25 mM) has been shown to inhibit β-cell apoptosis (33). This experiment would also shed some light on the possible mechanisms of ryanodine-induced apoptosis. Notably, if 10 μM ryanodine were causing apoptosis by augmenting CICR, 25 mM glucose would be expected to potentiate ryanodine-induced cell death. However, a 2-day culture of islets in 25 mM glucose blocked ryanodine-stimulated calpain activity and ryanodine-induced apoptosis (Fig. 9). Thus, the beneficial effects of short-term exposure to high glucose on β-cell survival may be mediated by a novel interaction with RyR2. Accordingly, we also examined the role of the RyR2/calpain pathway in cell death caused by a lowered rate of β-cell metabolism. Apoptosis induced by in vitro hypoglycemia (2 mM glucose for 60 h) was completely absent in 102–/– islets (Fig. 10A). On the other hand, apoptosis was enhanced in 102–/– islets incubated in moderately low glucose (5 mM) but not at 10 mM or 25 mM glucose (Fig. 10B). These results suggest that calpain-10 plays an important role in apoptosis induced by exposure to low glucose concentrations but not apoptosis induced by prolonged exposure to high glucose concentrations. Thus, the RyR2/calpain-10 death pathway is turned on when β-cell metabolic activity is low and turned off by stimuli that increase metabolic activity.

Roles of RyR2 and Calpain-10 in Apoptosis Induced by Chronic Hyperglycemia—Unlike the pro-survival effects of short incubations in 25 mM glucose, chronic stimulation (i.e., 7 days) with high glucose induces apoptosis (1, 32). Apoptosis induced by chronic (7 day) hyperglycemia was not affected by calpain-10 knockout (Fig. 10C) or calpastatin over-expression (102 ± 15% versus wt, n = 6), suggesting that apoptosis mediated by high glucose does not involve a calpain-10-dependent pathway. Like thapsigargin-induced apoptosis, glucose toxicity is known to involve caspase-3 (32). Therefore, the RyR2/calpain-10 apoptosis pathway is separate from other known β-cell apoptosis pathways.
Interestingly, inhibiting RyR protected β-cells against apoptosis induced by 7-day culture in high glucose (Fig. 10D). Therefore, we tested whether the protective effect of ryanodine on apoptosis induced by chronic stimulation may be mediated by blocking CICR through RyR1. Apoptosis induced by chronic exposure to high glucose was inhibited by dantrolene (Fig. 10E), implicating RyR1 and CICR in this process. A prominent role for dantrolene-sensitive RyR in excitotoxic neuronal cell death has been proposed (34).

**DISCUSSION**

The present studies were undertaken to assess the role of RyR in the survival of pancreatic β-cells and to determine the mechanism by which these Ca²⁺ channels regulate apoptosis. Our findings indicate that inhibiting the RyR2 is associated with increased apoptosis, suggesting that maintenance of normal basal Ca²⁺ flux through this channel is essential for β-cell survival. To our knowledge, these are the first results in any cell type to suggest that blocking RyR can induce apoptosis, although excessive RyR activity has been linked to cell death in other tissues, including the brain and heart (30, 35). That RyR2 may be a central molecule in the control of programmed cell death is perhaps not surprising. RyR are very large proteins that can directly sense cytosolic Ca²⁺, luminal Ca²⁺, ATP, redox potential, and nitric oxide (36), thus placing them in a key position to integrate multiple signals known to influence apoptosis. The general importance of RyR2 activity is underscored by the embryonic lethality of RyR2⁻/⁻ mice after embryonic day 9.5 (37). In contrast, no differences in islet morphology or β-cell ultrastructure were observed in mice lacking both RyR1 and RyR3 (38). Previous studies have reported that the expression of RyR2 is reduced in islets from several rodent models of diabetes (22). Together, these results suggest that RyR2 may be important in apoptosis in the β-cell and, therefore, be involved in the impairment of insulin secretion and the pathogenesis of diabetes.

Our results define a number of novel aspects of the mechanisms of apoptosis in the pancreatic β-cell. We have clearly demonstrated the existence of multiple pathways leading to β-cell apoptosis (Fig. 11). In the pancreatic β-cell, as least two major two apoptosis pathways are apparent, based upon their requirement for calpain-10 or caspase-3. Thapsigargin-induced ER stress and chronic hyperglycemia are known to be associated with the classical caspase-3-dependent pathway (5, 9). On the other hand, a novel calpain-10-dependent apoptosis pathway mediates cell death induced by ryanodine, hypoglycemia, and palmitate. Whether the pathways associated with these apoptotic stimuli involved additional molecules other than calpain-10 remains to be determined. Although we present evidence that RyR2 participates in glucose-induced Ca²⁺ signaling, the link between RyR2 and palmitate remains unclear. Although other calpain isoforms have been suggested to regulate cell death in other tissues, our results are the first to demonstrate that any calpain isoform is involved in apoptosis in primary β-cells. Previously, we have shown that prolonged...
The RyR2/calpain-10 pathway plays a critical role in type 2 diabetes susceptibility gene by linkage studies and positional cloning (39). The demonstration that a RyR2/calpain-10 pathway plays a critical role in β-cell survival suggests novel mechanisms for the pathophysiology of β-cell dysfunction in type 2 diabetes as well as novel targets for therapeutic intervention to preserve β-cell function. Our results may also provide a new framework for the investigation of potential mechanisms whereby alterations in Ca²⁺ handling by RyR may lead to cell death in other pathological states, such as Alzheimer’s disease, ischemia/reperfusion injury, and heart failure.

Acknowledgments—We thank the Juvenile Diabetes Research Foundation Human Islet Isolation Core and the Diabetes Research and Training Center at Washington University School of Medicine. We thank Eric Ford and Hung Tran for expert technical assistance.

REFERENCES
1. Johnson, J. D., Ahmed, N. T., Luciani, D. S., Han, Z., Tran, H., Fujita, J., Miser, S., Edlund, H., and Polonsky, K. S. (2003) J. Clin. Invest. 111, 1147–1160
2. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) Diabetes 52, 102–110
3. Mathis, D., Vence, L., and Benoist, C. (2001) Nature 414, 792–798
4. Federici, M., Hribal, M., Pan, C. Y., Matz, W., Mei, F., Zhou, Y. P., Sreenan, S. K., Otani, K., Hansen, P. A., Currie, K. P., Pan, C. Y., Lehmann, R., Candinas, D., Gassmann, M., and Weber, M. (2002) FASEB J. 16, 745–747
5. Shimabukuro, M., Zhou, Y. T., Levi, M., and Unger, R. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2498–2502
6. Zhou, Y. P., Teng, D., Draluyk, F., Ostrega, D., Roe, M. W., Philpous, L., and Polonsky, K. S. (1998) J. Clin. Invest. 101, 1623–1632
7. Araki, E., Oyadomari, S., and Mori, M. (2003) Intern. Med. 42, 7–14
8. Federici, M., Hribal, M., Pan, C. Y., Matz, W., Mei, F., Zhou, Y. P., Sreenan, S. K., Otani, K., Hansen, P. A., Currie, K. P., Pan, C. Y., Lehmann, R., Candinas, D., Gassmann, M., and Weber, M. (2002) FASEB J. 16, 745–747
9. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Surcinelli, M. D., Pozzan, T., and Korovsky, S. J. (2003) Science 300, 135–139
10. Johnson, J. D., and Chang, J. P. (2000) Biochem. Cell. Biol. 78, 217–240
11. Johnson, J. D., and Miser, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14566–14571
12. Holo, G. G., Lech, C. A., Heller, R. S., Castonguay, M., and Habener, J. F. (1999) J. Biol. Chem. 274, 14417–14456
13. Johnson, J. D., Kuang, S., Miser, S., and Polonsky, K. S. (2004) FASEB J. 18, 788–800
14. Pacher, P., Thomas, A. P., and Hajnoczky, G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2380–2385
15. Tsuchii, T., da Silva Xavier, G., Hohl, G. G., Jeannin, L. S., Thomas, A. P., and Rutter, G. A. (2003) Biochem. J. 369, 287–299
16. Zhao, F., Li, P., Chen, S. R., Liou, C. F., and Fruen, B. R. (2001) J. Biol. Chem. 276, 13810–13816
17. Islam, M. S. (2002) Diabetes 51, 1299–1309
18. Adachi, R., and Kagawa, H. (2003) Mol. Gen. Genet. 270, 797–806
19. Yang, H. T., Tweedie, D., Wang, S., Guia, A., Vinogradova, T., Bogdanov, K., Allen, P. D., Stern, M. D., Lakatta, E. G., and Rober, K. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9225–9230
20. Sreenan, S. K., Zhou, Y. P., Otani, K., Hansen, P. A., Currie, K. P., Pan, C. Y., Lee, J. P., Ostrega, D. M., Pan, C. Y., Horikawa, Y., Cox, N. J., Hanis, C. L., Burant, C. F., Fox, A. P., Bell, G. I., and Polonsky, K. S. (2003) Diabetes 53, 2103–2020
21. Zhou, Y. P., Sreenan, S., Pan, C. Y., Currie, K. P., Bindokas, V. P., Horikawa, Y., Lee, J. P., Ostrega, D. M., Pan, C. Y., Horikawa, Y., Cox, N. J., Hanis, C. L., Burant, C. F., Fox, A. P., Bell, G. I., and Polonsky, K. S. (2003) Diabetes 52, 528–534
22. Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M., and Poo, M. M. (2002) Nature 415, 83–88
23. Masuimya, H., Li, P., Zhang, L., and Chen, S. R. W. (2001) J. Biol. Chem. 276, 39727–39735
24. Mitchell, K. J., Pinton, P., Varadi, A., Taucchetti, C., Ainesov, E. K., Pozzan, T., Rizzuto, R., and Rutter, G. A. (2001) J. Cell Biol. 155, 41–51
25. Poll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) Physiol. Rev. 83, 731–801
26. Drucker, D. J. (2003) Mol. Endocrinol. 17, 161–171
27. Maeder, K., Spinaz, G., Lehmann, R., Sergeev, P., Weber, M., Fontana, A., Kaege, N., and Donath, M. Y. (2001) Diabetes 50, 1693–1699
28. Srinivasan, S., Bernal-Mizrachi, E., Ohashi, M., and Permutt, M. A. (2002) Am. J. Physiol. 283, E784–E793
29. Mody, I. and MacDonald, J. F. (1995) Trends Pharmacol. Sci. 16, 356–359
30. Marx, S. O., Reiken, S., Halsamut, Y., Tayaraman, T., Burkoff, D., Rosenblit, N., and Marks, A. R. (2000) Cell 101, 365–376
31. Fill, M., and Copello, J. A. (2002) Physiol. Rev. 82, 893–922
Additional References:

37. Takeshima, H., Komazaki, S., Hirose, K., Nishi, M., Noda, T., and Iino, M. (1998) *EMBO J.* **17**, 3309–3316
38. Komazaki, S., Ikemoto, T., Takeshima, H., Iino, M., Endo, M., and Nakamura, H. (1998) *Cell Tissue Res.* **294**, 467–473
39. Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E., del Bosque-Plata, L., Oda, Y., Yoshuchi, I., Cobilla, S., Polonsky, K. S., Wei, S., Concannon, P., Iwasaki, N., Schulze, J., Bauer, L. J., Bogardus, C., Groop, L., Boerwinkle, E., Hanis, C. L., and Bell, G. I. (2000) *Nat. Genet.* **26**, 163–175
40. Podesta, M., Pitto, A., Figari, O., Bacigalupo, A., Bruzzone, S., Guida, L., Franco, L., De Flora, A., and Zocchi, E. (2003) *FASEB J.* **17**, 310–312
41. Johnson, J. D., Wong, C. J., Yunker, W. K., and Chang, J. P. (2002) *Am. J. Physiol.* **282**, C635–C645
42. Johnson, J. D., Klausen, C., Habibi, H. R., and Chang, J. P. (2002) *Am. J. Physiol.* **282**, E810–E819
43. Johnson, J. D., and Chang, J. P. (2002) *J. Neuroendocrinol.* **14**, 144–155
