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A New Hypoglycemic Agent, JTT-608, Evokes Protein Kinase A-Mediated Ca^{2+} Signaling in Rat Islet β-Cells: Strict Regulation by Glucose, Link to Insulin Release, and Cooperation with Glucagon-Like Peptide-1(7-36)amide and Pituitary Adenylate Cyclase-Activating Polypeptide

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ABSTRACT

A new nonsulfonylurea oral hypoglycemic agent, JTT-608, has been reported to stimulate insulin release at elevated, but not low, glucose concentrations and consequently not to induce hypoglycemia in rats. Accordingly, this drug is potentially a safer antidiabetic agent than sulfonylureas. To explore the mechanisms underlying this glucose-dependent insulinotropism, the present study investigated the effects of JTT-608 on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and protein kinase A (PKA) activity in rat islet β-cells by microfluorometry using, respectively, fura-2 and a fluorescence PKA substrate, DR II. In the presence of glucose at normal and elevated concentrations (5.0–16.7 mM) JTT-608 (30–1000 μM) concentration dependently increased $[Ca^{2+}]_i$ in up to 88% of single β-cells, whereas at lower glucose concentrations (2.8 and 4.2 mM) it had little effect. The $[Ca^{2+}]_i$ responses were inhibited under Ca^{2+}-free conditions and by nitrendipine, an L-type Ca^{2+} channel blocker. JTT-608 rapidly activated PKA and a PKA inhibitor, H89, inhibited $[Ca^{2+}]_i$ responses to JTT-608. JTT-608 also stimulated insulin release from rat islets in a glucose- and Ca^{2+}-dependent manner. The glucose-unresponsive β-cells, which failed to respond to 8.3 mM glucose with increases in $[Ca^{2+}]_i$, were frequently recruited to $[Ca^{2+}]_i$ increases by JTT-608. JTT-608 also induced oscillations of $[Ca^{2+}]_i$. Glucagon-like peptide-1(7-36)amide (GLP-1), pituitary adenylate cyclase-activating polypeptide (PACAP), and acetylcholine (ACh) enhanced the action of JTT-608 on $[Ca^{2+}]_i$. In conclusion, JTT-608 evokes PKA-mediated Ca^{2+} influx and Ca^{2+} signaling in rat islet β-cells in a glucose-regulated manner, which may account for its glucose-dependent insulinotropism. JTT-608 and neurohormones may cooperatively activate islet β-cells under physiological conditions.

Type 2 diabetes is characteristically associated with impaired insulin secretion and/or insulin resistance (O'meara and Polonsky, 1994; Porte and Kahn, 1995). Sulfonylureas (SUs), such as tolbutamide, glibenclamide (glyburide), and glipizide have been widely used to treat patients with type 2 diabetes. They inhibit the β-cell ATP-sensitive K^+ (K_{ATP}) channel, a complex of the SU receptor and an inward rectifier K^+ channel (Inagaki et al., 1995), which leads to stimulation of insulin release and consequent reduction of blood glucose (Gylfe et al., 1984; Panten et al., 1992). However, SUs also have disadvantages: profound hypoglycemia resulting from a long-lasting and glucose-independent action to stimulate insulin release (Henquin, 1990), and the secondary failure to treatment with SUs (Groop et al., 1986), which may be due to the β-cell exhaustion resulting from prolonged stimulation (Greco et al., 1992). To minimize these disadvantages, non-SU oral hypoglycemic agents, including nateglinide (Fujitani and Yada, 1994; Hirose et al., 1994; Akiyoshi et al., 1995), KAD-1229 (Ohnota et al., 1994), and repaglinide (Gromada et al., 1995; Ladriere et al., 1997), members of the meglitinide family (Malaisse, 1995), have recently been developed. Their advantages are rapid and shorter lasting insulinotropic and hypoglycemic effects. These compounds,

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ABBREVIATIONS: SU, sulfonylurea; K_{ATP}, ATP-sensitive K^+; JTT-608, *trans*-4-(4-methylcyclohexyl)-4-oxobutyric acid; GLP-1, glucagon-like peptide-1(7-36)amide; ACh, acetylcholine; KRB, Krebs-Ringer bicarbonate buffer; PKA, protein kinase A; PACAP, pituitary adenylate cyclase-activating polypeptide; db-cAMP, dibutyryl adenosine-3′:5′-monophosphate; H89, *N*-[2-(*p*-bromocinnamylamine)ethyl]-5-isoquinoline-sulfonamide.

however, act on β-cells via similar mechanisms as SUs and potentially have an ability to stimulate insulin release at hypoglycemic states (Fujitani and Yada, 1994; Hirose et al., 1994; Ohnota et al., 1994; Akiyoshi et al., 1995; Gromada et al., 1995; Malaisse, 1995; Ladriere et al., 1997). Accordingly, a novel compound that activates β-cells to release insulin only at hyperglycemic states has been long awaited.

A new non-SU oral hypoglycemic agent, *trans*-4-(4-methylcyclohexyl)-4-oxobutyric acid (JTT-608), when administered before glucose loading, potentiates insulin release and improves glucose tolerance without causing a decrease in fasting glucose levels in normal rats, Goto-Kakizaki, and neonatally streptozotocin-treated type 2 diabetic rats (Shinkai et al., 1998; Ohta et al., 1999a,b). JTT-608 enhances insulin secretion only with glucose loading, whereas SUs enhance it irrespective of glucose levels (Ohta et al., 1999a,b). In isolated perfused pancreas from normal, neonatally streptozotocin-treated, and Goto-Kakizaki rats, JTT-608 enhances glucose-stimulated, but not basal, insulin secretion (Shinkai et al., 1998; Ohta et al., 1999a,b). In a mouse insulinoma cell line, MIN6 cells, JTT-608 potently enhances insulin secretion at elevated glucose levels without inhibiting the binding of [^{3}H]glibenclamide to membrane fractions (Furukawa et al., 1999), suggesting that JTT-608 enhances glucose-stimulated insulin secretion via a different mechanism from SUs. These studies on experimental animals suggest a possible beneficial role of JTT-608 in achievement of an improved glycemic control in patients with type 2 diabetes.

The glucose-dependent stimulation of insulin release by JTT-608 appears to account for its ability to correct hyperglycemia without inducing hypoglycemia. However, the mechanisms underlying the glucose-dependent action of JTT-608 are yet largely unknown. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) regulates insulin release in β-cells (Wollheim and Sharp, 1981; Ammala et al., 1993). The first aim of the present study was to examine whether JTT-608 increases $[Ca^{2+}]_i$ in β-cells and, if so, whether this process is tightly regulated by glucose and linked to insulin release. Furthermore, possible involvement of cAMP-PKA pathway in the production of $[Ca^{2+}]_i$ signals was investigated because this pathway has well been recognized as the potentiator of glucose-induced insulin release.

Oscillations of $[Ca^{2+}]_i$ in islet β-cells have been suggested to be involved in the pulsatile insulin secretion, which contributes to the physiological control of glucose metabolism (Matthews et al., 1983; O'rahilly et al., 1988; Hellman et al., 1992; Gilon et al., 1993; O'meara and Polonsky, 1994; Bergsten, 1995). Heterogeneity of pancreatic β-cells, with respect to electrical, metabolic, $[Ca^{2+}]_i$, and secretory responses to glucose, has been observed both in vitro and in vivo, and the existence of glucose-unresponsive β-cells has been evidenced (Pipeleers, 1987; Giordano et al., 1991; Holz et al., 1993; Yada et al., 1997). In type 2 diabetic animals, an increased fraction of glucose-unresponsive β-cells is suggested to partly account for the impaired glucose-induced insulin release (Tsuura et al., 1993; O'meara and Polonsky, 1994). Islet β-cells in vivo are perfused with physiological potentiators of insulin release, which include the intestinal peptide GLP-1(7-36)amide (Yada et al., 1993; Holz et al., 1995; Wang et al., 1995; Nauck, 1998), the pancreatic peptide PACAP38 (Yada et al., 1994, 1997; Filipsson et al., 1999), and the parasympathetic neurotransmitter ACh (Bergman and Miller, 1973; Garcia et al., 1988; Yada et al., 1995). The second aim of the present study was to examine whether JTT-608 could evoke $[Ca^{2+}]_i$ oscillations, recruit glucose-unresponsive β-cells into $[Ca^{2+}]_i$ increases, and cooperate with physiological neurohormones.

Materials and Methods

Preparation of Islets and Single β-Cells. Islets and single β-cells were prepared as previously reported (Yada et al., 1992, 1994, 1995). Briefly, islets of Langerhans were isolated from Wistar rats aged 10 to 16 weeks by collagenase digestion. Animals were anesthetized with an intraperitoneal injection of pentobarbitone at 80 mg/kg. The abdomen was opened, and collagenase (3 mg/ml) dissolved in 5 mM Ca^{2+}-containing Krebs-Ringer-bicarbonate buffer (KRB) was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The rats were killed by cervical dislocation. The pancreas was dissected out and incubated at 37°C for 17 min. Islets were hand-collected.

The isolated islets were dispersed into single cells by treatment with Ca^{2+}-free KRB with 0.1 mM EGTA. The single cells were plated on coverslips and maintained in a short-term culture for up to 2 days in Eagle's minimum essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 μU/ml penicillin at 37°C in a 95% air and 5% CO_2 atmosphere. The cells during this culture period responded to the test agents in a consistent manner. β-Cells were selected by immunostaining with antiserum against insulin or by the morphological and physiological criteria for these β-cells as reported previously (Yada et al., 1995, 1997).

Measurements of Insulin Release from Islets. Measurements of insulin release were carried out as previously described (Yada et al., 1994, 1997). Briefly, groups of nine isolated islets were first incubated for 30 min in KRB containing 2.8 mM glucose for stabilization. Islets were then incubated at 37°C for 30 min in 1 ml of KRB. Insulin concentration was determined by enzyme immunoassay using a kit (Morinaga, Yokohama, Japan).

Measurements of $[Ca^{2+}]_i$ in Single β-Cells. $[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorometry combined with digital imaging as previously reported (Yada et al., 1992, 1994). Briefly, cells on coverslips were loaded with fura-2 by incubation with 2 μM fura-2 acetoxymethylester in KRB containing 2.8 mM glucose for 30 min at 37°C. The cells were then mounted in a chamber and superfused with KRB at a rate of 1 ml/min at 37°C. The cells were excited at 340 and 380 nm alternately every 2.5 s, emission signals at 510 nm were detected with an intensified charge-coupled device camera, and ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to calibration curves (Yada et al., 1992).

Measurements of PKA Activity in Single β-Cells. PKA activity was measured using the newly developed fluorescence probe DR II according to the original report (Higashi et al., 1997) with slight modification. DR II is a fluorescence PKA substrate obtained by conjugating a fluorescence probe to a partial amino acid sequence of PKA regulatory domain II that contains a specific autophosphorylation site. Its fluorescence intensity was shown to decrease in response to a rise in intracellular cAMP concentration (Higashi et al., 1997). The single islet cells were incubated with 25 μg/ml DR II in KRB for 120 min at 23°C. The cells were superfused under the same condition as that for $[Ca^{2+}]_i$ measurements, excited at 380 nm every 5 s, the emission signal at 475 nm was detected with a cooled charge-coupled device camera, and digital images were produced by a HiSCA system (Hamamatsu Photonics).

Solutions and Chemicals. KRB was composed of 121.7 mM NaCl, 4.4 mM KCl, 1.2 mM KH_2PO_4, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.0 mM $NaHCO_3$, and 10 mM HEPES at pH 7.4 with NaOH supplemented with 0.1% bovine serum albumin.

JTT-608 was synthesized and provided by Japan Tobacco Inc., Central Pharmaceutical Research Institute (Osaka, Japan). Fura-2 and fura-2 acetoxymethylester were obtained from Molecular Probes (Eugene, OR). DR II was obtained from Dojin (Kumamoto, Japan). PACAP38 was purchased from Peptide Institute (Osaka, Japan). Fetal bovine serum was obtained from Life Technologies Inc. (New York, NY). Nitrendipine was a gift from Yoshitomi Pharmaceutical (Osaka, Japan). Dibutyryl adenosine-3′:5′-monophosphate (db-cAMP) was from Boehringer (Indianapolis, IN). H89 [*N*-[2-(*p*-bromocinnamylamine)ethyl]-5-isoquinoline-sulfonamide] was from Seikagaku Kogyo (Tokyo, Japan). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis. The calculated values were expressed as the mean ± S.E.M. (n = number of cells). The statistical analysis was carried out with the Student's t test. Differences were considered statistically significant when $P < 0.05$.

Results

Concentration- and Glucose-Dependent Effects of JTT-608 to Increase $[Ca^{2+}]_i$ in Single β-Cells. A rise in glucose concentrations from 2.8 to 8.3 mM increased $[Ca^{2+}]_i$ in a biphasic manner: an initial large increase followed by a moderate elevation that was occasionally superimposed with an oscillation (Fig. 1A). In the presence of 8.3 mM glucose, administration of JTT-608 at 300 μM, but not 30 μM, in the superfusion solution increased $[Ca^{2+}]_i$ in a rat pancreatic β-cell (Fig. 1A), thus showing a concentration-dependent effect. JTT-608 at 300 μM increased $[Ca^{2+}]_i$ in the presence of 8.3 mM but not 2.8 mM glucose (Fig. 1B). The mean amplitude of the $[Ca^{2+}]_i$ response to 300 μM JTT-608 at 8.3 mM glucose was 390 ± 15 nM (n = 31). The drug at a supraphar-macological concentration of 1000 μM induced a small increase in $[Ca^{2+}]_i$ [amplitude 63 ± 15 nM (n = 3)] only in 10% (3 of 31) of cells at 2.8 mM glucose, whereas at 8.3 mM glucose it induced a much larger increase in $[Ca^{2+}]_i$ [467 ± 16 nM (n = 15)] in 88% (15 of 17) of cells (Fig. 1C). The fraction of β-cells with $[Ca^{2+}]_i$ responses to JTT-608 is expressed as percentage (Fig. 2, A and B): at 8.3 and 16.7 mM glucose JTT-608 (30–1000 μM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner, whereas at 2.8 mM glucose only 1000 μM JTT-608 exerted a significant effect. In sharp contrast, a sulfonylurea tolbutamide at 30 μM increased $[Ca^{2+}]_i$ in 30% (3 of 10) of single β-cells at 2.8 mM glucose and in 55% (5 of 11) of cells in 8.3 mM glucose (Fig. 2B). Tolbutamide at 300 μM increased $[Ca^{2+}]_i$ in 94% (15 of 16) and 95% (19 of 20) of cells at 2.8 and 8.3 mM glucose, respectively (Fig. 2B). Thus, the effect of tolbutamide on $[Ca^{2+}]_i$ was only loosely related to the glucose concentration, whereas that of JTT-608 was tightly glucose-dependent.

These results indicated that the ability of JTT-608 to increase $[Ca^{2+}]_i$ requires the glucose concentration to be elevated to a critical level between 2.8 and 8.3 mM. Then the critical level of glucose concentration above which JTT-608 exerts its effect on $[Ca^{2+}]_i$ was studied. At 4.2 mM glucose JTT-608 had little effect on $[Ca^{2+}]_i$, whereas it increased $[Ca^{2+}]_i$ once glucose concentration was raised to 5.0 mM in the same cell (Fig. 3A), and it occurred in 9% of β-cells (Fig. 3B). A small rise in glucose concentration to 5.6 mM dramatically increased the fraction of β-cells that responded to JTT-608 (29%) (Fig. 3B). Thus, the level of 5.0 to 5.6 mM appears to be the threshold concentration above which glucose supports the action of JTT-608 on $[Ca^{2+}]_i$.

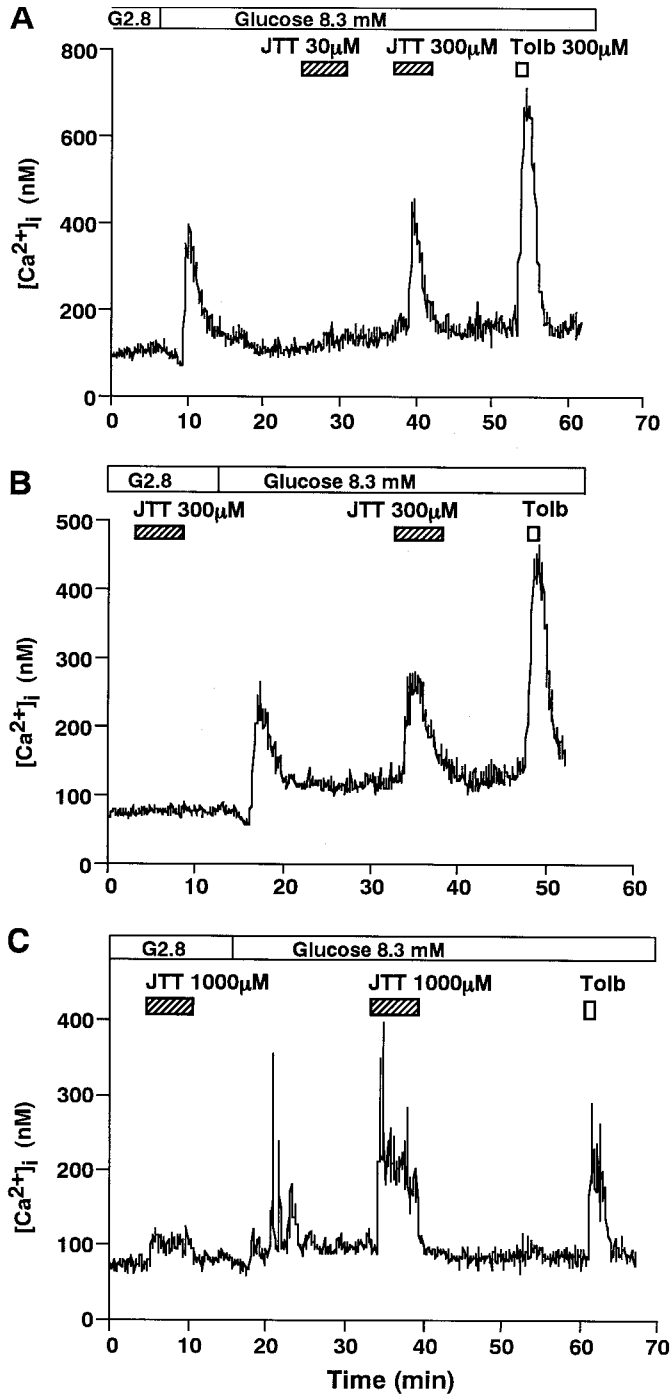

Fig. 1. Concentration- and glucose-dependent effects of JTT-608 (JTT) to increase $[Ca^{2+}]_i$ in rat single β-cells. A, in the presence of 8.3 mM glucose, JTT concentration dependently increased $[Ca^{2+}]_i$ in a single β-cell that also responded to 8.3 mM glucose and 300 μM tolbutamide (Tolb). B, JTT at 300 μM increased $[Ca^{2+}]_i$ in a single β-cell in the presence of 8.3 mM (G8.3), but not 2.8 mM, glucose. C, JTT at high concentration of 1000 μM increased $[Ca^{2+}]_i$ in both 2.8 and 8.3 mM glucose, and the amplitude of the $[Ca^{2+}]_i$ increase was much smaller in 2.8 mM glucose. Among 17 cells examined, three cells responded to 1000 μM JTT at both 2.8 and 8.3 mM glucose, whereas 14 cells responded to JTT only at 8.3 mM glucose. $[Ca^{2+}]_i$ was measured under superfusion conditions. The results shown are representative of 10 cells from three independent experiments in A, 59 from five in B, and three from two in C.

Inhibition of JTT-608-Induced $[Ca^{2+}]_i$ Increase under a Ca^{2+}-Free Condition and by a Ca^{2+} Channel Blocker. In a Ca^{2+}-free condition achieved in KRB with 0.1

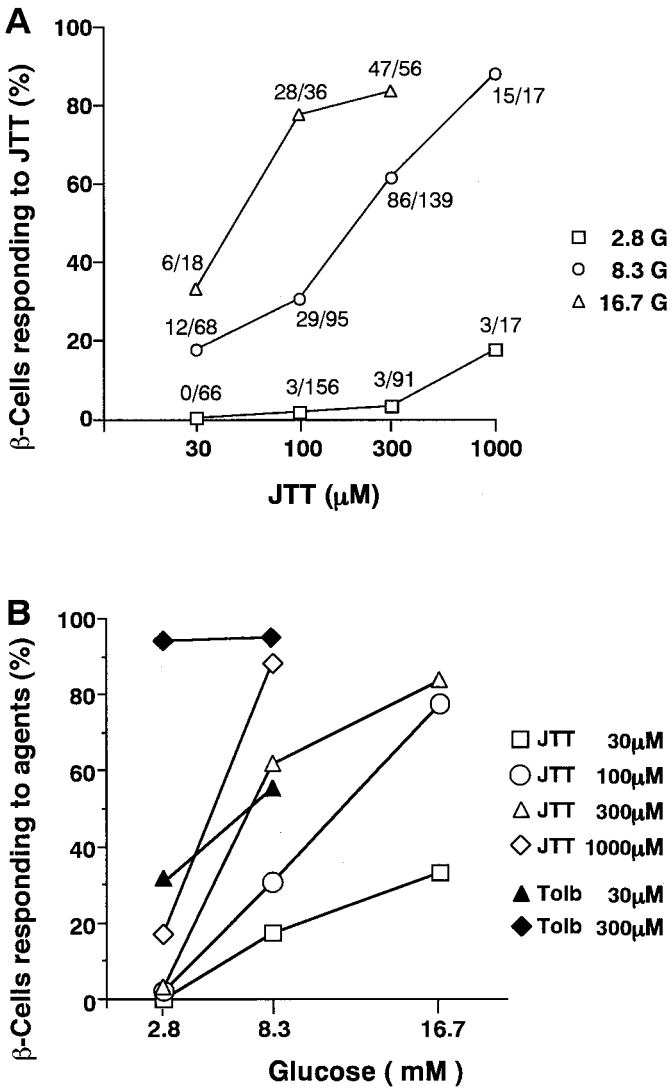

Fig. 2. Percentage of β-cells that responded to JTT and Tolb with increases in $[Ca^{2+}]_i$ is expressed as a function of the concentration of JTT in the presence of 2.8, 8.3, and 16.7 mM glucose (A) and as a function of glucose concentration with 30 to 1000 μM JTT (B). Numbers near each point indicate the number of cells responded over that examined.

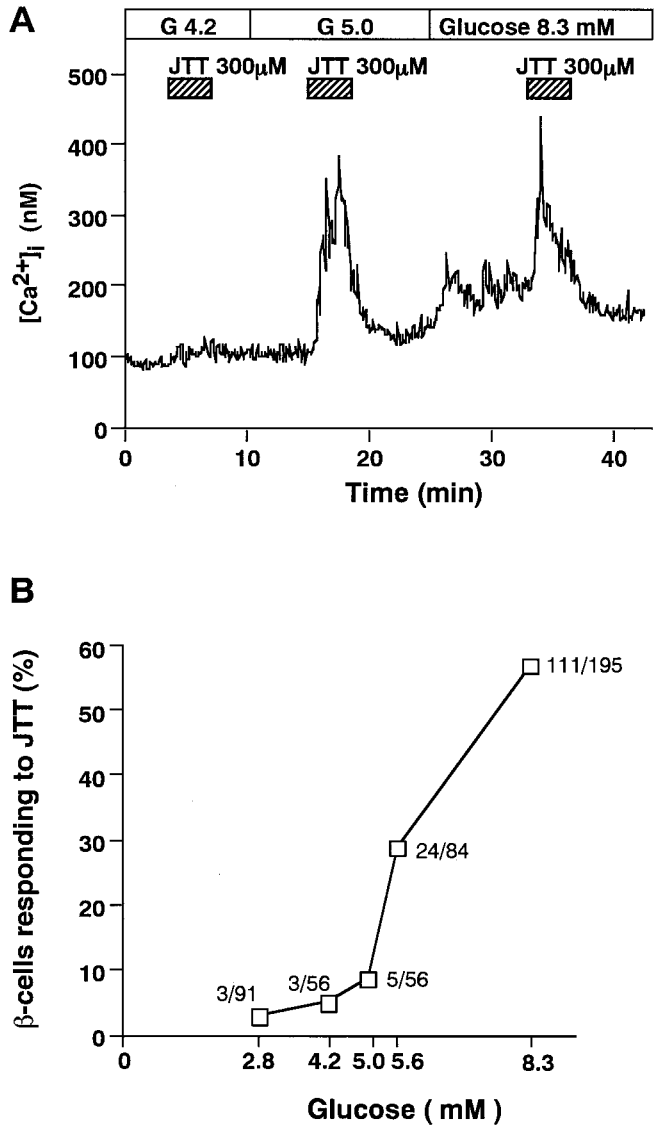

Fig. 3. Small changes in glucose concentration around 5 mM turn on and off the effect of JTT to increase $[Ca^{2+}]_i$. A, at 4.2 mM glucose JTT failed to significantly increase $[Ca^{2+}]_i$, whereas it increased $[Ca^{2+}]_i$ once glucose concentration was raised to 5.0 mM, as well as 8.3 mM. The results shown are representative of five cells from two independent experiments. B, percentage of β-cells that responded to 300 μM JTT with increases in $[Ca^{2+}]_i$ is expressed as a function of glucose concentration. Numbers near each point indicate the number of cells responded over that examined.

mM EGTA and no added Ca^{2+}, 300 μM JTT-608 failed to increase $[Ca^{2+}]_i$, and after bringing 1 mM Ca^{2+} back, the response to JTT-608 was observed (n = 7) (Fig. 4A). In the presence of 10 μM nitrendipine, a blocker of the L-type Ca^{2+} channel in β-cells, the $[Ca^{2+}]_i$ response to JTT-608 was completely inhibited in a reversible manner (n = 13) (Fig. 4B).

JTT-608 Activates PKA, a PKA Inhibitor Blocks the Effect of JTT-608, and a PKA Activator Mimics JTT-608 in Increasing $[Ca^{2+}]_i$ in Single β-Cells. In the presence of 8.3 mM glucose, administration of an adenylate cyclase activator, forskolin (10 μM), decreased the DR II fluorescence in a single β-cell (Fig. 5A). Administration of 300 μM JTT-608 also evoked a rapid decrease in the DR II fluorescence in a single β-cell, in a manner similar to that induced by forskolin (Fig. 5B). The reduction of DR II fluorescence indicates an activation of PKA (Higashi et al., 1997).

Whether the activation of PKA is linked to the increase in $[Ca^{2+}]_i$ in response to JTT-608 was examined. In the β-cells that had been pretreated for 120 min with a specific PKA inhibitor, H89 (60 μM), the $[Ca^{2+}]_i$ increase in response to db-cAMP was markedly inhibited (Fig. 5C): it was obtained only in 3 of 22 cells (14%), whereas it was observed in 9 of 13 cells (70%) under control conditions. Furthermore, $[Ca^{2+}]_i$ responses to tolbutamide were unaltered with the H89 treatment (Fig. 5C), verifying that the inhibition by H89 was specific for the cAMP-PKA pathway. Under these conditions, $[Ca^{2+}]_i$ responses to JTT-608 were markedly inhibited (Fig. 5C) and they were obtained only in 5 of 22 β-cells (23%).

Furthermore, db-cAMP increased $[Ca^{2+}]_i$ in a strictly glucose-dependent manner (Fig. 5, D and E), and it was completely blocked by nitrendipine (Fig. 5E) as previously reported (Yada et al., 1993; Yaekura et al., 1996). Thus, db-cAMP strikingly mimicked JTT-608.

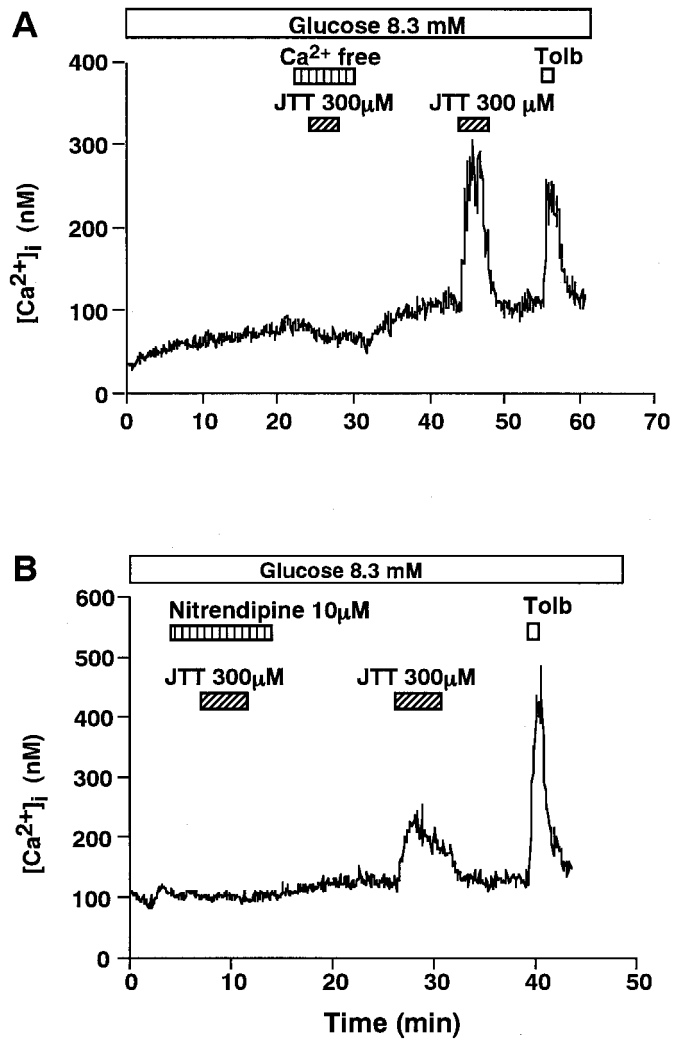

Fig. 4. Inhibition of $[Ca^{2+}]_i$ responses to 300 μM JTT under a Ca^{2+}-free condition (A) and by 10 μM nitrendipine (B). The Ca^{2+}-free condition was achieved in KRB with 0.1 mM EGTA and no added Ca^{2+}. Recovery of response was obtained after restoring 2 mM Ca^{2+} and upon washing out nitrendipine. The cells subsequently responded to Tolb. Glucose concentration is 8.3 mM. The results shown are representative of seven cells from two independent experiments in A and 13 from two in B.

JTT-608 Glucose Dependently Stimulates Insulin Release from Islets and Its Inhibition under a Ca^{2+}-Free Condition. Insulin release from isolated islets under static incubation was stimulated by 8.3 mM glucose. The glucose-stimulated insulin release was further increased by 300 μM JTT-608 significantly ($P < 0.01$), whereas it had no effect on the basal insulin release at 2.8 mM glucose (Fig. 6). Next, to examine the role of extracellular Ca^{2+} under conditions that might not chelate intracellular Ca^{2+}, we used a nominal Ca^{2+}-free KRB made with no added Ca^{2+} but without EGTA. In this nominal Ca^{2+}-free condition, the increase in insulin release by JTT-608 was significantly ($P < 0.01$) reduced (Fig. 6). Thus, JTT-608 increased insulin release in a glucose- and Ca^{2+}-dependent manner.

JTT-608 Recruits Glucose-Unresponsive β-Cells into $[Ca^{2+}]_i$ Responses. The effect of JTT-608 on glucose-unresponsive β-cells was investigated. Stimulation with 8.3 mM glucose failed to increase $[Ca^{2+}]$ in a single β-cell (Fig. 7A). This cell was subsequently confirmed to be the β-cell by its positive staining with insulin antibody (Fig. 7B) and by the $[Ca^{2+}]_i$ response to 300 μM tolbutamide (Fig. 7A), thereby exhibiting the property of glucose-unresponsive β-cells. Administration of JTT-608 recruited these glucose-unresponsive β-cells into $[Ca^{2+}]_i$ increases in a concentration-dependent manner (Fig. 7, A and C), whereby 300 μM JTT-608 aroused $[Ca^{2+}]_i$ responses in as many as 50% of glucose-unresponsive β-cells.

JTT-608 Induces $[Ca^{2+}]_i$ Oscillations in β-Cells. Whether JTT-608 could induce $[Ca^{2+}]_i$ oscillations in β-cells was examined. In the present study, when $[Ca^{2+}]_i$ went up and down four times or more in a repetitive manner, it was considered as the $[Ca^{2+}]_i$ oscillation. In the presence of 8.3 and 5.6 mM glucose, administration of JTT-608 at 300 to 1000 μM induced $[Ca^{2+}]_i$ oscillations (Fig. 8, A and B). JTT-608 also provoked $[Ca^{2+}]_i$ oscillations in β-cells that failed to respond to 8.3 mM glucose, the glucose-unresponsive β-cells (data not shown). These $[Ca^{2+}]_i$ oscillations were characterized by a slow frequency component that was occasionally superimposed with a fast frequency component. JTT-608 induced $[Ca^{2+}]_i$ oscillations in a concentration-dependent manner (Fig. 8C).

JTT-608 Acts in Concert with PACAP, GLP-1, and ACh to Increase $[Ca^{2+}]_i$. In a β-cell in which neither JTT-608 at relatively low concentration (100 μM) nor PACAP38 (1 nM) affected $[Ca^{2+}]_i$, the combination of these two agents induced a large increase in $[Ca^{2+}]_i$ (Fig. 9A), and it occurred in 15 of 57 cells. Similar effects were observed for the combination of JTT-608 and GLP-1 (1 nM) in 5 of 22 cells (Fig. 9B) and for the combination of JTT-608 and ACh (100 nM) in 22 of 55 cells (Fig. 9C). The cooperative effect was observed not only in the glucose-responsive but also in the glucose-unresponsive β-cells with GLP-1 ($n = 4$) (Fig. 9B), PACAP38 ($n = 3$), and ACh ($n = 3$) (data not shown).

Discussion

JTT-608 increased $[Ca^{2+}]_i$ and stimulated insulin release in rat islet β-cells in the presence of 8.3 mM, but not 2.8 mM, glucose. The increase in $[Ca^{2+}]_i$ and stimulation of insulin release in response to JTT-608 were inhibited under Ca^{2+}-free conditions. These results strongly suggest that JTT-608-induced $[Ca^{2+}]_i$ increase is linked to insulin release. Further analysis revealed that the ability of JTT-608 to increase $[Ca^{2+}]_i$ is strictly glucose-dependent (Figs. 1–3): as judged by the fraction of β-cells with $[Ca^{2+}]_i$ responses to 300 μM JTT-608, a nearly half-maximal effect of JTT-608 (29%) was obtained in the presence of 5.6 mM glucose, whereas in 4.2 mM glucose only a minor effect (5.4%) was observed (Fig. 3B). Thus, the threshold glucose concentration which turns on the ability of JTT-608 to increase $[Ca^{2+}]_i$ appears to exist around 5 mM, a value that corresponds to the normal blood glucose level. The results indicate that JTT-608 activates $[Ca^{2+}]_i$ signaling to a substantial extent only when the glucose level is above the normal, whereas the activation ceases when the glucose level is below the normal. These results indicate that the production of $[Ca^{2+}]_i$ signals is the process strictly regulated by glucose. This provides a mechanistic basis for the previous observation in rats that JTT-608 stimulates insulin release only at normoglycemic and hyperglycemic states and therefore does not induce hypoglycemia (Shinkai et al., 1998; Ohta et al., 1999a,b).

Our study demonstrates that JTT-608 activates PKA and a PKA inhibitor antagonizes the effect of JTT-608 on $[Ca^{2+}]_i$ (Fig. 5, B and C). Furthermore, an activator of cAMP-PKA

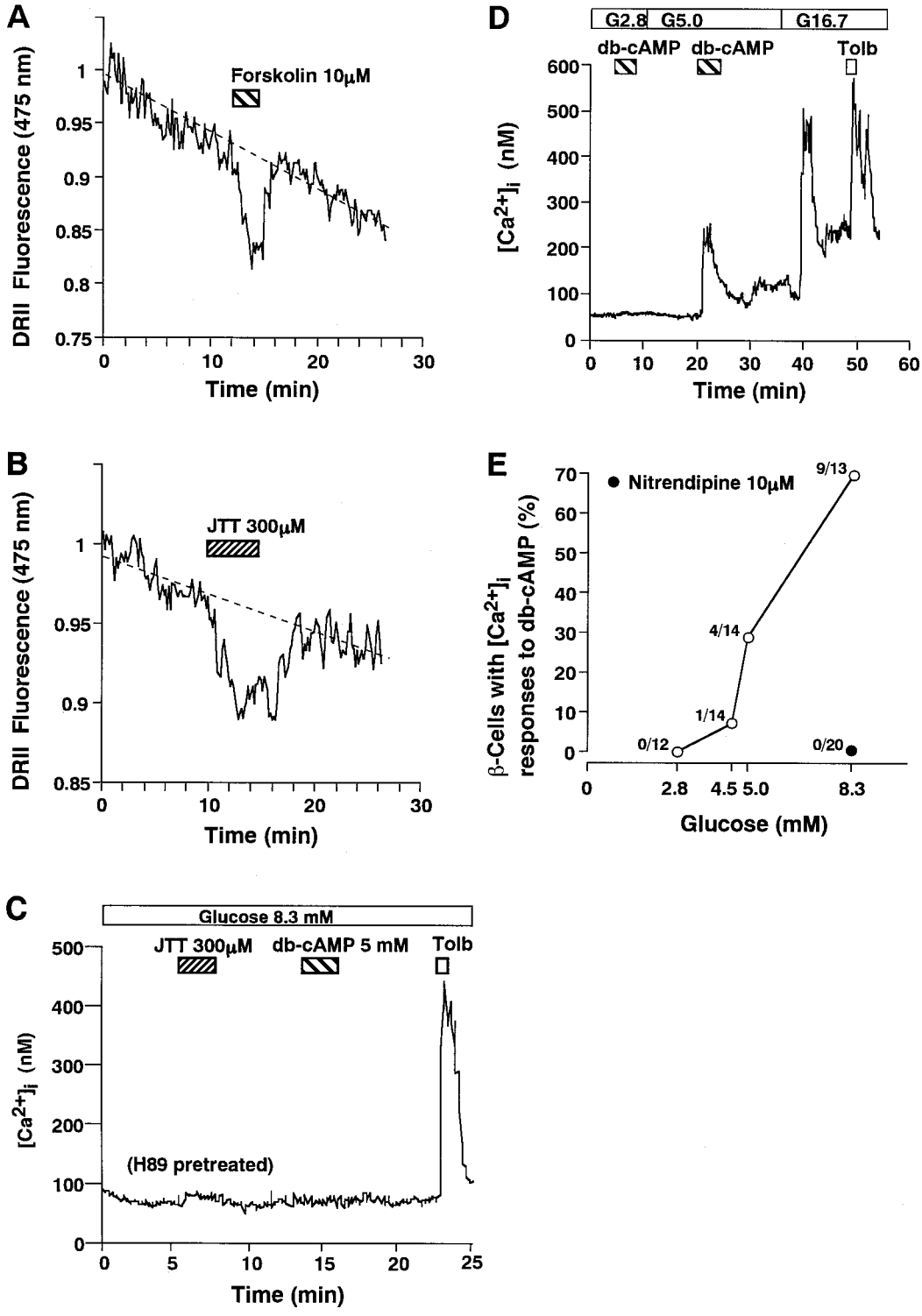

Fig. 5. Coupling of PKA pathway to Ca^{2+} signaling in response to JTT. A and B, administration of forskolin (10 μM) (A) and JTT (300 μM) (B) in the superfusion solution containing 8.3 mM glucose rapidly decreased the DR II fluorescence in single β-cells. A decrease in fluorescence corresponds to an increase in PKA activity. Dotted lines indicate the extrapolated baseline. The results shown are representative of 28 cells from two independent experiments in A and 25 from two in B. C, in a β-cell that had been pretreated for 120 min with H89 (60 μM), a specific PKA inhibitor, JTT, and db-cAMP failed to increase $[Ca^{2+}]_i$. The results shown are representative of 15 cells from two independent experiments.

pathway mimicked JTT-608: db-cAMP and JTT-608 both increased $[Ca^{2+}]_i$ in a manner that is dependent on glucose concentration and Ca^{2+} influx through L-type Ca^{2+} channels (Fig. 5, D and E). These results strongly suggest that the effect of JTT-608 on $[Ca^{2+}]_i$ is mediated, at least partly, by cAMP-PKA pathway.

The present study shows that a substantial population (5–25%) of insulin-containing β-cells from normal rats is glucose-unresponsive (Fig. 7), thereby confirming previous reports of the heterogeneity of pancreatic β-cells and existence of glucose-unresponsive β-cells (Pipeleers, 1987; Giordano et al., 1991; Holz et al., 1993; Yada et al., 1997). We found that many (24–50%) of the glucose-unresponsive β-cells are recruited into $[Ca^{2+}]_i$ increases by JTT-608. Notably, it occurred with JTT-608 at 100 to 300 μM, the concentration range that was used and shown to be effective in the perfused pancreas (200 μM) and in vivo experiments (50–500 μmol/kg) in rats (Shinkai et al., 1998; Ohta et al.,

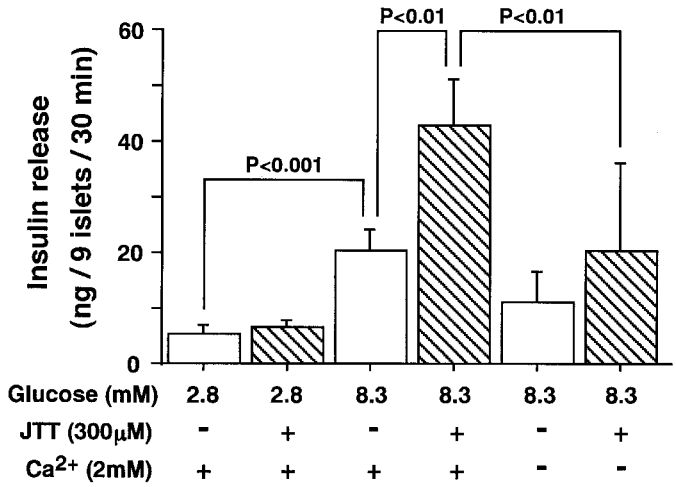

Fig. 6. Glucose-dependent effect of JTT to stimulate insulin release from rat islets under static incubation. In the presence of 8.3 mM, but not 2.8 mM, glucose, 300 μM JTT increased insulin release, and it was attenuated under the nominal Ca^{2+}-free condition achieved in KRB with no added Ca^{2+}. The results are expressed as the mean ± S.E.M. of five independent experiments.

1999a,b). A larger number of glucose-unresponsive β-cells observed in type 2 diabetic animals is suggested to partly account for the impaired glucose-induced insulin release characteristically associated with this disease (Tsuura et al., 1993; O'meara and Polonsky, 1994). Therefore, the ability of JTT-608 to arouse $[Ca^{2+}]_i$ responses in the glucose-unresponsive β-cells may be a beneficial property for a potential therapeutic agent for type 2 diabetes.

The present study shows that JTT-608 induces $[Ca^{2+}]_i$ oscillations in a substantial fraction (26–40%) of β-cells (Fig. 8C). $[Ca^{2+}]_i$ oscillations in islet β-cells have been implicated in the pulsatile insulin secretion, which contributes to the physiological control of glucose metabolism (Matthews et al., 1983; O'rahilly et al., 1988; Hellman et al., 1992; Gilon et al., 1993; O'meara and Polonsky, 1994; Bergsten, 1995). It has recently been shown that glucose-induced oscillations in $[Ca^{2+}]_i$ in a β-cell line, MIN6 cells, are linked to a long-lasting increase in mitochondrial Ca^{2+} (Nakazaki et al., 1998), a factor that is considered to be important in the maintenance of the β-cell metabolism (Civelek et al., 1996; Kennedy et al., 1996; Nakazaki et al., 1998). Regulation of gene expression by $[Ca^{2+}]_i$ oscillations has also been demonstrated (Dolmetsch et al., 1998; Li et al., 1998). Therefore, it is likely that induction of $[Ca^{2+}]_i$ oscillations by JTT-608 is related to the enhanced metabolism of β-cells, increased efficiency of insulin release, and, possibly, insulin biosynthesis.

An interesting finding of this study is that the Ca^{2+} signaling is elicited more effectively by a cooperative action of JTT-608 with physiological neurohormones GLP-1,

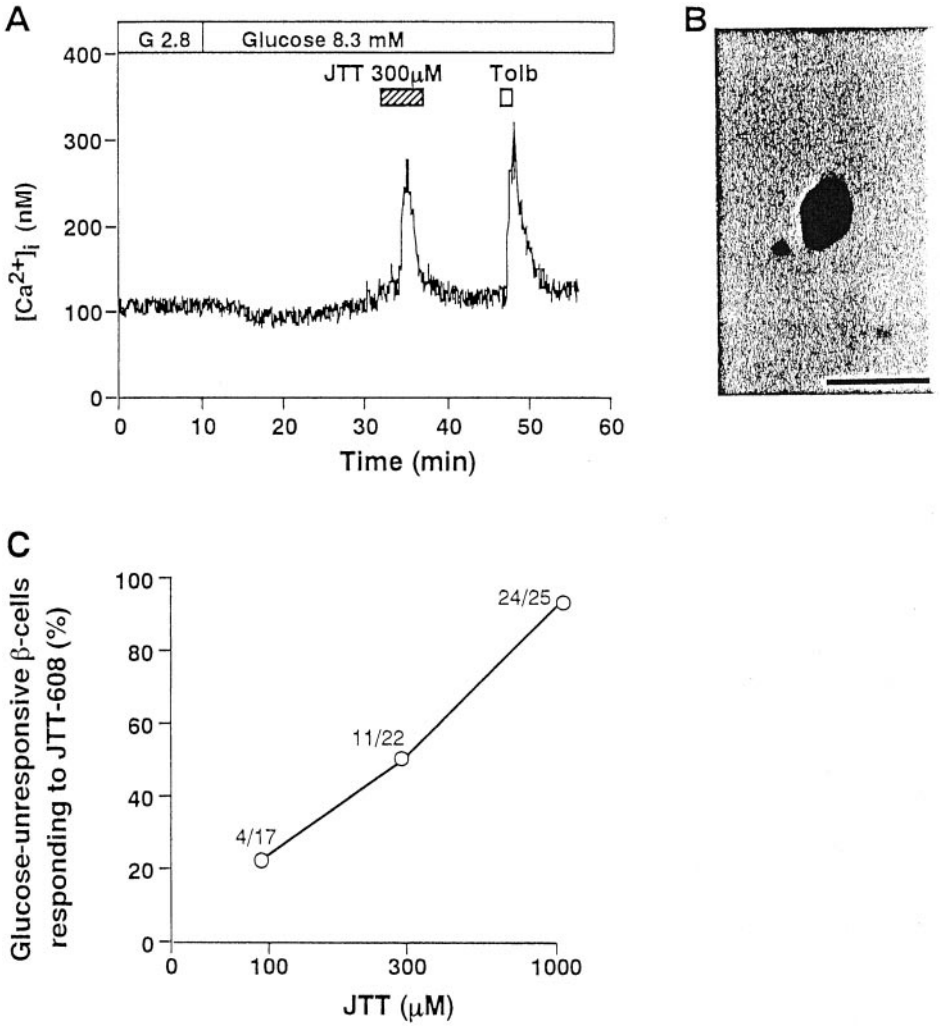

Fig. 7. Effects of JTT on $[Ca^{2+}]_i$ in glucose-unresponsive β-cells. A and B, JTT at 300 μM increased $[Ca^{2+}]_i$ in a single β-cell that had failed to respond to 8.3 mM glucose but responded to Tolb (A). This cell was subsequently stained positively with insulin antibody (B). The results shown are representative of 15 cells from three independent experiments. C, percentage of glucose-unresponsive β-cells that responded to JTT with increases in $[Ca^{2+}]_i$ is expressed as a function of the concentration of JTT. Glucose concentration is 8.3 mM. Numbers near each point indicate the number of the cells that responded to JTT over the glucose-unresponsive β-cells examined.

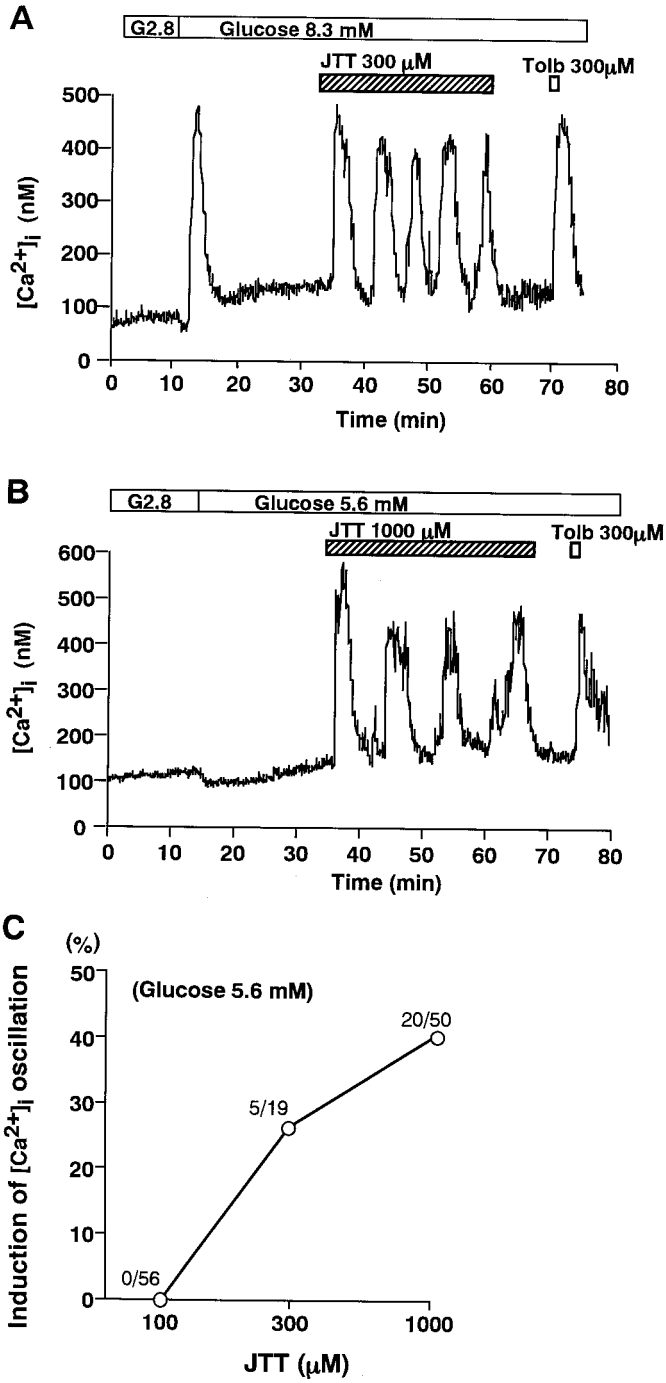

Fig. 8. Induction of $[Ca^{2+}]_i$ oscillations by JTT in β-cells. A, JTT at 300 μM induced $[Ca^{2+}]_i$ oscillations in a single β-cell in the presence of 8.3 mM glucose. B, JTT at a higher concentration of 1000 μM induced $[Ca^{2+}]_i$ oscillations in a single β-cell in the presence of 5.6 mM glucose. C, percentage of β-cells that exhibited induction or potentiation of $[Ca^{2+}]_i$ oscillations in response to 300 μM JTT in the presence of 5.6 mM glucose. Numbers near each point indicate the number of β-cells with induction or potentiation of $[Ca^{2+}]_i$ oscillations over that examined. The results shown are representative of five cells from three independent experiments in A and 20 from three in B.

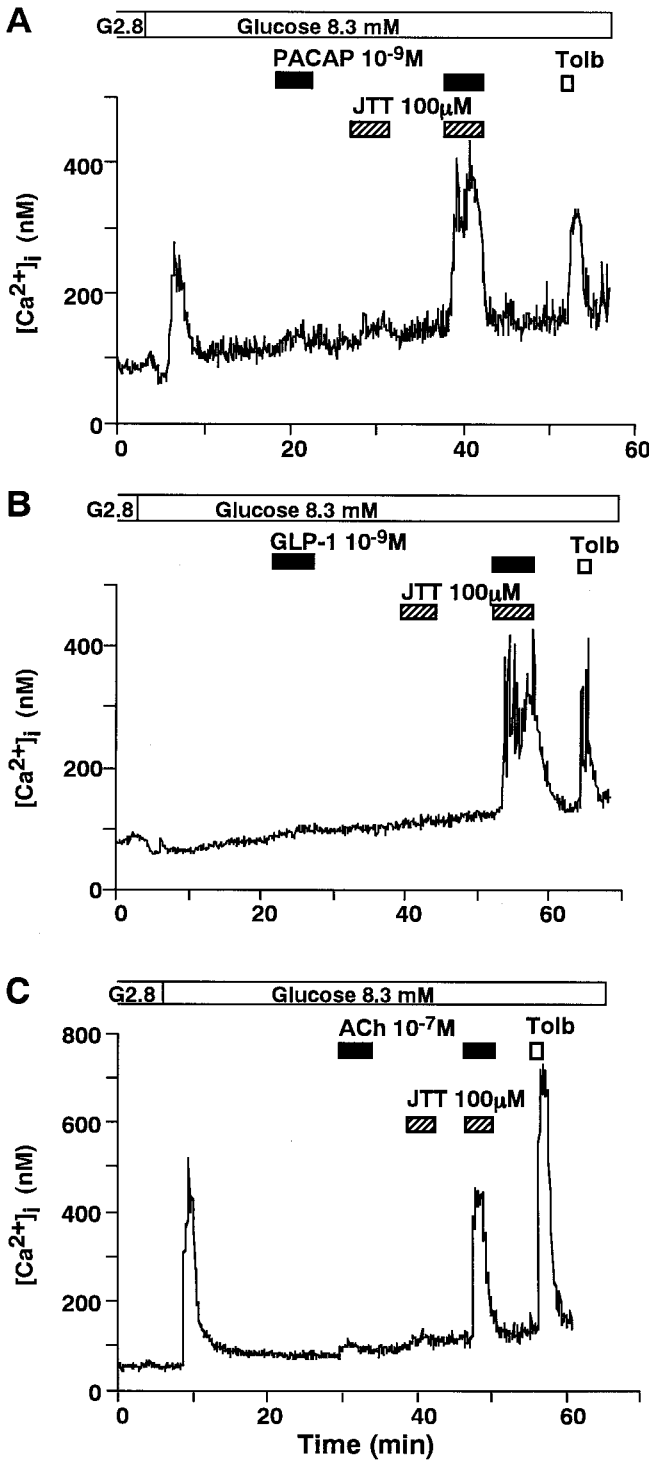

Fig. 9. Cooperative effects of JTT and insulinotropic neurohormones. A, neither PACAP38 (10^{-9} M) nor JTT (100 μM) had an effect on $[Ca^{2+}]_i$, whereas these two agents in combination increased $[Ca^{2+}]_i$ in a β-cell that responded to 8.3 mM glucose and Tolb. B, neither GLP-1 (10^{-9} M) nor JTT (100 μM) had an effect on $[Ca^{2+}]_i$, but the combination of the two increased $[Ca^{2+}]_i$ in a glucose-unresponsive β-cell. C, neither ACh (10^{-7} M) nor JTT (100 μM) had an effect on $[Ca^{2+}]_i$, but the combination of the two increased $[Ca^{2+}]_i$ in a β-cell. The results shown are representative of 15 cells from two independent experiments in A, four from two in B, and 22 from two in C.

PACAP38, and ACh, whose insulinotropic effects are also glucose-dependent (Fig. 9). The cooperative effect between JTT-608 and neurohormones is observed not only in glucose-responsive but also in glucose-unresponsive β-cells. GLP-1 is an intestinal incretin hormone currently under investigation as a novel therapeutic agent in the treatment of type 2 diabetes (Nauck, 1998). PACAP38 has been demonstrated to be a pancreatic peptide that acts as an endogenous intraislet

amplifier of glucose-induced insulin secretion (Yada et al., 1997; Filipsson et al., 1999). ACh is a neurotransmitter that innervates pancreatic islets. Therefore, the present finding suggests that the β-cells in pancreatic islets perfused with these physiological neurohormones may be activated more effectively by JTT-608 than single β-cells.

Conclusion. JTT-608 evokes protein kinase A-mediated increase in $[Ca^{2+}]_i$ in rat islet β-cells in a strictly glucose-dependent manner. The $[Ca^{2+}]_i$ increase is linked to stimulation of insulin release. JTT-608 also recruits glucose-unresponsive β-cells into $[Ca^{2+}]_i$ increases and induces oscillations of $[Ca^{2+}]_i$, which may also partly contribute to the insulin release by JTT-608. JTT-608 may act in concert with physiological neurohormones GLP-1, PACAP, and ACh.

Note Added in Proof. Since submission of this article, it has now been shown by Mukai et al. (2000) also that JTT-608-induced insulin release involves Ca^{2+} influx due to inhibition of phosphodiesterase in rat islets.

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References

Akiyoshi M, Kakei M, Nakazaki M and Tanaka H (1995) A new hypoglycemic agent, A-4166, inhibits ATP-sensitive potassium channels in rat pancreatic beta-cells. *Am J Physiol* **268:**E185–E193.

Ammala C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM and Rorsman P (1993) Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *J Physiol (Lond)* **472:**665–688.

Bergman R and Miller RE (1973) Direct enhancement of insulin secretion by vagal stimulation of the isolated pancreas. *Am J Physiol* **225:**481–486.

Bergsten P (1995) Slow and fast oscillations of cytoplasmic Ca^{2+} in pancreatic islets correspond to pulsatile insulin release. *Am J Physiol* **268:**E282–E287.

Civelek VN, Deeney JT, Shalosky NJ, Tornheim K, Hansford RG, Prentki M and Corkey BE (1996) Regulation of pancreatic beta-cell mitochondrial metabolism: Influence of Ca^{2+}, substrate and ADP. *Biochem J* **318:**615–621.

Dolmetsch RE, Xu K and Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature (Lond)* **392:**933–936.

Filipsson K, Sundler F and Ahren B (1999) PACAP is an islet neuropeptide which contributes to glucose-stimulated insulin secretion. *Biochem Biophys Res Commun* **256:**664–667.

Fujitani S and Yada T (1994) A novel D-phenylalanine derivative hypoglycemic agent A-4166 increases cytosolic free Ca^{2+} in rat pancreatic β-cells by stimulating Ca^{2+} influx. *Endocrinology* **134:**1395–1400.

Furukawa N, Ohta T, Noguchi T, Yonemori F and Wakitani K (1999) Glucose-dependent insulinotropic effects of JTT-608, a novel antidiabetic compound. *Eur J Pharmacol* **371:**51–58.

Garcia MC, Hermans MP and Henquin JC (1988) Glucose-, calcium- and concentration-dependence of acetylcholine stimulation of insulin release and ionic fluxes in mouse islets. *Biochem J* **254:**211–218.

Gilon P, Shepherd RM and Henquin JC (1993) Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. *J Biol Chem* **268:**22265–22268.

Giordano E, Bosco D, Cirutit V and Meda P (1991) Repeated glucose stimulation reveals distinct and lasting secretion patterns of individual rat pancreatic B cells. *J Clin Invest* **87:**2178–2185.

Greco AV, Caputo S, Bertoli A and Ghirlanda G (1992) The beta cell function in NIDDM patients with secondary failure. *Horm Metab Res* **24:**280–283.

Gromada J, Dissing S, Kofod H and FrokjaeR-Jensen J (1995) Effects of the hypoglycaemic drugs repaglinide and glibenclamide on ATP-sensitive potassium-channels and cytosolic calcium levels in beta TC3 cells and rat pancreatic beta cells. *Diabetologia* **38:**1025–1032.

Groop LC, Pelconen R, Koskimies S, Bottazzo GF and Doniach D (1986) Secondary failure to treatment with oral antidiabetic agents in non-insulin-dependent diabetes. *Diabetes Care* **9:**129–133.

Gylfe E, Hellman B, Sehlin J and Taljedal IB (1984) Interaction of sulphonylurea with the pancreatic β-cell. *Experientia* **40:**1126–1134.

Hellman B, Gylfe E, Grapengiesser E, Lund PE and Berts A (1992) Cytoplasmic Ca^{2+} oscillations in pancreatic β-cells. *Biochim Biophys Acta* **1113:**295–305.

Henquin JC (1990) Established, unsuspected and novel pharmacological insulin secretagogues, in *New Antidiabetic Drugs* (Bailey CJ and Flatt PR eds) pp 93–106, Smith-Gordon, London.

Higashi H, Sato K, Ohtake A, Omori A, Yoshida S and Kudo Y (1997) Imaging of cAMP-dependent protein kinase activity in living neural cells using a novel fluorescent substrate. *FEBS Lett* **414:**55–60.

Hirose H, Maruyama H, Ito K, Seto Y, Kido K, Koyama K, Dan K, Saruta T and Kato R (1994) Effects of N-[(trans-4-isopropylcyclohexyl)-carbonyl]-D-phenylalanine (A-4166) on insulin and glucagon secretion in isolated perfused rat pancreas. *Pharmacology* **48:**205–210.

Holz GG, Kuhtreiber WM and Habener JF (1993) Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1 (7–37). *Nature (Lond)* **361:**362–365.

Holz GG, Leech CA and Habener JF (1995) Activation of a cAMP-regulated Ca^{2+} signaling pathway in pancreatic β-cells by the insulinotropic hormone glucagon-like peptide-1. *J Biol Chem* **270:**17749–17757.

Inagaki N, Gonoi T, Clement Iv JP, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S and Bryan J (1995) Reconstitution of I_{KATP}: An inward rectifier subunit plus the sulfonylurea receptor. *Science (Wash DC)* **270:**1166–1170.

Kennedy ED, Rizzuto R, Theler JM, Pralong WF, Bastianutto C, Pozzan T and Wollheim CB (1996) Glucose-stimulated insulin secretion correlates with changes in mitochondrial and cytosolic Ca^{2+} in aequorin-expressing INS-1 cells. *J Clin Invest* **98:**2524–2538.

Ladriere L, Malaisse-Lagae F, Fuhlendorff J and Malaisse WJ (1997) Repaglinide, glibenclamide and glimepiride administration to normal and hereditarily diabetic rats. *Eur J Pharmacol* **335:**227–234.

Li W, Llopis J, Whitney M, Zlokarnik G and Tsien RY (1998) Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature (Lond)* **392:**936–941.

Malaisse WJ (1995) Stimulation of insulin release by non-sulphonylurea hypoglycemic agents: The meglitinide family. *Horm Metab Res* **27:**263–266.

Matthews DR, Naylor BA, Jones RG, Ward GM and Turner RC (1983) Pulsatile insulin has greater hypoglycemic effect than continuous delivery. *Diabetes* **32:**617–621.

Mukai E, Ishida H, Fujimoto S, Kajikawa M, Okamoto Y, Fujita J, Hamamoto Y, Tsuura Y, Yamada Y, Furukawa N, Ohta T and Seino Y (2000) The insulinotropic mechanism of the novel hypoglycaemic agent JTT-608: Direct enhancement of Ca^{2+} efficacy and increase of Ca^{2+} influx by phosphodiesterase inhibition. *Br J Pharmacol* **129:**901–908.

Nakazaki M, Ishihara H, Kakei M, Inukai K, Asano T, Miyazaki JI, Tanaka H, Kikuchi M, Yada T and Oka Y (1998) Repetitive mitochondrial Ca^{2+} signals synchronize with cytosolic Ca^{2+} oscillations in the pancreatic beta-cell line, MIN6. *Diabetologia* **41:**279–286.

Nauck MA (1998) Glucagon-like peptide 1 (GLP-1): A potent gut hormone with a possible therapeutic perspective. *Acta Diabetol* **3 5:**117–129.

Ohnota H, Koizumi T, Tsutsumi N, Kobayashi M, Inoue S and Sato F (1994) Novel rapid- and short-acting hypoglycemic agent, a calcium (2s)-2-benzyl-3-(cis-hexahydro-2-isoindolinylcarbonyl) propionate (KAD-1229) that acts on the sulfonylurea receptor: Comparison of effects between KAD-1229 and gliclazide. *J Pharmacol Exp Ther* **269:**489–495.

Ohta T, Furukawa N, Komuro G, Yonemori F and Wakitani K (1999a) JTT-608 restores impaired early insulin secretion in diabetic Goto-Kakizaki rats. *Br J Pharmacol* **126:**1674–1680.

Ohta T, Furukawa N, Yonemori F and Wakitani K (1999b) JTT-608 controls blood glucose by enhancement of glucose-stimulated insulin secretion in normal and diabetes mellitus rats. *Eur J Pharmacol* **367:**91–99.

O'meara NM and Polonsky KS (1994) Insulin secretion in vivo, in *Joslin's Diabetes Mellitus* (Kahn CR and Weir GC eds) pp 81–96, Lea & Febigar, Philadelphia.

O'rahilly S, Turner RC and Matthews DR (1988) Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. *N Engl J Med* **318:**1225–1230.

Panten U, Schwanstecher M and Schwanstecher C (1992) Pancreatic and extrapancreatic sulphonylurea receptors. *Horm Metab Res* **24:**549–554.

Pipeleers D (1987) The biosociology of pancreatic B cells. *Diabetologia* **30:**277–229.

Porte D Jr and Kahn SE (1995) The key role of islet dysfunction in type II diabetes mellitus. *Clin Invest Med* **18:**247–254.

Shinkai H, Ozeki H, Motomura T, Ohta T, Furukawa N and Uchida I (1998) 4-(trans-4-Methylcyclohexyl)-4-oxobutyric acid (JTT-608). A new class of antidiabetic agent. *J Med Chem* **41:**5420–5428.

Tsuura Y, Ishida H, Okamoto Y, Kato S, Sakamoto K, Horie M, Ikeda H, Okada Y and Seino Y (1993) Glucose sensitivity of ATP-sensitive K^+ channels is impaired in beta-cells of the GK rat. A new genetic model of NIDDM. *Diabetes* **42:**1446–1453.

Wang Z, Wang RM, Owji AA, Smith DM, Ghayei MA and Bloom SR (1995) Glucagon-like peptide-1 is a physiological incretin in rat. *J Clin Invest* **95:**417–421.

Wollheim CB and Sharp GWG (1981) Regulation of insulin release by calcium. *Physiol Rev* **61:**914–973.

Yada T, Hamakawa N and Yaekura K (1995) Two distinct modes of Ca^{2+} signalling by ACh in rat pancreatic β-cells: Concentration, glucose dependence and Ca^{2+} origin. *J Physiol (Lond)* **488:**13–24.

Yada T, Itoh K and Nakata M (1993) Glucagon-like peptide-1-(7-36)amide and a rise in cyclic AMP increase cytosolic free Ca^{2+} in rat pancreatic β-cells by enhancing Ca^{2+} channel activity. *Endocrinology* **133:**1685–1692.

Yada T, Kakei M and Tanaka H (1992) Single pancreatic β-cells from normal rats exhibit an initial decrease and subsequent increase in cytosolic free Ca^{2+} in response to glucose. *Cell Calcium* **13:**69–76.

Yada T, Sakurada M, Ihida K, Nakata M, Murata F, Arimura A and Kikuchi M (1994) Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet β-cells. *J Biol Chem* **269:**1290–1293.

Yada T, Sakurada M, Ishihara H, Nakata M, Shioda S, Yaekura K, Hamakawa N, Yanagida K, Kikuchi M and Oka Y (1997) Pituitary adenylate cyclase-activating polypeptide (PACAP) is an islet substance serving as an intra-islet amplifier of glucose-induced insulin secretion in rats. *J Physiol (Lond)* **505:**319–328.

Yaekura K, Kakei M and Yada T (1996) cAMP-signaling pathway acts in selective synergism with glucose or tolbutamide to increase cytosolic Ca^{2+} in rat pancreatic β-cells. *Diabetes* **45:**295–301.

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