A Novel Enhancer of Insulinotrophic Action by High Glucose (JTT-608) Stimulates Insulin Secretion from Pancreatic β-Cells via a New Cellular Mechanism

NAOKI ITABASHI, KOJI OKADA, SHIGEAKI MUTO, NOBUYA FUJITA, TAKESHI OHTA, JUN-ICHI MIYAZAKI, YASUSHI ASANO, and TOSHIKAZU SAITO

Division of Endocrinology and Metabolism (N.I., K.O., N.F., T.S.) and Nephrology (S.M., Y.A.), Department of Medicine, Jichi Medical School, Minamikawachi Tochigi, Japan; Central Pharmaceutical Research Institute, Japan Tobacco, Inc., Osaka, Japan (T.O.); and Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Osaka, Japan (J.M.)

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ABSTRACT

Insulin secretion from MIN6 cells (a pancreatic β-cell line) induced by high glucose (greater than 16.8 mM) was potentiated by a novel hypoglycemic agent [trans-4-(4-methylcyclohexyl)-4-oxobutyric acid (JTT-608)] (but not glibenclamide, a sulfonylurea). The extracellular Ca\(^{2+}\)-free condition, a L-type Ca\(^{2+}\) channel blocker (nifedipine) and an ATP-sensitive K\(^{+}\) channel opener, diazoxide, completely inhibited increases in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and insulin secretion evoked by JTT-608 in the presence of extracellular Ca\(^{2+}\). An electrophysiological study using single-barreled microelectrode techniques demonstrated that membrane potential (V\(_m\)) and input resistance of the cell membrane (R\(_m\)) are depolarized and increased by JTT-608, respectively. The apparent transference number for K\(^{+}\) was also significantly decreased after the addition of JTT-608. These effects immediately occurred after addition of JTT-608 and very rapidly disappeared after removal of JTT-608, which has not been observed in sulfonylureas. Also, these effects of JTT-608 were diminished, but not completely by diazoxide. JTT-608 did not affect the specific binding of [\(^3\)H]glibenclamide to the sulfonylurea receptor. These findings suggest that JTT-608 mainly inhibits ATP-sensitive K\(^{+}\) channel activity via a binding site distinct from the sulfonylurea receptor and then depolarizes V\(_m\) to open voltage-dependent L-type Ca\(^{2+}\) channels. Subsequently, these events stimulate Ca\(^{2+}\) entry to increase [Ca\(^{2+}\)]\(_i\) and induce insulin secretion from MIN6 cells. Therefore, JTT-608 is a unique hypoglycemic agent that enhances high glucose-induced insulin secretion. The present findings indicate that JTT-608 is a more useful new class of therapeutic drug for patients with non-insulin-dependent diabetes mellitus, compared with sulfonylurea derivatives.

Impaired insulin secretion from pancreatic β-cells in response to glucose, particularly loss of the first phase of insulin secretion, is an important feature in the pathology of non-insulin-dependent diabetes mellitus (NIDDM) (Polonsky et al., 1988; Porte, 1991; Taylor et al., 1994). This defect contributes to the cause of postprandial hyperglycemia in patients with NIDDM (Firth et al., 1986; Kelley et al., 1994). To compensate for this defective insulin release, sulfonylurea derivatives are the most widely used hypoglycemic agents (Gerich, 1989; Groop, 1992). Sulfonylurea derivatives induce insulin release by inhibition of the ATP-sensitive K\(^{+}\) channel of the pancreatic β-cells after binding to the sulfonylurea receptor (Rajan et al., 1990), but can not ameliorate the impairment of the first phase of insulin secretion in response to high glucose, resulting in failure to improve postprandial hyperglycemia in patients with NIDDM (Groop et al., 1986; Panten et al., 1992). Furthermore, there are several disadvantages to sulfonylurea therapy: severe and prolonged hypoglycemia because of lengthy duration of glucose-independent action (Jackson and Bressler, 1981; Ferner and Neil, 1988; Gerich, 1989; Jennings et al., 1989), and failure of response to sulfonylurea derivatives (secondary failure) and degeneration of pancreatic β-cells after chronic therapy (Dunbar and Foa, 1974; Groop et al., 1986; Sodoyez et al., 1990; Davalli et al., 1992). Therefore, a new class of hypoglycemic agent that improves insulin secretion in response to high plasma glucose levels by restoring pancreatic β-cells sensitivity to glucose would be beneficial for the treatment of patients with NIDDM.

JTT-608 [trans-4-(4-methylcyclohexyl)-4-oxobutyric acid] was developed by Japan Tobacco Inc., Central Pharmaceutical Institute (Osaka, Japan) as a drug that can improve glucose tolerance by restoring pancreatic β-cell sensitivity to glucose (Shinkai et al., 1998; Ohta et al., 1999a,b). The oral administration of this compound did not affect fasting blood glucose levels because of the minor insulinotropic effect

ABBREVIATIONS: NIDDM, non-insulin-dependent diabetes mellitus; JTT-608, trans-4-(4-methylcyclohexyl)-4-oxobutyric acid; DX, diazoxide; V\(_m\), membrane potential; R\(_m\), input resistance of the cell membrane; tk\(_{\text{app}}\), apparent transference number for K\(^{+}\); KRBB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin.
under low glucose conditions and improved glucose tolerance by enhancing both the first and second phases of insulin secretion from pancreatic β-cells in response to high glucose in neonatal streptozotocin rats, a model of NIDDM (Portha et al., 1974; Weir et al., 1981). These rats have a low insulin response to glucose and show postprandial hyperglycemia, as well as an oral glucose tolerance in the diabetic range. In contrast, sulfonylurea derivatives (tolbutamide and glibenclamide) caused a persistent decrease in fasting blood glucose levels due to marked stimulation of insulin secretion under low glucose conditions, but did not compensate glucose tolerance compared with that of JTT-608 because of the enhanced second phase of insulin secretion, but not the first phase in response to high glucose (Ohta et al., 1999b). Similar findings were obtained in diabetic Goto-Kakizaki rats, another genetic model of NIDDM (Ohta et al., 1999a). These observations suggested that JTT-608 is a useful and safe new class of therapeutic drug for patients with NIDDM. However, the cellular mechanisms by which JTT-608 stimulates insulin secretion from pancreatic β-cells and enhances high glucose-induced insulin secretion remain to be elucidated.

The present study was therefore undertaken to determine the cellular mechanisms of JTT-608 to evoke insulin secretion from MIN6 cells (a pancreatic β-cell line) by examining the effects of JTT-608 on insulin secretion, cytosolic free Ca$^{2+}$ ([Ca$^{2+}$]i), and binding to sulfonylurea receptors in MIN6 cells. We also examined the effects of JTT-608 on the membrane potential (V_m), input resistance of the cell membrane (R_i), and apparent transference number for K+ (t_K) using single-barreled microelectrode techniques.

**Materials and Methods**

**Drugs.** JTT-608 and glibenclamide were kindly provided by Japan Tobacco, Inc., Central Pharmaceutical Institute (Osaka, Japan) and Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan), respectively. Diazoxide (DX) was purchased from Sigma (St. Louis, MO).

**Cell Culture.** MIN6 cells were established from an insulinoma obtained by targeted expression of the simian virus 40 T antigen in transgenic mice as described previously (Miyazaki et al., 1990; Sakuma et al., 1995). MIN6 cells did not show inappropriate expression of brain-type glucose transporter, but showed exclusive expression of the liver-type glucose transporter. Moreover, MIN6 cells exhibit glucose-inducible insulin secretion, comparable with cultured normal mouse islet cells. Therefore, the MIN6 cell line retains physiological characteristics of normal β-cells (Miyazaki et al., 1990). Cells were cultured in a plastic flask in Dulbecco’s modified Eagle’s medium (Flow Laboratories, McLean, VA) containing 15% fetal bovine serum, 25 mM glucose, 75 µg/ml penicillin, and 50 µg/ml streptomycin. Cells were kept in a humidified incubator at 37°C in 95% air/5% CO_2_ and kept at 4°C until the assay for protein by the method of Lowry et al. (1951). JTT-608 and glibenclamide were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide (less than 0.1%) had no influence on insulin secretion from MIN6 cells.

**Insulin Secretion Studies.** The monolayer MIN6 cells grown on 35 × 10 mm culture dishes were used for insulin secretion studies. The cells were rinsed twice with 2 ml of Krebs-Ringer bicarbonate buffer (KRBB; 129 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO_4_, 5 mM NaHCO_3_, 1.2 mM MgSO_4_, 1.0 mM CaCl_2_, 10 mM HEPES, 2.8 mM glucose; pH 7.4) and incubated with KRBB containing 0.2% bovine serum albumin (BSA) (Sigma) (KRBB/0.2% BSA) for 1 h at 37°C. After incubation, cells were treated with 1 ml of KRBB/0.2% BSA containing various concentrations of glucose and effectors (JTT-608 and glibenclamide) for 30 min at 37°C. To study the effect of extra-cellular Ca$^{2+}$ on insulin secretion induced by JTT-608, cells were preincubated with Ca$^{2+}$-free KRBB containing 0.1 mM EGTA for 10 min, and then JTT-608 was added to the cells in Ca$^{2+}$-free KRBB/0.2% BSA containing 0.1 mM EGTA. The contribution of an ATP-sensitive K+ channel to insulin secretion induced by JTT-608 was examined using cells pretreated with an ATP-sensitive K+ channel opener, DX, for 10 min. Aliquots of medium were stored at −20°C until assay. Concentrations of insulin were measured by radioimmunoassay using Phadeseph insulin radioimmunoassay kits (Kabi Pharmacia Diagnostics AB Co., Uppsala, Sweden). Cells were dissolved in 1 ml of SDS-alkaline solution (0.1% SDS and 0.1 N NaOH) and kept at 4°C until the assay for protein by the method of Lowry et al. (1951). JTT-608 and glibenclamide were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide (less than 0.1%) had no influence on insulin secretion from MIN6 cells.

**Measurement of [Ca$^{2+}$]i.** The experimental procedure was similar to that used in our previous studies (Okada et al., 1993). The MIN6 cells grown on thin glass slides (13 mm in diameter) were rinsed twice with 1 ml of KRBB (2.8 mM glucose) and incubated in KRBB (2.8 mM glucose) containing 5 µM fura-2/ace-toxymethyl ester (Dojin Biochemicals, Kumamoto, Japan) for 60 min at 37°C. After aspiration of the fura-2/acetoxymethyl ester solution, the glass slides were rinsed and then placed in a 1 × 1 cm quartz cuvette with the aid of a special holder in a fluorescence spectrophotometer (CAF-110, Japan Spectroscopic Co., Tokyo, Japan). The dual wavelength excitation method for measurement of fura-2 fluorescence was used.

**Fig. 1.** Effects of JTT-608 and glibenclamide on insulin secretion from MIN6 cells. A, augmentation of glucose-induced insulin secretion from MIN6 cells by JTT-608. Open bars, control group; hatched bars, 10 µM JTT-608; closed bars, 0.1 mM JTT-608; double-hatched bars, 1 mM JTT-608. *p < 0.05, **p < 0.01. Values are mean ± S.E.M., n = 6. B, effect of glibenclamide on insulin secretion from MIN6 cells. Open bars, control group; hatched bars, 1 mM glibenclamide; closed bars, 10 mM glibenclamide; and double-hatched bars, 0.1 µM glibenclamide. *p < 0.05, **p < 0.01. Values are mean ± S.E.M., n = 4.
The fluorescence was monitored at 500 nm, with excitation wavelengths of 340 and 380 nm in the ratio mode. The effectors were added after a stable fluorescence signal (R) was achieved. To study the effect of Ca\(^{2+}\)-free conditions, the cells were pretreated with Ca\(^{2+}\)-free KRBB containing 0.1 mM EGTA for 5 min. From the ratio of fluorescence at 340 and 380 nm, [Ca\(^{2+}\)]\(_{i}\) was determined as described by Grynkiewicz et al. (1985).

**Measurement of \(V_m\).** Measurement of \(V_m\) was conducted using methods described previously (Muto and Asano, 1994). MIN6 cells grown on cover slips were placed on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan) and were then superfused with standard Ringer bicarbonate solution: 110 mM NaCl, 5.0 mM KCl, 25 mM NaHCO\(_3\), 10 mM NaAcetate, 0.8 mM Na\(_2\)HPO\(_4\), 0.2 mM NaH\(_2\)PO\(_4\), 1.0 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 8.3 mM D-glucose and 5.0 mM L-alanine. In some experiments, 45 mM NaCl was replaced with K\(^+\). All solutions had an osmolality between 285 and 295 mOsm/kg H\(_2\)O and were equilibrated with 95% O\(_2\)/5% CO\(_2\) adjusted to pH 7.4 at 37°C. \(V_m\) was measured with single-barreled microelectrodes, which were pulled from borosilicate glass capillaries (OD-1.5, 1.5 mm o.d., 1.0 mm i.d.; Narishige Scientific Laboratory, Tokyo, Japan) on a vertical puller (PE-2; Narishige). They were filled with a 0.5 mM KCl solution and had a resistance between 100 and 150 MΩ. They were fixed to a microelectrode holder containing an Ag/AgCl pellet and connected to a high-impedance electrometer (Duo 773; WPI, Sarasota, FL). To impale MIN6 cells, a microelectrode was positioned against the plasma membrane with a hydraulic micromanipulator (WR-6; Narishige), which was fixed to the stage of an inverted microscope (Diaphot, Nikon). The microelectrode was advanced into the cell using “tickle” current oscillations. Round-shaped cells were usually studied in the impalement experiments. The criteria for acceptable impalements were: 1) tip potentials <5 mV, 2) a stable \(V_m\) for more than 1 min with no further change in the input resistance of the microelectrode, and 3) the return of \(V_m\) to its baseline value ± 2 mV when the microelectrode was withdrawn. Some of the impalements were stable for up to 30 min. \(V_m\) was referenced to the bath and recorded on a four-pen chart recorder (model R64; Rikadenki, Tokyo, Japan). \(R\) was measured by injecting current (20–100 pA, 300-ms duration, 10-s intervals) through the microelectrode, where the series resistance was cancelled with a built-in bridge balance circuit (Duo 773; World Precise Instruments). The bath chamber had a volume of ~100 μl to permit rapid exchange of the bath solution within 5 s. The bath solution flowed by gravity at a rate of 5 to 15 ml/min from the reservoirs through a water jacket to stabilize the bath temperature at 37°C.

**Apparent Transference Number for K\(^+\).** Ion-substitution experiments were performed to evaluate the relative transmembrane K\(^+\) conductance by JTT-608. For this purpose, we observed the deflection of \(V_m\) (Δ\(V_m\)) upon abrupt increase in bath K\(^+\) from 5 to 50 mM in the absence and presence of JTT-608. The \(\Delta F\), which indicates the relative portion of a K\(^+\) conductance with respect to the overall conductance, was calculated according to the following equation: \(\Delta F = \Delta V_m / (RTzF) \times \ln([c1]/[c2])\), where c1 = 5 mM, c2 = 50 mM, z is valence, F is the Faraday constant, \(R\) is the gas constant, and \(T\) is the absolute temperature.

**Binding Experiments.** The cells grown on 35 × 10 mm culture dishes were used for competitive inhibition assays. MIN6 cells were washed twice with 2 ml of KRBB and incubated with 1 ml of KRBB containing 1 nM [\(^{3}H\)]glibenclamide (specific activity, 50.9 Ci/mmol, PerkinElmer Life Science Products, Boston, MA) and various concentrations of unlabeled glibenclamide and JTT-608 for 2 h at 37°C. The incubation was terminated by washing 4 times with 2 ml of KRBB, and the cells were dissolved with 2 ml of SDS-alkaline solution. The radioactivity of SDS-alkaline solution was measured using a liquid scintillation counter (Aloka LSC-671, Tokyo, Japan). The nonspecific binding was determined as residual binding in the presence of 1 μM unlabeled glibenclamide. Binding inhibition was expressed as a percentage of specific binding of [\(^{3}H\)]glibenclamide.

**Statistical Analysis.** All values were expressed as the mean ± S.E.M. The unpaired and paired Student’s t test and an analysis of multiple variance using the Scheffe method were used for statistical comparison. A p value of less than 0.05 was considered significant.

**Results**

**Effects of JTT-608 on Insulin Secretion from MIN6 Cells.** Figure 1, A and B, shows the effects of JTT-608 and glibenclamide on insulin secretion from MIN6 cells. Glucose (2.8–22.4 mM) stimulated insulin secretion from MIN6 cells in a dose-dependent manner. JTT-608 (10 μM) alone did not affect insulin secretion from MIN6 cells treated with low glucose concentrations (less than 11.2 mM), but significantly potentiated insulin secretion from MIN6 cells treated with high glucose concentrations (greater than 16.8 mM). Furthermore, JTT-608 at concentrations greater than 0.1 mM dose dependently increased insulin secretion even under low glucose conditions and also significantly enhanced high glucose-stimulated insulin secretion from MIN6 cells (Fig. 1A).

From the present findings, in the following studies, we used 1 mM JTT-608 to determine the cellular mechanisms responsible for the JTT-608-induced insulin secretion from MIN6 cells. In contrast, glibenclamide dose dependently increased insulin secretion from MIN6 cells under low glucose conditions, but failed to enhance insulin secretion induced by high glucose (Fig. 1B).

The roles of extracellular Ca\(^{2+}\) and ATP-sensitive K\(^+\) channel in insulin secretion produced by JTT-608 were then examined using extracellular Ca\(^{2+}\)-free solution containing 0.1 mM EGTA and an opener of ATP-sensitive K\(^+\) channel, DX, respectively (Fig. 2). The extracellular Ca\(^{2+}\)-free condition decreased basal insulin secretion and completely diminished insulin secretion stimulated by 1 mM JTT-608. The treatment of cells with DX for 10 min concentration-dependently inhibited JTT-608 (1 mM)-induced insulin secretion in the presence of extracellular Ca\(^{2+}\).

**Effect of JTT-608 on [Ca\(^{2+}\)]\(_{i}\) in MIN6 Cells.** The effects of JTT-608 on [Ca\(^{2+}\)]\(_{i}\) in MIN6 cells are shown in Fig. 3. In the presence of extracellular Ca\(^{2+}\), JTT-608 at concentra-
Effects of JTT-608 on $V_m$ and $R_i$ in MIN6 Cells. The above findings suggest the possibility that the JTT-608-induced insulin secretion and [Ca$^{2+}$]i elevation may occur through the inhibition of ATP-sensitive K$^+$ channel. To demonstrate this possibility, we examined the effects of JTT-608 on $V_m$ and $R_i$ in MIN6 cells using conventional microelectrode techniques. The basal values of resting $V_m$ in MIN6 cells were $-55.7 \pm 1.9$ mV ($n = 63$). These values are similar to those recorded using the perforated patch configuration in single pancreatic $\beta$-cells (Henquin and Meissner, 1981; Dunn et al., 1990; Smith et al., 1990; Miley et al., 1997). Figure 4A shows representative tracings of $V_m$ before and after addition of JTT-608, and Fig. 4B summarizes the effects of JTT-608 on $V_m$ and $R_i$. When 1 mM JTT-608 was added to MIN6 cells, $V_m$ was significantly depolarized from $-56.7 \pm 2.1$ to $-38.9 \pm 3.1$ mV ($n = 56$), and $R_i$ was significantly increased from $13.7 \pm 1.7$ to $26.9 \pm 3.0$ M ohm ($n = 32$). After removal of the drug, both $V_m$ and $R_i$ immediately returned to control levels.

To examine whether the effects of JTT-608 on $V_m$ and $R_i$ are due to changes in K$^+$ conductance, we observed changes in $V_m$ upon abrupt increase in bath K$^+$ concentration from 5 to 50 mM in the absence and presence of JTT-608. A representative tracing of $V_m$ is shown in Fig. 5A. In the absence of JTT-608, an abrupt increase in bath K$^+$ from 5 to 50 mM caused a rapid depolarization of $V_m$ from $-61.2 \pm 3.0$ to $-23.7 \pm 3.0$ mV ($p < 0.01, n = 17$). Upon abrupt increase of the bath K$^+$ in the presence of JTT-608, a significant depolarization of $V_m$ from $-43.2 \pm 3.5$ to $-23.7 \pm 3.0$ mV ($p < 0.01, n = 17$) was also observed. However, the changes in $V_m$ in the presence of JTT-608 (19.5 $\pm$ 2.9 mV, $p < 0.01, n = 17$) were significantly smaller than those in its absence (37.5 $\pm$ 2.9 mV, $n = 17$). The estimated $t_k$ after the addition of JTT-608 was also significantly decreased from 0.61 $\pm$ 0.05 to 0.34 $\pm$ 0.05 ($p < 0.01, n = 17$), as shown in Fig. 5B.

To further examine whether the effects of JTT-608 on $V_m$ and $R_i$ are affected by the addition of DX (the ATP-sensitive K$^+$ channel opener), DX was added to MIN6 cells in the

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Fig. 3. Effect of JTT-608 on Ca$^{2+}$ kinetics in MIN6 cells. Left, effect of extracellular Ca$^{2+}$ on an increase in [Ca$^{2+}$]i by JTT-608. Open bars, control group; hatched bars, Ca$^{2+}$-free KRBB containing 0.1 mM EGTA. *$p < 0.01$. Values are mean $\pm$ S.E.M., $n = 4$. Right, dose-dependent inhibitory effect of the ATP-sensitive K$^+$ channel opener, DX, on an increase in [Ca$^{2+}$]i, by 1 mM JTT-608. *$p < 0.01$. Values are mean $\pm$ S.E.M., $n = 4$.

Fig. 4. Effects of JTT-608 on $V_m$ and $R_i$ in MIN6 cells. JTT-608 at 1 mM was added to MIN6 cells. A, a representative tracing of $V_m$ before and after addition of JTT-608. B, summaries of effects of JTT-608 on $V_m$ and $R_i$. *$p < 0.01$, compared with control. The number of experiments for $V_m$ and $R_i$ were 56 and 32, respectively.
absence and presence of JTT-608. A representative tracing of $V_m$ is shown in Fig. 6A. When DX at 100 $\mu$M was added in the absence of JTT-608, $V_m$ significantly hyperpolarized from $-259.7 \pm 3.0$ to $-66.6 \pm 3.2$ mV ($p < 0.01$, $n = 14$), and $R_i$ significantly decreased from $12.3 \pm 2.0$ to $8.7 \pm 1.8$ $\text{M}\Omega$ ($p < 0.01$, $n = 11$). In the absence of DX, JTT-608 significantly depolarized $V_m$ from $-59.4 \pm 3.6$ to $-43.2 \pm 3.2$ mV ($p < 0.01$, $n = 14$) and significantly increased $R_i$ from $12.1 \pm 2.4$ to $26.3 \pm 4.38$ $\text{M}\Omega$ ($p < 0.01$, $n = 11$). In the presence of DX, we also observed that JTT-608 significantly depolarized $V_m$ from $-66.3 \pm 3.1$ to $-63.1 \pm 3.4$ mV ($p < 0.01$, $n = 14$) and significantly increased $R_i$ from $8.7 \pm 1.8$ to $11.1 \pm 1.9$ $\text{M}\Omega$ ($p < 0.01$, $n = 11$). However, JTT-608-induced changes in $V_m$ and $R_i$ in the presence of DX were significantly smaller than those in its absence, as shown in Fig. 6B.

**Binding of JTT-608 on Sulfonylurea Receptor in MIN6 Cells.** Finally, we examined the displacement of [$^3$H]glibenclamide from MIN6 cells by JTT-608 and unlabeled glibenclamide (Fig. 7). Increasing concentrations of unlabeled glibenclamide blocked the specific binding of [$^3$H]glibenclamide to sulfonylurea receptor of MIN6 cells. However, JTT-608 at concentrations that caused increases in [Ca$^{2+}$]i and insulin secretion did not affect the specific binding of [$^3$H]glibenclamide to sulfonylurea receptor.

**Discussion**

The present study showed that JTT-608 causes a dose-dependent enhancement of insulin secretion from MIN6 cells at high glucose concentrations and improves insulin secretion in response to high glucose concentrations by restoring pancreatic $\beta$-cells sensitivity to glucose. Low doses of JTT-608 had no insulinotrophic effect under low glucose conditions, but could enhance high glucose-stimulated insulin secretion from MIN6 cells. High doses of JTT-608 increased insulin secretion from MIN6 cells under low glucose conditions and caused a dose-dependent enhancement of insulin secretion from MIN6 cells in response to high glucose. In sharp contrast, glibenclamide, a sulfonylurea derivative, dose dependently increased insulin secretion under low glucose conditions, but failed to potentiate high glucose-stimulated insulin secretion from MIN6 cells.

These observations are in good agreement with previous in vivo and in vitro studies in normal and diabetic rats (Shinkai et al., 1998; Ohta et al., 1999a,b). JTT-608 improved glucose...
It has been widely established that an increase in plasma glucose levels evokes the depolarization of membrane potential by closing ATP-sensitive K⁺ channels, followed by increases in [Ca²⁺], through Ca²⁺ influx via voltage-dependent L-type Ca²⁺ channels, and subsequently insulin secretion occurs (Ashcroft et al., 1984, 1988; Rorsman and Turpe, 1985).

Initially, we examined the effects of JTT-608 on Ca²⁺ kinetics in MIN6 cells. JTT-608 caused a rapid elevation in [Ca²⁺], which was dependent on an increase in Ca²⁺ influx through voltage-dependent L-type Ca²⁺ channels because the extracellular Ca²⁺-free condition and the treatment of cells with a L-type Ca²⁺ channel blocker (nifedipine) completely inhibited this elevation in [Ca²⁺]. Moreover, an ATP-sensitive K⁺ channel opener, DX, completely blocked the increase in [Ca²⁺] by JTT-608 in the presence of extracellular Ca²⁺. The insulin secretion stimulated by JTT-608 was also completely diminished in the cells pretreated with extracellular Ca²⁺-free solution and DX. Mukai et al. (2000) reported that JTT-608 augments insulin secretion by enhancing Ca²⁺ efficacy and increasing Ca²⁺ influx resulting from the increased intracellular cyclic AMP concentration due to phosphodiesterase inhibition. We examined the effects of forskolin and 3-isobutyl-1-methylxanthine on [Ca²⁺] in MIN6 cells. Forskolin (5 μM), and 3-isobutyl-1-methylxanthine (0.5 mM) failed to affect [Ca²⁺] levels and did not influence the rapid increase in [Ca²⁺] by JTT-608 in the presence of extracellular Ca²⁺ (data not shown). These findings indicate that a rapid increase in [Ca²⁺] by JTT-608 is not associated with the production of cyclic AMP in MIN6 cells. These observations suggest the possibility that JTT-608 may inhibit ATP-sensitive K⁺ channels, and then activates voltage-dependent L-type Ca²⁺ channels and subsequently may stimulate Ca²⁺ entry from the extracellular space to increase [Ca²⁺], thus inducing insulin secretion from MIN6 cells.

The findings of insulin secretion and [Ca²⁺] demonstrated that the blockade of ATP-sensitive K⁺ channels is the first key step in insulinotrophic effects of JTT-608. Second, we examined the direct effects of JTT-608 on ATP-sensitive K⁺ channels by measuring Vₘ and Rᵢ in MIN6 cells because ATP-sensitive K⁺ channels are a major determinant of the resting membrane potential of pancreatic β-cells (Ashcroft and Kakei, 1989). The basal values of resting Vₘ in MIN6 cells were -55.7 ± 1.9 mV (n = 63). Vₘ was depolarized and Rᵢ was increased immediately after the addition of JTT-608 to MIN6 cells, and both Vₘ and Rᵢ were returned to control levels immediately after the removal of JTT-608 from the bathing solution. These findings indicate that the binding of JTT-608 to the site and the detachment of JTT-608 from the site are very quick, if there is a specific binding site for JTT-608 on the plasma membrane of MIN6 cells. The previous studies by patch clamp technique have shown that the membrane potential of pancreatic β-cells was immediately depolarized by sulfonylurea derivatives and an amino acid derivative, nateglinide (A-4166), and also slowly depolarized by a benzoic acid derivative of the meglitinide family, repaglinide (Akio yoshi et al., 1995; Gromada et al., 1995; Hu et al., 2000). The membrane potential was gradually recovered to control levels after the washout of these compounds, and the time for reversal of ATP-sensitive K⁺ channel inhibition of nateglinide (A-4166) was faster than those of glibenclamide and repaglinide (Hu et al., 2000). Therefore, the characteristics of JTT-608 with regard to binding at sulfonylurea recep-

Tolerance by stimulating both the first and second phases of insulin secretion from pancreatic β-cells in response to high plasma glucose levels without decreasing fasting blood glucose levels in neonatal streptozotocin rats and diabetic Goto-Kakizaki rats, both genetic models of NIDDM (Ohta et al., 1999a,b). In these rats, sulfonylurea derivatives (tolbutamide and glibenclamide) caused a persistent decrease in fasting blood glucose levels due to the marked stimulation of insulin secretion under low glucose conditions. However, these compounds could not compensate for postprandial hyperglycemia because they failed to enhance the first phase of insulin secretion in response to plasma high glucose levels (Ohta et al., 1999a,b).

The findings from these in vivo and in vitro studies suggest that JTT-608 becomes an enhancer of insulinotrophic action of glucose in pancreatic β-cells at high plasma glucose concentrations. Although sulfonylurea derivatives have been widely used in the treatment of patients with NIDDM, there are several disadvantages to sulfonylurea therapy, such as failure of improving postprandial hyperglycaemia, excessive hypoglycaemia, secondary failure, and exhaustion of pancreatic β-cells (Dunbar and Foa, 1974; Jackson and Bressler, 1981; Groop et al., 1986; Ferner and Neil, 1988; Gerich, 1989; Jennings et al., 1989; Sodoyez et al., 1990; Davalli et al., 1992). Therefore, JTT-608 is a safer and more useful drug for patients with NIDDM, compared with sulfonylurea derivatives.

The present findings of insulin secretion studies indicate that the insulinotrophic actions of JTT-608 may be different from those of sulfonylurea derivatives. However, the cellular mechanisms by which JTT-608 stimulates insulin secretion from pancreatic β-cells remain to be elucidated. JTT-608 may possess and modulate similar cellular mechanisms to those of glucose on insulin secretion from pancreatic β-cells, since insulin secretion studies have shown that JTT-608 enhances insulin secretion from MIN6 cells in response to high glucose.

![Figure 7. No displacement of [³H]glibenclamide from MIN6 cells by JTT-608. Open circles, JTT-608; closed circles, glibenclamide. Values are mean ± S.E.M., n = 3.](image-url)
tors are markedly different from other hypoglycemic agents, such as sulfonylurea derivatives (glibenclamide), nateglinide, and repaglinide. Furthermore, JTT-608 has an advantage as a therapeutic drug for patients with NIDDM without excessive hypoglycemia and exhaustion of pancreatic β-cells because of immediate occurrence and disappearance of effects on \( V_m \) and \( R_i \).

To examine whether the effects of JTT-608 on \( V_m \) and \( R_i \) are due to changes in \( K^+ \) conductance, changes in \( V_m \) after an abrupt 10-fold increase in bath \( K^+ \) concentrations in the absence or presence of JTT-608 were observed. This abrupt increase in bath \( K^+ \) caused a rapid depolarization of \( V_m \) by 37.5 mV. These findings indicate the presence of a large \( K^+ \) conductance in the plasma membrane of MIN6 cells. The changes in \( V_m \) by the abrupt increase in bath \( K^+ \) in the presence of JTT-608 were significantly smaller than those in its absence. The estimated \( t_6 \) after addition of JTT-608 was also significantly decreased. These findings indicate that JTT-608 inhibits \( K^+ \) conductance in the plasma membrane of MIN6 cells. We examined the effects of DX (the ATP-sensitive \( K^+ \) channel opener) on JTT-608-induced changes in \( V_m \) and \( R_i \). The treatment of MIN6 cells with DX almost diminished the JTT-608-induced changes in \( V_m \) and \( R_i \). These findings indicate that, in MIN6 cells, the effects of JTT-608 on \( V_m \) and \( R_i \) occur mainly via DX-sensitive processes, presumably via an inhibition of the ATP-sensitive \( K^+ \) channel. However, the possibility that the inhibitory effects of JTT-608 on \( K^+ \) conductance may be mediated by other types of \( K^+ \) channels cannot be excluded.

The ATP-sensitive \( K^+ \) channels of pancreatic β-cells are a complex of two proteins: an inward-rectifier \( K^+ \) channel subunit, Kir6.2, and the sulfonylurea receptor, SUR1. Kir6.2 acts as the pore-forming subunit of the ATP-sensitive \( K^+ \) channel, whereas SUR1 acts as a regulator of the ATP-sensitive \( K^+ \) channel activity, conferring sensitivity to sulfonylureas, diazoxide, and Mg-ADP (Ammala et al., 1996; Tucker et al., 1997). Although JTT-608 has no sulfonylamine moiety (Shinkai et al., 1998), several nonsulfonylurea hypoglycemic compounds were recently reported to inhibit ATP-sensitive \( K^+ \) channels via binding to sulfonylurea receptor of pancreatic β-cells (Ohnata et al., 1994; Akiyoshi et al., 1995). Finally, we examined whether JTT-608 binds to sulfonylurea receptors in MIN6 cells. Unlabeled glibenclamide dose-dependently blocked the specific binding of \([H]glibenclamide to sulfonylurea receptors of MIN6 cells. However, JTT-608 failed to detach this specific binding of \([H]glibenclamide to sulfonylurea receptors. These findings indicate that JTT-608 may secondarily inhibit ATP-sensitive \( K^+ \) channels via a binding site distinct from the sulfonylurea receptor of SUR1 or directly close an inward-rectifier \( K^+ \) channel. Also, JTT-608 may block ATP-sensitive \( K^+ \) channels after binding to other specific binding sites on the plasma membrane of pancreatic β-cells because a previous study proposed the existence of a specific binding site due to the strict structural specificity of JTT-608 (Shinkai et al., 1998). The existence of specific binding sites on the plasma membrane of pancreatic β-cells for hypoglycemic agents have been reported (Ishida-Takahashi et al., 1996; Dickinson et al., 1997; Mourtada et al., 1997). The imidazoline-guanidine insulin releasing agents, such as efaroxan, cibegline, and BTS 67 582, appear to promote insulin release via a binding site distinct from the sulfonylurea receptor of pancreatic β-cells (imidazoline-guanidine receptor site) (Ishida-Takahashi et al., 1996; Dickinson et al., 1997; Mourtada et al., 1997).

In conclusion, JTT-608 mainly closed ATP-sensitive \( K^+ \) channels via a binding site distinct from the sulfonylurea receptor and then depolarized the membrane potential to open voltage-dependent L-type Ca2+ channels, subsequently stimulating Ca2+ entry from the extracellular space to increase \([Ca^{2+}]_i \) and induced insulin secretion from MIN6 cells. The occurrence and disappearance of the effects of JTT-608 on the membrane potential were immediate. The JTT-608 induced insulin secretion and improved the sensitivity of MIN6 cells to glucose under high glucose conditions. These observations strongly suggest that JTT-608 is a safe and useful new class of therapeutic drug for patients with NIDDM, compared with sulfonylurea derivatives. The mechanisms by which JTT-608 blocked ATP-sensitive \( K^+ \) channels in pancreatic β-cells at a site separate from that used by sulfonylureas will require further investigation.

References


Send reprint requests to: Dr. Koji Okada, Division of Endocrinology and Metabolism, Department of Medicine, Jichi Medical School, 3311-1 Minami-kawachi Tochigi 329-0498, Japan.