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Glucose-dependent insulinotropic effects of JTT-608, a novel antidiabetic compound

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Abstract

The effects of JTT-608 [*trans*-4-(4-methylcyclohexyl)-4-oxobutyric acid], a novel antidiabetic compound, on insulin secretion were investigated using mouse insulinoma cell line (MIN6 cells) and isolated, perfused rat pancreas. JTT-608 enhanced insulin secretion in MIN6 cells in a dose dependent (10–300 μM) and glucose concentration-dependent (2.8–16.7 mM) manner. Unlike sulphonylureas, JTT-608 minimally stimulated insulin secretion at low glucose concentrations but potently enhanced insulin secretion at high glucose concentrations. In isolated, perfused pancreas of normal rats, JTT-608 (100–300 μM) dose-dependently enhanced insulin secretion in the first and second phases at high glucose concentrations but minimally stimulated insulin secretion at a basal glucose concentration. In isolated, perfused pancreas of neonatally streptozotocin-induced non-insulin-dependent diabetes mellitus rats (nSTZ rats), JTT-608 (200 μM) normalized the first phase and doubled the second phase of insulin secretion. In MIN6 cells, JTT-608 did not inhibit the binding of [^{3}H]glibenclamide to membrane fractions but enhanced K^+-ATP channel-independent insulin secretion. These results suggest that JTT-608 enhances insulin secretion in a different manner and via a different mechanism from hypoglycemic sulphonylureas.

Keywords: JTT-608; Insulin secretion; MIN6 cell; Pancreas, perfused; Streptozotocin-induced diabetes; Sulphonylurea receptor

1. Introduction

In the pathogenesis of impaired glucose tolerance and non-insulin-dependent diabetes mellitus, it has been clearly demonstrated that the characteristic impairment of the acute insulin response to glucose causes a prolonged elevation of postprandial glucose levels (Eriksson et al., 1989; Dinneen, 1997). The resulting postprandial hyperglycemia plays a crucial role in the development of diabetes mellitus (Luzi and DeFronzo, 1989). Sulphonylurea compounds, now widely used in the treatment of non-insulin-dependent diabetes mellitus, do not ameliorate this impairment of the insulin response effectively (Shapiro et al., 1989; Groop et al., 1991). Furthermore, several disadvantages have also been demonstrated with sulphonylurea therapy. For example, sulphonylureas sometimes cause fasting hypoglycemia because of their long-lasting activity and glucose-independent mechanism of action (Asplund et al., 1983; Ferner and Neil, 1988). Secondary failure after chronic sulphonylurea therapy is also a common problem of using this class of hypoglycemic compounds (Groop et al., 1986, 1989).

JTT-608 [*trans*-4-(4-methylcyclohexyl)-4-oxobutyric acid] is a novel antidiabetic compound. We have demonstrated that JTT-608 improves glucose tolerance in normal and non-insulin-dependent diabetes mellitus rats at doses not causing fasting hypoglycemia (Ohta et al., 1999). Increased blood insulin levels have also been observed in glucose- or meal-challenged rats but not in fasting rats, suggesting that JTT-608 enhances pancreatic insulin secretion at postprandial blood sugar levels but not at fasting blood sugar levels. The aim of the present study was to compare the direct effects of JTT-608 on pancreatic insulin secretion in vitro, to the effects of hypoglycemic sulphonylureas, using mouse insulinoma cell line (MIN6 cells) and isolated, perfused rat pancreas.

2. Materials and methods

2.1. Chemicals

JTT-608 was synthesized at Japan Tobacco, Central Pharmaceutical Research Institute (Osaka, Japan). Tolbu-

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tamide and glibenclamide were purchased from Wako (Osaka, Japan) and Research Biochemicals International (Natick, MA), respectively. All test compounds were dissolved in dimethylsulfoxide and added to each assay system to yield a solvent concentration of 0.1% for insulin secretion studies and 1.0% for binding assay studies.

2.2. Insulin secretion in MIN6 cells

Insulin secreting MIN6 cells established from pancreatic β cells of mice (Miyazaki et al., 1990) were kindly donated by Dr. Jun-ichi Miyazaki from Tokyo University. MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) containing 15% fetal calf serum, 60 unit ml^{-1} streptomycin, 60 unit ml^{-1} penicillin and 0.1 μM 2-mercaptoethanol, and used at passage 18 and 19. Insulin secretion was determined according to the method of Ishihara et al. (1993). The cells were seeded in DMEM (25 mM glucose) in a 24-well culture plate (Falcon) at 2×10^5 cells $well^{-1}$ or in a 96-well culture plate at 5×10^4 cells $well^{-1}$ and cultured for 2 days. Culture medium was replaced by HEPES-balanced Krebs Ringer bicarbonate buffer (KRB buffer: 119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM HEPES; pH 7.4) containing 5.6 mM glucose and 0.2% bovine serum albumin (fraction V, Sigma, St. Louis, MO) and incubated for 30 min at 37°C. KRB buffer was then replaced by the same buffer but containing 0.2% bovine serum albumin, varying concentrations of glucose (0–25 mM) and test compounds, and the cells were incubated for 30 or 60 min at 37°C. Incubations were terminated by cooling on ice. Insulin concentrations released into the KRB buffer were then measured with a radioimmunoassay kit from Pharmacia Upjohn (Uppsala, Sweden) using rat insulin as a standard.

2.3. Animals

These experiments complied with the Guidelines for Animal Experimentation of our laboratories. Male Wistar rats and pregnant Sprague–Dawley rats were purchased from Charles River Japan (Tokyo, Japan). Male Wistar rats (7 and 9 weeks old) were used as normal rats. Neonatally streptozotocin-induced non-insulin-dependent diabetes mellitus rats (nSTZ rats) rats were prepared according to a previously described method (Portha et al., 1974; Weir et al., 1981) with some modifications. In brief, male Sprague–Dawley pups (1.5 days-old) received a single subcutaneous injection of 120 mg kg^{-1} streptozotocin (Sigma, St. Louis, MO) freshly dissolved in citrate buffer (pH 4.3). After weaning, the rats were housed for 3–4 weeks. Urinary sugar-negative animals were selected and randomized according to blood glucose profiles after oral glucose challenge (2.0 g kg^{-1}), and used in the experiment at 14 weeks of age. Age-matched Sprague–Dawley rats were used as normal control animals.

2.4. Perfusion of isolated pancreas of rats

Rat pancreas was isolated and perfused according to the method of Grodsky and Fanska (1975) with slight modifications. In brief, the pancreas and associated spleen and duodenum were isolated under sodium pentobarbital anesthesia (50 mg kg^{-1} i.p.). The isolated pancreas was perfused through the celiac artery at a flow rate of 3.5 ml min^{-1} with basal Krebs Ringer bicarbonate buffer (KRB buffer: 119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$,

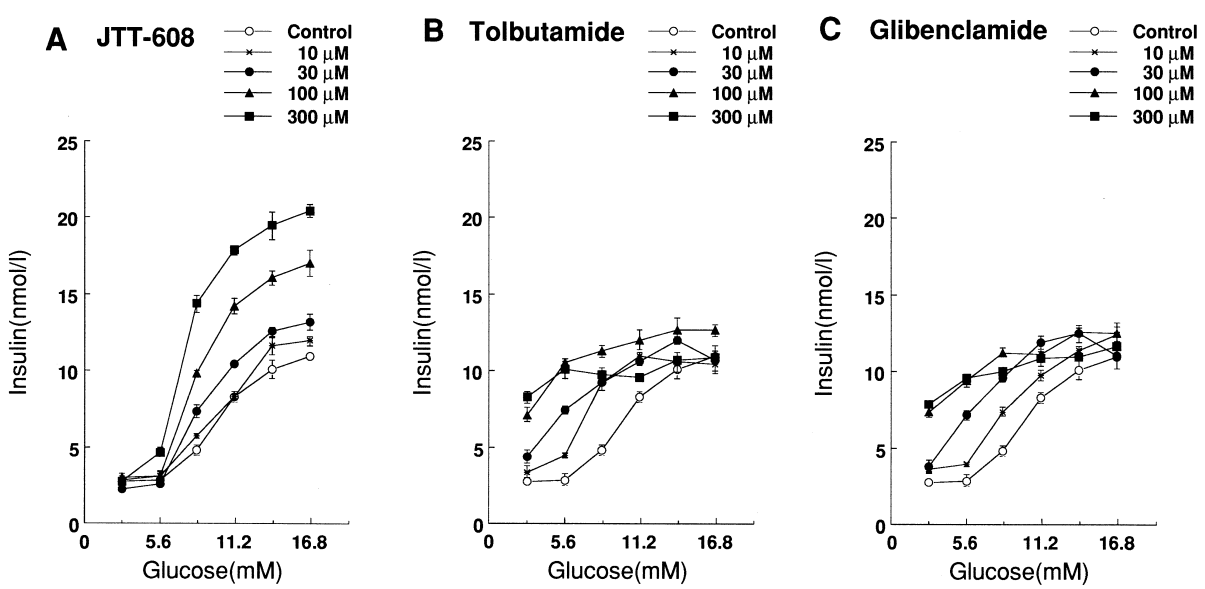

Fig. 1. Dose dependence of the effects of JTT-608 (A), tolbutamide (B) and glibenclamide (C) on insulin secretion in MIN6 cells at various glucose concentrations. Values are means ± S.E.M. ($N = 4$).

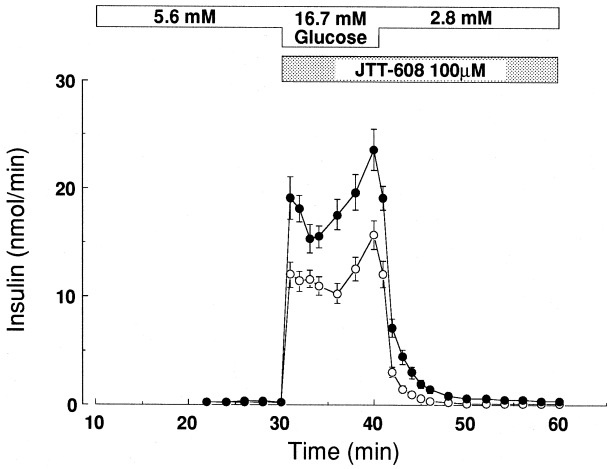

Fig. 2. Effect of JTT-608 (100 μM) on insulin secretion at 16.7 mM glucose, and insulin secretion in response to 2.8 mM glucose in isolated, perfused pancreas of fed Wistar rats (9w). Values are means ± S.E.M. ($N = 9$–11).

1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4 and 25 mM $NaHCO_3$; pH 7.4; equilibrated with 95% O_2, 5% CO_2) containing 2.8 or 5.6 mM glucose, 0.2% bovine serum albumin (fraction V, Sigma, St. Louis, MO) and 4.0% dextran T-70 (Pharmacia Biotech, Uppsala, Sweden). The preparation was placed in an acryl chamber filled with basal KRB buffer and kept at 37°C. The effluent perfusate from a portal vein cannula was collected at 1-min intervals into a fraction tube containing aprotinin (1000 U/tube). Collected samples were stored at −20°C and insulin concentration was measured with a radioimmunoassay kit from Pharmacia Upjohn (Uppsala, Sweden) using rat insulin as a standard. In the experiments with fasted rats, series of perfusates were divided into three phases consisting of basal, first and second phase, according to perfusion conditions and secretion pattern. The area under the curve of secreted insulin was integrated to compare the amount of insulin secreted during each phase. After 20 min of equilibration, the basal perfusate was changed to basal KRB buffer containing a test compound and this was perfused for 10 min (basal phase, 21–30 min). The perfusate was then changed to KRB buffer containing the same concentration of the test compound and high concentrations of glucose (7.0 or 11.1 mM), and this was perfused for 30 min (first phase, 31–35 min, second phase, 36–60 min).

2.5. Sulphonylurea receptor binding assay

Binding of [^{3}H]glibenclamide to MIN6 cell membranes was measured according to the method of Schmid-Antomarchi et al. (1987). MIN6 cells cultured subconfluently at passage 19 were washed and scraped off with ice-cold 40 mM HEPES/NaOH buffer (pH 7.5) containing 0.3 M sucrose. The cells were homogenized in a Potter-Elvehjem homogenizer (0°C, 1000 rpm, 5 strokes) and were centrifuged at $900 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $7900 \times g$ for 25 min at 4°C and the microsome pellet was resuspended in 20 mM HEPES/NaOH buffer (pH 7.5). For equilibrium binding assays, the microsomes were incubated for 3 h at 0°C in 1 ml of 20 mM HEPES/NaOH buffer (pH 7.5) with various concentrations of [^{3}H]glibenclamide. Incubations were terminated by rapid filtration through Whatman GF/B filters (Whatman, Maidstone, England) under reduced pressure. The filters were washed with ice-cold 100 mM Tris/HCl buffer (pH 7.5). Protein content of membranes was determined with a Bio-Rad protein assay kit (Ritchmond, CA, USA). Non-specific binding was measured in the presence of 1 μM glibenclamide. [^{3}H]glibenclamide binding was proportional to membrane protein concentrations between 25 and 200 μg ml^{-1}. K_d and B_{max} values were estimated by Scatchard analysis. In the displacement study, 25 μg of microsomes was incubated with 0.87 nM of [^{3}H]glibenclamide and various concentrations of test compounds.

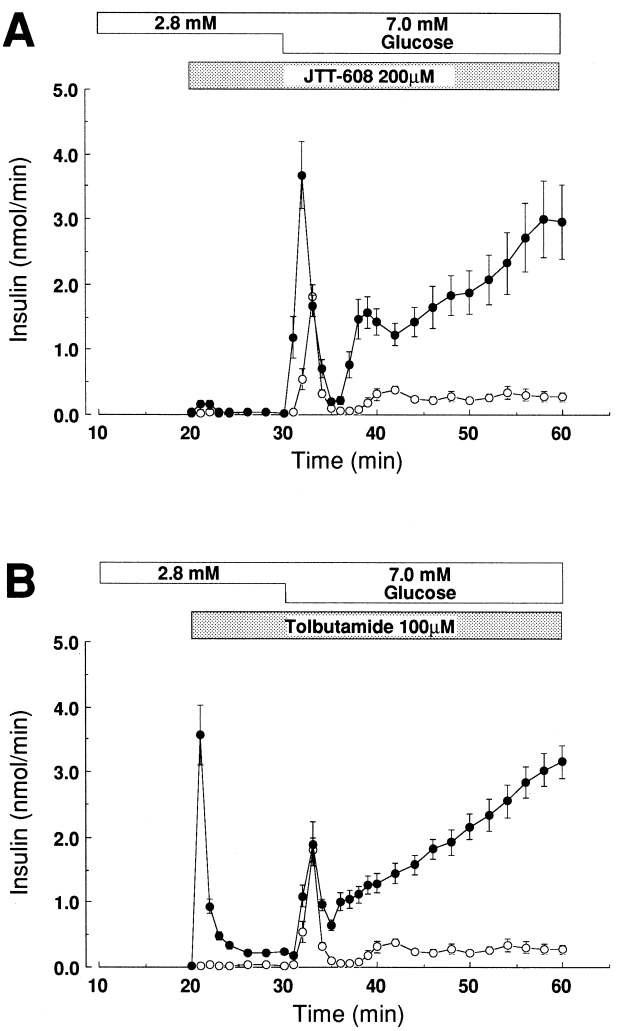

Fig. 3. Effects of JTT-608 (200 μM) (A) and tolbutamide (100 μM) (B) on insulin secretion at 2.8 mM glucose, and insulin secretion in response to 7.0 mM glucose in isolated, perfused pancreas of fasted Wistar rats (7w). Control (○), drug (●). Values are means ± S.E.M. ($N = 8$).

2.6. Statistical analysis

The data are expressed as means ± S.E.M., unless otherwise stated. The statistical significance of differences was evaluated using an one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed test or unpaired *t*-test.

3. Results

3.1. Effects on insulin secretion in MIN6 cells

Fig. 1 shows the effects of JTT-608 (10–300 μM) and two sulphonylureas (tolbutamide and glibenclamide) on insulin secretion in MIN6 cells at various glucose concentrations. In MIN6 cells treated with glucose alone, insulin secretion was clearly glucose concentration-dependent. JTT-608 markedly enhanced insulin secretion dose dependently at high glucose concentrations (8.3 mM or higher). JTT-608 (300 μM) doubled insulin secretion at 16.7 mM glucose. In contrast, tolbutamide (10–300 μM) and glibenclamide (1–30 nM) enhanced insulin secretion dose-dependently at low glucose concentrations (8.3 mM or lower), while minimal enhancement was observed at high glucose concentrations (11.1 mM or higher).

3.2. Effects on insulin secretion in isolated, perfused pancreas of normal rats

First, the effect of JTT-608 on insulin secretion was investigated using isolated, perfused pancreas of normal fed rats. As shown in Fig. 2, marked insulin secretion was observed after the glucose concentration was raised, and JTT-608 (100 μM) potently enhanced insulin secretion. The effect of JTT-608 was reversible since it was diminished within 10 min after the glucose concentration had fallen. The effect of JTT-608 on the secretion pattern was not clear in fed rats because the high second phase of secretion appeared so early that it overlapped the first phase of secretion.

Perfusion studies were next performed with the isolated pancreas of overnight-fasted rats in order to account for the results obtained from the in vivo study (Ohta et al., 1999), and to clarify the effects of JTT-608 on the secretion pattern. As shown in Fig. 3, characteristic biphasic insulin secretion consisting of a transient first phase and persistent second phase was observed in the control group. JTT-608 (200 μM) caused minimal insulin secretion at a basal 2.8 mM glucose but markedly enhanced insulin secretion in response to 7.0 mM glucose in both the first and second phases (Fig. 3A). On the other hand, tolbutamide (100 μM) markedly stimulated insulin secretion at a basal 2.8 mM glucose and enhanced insulin secretion in the second phase (Fig. 3B).

Concentration-dependent effects of JTT-608 (100, 200 and 300 μM) were compared with the effects of tolbutamide (100 μM) at several glucose concentrations (Table 1). Insulin secretion at a basal glucose concentration was negligible at doses up to 300 μM of JTT-608. On the other hand, a similar dose-dependent enhancement was observed in both the first and second phase of insulin secretion in response to 7.0 and 11.1 mM glucose. In contrast, en-

Table 1
Effects of JTT-608 and tolbutamide on insulin secretion at 2.8 mM glucose, and insulin secretion in response to 7.0 or 11.1 mM glucose in isolated, perfused pancreas of fasted rats

Rats	*N*	High glucose (mM)	Compound	Concentration (μM)	AUC of basal phase (nmol)	AUC of first phase (nmol)	AUC of second phase (nmol)
Wistar	7 to 8	7.0	Control	–	0.32 ± 0.032	2.8 ± 0.28	6.3 ± 1.41
			JTT-608	100	0.33 ± 0.044	6.0 ± 0.87	17.1 ± 4.59
				200	0.60 ± 0.151	7.4 ± 1.03[b]	45.6 ± 7.88[b]
				300	0.60 ± 0.288	9.2 ± 1.42[b]	69.2 ± 12.54[b]
			Tolbutamide	100	6.63 ± 0.815[d]	4.6 ± 0.62[c]	49.3 ± 4.52[d]
Wistar	4	11.1	Control	–	0.33 ± 0.050	5.7 ± 0.60	99.3 ± 18.69
			JTT-608	100	0.34 ± 0.053	8.2 ± 0.81	151.7 ± 24.32
				200	0.45 ± 0.073	16.0 ± 2.31[b]	246.0 ± 47.91[a]
				300	0.40 ± 0.024	14.9 ± 1.44[b]	339.6 ± 32.27[b]
			Tolbutamide	100	5.59 ± 0.746[d]	6.2 ± 0.85	145.9 ± 27.97
Sprague–Dawley	3	11.1	Control	–	0.42 ± 0.044	15.0 ± 0.95	160.5 ± 29.38
nSTZ (Sprague–Dawley)	4	11.1	Control	–	0.27 ± 0.046	4.8 ± 0.79	26.7 ± 1.90
			JTT-608	100	0.50 ± 0.135	7.3 ± 0.90	41.0 ± 8.14
				200	3.00 ± 1.098	15.4 ± 2.54[b]	65.4 ± 13.82
				300	3.00 ± 1.762	14.4 ± 2.98[a]	83.0 ± 31.70
			Tolbutamide	100	9.33 ± 1.823[d]	6.2 ± 0.65	39.6 ± 5.18

The areas under the curve (AUC) of released insulin were integrated in every phase (21–30 min, basal phase; 31–35 min, first phase; 36–60 min, second phase).
Values are means ± S.E.M. [a]$P < 0.05$, [b]$P < 0.01$ vs. control (Dunnett's two-tailed test), [c]$P < 0.05$, [d]$P < 0.01$ vs. control (unpaired *t*-test).

hancement of the second phase by tolbutamide diminished at 11.1 mM glucose.

3.3. Effects of insulin secretion in isolated, perfused pancreas of non-insulin-dependent diabetes mellitus rats

As shown in Fig. 4, insulin secretion in response to 11.1 mM glucose was severely impaired in both first and second phases in nSTZ rats compared with age-matched normal rats. JTT-608 (200 μM) almost normalized the first phase of secretion and doubled the second phase (Fig. 4A). Slight stimulation of insulin secretion was observed at a basal 2.8 mM glucose. In contrast, tolbutamide (100 μM) stimulated insulin secretion at a basal 2.8 mM glucose, but there was only a slight improvement in insulin secretion in response to 11.1 mM glucose (Fig. 4B). JTT-608 was nearly as potent and efficacious on insulin secretion as in normal fasted rats (Table 1).

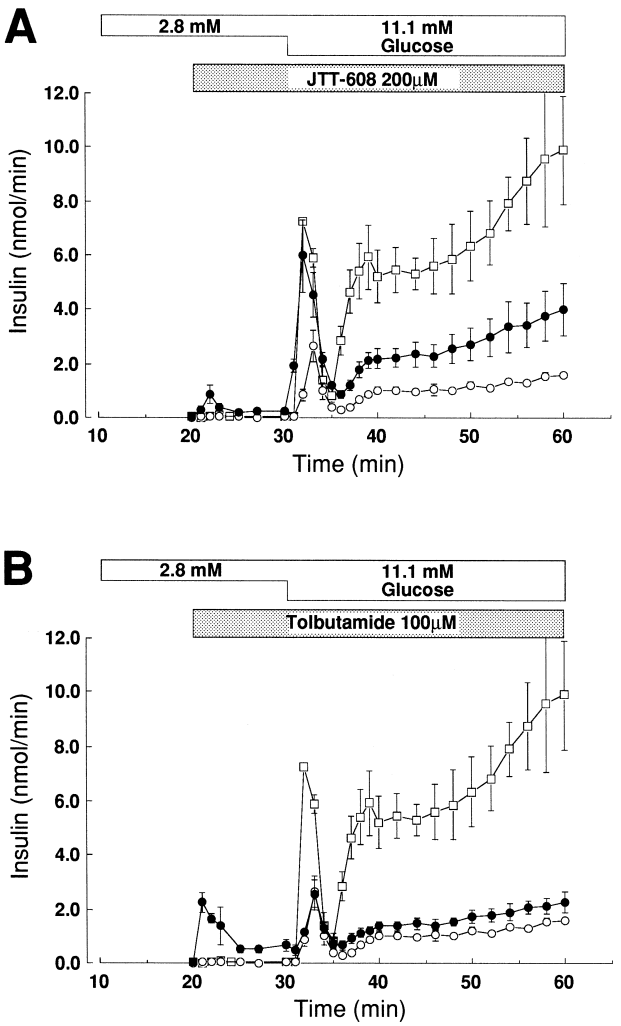

Fig. 4. Effects of JTT-608 (200 μM) (A) and tolbutamide (100 μM) (B) on insulin secretion at 2.8 mM glucose, and insulin secretion in response to 11.1 mM glucose in isolated, perfused pancreas of fasted nSTZ rats (14w). Age-matched normal rats (□), Control (○), drug (●). Values are means ± S.E.M. ($N = 3–4$).

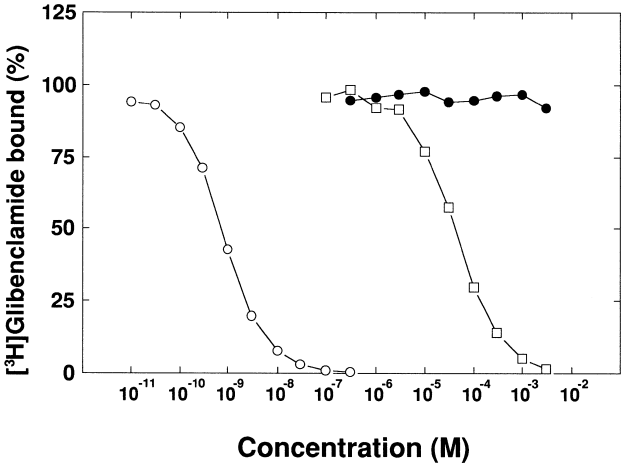

Fig. 5. Displacement of [^{3}H]glibenclamide binding to MIN6 cell membranes by JTT-608 and sulphonylurea compounds. Values are the means of three determinations. Glibenclamide (○), tolbutamide (□), JTT-608 (●).

3.4. Sulphonylurea receptor binding assay

To clarify whether JTT-608 has the same mechanism of action as sulphonylureas on insulin secretion, the inhibitory effect of JTT-608 on [^{3}H]glibenclamide binding to MIN6 membrane fractions was investigated. Scatchard analysis of specific binding of [^{3}H]glibenclamide to MIN6 membranes indicated a single type of high-affinity binding site with a binding constant (K_d) of 0.43 nM and maximum binding capacity (B_{max}) of 2.92 pmol mg^{-1} protein (data not shown). Specific binding of [^{3}H]glibenclamide was inhibited by unlabeled glibenclamide and tolbutamide in a concentration-dependent manner (Fig. 5). IC_{50} values for glibenclamide and tolbutamide were 0.8 nM and 50 μM, respectively. In contrast, JTT-608 did not inhibit the binding of [^{3}H]glibenclamideto MIN6 membranes even at 3 mM, the highest soluble concentration (Fig. 5).

3.5. Characteristics of the effect of JTT-608 on insulin secretion in MIN6 cells

In order to characterize the action of JTT-608, the effects of various compounds on the ability to enhance insulin secretion were examined using MIN6 cells. As shown in Table 2, diazoxide, a potassium channel opener, completely abolished the insulin secretion produced by high glucose (25 mM) plus JTT-608 (300 μM). Nifedipine, a Ca^{2+} channel blocker, or the removal of extracellular Ca^{2+} also completely abolished the insulin secretion produced by glucose plus JTT-608 (Table 2, upper panel).

The effect of JTT-608 on K^+-ATP channel-independent insulin secretion was also examined. JTT-608 dose dependently enhanced the insulin secretion produced by high-K^+ induced membrane depolarization in the presence of diazoxide (300 μM) and glucose (16.7 mM). On the other

Table 2
Characteristics of the effect of JTT-608 on insulin secretion in MIN6 cells

Incubation (min)	*N*	Glucose (mM)	Compound	Concentration (μM)	$[Ca^{2+}]$ (mM)	Addition	Insulin (nmol l^{-1})
60	8	0	JTT-608	0	2.54	–	1.1 ± 0.05
		25		0		–	6.9 ± 0.32
		25		300		–	10.9 ± 0.56
	4	25		300		Diazoxide 300 μM	1.3 ± 0.24[a]
		25		300		Nifedipine 10 μM	1.7 ± 0.38[a]
		25		300	0	–	2.9 ± 0.40[b]
		25		300	0	EGTA 1 mM	2.0 ± 0.17[b]
30	4	16.7	JTT-608	0	2.54	Diazoxide 300 μM + KCl 30 mM	17.2 ± 0.83
				30			20.1 ± 1.89
				100			28.9 ± 1.43[d]
				300			27.6 ± 3.53[c]
	4	16.7	Tolbutamide	0	2.54	Diazoxide 300 μM + KCl 30 mM	17.8 ± 1.32
				30			20.7 ± 1.34
				100			20.1 ± 1.18
				300			21.1 ± 1.04
	4	16.7	Glibenclamide	0	2.54	Diazoxide 300 μM + KCl 30 mM	18.6 ± 1.36
				0.003			21.8 ± 1.93
				0.01			16.7 ± 1.02
				0.03			16.1 ± 0.37

MIN6 cells were cultured in 96-well plates and incubated in KRB buffer under various conditions. Values are means ± S.E.M.
[a] $P < 0.01$ vs. glucose + JTT-608, [b] $P < 0.01$ vs. glucose + JTT-608 + Ca^{2+} (unpaired *t*-test). [c] $P < 0.05$, [d] $P < 0.01$ vs. with no added compounds (Dunnett's two-tailed test).

hand, no significant enhancement was observed with tolbutamide and glibenclamide (Table 2, lower panel).

4. Discussion

JTT-608 is a novel antidiabetic compound which was selected on the basis of its insulinotropic and hypoglycemic profiles in the postprandial state but not in the fasting state (Ohta et al., 1999). In the present study, potent insulinotropic effects of JTT-608 were demonstrated in both the mouse pancreatic β cell line (MIN6) and isolated, perfused pancreas of rats. These results clearly suggest that JTT-608 has direct effects on the pancreas, especially on pancreatic β cells. This is because MIN6 cells retain many differential functions of normal β cells and respond to glucose and sulphonylureas in a way similar to normal islets (Miyazaki et al., 1990; Ishihara et al., 1993).

The patterns of enhancement by JTT-608 and sulphonylureas were quite different in both MIN6 cells and perfused pancreas experiments. The effects of JTT-608 were pronounced at high glucose concentrations of postprandial blood sugar levels ($\geq$ 7.0 mM), but not at low glucose concentrations, i.e., less than fasting blood sugar level ($\leq$ 5.6 mM). On the contrary, the effects of sulphonylureas were more pronounced at lower glucose concentrations ($\leq$ 8.3 mM). In the results obtained from MIN6 cells (Fig. 1), sulphonylureas shifted the insulin response curve leftward (to lower glucose concentrations) but caused little increase in the maximal response at 16.7 mM glucose. JTT-608, on the other hand, caused a slight leftward shift but increased the maximal response at high glucose concentrations. All these effects of JTT-608 were observed at concentrations in a therapeutic range because the maximal blood concentration was about 300 μM when 30 mg kg^{-1} of JTT-608 was administered orally to normal fasting rats (data not shown). These glucose-dependent insulinotropic effects of JTT-608 should account for the antihyperglycemic effects observed selectively in the postprandial state (Ohta et al., 1999).

It is important that JTT-608 preserved the same action profiles not only in MIN6 cells but also in models with impaired insulin secretion. In the main part of the perfusion studies, fasted rats were used in order to account for the results obtained from the in vivo study (Ohta et al., 1999). Only in fasted animals could the absorption of drugs, the effects on fasting blood glucose level and the effects on postprandial increase in blood glucose level after fasting be properly evaluated. Fasted rats exhibit an impaired insulin secretion with some similarities to that of non-insulin dependent diabetes mellitus (Zawalich et al., 1979). nSTZ rats were used as a correspondent model of non-insulin-dependent diabetes mellitus. nSTZ rats have been demonstrated to have features of non-insulin-dependent diabetes, e.g., deterioration of glucose-induced insulin secretion and impaired glucose tolerance (Giroix et al., 1983; Tsuji et al., 1988). In the present study, the glucose-induced insulin secretion of fasted nSTZ rats was impaired more than that of normal fasted rats (Fig. 4). JTT-608 was equally efficient in both these models but tolbutamide was not, although the representative concentration of tolbu-

tamide (100 μM) had been demonstrated to close the K^+-ATP channel considerably (Akiyoshi et al., 1995; Fujita et al., 1996) and was therefore expected to be high enough to show a characteristic feature of sulphonylureas without any reversal effects, as shown in MIN6 cells (Fig. 1B).

A further conspicuous property of JTT-608 was the amelioration of the first phase of secretion in perfused pancreas. The therapeutic range of JTT-608 almost normalized the impaired first phase of secretion in the perfused pancreas of nSTZ rats. Tolbutamide, however, only minimally ameliorated both phases of insulin secretion in nSTZ rats. This property of JTT-608 contributes to its superiority over tolbutamide for the improvement of glucose tolerance in nSTZ rats (Ohta et al., 1999), and emphasizes the importance of the first phase of insulin secretion in controlling postprandial hyperglycemia. Because of all these properties, JTT-608 is expected to be a useful antidiabetic agent for non-insulin-dependent diabetes mellitus patients, without causing fasting hypoglycemia.

ATP sensitive K^+ channels (K^+-ATP channels) play an important role in the process of pancreatic insulin secretion produced by glucose and sulphonylureas. The increase of intracellular ATP concentration (or a rise in the ATP/ADP ratio), which is a result of glucose metabolism, causes the closure of the K^+-ATP channel. The closure of the K^+-ATP channel leads to membrane depolarization, Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCC) and activation of insulin exocytosis (Ashcroft et al., 1984; Prentki and Matschinsky, 1987).

Sulphonylureas bind directly to the sulphonylurea receptor and close the K^+-ATP channel (Schmid-Antomarchi et al., 1987), resulting in fasting hypoglycemia. In contrast, JTT-608 caused no inhibition of [^{3}H]glibenclamide binding to membrane fractions of MIN6 cells, on which one of the sulphonylurea receptors was shown (Inagaki et al., 1995). This observation clearly suggested that JTT-608 enhanced insulin secretion via a mechanism quite different from that of sulphonylureas and meglitinides, short-acting insulinotropic compounds, which also binds to the sulphonylurea receptor directly (Mogami et al., 1994; Fujita et al., 1996; Ikenoue et al., 1997).

The action of JTT-608 was glucose-dependent and abolished by diazoxide, nifedipine or omission of extracellular Ca^{2+}, suggesting it requires K^+-ATP channel closure and influx of extracellular Ca^{2+}, and mostly, glucose. In addition to the reversibility of the action, these findings suggest that JTT-608 acts to stimulate the exocytotic process per se but does not stimulate insulin secretion by a toxic effect on β cells.

Recently, K^+-ATP channel-independent mechanisms which can augment the distal signals of Ca^{2+} influx have been demonstrated for insulin secretion produced by glucagon, cholinergic agonists and glucose itself (Prentki and Matschinsky, 1987; Gembal et al., 1992; Sato et al., 1992). These K^+-ATP channel independent insulinotropic actions have been evaluated as effects on insulin secretion produced by high-K^+ induced membrane depolarization in the presence of sufficient amounts of diazoxide, which blocks the actions on K^+-ATP channels (Gembal et al., 1992; Sato et al., 1992). Under normal conditions, this glucose effect may contribute to the actions of sulphonylureas in the presence of glucose, but the maximal response to glucose might not be affected by sulphonylureas in the presence of glucose in concentrations high enough to itself fully close the K^+-ATP channel.

In the present study, tolbutamide and glibenclamide produced little enhancement in the K^+-ATP channel-independent insulin secretion, and minimal enhancement in insulin secretion at higher glucose concentrations under normal conditions in MIN6 cells (Fig. 1, Table 2). Tolbutamide also minimally enhanced insulin secretion in isolated, perfused pancreas at higher glucose concentrations (Table 1). These results suggest that sulphonylureas mainly affect the K^+-ATP channel to stimulate insulin secretion. On the other hand, JTT-608 enhanced the K^+-ATP channel-independent insulin secretion, and enhanced the maximal glucose response under normal conditions. All these findings suggest that the effects on post-K^+-ATP channel signalings, supposedly post-Ca^{2+} signalings, are involved in the action of JTT-608, although the sites of action remain unknown.

Several effects of JTT-608 on binding capacity to known receptors, cAMP content, intracellular calcium signaling and exocytotic pathway are under investigation to clarify the mechanisms of action of JTT-608.

In conclusion, it was demonstrated that JTT-608, unlike sulphonylureas, enhanced insulin secretion in a glucose concentration-dependent manner in MIN6 cells, and in isolated, perfused pancreas of normal and in non-insulin-dependent diabetes mellitus rats. Its inability to bind to the sulphonylurea receptor and the ability to enhance the K^+-ATP channel-independent insulin secretion suggest that JTT-608 enhanced insulin secretion by a quite different mechanism from that of hypoglycemic sulphonylureas.

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