ABSTRACT

Nateglinide and mitiglinide (glinides) are characterized as rapid-onset and short-acting insulinotropic agents. Although both compounds do not have a sulfonylurea structure, it has been postulated that insulin secretion is preceded by their binding to Kir6.2/SUR1 complex, and a mechanism of insulin secretion of glinides has been accounted for by this pathway. However, we hypothesized the involvement of additional mechanisms of insulin secretion enhanced by glinides, and we analyzed the pattern of time course of insulin secretion from MIN6 cells with the existence of agents that have specific pharmacologic actions. Dose-dependent effects of tolbutamide, glibenclamide, nateglinide, and mitiglinide were observed. Insulin secretion induced by 3 μM tolbutamide and 1 nM glibenclamide was completely inhibited by 10 μM diazoxide and 3 μM verapamil, although the latter half-component of insulin secretion profile induced by 3 μM nateglinide or 30 nM mitiglinide remained with the existence of those agents. Glinides enhanced insulin secretion even in Ca2+-depleted medium, and its pattern of secretion was same as the pattern with existence of verapamil. The latter half was suppressed by 1 μM dantrolene, and concomitant addition of verapamil and dantrolene completely suppressed the entire pattern of insulin secretion enhanced by nateglinide. Thus, we conclude that glinide action is demonstrated through two pathways, dependently and independently, from the pathway through KATP channels. We also demonstrated that the latter pathway involves the intracellular calcium release from endoplasmic reticulum via ryanodine receptor activation.

A decrease in the initial insulin secretion observed in an early stage of type 2 diabetes induces postprandial hyperglycemia. A large-scale clinical trial has demonstrated that postprandial hyperglycemia is closely related to complications of diabetes mellitus, particularly cardiovascular disorders affecting survival, and that the correction of postprandial hyperglycemia is important for the prevention of vascular events (DECODE Study Group, 2001).

Sulfonylureas, which are widely used for the treatment of diabetes mellitus, are considered to bind to Kir6.2/SUR1 present in pancreatic β cells and promote insulin secretion by closing the KATP channel and opening the voltage-dependent Ca2+ channel (Doyle and Egan, 2003). However, sulfonylureas, as the long-acting insulin secretagogues, probably cause hypoglycemia and body weight gain and are insufficient to control postprandial hyperglycemia (Shapiro et al., 1989; UK Prospective Diabetes Study Group, 1998). Furthermore, problems including secondary failure have been suggested previously (Salas and Caro, 2002).

Rapid-onset and short-acting insulinotropic agents (glinides), typically nateglinide and mitiglinide, are often used in an early stage of diabetes mellitus, because they alleviate postprandial hyperglycemia by quickly increasing insulin secretion after a meal and less frequently induce hypoglycemia than sulfonylureas (Sato et al., 1991). The insulin secretion-stimulating action of glinides is considered to be derived from a series of reactions triggered by their binding with Kir6.2/SUR1, similar to sulfonylurea (Akiyoshi et al., 1995).

Clinical effects of glinides, whose first site of action is considered to be the same as that of sulfonylureas, have been reported to appear significantly earlier and to disappear more rapidly than those of sulfonylureas (Hollander et al., 2001). These differences cannot be explained by the drug absorption rate (Ikenoue et al., 1997) or binding characteristics with SUR alone (Proks et al., 2002; Hu et al., 2003).
The presence of pathways dependent/not dependent on the K<sub>ATP</sub> channel has been clarified in the mechanism of glucose-stimulated insulin secretion (GSIS), and the results of electrophysiologic studies and studies of Ca<sup>2+</sup> influx suggest that the first phase is dependent on the K<sub>ATP</sub> channel, whereas the second phase, mediated by glucose metabolites, is not (Stroba and Sharp, 2002; Rorsman and Renstrom, 2003). However, the first phase of GSIS decreases but does not disappear in SUR1-defective patients, SUR1-knockout mice, and cells in culture (Seghers et al., 2000; Shiota et al., 2002; Shigeto et al., 2006) and is considered to be caused by a complicated insulin secretion mechanism mediated by metabolism.

In this study, we noted the differences in the characteristics of insulin secretion-promoting actions between sulfonylureas and glinides and evaluated the mechanism of insulin secretion at the cellular level using MIN6 cells, which have the same insulin secretion mechanism as isolated pancreatic β cells (Miyazaki et al., 1990; Ishihara et al., 1993). As a result, we demonstrated the presence of a pathway mediated by Ca<sup>2+</sup> release from endoplasmic reticulum, which is not dependent on the K<sub>ATP</sub> channel, in the mechanism of glinide-induced insulin secretion.

**Materials and Methods**

**Reagents.** Nateglinide and mitoglinide were generously provided from Ajinomoto Corp. (Tokyo, Japan) and Kissei Pharmaceutical Corp. (Nagano, Japan). Tolbutamide and glibenclamide were purchased from Sigma-Aldrich (St Louis, MO). Mouse insulin enzyme-linked immunosorbent assay kits were purchased from Shiba yagi (Gunma, Japan). Lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Takeara (Otsu, Japan). Dantrolene was purchased from Tocris Cookson Ltd. (Northpoint, UK). Caffeine, dibucaine, and verapamil were from Nakalai Tesque Inc. (Kyoto, Japan). Diaoxyzide and ryanodine were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals used were locally available and of analytic grade.

**MIN6 Cell Culture and Insulin Secretion Analysis.** MIN6 cells were kindly gifted from Dr. Junichi Miyazaki at Osaka University (Miyazaki et al., 1990). The cells (passages 20–30) were cultured with modified Dulbecco’s modified Eagle’s medium (containing 15% fetal bovine serum, 5 μl/l 2-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin, 3.3 g/l NaHCO<sub>3</sub>, and 25 mM glucose) in tissue culture flasks under humidified atmosphere at 37°C with 5% CO<sub>2</sub>/95% air. The culture medium was removed and replaced with fresh medium every 24 h, and the cells were used for experiments at 80% confluence.

For determination of insulin secretion, MIN6 cells were cultured in a 35-mm-diameter dish with 2 ml of the medium for 72 h and then preincubated for 30 min in 1 ml of 3 mM glucose KRBH buffer. The cells were pretreated with diazoxide, verapamil, dantrolene, and tetrodotoxin for 30 min in 1 ml of 3 mM glucose KRBH. The cells were incubated at 37°C for 5 min. The rate of [45Ca<sup>2+</sup>] uptake into the MIN6 cells was measured at a 30-s interval as the following procedure. The reaction was terminated by rapid aspiration of the buffer containing [45Ca<sup>2+</sup>]<sub>Cl</sub> followed by five times wash of the cells with ice-cold KRBH buffer, and then the cells were scraped off with 0.5 N NaOH. The procedure to stop the reaction was carried out within 40 s. An aliquot of alkaline-digested cells was neutralized with equimolar acetic acid and was subjected to measure the radioactivity accumulated in the cells with scintillation cocktail BioFluor (PerkinElmer Life and Analytical Sciences, Boston, MA) by liquid scintillation spectrometry. The content of protein in the alkaline-digested MIN6 cells was measured by the method of Lowry using bovine serum albumin as standard. The rate of calcium influx per each period (30 s) was calculated by the following equation: [calcium influx] = actual radioactivity/milligram protein — previous radioactivity/milligram protein.

**Immunohistochemical Study of Ryanodine Receptor.** Immunohistochemical study was carried out according to the method previously reported with a minor modification (Katsura et al., 2004). The cells were fixed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde, 0.2% picric acid, and 0.3% glutaraldehyde (4°C, 10 min) and then fixed again with 4% paraformaldehyde and 0.2% picric acid dissolved in 0.1 M PBS (4°C, 30 min). After washing five times with 15% sucrose containing 0.1 M PBS, the cells were incubated with the same sucrose buffer (4°C, 2 days) and 0.3% Triton X-100 containing 0.1 M PBS (4°C, 4 days). Immunostaining was performed by using ABC kit (Vector Laboratories, Burlingame, CA), with first antibody, and anti-ryanodine receptor 1, 2, and 3 antibodies (Chemicon International, Temecula, CA) were diluted with 0.01% Tween 20 containing PBS (1/200).

**Cytotoxicity Study.** The LDH in the per fusate was measured by LDH cytotoxicity detection kit in all of the experiments using reagents to assess the cell damage.

**Statistical Analysis.** Each value represents the means ± S.D. The experimental number is described in each figure legend, and each experiment was done in quartenion. The statistical analysis was determined by the method described in each figure legend following the application of the one-way analysis of variance.

**Results**

**Insulin Secretion by Sulfonylureas and Glinides.** We had already reported the dose-dependent manner of tolbutamide and glibenclamide on insulin secretion from MIN6 cells (Shigeto et al., 2006). Dose-dependent effects of nateglinide and miglitolide on insulin secretion from MIN6 cells were also confirmed in this study (Fig. 1). The pattern of insulin secretion induced by mitoglinide was same as the pattern with existence of nateglinide, with the exception that the concentration of mitoglinide was only 1/100 of nateglinide (Fig. 2). Our previous report demonstrated that tolbutamide-induced insulin secretion was initiated between 60 and 90 s after the addition of the drug with a peak at 120 s (Shigeto et al., 2006). On the other hand, insulin secretion induced by 3 μM nateglinide appeared earlier with a steep peak at 60 s and was observed until approximately 300 s. Both 3 μM tolbutamide-induced and 1 nM glibenclamide-induced insulin secretion from MIN6 cells were completely inhibited by

Measurement of [45Ca<sup>2+</sup>] Influx into the MIN6 Cells. [45Ca<sup>2+</sup>] influx into the MIN6 cells were measured by the method previously reported. MIN6 cells were preincubated for 30 min in 3 mM glucose KRBH buffer. Following the incubation with 3 mM glucose Ca<sup>2+</sup>-free KRBH buffer at 37°C for 10 min, the buffer was exchanged with fresh and warm (37°C) 3 mM glucose Ca<sup>2+</sup>-free KRBH buffer. The reaction was initiated by the addition of 2.7 mM [45Ca<sup>2+</sup>]Cl<sub>2</sub> [3.7 MBq of [45Ca<sup>2+</sup>]Cl<sub>2</sub> per dish], with or without 3 μM nateglinide. The cells were incubated at 37°C for 5 min. The rate of [45Ca<sup>2+</sup>] influx into the MIN6 cells was measured at a 30-s interval as the following procedure. The reaction was terminated by rapid aspiration of the buffer containing [45Ca<sup>2+</sup>]Cl<sub>2</sub> followed by five times wash of the cells with ice-cold KRBH buffer, and then the cells were scraped off with 0.5 N NaOH. The procedure to stop the reaction was carried out within 40 s. An aliquot of alkaline-digested cells was neutralized with equimolar acetic acid and was subjected to measure the radioactivity accumulated in the cells with scintillation cocktail BioFluor (PerkinElmer Life and Analytical Sciences, Boston, MA) by liquid scintillation spectrometry. The content of protein in the alkaline-digested MIN6 cells was measured by the method of Lowry using bovine serum albumin as standard. The rate of calcium influx per each period (30 s) was calculated by the following equation: [calcium influx] = actual radioactivity/milligram protein — previous radioactivity/milligram protein.

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the addition of 10 μM diazoxide, a K<sub>ATP</sub> channel opener, or 3 μM verapamil, an L-type voltage-dependent calcium channel blocker. However, the effects of diazoxide and verapamil on nateglinide-induced insulin secretion were quite different from the effects of those agents on sulfonylurea drug. The earlier half-component of nateglinide-induced insulin secretion was inhibited by the addition of diazoxide and verapamil, but the latter half-component still remained (Fig. 2).

**Insulin Secretion by Glinides under Ca<sup>2+</sup>-Depleted Condition.** Effects of nateglinide and mitiglinide on insulin secretion from MIN6 in the extracellular Ca<sup>2+</sup>-free condition were examined. Both 3 μM nateglinide and 30 nM mitiglinide stimulated insulin secretion with a peak at 150 s, even in the medium condition with Ca<sup>2+</sup> depletion by 1 mM EGTA, although the peak level was lower than the original one (Fig. 3). Interestingly, the pattern of insulin secretion in this condition was same as the pattern with the existence of 10 μM diazoxide or 3 μM verapamil as shown in Fig. 2.

**Tracing of Ca<sup>2+</sup> Ion after Nateglinide Stimulation.** Effect of nateglinide on the intracellular Ca<sup>2+</sup> influx is shown in Fig. 4. The addition of 3 μM nateglinide evoked a rapid influx of Ca<sup>2+</sup> ion into MIN6 cells, with a significant peak between 60 and 90 s after an onset of nateglinide stimulation. The duration until the induction of intracellular Ca<sup>2+</sup> influx by nateglinide coincided with the rapid peak of insulin secretion. We confirmed that 30 nM mitiglinide also induced a rapid influx of Ca<sup>2+</sup> ion into MIN6 cells as well as nateglinide (data not shown).

**Ca<sup>2+</sup> Handling at the Level of Endoplasmic Reticulum and Ryanodine Receptor Characterization.** The addition of 10 mM caffeine, which is known to be a ryanodine receptor agonist, induced insulin secretion from MIN6 cells, with a peak at 240 s after an onset of stimulation (Fig. 5A). The latter half of nateglinide-induced insulin secretion was completely blocked by 1 μM dantrolene, a nonspecific blocker of calcium efflux from endoplasmic reticulum (Fig. 5B). On the other hand, an addition of 5 mM caffeine augmented the latter half of insulin secretion, with a peak at 150 s (Fig. 5C). Nateglinide-stimulated insulin secretion was completely blocked by concomitant addition of 1 μM dantrolene and 3 μM verapamil (Fig. 6). In the same condition, the effect of 30 nM mitiglinide on insulin secretion was also completely inhibited (data not shown).

Immunohistochemical analysis revealed that MIN6 cells possessed ryanodine receptor types 1, 2, and 3 and also demonstrated the existence of cells that did not recognize the antibodies against ryanodine receptor types 1, 2, and 3 (Fig. 7). To confirm the role of ryanodine receptors on insulin secretion, we examined the effect of ryanodine on insulin secretion from MIN6 cells. As shown in Fig. 8A, ryanodine stimulated insulin secretion from MIN6 cells in a dose-dependent manner. Insulin secretion induced by 1 nM ryano-
dine showed a peak at 120 s and was not affected by the addition of 3 μM verapamil. On the other hand, the addition of 1 μM dantrolene completely inhibited insulin secretion induced by ryanodine (Fig. 8B).

**Discussion**

There have been a number of reports suggesting that the response of MIN6 cells to glucose and various drugs is nearly identical to that of pancreatic islets (Miyazaki et al., 1990; Ishihara et al., 1993; Shigeto et al., 2006). Therefore, the results obtained in this study are considered to be important for the clarification of in vitro responses.

Whereas glinides have been considered to induce insulin secretion by binding with sulfonylurea receptors, the present study showed that insulin secretion induced by glinides appeared significantly earlier than that by sulfonylurea, sug-
gesting different mechanisms of action between two insulin secretagogues, including binding character to sulfonylurea receptors. Our study first demonstrated the presence of a pathway other than the K<sub>ATP</sub> channel-dependent pathway in the mechanism of induction of insulin secretion by glinides. Direct evidence of this finding is that neither diazoxide nor verapamil, which are inhibitors of the K<sub>ATP</sub> channels and voltage-dependent Ca<sup>2+</sup> channels, completely inhibited glinide-induced insulin secretion. There may be an argument that the suppression of glinide action was insufficient because of the low concentrations of these inhibitors, but this possibility can be rejected for the following reasons. 1) The inhibitors used in this study had been confirmed to completely inhibit the action of sulfonylureas at the same concentrations. 2) Because responses similar to those observed with the addition of verapamil were obtained even without the presence of extracellular Ca<sup>2+</sup>, verapamil is considered to sufficiently inhibit the influx of extracellular Ca<sup>2+</sup>. Therefore, the concentrations of inhibitors are considered to have been appropriate, because they were sufficient to block their target pathway and because no sign of toxicity was noted.

It is noteworthy that the optimal concentration of the drugs used in this study for stimulation of insulin secretion was lower than the concentrations used in many previous studies. We determined the drug concentrations based on in vitro results (Shigeto et al., 2006) after confirming that the drugs do not damage MIN6 cells at any of these concentrations. Moreover, the optimal concentration obtained in this study was close to the circulating drug concentrations in clinical doses (Ikenoue et al., 1997; Doyle and Egan, 2003). In addition, the insulin secretion patterns of MIN6 cells under stimulation with nateglinide and glibenclamide were similar to clinical changes in the blood insulin concentration (Hu et al., 2001). These observations suggest that the results obtained in this study reflect in vivo pharmacological actions and are extremely useful for the analysis of action mechanisms of various drugs.

In SUR-defective patients or SUR1- and Kir6.2-knockout mice, sulfonylureas failed to stimulate insulin secretion (Seghers et al., 2000; Grimberg et al., 2001), indicating that sulfonylureas act via a K<sub>ATP</sub> channel-dependent pathway alone also in vivo. On the other hand, with glinides, the latter half of insulin secretion (component 2), but not the early half (component 1), persisted even in the absence of extracellular Ca<sup>2+</sup>, strongly suggesting the existence of K<sub>ATP</sub> channel-independent pathway.

An increase in the intracellular Ca<sup>2+</sup> concentration is generally considered necessary for insulin secretion from pan-

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**Fig. 6.** Effect of concomitant addition of 3 μM verapamil and 1 μM dantrolene on insulin secretion from MIN6 cells stimulated by nateglinide. Each value represents the mean ± S.D. of four separate experiments.

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**Fig. 7.** Immunohistochemical study to demonstrate the presence of ryanodine receptor (RyR) types 1, 2, and 3 in MIN6 cells. A, control. B, ryanodine receptor type 1. C, ryanodine receptor type 2. D, ryanodine receptor type 3. E, ryanodine receptor types 1, 2, and 3.
creatic β cells (Rorsman and Renstrom, 2003), and we evaluated the possibility that component 2 was induced by an increase in the intracellular Ca\textsuperscript{2+} concentration due to Ca\textsuperscript{2+} release from endoplasmic reticulum. As a result, component 2 was affected by the addition of dantrolene and caffeine; thus, we concluded that component 2 is due to the Ca\textsuperscript{2+} release from endoplasmic reticulum via ryanodine receptors. Experimental results concerning the Ca\textsuperscript{2+} influx also supported this conclusion. Dantrolene suppressed GSIS according to O’Mara et al. (1995) but did not affect GSIS according to Johnson et al. (2004). However, both reports were based on an evaluation at high concentrations; whether they reflect physiologic phenomena is questionable.

Ryanodine was known to stimulate insulin secretion associated with an increase in intracellular Ca\textsuperscript{2+} concentration (Johnson et al., 2004). We also confirmed that ryanodine stimulated insulin secretion from MIN6 cells dose-dependently, and the effect of ryanodine was completely inhibited by the addition of dantrolene but not by verapamil. Dan-

trolene has been used as a ryanodine receptor blocker in several organ tissues, such as skeletal muscle cells, cardiac muscle cells, and nerve cells (Brillantes et al., 1994; Parness and Palnitkar, 1995; Lee et al., 2005; Zhang and Bourque, 2006). On the other hand, the existence of ryanodine receptor types 1 and 2 has been demonstrated in pancreatic β cells (Johnson et al., 2004), and we also confirmed the presence of the receptor types 1, 2, and 3 in MIN6 cells. In this study, we demonstrated that glinide-induced insulin secretion was partly inhibited by dantrolene. Thus, it is obvious that action mechanism of glinides on insulin secretion from MIN6 cells involves a pathway through the ryanodine receptors. Okamoto and Takasawa (2002) demonstrated that ryanodine receptors and cADP ribose play an important role in GSIS under physiological conditions; this finding is considered to be useful for explaining GSIS, in which glucose metabolites are considered to be involved in a complicated manner. In our evaluation, significant suppression also was observed, with low-concentration dantrolene in agreement with this observation.

A decrease in the ryanodine receptor function has been demonstrated in multiple diabetic animal models and patients with type 2 diabetes (Islam, 2002; Patti et al., 2003). Thus, glinides, which stimulate the ryanodine receptor function, may be considered to enhance insulin secretion by a more physiologic mechanism than sulfonylureas.

**References**


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