Original Article

Imaging Docking and Fusion of Insulin Granules Induced by Antidiabetes Agents

Sulfonylurea and Glinide Drugs Preferentially Mediate the Fusion of Newcomer, but Not Previously Docked, Insulin Granules

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Sulfonylurea and glinide drugs, commonly used for antidiabetes therapies, are known to stimulate insulin release from pancreatic β-cells by closing ATP-sensitive K^+ channels. However, the specific actions of these drugs on insulin granule motion are largely unknown. Here, we used total internal reflection fluorescence (TIRF) microscopy to analyze the docking and fusion of single insulin granules in live β-cells exposed to either the sulfonylurea drug glibenclamide or the glinide drug mitiglinide. TIRF images showed that both agents caused rapid fusion of newcomer insulin granules with the cell membrane in both control and diabetic Goto-Kakizaki (GK) rat pancreatic β-cells. However, in the context of β-cells from sulfonylurea receptor 1 (SUR1) knockout mice, TIRF images showed that only mitiglinide, but not glibenclamide, caused fusion of newcomer insulin granules. Compositely, our data indicate that *1*) the mechanism by which both sulfonylurea and glinide drugs promote insulin release entails the preferential fusion of newcomer, rather than previously docked, insulin granules, and that *2*) mitiglinide can induce insulin release by a mechanism independent of mitiglinide binding to SUR1. *Diabetes* 55:2819–2825, 2006

In type 2 diabetic pancreatic β-cells, the glucose metabolic and insulin exocytotic processes are impaired, resulting in insufficient insulin release (1–4). Treatments to increase insulin release from diabetic β-cells include pharmaceutical agents targeting ATP-sensitive K^+ channels (K_{ATP} channels) (5,6) because these provide a critical regulatory function in insulin release (7–9). Drugs directed to inhibit K_{ATP} channel function include sulfonylureas and glinides. Sulfonylurea drugs such as glibenclamide have long been used clinically to increase insulin release in type 2 diabetes (10,11). More recently, nonsulfonylurea drugs, including glinides such as nateglinide (A-4166) and mitiglinide (KAD-1229), have become available for clinical use (12–14). It is generally accepted that both sulfonylureas and glinides induce K_{ATP} channel closure, resulting in downstream increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent insulin release. However, several details in the mechanism by which sulfonylureas and glinides control not only K_{ATP} channel function but also the insulin exocytotic process are still unknown.

K_{ATP} channel function is mediated by the pore-forming subunit Kir6.2 and the sulfonylurea receptor subunit 1 (SUR1) (15). The SUR subunit, a member of the ABC transporter family, is considered responsible for the Kir6.2/SUR1 complex sensitivity to sulfonylurea and glinide drugs (16–18). It has been concretely shown that sulfonylureas bind the SUR subunit and that the SUR moiety on sulfonylureas, which functions as a ligand for SUR receptors, is required for causing insulin release (11,19). Glinides such as mitiglinide lack the SUR moiety present on sulfonylureas, which facilitate these ligands binding to their cognate receptors (20,21). Nevertheless, mitiglinide was shown to inhibit [^{3}H]glibenclamide binding to SUR1 and caused increased $[Ca^{2+}]_i$ (22). Therefore, these drugs exhibit similar downstream effects but are structurally distinct and may use divergent mechanisms when compared with each other or with glucose.

To clarify mechanistic differences between these drug types, as well as between the drugs and glucose-mediated insulin release, the investigation was focused on the action of these agents on insulin granule motion, using total internal reflection fluorescence (TIRF) microscopy. We recently reported use of this system for studying insulin exocytosis in response to glucose (23–25). Using green fluorescent protein (GFP)-tagged insulin granules (23,26) and the TIRF microscopy technique originally developed by Axelrod (27), we could observe the biphasic kinetics of insulin release in real time, including a first phase, where a small population of insulin granules already docked at the plasma membrane was released, and a second phase, where newly recruited, or "newcomer," granules were

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K_{ATP} channel, ATP-sensitive K^+ channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCD, charge-coupled device; GFP, green fluorescent protein; KRB, Krebs-Ringer buffer; SUR, sulfonylurea receptor; TIRF, total internal reflection fluorescence.

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targeted to, and released from, the plasma membrane (24). Thus, this system is a powerful tool to reveal how these agents act on the insulin exocytotic process. In the present study, therefore, we used TIRF microscopy to analyze insulin exocytosis in response to sulfonylurea and glinide drugs in control, diabetic, and SUR1 knockout β-cells. Specifically, we investigated how the sulfonylurea drug glibenclamide and the glinide drug mitiglinide influenced the docking and fusion of insulin granules at the cell membrane. Our results indicate that these agents initiate rapid fusion of newcomer insulin granules with the cell membrane, rather than mimicking the glucose-mediated first phase of chiefly releasing previously docked granules. Finally, surprisingly, we found that mitiglinide in particular can cause insulin fusion in the absence of SUR1.

RESEARCH DESIGN AND METHODS

Diabetic Goto-Kakizaki (GK) rats, nondiabetic male Wistar rats, and C57BL/6J male mice were obtained from a commercial breeder (Oriental Yeast, Tokyo, Japan). SUR1 knockout mice were kindly provided by Joseph Bryan (Baylor College of Medicine, Houston, TX), and its generation and characterization were described previously (28,29). The animals were given free access to food and water until the start of experiments requiring intake control, using 10- to 11-week-old male rats or 10- to 11-week-old male mice. Blood glucose levels were determined by a glucose oxidase method (Toecho Super, Kyoto Daiichi Kagaku, Kyoto, Japan). Pancreatic islets of Langerhans were isolated by collagenase digestion with minor modifications (3). Isolated islets were dissociated into single cells by incubation in Ca^{2+}-free Krebs-Ringer buffer (KRB) containing 1 mmol/l EGTA, and then they were cultured on a fibronectin-coated high-refractive glass coverslip (Olympus) as described previously (24). To label the insulin secretory granules, pancreatic β-cells were infected with the recombinant adenovirus Adex1CA insulin-GFP as described previously (24). On the day of the experiments, infected β-cells were treated with glucose and/or glibenclamide (Sigma) or mitiglinide (synthesized at the pharmacological laboratory of Kissei Pharmaceutical, Nagano, Japan), as indicated in each figure legend, and TIRF experiments were performed as described elsewhere (23). The secretagogues were first dissolved as concentrated stock solutions in DMSO (Fluka, Buchs, Switzerland); the final concentration of DMSO was <0.5% (vol/vol).

TIRF microscopy. The Olympus TIRF system was used with an inverted microscope (IX70; Olympus) and a high-aperture objective lens (Apo 100× OHR, NA 1.65; Olympus), as previously described (23). To observe GFP, we used a 488-nm laser line for excitation and a 515-nm long-pass filter for the barrier. The images were projected onto a cooled charge-coupled device (CCD) camera (DV887DCSBV; Andor Technology, Belfast, Northern Ireland) operated with Metamorph version 6.2 (Universal Imaging, Downingtown, PA). Images were acquired at 300-ms intervals. The space constant for the exponential decay of the evanescent field was ~43 nm, as previously described (24). For the study, by TIRF microscopy of real-time images of GFP-tagged insulin granule motion, infected β-cells were placed on the high–refractive index glass, mounted in an open chamber, and incubated for 30 min at 37°C in KRB containing 110 mmol/l NaCl, 4.4 mmol/l KCl, 1.45 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.3 mmol/l calcium gluconate, 4.8 mmol/l $NaHCO_3$, 2.2 mmol/l glucose, 10 mmol/l HEPES (pH 7.4), and 0.3% BSA. Cells were then transferred to the thermostat-controlled stage (37°C) of a TIRF microscope. Stimulation was achieved by addition of the indicated concentrations of secretagogues into the chamber.

Image acquisition and analysis. Most analyses of images collected by a CCD camera, including tracking (the single projection of different images), were performed using Metamorph software. To analyze the data, we manually selected fusion events and then calculated the average fluorescence intensity of individual granules in a 1-μm × 1-μm square placed over the granule center. The number of fusion events was manually counted while looping 15,000 frame time lapses. Sequences were exported as single TIFF files and further processed using Adobe Photoshop 6.0, or they were converted into QuickTime movies. In all experiments numbering fusion events, we defined "exocytosis" and "fusion" as follows. Exocytosis involves the sequential stages within the exocytotic pathway: physical movement of vesicles to the subplasmalemmal region of the cell, tethering and then docking at release sites on the plasma membrane, conversion to a fully releasable state, triggered membrane fusion, and release of granule contents. Fusion refers only to the final step of exocytosis: fusion of vesicle membrane with plasma membrane, allowing release of granule contents.

Measurement of $[Ca^{2+}]_i$. Pancreatic β-cells were loaded with 2 μmol/l fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes) for 30 min at 37°C in KRB (2.2 mmol/l glucose), followed by washing and an additional 15-min incubation with KRB. Then, the coverslips were mounted on an ARGUS/HiSCA system (Hamamatsu Photonics, Hamatsu, Japan) and then stimulated with indicated secretogogues. Fura-2 fluorescence was detected by a cooled CCD camera after excitation at wavelengths of 340 nm (F340) and 380 nm (F380), and the ratio image (F340/F380) was calculated by use of the ARGUS/HiSCA system.

Statistical analysis. Results are the means ± SE, and statistical analysis was performed by ANOVA followed by Fisher's test and regression analysis, using StatView software (Abacus Concepts, Berkeley, CA).

RESULTS

TIRF analysis of docking and fusion of insulin granules in mitiglinide- and glibenclamide-treated rat pancreatic β-cells. Primary rat pancreatic β-cells were used to study sulfonylurea or glinide action on insulin granule motion. Rat pancreatic β-cells harboring GFP-labeled insulin granules were exposed to 5.5 mmol/l glucose or 22 mmol/l high glucose alone, or they were exposed to 5.5 mmol/l glucose plus sulfonylurea and glinide drugs. We used 500 nmol/l glibenclamide as a representative sulfonylurea drug, and we used 5 μmol/l mitiglinide as a representative glinide drug. Cells were monitored by TIRF microscopy for real-time images of docking and fusion of single insulin granules with sequential images acquired every 300 ms (Fig. 1). We found that in agreement with our previous report (24), 22 mmol/l high glucose stimulated the highest fusion of previously docked granules, the so-called "residents" that were visible before stimulation (Fig. 1*A*, *resident*) during the first phase of 0–4 min poststimulation. Fusion was observed as a suddenly brightening and vanishing fluorescent spot. In contrast, in the >4-min second phase, the fusion events rather arose from newcomer granules (Fig. 1*A* and supplemental movie 1, which is detailed in the online appendix [available at http://diabetes.diabetesjournals.org]). Stimulation by 5.5 mmol/l glucose only resulted in an overall small number of fusion events, mostly from newcomers (Fig. 1*B* and supplemental movie 2). On the other hand, 5 μmol/l mitiglinide under 5.5 mmol/l glucose significantly stimulated rapid fusion events, involving mostly newcomer granules, in addition to some previously docked granule fusion events (fusion events from previously docked granules 9.00 ± 1.61 per 200 μm^2 in 0–4 min, $n = 5$ cells) (Figs. 1*C* and 2*C* and supplemental movie 3). Treatment with 500 nmol/l glibenclamide under 5.5 mmol/l glucose also stimulated rapid fusion events from newcomers (Fig. 1*D* and supplemental movie 4). As shown in Fig. 2, fusion events stimulated by mitiglinide and glibenclamide were markedly increased compared with 5.5 mmol/l glucose stimulation (total fusion events: 44.2 ± 7.60 vs. 9.02 ± 0.90 per 200 μm^2 for mitiglinide vs. 5.5 mmol/l glucose only, 0–4 min, $P < 0.001$, $n = 5$ cells; and total fusion events: 25.4 ± 5.24 vs. 9.02 ± 0.90 per 200 μm^2 for glibenclamide vs. 5.5 mmol/l glucose, 0–4 min, $P < 0.0005$, $n = 5$ cells). In the histogram of fusion events, when the number of fusion events is small, the error bar is large. This is due to counting the number of fusion events as zero when no fusion occurred. The kinetics of these two drugs varied in that the largest number of fusion events was observed 61–120 s after the addition of mitiglinide, versus 121–180 s after the addition of glibenclamide. Thus, the time for responding to mitiglinide was rapid compared with that of glibenclamide. We also noted that the number of fusion events initiated by mitiglinide in 0–4 min was larger than

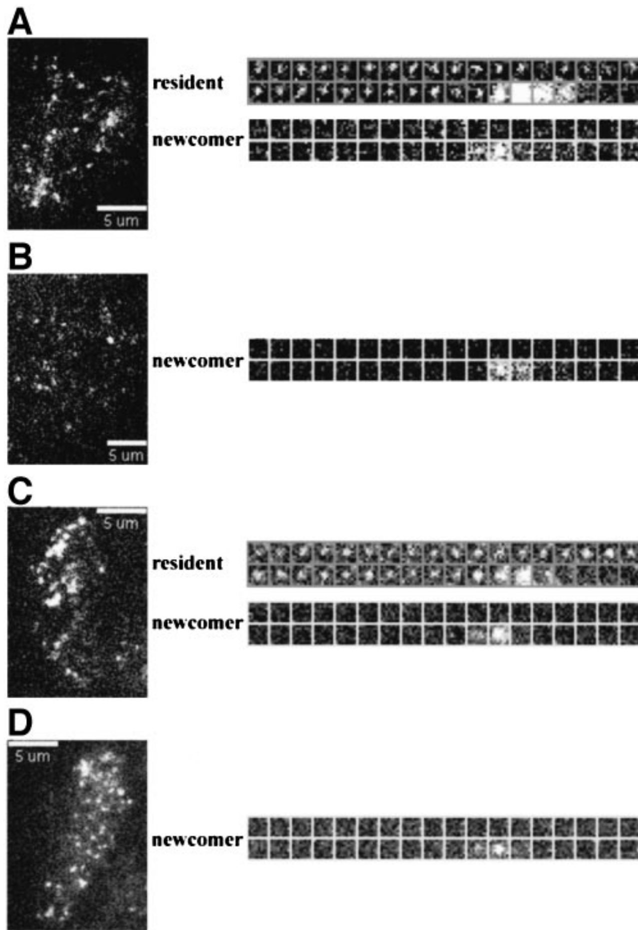

FIG. 1. Mitiglinide and glibenclamide preferentially cause fusion of newcomer insulin granules with the plasma membrane. Rat pancreatic β-cells were infected with Adex1CA insulin-GFP, preincubated with KRB including 2.2 mmol/l glucose for 30 min, and then stimulated with glucose or antidiabetes agents: 22 mmol/l glucose (*A*), 5.5 mmol/l glucose (*B*), 5 μmol/l mitiglinide under 5.5 mmol/l glucose (*C*), and 500 nmol/l glibenclamide under 5.5 mmol/l glucose (*D*). GFP-labeled insulin granules were imaged in 300-ms intervals. (See supplemental movies 1 [22 mmol/l glucose], 2 [5.5 mmol/l glucose], 3 [5 μmol/l mitiglinide under 5.5 mmol/l glucose], and 4 [500 nmol/l glibenclamide under 5.5 mmol/l glucose], which are detailed in the online appendix.) Sequential images (1 μm × 1 μm) of a granule docking and fusion with the plasma membrane under stimulation are presented.

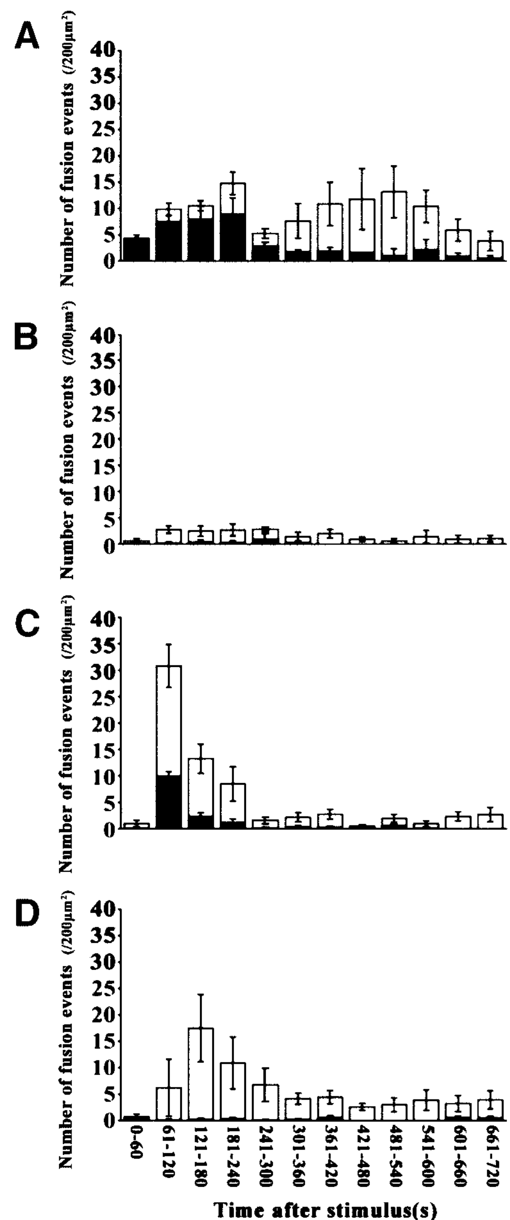

FIG. 2. Histogram of the number of fusion events under treatments. Shown is the number of fusion events per 200 μm² at 60-s intervals poststimulation. *A*: 2 mmol/l glucose. *B*: 4.4 mmol/l glucose. *C*: 5 μmol/l mitiglinide under 5.5 mmol/l glucose. *D*: 500 nmol/l glibenclamide under 5.5 mmol/l glucose. ■, fusion of previously docked granules (resident); □, fusion of newcomers.

those initiated by 22 mmol/l glucose and 500 nmol/l glibenclamide (44.2 ± 7.60 vs. 29.8 ± 4.05 per 200 μm^2 for 5 μmol/l mitiglinide vs. 22 mmol/l glucose, respectively, $P < 0.001$, $n = 5$ cells; and vs. 25.4 ± 5.24 per 200 μm^2 for glibenclamide, $P < 0.001$, $n = 5$ cells) (Fig. 2).

Figure 3 shows histograms of fusion events stimulated in a dose-dependent manner by both mitiglinide and glibenclamide. Under 50 nmol/l concentrations of these agents, little effect on fusion was observed. Higher 500-nmol/l concentrations of both mitiglinide and glibenclamide caused rapid fusion, mostly from newcomer granules. Although the maximum effect of glibenclamide on the fusion event was observed at 500-nmol/l concentrations, treatment with 5 μmol/l glibenclamide still did not cause fusion of previously docked granules with the cell membrane. As shown in Fig. 4, both 5 μmol/l mitiglinide and 5 μmol/l glibenclamide increased $[Ca^{2+}]_i$, which then remained at a plateau level during stimulation, but no marked differences in the pattern of calcium induction were observed between mitiglinide and glibenclamide treatment.

Mitiglinide and glibenclamide can induce fusion of granules in diabetic GK β-cells. We examined the effect of mitiglinide and glibenclamide on insulin granule motion in diabetic rat GK β-cells. The plasma glucose concentration in the fed status was 210 ± 22 mg/dl ($n = 4$) in GK rats and 112 ± 18 mg/dl ($n = 4$) in control rats ($P < 0.0001$). In agreement with our previous data (24), the number of fusion events stimulated by 22 mmol/l glucose was markedly reduced in diabetic β-cells during the first phase (0–4 min) (Fig. 5*D*). Although 5.5 mmol/l glucose caused few fusion events in diabetic β-cells (Fig. 5*C*), supplementing the 5.5 mmol/l glucose with 500 nmol/l mitiglinide induced a number of rapid fusion events involving newcomers (41.9 ± 7.00 vs. 10.4 ± 1.54 per 200 μm^2 for mitiglinide vs. 5.5 mmol/l glucose, respectively, 0–4 min, $P < 0.001$, $n =$

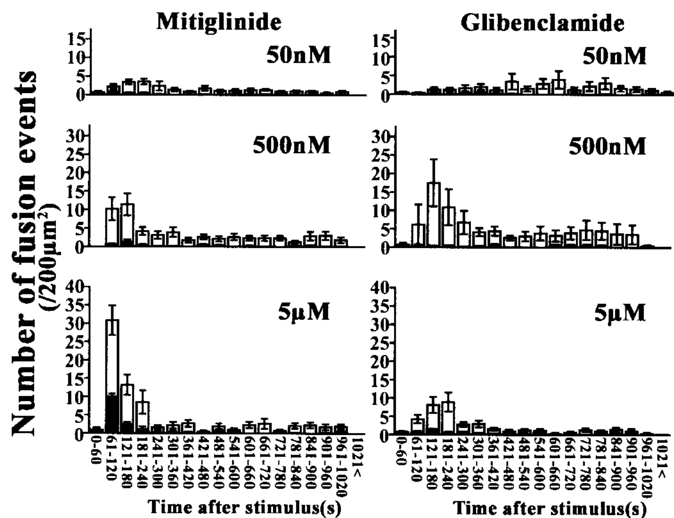

FIG. 3. Dose-dependent fusion events in mitiglinide- and glibenclamide-stimulated cells. Rat pancreatic β-cells prepared as in Fig. 1 were stimulated with mitiglinide and glibenclamide under 5.5 mmol/l glucose. The histogram shows the number of fusion events ($n = 5$ cells) at 60-s intervals poststimulation. ■, fusion of previously docked granules; □, fusion of newcomers.

6 cells) (Fig. 5*A*). In the case of 500 nmol/l glibenclamide treatment, the number of fusion events noted in 0–4 min also increased (37.8 ± 6.67 vs. 10.4 ± 1.54 per 200 μm^2 for glibenclamide vs. 5.5 mmol/l glucose, respectively, $P < 0.0001$, $n = 5$ cells) (Fig. 5*B*). In contrast to the rapid fusion events induced by mitiglinide, which quickly decayed, glibenclamide-induced fusion events continued until 5 min after stimulation.

Mitiglinide, but not glibenclamide, induced fusion of granules in SUR1 knockout mouse β-cells. It is generally accepted that mitiglinide and glibenclamide bind the SUR1, resulting in K_{ATP} channel closure (19–22). To directly explore the relationship between SUR1 and glib-

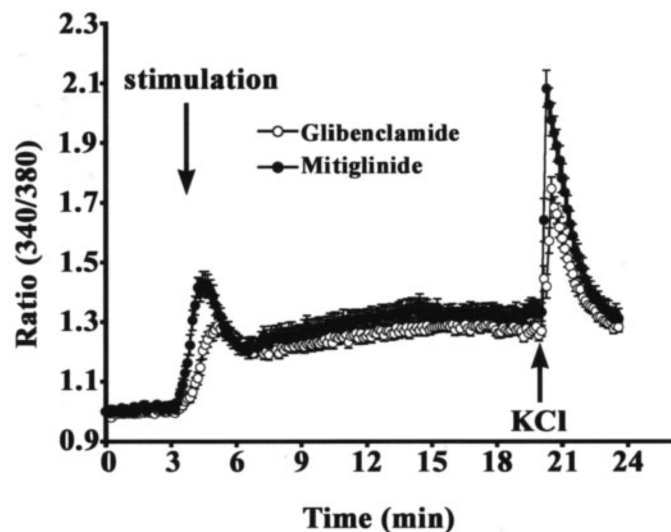

FIG. 4. $[Ca^{2+}]_i$ responses to mitiglinide and glibenclamide in rat pancreatic primary β-cells. $[Ca^{2+}]_i$ was measured using fura-2 acetoxymethyl ester by an Argus calcium imaging system in pancreatic β-cells, represented by a ratio of 340 to 380 nm. The initial preincubation period of 30 min under 2.2 mmol/l glucose is not shown. At 3 min after the recording was started, 5 μmol/l concentrations of mitiglinide and glibenclamide were applied under 5.5 mmol/l glucose. At the end of stimulation, 50 mmol/l KCl was added. Values are the means ± SE for 15 cells from four separate experiments.

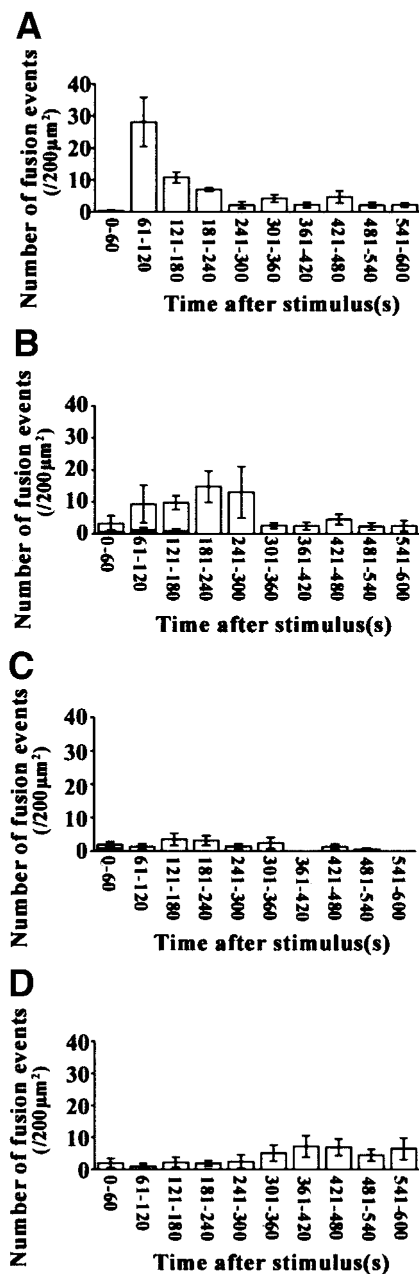

FIG. 5. TIRF analysis of insulin granule motion in diabetic β-cells stimulated by mitiglinide and glibenclamide. Diabetic β-cells prepared from 12-week-old GK rats were infected with Adex1CA insulin-GFP and exposed to 500 nmol/l mitiglinide (*A*), 500 nmol/l glibenclamide under 5.5 mmol/l glucose (*B*), 5.5 mmol/l glucose (control) (*C*), and 22 mmol/l glucose (control) (*D*). TIRM images were acquired every 300 ms (fusion events per 200 μm^2, $n = 5$ cells each). ■, fusion from previously docked granules; □, fusion from newcomers.

enclamide or mitiglinide effect on the fusion of insulin granules, we examined the action of these agents in SUR1 knockout mouse β-cells by TIRF imaging. As shown in Fig. 6*C*, TIRF results confirmed our previous report that SUR1 knockout β-cells exhibit rare fusion events stimulated by 22 mmol/l glucose (29). Here, we observed that 500 nmol/l mitiglinide stimulated fusion from newcomer insulin granules in these cells (Fig. 6*A*). The number of fusion events that occurred 0–4 min after stimulation with mitiglinide was significantly increased relative to the number after 22 mmol/l glucose (14.0 ± 1.92 vs. 6.12 ± 1.12 per 200 μm^2 for mitiglinide vs. 22 mmol/l glucose, respectively, $P < 0.001$,

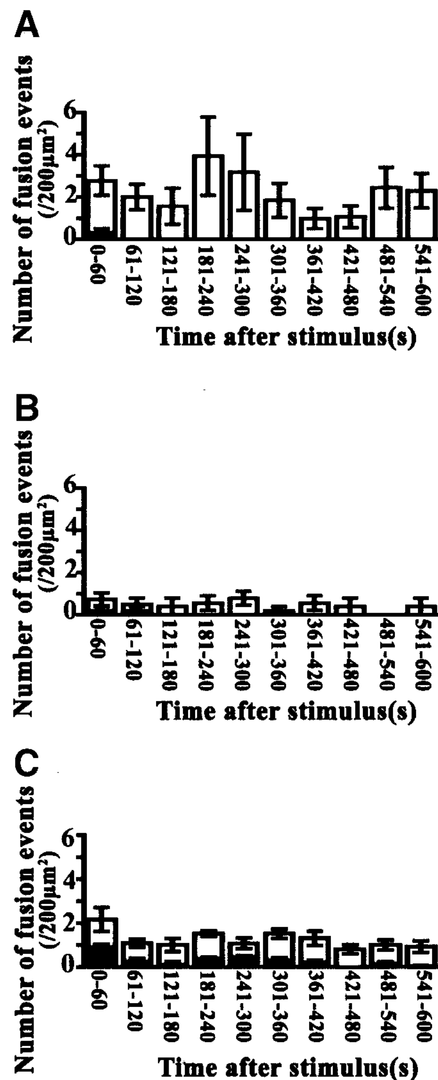

FIG. 6. Mitiglinide still causes insulin fusion events in SUR1 knockout mouse pancreatic β-cells. SUR1 knockout β-cells were prepared as in Fig. 1. Cells were stimulated by 500 nmol/l mitiglinide (*A*), 500 nmol/l glibenclamide under 5.5 mmol/l glucose (*B*), or 22 mmol/l glucose (*C*). TIRF images were acquired every 300 ms (fusion events per 200 μm², *n* = 5 cells). ■, fusion from previously docked granules; □, fusion from newcomers.

n = 5 cells). In contrast, it should be noted that 500 nmol/l glibenclamide did not stimulate fusion in SUR1 knockout β-cells. Thus, our data indicate that mitiglinide has a SUR1-independent pathway for the stimulation of insulin exocytosis.

DISCUSSION

Sulfonylurea and glinide drugs are commonly used to treat type 2 diabetes; however, the mechanism of these drugs' action on the translocation, docking, and fusion of insulin secretory granules is largely unknown. Here, we used TIRF microscopy to investigate the action of these drugs on insulin granule motion and to provide the first report that these drugs activate the translocation of insulin granules from an inner pool to subsequent fusion with the plasma membrane. These results add details, and raise additional questions, regarding the mechanism by which these drugs stimulate insulin release from pancreatic β-cells.

We found that both glibenclamide and mitiglinide caused rapid fusion in a time course nearly identical to the first phase of glucose-stimulated insulin release, mostly from newcomer insulin granules, rather than docked insulin granules, in both control and diabetic pancreatic β-cells. It is intriguing that both agents had little affect on the status of previously docked insulin granules, even though sulfonylureas were originally thought to mimic the first phase of glucose-induced insulin release, in which previously docked granules are the major type released (24,25). The reason for this mechanistic difference is currently unknown, but we anticipate that the intracellular site of SUR1 and K_{ATP} channels might be involved in the action of these agents. Indeed, several reports have indicated that there are intracellular actions of sulfonylureas (30–32). Recently, Geng et al. (33) demonstrated that SUR1 and K_{ATP} channels are present on the insulin secretory granules. Renstrom et al. (34) proposed the model that sulfonylurea binding to the secretory granule membrane facilitates lowering of granule pH, resulting in increased exocytosis. If that is the case, intracellular interaction of sulfonylureas and glinides with insulin granules may accelerate granule translocation, priming, and exocytosis and may account for the drug-induced fusion of newcomer granules. On the other hand, it should be noted that only mitiglinide, but not glibenclamide, caused fusion in SUR1 knockout β-cells, indicating that action of glibenclamide is mainly mediated through SUR1, but action of mitiglinide is not. Thus, there must be not only the mechanistic difference between mitiglinide and glibenclamide, but also unknown mechanisms to be anticipated for this phenomenon. In addition, the question of why the $[Ca^{2+}]_i$ increase by both agents does not cause fusion from previously docked granules remains to be determined. We speculate that there must be differences in the exocytotic process and/or in sites relevant to increased $[Ca^{2+}]_i$ between fusion from previously docked granules and fusion from newcomers, but further studies are required to dissect these differences.

Our results show that fusion of insulin granules induced by mitiglinide was more rapid than that induced by glibenclamide. This result is incongruent with a generally accepted mechanism in which mitiglinide acts solely through interaction with SUR1. Because mitiglinide's affinity for SUR1 has been shown to be weaker than glibenclamide's affinity for SUR, the more rapid fusion induced by mitiglinide is inexplicable in terms of binding affinity toward SUR1 (22). Furthermore, because the increase in $[Ca^{2+}]_i$ induced by mitiglinide and glibenclamide was almost similar, we cannot explain the faster fusion caused by mitiglinide in terms of $[Ca^{2+}]_i$ change. One consideration is that these drugs might target disparate molecules and protein interactions. Previous studies showed that glibenclamide quickly crosses the lipid bilayer (35); we do not know the comparative movement of mitiglinide across the membrane or what cellular molecules besides SUR1 may interact with mitiglinide. Given the lipophilic nature of mitiglinide, it seems likely that mitiglinide may rapidly enter the cytosol and interact with mitiglinide-targeted molecules associated with intracellular insulin granule trafficking, thereby facilitating efficient insulin granule translocation and subsequent fusion.

The likelihood that mitiglinide interacts with an insulin secretion/regulatory cellular protein other than SUR1 is supported by our finding that only mitiglinide, but not glibenclamide, induced fusion events in SUR1 knockout

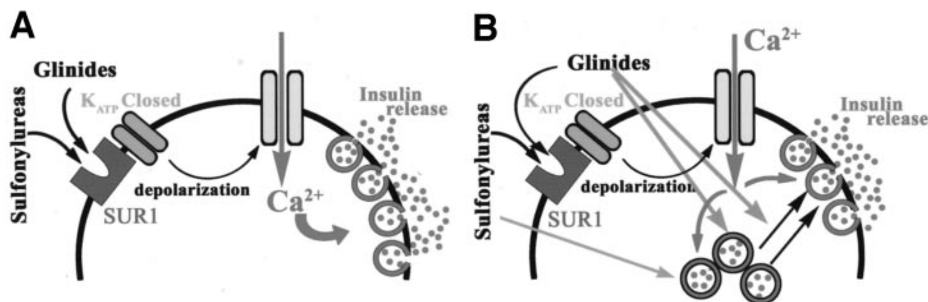

FIG. 7. Mechanism by which sulfonylurea and glinide drugs act on insulin exocytosis. ***A*****: Proposed model. It is generally supposed that sulfonylureas and glinides bind SUR1, resulting in K_{ATP} channel closure, and subsequently opening Ca^{2+} channels. Increased $[Ca^{2+}]_i$ triggers the fusion of previously docked granules, resembling the first phase of glucose-induced insulin release.** ***B*****: Our model based on TIRF data. Our TIRF imaging analysis revealed that these drugs directly activate the insulin granules to accelerate the translocation from intracellular pool to the plasma membrane for subsequent fusion. Mitiglinide appears to have a SUR1-independent pathway for modulating insulin granule motion.**

β-cells. At the least, this suggests that mitiglinide affects a molecule(s) not affected by glibenclamide. Although glibenclamide action is mostly mediated via SUR1, mitiglinide appears to have alternative interacting proteins or pathways besides SUR1 available for regulating insulin granule motion. Based on the differences these drugs display in efficiency of insulin release in rat β-cells and in ability to release insulin from SUR1 knockout mouse β-cells, we speculate that mitiglinide might bind an unidentified intracellular molecule closely associated with vesicle trafficking. We propose the following as possible mechanisms by which mitiglinide increases fusion events from newcomer insulin granules: *1*) mitiglinide might affect cytoskeletal elements such as actin or myosin because myosin Va was recently reported to be involved in insulin granule movement during the second phase of insulin release (36); *2*) mitiglinide might directly affect the granular pH, which was recently reported to regulate insulin exocytosis (34,37,38); or *3*) mitiglinide might increase insulin biosynthesis.

From a clinical perspective, our finding that sulfonylurea and glinide drugs induced the fusion of newcomer granules confirms the beneficial role of these drugs in treating type 2 diabetes. We previously reported that diabetic GK rat β-cells showed a marked reduction in the number of docked insulin granules, which may be one of the factors causing the reduced fusion events during the first phase of insulin release (4,24). This implies that drugs driving the fusion of previously docked granules with the plasma membrane may be less effective in type 2 diabetes therapy than drugs that drive the fusion of newcomers, as seen here for glibenclamide and mitiglinide. Our finding showing that mitiglinide caused increased fusion in diabetic GK β-cells relative to that observed in normal β-cells (fusion events: 41.9 ± 7.00 vs. 29.8 ± 4.95 per 200 μm^2, respectively, in 0–4 min, $P < 0.0001$, $n = 5$ cells) also supports the specific application of this drug as a diabetic therapy. We consider it possible that an unidentified target molecule for mitiglinide may be upregulated in diabetic β-cells, therefore promoting drug efficacy or efficiency. We propose a possible mechanism by which sulfonylurea and glinide drugs activate insulin granule motion and subsequent fusion, as shown in Fig. 7.

In conclusion, our TIRF imaging analysis revealed that the mechanism of action for both the sulfonylurea and glinide drugs tested here preferentially induced fusion of newcomer insulin granules; previously docked granules in both normal and diabetic β-cells received little or no influence from these drugs. Because the mechanism of mitiglinide action singularly appeared to achieve this effect even in the absence of SUR1, a new door is opened for investigating SUR1-independent pathways.

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