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Probing of Specific Binding of Synthetic Sulfonylurea with the Insulinoma Cell Line MIN6

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To overcome the limitation of conventional sulfonylurea (SU) for investigation of biological mechanisms related to K_{ATP} channels, a hypoglycemic sulfonylurea (SU) was conjugated with a non-reducing glucose bearing polystyrene (PS) derivative to provide enhanced interaction with an insulinoma cell line (MIN6). The specific interaction between the SU (K^+ channel closer)-conjugated copolymer and MIN6 cells was confirmed by confocal laser microscopic images using rhodamine B isothiocyanate (RITC)–labeled SU-conjugated polymer, which revealed the specific interaction between SU-conjugated polymer and MIN6 cells. Moreover, the location of labeled polymer and the site of Ca^{2+} ion mobilization obtained from the same MIN6 cells were identical. Based on the specificity and insulinotropic activity, the SU-conjugated polymer is expected to be useful tool for the study of biological mechanisms of K_{ATP} channels.

Key words: Ca^{2+}, K_{ATP} channels, MIN6 cells, specific interaction, sulfonylurea.

Among the important drugs regulating biological functions of pancreatic β cells are hyperglycemic sulfonylureas (SUs), which have been used for the treatment of type 2 diabetes mellitus because of their insulinotropic activity on pancreatic islets (*1*, *2*). Their principle target is the ATP-sensitive potassium channel, which plays a major role in controlling the β cell membrane potential. Inhibition of K_{ATP} channels by glucose (*3*, *4*) or SUs (*5*) causes depolarization of the β cell membrane; in turn, this triggers the opening of voltage-gated Ca^{2+} channels (*6*, *7*), leading to Ca^{2+} influx and a rise in its intracellular concentration, which stimulates the exocytosis of insulin-containing secretory granules (*8*). Binding sites for these agents were shown to have a very similar tissue distribution to the ATP-sensitive potassium channel, and the interaction mechanism of SU with the K^+ channel of cells was clarified (*9*, *10*). Thus, in pancreatic β cells, the drug binds to sulfonylurea receptor (SUR), which forms an ATP-sensitive K^+ (K_{ATP}) channel; and blocking of K_{ATP} channels by sulfonylureas (SU) is influenced by ATP, ADP, and divalent cations (*11*). Although the mechanism by which SU for triggers insulin secretion has been intensively studied, studies are limited by the need to use insulinoma model cell lines or primary single cells enzymatically digested from intact islets. To address this issue and to establish a new methodology that allows investigation of the binding behavior of SU with an inuslinoma cell line, we utilized a SU-conjugated polymer (*12*, *13*).

In this study, MIN6 cells, one of several pancreatic β cell lines, were used instead of primary islets to examine the effectiveness of SU-conjugated polymers in promoting insulin secretion and to provide more direct evidence of the mechanism by which the β cell interacts with SU-conjugate polymer. To probe the interaction between a sulfonylurea and its receptor, we attempted a novel approach using a sulfonylurea derivative. To firmly determine whether the SU-conjugated polymer is a comparable agent (drug) to β-cells, a competition test with glibenclamide was performed. Finally, the interaction and Ca^{2+} ion release from MIN6 stimulated by SU-conjugated polymer was visualized by confocal laser microscopy using rodamine B isothiocyanate (RITC)–labeled polymer. This experiment provides clear evidence of K_{ATP} channel modulation by sulfonylurea and also can offer a new tool for examining the biological events involving K_{ATP} channels.

MATERIALS AND METHODS

Preparation of Grafted SU Derivative—An antidiabetic SU analog was synthesized as follows. Briefly, *p*-aminoethyl benzene sulfonamide (1 g, 6 mmol) and acryloyl chloride (0.45 g, 6 mmol) dissolved in a mixture of 5 ml of acetone and 5 ml of 1 N NaOH aqueous solution were reacted for 2 h at room temperature, and the product was recrystallized twice from MeOH. The resultant 4-[(2)′-acrylamido-ethyl] benzene sulfonylamide (AEBSA) (2.7 g, 7 mmol) was swollen in acetone and poured into 7 ml of 1 N NaOH aqueous solution; then cyclohexylisocyanate (0.9 ml, 7 mmol) dissolved in 3.5 ml of acetone was reacted with the AEBSA solution for 16 h at room temperature with stirring. The reaction mixture was then poured into 1 N HCl aqueous solution (7 ml), and the resultant precipitate was vacuum-dried. A mixture of *N*-*p*-vinylbenzyl D-maltonamide (VMA) and cyclohexyl 4-[(2)′-acrylamido-ethyl] benzene SU and AIBN as an initiator dissolved in DMSO were poured into a glass ampule.

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The sealed ampule was placed in a thermostat at 60°C for 6 h. After polymerization, the solution was poured into methanol to obtain a precipitate. The product was reprecipitated from methanol and freeze-dried. The chemical structures of SU-incorporated polymer synthesized in this study were confirmed by ^{13}C-NMR.

Synthesized polymer was labeled with 50 mg of rhodamine B isothiocyanate (RITC). Fifteen micrograms of dilaurated dibutyltin was added, and the mixture was allowed to stand for 2 h at 90°C. After cooing, the mixture was added to an excess of ethanol to precipitate fluorescence labeled polymer. Finally, the polymer was dissolved in 30 ml water and dialyzed against 1,000 ml of mild alkaline water (pH 8.0 with NaOH) for 1 day, and then against distilled water for 3 days, exchanging water three times. RITC-labeled polymers were obtained by freeze-drying. On average, one RITC molecule was introduced approximately per hundred repeat units for each copolymer, as determined by use of a microplate reader (FL600, Bio-Tek, USA).

Cell Culture and Binding Affinity Test—MIN6 cells were kindly provided by Dr. Jun-ichi Miyazaki. MIN6 cells were cultured in DMEM (25 mM glucose) equilibrated with 5% CO_2 and 95% air at 37°C. The medium was supplemented with 15% fetal calf serum, 50 mg/liter streptomycin and 75 mg/liter penicillin sulphate. MIN6 cells (5×10^5) suspended in DMEM containing 25 mM glucose were seeded onto a polystyrene culture dish and precultured for 3 weeks. MIN6 cells in confluent state were detached by treatment with phosphate-buffered saline (PBS) containing 0.5 mM EDTA for 5 min.

Flow Cytometry Analysis—Subcultured MIN6 cells were treated with trypsin–EDTA solution and incubated with FITC-labeled polymers at 4°C for 1 h. The cells were washed with PBS solution three times and subjected to flow cytometric analysis. All measurements were performed with a Cyto ACE-150 apparatus(JASCO, Tokyo, Japan). Each sample of 10,000 cells was analyzed by logarithmic amplication of fluorescence intensity (FI).

Calcium Imaging—Subcultured MIN6 cells were incubated with 2 μM Fluo-3 AM (Molecular Probes, Inc., Eugene, OR) in HEPES-balanced KRBB solution for 1 h at 37°C without gas-phase supplement. After two washes with PBS, the cells were then incubated in 1 ml of 5.6 mM glucose HEPES-balanced KRBB solution and stimulated with 5 μl of 2 μM RITC-labeled polymers (final concentration of 10 nM) for 15 min at 37°C. The cells were then quickly rinsed with PBS and fixed with 4% formaldehyde solution for a few minutes. Confocal microscopic images were obtained by continuously switching fluorescence filters for the labeled polymer (RITC; 570 nm excitation and 595 nm emission) and for the Fluo-3 AM (FITC; 490 nm excitation and 520 nm emission). Images of calcium mobilization and labeled polymer bound to the MIN6 cells were displayed as a result of synchronous detection of RITC and FITC in the same cell.

Fluorescence Intensity Analysis—MIN6 cells (5×10^5) were plated in a 24-well tissue culture dish and cultured for 3 d. The solution of RITC-labeled polymers in DMEM medium was added, and cells were incubated at 37°C for 1 h. The cells were then washed three times with HEPES-balanced KRBB, and the fluorescence intensity of cells in 1 ml of HEPES-balanced KRBB was measured with a fluorescence microplate reader (FL600, Bio-Tek, USA).

Confocal Laser Microscopic Analysis—MIN6 cells in confluent state were detached by treatment with phosphate-buffered saline (PBS) containing 0.5 mM EDTA for 5 min. The detached MIN6 cells were preincubated in HEPES-balanced KRBB (pH 7.4, 3.3 mM glucose) equilibrated with 5% CO_2 and 95% air at 37°C for 20 min, then incubated in HEPES-balanced KRBB containing RITC-labeled at 4°C or 37°C for 1 h. The cells were then washed three times with HEPES-balanced KRBB and placed on a cover glass, which was gently placed on a slide glass. The lumination of MIN6 cells was observed using a confocal laser microscope (Leika, Heidelbulg, Germany). The cells were then replaced into 1 ml of HEPES-balanced KRBB from DMEM (25 mM glucose) and incubated with 2 ml of 1 mM Fluo-3 AM in DMSO fro 1 h at 37°C. After a wash with PBS, the cells were incubated in 1 ml of HEPES-balanced KRBB (3.3 mM glucose) and stimulated with 5 ml of 2 mM (concentration of SU stock solution) RITC-labeled polymer for 15 min at 37°C. They were then quickly rinsed with PBS and fixed with 4% formaldehyde solution for a few minutes. The MIN6 cell suspension was placed onto a cover glass, which was gently placed on a slide glass. Specific interaction between RITC-labeled SU polymer and MIN6 cells was observed by means of a confocal laser microscope (Leika, Heidelberg, Germany).

RESULTS AND DISCUSSION

Glibenclamide Competes with SU-Conjugated Copolymer for Binding to MIN6 Cells—In a previous study, the bioactivity of SU-conjugated copolymer was explored by adding the copolymer into MIN6 cell culture. The amount of the copolymer was adjusted to give a final concentration of 10 nM SU units in the medium. The SU-conjugated copolymer stimulated insulin secretion up to 2.5-fold at low glucose concentration and 1.3-fold at high glucose concentration compared with unstimulated MIN6 cells. This means that SU-conjugated polymer showed almost the same effect on insulin secretion as glibenclamide (*12*, *13*).

In order to correlate to the pharmacological effect of SU-conjugated polymer with that of a glibenclamide, a competition test was conducted between them. If SU-conjugated polymer triggers insulin secretion through a practically identical mechanism to the signal pathway of native glibenclamide, it will vie with glibenclamide for binding to one site, presumably the K_{ATP} channel. Figure 1 shows the flow cytometric profiles of MIN6 cells incubated with RITC-labeled polymers. In this experiment, concentration of 10 nM SU-conjugated polymer, together with 1 nM (a), 10 nM (b), and 100 nM glibenclamide (c) were employed. As shown in Fig. 1, histogram of MIN6 cells in flow cytometry at a ratio of SU-conjugated polymer to glibenclamide 10:1 was shifted to the right side. However, histogram of flow cytometry at ratios of 1:1 and 1:10 specific interaction with MIN6 cells was less. It was evident that when the higher glibenclamide concentration was applied, fluorescence of RITC-labeled polymer disappeared. This result clearly suggests that SU-conjugated polymer competes with glibenclamide for the binding to MIN6 cells.

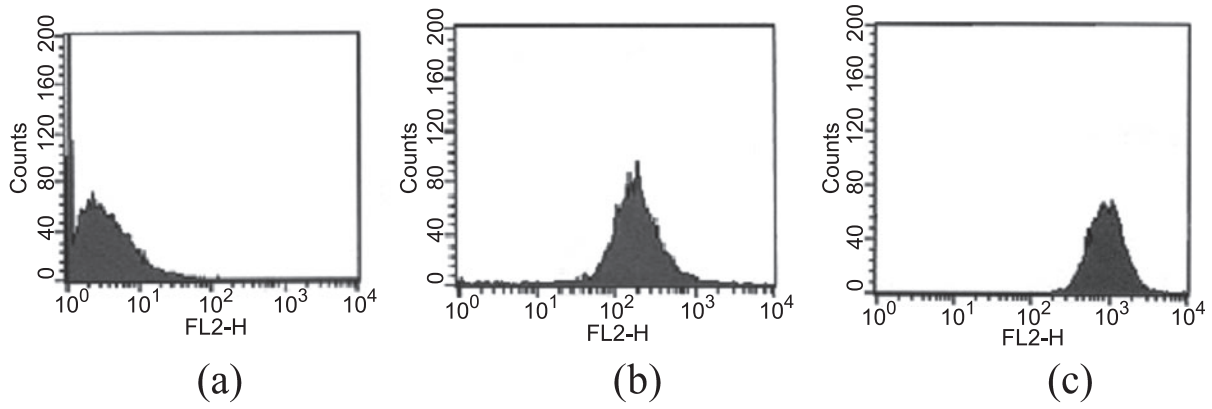

Fig. 1. **Flow cytometric profiles of MIN6 cells incubated with RITC-labeled polymers.** Each sample was incubated in HEPES-balanced KRBB (without BSA) with low glucose concentration for 1 h with labeled polymer and unlabeled glibenclamide. The feed ratios of SU-conjugated polymer to glibenclamide were: (a) 10:1, (b) 1:1, (c) 1:10.

Qualification test at the ratios of polymer to glibenclamide were 10:1, 1:1, and 1:10, shown in Fig. 2 (a), (b), and (c), respectively. As the concentration of glibenclamide increased (1 nM, 10 nM, and 100 nM), fluorescence intensity was significantly decreased. The fluorescence intensity of at the ratio of SU-conjugated polymer to glibenclamide of 10:1 was higher than at the other ratios shown in Fig. 2, being more than 5-fold highes than that at the ratio of 1:10. This result suggested that the binding process of SU-conjugated polymer to MIN6 cells is inhibited by glibenclamide. This can be explained by postulating that a large amount of glibenclamide interacted in advance of SU-conjugated polymer with sulfonylurea receptor (SUR) expressed on cell membrane.

Visualization of Specific Interaction—Fluorescence microscopic observation was used for probing the specific interaction between SU-conjugated polymer and MIN6 cells. In Fig. 3, glibenclamide and SU-conjugated polymer competed with each other. As the concentration of glibenclamide increased, the luminance of MIN6 cells gradually faded. The ratios of polymer to glibenclamide were 10:1, 1:1, and 1:10, shown in Fig. 3 (a), (b), and (c), respectively. In Fig. 3 (a), MIN6 cells were strongly luminated by interaction with FITC-labeled SU copolymer. In Fig. 3 (c), however, 100 nM glibenclamide so remarkably excluded 10 nM SU-conjugated polymer that the MIN6 cells were hardly distinguished from the basal noise. This result indicates that SU-conjugated polymer competes with glibenclamide.

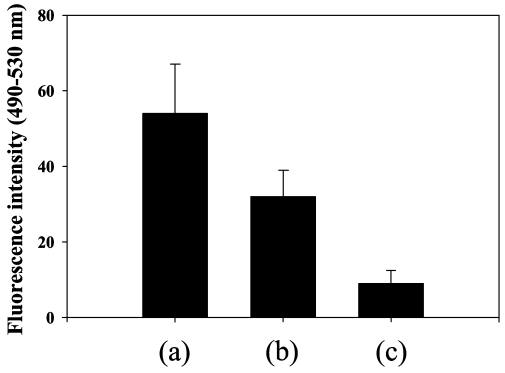

Fig. 2. **Competition test of SU-conjugated polymer to glibenclamide for binding to MIN6 cells.** The feed ratios of SU-conjugated polymer to glibenclamide were: (a) 10:1, (b) 1:1, (c) 1:10. The error bars represent mean ± SD.

Cytoplasmic Ca^{2+} Imaging of MIN6 Cells Interacting with SU-Conjugated Polymer—When the SU-conjugated polymer stimulates the β cells as glibenclamide does, the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of the cells must increase. It is well known that increase of the $[Ca^{2+}]_i$ stimulates a number of cellular events, including the release insulin from pancreatic β cells. The individual β cell serves as a biologic oscillator, responding to a glucose stimulus with 2- to 10-min oscillations of $[Ca^{2+}]_i$ (*14*). An increase of the glucose concentration triggers slow oscillations of $[Ca^{2+}]_i$ due to periodic opening of voltage dependent channels (*6*). These oscillations propagate *via* gap junctions among β cells in aggregates (*15*) and account for pulsatile release of insulin from individual pancreatic islets (*16*). Upon exposure of cells to SU (tolbutamide and glibenclamide), $[Ca^{2+}]_i$ and insulin secretion were elevated even in low glucose concentration. Thus, K_{ATP} channels were blocked and depolarized by the SU-conjugated polymer causing the elevation of $[Ca^{2+}]_i$ (*17*). Figure 4 illustrates (a) the localized RITC-labeled SU-conjugated polymer image, (b) the mobilized calcium ions detected by hydrolysis of Fluo-3 AM, and (c) the overlapped image. RITC-labeled SU-conjugated polymer directly interacted with the MIN6 cells. The polymer bound to the receptor of MIN6 cells so tightly that it was not subject to being washed out by rinsing twice with PBS. In Fig. 4 (b), intracellular calcium ion was mobilized, and the majority of fluoresced MIN6 cells were localized at the cellular membrane. Figure 4 (c) shows completely overlapped image of MIN6 cells by RITC-labeled glibenclamide polymers and Fluo-3 AM.

Inhibition of Specific Interaction between MIN6 Cells and SU-Conjugated Polymer by Pretreatment with Diazoxide—Figure 5 indicates that the interaction between RITC-labeled SU-conjugated copolymer and MIN6 cells was inhibited by the pretreatment of the cells with diazoxide. The fluorescence intensity significantly decreased when the cells were pretreated with diazoxide. Con-

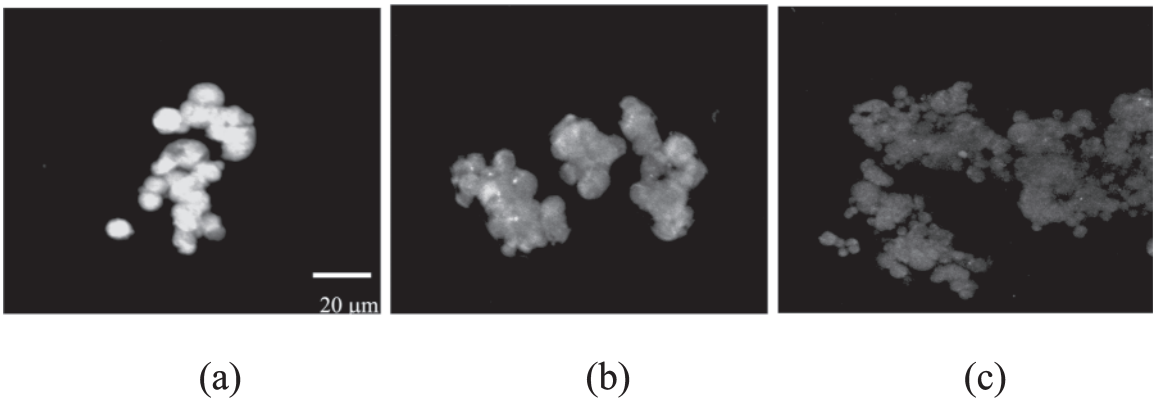

Fig. 3. **Confocal microscopic images showing that the synthetic polymer competed with glibenclamide and specifically interacted with MIN6 cells (×100, fluorescein isothiocyanate as a probe).** Each sample was incubated in HEPES-balanced KRBB (without BSA) with low glucose concentration for 1 h with labeled polymer and unlabeled glibenclamide. The feed ratios of SU-conjugated polymer to glibenclamide were: (a) 10:1, (b) 1:1, (c) 1:10.

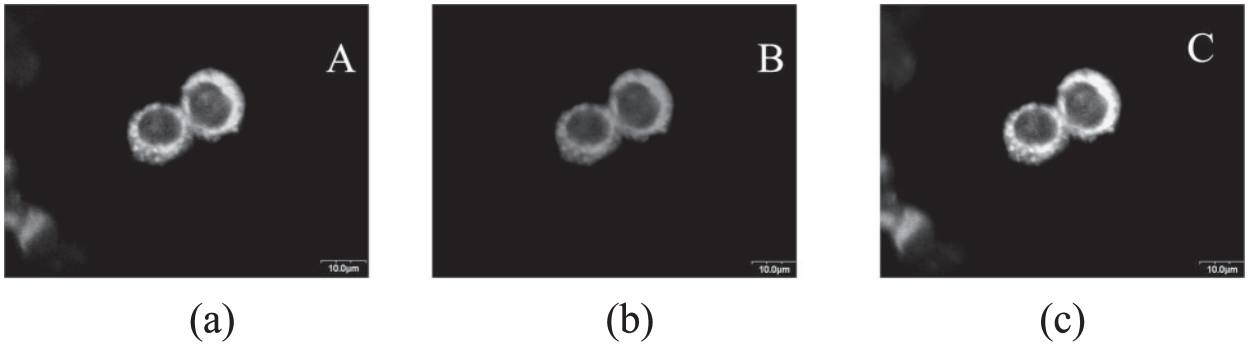

Fig. 4. **Cytoplasmic Ca^{2+} imaging stimulated by SU-conjugated polymer to MIN6 cells. Scale bar, 10 μm.** (a) Immunofluorescence of SU-conjugated polymer. Cells are visualized using confocal microscopy with suitable filters for rhodamine. (b) Binding of Fluo-3 AM to the same cells as in (a). Binding was visualized using suitable filters for fluorescein with confocal microscopy. (c): Yellow color indicates positive cells for both SU-conjugated polymer and fura-3 AM staining.

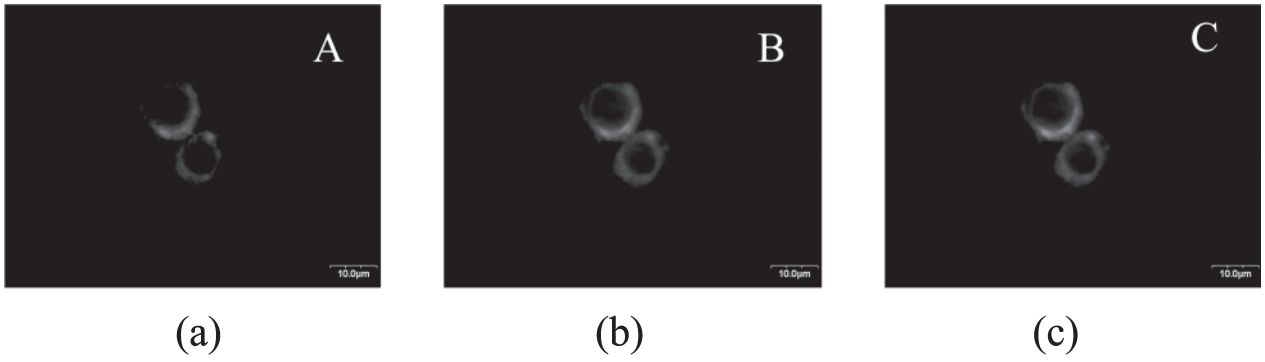

Fig. 5. **Inhibition of cytoplasmic Ca^{2+} of MIN6 cells pretreated with diazoxide for 20 min at 37°C.** The sample was incubated in a 3.3 mM glucose-containing HEPES-balanced KRBB buffer solution for 1 h with the labeled polymer. (a) Fluorescence of SU-conjugated polymer in MIN6 cells pretreated with diazoxide. (b) Binding of Fluo-3 AM to the same as cells in (a). (c) Yellow color indicates positive cells for both SU-conjugated polymer and Fluo-3 AM staining.

versely, diazoxide inhibits insulin secretion by opening K_{ATP} channels and eventually preventing glucose from depolarizing β-cells and raising $[Ca^{2+}]_i$ (*18*, *19*).

In conclusion, the pharmacological effects of the SU-conjugated polymer on β cell line of MIN6 were identical to those of glibenclamide. A study of the interaction of the SU-conjugated polymer (RITC-labeled) with MIN6 cells by direct visualization with confocal laser microscopy provided strong evidence of receptor-mediated interaction between MIN6 cells and the polymers bearing SU ligands. Moreover, location of labeled polymer and the site of Ca^{2+} ion mobilization obtained from the same MIN6 cells were identical. This result provides clear evidence of K_{ATP} channel modulation by SU and can offer a

new tool for investigating the biological events on K_{ATP} channels. Also, this SU-conjugated polymer could be used for imaging and diagnosis of pancreatic β cells. Consequently, the SU-conjugated polymer can be a useful tool for biomedical and pharmacological investigations into the physiology of intact islets or β cell lines.

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