J. Biochem. 131, 359–364 (2002)

Determination of the Specific Interaction between Sulfonylurea-Incorporated Polymer and Rat Islets

Keun-Hong Park,* Soo Chang Song,† and Toshihiro Akaike‡,[1]

**Department of Oral and Maxillofacial Surgery, Craniofacial Tissue Engineering Laboratory of BK21 Human Life Science and the Intellectual Biointerface Engineering Center, College of Dentistry, Seoul National University, 28-2 Yeongun-dong Jongro-ku, Seoul 151-100, Korea; †Life Sciences Division, Korea Institute of Science and Technology, Cheongryang, Seoul 130-650, Korea; and ‡Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8601*

Received August 16, 2001; accepted December 8, 2001

A SU derivative, mimicking glibenclamide in chemical structure, was synthesized to incorporate it into a water-soluble polymeric backbone as a biospecific and stimulating polymer for insulin secretion. The ability of insulin secretion was examined with different glucose concentrations (3.3 and 11.6 mM). Although the vinylated SU did not exhibit significant activity compared to the control, the SU-incorporated copolymer could enhance insulin secretion as much as or more than glibenclamide did. In this study, a polymer fluorescence-labeled with rodamine-B isothiocyanate was used to visualize the interactions and we found that the labeled polymer was strongly absorbed to rat islets, probably due to its specific interaction mediated by SU receptors on the cell membrane. To verify the specific interaction between the SU (K^+ channel closer)-incorporated copolymer and rat islets, cells were pretreated with diazoxide, an agonist of ATP-sensitive K^+ channels (K^+ channel opener), before adding the incorporated polymer to the cell culture medium. This treatment suppressed the action of SUs on rat islets. A confocal laser microscopic study further confirmed this interaction. The results of this study provided evidence that the SU-incorporated copolymer stimulates insulin secretion through specific interactions of SU moieties in the polymer with rat islets.

Key words: diazoxide, fluorescence, rat islets, specific interaction, sulfonylurea.

For decades, considerable efforts have been made to develop a biohybrid artificial pancreas (BAP) for the treatment of insulin-dependent diabetes mellitus (IDDM) (*1–3*). To develop a BAP, major approaches have involved islet macroencapsulation in chambers with vascular grafts (*4, 5*), and microencapsulation of islets within permselective membranes (*6–8*). However, several unsolved problems remained for the development of a BAP. Among them, one of the most significant is how to increase cell functionality such as insulin secretion when a BAP is implanted.

To address the problem associated with a large implant volume, it has been proposed to enhance insulin secretion by co-encapsulating islets of Langerhans with an insulinotropic agent, sulfonylurea (SU), followed by grafting onto water soluble polymers to prevent their diffusional loss from the capsules, with the expectation of a reduced number (or volume) of cells required for normoglycaemia after implantation (*9, 10*). In an *in vitro* experiment, the SU-incorporated polymers were able to stimulate insulin secretion from the islets, particularly at low glucose concentrations, *i.e.* up to 30%. It was thought that the conjugate specifically binds with β-cells in islets *via* SU-receptors, a part of the K^+ channel (SUR1), on the cell membrane (*11, 12*). This binding closes ATP-sensitive K^+ channels preventing K^+ outflux (*13*) and depolarizes the cell membrane potential, resulting in increasing calcium flux through voltage-dependent Ca^{2+} channels. The increase in cytoplasmic Ca^{2+} concentration eventually triggers insulin secretion from pancreatic islets (*14, 15*).

In a previous study, we reported a new concept regarding double ligands, which have sugar-bearing PS derivatives and the additional drug ligand of SU as the specific agent for islet or insulinoma cell lines (*16, 17*). A SU derivative, as one of the specific ligands for islets, was copolymerized with sugar-bearing PS derivatives which could specifically recognize SU-receptors of the cell surface. Insulin secretion from MIN6 cells attached to these copolymers was enhanced by the SU as a specific ligand.

In this study, a vinylated SU (a glibenclamide analogue) was copolymerized with a highly water-soluble sugar-containing monomer because the sugar-bearing copolymers significantly increased the water solubility of SU moieties in the copolymer and improved the binding at receptor sites due to their unique amphiphilic nature, and its bioactivity as to rat islets was examined. For more detailed observation of the interactions of the polymers with the cells, a fluorescence-labeled polymer was also used and the interactions were monitored by confocal microscopic analysis.

MATERIALS AND MATHODS

Materials—Diazoxide, ethylenediaminetetraacetic acid (EDTA), and rhodamine B isothiocyanate (RITC) were pur-

[1]To whom correspondence should be addressed.
E-mail: takaike@bio.titech.ac.jp, Fax: +81-45-924-5815

chased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA), fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), streptomycin, and penicillin sulphate were purchased from GIBCO BRL (Grand Island, NY). 4-(2-Aminoethyl)benzene sulfonamide, cyclohexyl-isocyanate, and dimethyl sulfoxide (DMSO)-d_6 were purchased from Aldrich Chemical (Milwaukee, WI), and DMSO and methanol were purchased from Showa Chemical (Tokyo). All other chemicals used were of reagent grade.

Methods—An antidiabetic SU analog was synthesized by the following method. 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 a 1 N NaOH aqueous solution were reacted for 2 h at room temperature, followed by recrystallization twice in MeOH. Then 4-[(2)′-acrylamido-ethyl]benzene sulfonylamide (AEBSA) (2.7 g, 7 mmol) swollen in acetone was poured into 7 ml of the 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. Finally, the reaction mixture was put into the 1 N HCl aqueous solution (7 ml) to obtain a precipitate, which was vacuum-dried.

The synthesized sulfonylurea (SU) monomer was characterized by FT-IR and ^{1}H-NMR. FT-IR spectra of SU showed characteristic peaks; -SO_2-: 1,350, 1,140 cm^{-1}. The structure of SU was confirmed by the amine peaks, in that a secondary amine (-NH-) shows one peak around 3,400 cm^{-1} due to only one proton of an amine group while a primary amine (-NH_2) exhibits two peaks around the corresponding position. SU gave the secondary amine peak, which was distinguished from the primary amine peak of aminoethylbenzene sulfonamide. ^{1}H-NMR spectra confirmed its structure, the characteristic peaks being as follows: protons on phenyl: 7.3–8.0 ppm, CONHCH_2CH_2: 8.7 ppm, CH_2CH_2: 2.9 ppm, CH_2CH_2: 3.5 ppm, SO_2NHCONH: 6.3 ppm, protons on cyclohexyl group: 1.1–1.6 ppm.

Preparation of Copolymers Containing SU—A mixture of vinylbenzyl maltonamide (VMA), cyclohexyl 4-[(2)′-acrylamido-ethyl]benzene SU and AIBN, as an initiator, dissolved in DMSO was poured into a glass ampule. The sealed ampule was placed in a thermostat at 60°C for 6 h. After polymerization, the mixture was poured into methanol to obtain a precipitate. The obtained products were reprecipitated with methanol and then freeze-dried. The molecular weights of SU-incorporated copolymers were determined by means of a static light scattering study [Zimm's plot (Malvern Instruments, Series 4700, at 488 nm with a 40–140° scattering angle and at least 10 measurements)]. This method revealed that M_rs of p(VMA-co-SU)-1, p(VMA-co-SU)-2, and p(VMA-co-SU)-3 were 810, 780, and 760K, respectively. The structures and characteristics of the prepared monomer and polymers are shown in Fig. 1 and Table I, respectively.

Fluorescent Labeling of Polymers—Each polymer was labeled with RITC since it is possible to react the C6 (CH_2OH) portion of sugar-bearing or SU-incorporated copolymers with RITC. To a solution of the polymer (500 mg) in 5 ml of DMSO, 50 mg of RITC and 15 mg of dibutyltin dilaurate were added, followed by standing for 2 h at 90°C. After cooling to room temperature, the mixture was added to excess ethanol to precipitate the fluorescence-labeled

TABLE I. **Characteristics of the prepared polymers.**

Polymer	Feed ratio of VMA to SU (w/w)	Composition of SU (weight %)[a]	$[\eta]^b$	Yield (%)
PVMA	100:0	0	0.15	93
p(VMA-co-SU)-1	88:12	7.5	0.25	93
p(VMA-co-SU)-2	77:23	17.4	0.15	92
p(VMA-co-SU)-3	70:30	23.0	0.25	94

[a]Composition of SU in the copolymer estimated by ^{13}C-NMR measurement. [b][η] was measured in H_2O at 25°C.

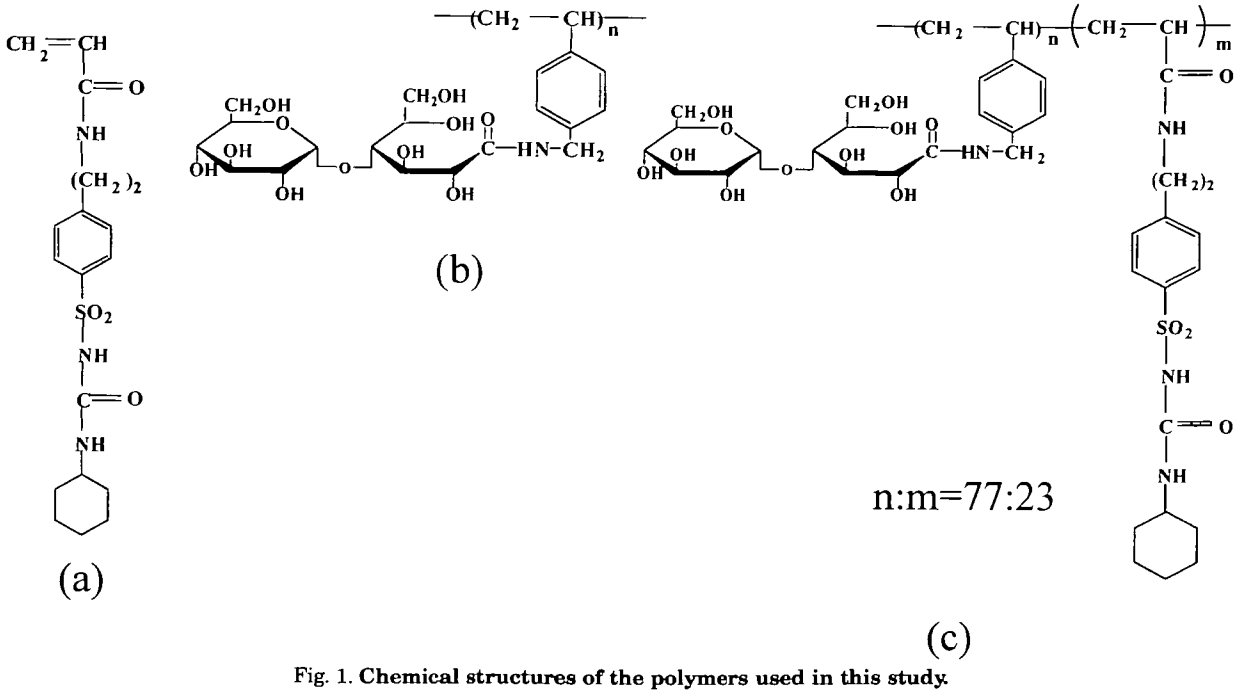

Fig. 1. **Chemical structures of the polymers used in this study.**

polymer. The polymer was then dissolved in 30 ml water and dialyzed against 1,000 ml of mild alkaline water (pH 8.0 with NaOH) for one day, and then against distilled water for two weeks, with exchanging of the water every day, at 4°C. The product of RITC-labeled polymers was obtained by freeze-drying. On average, one RITC molecule was introduced per approximately hundred repeat units for each copolymer, as determined with a microplate reader (FL600; Bio-Tek, USA).

Rat Islet Isolation—Rat islets were isolated by a modification of the method of Lancy and Kostianovsky (*18*). Collagenase type V was dissolved in Hank's balanced salt solution at a concentration of 1.1 mg/ml. The solution was filtered through a 0.22-μm syringe filter to sterilize it. Islets were isolated from the pancreases of male Sprague-Dawley (SD) rats (200–250 g) by the standard collagenase (type V) digestion method with minimal modification, using 11 ml of a collagenase solution. The whole procedure was carried out after each rat had been anesthetized with a xylazine/ketamine mixture (0.33 ml/g body weight of the animal). Approximately 400–600 islets were obtained from each pancreas. The isolated islets were purified by the Ficoll gradient method (11, 20, 23, and 27% solutions in HBSS), and then cultured in an RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 5 mM sodium bicarbonate, 20 mM HEPES, and 2 g/liter of D-glucose under humidified air containing 5% CO_2 at 37°C. Freshly isolated islets were stabilized by culturing in the RPMI 1640 medium with 11.6 mM glucose for 3 days, and spherical islets were hand-picked for further experiments.

Test for the Insulinotropic Activity of the Synthetic Polymers—Islets (n = 50) were embedded in each well of a 24-well culture plate with 1 ml of RPMI 1640 medium, in which the glucose concentration was 3.3 mM. The concentration of sulfonylurea in synthetic polymers to stimulate islets was 1 μM, so the final SU concentration in 1 ml medium was 10 nM on the addition of 10 μl of polymer suspension in basic phosphate-buffered saline (PBS). As controls, glibenclamide and the synthetic SU monomer were simultaneously used. After 2 h activation, 750 μl aliquots of medium were placed in 1.5 ml tubes and then centrifuged for 5 min at 1,000 rpm. Each supernatant (400 μl) was transferred to another 1.5 ml tube and then cryopreserved at –20°C till radioimmunoassaying (RIA). RIA was carried out with a typical two-antibody system with ^{125}I-labeled rat insulin (Rat Insulin RIA Kit from LINCO Research), and Student's t-test and the z-test were performed to analyze the data obtained for duplicate samples.

Test for Inhibition of the SU-Incorporated Copolymer by Diazoxide—Islets (n = 50) that had been cultured in a 11.6 mM glucose-containing RPMI 1640 medium were incubated in a 3.3 mM glucose-containing HEPES-buffered Krebs solution (HK solution; 130 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES) for 2 h. Diazoxide (20 mM, 5 μl) was then added to 1 ml of the RPMI 1640 medium (glucose concentration, 3.3 or 11.6 mM) containing 50 starved islets. After 20 min, glibenclamide, the synthesized SU derivative, or the SU-incorporated polymer was added to pretreated islets. After 2 h incubation under 5% CO_2 and 95% humidified air, insulin secreted by the rat islets was detected by RIA as already described.

Statistical Analysis—The statistical significance of differences between the experimental groups was assessed by a two-tailed Student's t-test. In Figs. 2, 3, and 4, multiple comparisons were made with Bonferroni adjustment to calculate the p level of significance (*19*).

Confocal Laser Microscopic Analysis—Islets (n = 30) precultured in 11.6 mM RPMI 1640 medium for 3 days were incubated in a 3.3 mM HK solution for 1 h. After the solution had been replaced with a fresh 5.6 mM HK solution, the islets were simultaneously stimulated with 5 μl of RITC-labeled polymer. After 1 h at 37°C, the islets were collected by concentration at 1,000 rpm and then rinsed with PBS twice to remove unbound labeling polymers. The islets were then fixed with a 4% formaldehyde solution in 10× PBS for a few minutes, and then placed on a slide glass. Confocal microscopic images of the islets were obtained with a Leika confocal laser microscope system (magnification, ×100) within 6 h after sampling.

RESULTS AND DISCUSSION

Bioactivity Test for SU-Carrying Copolymers—It is well known that the solubility of SU derivatives in an aqueous milleu is limited because of their high hydrophobicity. To evaluate the bioactivity of the SU compounds, SU solutions in DMSO were added to the rat islet cell culture to make the SU unit concentration in the medium around 10 nM. Rat islets were then incubated with the SU compounds for 2 h at 37°C. Figure 2 shows the level of insulin secretion from rat islets stimulated by the presence and absence of the SU ligand. In a bioactivity test, about 50% insulin secretion was observed with glibenclamide, a second generation SU drug, with the low glucose concentration (3.3 mM), as compared with in the case of unstimulated cells. Although the SU derivatives enhanced the insulin secretion by rat islets with the low glucose concentration, the bioactivity of SU derivatives was less for insulin secretion with the high glucose concentration (11.6 mM) because glucose is the one of the stimulants in rat islet β cells. Thus, the insulin secretion by cells was affected by glucose and SU derivatives, while the insulin secretion was only affected by the SU derivatives with the low glucose concentration. However, the synthesized SU monomer did not have a significant effect on insulin secretion from rat islets. In contrast to in the case of unstimulated cells, the bioactivity of

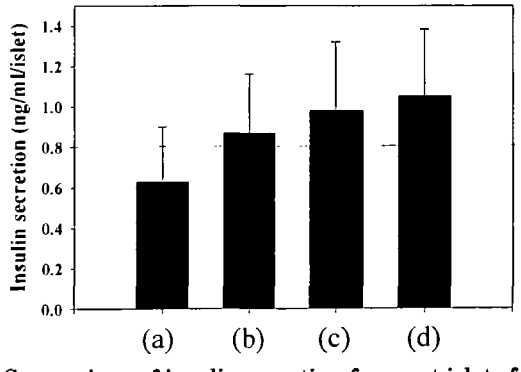

Fig. 2. **Comparison of insulin secretion from rat islets for sulfonylurea derivatives with a low glucose concentration (3.3 mM) after 2 h.** (a) Control (without any stimulant), (b) SU-monomer, (c) glibenclamide, and (d) p(VMA-co-SU)-3. The error bars represent means ± SD (n = 5).

p(VMA-co-SU) showed an approximately 60% increase. Judging from the above results, the SU-incorporated copolymer has a greater effect on insulin secretion from rat islets than the monomer or unstimulated cells. It is suggested that when the SU is added to an islet cell culture, SU is partitioned into a lipid phase of the cellular membrane or in the receptors because of their high hydrophobicity. Also, SU receptors on the ATP-sensitive K^+ channels are located in the lipid phase of the cellular membrane, and thus SUs are likely to gain access to their receptors in the lipid phase of the β cell membrane *via* lateral diffusion (*22*).

In addition, although the vinylated SU did not exhibit significant activity compared to the control (without the addition of any stimulant), the SU-incorporated copolymer could enhance insulin secretion as much as glibenclamide did. Thus, the sugar moieties in the SU-incorporated copolymer showed a synergistical effect on the specific interaction increasing the insulin secretion (data not shown). This means that the sugar moiety incorporated with the SU ligand plays an important role as to the affinity with rat islets because receptors or transporters of rat islets in β-cells can be recognized through specific interactions between them.

Figure 3 shows the time course of stimulation by the SU-incorporated copolymer and glibenclamide of rat islets over a period of 5–60 min. It has been reported that SU receptors on the ATP-sensitive K^+ channels are located in the lipid phase of the cellular membrane, and thus SUs are likely to gain access to their receptors in the lipid phase of the cell membrane *via* lateral diffusion after partitioning in the cell membrane bilayer (*20*). After 10 min, significant intensity was observed. This intensity after 60 min increased with time and reached more 1.5-fold that at 10 min. In parallel, it was found that the increase in insulin secretion caused by glibenclamide was higher for p(VMA-co-SU) in a short-term culture. However, the insulin secretion caused by p(VMA-co-SU) was not different from that by glibenclamide in 1 h. This suggested that the process of binding of the SU-incorporated copolymer to rat islets is time-dependent. This can be explained by that the SU-incorporated polymer located near the cells is partitioned into the cell membrane in 10 min, resulting in a decrease in the local SU concentration, which causes diffusion of the polymer from the bulk to the cell surface leading to more binding with time.

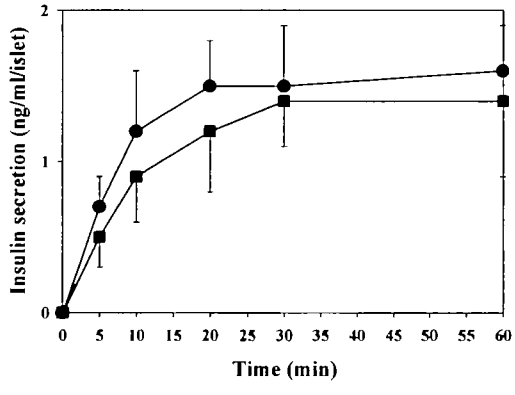

Fig. 3. **Time-course of insulin secretion from rat islets caused by a sulfonylurea derivative [p(VMA-co-SU)-3].** The error bars represent means ± SD. ●: glibenclamide, and ■: p(VMA-co-SU)-3.

TABLE II. **Bioactivity of sulfonylurea analogues as to insulin secretion.**

Sample (3 μg/ml)	SI*
Control (without polymer)	1.0
p(VMA-co-SU) (SU content: 7.5 mol%)	1.10 ± 0.05
p(VMA-co-SU) (SU content: 17.4 mol%)	1.25 ± 0.08
p(VMA-co-SU) (SU content: 23 mol%)	1.46 ± 0.12

The sample was incubated in a 3.3 mM glucose-containing HK buffer solution for 2 h with the SU polymer or without the polymer. *SI = (insulin secretion with sulfonylurea polymer)/(insulin secretion without polymer). The data are presented as means ± SD (n = 5).

Table II summarizes the effects of the SU content on insulin secretion from rat islets. With higher SU contents of p(VMA-co-SU), greater insulin secretion was observed. This suggested that the insulin secretion from rat islets is dependent on the SU content in the p(VMA-co-SU) polymer, indicating that a higher SU content of p(VMA-co-SU) may lead to a higher probability of insulin secretion from rat islets.

Suppression of Binding Affinity by Diazoxide—ATP-sensitive potassium channels play a central role in regulating physiological insulin secretion from β-cells in the islets of Langerhans. SU is a K^+ channel closer (antagonist) and stimulates insulin secretion (*21*). Diazoxide behaves in the opposite way by opening K^+ channels (agonist), which then inhibits insulin secretion (*22*). It is known that binding of diazoxide to its receptor can partially suppress the binding of SU to SUR1, and disturbs the effects of SU on both electrical activity and ion fluxes (*23*). Diazoxide is also known to suppress ATP synthesis in the cells. When diazoxide was added 20 min after the start of the incubation, by which time ATP in the cells with a high glucose concentration is expected to be completely converted into ADP through hydrolysis, it reduced glibenclamide binding to the same extent as that found when it was present from the start of the incubation (*24, 25*). For this reason, in this study, diazoxide was used for inhibition of the fluorescence intensity on the cells. Figure 4 illustrates that the suppression of insulin secretion was observed on pretreatment with diazoxide (300 μM) in the presence of a low glucose concentration (3.3

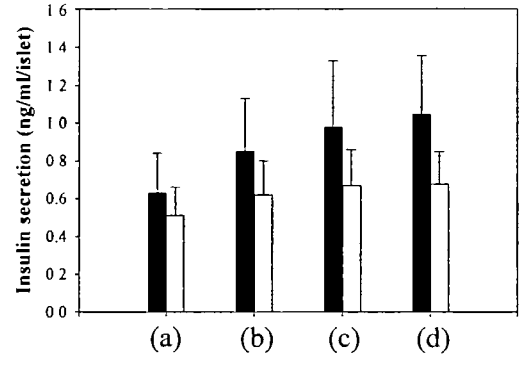

Fig. 4. **Inhibition test involving diazoxide, a potassium channel opener.** Each datum was determined by RIA after 30 islets had been incubated in HEPES-buffered Kreb's (HK) solution for 1 h at 37°C. (a) Control (without any stimulant), (b) SU-monomer, (c) glibenclamide, and (d) p(VMA-co-SU)-3. ■: diazoxide-free conditions, □: pretreatment with diazoxide.

mM). Diazoxide suppressed the effect of the SU-incorporated copolymer on the binding affinity as to rat islets by approximately 50% compared to the diazoxide-free conditions, due to the inhibition acting to the SU moieties on the SU-receptor. This suggested that the diazoxide pretreatment abolished the responsiveness of rat islets to the SU moieties of the copolymer.

To prove that the SU-incorporated polymer retained the ability to bind with rat islets, fluorescence microscopic observation of islet cells was performed to visualize the specific interaction. In order to examine the specific interaction between the SU-incorporated copolymer and rat islets, a 16-sectioned rat islet fluorescence-labeled with the SU-incorporated copolymer was viewed to illustrate the whole rat islet (Fig. 5). In this study, the SU-incorporated copolymer strongly interacted with the β-cells of rat islets. It could be suggested that the water-soluble SU-incorporated copolymer easily penetrates the collagen mesh of islets and selectively interacted with the β-cells of the islets, but not with the α- and δ-cells of the islets.

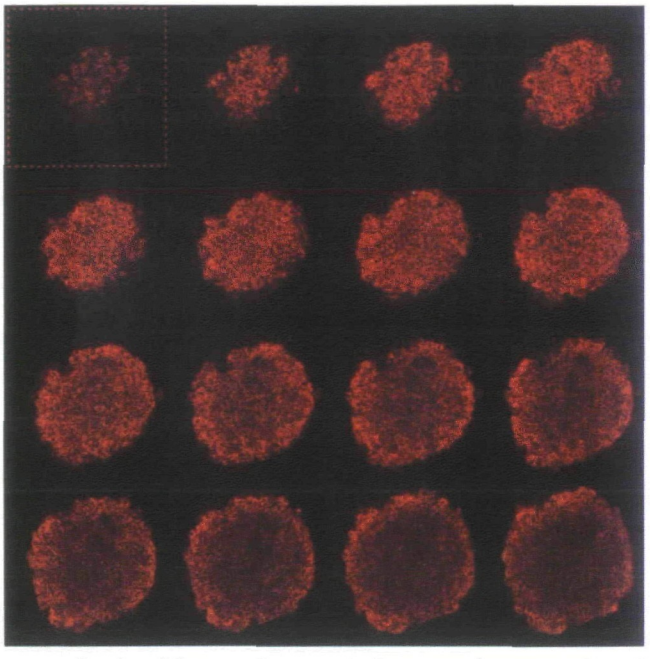

Fig. 5. **Confocal laser microscopic images of a 16-sectioned rat islet that had interacted with the SU-incorporated copolymer [p(VMA-co-SU)-3].** The sample was incubated in a 3.3 mM glucose-containing HK buffer solution for 2 h with the labeled polymer.

In Fig. 6, the total islet picture was viewed to clarify the specific interaction of the SU-incorporated copolymer with the β-cells of islets. In this picture, the position of β-cells in the rat islet was strongly labeled due to interaction with the RITC-labeled SU-incorporated copolymer. However, in the control experiment (RITC-labeled PVMA polymer), labeling of rat islet cells that had interacted with the PVMA homopolymer, which did not incorporate the SU ligands, was not observed. This indicates that the SU-incorporated copolymer undergoes negligible non-specific interaction with rat islet cells. This finding supports that the SU moieties on the copolymer strongly associated with the SU receptors on the cell membrane *via* a receptor-mediated specific interaction. Figure 6c visually demonstrates that the specific interaction between the RITC-labeled SU-incorporated copolymer and rat islets was inhibited by pretreatment of the cells with diazoxide. Since the binding affinity of diazoxide is known to be weaker than the SU binding affinity and their mutual influence on the affinity of both compounds, the rat islets were allowed to interact with diazoxide before exposure to SU. Therefore, the labeling of rat islets that had interacted with the RITC-labeled SU-incorporated copolymer was significantly decreased when the cells were pretreated with diazoxide. Thus, confocal microscopy revealed that the synthetic SU-incorporated copolymer bound to specific receptors in islets, which are known as ATP-sensitive potassium channels (K_{ATP} channels).

In conclusion, a novel synthetic SU-incorporated polymer effectively stimulated insulin secretion from isolated rat islets. The specific interaction between a synthetic polymer and K_{ATP} channels was confirmed by confocal microscopy and by an inhibition test involving diazoxide. Confocal microscopic images well agreed with the fact previously reported. Based on the results of the present study, the SU-incorporated polymer could be a useful tool for elucidating the physiological action of K_{ATP} and modulation.

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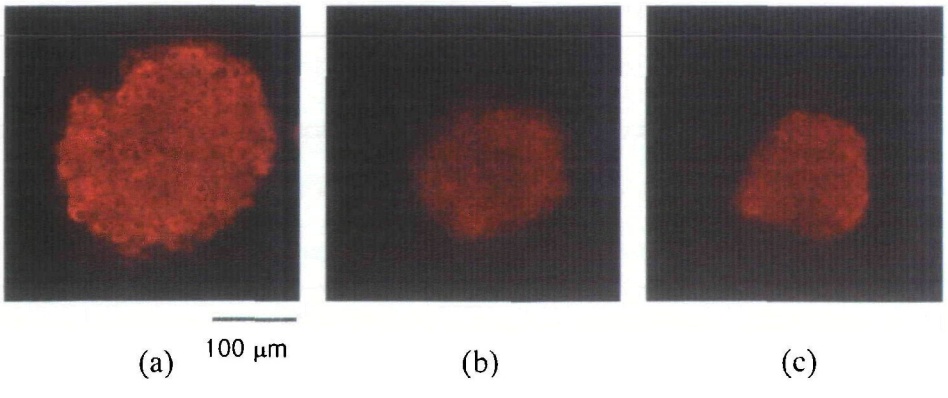

Fig. 6. **Confocal laser microscopic view of rat islets that had interacted with the RITC-labeled polymer in the presence or absence of the SU ligand at 37°C.** The sample was incubated in a 3.3 mM glucose-containing HK buffer solution for 2 h with the labeled polymer. (a) p(VMA-co-SU)-3, (b) PVMA, and (c) pretreatment with diazoxide (100 μm) for 20 min to inhibit the interaction between p(VMA-co-SU)-3 and islets.

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