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Chronic Exposure to SCO-267, an Allosteric GPR40 Full Agonist, Is Effective in Improving Glycemic Control in Rats[S]

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ABSTRACT

Full agonist-mediated activation of free fatty acid receptor 1 (FFAR1/GPR40) alleviates diabetes in rodents. Considering that diabetes is a chronic disease, assessment of treatment durability of chronic exposure to a GPR40 full agonist is pivotal for treating patients with diabetes. However, the physiologic significance of chronic in vitro and in vivo exposure to GPR40 full agonists is largely unclear. Here, we evaluated the in vitro and in vivo effects of chronic treatment with SCO-267, a GPR40 full agonist, on signal transduction and glucose control. In vitro experiments showed that SCO-267 is an allosteric full agonist for GPR40, which activates the $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ pathways and β-arrestin recruitment. The calcium signal response was largely sustained in GPR40-overexpressing CHO cells even after prolonged incubation with SCO-267. To evaluate the in vivo relevance of chronic exposure to GPR40 full agonists, SCO-267 (1 and 10 mg/kg) was administered once daily to neonatally streptozotocin-induced diabetic rats for 15–33 days, and glucose control was evaluated. After 15 days of dosing followed by the drug washout period, SCO-267 improved glucose tolerance, most likely by increasing insulin sensitivity in rats. After 33 days, repeated exposure to SCO-267 was highly effective in improving glucose tolerance in rats. Furthermore, chronic exposure to SCO-267 increased pancreatic insulin content. These results demonstrated that even after chronic exposure, SCO-267 effectively activates GPR40 in cells and rats, suggesting the clinical application of SCO-267 in treating chronic diseases including diabetes.

SIGNIFICANCE STATEMENT

GPR40 is a validated therapeutic target for diabetes. This study showed that even after chronic exposure, SCO-267, an allosteric GPR40 full agonist, effectively activates GPR40 in cells and rats; these results suggest a durable efficacy of SCO-267 in patients.

Introduction

Free fatty acid receptor 1 (FFAR1/GPR40) is a G protein–coupled receptor (GPCR) that is endogenously activated by medium-to-long chain fatty acids (Briscoe et al., 2003; Itoh et al., 2003). The receptor potentiates the secretion of glucose-dependent insulin from pancreatic β cells and stimulates the secretion of incretins such as glucagon-like peptide 1 (GLP-1) from intestinal endocrine cells (Mancini and Poitout, 2013; Pais et al., 2016). Fasiglifam, a partial agonist of GPR40, which improves glucose control mainly by stimulating insulin secretion (Tsujihata et al., 2011), showed a glucose-lowering effect in clinical studies on patients with type 2 diabetes mellitus (T2DM) (Burant et al., 2012; Kaku et al., 2016). The results of these clinical trials indicate that GPR40 is a promising therapeutic target for T2DM. Since the report of the superior glucose-lowering efficacy of a full GPR40 agonist, AM-1638, over a partial GPR40 agonist (Lin et al., 2012), various synthetic full GPR40 agonists have been investigated as new drug candidates (Li et al., 2016, 2020). These full agonists bind to the allosteric binding site of the receptor independent of binding sites for endogenous ligands or fasiglifam (Lin et al., 2012; Lu et al., 2017; Ho et al., 2018). Furthermore, in contrast to the partial agonists activating the $G\alpha_q$ signal, these full agonists activate not only the $G\alpha_q$ signal but also the $G\alpha_s$ (Hauge et al., 2014) and $G\alpha_{12/13}$ signals (Rives et al., 2018), which may explain its robust incretin stimulation and maximal efficacy in preclinical models (Defossa and Wagner, 2014). Based on these observations, GPR40 full agonists have been suggested as a novel strategy to treat diabetes (Li et al., 2018).

Considering that diabetes is a chronic disease with metabolic dysfunctions, the durability of drug efficacy is highly important (Kahn et al., 2006), and this is also the case with full agonists for GPR40. Generally, chronic agonist exposure causes GPCR desensitization and internalization, and the response is reduced (Drake et al., 2006; Kelly et al., 2008). These effects occur within a few minutes to hours, depending on the GPCR and agonist ligands. For example, relaxin family peptide receptor 1 demonstrates prolonged agonist-induced cAMP response by poor internalization and a lack of β-arrestin interaction (Callander et al., 2009). In addition, the neuropeptide FF-activated proto-oncogene Mas can be restimulated

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ABBREVIATIONS: BSA, bovine serum albumin; DC_{50}, half-maximal desensitization concentration; FFAR1/GPR40, free fatty acid receptor 1; GLP-1, glucagon-like peptide 1; GPCR, G protein–coupled receptor; HTRF, homogeneous time-resolved fluorescence; IP1, myo-inositol 1 phosphate; ITT, insulin tolerance test; N-STZ rat, neonatally streptozotocin-induced diabetic rat; OGTT, oral glucose tolerance test; SRF-RE, serum response factor response element; STZ, streptozotocin; T2DM, type 2 diabetes mellitus.

in calcium response, whereas the receptor activated by non-peptide ligands cannot be stimulated (Tirupula et al., 2014). A durable glucose control effect of GPR40 partial agonists in preclinical models has been reported (Chen et al., 2016, 2020), and patients with T2DM treated with fasiglifam continued to exhibit reduced HbA1c for 52 weeks (Kaku et al., 2016). However, the in vitro and in vivo effect of chronic exposure to GPR40 full agonist on downstream signaling of GPR40 is still unclear. Therefore, evaluating the downstream signaling of GPR40 upon chronic treatment with GPR40 full agonists is of importance when considering the application of this class of compounds for treating chronic metabolic diseases in clinical settings.

The present study was conducted to reveal the effect of chronic exposure to SCO-267, a GPR40 full agonist, on downstream signaling of GPR40 in vitro and in vivo. The signal transduction and allosteric properties of SCO-267 were evaluated using a recombinant expression system. In addition, the chronic effect of SCO-267 was investigated with respect to the $G\alpha_q$ signal in cell models. Finally, the chronic effects of SCO-267 on glycemic control were evaluated in a rat model.

Materials and Methods

Materials. SCO-267, fasiglifam, and AM-1638 were obtained from SCOHIA PHARMA (Fujisawa, Japan). γ-Linolenic acid was purchased from Sigma-Aldrich (Tokyo, Japan). For in vitro studies, compounds were dissolved in dimethyl sulfoxide, except for γ-linolenic acid, which was dissolved in ethanol. For in vivo studies, compounds were suspended in 0.5% methylcellulose solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Myo-Inositol 1 Phosphate Homogeneous Time-Resolved Fluorescence Assay for $G\alpha_q$ Signaling. CHO dihydrofolate reductase–deficient cells stably expressing human *FFAR1* (mRNA for GPR40) with different receptor mRNA expression levels were established previously (Yabuki et al., 2013). The mRNA copy number in high (clone 104) and low (clone 2) *FFAR1*-expressing cells was quantified by quantitative polymerase chain reaction as reported previously (Yabuki et al., 2013). These cells were cultured in minimum essential medium-α (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% dialyzed FBS (GE Healthcare, Chicago, IL), 100 U/ml penicillin-streptomycin (FUJIFILM Wako), and 10 mM HEPES solution (FUJIFILM Wako) and were tested for mycoplasma contamination before the experiment. The day before the assay, human *FFAR1*-expressing CHO cells were plated at 5000 cells per well in poly-D-lysine–coated 384-well white plates. After culturing overnight, the cells were treated with compounds in stimulation buffer [included in the IP-One homogeneous time-resolved fluorescence (HTRF) assay kit; PerkinElmer, Waltham, MA] containing 0.01% fatty acid–free bovine serum albumin (BSA) at varying concentrations and incubated at 37°C for 30 minutes. Intracellular myo-inositol 1 phosphate (IP1) level was measured using the IP-One HTRF Assay Kit (PerkinElmer) according to the manufacturer's protocol. HTRF signals were detected using the EnVision multimode plate reader (PerkinElmer). For the desensitization assay, CHO cells expressing high levels of human *FFAR1* (clone 104) were pretreated with the compounds in culture medium for 4 hours at 37°C. To remove excess compound, the cells were washed twice with Dulbecco's phosphate-buffered saline and treated with compounds in stimulation buffer containing 0.1% fatty acid–free BSA for 30 minutes at 37°C. Raw data or corrected data were analyzed using Prism 7 (GraphPad Software, San Diego, CA), and a four-parameter logistic fit equation was used to determine EC_{50} and half-maximal desensitization concentration (DC_{50}) for the desensitization analysis.

cAMP HTRF Assay for $G\alpha_s$ Signaling. Stable human glucagon-like peptide 1 receptor (GLP-1R)–expressing CHO-K1 cells were generated by transfection of pRP[Exp]-Neo-CMV>*hGLP1R* (VectorBuilder Inc., Chicago, IL) and selection of geneticin (0.5 mg/ml; Thermo Fisher Scientific). Stable human GLP-1R–expressing CHO-K1 cells and CHO cells expressing high levels of human *FFAR1* (clone 104, mycoplasma tested) were plated at a density of 30,000 cells in poly-D-lysine–coated 96-well plates and incubated overnight at 37°C under 5% CO_2. The culture medium was replaced with assay buffer [Hanks' balanced salt solution containing 10 mM HEPES (pH 7.5), 0.1% fatty acid–free BSA, and 0.5 mM 3-isobutyl-1-methylxanthine]. The cells were then stimulated with drugs for 30 minutes at 37°C. For the GLP-1R desensitization assay, the cells were pretreated with the compounds in Ham's F-12 (FUJIFILM Wako) supplemented with 10% FBS (Thermo Fisher Scientific) at 37°C for 4 hours. To remove excess compound, the cells were washed twice with Dulbecco's phosphate-buffered saline and treated with compounds at 37°C for 30 minutes. Intracellular cAMP level was determined using the HTRF cAMP Gs Dynamic Kit (PerkinElmer) according to the manufacturer's instructions. HTRF signals were detected using EnVision (PerkinElmer). Raw data or corrected data were analyzed using Prism 7, and a four-parameter logistic fit equation was used to determine EC_{50} and DC_{50} for the desensitization analysis.

Serum Response Factor Response Element Reporter Gene Assay for $G\alpha_{12/13}$ Signaling. CHO cells expressing high levels of human *FFAR1* (clone 104) were transfected with pGL4.34 (E1350, Promega, Madison, WI, USA), which contains a luciferase gene with serum response factor response element (SRF-RE) in response to serum response factor through the $G\alpha_{12/13}$-RhoA–mediated pathway, using Lipofectamine 3000 (Thermo Fisher Scientific). The transfected cells were plated at 15,000 cells per well in poly-D-lysine–coated 384-well white plates. After culturing for 4 hours, the culture medium was replaced with assay medium [minimum essential medium-α containing 10 mM HEPES (pH 7.5) and 2% FBS] before overnight incubation at 37°C in the presence of 5% CO_2. The cells were stimulated with the drugs in the assay medium for 6 hours at 37°C, and luciferase activity was measured using EnVision (PerkinElmer) with the Steady-Glo luciferase assay system (Promega). Raw data were analyzed using Prism 7, and a four-parameter logistic fit equation was used to determine EC_{50}.

β-Arrestin Recruitment Assay. The PathHunter β-arrestin assay (DiscoverX, Fremont, CA) was used to assess β-arrestin recruitment activity. PathHunter HEK293 cells stably expressing human GPR40, obtained from Takeda Pharmaceutical Company Limited (Tokyo, Japan; mycoplasma tested), were added into poly-D-lysine–coated 384-well white plates at 10,000 cells per well in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed FBS, 0.05 mg/ml hygromycin B (FUJIFILM Wako), 0.25 mg/ml geneticin (FUJIFILM Wako), and 100 U/ml penicillin-streptomycin. After overnight incubation at 37°C in the presence of 5% CO_2, the medium was replaced with Opti-MEM I (Thermo Fisher Scientific) containing 0.1% fatty acid–free BSA. Thereafter, compound stimulation was performed for 4 hours at 37°C, followed by incubation with the PathHunter Detection Reagent Solution at 22–26°C for 1 hour. Luminescence was measured using EnVision (PerkinElmer). Raw data were analyzed using Prism 7, and a four-parameter logistic fit equation was used to determine EC_{50}.

Animals. Male neonatally streptozotocin-induced (N-STZ) diabetic rats were developed by subcutaneous administration of 120 mg/kg streptozotocin (STZ) to Wistar Kyoto rats (RABICS, LTD., Kanagawa, Japan) at 1.5 days after birth. Saline-injected rats were used as normal control rats. N-STZ rats have been reported to show dysfunction of insulin secretion and action, which is similar to the pathology of human T2DM (Portha et al., 2007). All animals were housed in rooms under a 12-hour light/dark cycle (light on at 7:00 AM) and had ad libitum access to standard laboratory chow diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and tap water. The care of the animals and use of the experimental protocols were approved by the

Institutional Animal Care and Use Committee of Shonan Health Innovation Park accredited by the American Association for Accreditation of Laboratory Animal Care. For animal experiments, 0.5% methylcellulose was used as the vehicle. All blood samples used in the present study were obtained via the tail vein of the animals.

Subchronic Study of SCO-267 for Evaluating Insulin and Glucose Tolerance. Twenty-five-week-old N-STZ rats were randomized into groups based on body weight, fasting glucose level, and glycosylated hemoglobin ($n = 6$). The animals were orally administered either SCO-267 (1 and 10 mg/kg) or vehicle once a day for 15 days, followed by a washout period of 3 days. The first day of treatment was designated as day 1. The insulin tolerance test (ITT) and oral glucose tolerance test (OGTT) were performed on day 18 after overnight fasting (17 hours). In the ITT, insulin (0.25 IU/kg; Novo-Nordisk, Bagsvaerd, Denmark) was injected subcutaneously, and plasma glucose level was determined at the indicated time points. In the OGTT, glucose (1.5 g/kg) was orally administered, and blood glucose and insulin levels were determined at the indicated time points. The plasma level of SCO-267 was determined before administering glucose in the OGTT.

Chronic Study of SCO-267 for Evaluating Glucose Tolerance. Twenty-five-week-old N-STZ rats were fasted for 18 hours. The rats were then randomized into groups ($n = 6$) based on body weight, fasting glucose levels, and glycosylated hemoglobin. The average body weight of N-STZ rats and normal rats was 379 ± 7 and 442 ± 16 g, respectively. The rats were then orally administered test materials (SCO-267, 1 and 10 mg/kg; glibenclamide, 10 mg/kg) or vehicle 60 minutes before oral glucose loading (1.5 g/kg). The first treatment day was designated as day 1. Glibenclamide, a sulfonylurea that stimulates insulin secretion (Levetan, 2007), was used as a reference drug. Thereafter, SCO-267, glibenclamide, or vehicle was repeatedly administered once daily. After the 32nd dose, the rats were fasted for 18 hours. Glucose (1.5 g/kg) was orally administered 1 hour after the 33rd dose of each material, and plasma parameters were determined at the indicated time points (day 33). A pharmacokinetic study with 1 and 10 mg/kg SCO-267 was conducted under the same experimental conditions ($n = 3$; after the 33rd dosing). After the drug washout period (days 38–42), the rats were fasted for 16 hours (day 43), and the entire pancreas was isolated and homogenized in 75% (v/v) ethanol containing 0.15 M HCl. The homogenized tissues were centrifuged at 8200*g* for 5 minutes at 4°C. The supernatants were then diluted with phosphate-buffered saline containing 0.1% BSA, and the total insulin level in the supernatants was determined.

Measurement of In Vivo Parameters. Plasma glucose level was measured using an Accu-Chek ST glucometer (Roche Diagnostics, Manheim, Germany) or a 7180 Clinical Analyzer (Hitachi, Tokyo, Japan). Glycosylated hemoglobin was determined using the HLC-723 G8 automated glycosylated hemoglobin analyzer (Tosoh, Tokyo, Japan). Insulin level was determined using an insulin ELISA kit (Cat. No. M1101; Morinaga Institute of Biologic Science, Inc., Yokohama, Japan).

Statistical Analysis. The experiments performed in this study were exploratory in nature and designed to evaluate the profiles of SCO-267. The current study did not employ a predefined study design; as such, reported *P* values are descriptive. Statistical significance was analyzed using Bartlett's test for homogeneity of variances, followed by Williams' test ($P > 0.05$) and the Shirley-Williams test ($P \leq 0.05$) for evaluating the dose-dependent effects of SCO-267. Alternatively, statistical significance was analyzed using the *F* test for homogeneity of variances, followed by Student's *t* test ($P > 0.2$) or the Aspin-Welch test ($P \leq 0.2$) for evaluating the effect of glibenclamide. All tests were conducted using a two-tailed significance level of 5% (0.05). All data are presented as means ± S.D.

Results

SCO-267 is a Full Agonist for GPR40 Activating the $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ Pathways and β-Arrestin Recruitment. To determine the potential signaling pathways of SCO-267, the cell-based IP1 HTRF (for $G\alpha_q$), cAMP HTRF (for $G\alpha_s$), SRF-RE reporter gene (for $G\alpha_{12/13}$), and β-arrestin recruitment assays were conducted (Fig. 1; Table 1). In these assays, AM-1638, fasiglifam, and γ-linolenic acid were used as a representative full allosteric agonist, partial agonist, and endogenous ligand, respectively. In the $G\alpha_q$-mediated IP1 accumulation assay, SCO-267, AM-1638, fasiglifam, and γ-linolenic acid elevated the IP1 level in cells expressing high levels of human *FFAR1* (Fig. 1A). GPR40 full agonists can activate $G\alpha_q$ signaling even in cells expressing low levels of GPR40 (Brown et al., 2012). As shown in Fig. 1B, SCO-267 and AM-1638 were effective (EC_{50} = 0.91, 26 nM, respectively) with similar E_{max} in CHO cells expressing low levels of human *FFAR1*, whereas fasiglifam and γ-linolenic acid were very weak ($EC_{50} > 10$ and 150 μM, respectively). These data confirmed that SCO-267 is a full agonist, which is consistent with the findings of a previous study (Ueno et al., 2019). In terms of other signals, SCO-267 and AM-1638 showed potent activity in all assays (Fig. 1, C–E), suggesting that these compounds potentiate the $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ pathways and β-arrestin recruitment. In addition, the EC_{50} value of SCO-267 was over 10 times lower than that of AM-1638 in all assays.

SCO-267 Is Allosteric with Fasiglifam and an Endogenous Ligand. GPR40 has three known binding sites: one is an endogenous fatty acid-binding site, the second is where partial agonists such as fasiglifam bind, and the third is where full agonists such as AM-1638 and AP8 bind (Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014; Srivastava et al., 2014; Lu et al., 2017). As shown in Fig. 1B, SCO-267, fasiglifam, and γ-linolenic acid effectively elevated the IP1 level in CHO cells expressing GPR40. To explore the binding site of SCO-267, a titration study of two compounds in a two-dimensional matrix format was conducted (Fig. 2). For this matrix analysis, the IP1 HTRF assay was selected because it was identified as the most robust and accurate method compared with the evaluation of other signals (e.g., cAMP and β-arrestin). The presence of fasiglifam (Fig. 2A) or γ-linolenic acid (Fig. 2B) significantly shifted the dose response curve of SCO-267 toward a lower concentration [from 4.6 (95% confidence interval, 3.6–6.0 nM) to 0.44 nM (95% confidence interval, 0.31–0.62 nM), or 5.0 (95% confidence interval, 3.5–6.9 nM) to 1.7 nM (95% confidence interval, 1.0–2.7 nM) of EC_{50}, respectively]. This indicated that SCO-267 binds at a site different from those for fasiglifam and γ-linolenic acid and induces positive cooperative effects with these compounds.

SCO-267 Activates Downstream Signaling After Chronic Exposure in Cells. To assess the functional desensitization of GPR40 by SCO-267, the effect of pretreatment with SCO-267 on reactivation of the receptor was examined using the IP1 assay. Chronic exposure to SCO-267 at 37°C for 4 hours at less than 10 nM concentration did not cause signal loss compared with the restimulation response in the control (Fig. 3A). When the cells were pretreated with 1 μM SCO-267, the restimulation response remained at approximately 70%. The desensitization potency of SCO-267 (DC_{50} = 45 nM) was approximately 300 times higher than its EC_{50}. To compare the rate of desensitization with GLP-1 agonism, which has been demonstrated to be effective in clinical settings when chronically exposed (Buse et al., 2004), the effect of extendin-4 on the restimulation of the GLP-1 receptor was examined using the cAMP assay. When the cells were pretreated with 100 nM

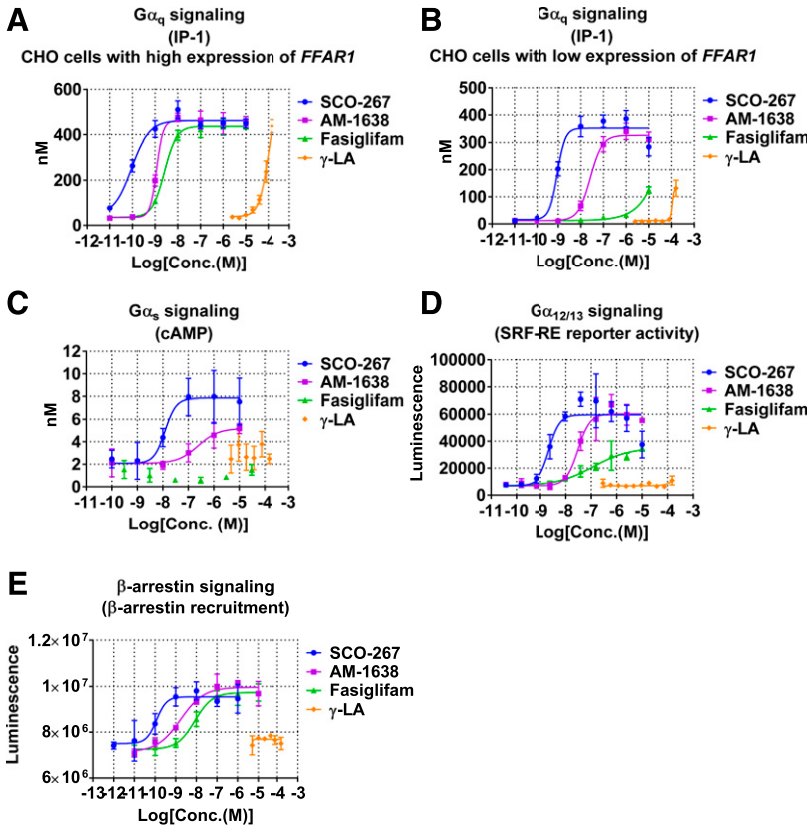

Fig. 1. Effects of SCO-267 on the $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ signals and β-arrestin recruitment. The effects of SCO-267, AM-1638, fasiglifam, and γ-linolenic acid were analyzed by IP1 accumulation in CHO cells expressing high (A) and low (B) levels of human *FFAR1*, cAMP production (C), SRF-RE response (D), and β-arrestin recruitment (E). Representative graphs from two [for (C–E)] or three [for (A and B)] independent experiments are shown. The data are presented as means ± S.D. of four technical replicates (A, B, and E), three technical replicates, except SCO-267 with five technical replicates (C), and three technical replicates (D). γ-LA, γ-linolenic acid.

extendin-4, the restimulation response remained at approximately 70% (Fig. 3B). The desensitization potency of extendin-4 (DC_{50} = 100 pM) was approximately four times higher than its EC_{50}. In contrast, the residual response to chronic exposure to fasiglifam, which showed durable efficacy in a 52-week clinical study (Kaku et al., 2016), at 30 μM was approximately 30% (Supplemental Fig. 1).

SCO-267 Improves Insulin Sensitivity in N-STZ Rats. To explore the effect of chronic exposure to SCO-267 on glucose tolerance and insulin sensitivity, SCO-267 was administered to diabetic N-STZ rats for 15 days, and the glucose tolerance and insulin sensitivity were evaluated after the drug washout period (Fig. 4). The plasma level of SCO-267 (1 and 10 mg) in rats after the drug washout period (day 18) was 0.22 and 0.35 ng/ml, respectively. The unbound SCO-267 concentration calculated using the rat plasma protein binding activity (Ueno et al., 2019) was 1.4 (1 mg/kg SCO-267) and 2.3 pM (10 mg/kg SCO-267), both of which are unlikely to activate GPR40. In fact, the insulin level was not increased in N-STZ rats subchronically treated with SCO-267 upon glucose loading (Fig. 4A), and this confirmed the complete removal of SCO-267. In contrast, N-STZ rats subchronically treated with SCO-267 (10 mg/kg) showed improved glucose tolerance (Fig. 4B). In addition, the ITT revealed that N-STZ rats subchronically treated with SCO-267 (10 mg/kg) showed increased insulin sensitivity (Fig. 4C).

SCO-267 Exerts Sustained Glucose-Lowering Effect After Administration in N-STZ Rats. To explore whether chronic exposure to SCO-267 is effective in improving glycemic control in vivo, glucose tolerance was evaluated after the first and repeated dosing of SCO-267 (1 and 10 mg/kg) in N-STZ rats. In this experiment, food intake levels were lower and body weight was decreased in the 10 mg/kg SCO-267 dose group (Fig. 5, A and B). In the OGTT, after the first dose, SCO-267 significantly increased insulin secretion and improved glucose tolerance, which were superior to those in normal rats (Fig. 5, C and D). As shown in Fig. 5E, the plasma level of SCO-267 was 28.8 ± 1.5 and 24.2 ± 2.3 ng/ml before the 33rd dose of

TABLE 1
Pharmacological potencies of SCO-267

	$G\alpha_q$ (IP1) high *FFAR1* expression	$G\alpha_q$ (IP1) low *FFAR1* expression	$G\alpha_s$ (cAMP)	$G\alpha_{12/13}$ (SRF-RE reporter activity)	β-Arrestin (β-arrestin recruitment)
Test Material			EC_{50} [95% CI] nM		
SCO-267	0.093 [0.035–0.14]	0.91 [0.52–1.2]	12 [5.8–30]	2.1 [1.1–3.7]	0.12 [0.032–0.31]
AM-1638	1.2 [1.1–1.5]	26 [19–35]	240 [65–8900]	30 [22–42]	1.7 [0.61–3.8]
Fasiglifam	2.6 [1.9–3.4]	>10,000	>30,000	120 [42–1500]	8.5 [4.4–15]
γ-LA	N.A.	>150,000	>150,000	>150,000	>150,000

Data are representative of two experiments performed in three or four technical replicates. γ-LA, γ-linolenic acid; CI, confidence interval; N.A., not applicable.

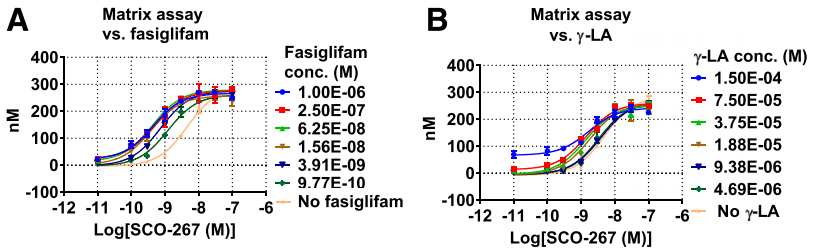

Fig. 2. Effects of increasing concentrations of fasiglifam or γ-linolenic acid on the dose-response curve of SCO-267. The IP1 accumulation assay of SCO-267 in the presence of fasiglifam (A) or γ-linolenic acid (B) at various concentrations using CHO cells expressing low levels of human *FFAR1* (clone 2). Representative graphs of two independent experiments are shown. The data are presented as means ± S.D. of two technical replicates. γ-LA, γ-linolenic acid.

10 mg/kg SCO-267 (time = 0) and after 24 hours. In the OGTT after the 33rd dosing (day 33), SCO-267 still increased insulin secretion and improved glucose tolerance, which were superior to those in normal rats (Fig. 5, F and G). Glibenclamide showed a trend of improvement in glucose tolerance after the first dose and impaired glucose tolerance after the 33rd dose (Fig. 5, C and D, F and G). In addition to the sustained glucose-lowering effect, SCO-267 increased pancreatic insulin level at the end of the study (Fig. 5H).

Discussion

In this study, SCO-267, a GPR40 full allosteric agonist, was effective in activating downstream signaling after chronic exposure in vitro and in vivo. The in vitro experiments showed that SCO-267 activated the $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ pathways and β-arrestin recruitment and binds to a site different from that of fasiglifam and endogenous ligand with positive cooperativity. The in vitro desensitization analysis using GPR40-overexpressing cells showed that GPR40 can be activated by SCO-267 after 4 hours of exposure to SCO-267. Our experiment using N-STZ rats showed that SCO-267 treatment of 15 days improved glucose tolerance by increasing insulin sensitivity. A 33-day repeated dose study, in which GPR40 was constantly exposed to SCO-267, revealed that repeated dosing with SCO-267 was effective in inducing a durable therapeutic efficacy in lowering glucose level and increasing insulin level in N-STZ rats.

In the IP1 accumulation assay, the E_{max} of SCO-267 was as high as that of AM-1638, a well studied GPR40 full agonist, in CHO cells expressing low levels of human *FFAR1*, indicating that SCO-267 is a GPR40 full agonist. In addition, SCO-267 showed positive cooperativity with fasiglifam or γ-linolenic acid in the IP1 accumulation assay, indicating that SCO-267 is allosteric with either fasiglifam or the endogenous ligand. These results demonstrated that SCO-267 is an allosteric full agonist of GPR40.

SCO-267 was efficacious in activating downstream signaling even after chronic exposure in human GPR40-expressing CHO cells, similar to exendin-4. Pretreatment of cells with SCO-267 for 4 hours at high concentrations (≥100 nM) caused only 30% loss of restimulation response, similar to that of exendin-4. The loss rate of the re-stimulation response was higher with fasiglifam, which showed a 70% loss of restimulation response. These findings indicate that SCO-267–mediated chronic activation of GPR40 may not be efficacious in desensitizing downstream signaling, which is likely an important characteristic of an agonistic drug candidate. Typically, GPCRs undergo internalization and desensitization upon chronic exposure to agonists through phosphorylation by G protein–coupled receptor kinase and β-arrestin recruitment (Kelly et al., 2008). It has been reported that GPR40 undergoes rapid linoleic acid–induced internalization through arrestin-3 and GPCR kinase 2, and the internalized receptors are recycled to the cell surface via recycling endosomes in GPR40-overexpressed HEK293 cells (Qian et al., 2014). These recycled receptors on the cell surface may allow the restimulation response of SCO-267.

In the chronic dose study in rats, the plasma SCO-267 concentration immediately before and 24 hours after the 33rd dose of 10 mg/kg SCO-267 was 28.8 and 24.2 ng/ml, respectively. In a previous study, N-STZ rats dosed with SCO-267 (0.3 mg/kg, C_{max} = 22.7 ng/ml) potently stimulated insulin secretion and improved glucose tolerance (Ueno et al., 2019).

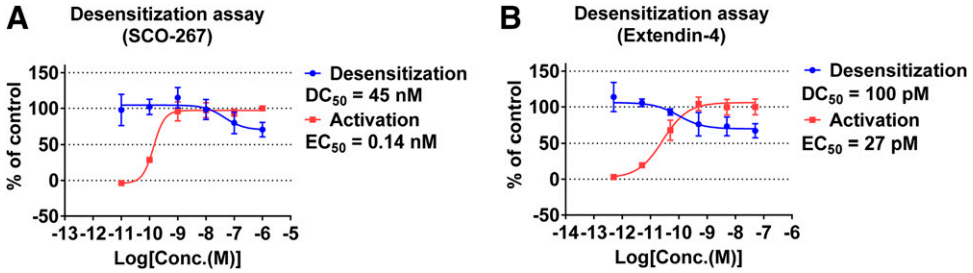

Fig. 3. Prolonged stimulation effect of SCO-267 in the IP1 accumulation assay. (A) CHO cells stably expressing high levels of human *FFAR1* (clone 104) were pretreated with the indicated concentrations of SCO-267 for 4 hours at 37°C before excess ligand was removed by washing. The cells were then restimulated with 300 nM SCO-267 for 30 minutes at 37°C, and the IP1 level was measured (● Desensitization). At the same time, the cells were also stimulated with various concentrations of SCO-267 after DMSO pretreatment (■ Activation). IP1 response is expressed as a percent of control, in which the cells treated with 1000 nM SCO-267 for 30 minutes after DMSO pretreatment were used as 100% controls (Top) and the cells treated with DMSO for 30 minutes after DMSO pretreatment were used as 0% controls (bottom). (B) CHO cells stably expressing human GLP-1R were pretreated with the indicated concentrations of extendin-4 for 4 hours at 37°C before excess ligand was removed by washing. The cells were restimulated with 10 nM extendin-4 for 30 minutes at 37°C, and the intracellular cAMP level was measured (● Desensitization). At the same time, the cells were also stimulated with extendin-4 at various concentrations after DMSO pretreatment (■ Activation). cAMP response was expressed as a percent of control, in which the cells treated with 10 nM extendin-4 for 30 minutes after DMSO pretreatment were used as 100% controls (Top) and the cells treated with DMSO for 30 minutes after DMSO pretreatment were used as 0% controls (bottom). Representative graphs of two independent experiments are shown. The data are presented as means ± S.D. of three technical replicates.

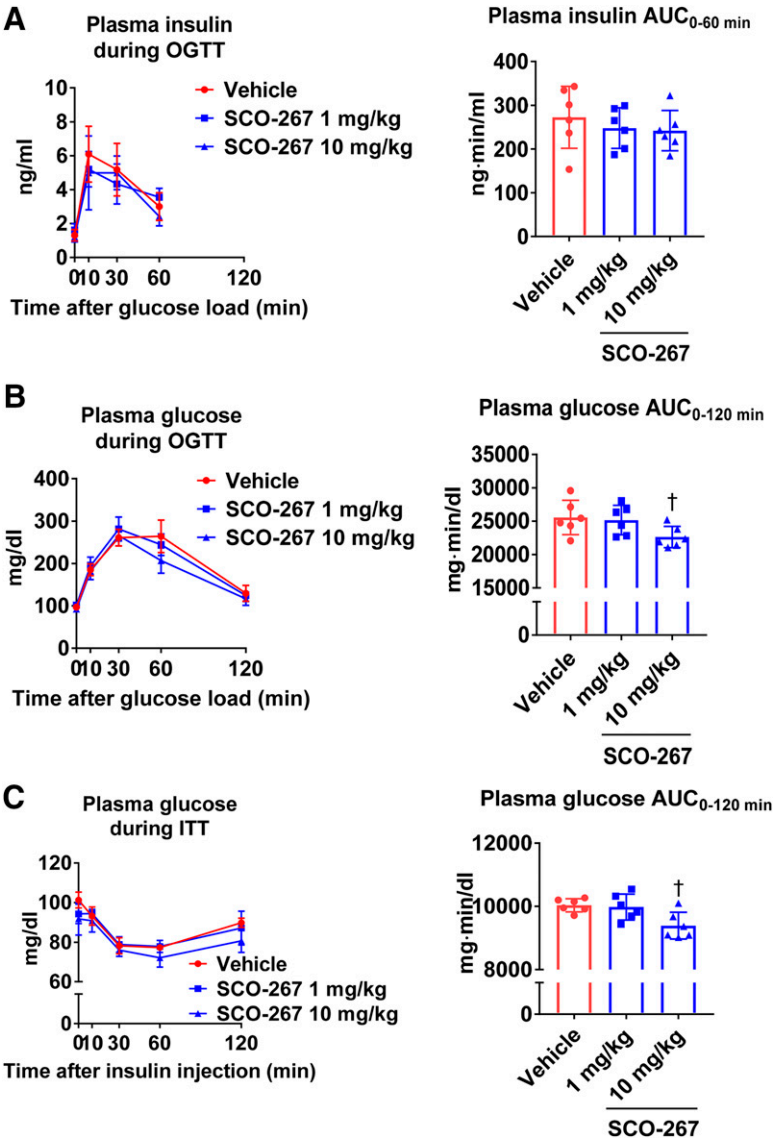

Fig. 4. Effect of subchronic administration of SCO-267 on glucose tolerance and insulin sensitivity in N-STZ rats. Vehicle or SCO-267 (1 or 10 mg/kg) was orally administered once a day for 15 days after a drug washout period of 3 days. (A) Plasma insulin level and AUC during the OGTT. (B) Plasma glucose level and AUC during the OGTT. (C) Plasma glucose level and AUC during the ITT. †$P < 0.05$ vs. vehicle by Williams' test. Values are presented as means ± S.D. (n = 6, biologic replicates). AUC, area under the curve.

This suggests that the plasma level of exposure achieved by 10 mg/kg SCO-267 was high enough to activate GPR40 throughout the day in our chronic dosing study in N-STZ rats. Even under these conditions, the sustained efficacy of SCO-267 on the glucose-lowering effect, which was superior to that in normal rats, was observed upon drug dosing. The continuous glucose-lowering effect of exendin-4 was confirmed in patients with T2DM after 30 weeks of treatment (Drucker et al., 2008). In addition, the effect of fasiglifam has been confirmed in rats treated for 6 weeks (Ito et al., 2013) and in patients with T2DM after 52 weeks of treatment (Kaku et al., 2016). Taken together with the present in vitro observations, in which SCO-267 showed equal or less desensitization to exendin-4 and fasiglifam, SCO-267 may induce similar durability of therapeutic efficacy in patients.

Notably, after the drug washout period, N-STZ rats treated with SCO-267 for 15 days showed increased insulin sensitivity. In the present study, food intake and body weight were lowered in SCO-267–treated N-STZ rats. Hence, increased insulin sensitivity may be the indirect result of weight loss. In addition, GLP-1 stimulation by SCO-267 may have contributed to the increased insulin sensitivity. Our previous data showed that SCO-267 stimulated GLP-1 in N-STZ rats (Ueno et al., 2019). GLP-1 is known to promote peripheral glucose uptake and reduce hepatic glucose production partially through the central nervous system (Sandoval and D'Alessio, 2015). Further studies are required to investigate the mechanism of SCO-267 dosing on increased insulin sensitivity.

STZ treatment causes abnormalities in insulin secretion and β-cell function (Bonner-Weir et al., 1981). Interestingly, chronic exposure of N-STZ rats to SCO-267 significantly increased the pancreatic insulin level. Hyperglycemia induces glucotoxicity, which results in β-cell dysfunction (Kaiser et al., 2003). This may have been caused by a decrease in glucotoxicity via the glucose-lowering activity of SCO-267. Furthermore, it has been reported that vincamine, a monoterpenoid indole alkaloid, which activates GPR40, protected STZ-treated INS-832/13 cells, a rat insulinoma cell line, through GPR40 activation (Du et al., 2019) and that CNX-011-67, a GPR40 agonist, reduces inflammation-induced apoptosis of NIT1 cells, a mouse pancreatic β-cell line (Verma et al., 2014).

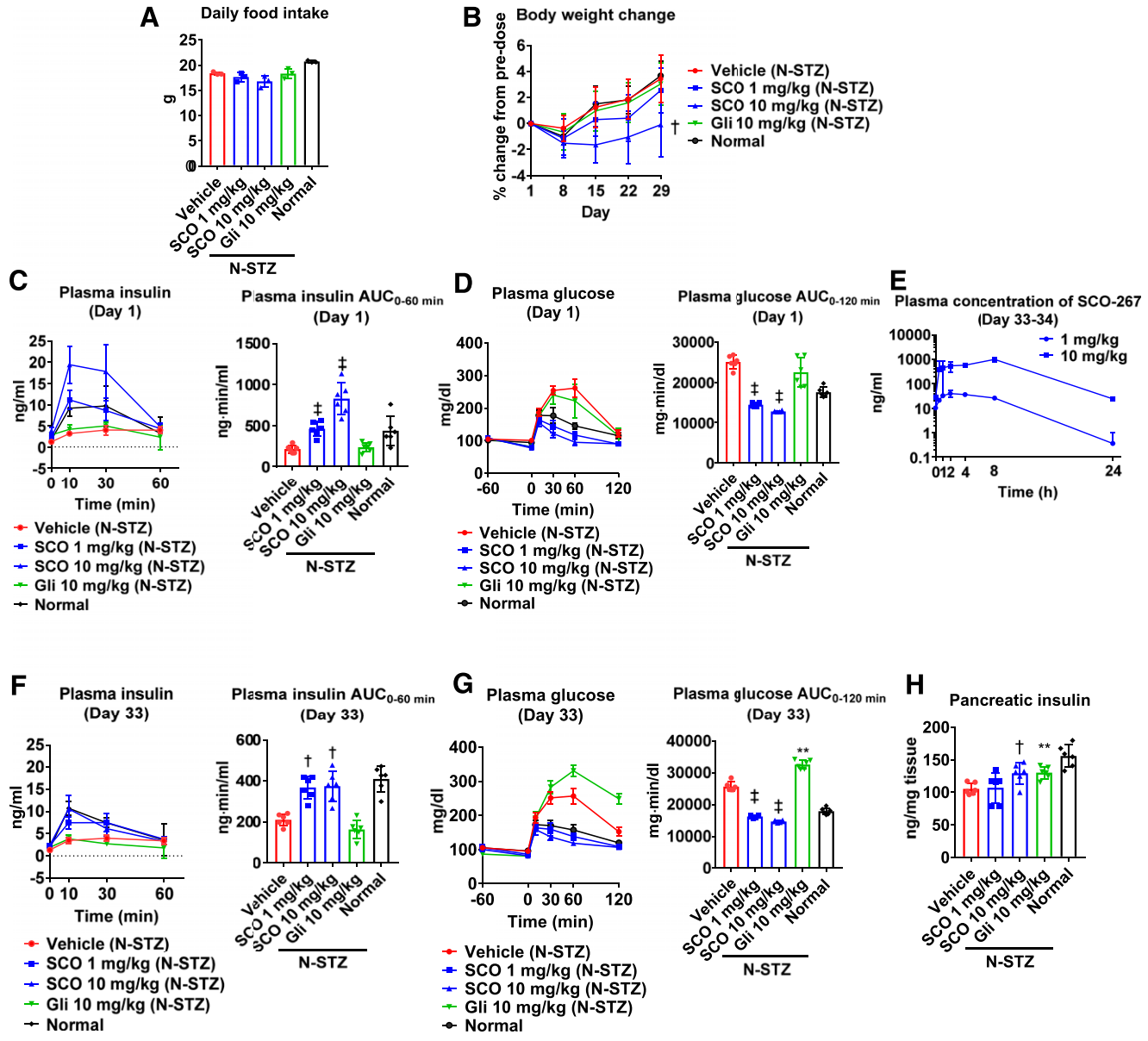

Fig. 5. Effect of chronic administration of SCO-267 in N-STZ rats. Vehicle, SCO-267 (1 or 10 mg/kg), or glibenclamide (10 mg/kg) was repeatedly administered to N-STZ rats. (A) Daily food intake. (B) Body weight change (the average baseline body weight of N-STZ rats and normal rats was 379 ± 7 and 442 ± 16 g, respectively). Plasma insulin level (C) and plasma glucose level (D) during the OGTT on day 1. (E) Pharmacokinetics analysis of SCO-267 in N-STZ rats on days 33 and 34. Plasma insulin level (F) and plasma glucose level (G) during the OGTT on day 33. Pancreatic insulin level on day 43 (H). †$P < 0.05$ and ‡$P < 0.05$ vs. vehicle by Williams' test and the Shirley-Williams test, respectively. **$P < 0.01$ vs. vehicle by Student's t test. Values are presented as means ± S.D. ($n = 6$ for chronic dose study except $n = 3$ for daily food intake and $n = 3$ for pharmacokinetic study, biologic replicates). SCO, SCO-267. Gli, glibenclamide. AUC, area under the curve.

Overall, SCO-267 may improve β-cell function via a direct GPR40-mediated effect.

In the present study, we were unable to determine the components of GPR40 and hormones that are important for the in vivo observations with SCO-267, which showed a sustained improvement in glucose tolerance in diabetic rats. Thus, future studies using specific antagonists/inhibitors and gene knockout models are needed. In addition, we evaluated only SCO-267 as a GPR40 full agonist, and it is uncertain if the current findings are universal to GPR40 full agonists. Thus, other GPR40 full agonists should be investigated in future studies.

In conclusion, even after chronic exposure, SCO-267 effectively activates GPR40 in cells and rats. In diabetic rats, chronic exposure to SCO-267 was highly effective in improving glucose tolerance. These findings suggest that sustained exposure to SCO-267 likely induces a durable glucose-lowering effect without tachyphylaxis in patients with diabetes.

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Authorship Contributions

Participated in research design: Koyama, Ookawara, Watanabe, Moritoh.

Conducted experiments: Koyama, Ookawara.

Performed data analysis: Koyama, Ookawara, Moritoh.

Wrote or contributed to the writing of the manuscript: Koyama, Moritoh.

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Supplemental file

Chronic exposure to SCO-267, an allosteric GPR40 full agonist, is effective in improving glycemic control in rats

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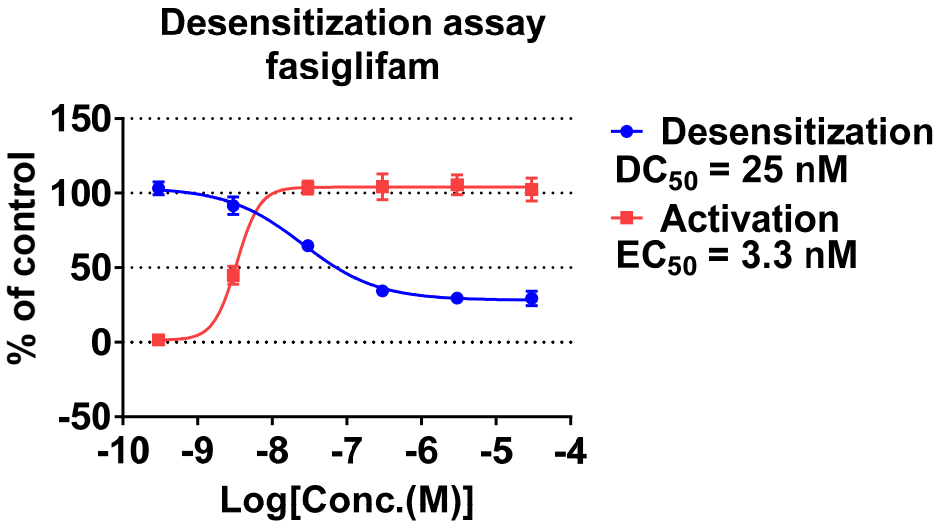
Molecular Pharmacology

Supplemental Figure



Supplemental Figure 1. Prolonged stimulatory effect of fasiglifam in the IP1 accumulation assay. CHO cells stably expressing human *FFAR1* were pretreated with the indicated concentrations of fasiglifam for 4 h at 37°C before excess ligand was removed by washing. The cells were re-stimulated with 3 μM fasiglifam for 30 min at 37°C, and the IP1 level was measured (● Desensitization). At the same time, the cells were also stimulated with fasiglifam at various concentrations after DMSO pretreatment (■ Activation). The data are presented as mean ± S.D. of three technical replicates.