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Rediocide A, an Insecticide, Induces G-Protein-Coupled Receptor Desensitization via Activation of Conventional Protein Kinase C

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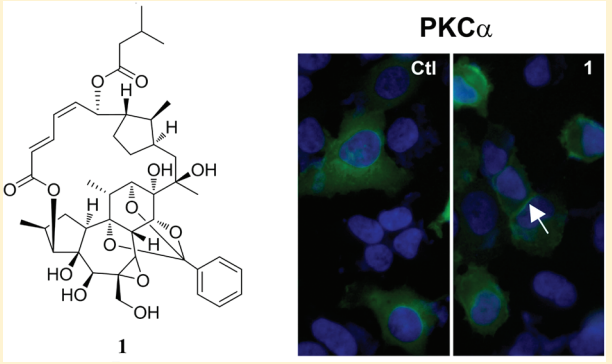
Supporting Information

ABSTRACT: In order to identify small-molecule antagonists of Methuselah (Mth), a *Drosophila* G-protein-coupled receptor (GPCR) involved in life-span control, a library of natural compounds was screened, and it was found that rediocide A (**1**), a daphnane ester from the roots of *Trigonostemon reidioides* and used currently for flea control, potently inhibited calcium mobilization mediated by this receptor. Compound **1** inhibited calcium mobilization in GPCRs other than Mth, indicating that the inhibitory effect was not due to receptor antagonism but rather to a more general mechanism. It was found that **1** can induce GPCR desensitization and internalization, and such effects were mediated by the activation of conventional protein kinase C.



G-protein-coupled receptors (GPCRs) constitute a superfamily of receptors characterized by a signature seven-transmembrane configuration, with about 900 members having been discovered.[1] GPCRs respond to a diverse array of sensory and chemical stimuli, such as light, odor, taste, pheromones, hormones, and neurotransmitters.[2] Due to the variety of physiological and pathological functions regulated by GPCRs, they are frequently used drug targets in the pharmaceutical industry, and approximately 40% to 50% of all currently marketed drugs target GPCRs.[3,4]

Methuselah (*mth*) is an important gene encoding a GPCR (Mth) involved in stress responses and biological aging in *Drosophila*.[5] It is reported that disturbing the gene by P-element insertion not only prolongs the life span of *Drosophila* at higher temperatures but also increases their resistance to paraquat and starvation. Genetic ablation of the endogenous ligand[6] and overexpression of a peptide antagonist[7] both elongated the life span of *Drosophila*.

To identify small-molecule antagonists of the Mth receptor, a library consisting of 2800 natural products (provided by the Chinese National Compound Resource Center, Shanghai, People's Republic of China) was screened with HEK-293 cells stably expressing Mth-B, the longer splicing variant of the Mth receptor. Rediocide A (**1**), a daphnane ester, was found to inhibit a Mth agonist (SunA1-30)-induced calcium response potently.

Rediocides may be isolated from a methanol extract of the roots of the plant *Trigonostemon reidioides* Craib (Euphorbiaceae). Up to the present, seven rediocides have been identified and characterized from this plant.[8,9] All rediocides are reported to be among the most potent groups of antiflea compounds, although their molecular mechanism of action remains unclear. Rediocide A (**1**) is the first compound isolated in this series and represents a new chemotype with complex daphnane ester diterpenoid structure.[8,10] This substance has also been reported to exhibit antitoxin activity against cobra venom[11] and a cytotoxic effect against various cancer cell lines.[9,12] Herein it is reported that **1** potently inhibits calcium responses mediated by Mth-B and other types of GPCRs. It was found that **1** induces GPCR desensitization and internalization by activating conventional PKC isoforms.

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RESULTS AND DISCUSSION

Rediocide A (1) Inhibits the GPCR-mediated Calcium Response. Mth is a Gq-coupled GPCR, so the effect of **1** was tested on calcium flux induced by the Mth agonist SunA1-30 in

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HEK293 cells stably transfected with Mth-B (HEK293-Mth-B), the longest splicing form of the Mth receptor. As shown in Figure 1A, pretreating the cells with **1** blocked SunA1-30-induced intracellular calcium elevation in HEK293-Mth-B cells in a dose-dependent manner (IC_{50} 88 nM). This compound was then tested on other GPCRs to verify whether this inhibitory effect is specific to Mth. Compound **1** displayed dose-dependent inhibitory effects on all four GPCRs in which it was evaluated, comprising adrenergic receptor alpha 1a (ADRA1A), adrenergic receptor alpha 1b (ADRA1B), chemokine receptor CCR6 (CCR6), and glucagon receptor (GCGR), with IC_{50} values of 13, 215, 261, and 4 nM, respectively (Figure 1B and C). ADRA1A and ADRA1B are activated by small-molecule agonists, and CCR6 and GCGR are activated by peptide ligands. With GCGR in the secretin receptor family (family B) and ADRA1A/B and CCR6 in the α- and γ-group of the rhodopsin receptor family (family A), these receptors have a remote phylogenetic relationship.[13,14] The results showing that **1** blocked the calcium response mediated by these different GPCRs indicate that the inhibitory effects are not likely due to receptor antagonism, but to a more general mechanism.

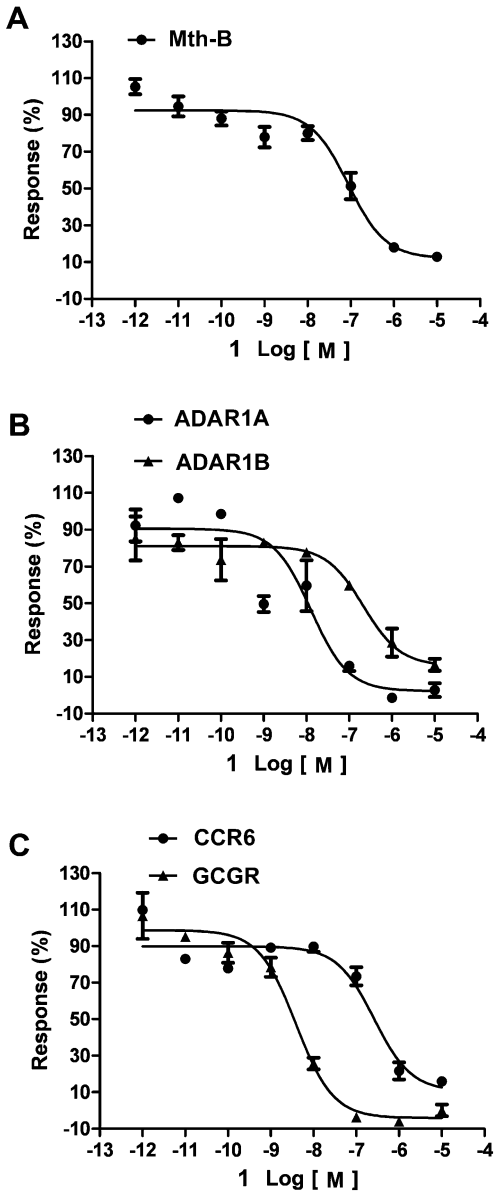

Figure 1. Rediocide A (**1**) inhibits GPCR-mediated calcium mobilization. (A) Rediocide A (**1**) inhibits SunA1-30-induced calcium elevation in a dose-dependent manner in HEK293 cells stably expressing Mth-B, with an IC_{50} value of 88 nM. (B) Rediocide A (**1**) inhibits ADAR1A- and ADAR1B-mediated calcium responses. (C) Rediocide A (**1**) inhibits CCR6- and GCGR-mediated calcium responses. Data are presented as means ± SEM (n = 3).

Rediocide A (1) Induces Desensitization and Internalization of GPCR. One important feature of the GPCR signaling systems is that they are not constant but exhibit a memory effect as a result of prior activation or signaling.[15] Thus, high activation of a receptor leads to a reduced ability to be stimulated in the future (desensitization), whereas low activation leads to an increased ability to be stimulated (sensitization).[16] Accordingly, desensitization is another major mechanism that might cause the loss of function in GPCRs.[17] To assess whether **1** could induce GPCR desensitization, HEK293 cells stably expressing GCGR were first stimulated with either 100 nM glucagon or 1 μM **1**, with exposure to 1% dimethyl sulfoxide (DMSO) serving as a negative control (Figure 2A, first arrow). While glucagon induced a robust calcium response, **1** failed to cause any intracellular calcium elevation, indicating that it is not an agonist of GCGR. After a 30 min incubation, cells were washed and restimulated with 100 nM glucagon (Figure 2A, second arrow). Prestimulation with both glucagon and **1** led to receptor desensitization, i.e., reduced calcium response at the second stimulation with glucagon. Receptor internalization is a common phenomenon following GPCR stimulation, and it plays a key role in receptor desensitization and resensitization.[18,19] By using GCGR tagged with a red fluorescent protein, the effect of **1** was studied on GPCR internalization. As shown in Figure 2B–E, at resting stage, the GCGRs were mainly located on the surface of the cells (arrows). Thirty minutes after glucagon stimulation, the receptors internalized and formed bright intracellular vesicles (arrowheads). Similarly, **1** also induced robust internalization of GCGR (arrowheads).

Rediocide A (1) Induces Phosphorylation and Translocation of Conventional PKC. There are two types of desensitization in GPCRs: homologous desensitization via ligand stimulation and subsequent G-protein-coupled receptor kinase (GRK) activation and heterologous desensitization that does not require specific ligand binding.[16,20,21] As demonstrated above, **1** induced GPCR desensitization and internalization but did not interact with a specific receptor, so it was speculated that this daphnane ester might activate the heterologous desensitization pathway by modulating second messenger-activated protein kinases. It is generally accepted that PKC can phosphorylate consensus serine and threonine residues within the intracellular loop and carboxyl-terminal domains of GPCRs.[2,22,23] Phosphorylation of the receptor will lead to the recruitment of β-arrestins, which then target the receptors to clathrin-coated internalization vesicles.[24]

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) is a well-known compound that activates PKC and induces desensitization and internalization of GPCRs.[25] The structures of rediocide A (**1**) and TPA share a biogenetically related diterpenoid skeleton, so the ability of **1** to induce PKC phosphorylation and activation was assessed. HEK293 cells were treated with 1 μM **1** for various durations, and PKC phosphorylation was assessed with Western blotting and an antibody that detects conventional and novel PKC isoforms

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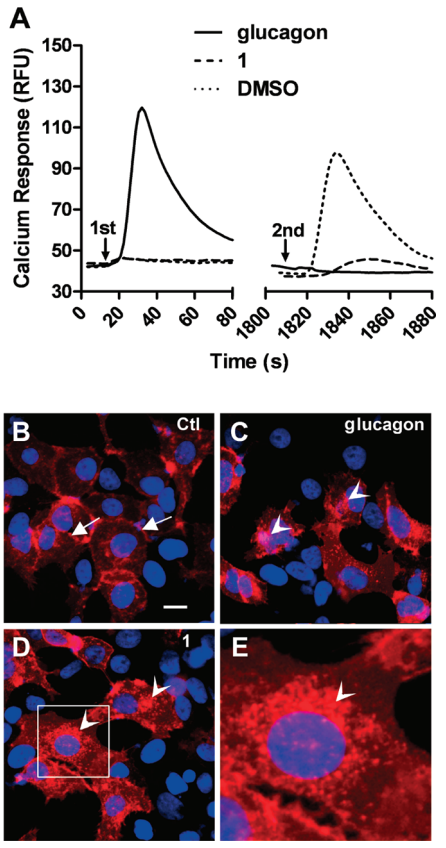

Figure 2. Rediocide A (**1**) induces the desensitization and internalization of GPCR. (A) HEK293 cells that stably express GCGR were cultured in 96-well plates and loaded with Fluo-4 AM and stimulated (first arrow) with 100 nM glucagon, 1 μM **1**, or DMSO (negative control). After 30 min incubation, cells were washed and restimulated (second arrow) with 100 nM glucagon. The intracellular calcium levels were measured. The curves shown are representatives of three independent experiments, each carried out in triplicate. (B–E) HEK293 cells were transfected with pDsRed-GCGR (red) and stimulated with vehicle control (B), 100 nM glucagon (C), or 1 μM **1** (D) for 30 min, and the internalization of the receptor was observed. Cell nuclei were stained with Hoechst 33342 (blue). The boxed area in D is enlarged in E. Arrows indicate the receptors located on the cell surface, and arrowheads indicate the internalized receptors. Scale bar: 10 μm.

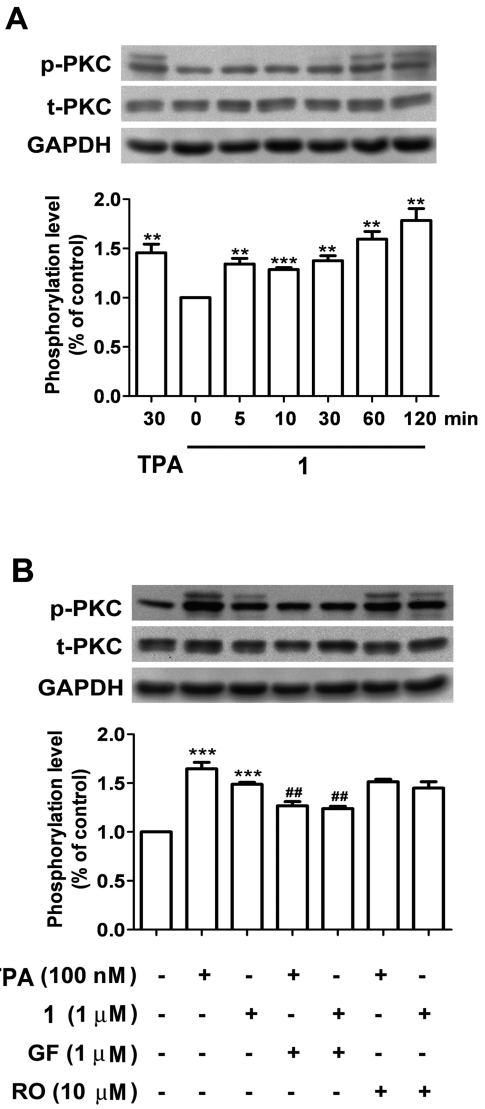

Figure 3. Rediocide A (**1**) induces phosphorylation of conventional PKC. (A) Western blots and statistical analysis of PKC phosphorylation levels after stimulation with 1 μM **1**. (B) Effect of GF109203 X (1 μM) and rottlerin (10 μM) on TPA (100 nM) or **1** (1 μM)-induced PKC phosphorylation. Data are presented as means ± SEM (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$, versus vehicle control; $^{\#\#}p < 0.01$, versus TPA alone or **1** alone. PKC phosphorylation levels were normalized to the GAPDH level in the same sample.

when phosphorylated at a carboxy-terminal residue homologous to Ser660 of PKCβII/δ. It was found that **1** induced a time-dependent phosphorylation of PKC (Figure 3A). TPA was used as a positive control. To clarify which isoform was phosphorylated by **1**, two PKC inhibitors, GF109203 X and rottlerin, were used. GF109203 X inhibits both conventional and novel PKCs, and rottlerin is a selective inhibitor for the novel PKCs.[26,27] Pretreatment with GF109203 X significantly reduced the phosphorylation of PKC induced by **1**, whereas rottlerin had no significant effects (Figure 3B).

In addition to phosphorylation, PKC translocation from the cytoplasm toward the membrane is another sign of activation. As shown in Figure 4, the GFP-tagged PKCα was located in the cytosol in the resting condition. It was recruited to the cell membrane after 30 min exposure to **1** (arrows). Pretreatment of GF109203 X completely abolished PKC translocation induced by **1** (Figure 4C). Thus, rediocide A (**1**) can induce conventional PKC phosphorylation and translocation.

Rediocide A (1) Induces Desensitization of GPCR via Activation of Conventional PKC. To further explore which PKC isoform might be involved in **1**-induced GPCR desensitization, the effects of GF109203 X (blocker for both conventional and novel PKC) and rottlerin (novel PKC blocker) were tested in a desensitization assay. Cells stably expressing GCGR were preincubated with 10 μM GF109203 X for 15 min (Figure 5A). Then, the cells were first stimulated with either 100 nM glucagon or 1 μM **1**, and 1% DMSO served as a negative control (Figure 5A, first arrow). Similar to the effect shown in Figure 2A, glucagon induced a strong calcium response, but **1** had no stimulatory effects. Cells were then washed and restimulated with 100 nM glucagon (Figure 5A, second arrow). Different from what was observed in Figure 2A, glucagon elicited a robust calcium response in cells stimulated with **1**, indicating that preincubation with GF109203 X

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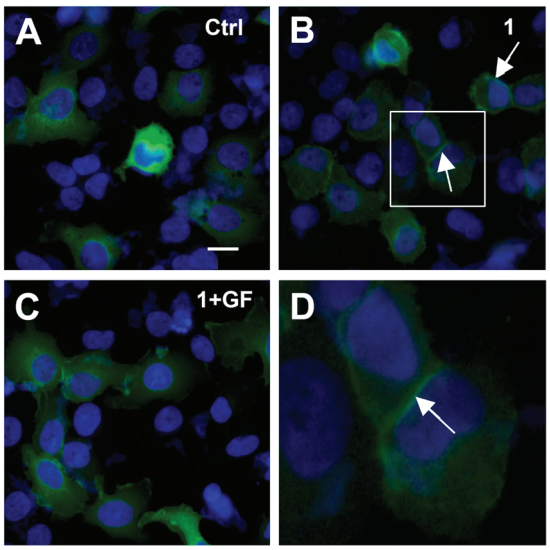

Figure 4. Rediocide A (**1**) induces membrane translocation of conventional PKC. Translocation of PKCα was studied with HEK293 cells transfected with GFP-tagged PKCα (green). Cells were stimulated with vehicle control (A), 1 μM **1** (B), or 1 μM **1** in the presence of GF109203 X (C). Cell nuclei were stained with Hoechst 33342 (blue). Stimulation with **1** led to the cytoplasmic membrane translocation (arrows) of PKCα, while GF109203 X inhibited **1**-induced PKCα translocation. The boxed area in B is enlarged in D. Scale bar: 10 μm.

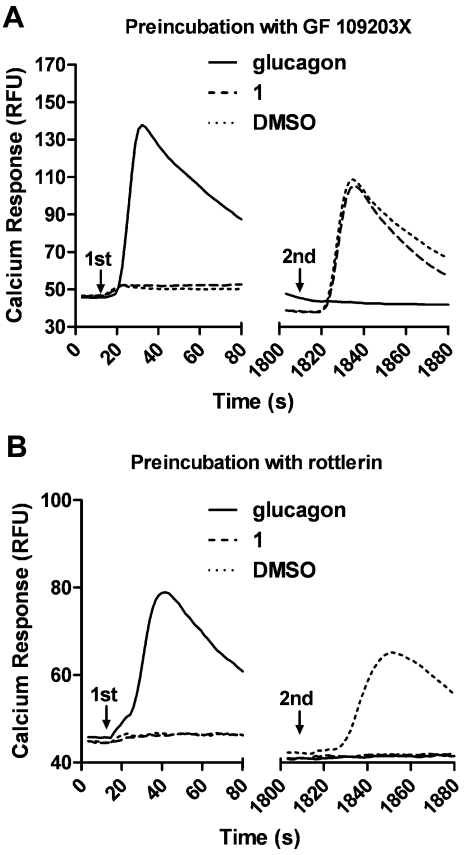

Figure 5. A conventional but not a novel PKC inhibitor blocks rediocide A (**1**)-induced GPCR desensitization. HEK293 cells that stably express GCGR were cultured in 96-well plates, loaded with Fluo-4 AM, and then incubated with 10 μM GF109203X (A) or 10 μM rottlerin (B) for 15 min. Cells were then stimulated (first arrow) with 100 nM glucagon, 1 μM **1**, or DMSO (negative control). After a 30 min incubation, cells were washed and restimulated (second arrow) with 100 nM glucagon, and intracellular calcium was measured. The curves shown are representatives of three independent experiments, each carried out in triplicate.

completely abolished **1**-induced receptor desensitization. In contrast, preincubation with rottlerin did not alleviate **1**-induced receptor desensitization (Figure 5B), indicating that rediocide A (**1**) induces GPCR desensitization mainly via the activation of conventional PKC. It is interesting to note that while it completely blocked **1**-induced receptor desensitization, GF109203 X had no effect on glucagon-mediated receptor desensitization, indicating that glucagon induces GCGR desensitization mainly via the GRK pathway, i.e., the homologous desensitization pathway.

In summary, a new biological function of rediocide A (**1**) is reported in that it can activate conventional PKC. Also via PKC activation, **1** induces GPCR desensitization, internalization, and possibly many other cellular events. The phorbol esters were the first tumor-promoting agents isolated from natural sources and are known to be powerful activators of PKC.[28] However, some of these diterpenes are not tumor promoters and instead are very effective anticancer or antiviral (HIV-1) agents.[29] Meanwhile, modulation of conventional and novel PKC activity has also been reported to underlie the antitumor activity of certain compounds.[30,31] Thus the relationship between the cytotoxic activity of **1** and the activation of conventional PKC warrants a closer investigation.

EXPERIMENTAL SECTION

Chemicals and Reagents. Rediocide A (**1**) (98% pure by HPLC) was purchased from Biobiopha (Kunming, People's Republic of China), and phenylephrine was purchased from Tokyo Kasei (Tokyo, Japan). MIP-3α was purchased from Peprotech (London, UK), and glucagon was purchased from GL Biochem (Shanghai, People's Republic of China). Hoechst 33342 sulfinpyrazone and rottlerin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PKC inhibitor GF109203 X was obtained from Enzo Life Science (Farmingdale, NY, USA). Antibodies against PKC or phosphorylated PKC isoforms and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA, USA). The HEK293-Mth-B cell line was kindly provided by Dr. Xinyun Huang (Cornell University). Mammalian expression vectors encoding Gα16, ADRA1A, ADRA1B, CCR6, and GCGR were purchased from the UMR cDNA Resource Center. Plasmid encoding PKCα was provided by Dr. Lars Kaestner (Saarland University).

Cell Culture and Transfection. HEK293 cells obtained from American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. HEK293 cells were cotransfected with plasmids encoding various GPCRs and Gα16 by electroporation. To generate stable cell lines, transfected cells were seeded onto 10 cm dishes, and 1 mg/mL G418 and 40 μg/mL blasticidin were added to the culture medium 24 h later. The selection medium was changed every 3 days until colonies formed. A single colony was isolated, expanded, and tested with a calcium mobilization assay to confirm the expression and proper function of the transfected genes.

Calcium Mobilization Assay. Cells were seeded onto 96-well plates at a density of 3×10^4 cells/well and cultured overnight. Cells were then incubated with 2 μM Fluo-4 AM in HBSS (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose, and 250 μM sulfinpyrazone, pH 7.4) at 37 °C for 45 min. After a thorough washing, 50 μL of HBSS containing either antagonists or 1% DMSO (negative control) was added. After incubation at room

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temperature for 10 min, 25 μL of agonist (depending on the receptor) was dispensed into the well using a FlexStation III microplate reader (Molecular Devices), and intracellular calcium change was recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

Western Blotting. HEK293 cells were serum starved for 4 h and then treated with 1 μM **1** or 100 nM TPA for the indicated duration at 37 °C. Cells were lysed, sonicated, and boiled at 95 °C for 5 min in sample buffer (62.5 mM tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenyl blue; pH 6.8). Aliquots of protein were fractionated by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated first with blocking buffer (TBS with 0.05% Tween 20 and 5% nonfat milk) for 1 h at room temperature and then incubated overnight at 4 °C in buffer containing anti-GAPDH (1:10 000) and anti-PKC antibodies (1:1000). The membranes were washed three times and incubated with goat anti-rabbit IgG HRP (1:10 000) for 1 h. After washing, immunostaining was visualized using Amersham ECL Plus Western blotting detection reagents (GE Healthcare).

Fluorescent Image Collection. For the GCGR internalization experiment, HEK293 cells transfected with pDsRed-GCGR were seeded onto 24-well plates with coverslips at a density of 8×10^4 per well. After overnight incubation, cells were stimulated with 1 μM glucagon, **1**, or 1% DMSO (negative control) at 37 °C for 30 min. For the PKCα translocation assay, HEK293 cells transfected with pCR259-hPKCα-GFP were seeded onto 24-well plates with coverslips at a density of 8×10^4 per well. After overnight incubation, cells were pretreated with GF109203 X or vehicle at 37 °C for 15 min and then stimulated with 1 μM **1** for 30 min. After fixation with 4% formaldehyde in phosphate-buffered saline, cells were washed and the nuclei were stained with Hoechst 33342 for 10 min at room temperature. Fluorescent images were obtained with an Olympus IX51 inverted fluorescent microscope.

Data Analysis. Data were analyzed with GraphPad Prism software (GraphPad). Nonlinear regression analysis was performed to generate dose−response curves and calculate concentration for 50% inhibition (IC_{50}) values. Means ± SEM were calculated using this software. Two-tailed Student's *t* tests were performed to determine statistically significant differences.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Author Contributions

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Notes

The authors declare no competing financial interest.

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