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ORIGINAL ARTICLE

Identification of a small molecule activator of novel PKCs for promoting glucose-dependent insulin secretion

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Using an image-based screen for small molecules that can affect Golgi morphology, we identify a small molecule, Sioc145, which can enlarge the Golgi compartments and promote protein secretion. More importantly, Sioc145 potentiates insulin secretion in a glucose-dependent manner. We show that Sioc145 selectively activates novel protein kinase Cs (nPKCs; δ and ε) but not conventional PKCs (cPKCs; α, βI and βII) in INS-1E insulinoma cells. In contrast, PMA, a non-selective activator of cPKCs and nPKCs, promotes insulin secretion independent of glucose concentrations. Furthermore, we demonstrate that Sioc145 and PMA show differential abilities in depolarizing the cell membrane, and suggest that Sioc145 promotes insulin secretion in the amplifying pathway downstream of K_{ATP} channels. In pancreatic islets, the treatment with Sioc145 enhances the second phase of insulin secretion. Increased insulin granules close to the plasma membrane are observed after Sioc145 treatment. Finally, the administration of Sioc145 to diabetic GK rats increases their serum insulin levels and improves glucose tolerance. Collectively, our studies identify Sioc145 as a novel glucose-dependent insulinotropic compound via selectively activating nPKCs.

Keywords: PKC; insulin secretion; glucose dependency; Golgi

Introduction

Regulation of insulin secretion plays a critical role in glucose homeostasis. Two signaling pathways have been identified that play major roles in mediating glucose-stimulated insulin secretion (GSIS), the triggering pathway and the amplifying pathway [1, 2]. Under physiological conditions, the triggering pathway plays a primary role in determining whether insulin is secreted

[3]; whereas the amplifying pathway, manifesting itself only after the triggering signal has been produced, serves to optimize the secretory response [2, 4]. The threshold concentration of glucose for activating the triggering pathway is much higher than that required for the amplifying pathway [2, 4]. The hierarchy between the two pathways ensures that no insulin is inappropriately secreted under the conditions of low glucose concentrations. The current insulinotropic therapy using sulfonylureas alters the hierarchy and thus suffers a mechanism-based side effect of inducing hypoglycemia [2]. Selective activation of regulators distal to the production of triggering signals is believed to be an important strategy for enhancing glucose-dependent insulin secretion.

Several protein kinase C (PKC) isoforms are expressed in pancreatic β-cells and have been implicated in the regulation of GSIS [5-8]. Different PKC isoforms are speculated to play different roles, but the exact functions of individual PKC isoforms in regulating insulin secretion are still controversial [9, 10]. PMA, a non-selective

phorbol ester activator of both conventional (c) PKCs (α, βI, βII and γ) and novel (n) PKCs (δ, ε, η/l and θ), stimulates insulin secretion independent of glucose concentrations [6]. As far as we know, no selective PKC activator that is capable of regulating glucose-dependent insulin secretion has been reported.

Recently, we have developed an image-based assay using YFP-tagged p58 as a Golgi marker to identify small molecules as regulators of protein secretion [11]. Using this assay, we isolated a small molecule, Sioc145, which can enlarge the Golgi compartments and promote protein secretion. We show that in pancreatic islets and a selected β-cell line, Sioc145 potentiates insulin secretion in a glucose-dependent manner. Furthermore, we show that Sioc145 selectively activates nPKCs but not cPKCs, and provide evidence that acute nPKCs activation potentiates GSIS in the amplifying pathway downstream of K_{ATP} channels.

Results

Identification of Sioc145 as a modulator of Golgi morphology

Since the Golgi plays a central role in mediating protein modification and secretion, we designed an image-based screen to isolate small molecules that affect the morphology of the Golgi [11]. H4 human glioblastoma cell line was made to stably express a p58-YFP fusion protein (H4-p58-YFP), in which p58, a cargo receptor involved in transporting proteins from endoplasmic reticulum to the Golgi complex, was used as a marker for the vesicular tubular clusters and the *cis*-Golgi [12].

Using H4-p58-YFP cells and fluorescent microscopy, we screened for compounds that could affect the fluorescent signal of p58-YFP in the SIOC Chemical Library containing 200 small molecule compounds that are speculated to have high bioactivity [13, 14]. p58-YFP normally appeared as peripheral punctate structures close to the *cis*-Golgi (Figure 1A). Sioc145, a derivative of benzolactam-V8 designed to be a PKC activator [13, 14], can significantly enhance the fluorescent signal of p58-YFP (Figure 1A). Interestingly, this effect of Sioc145 is fully reversible: the levels of size and intensity of p58-YFP signal returned to normal after the Sioc145-treated H4 cells were cultured in media without Sioc145 for 24 h.

We measured the images for the size and intensity of p58-YFP signal using high-throughput fluorescent microscopy. The intensity and area of p58-YFP spots in Sioc145-treated H4 cells were enlarged in a dose- and time-dependent manner with an $EC_{50} \approx 0.78$ μM (Figure 1B and 1C). The maximum increase in the area of p58-YFP was up to 7-8 times than that of the negative con-

trol. We also analyzed the effect of Sioc145 on cell viability. Treatment with up to 25 μM of Sioc145 for 24 h had no obvious effect on cell survival, whereas the cells died after treatment with 50 μM Sioc145 for 24 h (Supplementary information, Figure S1). Thus, the difference between EC_{50} and LD_{50} of Sioc145 on H4 cells is at least 25-fold.

We conducted a structure activity relationship (SAR) study to analyze the structural requirement for the effect of Sioc145 in enhancing p58-YFP signal. We found that Sioc111, a close diastereoisomer analog of Sioc145 (Figure 1D), had a significantly reduced effect on the fluorescent signal of p58-YFP (Figure 1E), suggesting a stringent SAR relationship. Consistent with the role of PKCs in regulating the Golgi morphology, PMA treatment also significantly increased the p58-YFP signal (Figure 1E).

To test whether the enhancement of p58-YFP signal by Sioc145 relies on protein synthesis, we treated H4-p58-YFP cells with cycloheximide (CHX), an inhibitor of protein biosynthesis. Treatment with CHX alone did not have any obvious effect on the signal marked by p58-YFP in control cells (Figure 1F and Supplementary information, Figure S2). Furthermore, the increases in size and intensity of p58-YFP signal in Sioc145-treated cells were not reduced in the presence of CHX. Thus, the enhancing effect of Sioc145 on p58-YFP signal is independent of protein synthesis.

Effect of Sioc145 on morphology and structure of the Golgi

To further explore the effect of Sioc145 on the Golgi, we examined the Golgi morphology in parental H4 cells by immunofluorescence using an antibody against a Golgi matrix protein GM130, which is peripherally associated with the *cis*-Golgi compartment [15]. As shown in Figure 2A, the *cis*-Golgi compartment marked by anti-GM130 in H4 cells treated with Sioc145 grew larger in a time-dependent manner. A significant increase in the Golgi sizes was also observed for the Sioc145-treated H4 cells expressing CFP-tagged GalT, which is a marker for *trans*-Golgi (Supplementary information, Figure S3). These data suggest that Sioc145 has a strong enlargement effect on the size of both the *cis*- and *trans*-Golgi.

We also used electron microscopy to assess the effect of Sioc145 on the ultra-structure of the Golgi stacks and vesicles. Comparing to that of the untreated cells, the numbers of Golgi stacks and vesicles were increased significantly in Sioc145-treated H4 cells (Figure 2B). Furthermore, a large number of vesicles with big granules were found around the Golgi stacks, suggesting that Sioc145 not only enlarges the Golgi size but may also promote vesicular transport.
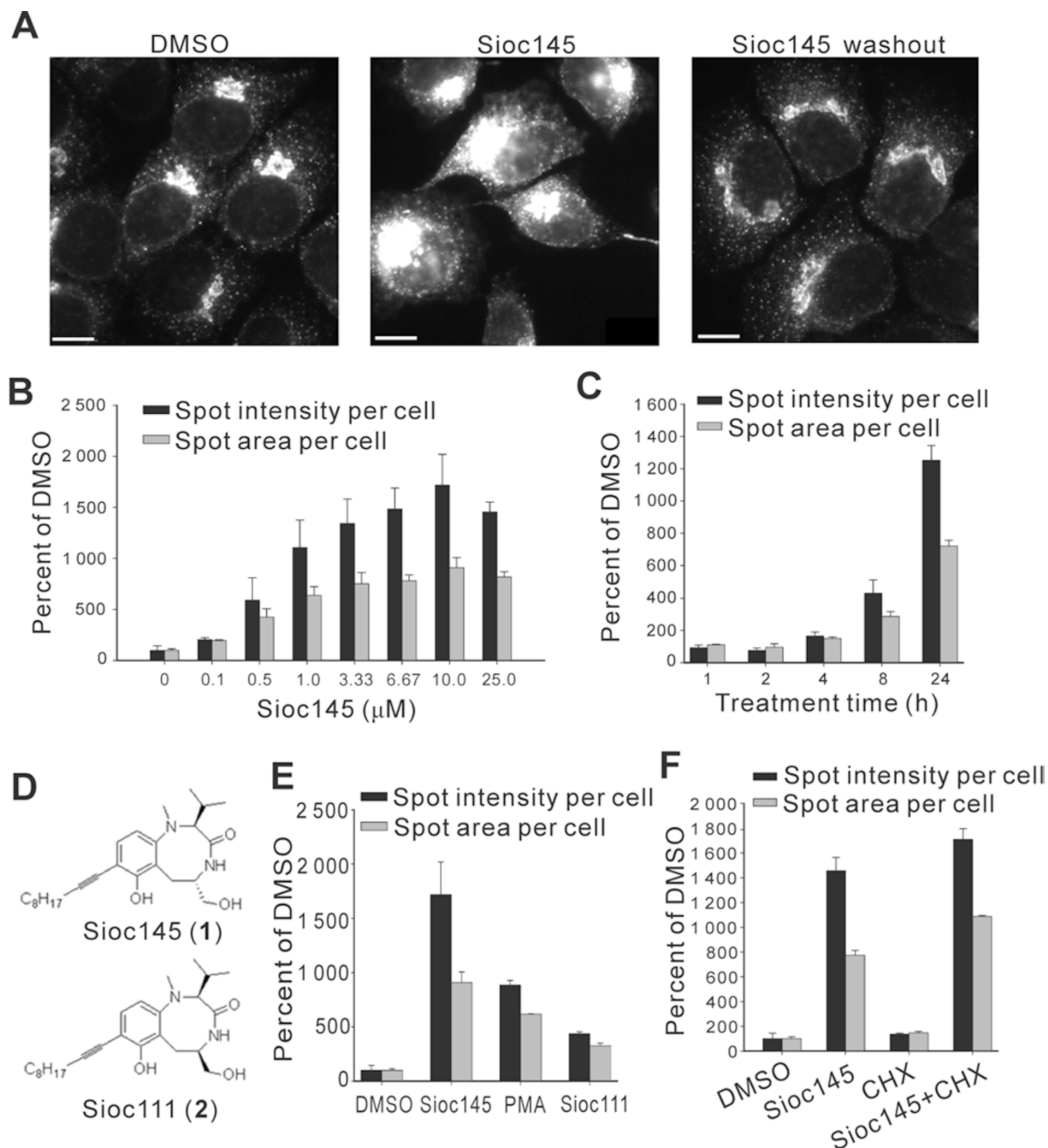
Figure 1 Sioc145 enhances the signal of p58-YFP in H4 cells. **(A)** H4-p58-YFP cells were treated with DMSO or 5 μM Sioc145 for 24 h or then exchanged with fresh medium without Sioc145 for an additional 24 h. Images were analyzed for spots' intensity and area per cell. Scale bar, 10 μm. **(B)** Dose-response of Sioc145 in H4-p58-YFP cells treated for 24 h. **(C)** Time course response of 5 μM Sioc145 in H4-p58-YFP cells. **(D)** Chemical structures of Sioc145 and Sioc111. **(E)** p58-YFP signal in cells treated with DMSO, 5 μM Sioc145, 5 μM Sioc111 or 100 ng/ml PMA. **(F)** p58-YFP signal in cells treated with DMSO, 5 μM Sioc145, 10 μg/ml CHX alone or CHX together with 5 μM Sioc145 for 24 h. All graphics represent means ± SD obtained from three independent experiments.

Sioc145 promotes SEAP secretion in a PKC-dependent manner

Based on the above results, we surmise that Sioc145 may be able to promote protein secretion. To examine this hypothesis, we established a stable H4 cell line that expresses a secretory form of the human placental alka-
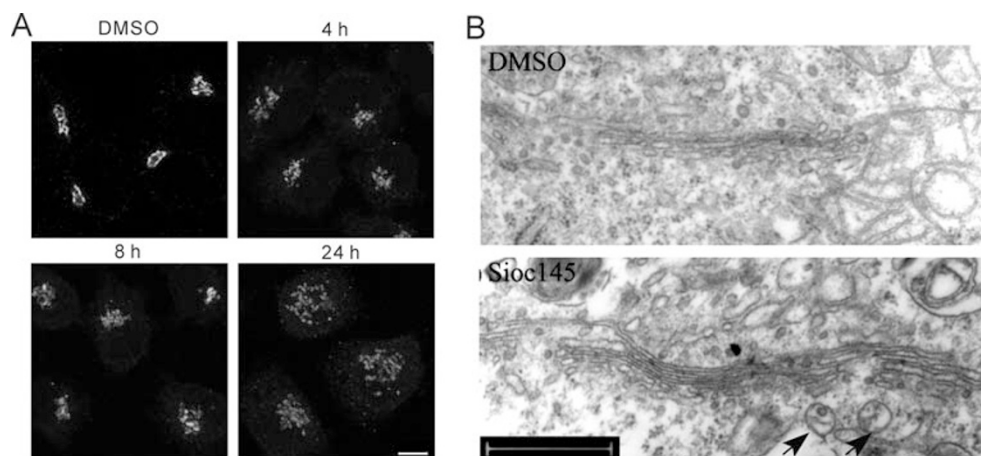
Figure 2 Effect of Sioc145 on the morphology and structure of the Golgi. **(A)** Immunofluorescence images of H4 cells treated with DMSO, 5 μM Sioc145 for 4 h, 8 h or 24 h using GM130 antibody. Scale bar, 10 μm. **(B)** Representative electron microscopy images of the Golgi in H4 cells treated with DMSO or 5 μM Sioc145 for 24 h. Arrows indicate the representatives of big secretory vesicles. Scale bar, 500 nm.

line phosphatase (SEAP). Secretion of SEAP can be used as an assay to determine the rate of protein secretion [16, 17]. Using this assay, we found that Sioc145 treatment promoted SEAP secretion in a dose-dependent manner (Figure 3A). We determined the effective concentration for the half-maximum response (EC_{50}) of Sioc145-mediated SEAP secretion to be 0.62 μM, which is in good agreement with its EC_{50} in enlarging the Golgi compartment (0.78 μM). Consistent with the ability of Sioc145 in promoting secretion, the intracellular levels of SEAP (endo-

AP) were decreased after the treatment with Sioc145 for 24 h (Figure 3A and 3B).

To further explore the roles of PKCs in mediating the effects of Sioc145, we employed different PKC activators and inhibitors (Figure 3C). Consistent with the role of PKC, the treatment with PMA alone increased SEAP secretion. Rottlerin, Gö6976, Ro318220 and GF109203X (GFX), all of which are PKC inhibitors, had no apparent effect on SEAP secretion when used alone. However, both pan PKC inhibitors, Ro318220 and GFX, signifi-
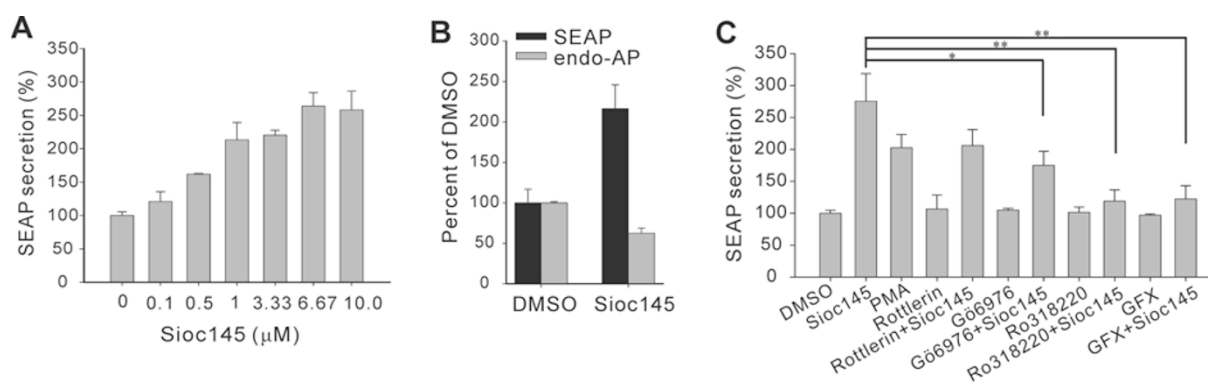

Figure 3 Sioc145 promotes SEAP secretion via a PKC-dependent pathway. **(A)** Dose-response of Sioc145 in promoting SEAP secretion in H4-SEAP cells treated for 24 h. **(B)** H4-SEAP cells were treated with DMSO or 5 μM Sioc145 for 24 h. The levels of alkaline phosphatase enzyme in culture medium (SEAP) or total cell lysate (endo-AP) were analyzed. **(C)** H4-SEAP cells were treated with DMSO, 5 μM Sioc145 or 100 ng/ml PMA in the presence or absence of 10 μM Rottlerin, 2 μM Gö6976, 2 μM Ro318220 or 2.5 μM GFX for 24 h as indicated. Secreted SEAP was measured. Data are normalized to that of DMSO-treated cells, which is set as 100% and error bars represent SD from three independent experiments. *$P < 0.05$; **$P < 0.01$.

cantly inhibited the enhancing effect on SEAP secretion induced by Sioc145. In addition, Gö6976, which inhibits cP-KCs, or Rottlerin, which inhibits PKC δ selectively, partially inhibited the effect of Sioc145 on SEAP secretion. These results suggest that the enhancement of SEAP secretion in H4 cells by Sioc145 treatment requires PKC activation.

Sioc145 potentiates insulin secretion in a glucose-dependent manner

To investigate the promoting effect of Sioc145 on secretion in a physiological relevant setting, we examined whether Sioc145 has an insulinotropic effect in rat insulinoma INS-1E cells. We treated INS-1E cells for a short time of 30 min with Sioc145 under the condition of 2.8 mM (low) or 16.7 mM (high) glucose. The result showed that Sioc145 potentiated high glucose-stimulated insulin secretion in a dose-dependent manner, whereas it did not enhance insulin secretion under the condition of 2.8 mM glucose (Figure 4A). Similar results were also obtained from isolated rat pancreatic islets, although the effect was quantitatively less dramatic than that in INS-1E cells (Figure 4B).

Since a previous report indicated that PMA was an insulinotropic compound without glucose dependency [6], the relationship between glucose concentration and the stimulatory action of Sioc145 was further analyzed in comparison with PMA. Consistent with published results [6], PMA could trigger insulin secretion under conditions of low (2.8 mM) or even no glucose, whereas Sioc145 potentiated insulin secretion only under the conditions of high stimulatory glucose concentrations (5.6 mM and 16.7 mM; Figure 4C). These data indicate that Sioc145 may promote insulin secretion in a glucose concentration-dependent manner.

Selective activation of nPKCs is required for insulinotropic action of Sioc145

Since the phosphorylation of MARCKS protein has been used as a marker of PKC activation [18], we examined the status of MARCKS phosphorylation in Sioc145-treated INS-1E cells. Consistent with the activation of PKCs, the treatment with Sioc145 led to a rapid increase in the phosphorylation of MARCKS within 2 min (Figure 5A). On the other hand, our results showed that Sioc145 increased the phosphorylation of MARCKS under the conditions of both 2.8 mM and 16.7 mM glucose, suggesting that PKC activation mediated by Sioc145 is independent of glucose concentrations.

To explore the mechanism of Sioc145 action on insulin secretion, we analyzed the effect of Sico145 in the presence of different PKC inhibitors. Rottlerin, GFX or Ro318220 were administered to Sioc145-treated INS-1E cells at 16.7 mM glucose concentration. The results showed that all three inhibitors reduced the insulinotropic effect of Sioc145 to different extents (Figure 5B). In addition, Sioc145 and PMA did not have synergistic effect in potentiating GSIS; and Sioc111 did not have any obvious effect on insulin secretion (Figure 5B), consistent with the results in H4-p58-YFP cells (Figure 1E). These data suggest that Sioc145 potentiated insulin secretion through activating PKC.

To elucidate why Sioc145 and PMA had different glucose dependency in promoting insulin secretion, we analyzed their performances in activating PKC isoforms. Since the translocation of PKCs from cytosol to cell membrane has generally been considered as the hallmark of PKC activation [19, 20], we analyzed the translocation patterns of different PKC isoforms under the Sioc145 or PMA treatments. The immunoblotting
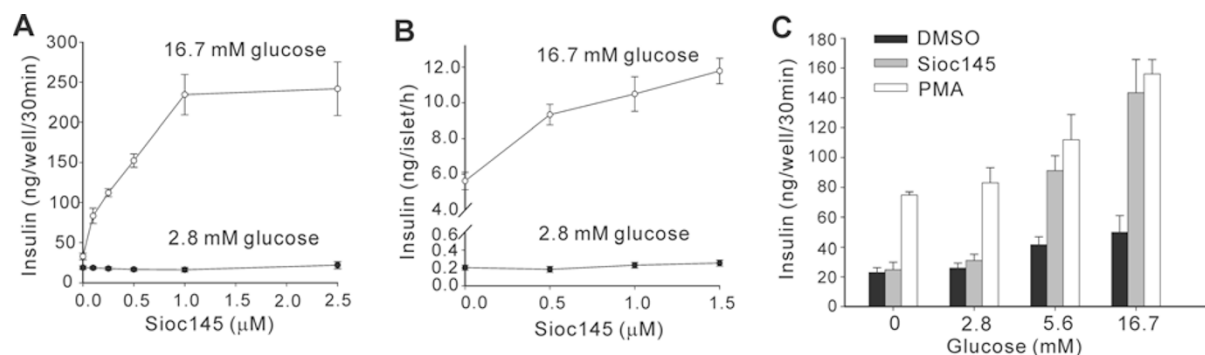

Figure 4 Sioc145 potentiates insulin secretion in a glucose-dependent manner. **(A)** Insulin secretion from INS-1E cells stimulated with either 2.8 mM or 16.7 mM glucose for 30 min in the presence of Sioc145 at indicated concentrations. **(B)** Dose-response of Sioc145 in rat pancreatic islets stimulated with 2.8 mM or 16.7 mM glucose concentration (see Materials and Methods). **(C)** Insulin secretion in response to various concentrations of glucose was examined in the presence of 1 μM Sioc145 or 0.05 μM PMA in INS-1E cells. Error bars represent SEM from three independent experiments.
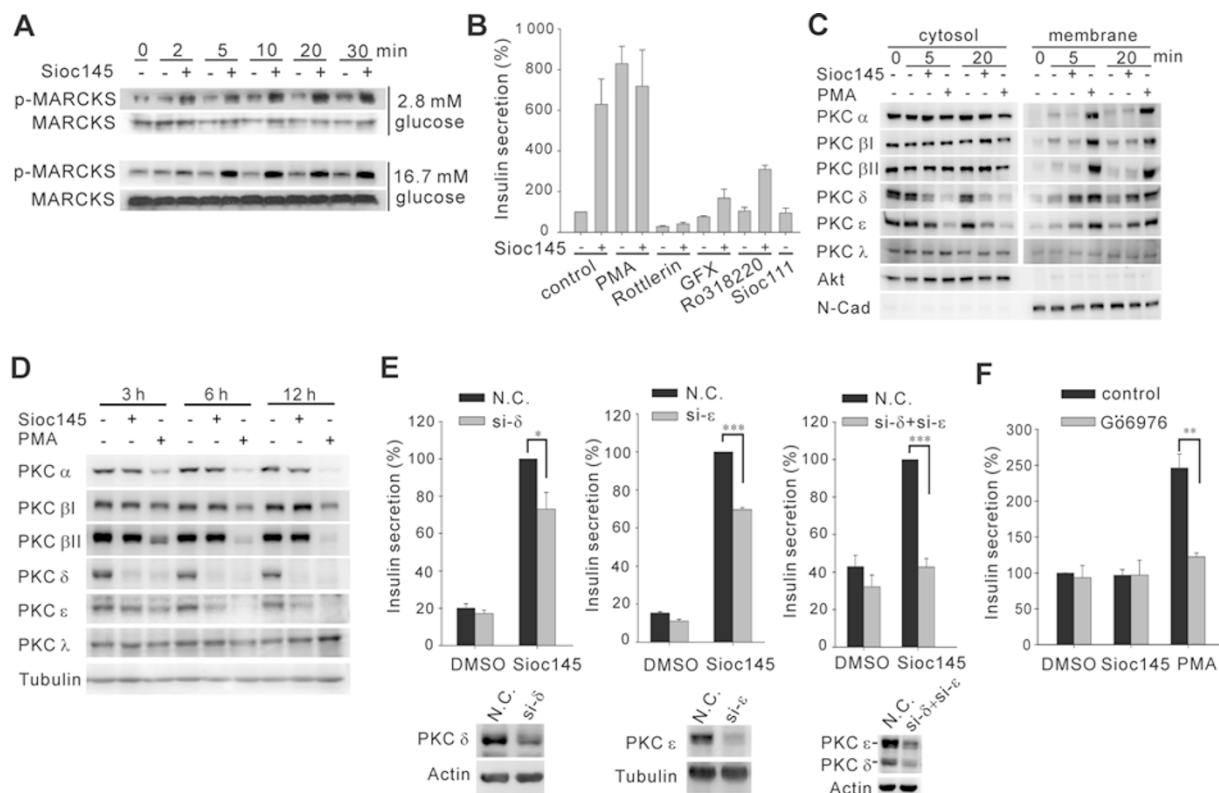
Figure 5 Selective activation of nPKCs is required for insulinotropic action of Sioc145. **(A)** MARCKS phosphorylation in 2.8 mM or 16.7 mM glucose was analyzed in INS-1E cells treated with 1 μM Sioc145 for indicated time points. **(B)** 16.7 mM glucose-stimulated insulin secretion from INS-1E cells treated with Sioc145 (1 μM) alone or together with indicated compounds: PMA, 0.05 μM; Rottlerin, 5 μM; GFX, 2.5 μM; Ro318220, 2 μM. Data are normalized to that of DMSO-treated cells which is set as 100%. **(C)** The translocation of PKC isoforms in INS-1E cells treated with Sioc145 (1 μM) or PMA (0.05 μM). N-cadherin (N-cad) and Akt were used as membrane and cytosol markers, respectively. **(D)** Effect of prolonged treatment with Sioc145 (1 μM) or PMA (0.05 μM) on protein levels of PKC isoforms. **(E)** Insulin secretion in PKC δ and/or ε downregulated cells. Cells transfected with indicated siRNA(s) were stimulated with DMSO or 1 μM Sioc145 at 16.7 mM glucose concentration. Insulin secretion from Sioc145-treated control cells (N.C.) were set as 100%. **(F)** Gö6976 abolished PMA-induced secretion. INS-1E cells treated with Sioc145 (1 μM) or PMA (0.05 μM) were co-treated with Gö6976 (1 μM) for 30 min in 2.8 mM glucose. Error bars represent SEM from at least three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

results showed that the expression of three cPKCs (α, βI and βII), two nPKCs (δ and ε) and one atypical PKC (λ) was detectable in INS-1E cells (Figure 5C). Interestingly, only nPKCs demonstrated membrane translocation in Sioc145-treated cells, whereas the treatment with PMA promoted the membrane translocation of both cPKCs and nPKCs (Figure 5C). Both Sioc145 and PMA did not affect the localization of PKC λ, which could not bind PMA [21].

It was reported that chronic activation of PKCs resulted in the downregulation of their protein levels [20]. Consistent with the translocation/activation data, nPKCs were downregulated after prolonged Sioc145 treatment, whereas Sioc145 had little effect on the levels of cPKCs, which were significantly downregulated in the PMA-treated cells (Figure 5D). Collectively, the results of membrane translocation and downregulation of PKCs indicate that Sioc145 selectively activates nPKCs in INS-1E cells.

To further confirm the involvement of nPKCs (PKC δ and ε) in the insulinotropic effect of Sioc145, we conducted RNAi-mediated loss-of-function experiments with chemically synthesized siRNAs (si-δ, si-ε and si-ε-2) and lentivirus-mediated transfection of shRNAs (sh-δ-1 and sh-δ-2; Figure 5E and Supplementary information, Figure S4). The results showed that the downregulation of either PKC δ or PKC ε by RNAi approach resulted in a significant inhibition of the insulinotropic effect of Sioc145. In addition, the PKC δ and ε double knockdown synergistically inhibited Sioc145 insulino-

tropic effect. Taken together, we conclude that Sioc145 selectively activates PKC δ and PKC ε, which are both required for its insulinotropic action.

Since cPKCs were activated by PMA but not by Sioc145, we speculated that the activation of cPKCs is required for PMA to trigger insulin secretion under low glucose conditions. The cPKCs inhibitor Gö6976 abolished PMA-triggered insulin secretion under the 2.8 mM glucose condition (Figure 5F), suggesting that cPKCs play a critical role in mediating PMA-induced glucose-independent secretion of insulin. Taken together, the difference in specificity of activating different PKC isoforms might explain the difference in the glucose dependency between Sioc145 and PMA in promoting insulin secretion.

Cell membrane depolarization is a prerequisite for insulinotropic effect of Sioc145

Cell membrane depolarization achieved via K_{ATP} channel inhibition is known as an upstream event for insulin secretion in the triggering pathway [1]. Therefore, we examined the effects of Sioc145 and PMA on cell membrane depolarization. The results showed that the PMA treatment significantly depolarized the cells in the presence of 2.8 mM glucose, which was inhibited by Gö6976, whereas the Sioc145 treatment did not lead to depolarization under the same concentration of glucose (Figure 6A).

We employed diazoxide (DZX), an agonist of K_{ATP} channels, to further characterize the role of cell membrane depolarization in the insulinotropic actions of Sioc145 and PMA. The results showed that DZX abolished the insulinotropic effect of Sioc145 under 16.7 mM glucose stimulation, but did not abolish that of PMA in both high and low glucose concentrations (Figure 6B). In

the presence of 30 mM KCl, which depolarizes cells [4], Sioc145 enhanced insulin secretion in both 2.8 mM and 16.7 mM glucose concentrations, even in the presence of DZX (Figure 6B). Insulin secretion stimulated by high glucose in the presence of KCl and DZX reflects the effect of glucose in the amplifying pathway [22]. Taken together, these data indicate that Sioc145 regulates insulin secretion in the amplifying pathway downstream of K_{ATP} channels, and that the cell membrane depolarization is a prerequisite for Sioc145 to execute its insulinotropic action.

Characterization of secretion kinetics and insulin granule distributions in Sioc145-treated pancreatic islets

GSIS in pancreatic islets is biphasic: a transient first phase elicited by the triggering pathway, followed by a gradually increasing but sustained second phase elicited by the amplifying pathway acting in synergy with the triggering pathway [1]. We analyzed the kinetics of insulin secretion and the distribution of insulin granules of isolated rat pancreatic islets treated with Sioc145. Interestingly, a pretreatment with Sioc145 significantly enhanced the second phase of GSIS (Figure 7A). Morphologically, the treatment with Sioc145 markedly increased the density of granules close to the plasma membrane (Figure 7B and 7C), while the average density of granules in cytoplasm was not altered significantly (Figure 7D). These results support the above conclusion that Sioc145 does not act on the initial triggering pathway of insulin secretion.

Sioc145 treatment increases plasma insulin levels in diabetic rats

To test the insulinotropic action of Sioc145 *in vivo*, we analyzed the profile of insulin secretion and glucose
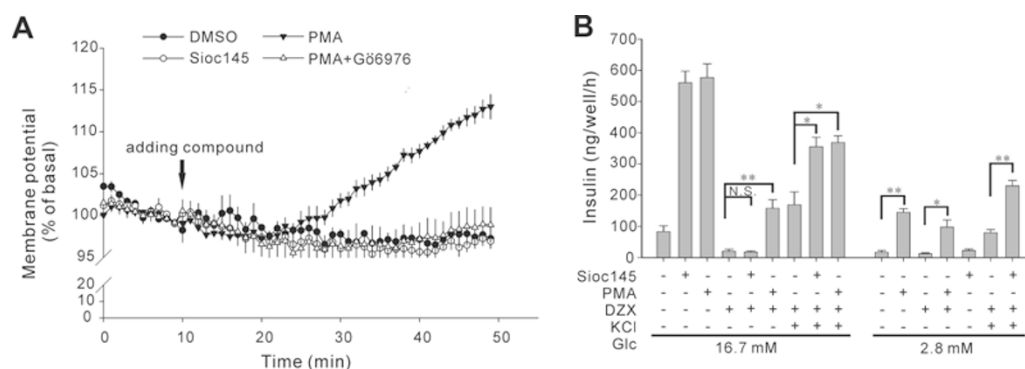

Figure 6 Cell membrane depolarization is a prerequisite for insulinotropic effect of Sioc145. **(A)** Cell membrane potential was monitored as bisoxonol fluorescence in INS-1E cells stimulated with Sioc145 (1 μM), PMA (0.05 μM) or PMA plus Gö6976 (1 μM), in the presence of 2.8 mM glucose. Arrow indicates the time point when indicated compounds were added. **(B)** INS-1E cells were treated with indicated combinations of Sioc145 (1 μM), PMA (0.05 μM), DZX (250 μM) and KCl (30 mM), in the presence of 2.8 mM or 16.7 mM glucose for 1 h. Data are means ± SEM from three independent experiments. N.S., not significant; *$P < 0.05$; **$P < 0.01$.
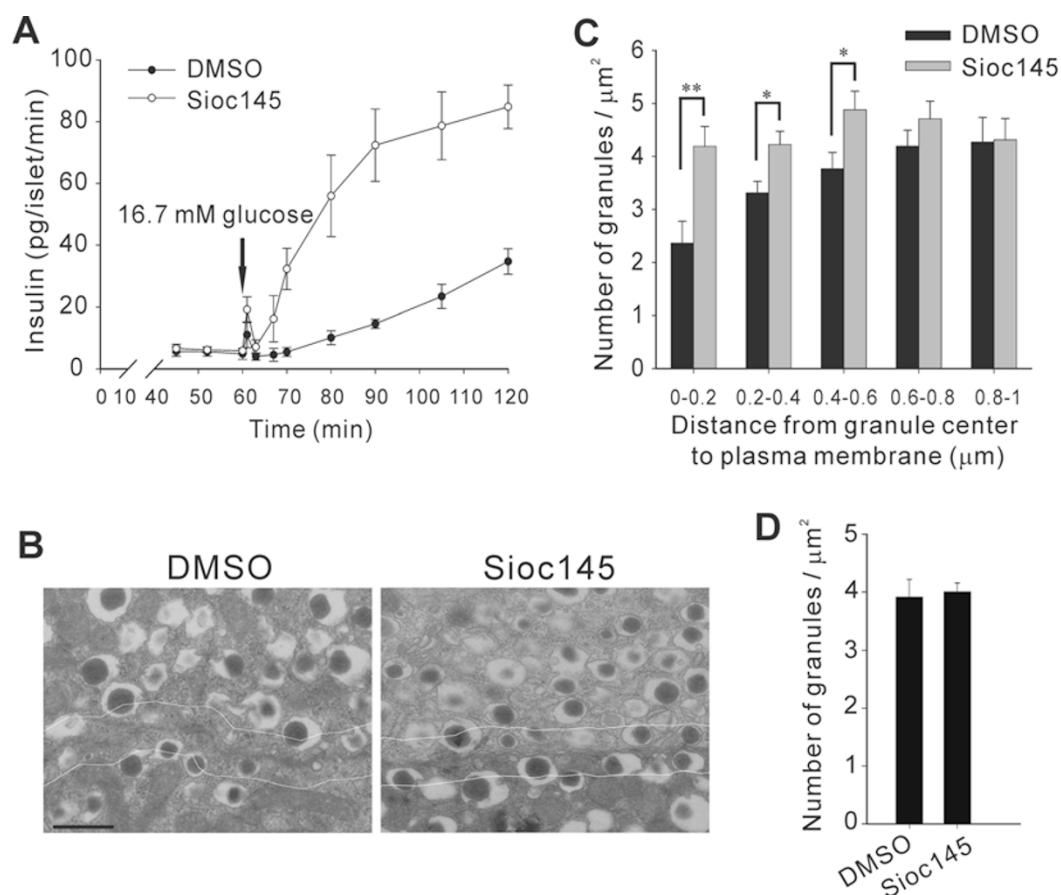
Figure 7 Characterization of secretion kinetics and insulin granule distribution in Sioc145-treated rat pancreatic islets. **(A)** Rat pancreatic islets pretreated with 5 μM Sioc145 or DMSO for 30 min were perifused with 16.7 mM glucose buffer for 1 h, and insulin secretion was measured at indicated time points (see Materials and Methods). **(B)** Islets were treated with DMSO or 5 μM Sioc145 for 30 min, followed by a 60 min equilibration in 2.8 mM glucose buffer. Representative electron micrographs of islet β-cell sections are shown. Scale bar, 0.5 μm. White lines indicate a distance of 0.2 μm from the plasma membrane. **(C)** Density of insulin granules located in 0.2 μm concentric shells within the first 1 μm area from the plasma membrane. Granules located in the single section were categorized according to their distance from the granule center to the plasma membrane. **(D)** The average density of granules in cytoplasm. The area of cytoplasm is calculated as the cell area minus the nuclear area. Error bars represent SEM from three independent experiments **(A)** or 10 individual β-cells derived from four rats **(C, D)**. *$P < 0.05$; **$P < 0.01$.

tolerance in Goto-Kakizaki (GK) rats, a model for type 2 diabetes, under Sioc145 treatment. Sioc145 treatment (0.414 mg/kg) led to a significant increase in the levels of serum insulin in response to glucose administration (Figure 8A), whereas the improvement in glucose tolerance was relatively mild (Figure 8B). In addition, to analyze whether Sioc145 induces hypoglycemia, we treated the fasted Wistar rats with Sioc145 or tolbutamide, a sulfonylurea derivative drug used in the management of type 2 diabetic patients. While the treatment with tolbutamide could further decrease the levels of serum glucose under the fasting condition, Sioc145 had no hypoglycemic effect in fasting rats at the dose of 0.414 mg/kg during 2 h

of observation (Supplementary information, Figure S5). Thus, consistent with its insulinotropic effect *in vitro*, Sioc145 treatment could increase plasma insulin levels *in vivo* in response to glucose loading.

Discussion

Sioc145 treatment might generate a "competent-for-secretion" state of insulin-secreting cells

In the present study, we show that the action of Sioc145 is downstream of K_{ATP} channels and, in contrast to PMA, does not lead to membrane depolarization (Figure 6). Since membrane depolarization is an early triggering
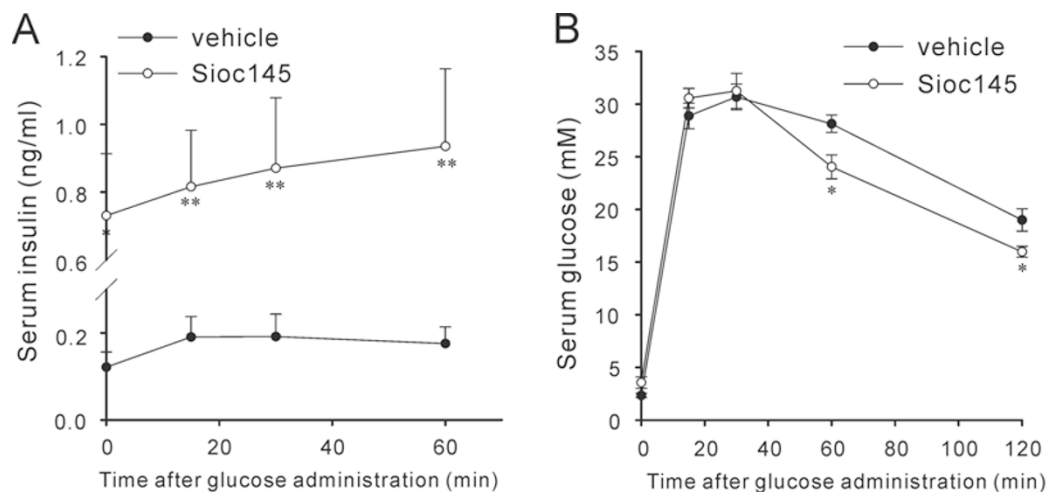
Figure 8 Effect of Sioc145 in diabetic GK rats. Fasted GK rats were administered intraperitoneally with Sioc145 (0.414 mg/kg) or vehicle 2 h before glucose loading (2 g/kg, i.p). Sera were collected before or at indicated time points after glucose administration to measure insulin levels **(A)** and glucose levels **(B)**. $n \geq 5$. Data shown are means ± SEM (*$P < 0.05$; **$P < 0.01$ vs vehicle; Student's t-test).

event for insulin secretion, this observation potentially explains why the treatment with Sioc145 *per se* does not initiate insulin secretion. In insulin-secreting cells, the ability of Sioc145 to promote insulin secretion requires the presence of high glucose. Since Sioc145 treatment under low glucose conditions activates PKC and increases the number of insulin granules close to the plasma membrane, we speculate that Sioc145 prepares cells to a "competent-for-secretion" state without triggering secretion. Only when cells are depolarized by other stimuli such as glucose, Sioc145 can execute its insulinotropic actions. This hypothesis is supported by our observation that Sioc145 pretreatment sensitized INS-1E cells to respond to later Sioc145-free glucose stimulation (data not shown). This is also consistent with a reported role of PKC in mediating time-dependent potentiation of insulin secretion [23].

The lack of insulinotropic effect of Sioc145 under low glucose conditions is most probably correlated with its inability to activate cPKCs. cPKCs are required for PMA-induced insulin secretion because Gö6976 could totally inhibit PMA-induced secretion [6, 24] and membrane depolarization. In addition, PMA-triggered insulin secretion is significantly inhibited by overexpressing kinase-dead PKC α, whereas it is increased by overexpressing wild-type PKC α [24]. The inability of Sioc145 to depolarize the membrane provides direct evidence that PKC δ and ε are downstream regulators of insulin secretion.

The amplifying pathway is the major action target of Sioc145. Sioc145 potentiates GSIS in pancreatic islets via enhancing the second phase of secretion. This is consistent with a previous report that islets from PKC δ knockout mice exhibited normal Ca²⁺ influx but decreased second phase of GSIS [7]. However, Biden's lab reported recently that PKC ε deletion enhances GSIS via the amplifying pathway from mice islets, but this occurs selectively after prolonged lipid exposure [25, 26]. The seeming discrepancy between this report and our observations implies that prolonged or acute PKC ε activation might have different effects on β-cell function.

Our study suggests that Sioc145 is a novel glucose-dependent insulinotropic compound due to its selective activation of nPKCs without activating cPKCs. Previous gain-of-function studies on the mechanisms by which PKC regulates secretion were mostly based on experiments applying PMA as a PKC activator. Since cPKCs and nPKCs play quite different roles in regulating insulin secretion as suggested by our data, further studies on the actions of Sioc145 in comparison with that of PMA may offer useful clues in understanding different functions of PKC isoforms, and shed light on developing glucose concentration-dependent insulinotropic drugs.

The potential roles of nPKCs in drug targeting and therapeutics of type 2 diabetes

It has been reported that several PKC isoforms are expressed in pancreatic β-cells [5-8], and different PKC isoforms are speculated to play different roles [9, 10]. The complexity of *in vivo* roles of PKCs in insulin secretion was highlighted by the finding that the treatment with either a general PKC inhibitor, bisindolylmaleim-

ide, or a structurally unrelated PKC inhibitory peptide PKC19-31 restored insulin secretion efficacy in GK rats, whereas the general PKC activator, PMA, did not generate the opposite results [27]. In the present study, Sioc145 treatment significantly increased plasma insulin levels in GK rats (Figure 8A), suggesting that Sioc145 might be able to selectively activate nPKCs in β-cells *in vivo*, and the activation of nPKCs in GK rats could promote insulin secretion under the glucose stimulation. This is supported by a previous report that PKC δ knockout mice demonstrated impaired glucose tolerance due to impaired GSIS [7]. Our results suggest that nPKCs could be used as potential drug targets for regulating glucose-dependent insulin secretion.

It should be noted that although the serum insulin levels increased significantly in Sioc145-treated GK rats, the improvement in glucose tolerance was relatively mild (Figure 8B). There are two potential explanations for this result. First, since GK rat is a typical type 2 diabetic model, and these animals had already suffered from insulin resistance besides β-cell dysfunctions [28], the Sioc145 treatment might not be able to improve insulin sensitivity in the peripheral tissues of GK rats, which led to the relatively minor improvement in glucose tolerance. Second, the Sioc145 treatment, while promoting insulin secretion in pancreas of GK rats, might increase insulin resistance in the muscle or other peripheral tissues, since insulin resistance has been reported to be associated with PKC activation, especially activation of novel PKCs [29-32]. It is known that activation of novel PKC isoforms is involved in the impairment of insulin sensitivity induced by free fatty acids (FFAs; reviewed in [33]). In addition, diet containing peptide inhibitors of PKC ε protected muscle IRS-1 from PKC ε-induced serine phosphorylation, abrogating insulin resistance in a rat model (*Psammomys*) of type 2 diabetes [34].

Taken together, the complex effects of nPKCs on glucose tolerance of diabetic animal model uncovered in this study and previous ones suggest that a simple activation of nPKCs would not work well as a therapeutic strategy. To do so, one needs to find a way to de-couple the two opposite consequences of nPKC activation, i.e., enhanced insulin secretion and impaired insulin sensitivity. This perhaps could be achieved by more selective activation of particular PKC isoforms or by finding a way to block the inhibitory effect of nPKC activation on insulin sensitivity.

Materials and Methods

Plasmids, siRNAs and antibodies

Plasmid expressing p58-YFP was provided by Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, USA). Specific siRNA oligonucleotides against PKC δ and ε were synthesized by Shanghai GenePharma Co. or from Ambion Inc. The sequences are as follows: si-δ, 5′-GCAACGCUGCCAUCCA-UAAUU-3′; si-ε, 5′-GCCCCUAAAGACAAUGAAGTT-3′; si-ε-2, 5′-AGCCGAGAAUUAAAACCAATT-3′. To establish stable PKC δ knockdown cells, a retroviral-based siRNA expression system was used. The target sequences of PKC δ, sh-δ-1, 5′-GCAGT-ATTTC CTGGAGGAT-3′; sh-δ-2, 5′-CCTTGAGAACTTCAC-CTTC-3′, were cloned into pSIREN-Retro-Q vector and shuttled into Knockout™ RNAi Systems (Clontech Laboratories Inc.). A sequence of Shuffle: 5′-AACTAGAGCCACAACTACC-3′ was used as control. The following primary antibodies were used: anti-GM130 (BD Biosciences), anti-MARCKS (Novus), anti-phospho-MARCKS (Cell Signaling), anti-PKC α, δ, ε, λ (BD Biosciences), anti-PKC βI, βII (Santa Cruz Biotechnology), anti-Akt (Cell Signaling), anti-N-Cadherin (BD Biosciences), anti-actin or anti-tubulin (Sigma).

Cell culture and transfection

H4 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine at 37 °C, 5% CO_2. Rat insulinoma INS-1E cells, kindly provided by Drs CB Wollheim and P Maechler (University Medical Center, Switzerland), were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin/streptomycin, as described previously [35]. Transfection was done using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Stable H4 cell lines expressing p58-YFP or SEAP were established in the presence of 1 mg/ml G418 or Zeocin (Invitrogen). Stable knockdown of PKC δ was obtained by lentivirus-mediated transduction of short hairpins against PKC δ mRNA and cells were selected with puromycin (4 μg/ml).

Phenotypic screen and image analysis

H4-p58-YFP cells were plated in 96-well plates at 3 000 cells per well, and treated individually with compounds in the SIOC Compounds Library in duplication. The cells were examined using fluorescence microscopy (IX81, Olympus) after incubating with the compounds for 4 h, 8 h and 24 h. Images analysis was performed by high-throughput fluorescent microscopy as described previously [36].

Immunofluorescence and electron microscopy

Cells cultured on coverslips were washed with PBS, fixed in 3.8% paraformaldehyde for 20 min at room temperature. After blocking with 1% BSA in TBST, cells were incubated with GM130 antibody for 2 h, then with secondary antibody conjugated with Texas Red or FITC (Rockland Immunochemicals) for 1 h. DNA was stained with DAPI (1 μg/ml). Cells were imaged using a confocal microscopy LSM 510 (Carl Zeiss) with a 100× oil 1.35 NA objective. For electron microscopy, pretreated pancreatic islets or H4 cells were fixed in 2.5% glutaraldehyde, dehydrated and embedded. Ultrathin sections (90 nm) were cut, stained and analyzed by a JEM 1230 electron microscope (JEOL). To determine insulin granule distribution, cell area and distance of the granule center to the plasma membrane were measured using Image-Pro Plus 6.0.

Measurement of alkaline phosphatase secretion

The Great EscAPe SEAP assay was used following the manufacturer's instructions (Clontech) using the chemiluminescent substrate CSPD and signal was detected with a plate luminometer.

Pancreatic islets isolation and insulin secretion

Pancreatic islets were isolated from male Wistar rats (250-300 g) by collagenase digestion [37]. In static incubation experiments, size-matched five islets were grouped, and equilibrated with Krebs-Ringer bicarbonate (KRB) buffer containing 2.8 mM glucose for 1 h, then incubated with 2.8 mM glucose buffer in the presence of Sioc145 at indicated concentrations for another 1 h. Finally, the islets were stimulated with 2.8 mM or 16.7 mM glucose buffer in the continuous presence of indicated Sioc145 concentrations for 1 h. Supernatants in the last step were collected for insulin secretion assay. Three independent experiments were carried out in triplicates. KRB buffer is composed of 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 0.1% BSA, pH 7.4. In perifusion studies, size-matched 50 islets were loaded onto each column immediately after the isolation as described previously [38]. Islets were perifused with KRB buffer at a flow rate of 0.6 ml/min for 30 min in the presence of 2.8 mM glucose for equilibration, then with 2.8 mM glucose buffer supplemented with Sioc145 or DMSO for 30 min. Next, the islets were perifused in 2.8 mM glucose buffer for 1 h, finally stimulated with 16.7 mM glucose for 1 h. Perifusate solutions were gassed with 95% O_2/5% CO_2 and maintained at 37 °C.

For insulin secretion from INS-1E cells, the cells were preincubated for 30 min at 37 °C in KRB buffer supplemented with 10 mM HEPES (KRBH buffer) and 2.8 mM glucose. The cells were then incubated in KRBH buffer supplemented with indicated stimuli for 30 min unless otherwise indicated. When inhibitors were used, they were added 30 min before the stimuli. Insulin in the media was measured by rat insulin ELISA kit (Mercodia, Sweden).

PKC translocation and western blotting assays

After preincubation in KRBH buffer containing 2.8 mM glucose for 1 h, INS-1E cells were stimulated with indicated compounds. The cells were fractionated following the protocol described previously [5]. Briefly, the cells were scraped with cold homogenization buffer (10 mM Tris, pH 7.4, 4.5 mM EDTA, 2.5 mM EGTA, 2.3 mM β-mercaptoethanol and protease inhibitors cocktail (Sigma)) and sonicated on ice. The resulting homogenate was centrifuged at 100 000× g for 30 min to separate the cytosolic fraction from the particular fraction. The pellet was briefly sonicated in homogenization buffer containing 0.5% Triton X-100 and extracted for 30 min at 4 °C. The resulting homogenate was centrifuged at 16 000× g for 10 min, and the supernatants were taken as membrane fraction. Equal volume of each sample was subjected to western blotting using indicated antibodies, and signals were detected with Immobilon chemiluminescent HRP substrate (Millipore).

Membrane potential analysis

Cell membrane potential was monitored using 2 μM of the fluorescent probe $DiBAC_4(3)$ (Dojindo) in thermostated (37 °C) plate reader fluorimeter (Berthold Technologies). Filters used for excitation and emission were 485 nm and 535 nm, respectively. Gö6976 was added 30 min before the time lapse reading.

Statistics

Statistical analysis was performed using Sigmaplot and Microsoft Excel software. The level of significance for the difference between data sets was assessed using Student's *t*-test.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)