

# Potential of Sulfonylurea Action by an EPAC-selective cAMP Analog in INS-1 Cells: Comparison of Tolbutamide and Gliclazide and a Potential Role for EPAC Activation of a 2-APB-sensitive $\text{Ca}^{2+}$ Influx<sup>□</sup>

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## ABSTRACT

Tolbutamide and gliclazide block the  $\text{K}_{\text{ATP}}$  channel  $\text{K}_{\text{ir}}6.2/\text{Sur}1$ , causing membrane depolarization and stimulating insulin secretion in pancreatic beta cells. We examined the ability of the EPAC-selective cAMP analog 8-pCPT-2'-O-Me-cAMP-AM to potentiate the action of these drugs and the mechanism that might account for it. Insulin secretion stimulated by both 200  $\mu\text{M}$  tolbutamide and 20  $\mu\text{M}$  gliclazide, concentrations that had equivalent effects on membrane potential, was inhibited by thapsigargin (1  $\mu\text{M}$ ) or the L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (2  $\mu\text{M}$ ) and was potentiated by 8-pCPT-2'-O-Me-cAMP-AM at concentrations  $\geq 2 \mu\text{M}$  in INS-1 cells.  $\text{Ca}^{2+}$  transients stimulated by either tolbutamide or gliclazide were inhibited by thapsigargin or nifedipine and were significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at 5  $\mu\text{M}$  but not 1  $\mu\text{M}$ . Both tolbutamide and gliclazide stimulated phospholipase C activity; however, only gliclazide did so independently of its

activity at  $\text{K}_{\text{ATP}}$  channels, and this activity was partially inhibited by pertussis toxin. 8-pCPT-2'-O-Me-cAMP-AM alone (5  $\mu\text{M}$ ) did not stimulate insulin secretion, but did increase intracellular  $\text{Ca}^{2+}$  concentration significantly, and this activity was inhibited by 25  $\mu\text{M}$  2-aminoethoxydiphenylborate (2-APB) or the removal of extracellular  $\text{Ca}^{2+}$ . 8-pCPT-2'-O-Me-cAMP-AM potentiation of insulin secretion stimulated by tolbutamide was markedly inhibited by 2-APB (25  $\mu\text{M}$ ) and enhanced by the PKC inhibitor bisindolylmaleimide I (1  $\mu\text{M}$ ). Our data demonstrate that the actions of both tolbutamide and gliclazide are strongly potentiated by 8-pCPT-2'-O-Me-cAMP-AM, that gliclazide can stimulate phospholipase C activity via a partially pertussis toxin-sensitive mechanism, and that 8-pCPT-2'-O-Me-cAMP-AM potentiation of tolbutamide action may involve activation of a 2-APB-sensitive  $\text{Ca}^{2+}$  influx.

## Introduction

Sulfonylurea drugs, such as tolbutamide and gliclazide, have been used for decades to stimulate insulin secretion and decrease blood glucose levels in type 2 patients with diabetes with insufficient endogenous insulin secretion (Groop, 1992). These drugs bypass the metabolic steps required for glucose-stimulated insulin secretion and mimic the effect of an increase in ATP/ADP ratio (Rorsman and Trube, 1985; Dunne and Petersen, 1986) on the  $\text{K}_{\text{ATP}}$  channel composed of the

$\text{K}_{\text{ir}}6.2$  and SUR1 subunits (Babenko et al., 1998). Binding of sulfonylureas to the  $\text{K}_{\text{ATP}}$  channel favors channel closing (Schmid-Antomarchi et al., 1987) and subsequent membrane depolarization because open  $\text{K}_{\text{ATP}}$  channels permit the efflux of  $\text{K}^{+}$  ions and the maintenance of the membrane potential close to the equilibrium potential for  $\text{K}^{+}$  (Cook and Hales, 1984). Depolarization of the membrane potential of pancreatic beta cells leads to the activation of several varieties of voltage-gated  $\text{Ca}^{2+}$  channels (Horvath et al., 1998), including the L-type channels  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  (Seino et al., 1992). The subsequent rise in intracellular  $\text{Ca}^{2+}$  triggers the exocytosis of insulin (Wollheim et al., 1975) via dense core vesicles that are shuttled to (Van Obberghen et al., 1975), and eventually fuse with, the plasma membrane (Barg et al., 2001; Shibasaki et al., 2007).

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**ABBREVIATIONS:** 2-APB, 2-aminoethoxydiphenylborate; BIS, bisindolylmaleimide I; Carbachol-2, (trimethylazaniumyl)ethyl carbamate chloride; diazoxide, 7-chloro-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dione; EPAC, exchange protein directly activated by cAMP; ESCA, EPAC-selective cAMP analog; gliclazide, 1-[(4-methylbenzene)sulfonyl]-3-[octahydrocyclopenta[c]pyrrol-2-yl]urea; IP<sub>1</sub>, inositol monophosphate; nifedipine, 3-[2-[benzyl(methyl)amino]ethyl] 5-methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate; 8-pCPT, 2'-O-Me-cAMP-AM-*para*-chlorophenylthio-2'-O-methyladenosine-3'-5'-cyclic monophosphate, acetoxymethyl ester; Rp-cAMPs, adenosine-3'-5'-cyclic monophosphorothioate, Rp-isomer; SERCA, sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; tolbutamide, 3-butyl-1-[(4-methylbenzene)sulfonyl]urea; TRP, transient receptor potential; U73122, 1-[6-[[[(17 $\beta$ )-3-methoxyestra-1,3,5(10)-trien-17-yl]- amino]hexyl]-1H-pyrrole-2,5-dione; U73343, 1-[6-[[[(17 $\beta$ )-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione.

Recently, exchange protein directly activated by cAMP 2 (EPAC2) was identified as a novel cellular target for some members of the sulfonylurea drug class (Zhang et al., 2009). EPAC2 is a guanine nucleotide exchange factor (GEF) for the low molecular weight GTP binding protein Rap1 (Kawasaki et al., 1998). In the cAMP-bound form, EPAC2 accelerates the exchange of GDP for GTP at the nucleotide binding site of Rap1 (Kawasaki et al., 1998). Along with the activation of protein kinase A (Ding and Gromada, 1997), activation of EPAC2 contributes to the potentiation of glucose-stimulated insulin secretion by incretin hormones that stimulate adenylyl cyclase activity, such as GLP-1 (Leech et al., 2010a). The GTP-bound form of Rap1 is implicated in the activation of phospholipase C- $\epsilon$  (Dzhura et al., 2010) in pancreatic beta cells, in augmenting the number of insulin granules in close proximity to sites of exocytosis on the plasma membrane (Shibasaki et al., 2007), and in the priming of insulin granules for exocytosis (Eliasson et al., 2003).

EPAC2 is thought to exist in a signaling complex with several other peripheral membrane proteins. EPAC2 interacts directly with SUR1 (Shibasaki et al., 2004) and with the CAZ protein piccolo (Fenster et al., 2000), which forms a dimer with the related protein RIM2 (Wang et al., 2000), in a  $\text{Ca}^{2+}$ -dependent manner (Fujimoto et al., 2002). In addition, the voltage-gated  $\text{Ca}^{2+}$  channel  $\text{Ca}_v1.2$  interacts with the C2 domains of RIM2 and piccolo via the intracellular II-III loop (Shibasaki et al., 2004). This signaling complex of scaffolding proteins (RIM2, piccolo, cAMP effector (EPAC2), and ion channel subunits (SUR1,  $\text{Ca}_v1.2$ ) is of special significance because membrane depolarization-dependent calcium influx via  $\text{Ca}_v1.2$  channels has been implicated in triggering  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the ER in pancreatic beta cells (Liu et al., 2006), a process that is amplified by cAMP, at least in part, through EPAC2 (Kang et al., 2003; Liu et al., 2006). The role of intrinsic EPAC2 stimulation by sulfonylureas on insulin secretion or the underlying  $\text{Ca}^{2+}$  dynamics in beta cells is currently unknown.

Tolbutamide and gliclazide, which both stimulate insulin secretion from pancreatic beta cells by block of  $\text{K}_{\text{ATP}}$  channels, differ in their ability to bind and activate EPAC2. Zhang et al. (2009), reported that tolbutamide at concentrations  $\geq 30 \mu\text{M}$  significantly activated Rap1 in an EPAC2-dependent manner, whereas gliclazide at concentrations up to 30 nM did not. We used electrophysiological analysis to identify concentrations of tolbutamide and gliclazide that had equivalent effects on  $\text{K}_{\text{ATP}}$  channel activity both in terms of block of current and induction of membrane depolarization. We then used these concentrations of tolbutamide and gliclazide to compare stimulation of insulin secretion, stimulation of increases in intracellular  $\text{Ca}^{2+}$  concentration, and potentiation of these activities by the EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP-AM in INS-1 cells. Because activation of phospholipase C is a consequence of EPAC2 activation in pancreatic beta cells, we also assayed phospholipase C activation by tolbutamide and gliclazide in the absence and presence of diazoxide to prevent membrane depolarization and  $\text{Ca}^{2+}$  influx via voltage-gated  $\text{Ca}^{2+}$  channels. To gain insight into the mechanism whereby EPAC activation potentiates sulfonylurea action, we assayed 8-pCPT-2'-O-Me-cAMP-AM modulation of phospholipase C activity and intracellular  $\text{Ca}^{2+}$  concentration in INS-1 cells.

## Materials and Methods

**Chemicals.** U73122 and U73343 were from Tocris (Minneapolis, MN). 8-pCPT-2'-O-Me-cAMP and 8-pCPT-2'-O-Me-cAMP-AM were from Biolog (Bremen, Germany). Rp-cAMP was from Santa Cruz Biotechnology (Santa Cruz, CA). Ryanodine was from Calbiochem (San Diego, CA). All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** INS-1 cells were grown in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 11 mg/ml sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 50  $\mu\text{M}/\beta$ -mercaptoethanol at 37°C, 5%  $\text{CO}_2$ .

**Electrophysiological Assay.** Electrophysiological measurements in INS-1 cells were recorded at room temperature using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and filtered at 1 kHz (six-pole Bessel filter, -3 dB). Electrodes were pulled from borosilicate glass (VWR, West Chester, PA) and fire-polished to resistances of 2–5 M $\Omega$ . For current-clamp experiments and voltage-clamp experiments to measure  $\text{K}_{\text{ATP}}$  channel current, the intracellular solution contained (in mM) 90  $\text{K}_2\text{SO}_4$ , 10 NaCl, 1  $\text{MgCl}_2$ , 1.1 EGTA, 0.1  $\text{CaCl}_2$ , 5 HEPES, 0.3 ATP, 0.2 GTP. The extracellular solution used for measuring  $\text{K}_{\text{ATP}}$  currents contained (in mM) 138 NaCl, 5.6 KCl, 11.1 glucose, 10 HEPES, 1.2  $\text{MgCl}_2$ , 2.6  $\text{CaCl}_2$ , and the same solution with the addition of 2.5 mM glucose was used in current-clamp experiments. The pH of solutions was adjusted to 7.4 with NaOH, and the osmolality was adjusted to 290–300 mOsm. Whole cell  $\text{K}_{\text{ATP}}$  currents were elicited by 1.3-s steps of  $\pm 20$  mV from a holding potential of -70 mV. Data were acquired at a sampling frequency of 1 kHz. The membrane potential of INS-1 cells was measured using gap-free recording at a sampling frequency of 1 kHz in  $I = 0$  current-clamp mode. The  $\text{K}_{\text{ATP}}$  channel opener, diazoxide (300  $\mu\text{M}$ ), was transiently applied to maximally open  $\text{K}_{\text{ATP}}$  channels, before application of tolbutamide or gliclazide. Tolbutamide and gliclazide solutions were prepared from stocks dissolved in 0.1 M NaOH made fresh daily. Diazoxide solutions were prepared from stocks dissolved in DMSO. For recordings of voltage-gated  $\text{Ca}^{2+}$  channel currents, the bath solution contained (in mM) 150 Tris, 10  $\text{BaCl}_2$ , 4  $\text{MgCl}_2$ . The intracellular solution contained (in mM) 130 *N*-methyl-D-glucamine, 10 EGTA, 60 HEPES, 2 ATP, and 1  $\text{MgCl}_2$ . The pH of both solutions was adjusted to 7.3 with methanesulfonic acid, and the osmolality was corrected to 290–300 mOsm. Current-voltage relationship data were collected by applying 100-ms test depolarizations from -50 to +60 mV in 10-mV increments from a holding potential of -70 mV. Data were acquired at a sampling frequency of 10 kHz and filtered at 1 kHz.

**Insulin Secretion Assay.** INS-1 cells were plated in 24-well tissue culture plate at 50–70% confluency and incubated overnight in RPMI medium supplemented as described above at 37°C, 5%  $\text{CO}_2$ . Immediately before the assay, cells were washed twice in phosphate-buffered saline [PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl (pH 7.4)], and preincubated with a modified Krebs-Ringer buffer [KRBH: 134 mM NaCl, 3.5 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgSO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 5 mM  $\text{NaHCO}_3$ , 10 mM HEPES (pH 7.4)] supplemented with 0.05% fatty acid free BSA (KRBH buffer) alone or containing indicated concentrations of inhibitors for 30 min at 37°C, 5%  $\text{CO}_2$ . The buffer was decanted and replaced with fresh KRBH buffer alone (basal condition) or KRBH containing the indicated concentrations of sulfonylurea with or without inhibitors for 1 hour at 37°C, 5%  $\text{CO}_2$ . Secreted insulin was assayed using an ELISA for rat insulin (High-Range EIA kit, ALPCO Diagnostics, Salem, NH). Cells were lysed in 20 mM  $\text{Na}_2\text{HPO}_4$ , 150 mM NaCl, 0.1% Triton X-100, 800 nM aprotinin, 50  $\mu\text{M}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  pepstatin, 1 mM benzamide, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride, 10  $\mu\text{g}/\text{ml}$  calpain inhibitor I, and 10  $\mu\text{g}/\text{ml}$  calpain inhibitor II (pH 7.4), and cellular protein in each well was determined using the BCA assay (Thermo Scientific, Rockford, IL).

**Intracellular  $\text{Ca}^{2+}$  Assays.** INS-1 cells were plated at 100% confluency in black-walled 96 well plates (Corning Life Sciences,

Lowell, MA) in RPMI supplemented as described above, and incubated overnight at 37°C, 5% CO<sub>2</sub>. Cells were washed twice with PBS and incubated with 5 μM Fura-2/acetoxymethyl ester (Fura-2 AM; Molecular Probes, Eugene, OR) diluted in KRBH for 1 hour at 37°C, 5% CO<sub>2</sub>. The KRBH containing Fura-2 AM was then removed, and the cells were washed twice with KRBH and equilibrated for 30 min at 37°C, 5% CO<sub>2</sub> in the KRBH alone, or with indicated concentrations of inhibitors or 8-pCPT-2'-O-Me-cAMP-AM. When pertussis toxin was used, 25 ng/well was added to cells in 96-well plates approximately 18 hours before assay. Cells were stimulated by injection of the indicated concentration of sulfonylurea or 8-pCPT-2'-O-Me-cAMP-AM (or buffer control), and changes in intracellular Ca<sup>2+</sup> concentrations were measured by recording the ratio of fluorescence intensities at 508/20 nm resulting from excitation of Fura-2 at 340/11 nm or 380/20 nm (center/band pass) using a Synergy 4 multi-mode microplate reader (BioTek, Winooski, VT). For experiments injecting sulfonylureas, ratios were acquired every 0.7 s for 15 s before injection and 2 minutes after injection of stimuli. For experiments injecting 8-pCPT-2'-O-Me-cAMP-AM, ratios were acquired every 5 s for 2 minutes before injection and at least 8 minutes after injection. Data were corrected for any injection artifact by subtracting the change in fluorescence ratio measured in cells injected with KRBH alone.

**IP<sub>1</sub> Assays.** INS-1 cells were plated at approximately 65,000 cells/well in white 96-well tissue culture plates (PerkinElmer, Waltham, MA) in the presence of 2.5 mM glucose overnight at 37°C and 5% CO<sub>2</sub>. A prestimulation buffer was added to the cells for 1 hour before stimulation [10 mM HEPES, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 4.2 mM KCl, 146 mM NaCl (pH 7.4)]. The prestimulation buffer was decanted, and treatments were made in a stimulation buffer containing LiCl to block inositol phosphate degradation [10 mM HEPES, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 4.2 mM KCl, 146 mM NaCl, 5 mM LiCl (pH 7.4)]. Cells were incubated with treatments for 1 hour at 37°C, 5% CO<sub>2</sub>. When pertussis toxin was used, 25 ng/well was added to cells in 96-well plates approximately 18 hours before assay. IP<sub>1</sub> levels were measured using the IP-One Tb Homogeneous Time-Resolved Fluorescence Resonance Energy Transfer (HTRF-FRET) Kit from Cisbio (Bedford, MA) according to the manufacturer's instructions. The 620 nm/650 nm fluorescence ratio (excitation wavelength of 330 nm) was measured using a Synergy 4 fluorescent plate reader (BioTek). The concentration of IP<sub>1</sub> in each sample was determined by comparison of the 620 nm/650 nm ratio to those for a standard curve of IP<sub>1</sub> concentrations.

**Data Analysis.** Data were analyzed using SigmaPlot 11.0. Data are shown as means ± S.E. Statistical significance was determined using one-way analysis of variance and the Tukey's post hoc test unless otherwise indicated.  $P < 0.05$  was considered significant.

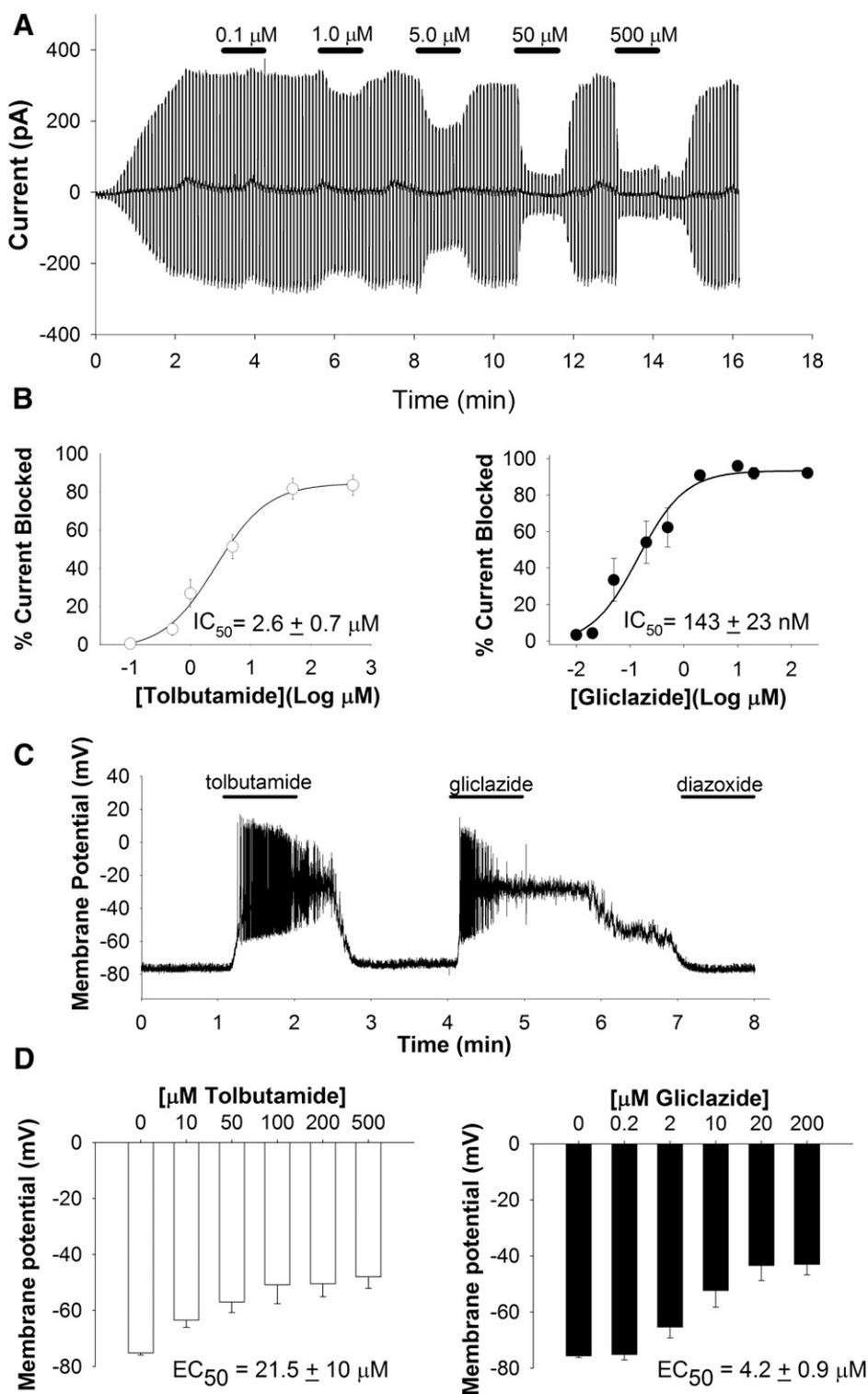
## Results

**Electrophysiological Characterization of Tolbutamide and Gliclazide Activity in INS-1 Cells.** Both tolbutamide and gliclazide bind to and block the K<sub>ATP</sub> channel in pancreatic beta cells. To compare the effects of these two drugs and determine whether tolbutamide might also activate EPAC2 in INS-1 cells, we determined the potency of K<sub>ATP</sub> channel block and the dose response curve for membrane depolarization in INS-1 cells (Fig. 1). In the whole cell voltage-clamp mode, an alternating voltage-step protocol (stepping to -50 mV or -90 mV from a holding potential of -70 mV) elicited inward (at -90 mV) and outward (at -50 mV) K<sup>+</sup> currents through K<sub>ATP</sub> channels (Fig. 1A). These currents were blocked by increasing concentrations of either sulfonylurea drug, as indicated by the decrease in current amplitude in both directions. Plots of the percent of current block at each of several different concentrations of either tolbutamide or gliclazide yielded dose response curves that were fit as

described in *Materials and Methods*. As expected, gliclazide was more potent in blocking of K<sub>ATP</sub> channel current than was tolbutamide (gliclazide IC<sub>50</sub> = 143 ± 23 nM; tolbutamide IC<sub>50</sub> = 2.6 ± 0.7 μM; Fig. 1B).

Because the block of K<sub>ATP</sub> channels leads to membrane depolarization in pancreatic beta cells, we also measured the dose dependence for the membrane depolarization induced by tolbutamide or gliclazide. In current-clamp recordings using the zero-current injection mode, the resting membrane potential of INS-1 cells was found to be -75.1 ± 0.75 mV. Application of high concentrations of either tolbutamide (500 μM) or gliclazide (200 μM) caused similar, strong depolarization of the membrane potential that often led to firing of action potentials at voltages greater than -50 mV (Fig. 1C). Membrane potential repolarization occurred rapidly upon washout of tolbutamide. Washout of gliclazide was slower, but could be accelerated by application of 300 μM diazoxide, a K<sub>ATP</sub> channel "opener" (Fig. 1C). To compare the potency of tolbutamide and gliclazide in stimulating membrane potential depolarization, increasing concentrations of either drug were applied to INS-1 cells under current clamp. Tolbutamide and gliclazide both led to progressively greater depolarization of the membrane potential (Fig. 1D). Similar to block of K<sub>ATP</sub> channel current, gliclazide induced membrane depolarization more potently than did tolbutamide. The EC<sub>50</sub> for membrane depolarization by tolbutamide was determined to be 21.5 ± 10 μM with a maximum depolarization of 27.1 ± 4.2 mV. The EC<sub>50</sub> for membrane depolarization by gliclazide was determined to be 4.2 ± 0.9 μM with a maximum depolarization of 32.6 ± 3.8 mV. Because the concentrations of 20 μM gliclazide and 200 μM tolbutamide were maximally effective in their ability to induce membrane depolarization and were within error of each other, we used these concentrations for subsequent experiments to compare the ability of these sulfonylurea drugs to stimulate activities in INS-1 cells.

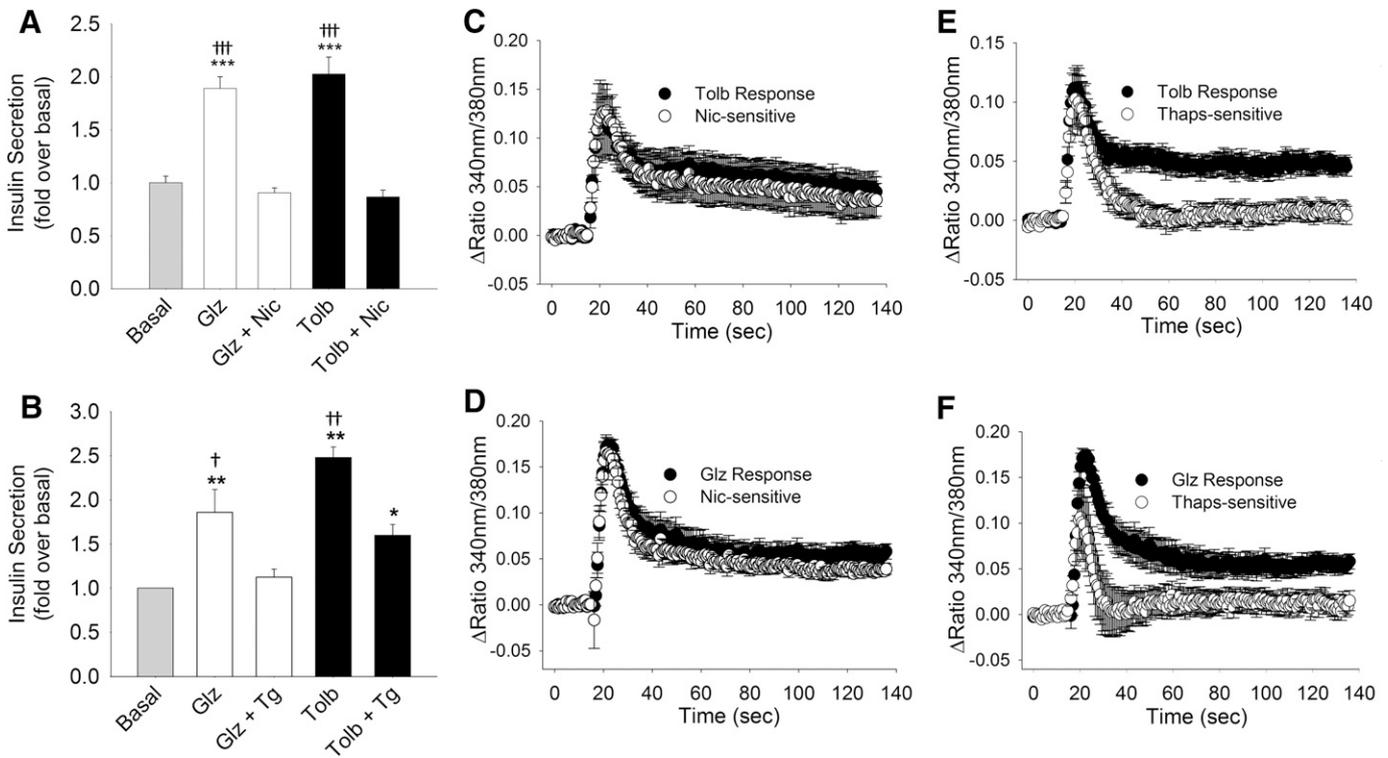
**Stimulation of Insulin Secretion and Ca<sup>2+</sup> Transients by Tolbutamide and Gliclazide.** The concentrations of 20 μM gliclazide and 200 μM tolbutamide were used to induce insulin secretion in INS-1 cells using the static incubation method (Fig. 2). As expected from the electrophysiological characterization of the drugs, these concentrations of the sulfonylureas stimulated insulin secretion that was significantly different from basal secretion. Secretion stimulated by both 20 μM gliclazide and 200 μM tolbutamide was completely inhibited by 2 μM nifedipine, an L-type-selective Ca<sup>2+</sup> channel blocker (Fig. 2A). Nifedipine was used at 2 μM because we had shown previously that it completely inhibits L-type channels but does not substantially inhibit P/Q-type Ca<sup>2+</sup> channels at this concentration (Lin et al., 2011). To assess the role of internal stores of Ca<sup>2+</sup> in sulfonylurea-stimulated insulin secretion, the ability of 1 μM thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA), to inhibit insulin secretion stimulated by either gliclazide or tolbutamide was determined. We found that thapsigargin completely inhibited secretion stimulated by 20 μM gliclazide and significantly, but incompletely, blocked insulin secretion stimulated by 200 μM tolbutamide (Fig. 2B). Thus, our results suggest that both influx of Ca<sup>2+</sup> via L-type Ca<sup>2+</sup> channels and release of internal stores of Ca<sup>2+</sup> are required for maximal insulin secretion in response to gliclazide and tolbutamide stimulation.



**Fig. 1.** Tolbutamide and gliclazide induce similar  $\text{K}_{\text{ATP}}$  current block and membrane depolarization in INS-1 cells. (A) representative trace of the dose-dependent block of  $\text{K}_{\text{ATP}}$  current measured using whole cell voltage clamp. Whole cell current was elicited by a  $\pm 20$ -mV step from a holding potential of  $-70$  mV. (B) percentage of the whole cell  $\text{K}_{\text{ATP}}$  current blocked by tolbutamide ( $n = 4-11$ ) or gliclazide ( $n = 3-8$ ).  $\text{IC}_{50}$  values were determined by fitting the data using the equation  $\text{Relative Current} = 1/[1 + 10^{(\log \text{IC}_{50} - [\text{drug}])}]$ . (C) representative trace of membrane potential depolarization elicited by tolbutamide ( $500 \mu\text{M}$ ) or gliclazide ( $200 \mu\text{M}$ ) and reversed by diazoxide ( $300 \mu\text{M}$ ). (D) dose-dependent effect of tolbutamide ( $n = 8-18$ ) or gliclazide ( $n = 7-27$ ) on membrane depolarization.  $\text{EC}_{50}$  values were determined by fitting the data to the equation  $\text{Relative Depolarization} = 1 - \{1/[1 + (\text{EC}_{50}/[\text{drug}])]\}$ .

Because sulfonylurea-stimulated insulin secretion was inhibited by nifedipine and thapsigargin, we examined the changes in intracellular  $\text{Ca}^{2+}$  concentration induced by tolbutamide or gliclazide. INS-1 cells in 96-well plates were loaded with the  $\text{Ca}^{2+}$  indicator Fura-2-AM, and changes in the ratio of fluorescence intensity at 510 nm after excitation at 340 nm and 380 nm (340 nm/380 nm ratio) were measured

upon injection of tolbutamide (final concentration of  $200 \mu\text{M}$ ) or gliclazide (final concentration  $20 \mu\text{M}$ ). The changes in 340 nm/380 nm ratio upon sulfonylurea injection were corrected by subtracting the changes in 340 nm/380 nm ratio measured in replicate wells of INS-1 cells upon injection of buffer only. The net change in fura2 fluorescence ratio, reflecting the net change in intracellular  $\text{Ca}^{2+}$  concentration stimulated by

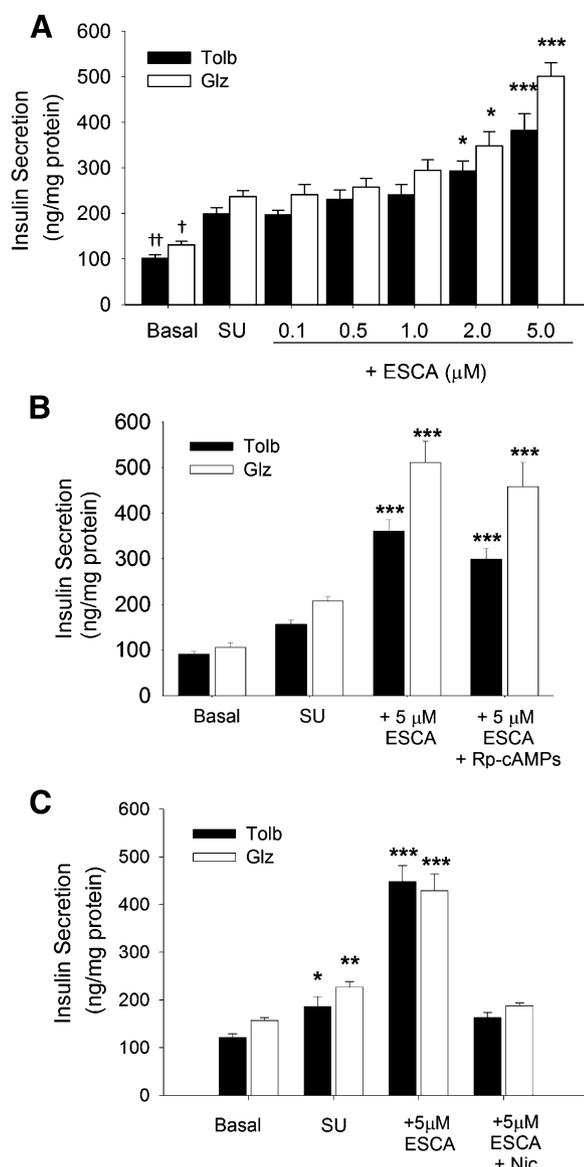


**Fig. 2.** Modulation of tolbutamide- and gliclazide-stimulated insulin secretion and  $\text{Ca}^{2+}$  transients by nicardipine and thapsigargin. (A) insulin secretion stimulated by either 20  $\mu\text{M}$  gliclazide (white bars) or 200  $\mu\text{M}$  tolbutamide (black bars) is completely inhibited by 2  $\mu\text{M}$  nicardipine. (B) insulin secretion stimulated by 20  $\mu\text{M}$  gliclazide (white bars) is completely inhibited, and secretion stimulated by 200  $\mu\text{M}$  tolbutamide (black bars) is partially inhibited by 1  $\mu\text{M}$  thapsigargin.  $***P < 0.001$ ,  $**P < 0.01$ ,  $*P < 0.05$  compared with basal;  $†††P < 0.001$ ,  $††P < 0.01$ ,  $†P < 0.05$  compared with nicardipine or thapsigargin. (C–F) increases in intracellular  $\text{Ca}^{2+}$  concentration are shown as increases in the ratio of intensity of fluorescence at 510 nm excited at 340 nm or 380 nm (340 nm/380 nm ratio). Black circles represent the response to 200  $\mu\text{M}$  tolbutamide (C and E) or 20  $\mu\text{M}$  gliclazide (D and F). Data shown were corrected by subtracting the increase in 340 nm/380 nm ratio observed upon addition of KRBH buffer alone. White circles in (C and E) and (D and F) represent the portion of the response to tolbutamide or gliclazide, respectively, inhibited by the indicated agent. Nicardipine (2  $\mu\text{M}$ ) completely inhibited the  $\text{Ca}^{2+}$  transient stimulated by either tolbutamide (C) or gliclazide (E). Preincubation with 2  $\mu\text{M}$  thapsigargin (1  $\mu\text{M}$  final concentration) selectively inhibited the rapid peak of the response stimulated by either tolbutamide (D) or gliclazide (F). Data shown are the mean  $\pm$  S.E. of at least three representative experiments performed in quadruplicate.

tolbutamide or gliclazide, is shown in Fig. 2. Both drugs induced a biphasic rise in intracellular  $\text{Ca}^{2+}$  concentration, with a rapid peak that decayed to an elevated plateau that persisted until the end of the 2-minute measurement (black circles: tolbutamide, Fig. 2, C and E; gliclazide, Fig. 2, D and F). Pretreatment of INS-1 cells with 2  $\mu\text{M}$  nicardipine for 30 minutes before injection of sulfonylureas completely inhibited this response. The portion of the response to each sulfonylurea that was sensitive to nicardipine [sulfonylurea response – (sulfonylurea + nicardipine response)] is shown as white circles in Fig. 2C (tolbutamide) and Fig. 2D (gliclazide). Pretreatment of INS-1 cells with 1  $\mu\text{M}$  thapsigargin for 30 minutes before injection of sulfonylureas selectively inhibited the rapid peak in intracellular  $\text{Ca}^{2+}$  concentration, with minimal effect on the sustained plateau phase stimulated by either tolbutamide or gliclazide. The portion of the response to each sulfonylurea that was sensitive to thapsigargin pretreatment is shown as white circles in Fig. 2E (tolbutamide) and Fig. 2F (gliclazide). Thus,  $\text{Ca}^{2+}$  transients stimulated by either tolbutamide or gliclazide are completely blocked by nicardipine, whereas thapsigargin selectively inhibits the early phase of the transient. The close correlation between block of insulin secretion and block of the early phase of the  $\text{Ca}^{2+}$  transient by nicardipine and thapsigargin suggests that this rapid peak in intracellular  $\text{Ca}^{2+}$  concentration is particularly

important in sulfonylurea-stimulated insulin secretion in INS-1 cells.

**Potentiation of Sulfonylurea-Stimulated Insulin Secretion by 8-pCPT-2'-O-Me-cAMP-AM.** Because tolbutamide is reported to directly activate EPAC2 (Zhang et al., 2009), we next asked if the insulin secretion stimulated by 200  $\mu\text{M}$  tolbutamide or 20  $\mu\text{M}$  gliclazide was differentially potentiated by the EPAC-selective cAMP analog 8-pCPT-2'-O-Me-cAMP-AM. As shown in Fig. 3A, insulin secretion was stimulated with 200  $\mu\text{M}$  tolbutamide in the absence or presence of 0.1, 0.5, 1.0, 2.0, or 5.0  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM. Tolbutamide-stimulated insulin secretion was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at concentrations  $\geq 2.0$   $\mu\text{M}$ . The potentiation of secretion over that stimulated by tolbutamide alone was 1.47-fold and 1.92-fold at 2  $\mu\text{M}$  and 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM, respectively. When the same concentrations of 8-pCPT-2'-O-Me-cAMP-AM were used in combination with 20  $\mu\text{M}$  gliclazide to stimulate insulin secretion (Fig. 3A), we found that significant potentiation of the response occurred at concentrations  $\geq 2.0$   $\mu\text{M}$  as well. The potentiation of secretion over that stimulated by gliclazide alone was 1.47-fold and 2.1-fold at 2  $\mu\text{M}$  and 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM, respectively. Moreover, the insulin secretion stimulated by 200  $\mu\text{M}$  tolbutamide ( $199.3 \pm 13$  ng insulin/mg protein;  $n = 14$ )



**Fig. 3.** Potentiation of tolbutamide- or gliclazide-stimulated insulin secretion by the EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP-AM. (A) 200  $\mu$ M tolbutamide and 20  $\mu$ M gliclazide both stimulated insulin secretion that was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM (ESCA) at concentrations  $\geq 2$   $\mu$ M.  $^{\dagger\dagger}P < 0.01$ ,  $^{\dagger}P < 0.05$  compared with sulfonylurea and sulfonylurea + all concentrations of 8-pCPT-2'-O-Me-cAMP-AM;  $^{***}P < 0.001$ ,  $^{*}P < 0.05$  compared with sulfonylurea alone ( $n = 9$ –14 in 3–5 independent experiments). (B) potentiation of tolbutamide- and gliclazide-stimulated insulin secretion by 8-pCPT-2'-O-Me-cAMP-AM is not blocked by the selective PKA inhibitor Rp-cAMPs (100  $\mu$ M).  $^{***}P < 0.001$  compared with sulfonylurea alone; sulfonylurea and ESCA  $\pm$  Rp-cAMPs were not significantly different ( $P = 0.089$  for Tolb,  $P = 0.91$  for Glz) ( $n = 9$  in 3 independent experiments). (C) potentiation of tolbutamide- and gliclazide-stimulated insulin secretion by 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM is completely blocked by the L-type  $Ca^{2+}$  channel blocker nicardipine (2  $\mu$ M).  $^{***}P < 0.001$ ,  $^{**}P < 0.01$ ,  $^{*}P < 0.05$  compared with basal ( $n = 9$  in 3 independent experiments).

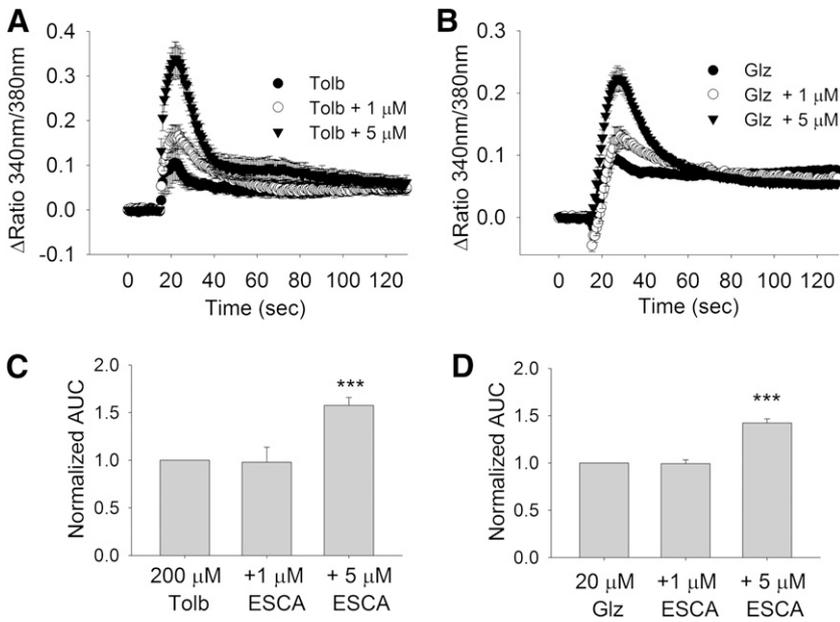
is not significantly different from that stimulated by 20  $\mu$ M gliclazide ( $237 \pm 13$  ng insulin/mg protein;  $n = 12$ ). 8-pCPT-2'-O-Me-cAMP-AM at concentrations up to 5  $\mu$ M had no effect on insulin secretion in the absence of sulfonylurea (basal =  $121 \pm 8$  ng insulin/mg protein, 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM only =  $156 \pm 6$  ng insulin/mg protein;  $n = 9$  in three separate experiments). Thus the concentration dependence

and extent of potentiation of both 200  $\mu$ M tolbutamide and 20  $\mu$ M gliclazide-stimulated insulin secretion by 8-pCPT-2'-O-Me-cAMP-AM are essentially identical.

To ensure that the potentiation of insulin secretion by 8-pCPT-2'-O-Me-cAMP-AM that we observed with both tolbutamide and gliclazide was not mediated by cross-activation of protein kinase A (PKA), we tested the ability of the PKA-specific inhibitor Rp-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPs) to inhibit the potentiation of sulfonylurea-stimulated insulin secretion by 8-pCPT-2'-O-Me-cAMP-AM. As shown in Fig. 3B, 100  $\mu$ M Rp-cAMPs did not significantly inhibit the potentiation of either tolbutamide- or gliclazide-stimulated insulin secretion by 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM. However, 2  $\mu$ M nicardipine completely blocked insulin secretion stimulated by tolbutamide or gliclazide in the presence of 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM (Fig. 3C). Thus, 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM potentiates insulin secretion stimulated by tolbutamide or gliclazide in a manner that does not involve PKA, but does require activation of L-type  $Ca^{2+}$  channels.

**Potentiation of Sulfonylurea-Stimulated Intracellular  $Ca^{2+}$  Transients by 8-pCPT-2'-O-Me-cAMP-AM.** Because 5  $\mu$ M, but not 1  $\mu$ M, 8-pCPT-2'-O-Me-cAMP-AM potentiated insulin secretion stimulated by either tolbutamide or gliclazide, we tested the ability of these concentrations of 8-pCPT-2'-O-Me-cAMP-AM to potentiate the  $Ca^{2+}$  transient stimulated by tolbutamide or gliclazide. Fura2-loaded INS-1 cells were pretreated with 0, 1, or 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM for 30 minutes prior to injection of either buffer only, 200  $\mu$ M tolbutamide, or 20  $\mu$ M gliclazide. The net change in fura2 fluorescence ratio (sulfonylurea response – buffer only response) is shown for a representative experiment with tolbutamide (Fig. 4A) or gliclazide (Fig. 4B). Pretreatment with 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM markedly and selectively increased the early peak of the  $Ca^{2+}$  transient stimulated by either sulfonylurea over pretreatment with buffer alone or 1  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM. Area under the curve (AUC) analysis for the entire post-injection time course of three separate experiments revealed that pretreatment with 5  $\mu$ M, but not 1  $\mu$ M, 8-pCPT-2'-O-Me-cAMP-AM significantly increased the  $Ca^{2+}$  integral induced by tolbutamide or gliclazide compared with control cells (Fig. 4, C and D, respectively). Thus, just as observed for potentiation of sulfonylurea-stimulated insulin secretion, 5  $\mu$ M, but not 1  $\mu$ M, 8-pCPT-2'-O-Me-cAMP-AM significantly increased the magnitude of the  $Ca^{2+}$  transient in response to sulfonylurea stimulation, and the concentration threshold for potentiation of the tolbutamide response did not differ from that for gliclazide.

**Activation of Phospholipase C by Tolbutamide or Gliclazide in INS-1 Cells.** Because tolbutamide, but not gliclazide, is reported to directly activate EPAC2, we compared the ability of gliclazide and tolbutamide to stimulate phospholipase C activity in these cells. The concentrations of inositol-1-phosphate ( $IP_1$ ) in the presence of LiCl [to inhibit inositol-1-phosphate phosphatase (Hallcher and Sherman, 1980)] after stimulation with the muscarinic receptor agonist carbachol (500  $\mu$ M) was measured in INS-1 cells using homogeneous time-resolved FRET (HTRF). Muscarinic acetylcholine receptor agonists, such as carbachol, stimulate phospholipase C activity in pancreatic beta cells (Yada et al., 1995) and INS-1 cells (Jacobo et al., 2009). The phospholipase



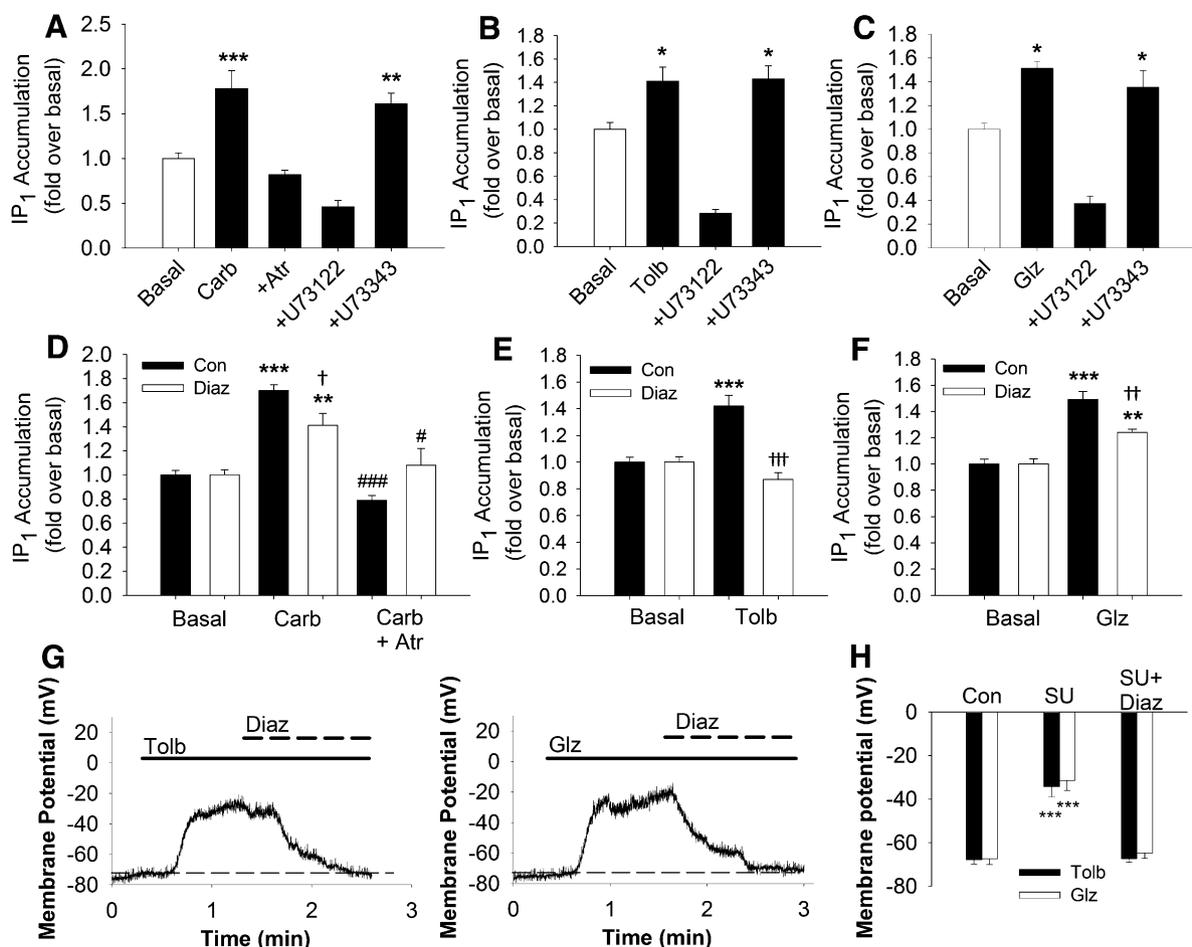
**Fig. 4.** Potentiation of  $\text{Ca}^{2+}$  transients by the EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP-AM. (A and B) increase in fura2 340 nm/380 nm ratio in response to application of 200  $\mu\text{M}$  tolbutamide (A) or 20  $\mu\text{M}$  gliclazide (B) to INS-1 cells preincubated with KRBH buffer only (black circles), 1  $\mu\text{M}$  (white circles), or 5  $\mu\text{M}$  (black triangles) 8-pCPT-2'-O-Me-cAMP-AM (ESCA). Data shown are mean  $\pm$  S.E. from a representative experiment done in quadruplicate and were corrected by subtracting the change in 340 nm/380 nm ratio stimulated by injection of KRBH buffer only on to cells given the same pretreatment. C and D, area under the curve analysis of the  $\text{Ca}^{2+}$  transients stimulated by tolbutamide or gliclazide alone or in the presence of 1  $\mu\text{M}$  or 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM. The  $\text{Ca}^{2+}$  integrals for the entire poststimulation period with preincubations of 1  $\mu\text{M}$  or 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM for three separate experiments were normalized to the  $\text{Ca}^{2+}$  integral for tolbutamide (C) or gliclazide (D) in cells preincubated with KRBH buffer alone. The  $\text{Ca}^{2+}$  integral for 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM + tolbutamide or gliclazide was significantly different from that for either sulfonylurea alone. \*\*\* $P < 0.001$  ( $n = 12$  in 3 separate experiments).

C inhibitor U73122 and its inactive analog U73343 were used to define specific phospholipase C activity stimulated by 500  $\mu\text{M}$  carbachol, 200  $\mu\text{M}$  tolbutamide, or 20  $\mu\text{M}$  gliclazide in the HTRF assay for  $\text{IP}_1$ . As shown in Fig. 5A, 500  $\mu\text{M}$  carbachol stimulated  $\text{IP}_1$  accumulation in INS-1 cells that was completely blocked by either 100  $\mu\text{M}$  atropine, a muscarinic receptor antagonist, or 10  $\mu\text{M}$  U73122, but not 10  $\mu\text{M}$  U73343. Stimulation of INS-1 cells with 200  $\mu\text{M}$  tolbutamide or 20  $\mu\text{M}$  gliclazide also resulted in a significant increase in  $\text{IP}_1$  accumulation over basal levels that was completely inhibited by 10  $\mu\text{M}$  U73122, but not 10  $\mu\text{M}$  U73343 (Fig. 5, B and C). Although U73122 and U73343 gave the expected results in our experiments measuring  $\text{IP}_1$  accumulation, we did not use these compounds in experiments measuring changes in intracellular  $\text{Ca}^{2+}$  concentration because we found that 10  $\mu\text{M}$  U73122 irreversibly blocks  $\sim 80\%$  and 10  $\mu\text{M}$  U73343 reversibly blocks  $\sim 50\%$  of the voltage-gated  $\text{Ca}^{2+}$  channel activity in INS-1 cells (Supplemental Fig. 1).

Because membrane depolarization is reported to stimulate phospholipase C activity in INS-1 cells (Thore et al., 2004), we further asked if carbachol, tolbutamide, or gliclazide could stimulate  $\text{IP}_1$  accumulation in the presence of the  $\text{K}_{\text{ATP}}$  channel opener diazoxide. As expected, 500  $\mu\text{M}$  carbachol stimulated a significant increase in  $\text{IP}_1$  accumulation over basal levels that was completely blocked by 100  $\mu\text{M}$  atropine in the presence or absence of 300  $\mu\text{M}$  diazoxide (Fig. 5D). However, diazoxide incompletely, but significantly, reduced the  $\text{IP}_1$  accumulation stimulated by carbachol, suggesting that membrane depolarization contributes to carbachol activation of phospholipase C activity. In contrast, diazoxide completely inhibited the ability of 200  $\mu\text{M}$  tolbutamide to stimulate  $\text{IP}_1$  accumulation above basal levels (Fig. 5E). Similar to what was observed with carbachol, 20  $\mu\text{M}$  gliclazide significantly stimulated  $\text{IP}_1$  accumulation over basal levels in the presence or absence of diazoxide, although this increase was significantly greater in the absence of diazoxide (Fig. 5F). To ensure that membrane potential depolarization was not contributing to the diazoxide-resistant  $\text{IP}_1$  accumulation observed with gliclazide, we measured the membrane potential in

INS-1 cells stimulated with either 200  $\mu\text{M}$  tolbutamide or 20  $\mu\text{M}$  gliclazide and subsequently treated with 300  $\mu\text{M}$  diazoxide in the continued presence of sulfonylurea drug. Figure 5G shows representative traces of whole cell current-clamp recordings of INS-1 cells depolarized with 200  $\mu\text{M}$  tolbutamide (Fig. 5G, left) or 20  $\mu\text{M}$  gliclazide (Fig. 5G, right). After the membrane depolarization induced by each drug had reached its maximum, 300  $\mu\text{M}$  diazoxide was coapplied with the sulfonylureas. With both tolbutamide and gliclazide, the resting membrane potential was rapidly re-established when 300  $\mu\text{M}$  diazoxide was present. Figure 5H summarizes the results of five or six separate experiments with 200  $\mu\text{M}$  tolbutamide or 20  $\mu\text{M}$  gliclazide and 300  $\mu\text{M}$  diazoxide and shows that the membrane potential in the presence of diazoxide and tolbutamide or gliclazide is not different from the resting membrane potential before application of either sulfonylurea drug. Taken together, our results show that gliclazide, but not tolbutamide, is capable of stimulating phospholipase C activity independently of its  $\text{K}_{\text{ATP}}$  channel-blocking activity.

**Evidence That Gliclazide Stimulates a  $\text{G}\alpha_{i/o}$  Protein-Coupled Receptor.** One possibility that might explain the  $\text{K}_{\text{ATP}}$  channel-independent activity of gliclazide is that it activates a G protein-coupled receptor that can stimulate phospholipase C activity. Therefore, we asked whether pertussis toxin, an inhibitor of  $\text{G}\alpha_{i/o}$  function, could interfere with the gliclazide stimulation of phospholipase C activity in the presence of diazoxide. Figure 6A shows that pretreatment of INS-1 cells in 96-well plates with 25 ng/well of pertussis toxin for 18 hours at 37°C, 5%  $\text{CO}_2$  significantly, but incompletely, inhibited the ability of 20  $\mu\text{M}$  gliclazide to stimulate  $\text{IP}_1$  accumulation in the presence of 300  $\mu\text{M}$  diazoxide. Increasing the amount of pertussis toxin used in the pretreatment to 50 ng/well did not further increase the extent of inhibition (data not shown). In contrast, pertussis toxin pretreatment had no effect on basal  $\text{IP}_1$  levels in the presence of 300  $\mu\text{M}$  diazoxide (Fig. 6A). If gliclazide is, in fact, able to stimulate phospholipase C activity in the absence of membrane depolarization via activation of a  $\text{G}\alpha_{i/o}$ -coupled receptor, then gliclazide should also stimulate a  $\text{Ca}^{2+}$

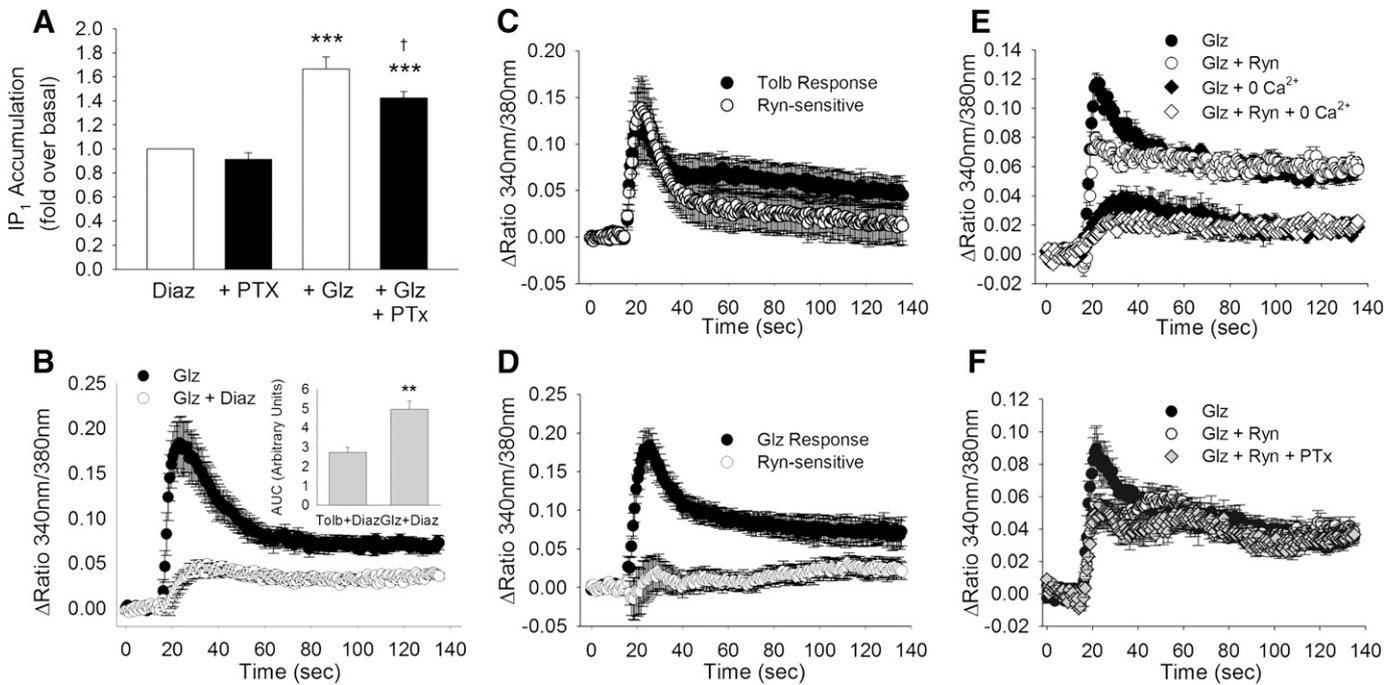


**Fig. 5.** Stimulation of phospholipase C activity by carbachol and the sulfonyleureas tolbutamide and gliclazide. (A) 500  $\mu$ M carbachol stimulated significant accumulation of IP<sub>1</sub> over basal levels in INS-1 cells that was completely blocked by either 100  $\mu$ M atropine or 10  $\mu$ M U73122, but not 10  $\mu$ M U73343. B and C, 200  $\mu$ M tolbutamide (B) or 20  $\mu$ M gliclazide (C) stimulated significant IP<sub>1</sub> accumulation over basal levels in INS-1 cells that was completely blocked by 10  $\mu$ M U73122, but not 10  $\mu$ M U73343. D and F, IP<sub>1</sub> accumulation stimulated by 500  $\mu$ M carbachol (D), 200  $\mu$ M tolbutamide (E), or 20  $\mu$ M gliclazide (F) in the presence (white bars) or absence (black bars) of 300  $\mu$ M diazoxide.  $***P < 0.001$ ,  $**P < 0.01$ ,  $*P < 0.05$  compared with basal;  $†††P < 0.001$ ,  $††P < 0.01$ ,  $†P < 0.05$  compared with indicated stimulus in the absence of diazoxide;  $###P < 0.001$ ,  $#P < 0.05$  compared with carbachol alone. (G) representative whole cell current-clamp traces of tolbutamide (200  $\mu$ M)- or gliclazide (20  $\mu$ M)-induced membrane depolarization and reversal by diazoxide. Solid lines represent the time during which the indicated sulfonyleurea was applied to the cell. Bold dashed lines represent the time during which 300  $\mu$ M diazoxide was applied to the cell. The thin dashed line represents membrane potential of the cell before perfusion of sulfonyleureas. (H) summarized data comparing the resting membrane potential (Con), the membrane potential in the presence of the indicated sulfonyleurea (SU), and membrane potential in the presence of sulfonyleurea and 300  $\mu$ M diazoxide (SU+Diaz). Tolbutamide: Con =  $-67.9 \pm 2$  mV; SU =  $-34.3 \pm 5$  mV; DU + Diaz =  $-67.3 \pm 2$  mV ( $n = 5-6$ ). Gliclazide: Con =  $-67.4 \pm 3$  mV; SU =  $-31.5 \pm 5$  mV; DU + Diaz =  $-64.8 \pm 2$  mV ( $n = 5-6$ ).  $***P < 0.001$  compared with control or SU + Diaz.

transient in the presence of diazoxide. Figure 6B is a representative experiment showing 20  $\mu$ M gliclazide stimulation of Ca<sup>2+</sup> transients in fura2-loaded INS-1 cells in the absence or presence of 300  $\mu$ M diazoxide. A rise in intracellular Ca<sup>2+</sup> concentration in the presence of diazoxide was clearly detectable. The magnitude of the response (area under the curve) to gliclazide in the presence of diazoxide was significantly greater than that stimulated by 200  $\mu$ M tolbutamide under the same conditions (Fig. 6B, inset).

We next examined the ability of ryanodine to block Ca<sup>2+</sup> transients stimulated by tolbutamide or gliclazide. We reasoned that Ca<sup>2+</sup> transients stimulated by Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release would be sensitive to ryanodine, but Ca<sup>2+</sup> release stimulated by activation of phospholipase C via IP<sub>3</sub> receptors would not. We found that pretreatment of INS-1 cells with 20  $\mu$ M ryanodine for 30 minutes before injection of

sulfonyleureas completely inhibited the early phase of the rise in intracellular Ca<sup>2+</sup> in response to 200  $\mu$ M tolbutamide (Fig. 6C; ryanodine-sensitive: white circles). However, pretreatment of INS-1 cells with 20  $\mu$ M ryanodine did not strongly inhibit the Ca<sup>2+</sup> transient stimulated by 20  $\mu$ M gliclazide (Fig. 6D; ryanodine-sensitive: white circles). We next tested the requirement for physiologic concentrations of extracellular Ca<sup>2+</sup> in the ryanodine-resistant transients stimulated by gliclazide. Figure 6E shows that in KRBH with zero added Ca<sup>2+</sup>, 20  $\mu$ M gliclazide was still able to stimulate a robust increase in intracellular Ca<sup>2+</sup> concentration in the presence or absence of 20  $\mu$ M ryanodine. To examine a possible role of G $\alpha_{i/o}$ -coupled receptors in this gliclazide-stimulated, ryanodine-insensitive Ca<sup>2+</sup> transient, we pretreated INS-1 cells in 96-well plates with 25 ng/well of pertussis toxin (PTx) for 18 hours at 37°C, 5% CO<sub>2</sub> and measured the Ca<sup>2+</sup> transients stimulated by



**Fig. 6.** Evidence for  $K_{ATP}$  channel-independent activities of gliclazide. (A) stimulation of phospholipase C activity in the presence of diazoxide by gliclazide in INS-1 cells is partially inhibited by pertussis toxin (PTx). In the absence of PTx (white bars), 20  $\mu$ M gliclazide (Glz) significantly stimulates  $IP_1$  accumulation in the presence of 300  $\mu$ M diazoxide (Diaz). In cells pretreated with PTx (black bars), basal  $IP_1$  accumulation in the presence of diazoxide is not different from non-pertussis toxin-treated cells, but gliclazide-stimulated  $IP_1$  accumulation is significantly reduced compared with control cells. Data shown are mean  $\pm$  S.E. from four separate experiments done in triplicate. (\*\*\*)  $P < 0.001$  compared with the corresponding control;  $^{\dagger}P < 0.05$  compared with Glz + Diaz without PTx pretreatment). (B) representative experiment illustrating the transient rise in intracellular  $Ca^{2+}$  concentration in INS-1 cells stimulated by 20  $\mu$ M Glz in the absence (black circles) or presence (white circles) of 300  $\mu$ M Diaz. (Inset) area under the curve analysis of  $Ca^{2+}$  transients measured in INS-1 cells stimulated with either tolbutamide (Tolb) or gliclazide in the presence of Diaz. (\*\*\*)  $P < 0.01$  ( $n = 6-8$  in 3-4 independent experiments). (C and D) increase in fura2 340 nm/380 nm ratio in response to application of 200  $\mu$ M tolbutamide (C) or 20  $\mu$ M gliclazide (D) to INS-1 cells preincubated with KRBH only (black circles). White circles in (C) and (D) represent the portion of the response to Tolb or Glz, respectively, inhibited by preincubation with 20  $\mu$ M ryanodine (Ryn). Preincubation with Ryn completely inhibited the early peak of the transient stimulated by Tolb (C) but not Glz (D). Data shown are mean  $\pm$  S.E. of representative experiments performed in quadruplicate. (E) removal of extracellular  $Ca^{2+}$  reduces, but does not eliminate, the  $Ca^{2+}$  transient stimulated by 20  $\mu$ M Glz or 20  $\mu$ M Glz + 20  $\mu$ M Ryn. In experiments labeled 0  $Ca^{2+}$ , KRBH with no added  $Ca^{2+}$  was used. Data shown are mean  $\pm$  S.E. for 6-15 independent experiments performed in quadruplicate. Area under the curve analysis for 6 (Glz alone, Glz + 0  $Ca^{2+}$ , Glz + Ryn + 0  $Ca^{2+}$ ) or 15 (Glz + Ryn) independent experiments revealed no significant difference between the  $Ca^{2+}$  transients stimulated by Glz or Glz + Ryn in either the absence or presence of 2 mM  $Ca^{2+}$ . However, reducing extracellular  $Ca^{2+}$  significantly reduced the  $Ca^{2+}$  transients stimulated by Glz alone or Glz + Ryn ( $P < 0.05$  and  $P < 0.01$ , respectively; not shown). (F) ryanodine-resistant  $Ca^{2+}$  transient stimulated by gliclazide is not inhibited by PTx. Increase in fura2 340 nm/380 nm ratio in response to application of 20  $\mu$ M gliclazide to INS-1 cells preincubated with KRBH only (black circles), 20  $\mu$ M Ryn (white circles), or 20  $\mu$ M Ryn after an 18 hours of pretreatment with PTx (gray diamonds). Data from cells pretreated with PTx only were omitted for clarity. Data shown are mean  $\pm$  S.E. and are representative of six independent experiments performed in quadruplicate. Area under the curve analysis of six independent experiments revealed no significant difference between Glz alone, Glz + PTx, Glz + Ryn, or Glz + Ryn + PTx (not shown).

20  $\mu$ M gliclazide in the presence of 20  $\mu$ M ryanodine. A representative of six independent experiments is shown in Fig. 6F. Area under the curve analysis of these six experiments, normalized to the gliclazide response, revealed that PTx pretreatment did not significantly reduce the ability of gliclazide to stimulate a rise in intracellular  $Ca^{2+}$  concentration in the presence of ryanodine. Together, the data in Fig. 6 suggest that gliclazide stimulates phospholipase C activity independently of  $K_{ATP}$  channel block and that this activity is, in part, mediated by a  $G\alpha_{i/o}$ -dependent mechanism. This unique activity of gliclazide is correlated with stimulation of a more robust  $Ca^{2+}$  transient in the presence of diazoxide compared with tolbutamide. Moreover, the unique ability of gliclazide to stimulate a  $Ca^{2+}$  transient independently of activation of ryanodine receptors does not involve a  $G\alpha_{i/o}$ -dependent mechanism and does not require influx of  $Ca^{2+}$  across the plasma membrane.

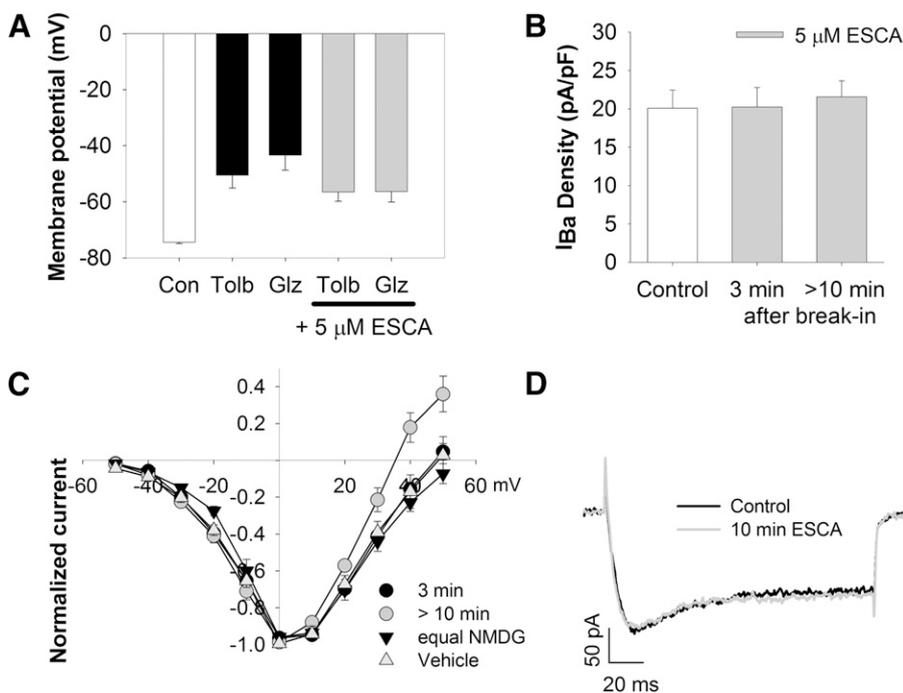
**8-pCPT-2'-O-Me-cAMP Does Not Enhance Membrane Depolarization Stimulated by 200  $\mu$ M Tolbutamide or**

**20  $\mu$ M Gliclazide or Voltage-Gated  $Ca^{2+}$  Channel Activity in INS-1 Cells.** To further elicit the mechanism of 8-pCPT-2'-O-Me-cAMP potentiation of insulin secretion stimulated by tolbutamide or gliclazide in INS-1 cells, we used whole cell current-clamp measurements of membrane potential depolarization induced by these sulfonylureas in the presence or absence of 8-pCPT-2'-O-Me-cAMP. In these experiments 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP, not the acetoxymethyl ester, was included in the pipette solution and allowed to dialyze into the cells for 10 min before either 200  $\mu$ M tolbutamide or 20  $\mu$ M gliclazide was applied via bath perfusion. We found that addition of 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP to the pipette solution did not significantly alter the extent of membrane potential depolarization induced by either sulfonylurea (Fig. 7A). The effect of 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP dialysis into the cells on whole cell voltage-gated  $Ca^{2+}$  channel currents was also assessed. Addition of 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP to the pipette solution did not change the  $Ba^{2+}$  current density or the voltages at which whole cell

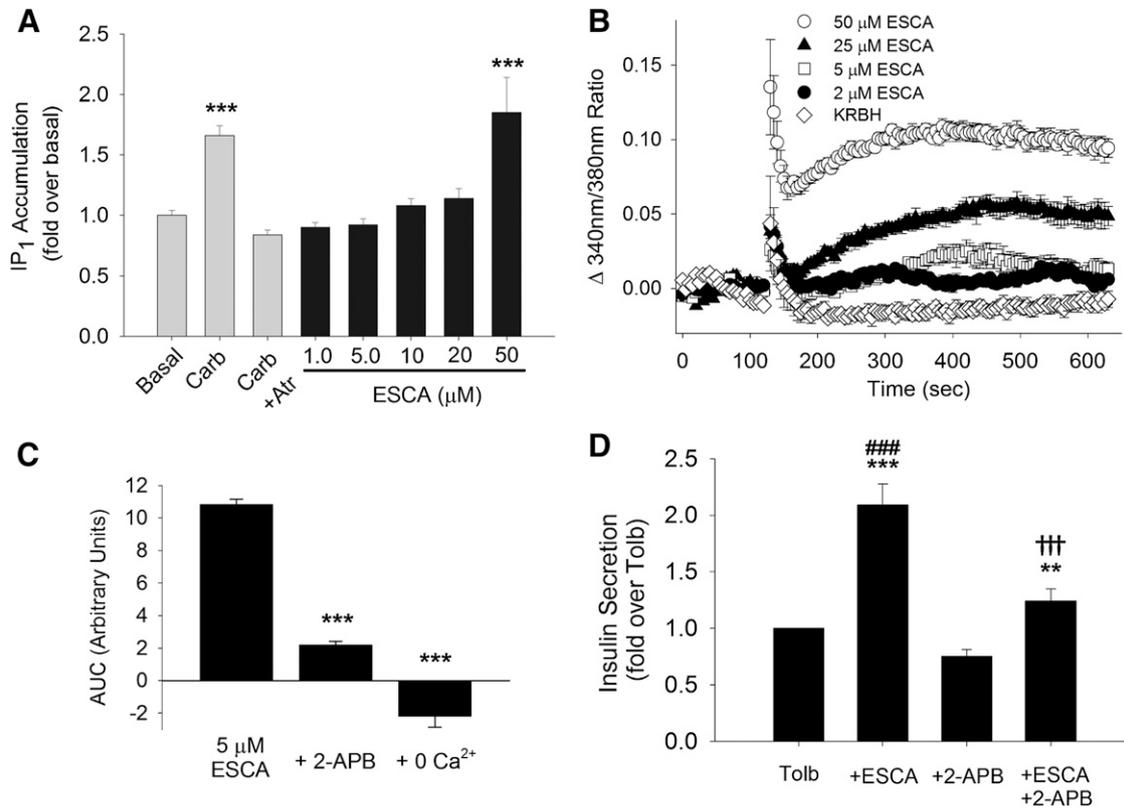
voltage-dependent  $Ba^{2+}$  currents activated, from a holding potential of  $-70$  mV, at either 3 min or  $>10$  min after break-in compared with control cells (Fig. 7, B and C). However, an approximately  $-15$  mV shift in the reversal potential of voltage-dependent  $Ba^{2+}$  current was observed after 10 minutes with  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP in the pipette (Fig. 7C). This shift in reversal potential was likely due to an increase in membrane permeability to the organic cation *N*-methyl-D-glucamine (NMDG) present in the intracellular solution, because it was abolished by equalizing the NMDG concentration in the intracellular and extracellular solutions (Fig. 7C). In contrast, no shift was observed in controls cells in which 0.1% DMSO (vehicle for 8-pCPT-2'-O-Me-cAMP) was included in the intracellular solution. The kinetics of current activation and inactivation were also unaffected by  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP as illustrated in Fig. 7D, which shows  $Ba^{2+}$  current measured at 0 mV from a holding potential of  $-70$  mV, in a single cell, at break-in and 10 minutes after break-in. Thus, at a concentration that significantly potentiated insulin secretion, 8-pCPT-2'-O-Me-cAMP had no detectable effect on membrane potential depolarization induced by either  $200 \mu\text{M}$  tolbutamide or  $20 \mu\text{M}$  gliclazide or on the amplitude and voltage dependence of voltage-gated  $Ca^{2+}$  channel activity in INS-1 cells. However,  $5 \mu\text{M}$  8-pCPT-2'-O-

Me-cAMP, applied via the patch pipette, did enhance membrane permeability to the organic cation NMDG.

**Activation of Phospholipase C Activity by 8-pCPT-2'-O-Me-cAMP-AM.** Because activation of EPAC2 leads to activation of PLC- $\epsilon$  via Rap1 (Dzhura et al., 2010), we examined the ability of the concentrations of 8-pCPT-2'-O-Me-cAMP-AM that gave significant potentiation of insulin secretion by tolbutamide or gliclazide to stimulate an increase in cellular phospholipase C activity. As expected,  $500 \mu\text{M}$  carbachol stimulated an increase in  $IP_1$  accumulation in INS-1 cells that was completely blocked by  $100 \mu\text{M}$  atropine (Fig. 8A). Concentrations of 8-pCPT-2'-O-Me-cAMP-AM from  $1$ – $20 \mu\text{M}$  did not stimulate  $IP_1$  accumulation above the basal level. However,  $50 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM applied to INS-1 cells significantly increased  $IP_1$  accumulation to an extent that was not different from that stimulated by  $500 \mu\text{M}$  carbachol (Fig. 8A). Because  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM was sufficient to strongly potentiate both insulin secretion and  $Ca^{2+}$  transients stimulated by tolbutamide or gliclazide, we looked for other evidence that  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM could, in fact, stimulate phospholipase C activity. If the HTRF assay for  $IP_1$  is not sensitive enough to detect very low concentrations of  $IP_1$  generated by  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM, we reasoned that a small rise in



**Fig. 7.** Effect of intracellular application of the EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP on sulfonylurea-stimulated membrane depolarization and voltage-dependent  $Ca^{2+}$  channel activity in INS-1 cells. (A) membrane potential changes measured using whole cell current clamp during application of tolbutamide or gliclazide in the absence (black bars) or presence (gray bars) of  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP (ESCA) ( $n = 6$ – $10$ ). 8-pCPT-2'-O-Me-cAMP was included in the intracellular solution of the patch pipette, and the cell was held for at least 10 min to allow for equilibration of 8-pCPT-2'-O-Me-cAMP into the cell. After 10 minutes, tolbutamide or gliclazide was applied and changes in membrane potential were recorded. (B) whole cell IBa density (pA/pF) measured at 0 mV from a holding potential of  $-70$  mV using whole cell voltage clamp. 8-pCPT-2'-O-Me-cAMP ( $5 \mu\text{M}$ ) was included in the intracellular solution of the patch pipette. Once consistent, currents were recorded 3 minutes after break-in and then again 10 minutes after break-in. ( $n = 11$ ). (C) current-voltage relationship curves of IBa measured using whole cell voltage clamp. Currents were elicited by 100-ms depolarizations from  $-50$  to  $+60$  mV in 10-mV increments, from a holding potential of  $-70$  mV, in the absence (control, gray triangles) or presence of  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP in the patch pipette 3 minutes (black circles) or 10 minutes (gray circles) after break-in ( $n = 15$ ). The shift in reversal potential observed  $>10$  min after break-in with 8-pCPT-2'-O-Me-cAMP in the patch pipette is abolished when the concentration of *N*-methyl-D-glucamine (NMDG) is equalized on both sides of the membrane by replacing 130 mM of Tris in the extracellular solution with 130 mM NMDG (black triangles) ( $n = 6$ ). The shift in reversal potential is not observed at  $>10$  minutes after break-in when 0.1% DMSO only (vehicle; gray triangles) is added to the intracellular solution. (D) representative whole cell voltage clamp trace of IBa measured at 0 mV, from a holding potential of  $-70$  mV, with  $5 \mu\text{M}$  of 8-pCPT-2'-O-Me-cAMP at 3 min after break-in (control) or 10 min after break-in in the same INS-1 cell.



**Fig. 8.** The EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP stimulates phospholipase C activity and a slow, persistent rise in intracellular  $\text{Ca}^{2+}$  in INS-1 cells. (A) 8-pCPT-2'-O-Me-cAMP-AM significantly stimulates IP<sub>1</sub> accumulation at 50  $\mu\text{M}$ , but not at 1, 5, 10, or 20  $\mu\text{M}$  in INS-1 cells (black bars). A positive control for phospholipase C activation, carbachol (500  $\mu\text{M}$ ), stimulates IP<sub>1</sub> accumulation that is completely blocked by 100  $\mu\text{M}$  atropine. \*\*\* $P < 0.001$  compared with basal (one-way analysis of variance with Tukey's post hoc test). (B) representative experiments showing fura2 340 nm/380 nm ratio over a 10-minute interval following application of 2, 5, 25, or 50  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM ( $n = 2-7$  in 7 independent experiments). Concentrations as low as 2  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM displayed a sustained rise in intracellular  $\text{Ca}^{2+}$ . At 7 minutes post injection, the  $\Delta 340 \text{ nm}/380 \text{ nm}$  ratio was significantly different from KRBH injection for all concentrations of 8-pCPT-2'-O-Me-cAMP-AM (2  $\mu\text{M}$ ,  $P < 0.05$ ; 5  $\mu\text{M}$ ,  $P < 0.05$ ; 25  $\mu\text{M}$ ,  $P < 0.001$ ; 50  $\mu\text{M}$ ,  $P < 0.001$ ). (C) area under the curve analysis of  $\text{Ca}^{2+}$  transients stimulated by 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM alone in the presence of 25  $\mu\text{M}$  2-APB or in the absence of extracellular  $\text{Ca}^{2+}$ . Data from 3-4 separate experiments performed in quadruplicate are shown as mean  $\pm$  S.E. \*\*\* $P < 0.001$  compared with 8-pCPT-2'-O-Me-cAMP-AM stimulation alone. (D) 25  $\mu\text{M}$  2-APB markedly reduces potentiation of tolbutamide-stimulated insulin secretion by 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM. Data were normalized to secretion stimulated by 200  $\mu\text{M}$  tolbutamide alone. Data shown are mean  $\pm$  S.E. of three independent experiments. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , compared with tolbutamide + 2-APB; ### $P < 0.001$ , compared with tolbutamide alone; ††† $P < 0.001$  compared with tolbutamide + ESCA + 2-APB.

intracellular  $\text{Ca}^{2+}$  stimulated by low levels of phospholipase C activation might be detectable. Therefore, we applied increasing concentrations of 8-pCPT-2'-O-Me-cAMP-AM to fura2-loaded INS-1 cells and measured the change in 340 nm/380 nm ratio over 8-10 minutes. This longer time scale was used because 8-pCPT-2'-O-Me-cAMP-AM must be deesterified intracellularly before it becomes active. We found that 8-pCPT-2'-O-Me-cAMP-AM dose dependently increased intracellular  $\text{Ca}^{2+}$  concentrations and that a significant increase over basal was observed at concentrations as low as 2  $\mu\text{M}$  and 5  $\mu\text{M}$  (Fig. 8B). The rise in intracellular  $\text{Ca}^{2+}$  stimulated by 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM was significantly blocked by preincubation of cells with 25  $\mu\text{M}$  2-aminoethoxydiphenylborate (2-APB) (Fig. 8C). Because 2-APB is able to block both IP<sub>3</sub> receptors and TRP channels (Bootman et al., 2002), we examined the ability of 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM to stimulate  $\text{Ca}^{2+}$  transients in the absence of extracellular  $\text{Ca}^{2+}$ . We found that 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM stimulation of  $\text{Ca}^{2+}$  transients in INS-1 cells was abolished by removal of  $\text{Ca}^{2+}$  from the extracellular solution (Fig. 8C). Thus, even though we were unable to detect an increase in IP<sub>1</sub> levels stimulated by 5  $\mu\text{M}$  8-pCPT-2'-O-

Me-cAMP-AM, it does stimulate a small, but detectable, increase in intracellular  $\text{Ca}^{2+}$  that could mediate the potentiation of sulfonylurea action.

Because 2-APB inhibits the 8-pCPT-2'-O-Me-cAMP-AM-stimulated increase in intracellular  $\text{Ca}^{2+}$  concentration, we tested the ability of 2-APB to inhibit 8-pCPT-2'-O-Me-cAMP-AM potentiation of tolbutamide-stimulated insulin secretion. We chose tolbutamide over gliclazide for these experiments to avoid the  $\text{K}_{\text{ATP}}$  channel-independent activity of gliclazide. As shown in Fig. 8D, 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM increased insulin secretion stimulated by 200  $\mu\text{M}$  tolbutamide by approximately twofold, whereas addition of 25  $\mu\text{M}$  2-APB alone did not significantly affect tolbutamide-stimulated insulin secretion. However, when INS-1 cells were treated with 25  $\mu\text{M}$  2-APB + 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM, tolbutamide-stimulated insulin secretion was not different from that measured in the absence of either compound. Thus, 25  $\mu\text{M}$  2-APB significantly blocked both the increase in intracellular  $\text{Ca}^{2+}$  concentration and the potentiation of tolbutamide-stimulated insulin secretion stimulated by 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM in INS-1 cells.

### Modulation of 8-pCPT-2'-O-Me-cAMP-AM Potentiation of Tolbutamide Action by Bisindolylmaleimide I.

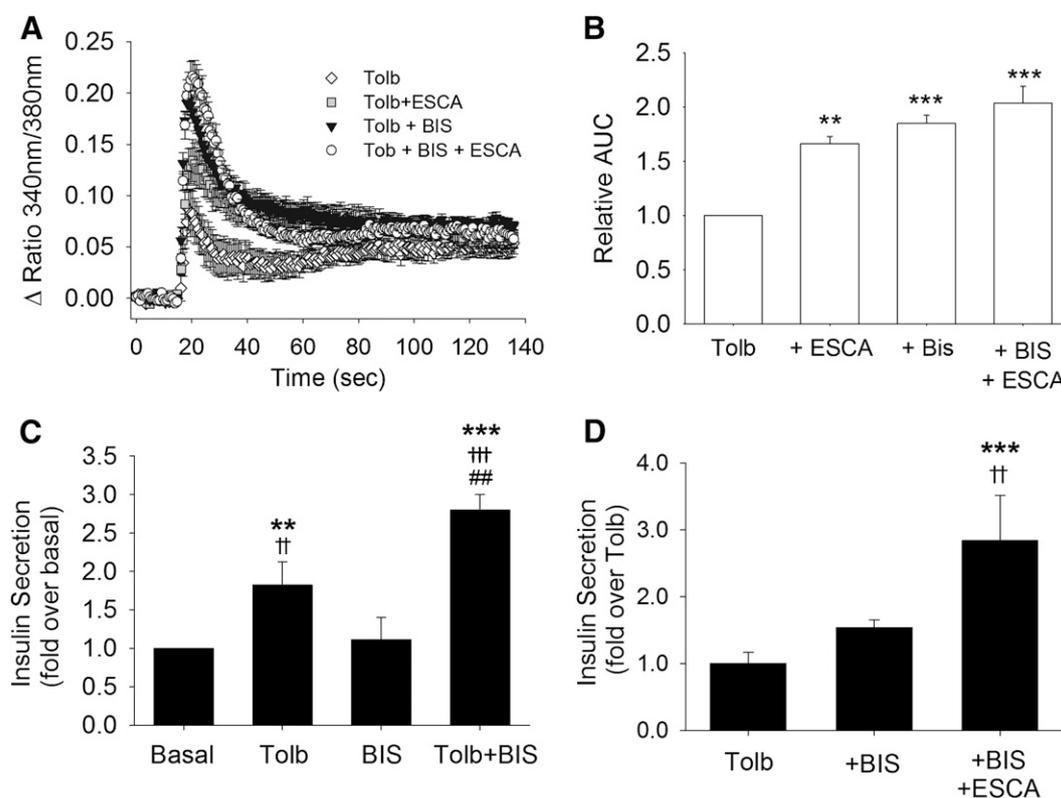
Protein kinase C (PKC) is a potential downstream effector of the small increase in intracellular  $\text{Ca}^{2+}$  stimulated by low concentrations of 8-pCPT-2'-O-Me-cAMP-AM. PKC is activated by  $\text{Ca}^{2+}$  and is reported to phosphorylate RYR2 and enhance  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in cardiac muscle downstream of EPAC activation (Oestreich et al., 2009). We therefore examined the ability of the broad-spectrum PKC inhibitor bisindolylmaleimide I (BIS) to inhibit potentiation of tolbutamide action by 8-pCPT-2'-O-Me-cAMP-AM (Fig. 9). As shown in Fig. 9A, preincubation with 1  $\mu\text{M}$  BIS significantly increased the amplitude of the  $\text{Ca}^{2+}$  transient stimulated by 200  $\mu\text{M}$  tolbutamide to a similar extent as 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM. The combination of BIS and 8-pCPT-2'-O-Me-cAMP-AM also significantly increased the amplitude of the  $\text{Ca}^{2+}$  transient stimulated by tolbutamide, but there was no further increase over that observed with BIS or 8-pCPT-2'-O-Me-cAMP-AM alone. We next examined the ability of BIS to potentiate insulin secretion stimulated by tolbutamide. Consistent with the modulation of  $\text{Ca}^{2+}$  transients, 1  $\mu\text{M}$  BIS also potentiated the insulin secretion stimulated by 200

$\mu\text{M}$  tolbutamide (Fig. 9C). In contrast to the experiments measuring  $\text{Ca}^{2+}$  transients, the combination of 1  $\mu\text{M}$  BIS + 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM potentiated insulin secretion stimulated by tolbutamide to a greater extent than 1  $\mu\text{M}$  BIS alone (Fig. 9D). Because inhibition of PKC potentiates both tolbutamide-stimulated  $\text{Ca}^{2+}$  transients and insulin secretion, it is unlikely that activation of PKC by EPAC2/Rap1 stimulation of phospholipase C activity plays a role in the potentiation of sulfonylurea-stimulated  $\text{Ca}^{2+}$  transients or insulin secretion.

## Discussion

### Potentiation of Tolbutamide- or Gliclazide-stimulated Insulin Secretion and $\text{Ca}^{2+}$ Transients by the EPAC-selective cAMP Analog 8-pCPT-2'-O-Me-cAMP Is Not Different in INS-1 Cells.

Previous studies reported that the sulfonylurea tolbutamide is able to directly activate EPAC2 while the structurally distinct sulfonylurea gliclazide is not (Zhang et al., 2009). We tested this conclusion in the rat pancreatic  $\beta$ -cell line INS-1 using a pharmacological approach. If tolbutamide activates EPAC2 in INS-1 cells, we



**Fig. 9.** Tolbutamide action is enhanced by the PKC inhibitor bisindolylmaleimide I. (A and B) increase in fura2 340 nm/380 nm ratio in response to application of 200  $\mu\text{M}$  tolbutamide (A) to INS-1 cells preincubated with KRBH buffer only (white diamonds), 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM (gray squares), 1  $\mu\text{M}$  bisindolylmaleimide (BIS) (black triangles) or both 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM (ESCA) and 1  $\mu\text{M}$  BIS (white circles). Data shown are mean  $\pm$  S.E. from a representative experiment done in quadruplicate (A). Area under the curve analysis of  $\text{Ca}^{2+}$  transients (B) that were normalized to the  $\text{Ca}^{2+}$  integrals for tolbutamide in the absence of any treatment. The  $\text{Ca}^{2+}$  integrals for 200  $\mu\text{M}$  tolbutamide plus 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM or 1  $\mu\text{M}$  bisindolylmaleimide or both 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM and 1  $\mu\text{M}$  bisindolylmaleimide were each significantly greater than tolbutamide alone. Data shown are mean  $\pm$  S.E. for three separate experiments ( $n = 9$ ).  $***P < 0.001$ ,  $**P < 0.01$ . (C) potentiation of tolbutamide-stimulated insulin secretion by 1  $\mu\text{M}$  BIS. Insulin secretion stimulated by 200  $\mu\text{M}$  tolbutamide in the presence of 1  $\mu\text{M}$  BIS was significantly greater than that stimulated by 200  $\mu\text{M}$  tolbutamide alone. Insulin secretion in the presence of 1  $\mu\text{M}$  BIS alone was not different from basal. Data shown are mean  $\pm$  S.E. for three separate experiments ( $n = 9$ ).  $***P < 0.001$ ,  $**P < 0.01$  compared with basal;  $†††P < 0.001$ ,  $††P < 0.01$  compared with BIS alone;  $\#P < 0.01$  compared with tolbutamide alone. (D) insulin secretion stimulated by 200  $\mu\text{M}$  tolbutamide + 1  $\mu\text{M}$  BIS is further increased by 5  $\mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM. Data shown are mean  $\pm$  S.E. for three independent experiments.  $***P < 0.001$ , compared with tolbutamide alone;  $††P < 0.01$  compared with tolbutamide + BIS.

would expect that tolbutamide might stimulate insulin secretion to a greater extent than gliclazide at concentrations of the drugs that are equivalent in terms of their activity at the  $K_{ATP}$  channels. In fact, we found that at such concentrations, tolbutamide and gliclazide stimulated insulin secretion to the same extent (Fig. 3A). In addition, if tolbutamide was able to activate EPAC2 directly in INS-1 cells, we would expect that gliclazide-stimulated secretion would be more sensitive to potentiation by 8-pCPT-2'-O-Me-cAMP-AM. However, we found that the concentration at which 8-pCPT-2'-O-Me-cAMP-AM potentiation of insulin secretion reached significance was identical for both sulfonylureas (i.e., 2  $\mu$ M; Fig. 3A). In addition, the  $Ca^{2+}$  transient stimulated by either sulfonylurea was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at 5  $\mu$ M, but not at 1  $\mu$ M (Fig. 4). These findings are inconsistent with a direct activation of EPAC2 by 200  $\mu$ M tolbutamide, but not 20  $\mu$ M gliclazide, in INS-1 cells.

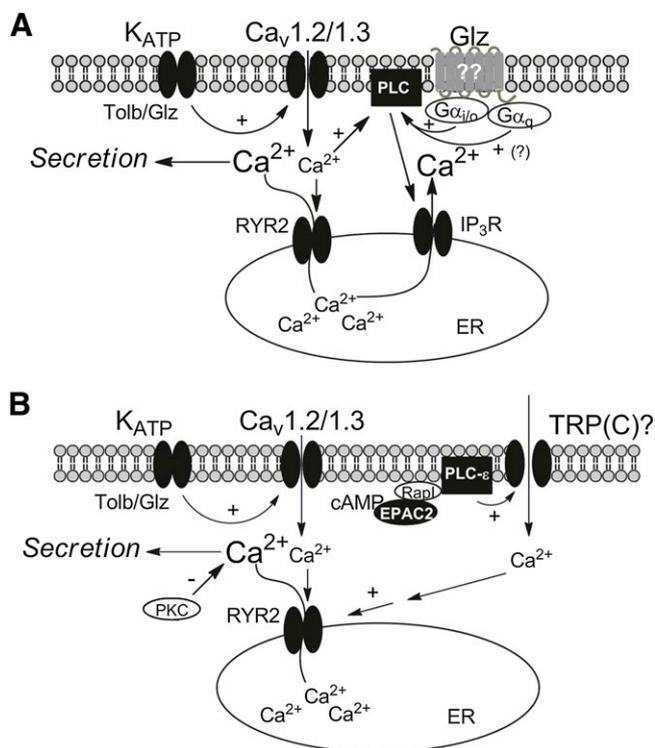
**8-pCPT-2'-O-Me-cAMP Potentiation of Sulfonylurea-Stimulated Insulin Secretion and  $Ca^{2+}$  Transients Does Not Require Activation of PKA or Enhancement of Membrane Depolarization.** The mechanism by which 8-pCPT-2'-O-Me-cAMP-AM potentiates insulin secretion by sulfonylureas was also examined. The concentrations of 8-pCPT-2'-O-Me-cAMP-AM used in this study are very likely to be selective for activation of EPAC over PKA because even 1 mM 8-pCPT-2'-O-Me-cAMP activated <25% of PKA activity at physiologic concentrations of the enzyme (Christensen et al., 2003). However, sulfonylurea stimulation of pancreatic  $\beta$ -cells has been reported to stimulate adenylyl cyclase activity (Grill and Cerasi, 1978). Therefore, endogenously produced cAMP, and activation of PKA, could potentially contribute to the activities of tolbutamide and gliclazide observed in this study. To test this possibility, we asked if the PKA-selective inhibitor Rp-cAMPs could interfere with 8-pCPT-2'-O-Me-cAMP-AM potentiation of sulfonylurea-stimulated insulin secretion; however, a role for PKA activity was excluded because 100  $\mu$ M Rp-cAMPs had no effect (Fig. 3). This result contrasts with a previous report that potentiation of glucose-stimulated insulin secretion by 8-pCPT-2'-O-Me-cAMP-AM was markedly attenuated by inhibitors of PKA in human islets (Chepurny et al., 2010). This discrepancy may result from differences between glucose stimulation of  $\beta$ -cells, which generates many glucose metabolites, and sulfonylurea stimulation, which more specifically regulates membrane potential.

A previous study (Leech et al., 2010b) found that 8-pCPT-2'-O-Me-cAMP-AM at concentrations of 10  $\mu$ M and 50  $\mu$ M increased the sensitivity of  $K_{ATP}$  channels in excised membrane patches to relatively low concentrations of tolbutamide. However, as channel block by tolbutamide approached saturation, this effect of 8-pCPT-2'-O-Me-cAMP-AM became insignificant. Our analysis of the electrophysiological effects of 5  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM on INS-1 cells did not reveal any potentiation of sulfonylurea-induced membrane depolarization or any enhancement of voltage-gated  $Ca^{2+}$  channel activity (Fig. 7). This is not surprising given that the concentrations of sulfonylureas were saturating both in terms of their effect on membrane potential and in block of whole cell  $K_{ATP}$  channel currents (Fig. 1). Moreover, the concentration of 8-pCPT-2'-O-Me-cAMP-AM used in the electrophysiological experiments reported here were lower than those used by Leech et al. (2010b). Thus, the ability of

8-pCPT-2'-O-Me-cAMP-AM to potentiate insulin secretion and  $Ca^{2+}$  transients in our experiments was independent of any enhancement of electrical activity stimulated by the sulfonylureas.

**Potentiation of Sulfonylurea-Stimulated Insulin Secretion by 8-pCPT-2'-O-Me-cAMP-AM Involves  $Ca^{2+}$  Influx via L-type Voltage-Gated  $Ca^{2+}$  Channels and Enhanced Release of  $Ca^{2+}$  from Internal Stores.** Insulin secretion and  $Ca^{2+}$  transients (Fig. 2) stimulated by tolbutamide and gliclazide were both blocked by the L-type  $Ca^{2+}$  channel blocker nifedipine. Moreover, insulin secretion stimulated by both sulfonylureas was substantially blocked by unloading intracellular stores of  $Ca^{2+}$  with thapsigargin (Fig. 2). Thapsigargin selectively inhibited the early peak of the  $Ca^{2+}$  transient stimulated by either tolbutamide or gliclazide (Fig. 2). Interestingly, the major effect of 8-pCPT-2'-O-Me-cAMP-AM on sulfonylurea-stimulated  $Ca^{2+}$  transients was to markedly increase the amplitude of this early peak (Fig. 4). These data suggest that  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels upon sulfonylurea stimulation of INS-1 cells causes a rapid release of  $Ca^{2+}$  from internal stores that is greatly amplified by 8-pCPT-2'-O-Me-cAMP-AM via EPAC2. This enhancement of  $Ca^{2+}$  release is likely the driving factor behind the enhancement of insulin secretion because the concentration threshold of 8-pCPT-2'-O-Me-cAMP-AM for both activities is essentially the same.

**Gliclazide, But Not Tolbutamide, Stimulates a Depolarization-independent Activation of Phospholipase C Activity in INS-1 Cells.** If tolbutamide were able to directly activate EPAC2 and Rap1 in INS-1 cells, it may have a unique ability to activate phospholipase C- $\epsilon$  and mobilize  $Ca^{2+}$  from internal stores via an RYR2-independent, phospholipase C/IP<sub>3</sub> receptor-dependent mechanism. We found, however, that both tolbutamide and gliclazide markedly stimulated phospholipase C activity as assessed by accumulation of IP<sub>1</sub>. It was previously reported that glucose stimulates phospholipase C activity that depends upon  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels and protein kinase C activation in INS-1 cells (Thore et al., 2004). However, our experiments measuring gliclazide- and tolbutamide-stimulated phospholipase C activity in the presence of diazoxide uncovered a unique ability of gliclazide to activate phospholipase C independently of its  $K_{ATP}$  channel blocking activity (Fig. 5). The mechanism accounting for this activity remains unknown. However, the unique activities of gliclazide that we have identified in this study, including its similarity to carbachol in stimulating IP<sub>1</sub> accumulation in the presence or absence of diazoxide, the partial sensitivity of this activity to pertussis toxin, the ability to stimulate a diazoxide-insensitive  $Ca^{2+}$  transient, and the ability to stimulate a ryanodine-insensitive  $Ca^{2+}$  transient in the presence or absence of physiologic concentrations of extracellular  $Ca^{2+}$  (Fig. 6), suggest that gliclazide may activate a GPCR that stimulates phospholipase C (Fig. 10A). Attractive possibilities include cannabinoid or purinergic receptors. CB<sub>1</sub> and CB<sub>2</sub> receptors are present and coupled to activation of phospholipase C and  $Ca^{2+}$  mobilization in the rat pancreatic  $\beta$ -cell line Rinm5F (De Petrocellis et al., 2007) and mouse pancreatic  $\beta$ -cells (Li et al., 2010). The CB<sub>1</sub> receptor inverse agonists rimonabant and ibipinabant are reported to act as  $K_{ATP}$  channel openers (Lynch et al., 2012) at low micromolar concentrations, suggesting some similarity in the structural requirements for  $K_{ATP}$  channel and CB<sub>1</sub>



**Fig. 10.** Model for the gliclazide  $K_{ATP}$  channel-independent activity of gliclazide and the EPAC-mediated potentiation of sulfonylurea action. (A) model for the  $K_{ATP}$  channel-independent activity of gliclazide. Both tolbutamide and gliclazide depolarize the membrane potential by blocking  $K_{ATP}$  channels. The resulting  $Ca^{2+}$  influx via L-type channels is coupled to  $Ca^{2+}$ -induced  $Ca^{2+}$  release from the ER and insulin secretion. Membrane depolarization (this study) and  $Ca^{2+}$  influx (Mogami et al., 2003; Jacobo et al., 2009) is also apparently coupled to activation of a phospholipase C activity. When membrane potential depolarization is prevented with diazoxide, the unique ability of gliclazide to activate PLC via a mechanism that is partially mediated by  $G_{\alpha_{i/o}}$  is unmasked. The observation that PTx only partially inhibits gliclazide-stimulated PLC activity in the presence of diazoxide suggests that another G protein (presumably  $G_{\alpha_{q/11}}$ ) is also involved. We hypothesize that gliclazide is an agonist at an as of yet unidentified G protein-coupled receptor. (B) model for EPAC-mediated potentiation of sulfonylurea action. Binding of cAMP to EPAC2 activates PLC- $\epsilon$  via Rap1 (Dzhura et al., 2010), leading to an influx of  $Ca^{2+}$  that can greatly amplify  $Ca^{2+}$ -induced  $Ca^{2+}$  release and insulin secretion stimulated by sulfonylureas. TRPC channels are proposed as potential effectors of EPAC2/Rap1 activation based upon their sensitivity to 2-APB and activation via a PLC-dependent mechanism.

receptor modulation. Additionally, various subtypes of  $P_2Y$  receptors are expressed in pancreatic beta cells, some of which activate PLC in response to binding of ATP and other nucleotides (Burnstock and Novak, 2012). Alternatively, our data do not exclude the possibility that gliclazide has a direct effect on PLC activity that is synergistic with  $G_i$  and is therefore partially inhibited by pertussis toxin. Whatever the mechanism, our results provide a potential explanation for some unique therapeutic advantages of gliclazide. For example, gliclazide carries a reduced risk for secondary beta cell failure in patients with type 2 diabetes compared with the sulfonylurea glibenclamide (Sato et al., 2005). In addition, two large clinical studies have reported a reduced risk of death by cardiovascular disease in type 2 diabetics taking gliclazide compared with similar patients taking other sulfonylureas including glibenclamide and tolbutamide (Jorgensen et al., 2010; Schramm et al., 2011). Interestingly, gliclazide is reported to inhibit several activities of oxidized low-density

lipoprotein in human endothelial cells (Li and Renier, 2009) and to reduce adhesion of monocytes to endothelial cells (Renier et al., 2003)—early steps in atherosclerosis. Determining whether these beneficial effects of gliclazide can be attributed to the non- $K_{ATP}$  channel-dependent activity reported here will clearly require further investigation.

The concentrations at which 8-pCPT-2'-O-Me-cAMP-AM began to potentiate insulin secretion ( $2 \mu\text{M}$ ) and the first phase of the  $Ca^{2+}$  transient stimulated by sulfonylureas ( $5 \mu\text{M}$ ) were an order of magnitude below the lowest concentration of 8-pCPT-2'-O-Me-cAMP-AM that activated phospholipase C as detected by the  $IP_1$  assay ( $50 \mu\text{M}$ ). A previous study reported activation of phospholipase C activity in INS-1 cells by  $10 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP-AM as detected by the translocation of a pleckstrin homology domain/GFP fusion (Leech et al., 2010b). Our data argue that concentrations as low as  $2 \mu\text{M}$  of 8-pCPT-2'-O-Me-cAMP-AM stimulate PLC activity, which is sufficient to stimulate a rise in intracellular  $Ca^{2+}$  concentration, but below the level of detection of the  $IP_1$  HTRF assay. The greater sensitivity of fluorescent  $Ca^{2+}$  indicators compared with the  $IP_1$  HTRF assay in detecting the activation of phospholipase C was previously reported (Liu et al., 2008). The 8-pCPT-2'-O-Me-cAMP-AM-stimulated  $Ca^{2+}$  rise is sensitive to both 2-APB and removal of extracellular  $Ca^{2+}$ , suggesting that influx of  $Ca^{2+}$  via a TRP channel may be involved (Fig. 10B). Indeed, inclusion of  $5 \mu\text{M}$  8-pCPT-2'-O-Me-cAMP in the pipette solution during voltage-clamp experiments activated an NMDG conductance in the plasma membrane of INS-1 cells (Fig. 7C). Interestingly, several members of the TRP superfamily of cation channels are reported to conduct NMDG, including TRPC (Hillyard et al., 2010), TRPA (Banke et al., 2010), and TRPV (Chung et al., 2008). TRPC channel subtypes are attractive candidates for this activity because they are blocked by 2-APB (Birbaumer, 2009) and many are positively regulated by PLC activity (Beech, 2012). Interestingly, TRPC1 and TRPC4 are expressed in INS-1 cells and rat  $\beta$ -cells (Li and Zhang, 2009), and TRPC4 is expressed in  $\beta\text{TC3}$  cells (Qian et al., 2002).

In summary, our data show that insulin secretion and  $Ca^{2+}$  transients stimulated by gliclazide or tolbutamide are potentiated equally by the EPAC selective cAMP analog 8-pCPT-2'-O-Me-cAMP in INS-1 cells. However, gliclazide, but not tolbutamide, exhibits  $K_{ATP}$  channel-independent stimulation of phospholipase C activity that is mediated, in part, by a  $G_{\alpha_{i/o}}$ -dependent mechanism. Uncovering differences in activities between gliclazide and other sulfonylureas is of therapeutic relevance because gliclazide has shown some clinical advantages over other second generation drugs. Our data also suggest a potential role for activation of a 2-APB-sensitive  $Ca^{2+}$  influx in the EPAC-dependent potentiation of sulfonylurea-stimulated insulin secretion in INS-1 cells. It will be of interest to further characterize this  $Ca^{2+}$  flux and identify the channel by which it is conducted.

#### Authorship Contributions

*Participated in research design:* Hockerman, Jarrard, Wang, Guerra.

*Conducted experiments:* Jarrard, Wang, Guerra, Salyer, Soderling, Pratt, Lange, Broderick.

*Performed data analysis:* Hockerman, Jarrard, Wang, Pratt, Guerra.

*Wrote or contributed to the writing of the manuscript:* Hockerman.

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