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Potentiation 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 Ca^{2+} influx.

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Running Title: EPAC modulation of sulfonylurea action

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Non-Standard Abbreviations:

8-pCPT-2'-O-Me-cAMP-AM- *para*- Chlorophenylthio-2'-O-methyladenosine-3'-5'-cyclic monophosphate, acetoxymethyl ester; ESCA- EPAC-selective cAMP analog; Rp-cAMPs- Adenosine-3'-5'-cyclic monophosphorothioate, Rp-isomer; EPAC- Exchange protein directly activated by cAMP; U71322- 1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1H-pyrrole-2,5-dione; U-73343- 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione; IP_1- inositol monophosphate; SERCA- sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; BIS- Bisindolylmaleimide I; 2-APB- 2-aminoethoxydiphenylborate; TRP- transient receptor potential; Tolbutamide- 3-butyl-1-[(4-methylbenzene)sulfonyl]urea; Gliclazide- 1-[(4-methylbenzene)sulfonyl]-3-{octahydrocyclopenta[c]pyrrol-2-yl}urea; Nicardipine- 3-{2-[benzyl(methyl)amino]ethyl} 5-methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate; Diazoxide- 7-chloro-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dione; Carbachol- 2-(trimethylazaniumyl)ethyl carbamate chloride.

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Abstract

Tolbutamide and gliclazide block the K_{ATP} channel $K_{ir}6.2$/Sur1, 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 μM tolbutamide and 20 μM gliclazide, concentrations that had equivalent effects on membrane potential, was inhibited by thapsigargin (1 μM) or the L-type Ca^{2+} channel blocker nicardipine (2 μM), and was potentiated by 8-pCPT-2'-O-Me-cAMP-AM at concentrations $\geq$ 2 μM in INS-1 cells. Ca^{2+} transients stimulated by either tolbutamide or gliclazide were inhibited by thapsigargin or nicardipine and were significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at 5 μM but not 1μM. Both tolbutamide and gliclazide stimulated phospholipase C activity; however, only gliclazide did so independently of its activity at K_{ATP} channels, and this activity was partially inhibited by pertussis toxin. 8-pCPT-2'-O-Me-cAMP-AM alone (5 μM) did not stimulate insulin secretion, but did increase intracellular Ca^{2+} concentration significantly, and this activity was inhibited by 25 μM 2-aminoethoxydiphenylborate (2-APB) or the removal of extracellular Ca^{2+}. 8-pCPT-2'-O-Me-cAMP-AM potentiation of insulin secretion stimulated by tolbutamide was markedly inhibited by 2-APB (25 μM), and enhanced by the PKC inhibitor Bisindolylmaleimide I (1 μM). 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 Ca^{2+} influx.

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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 diabetics 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 (Dunne and Petersen, 1986) (Rorsman and Trube, 1985) on the K_{ATP} channel composed of the $K_{ir}6.2$ and SUR1 subunits (Babenko et al., 1998). Binding of sulfonylureas to the K_{ATP} channel favors channel closing (Schmid-Antomarchi et al., 1987), and subsequent membrane depolarization since open K_{ATP} channels permit the efflux of K^+ ions and the maintenance of the membrane potential close to the equilibrium potential for K^+ (Cook and Hales, 1984). Depolarization of the membrane potential of pancreatic beta cells leads to the activation of several varieties of voltage-gated Ca^{2+} channels (Horvath et al., 1998), including the L-type channels $Ca_v1.2$ and $Ca_v1.3$ (Seino et al., 1992). The subsequent rise in intracellular Ca^{2+} triggers the exocytosis of insulin (Wollheim et al., 1975) via dense core vesicles which are shuttled to (Van Obberghen et al., 1975), and eventually fuse with, the plasma membrane (Barg et al., 2001) (Shibasaki et al., 2007).

Recently, EPAC2 (Exchange Protein directly Activated by cAMP 2) 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-ε (Dzhura et al., 2010) in pancreatic

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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 Ca^{2+}-dependent manner (Fujimoto et al., 2002). In addition, the voltage-gated Ca^{2+} channel $Ca_v1.2$ 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, $Ca_v1.2$) is of special significance since membrane depolarization-dependent calcium influx via $Ca_v1.2$ channels has been implicated in triggering Ca^{2+}-induced 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 Ca^{2+} dynamics in beta cells is currently unknown.

Tolbutamide and gliclazide, which both stimulate insulin secretion from pancreatic beta cells by block of K_{ATP} channels, differ in their ability to bind and activate EPAC2. Zhang et al. (Zhang et al., 2009), reported that tolbutamide at concentrations $\geq$30 μM significantly activated Rap1 in an EPAC2-dependent manner, while 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 K_{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 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. Since activation of phospholipase C is a

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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 Ca^{2+} influx via voltage-gated Ca^{2+} channels. In order 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 Ca^{2+} concentration in INS-1 cells.

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Materials and Methods

Chemicals- U73122 and U73433 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-cAMPs 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, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (HyClone, Logan, UT), 11 mg/ml sodium pyruvate, 10mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM/ β-mercaptoethanol at 37°C, 5% 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 to 5 MΩ. For current clamp experiments and voltage clamp experiments to measure K_{ATP} channel current, the intracellular solution contained (in mM) 90 K_2SO_4, 10 NaCl, 1 $MgCl_2$, 1.1 EGTA, 0.1 $CaCl_2$, 5 HEPES, 0.3 ATP, 0.2 GTP. The extracellular solution used for measuring K_{ATP} currents contained (in mM) 138 NaCl, 5.6 KCl, 11.1 glucose, 10 HEPES, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 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 K_{ATP} currents were elicited by 1.3 second steps of ± 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 K_{ATP} channel opener, diazoxide (300 μM), was transiently applied to maximally open K_{ATP} channels, before application of tolbutamide or gliclazide. Tolbutamide and gliclazide

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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 Ca^{2+} channel currents, the bath solution contained (in mM) 150 Tris, 10 $BaCl_2$, 4 $MgCl_2$. The intracellular solution contained (in mM) 130 N-methyl-D-glucamine, 10 EGTA, 60 HEPES, 2 ATP, and 1 $MgCl_2$. 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% 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 pre-incubated with a modified Kreb-Ringer buffer (KRBH: 134 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 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 minutes at 37°C, 5% 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 one hour at 37°C, 5% 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 Na_2HPO_4, 150 mM NaCl, 0.1% Triton X-100**,** 800 nM aprotinin, 50 μM leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine, 1 mM 4-(2-Aminoethyl)benzenesulfonylfluoride, 10 μg/ml calpain inhibitor I, 10 μg/ml calpain inhibitor II, (pH 7.4) and cellular protein in each well was determined using the BCA assay (Thermo Scientific, Rockford, IL).

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Intracellular 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_2. Cells were washed twice with PBS and incubated with 5 μM Fura-2AM (Molecular Probes, Eugene, OR) diluted in KRBH for one hour at 37°C, 5% CO_2. The KRBH containing Fura-2AM was then removed and the cells were washed twice with KRBH, and equilibrated for 30 minutes at 37°C, 5% CO_2 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 were 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^{2+} 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/bandpass) using a Synergy 4 multi-mode microplate reader (BioTek, Winooski, VT). For experiments injecting sulfonylureas, ratios were acquired every 0.7 secs for 15 sec before injection and two minutes after injection of stimuli. For experiments injecting 8-pCPT-2'-O-Me-cAMP-AM, ratios were acquired every 5 seconds 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_1 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 2.5mM Glucose overnight at 37°C and 5% CO_2. A pre-stimulation buffer was added to the cells for 1 hour before stimulation (10mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl (pH 7.4)). The pre-stimulation buffer was decanted, and treatments made in a stimulation buffer containing LiCl to block inositol phosphate degradation (10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5 mM LiCl (pH 7.4)), were added to cells. Cells were incubated with treatments for 1 hour at 37°C 5% CO_2. When pertussis toxin was used, 25 ng/well were added to cells in

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96 well plates approximately 18 hours before assay. IP_1 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 wave length of 330 nM) was measured using a Synergy 4 fluorescent plate reader (BioTek, Winooski, VT). The concentration of IP_1 in each sample was determined by comparison of the 620 nm/650 nm ratio to those for a standard curve of IP_1 concentrations.

Data Analysis- Data was analyzed using SigmaPlot 11.0. Data are shown as means ± standard error. Statistical significance was determined using One-way ANOVA and the Tukey *post-hoc* test unless otherwise indicated. $P < 0.05$ was considered significant.

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Results

Electrophysiological characterization of tolbutamide and gliclazide activity in INS-1 cells- Both tolbutamide and gliclazide bind to and block the K_{ATP} channel in pancreatic beta cells. In order to compare the effects of these two drugs and determine whether or not tolbutamide might also activate EPAC2 in INS-1 cells, we determined the potency of K_{ATP} channel block, and the dose response curve for membrane depolarization in INS-1 cells (Figure 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^+ currents through K_{ATP} channels (Figure 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_{ATP} channel current than was tolbutamide (gliclazide IC_{50} = 143 ± 23 nM; tolbutamide IC_{50} = 2.6 ± 0.7 μM; Figure 1B).

Since the block of K_{ATP} 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 (Figure 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_{ATP} channel "opener" (Figure 1C). To compare the potency

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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 (Figure 1D). Similar to block of K_{ATP} channel current, gliclazide induced membrane depolarization more potently than did tolbutamide. The EC_{50} 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_{50} for membrane depolarization by gliclazide was determined to be 4.2 ± 0.9 μM with a maximum depolarization of 32.6 ± 3.8 mV. Since 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^{2+} 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 (Figure 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 nicardipine, an L-type-selective Ca^{2+} channel blocker (Figure 2A). Nicardipine was used at 2 μM since we had shown previously that it completely inhibits L-type channels but does not substantially inhibit P/Q-type Ca^{2+} channels at this concentration (Lin et al., 2011). To assess the role of internal stores of Ca^{2+} in sulfonylurea-stimulated insulin secretion, the ability of 1 μM thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}- ATPase (SERCA), to inhibit insulin secretion stimulated by either gliclazide or tolbutamide was determined. We found

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that thapsigargin completely inhibited secretion stimulated by 20 μM gliclazide, and significantly, but incompletely, blocked insulin secretion stimulated by 200 μM tolbutamide (Figure 2B). Thus, our results suggest that both influx of Ca^{2+} via L-type Ca^{2+} channels and release of internal stores of Ca^{2+} are required for maximal insulin secretion in response to gliclazide and tolbutamide stimulation.

Since sulfonylurea-stimulated insulin secretion was inhibited by nicardipine and thapsigargin, we examined the changes in intracellular Ca^{2+} concentration induced by tolbutamide or gliclazide. INS-1 cells in 96-well plates were loaded with the Ca^{2+} indicator fura2-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 μM) or gliclazide (final concentration 20 μM). 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 Ca^{2+} concentration stimulated by tolbutamide or gliclazide, is shown in Figure 2. Both drugs induced a biphasic rise in intracellular Ca^{2+} concentration, with a rapid peak that decayed to an elevated plateau that persisted until the end of the two minute measurement (black circles: tolbutamide, Figure 2C&E; gliclazide, Figure 2D&F). Pre-treatment of INS-1 cells with 2 μM 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 Figure 2C (tolbutamide) and 2D (gliclazide). Pre-treatment of INS-1 cells with 1 μM thapsigargin for 30 minutes before injection of sulfonylureas selectively inhibited the rapid peak in intracellular 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 pre-treatment is shown

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as white circles in Figure 2E (tolbutamide) and 2F (gliclazide). Thus, Ca^{2+} transients stimulated by either tolbutamide or gliclazide are completely blocked by nicardipine, while 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 Ca^{2+} transient by nicardipine and thapsigargin suggests that this rapid peak in intracellular 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- Since tolbutamide is reported to directly activate EPAC2 (Zhang et al., 2009), we next asked if the insulin secretion stimulated by 200 μM tolbutamide or 20 μM gliclazide was differentially potentiated by the EPAC-selective cAMP analog 8-pCPT-2'-O-Me-cAMP-AM. As shown in Figure 3A, insulin secretion was stimulated with 200 μM tolbutamide in the absence or presence of 0.1, 0.5, 1.0, 2.0, or 5.0 μM 8-pCPT-2'-O-Me-cAMP-AM. Tolbutamide-stimulated insulin secretion was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at concentrations ≥ 2.0 μM. The potentiation of secretion over that stimulated by tolbutamide alone was 1.47 fold and 1.92 fold at 2 μM and 5 μM 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 μM gliclazide to stimulate insulin secretion (Figure 3A), we found that significant potentiation of the response occurred at concentrations ≥ 2.0 μM as well. The potentiation of secretion over that stimulated by gliclazide alone was 1.47 fold and 2.1 fold at 2 μM and 5 μM 8-pCPT-2'-O-Me-cAMP-AM, respectively. Moreover, the insulin secretion stimulated by 200 μM tolbutamide (199.3 ± 13 ng insulin/mg protein; n = 14) is not significantly different from that stimulated by 20 μM gliclazide (237 ± 13 ng insulin/mg protein; n = 12). 8-pCPT-2'-O-Me-cAMP-AM at concentrations up to 5 μM had no effect on insulin secretion in the absence of sulfonylurea (basal = 121 ± 8 ng insulin/mg protein, 5 μM 8-pCPT-2'-O-Me-cAMP-AM only = 156 ± 6 ng insulin/mg protein; n = 9

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in three separate experiments). Thus the concentration dependence and extent of potentiation of both 200 μM tolbutamide and 20 μM 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 Figure 3B, 100 μM Rp-cAMPs did not significantly inhibit the potentiation of either tolbutamide or gliclazide-stimulated insulin secretion by 5 μM 8-pCPT-2'-O-Me-cAMP-AM. However, 2 μM nicardipine completely blocked insulin secretion stimulated by tolbutamide or gliclazide in the presence of 5 μM 8-pCPT-2'-O-Me-cAMP-AM (Figure 3C). Thus, 5 μM 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- Since 5 μM, but not 1 μM, 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 pre-treated with 0, 1, or 5 μM 8-pCPT-2'-O-Me-cAMP-AM for 30 minutes prior to injection of either buffer only, 200 μM tolbutamide, or 20 μM gliclazide. The net change in fura2 fluorescence ratio (sulfonylurea response - buffer only response) is shown for a representative experiment with tolbutamide (Figure 4A) or gliclazide (Figure 4B). Pre-treatment with 5 μM 8-pCPT-2'-O-Me-cAMP-AM markedly, and selectively increased the early peak of the Ca^{2+} transient stimulated by either sulfonylurea over pre-

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treatment with buffer alone or 1 μM 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 pre-treatment with 5 μM, but not 1 μM, 8-pCPT-2'-O-Me-cAMP-AM significantly increased the Ca^{2+} integral induced by tolbutamide or gliclazide compared to control cells (Figure 4C&D, respectively). Thus, just as observed for potentiation of sulfonylurea-stimulated insulin secretion, 5 μM, but not 1 μM, 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- Since 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 μM) 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 C inhibitor U73122 and its inactive analog U73343 were used to define specific phospholipase C activity stimulated by 500 μM carbachol, 200 μM tolbutamide, or 20 μM gliclazide in the HTRF assay for IP_1. As shown in Figure 5A, 500 μM carbachol stimulated IP_1 accumulation in INS-1 cells that was completely blocked by either 100 μM atropine, a muscarinic receptor antagonist, or 10 μM U73122, but not 10 μM U73433. Stimulation of INS-1 cells with 200 μM tolbutamide or 20 μM gliclazide also resulted in a significant increase in IP_1 accumulation over basal levels that was completely inhibited by 10 μM U73122, but not 10 μM U73433 (Figure 5B&C). Though U73122 and U73433 gave the expected results in our experiments measuring IP_1 accumulation, we did not use these compounds in experiments

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measuring changes in intracellular Ca^{2+} concentration since we found that 10 μM U73122 irreversibly blocks ~80% and 10 μM U73433 reversibly blocks ~50% of the voltage-gated Ca^{2+} channel activity in INS-1 cells (Supplementary Figure 1).

Since 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 IP_1 accumulation in the presence of the K_{ATP} channel opener diazoxide. As expected, 500 μM carbachol stimulated a significant increase in IP_1 accumulation over basal levels that was completely blocked by 100 μM atropine in the presence or absence of 300 μM diazoxide (Figure 5D). However, diazoxide incompletely, but significantly reduced the 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 μM tolbutamide to stimulate IP_1 accumulation above basal levels (Figure 5E). Similar to what was observed with carbachol, 20 μM gliclazide significantly stimulated IP_1 accumulation over basal levels in the presence or absence of diazoxide, though this increase was significantly greater in the absence of diazoxide (Figure 5F). To ensure that membrane potential depolarization was not contributing to the diazoxide-resistant IP_1 accumulation observed with gliclazide, we measured the membrane potential in INS-1 cells stimulated with either 200 μM tolbutamide or 20 μM gliclazide and subsequently treated with 300 μM 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 μM tolbutamide (left panel) or 20 μM gliclazide (right panel). After the membrane depolarization induced by each drug had reached its maximum, 300 μM diazoxide was co-applied with the sulfonylureas. With both tolbutamide and gliclazide, the resting membrane potential was rapidly re-established when 300 μM diazoxide was present. Figure 5H summarizes the results of 5-6 separate experiments with

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200 μM tolbutamide or 20 μM gliclazide and 300 μM 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 K_{ATP} channel-blocking activity.

Evidence that Gliclazide stimulates a G$\alpha_{i/o}$-protein coupled receptor- One possibility that might explain the K_{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 or not pertussis toxin, an inhibitor of G$\alpha_{i/o}$ function, could interfere with the gliclazide stimulation of phospholipase C activity in the presence of diazoxide. Figure 6A shows that pre-treatment of INS-1 cells in 96-well plates with 25 ng/well of pertussis toxin for 18 hours at 37°C, 5% CO_2 significantly, but incompletely, inhibited the ability of 20 μM gliclazide to stimulate IP_1 accumulation in the presence of 300 μM diazoxide. Increasing the amount of pertussis toxin used in the pre-treatment to 50 ng/well did not further increase the extent of inhibition (data not shown). In contrast, pertussis toxin pre-treatment had no effect on basal IP_1 levels in the presence of 300 μM diazoxide (Figure 6A). If gliclazide is, in fact, able to stimulate phospholipase C activity in the absence of membrane depolarization via activation of a G$\alpha_{i/o}$ coupled receptor, then gliclazide should also stimulate a Ca^{2+} transient in the presence of diazoxide. Figure 6B is a representative experiment showing 20 μM gliclazide stimulation of Ca^{2+} transients in fura2-loaded INS-1 cells in the absence or presence of 300 μM diazoxide. A rise in intracellular Ca^{2+} 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 μM tolbutamide under the same conditions (Figure 6B, inset).

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We next examined the ability of ryanodine to block Ca^{2+} transients stimulated by tolbutamide or gliclazide. We reasoned that Ca^{2+} transients stimulated by Ca^{2+}-induced Ca^{2+} release would be sensitive to ryanodine, but Ca^{2+} release stimulated by activation of phospholipase C via IP_3 receptors would not. We found that pre-treatment of INS-1 cells with 20 μM ryanodine for 30 minutes before injection of sulfonylureas completely inhibited the early phase of the rise in intracellular Ca^{2+} in response to 200 μM tolbutamide (Figure 6C; ryanodine-sensitive: white circles). However, pre-treatment of INS-1 cells with 20 μM ryanodine did not strongly inhibit the Ca^{2+} transient stimulated by 20 μM gliclazide (Figure 6D; ryanodine-sensitive: white circles). We next tested the requirement for physiological concentrations of extracellular Ca^{2+} in the ryanodine-resistant transients stimulated by gliclazide. Figure 6E shows that in KRBH with zero added Ca^{2+}, 20 μM gliclazide was still able to stimulate a robust increase in intracellular Ca^{2} concentration in the presence or absence of 20 μM ryanodine. To examine a possible role of $G\alpha_{i/o}$-coupled receptors in this gliclazide-stimulated, ryanodine-insensitive Ca^{2+} transient, we pre-treated INS-1 cells in 96-well plates with 25 ng/well of pertussis toxin (PTx) for 18 hours at 37°C, 5% CO_2, and measured the Ca^{2+} transients stimulated by 20 μM gliclazide in the presence of 20 μM ryanodine. A representative of six independent experiments is shown in Figure 6F. Area under the curve analysis of these six experiments, normalized to the gliclazide response, revealed that PTx pre-treatment 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 Figure 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 to 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.

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8-pCPT-2'-O-Me-cAMP doesn't enhance membrane depolarization stimulated by 200 μM tolbutamide or 20 μM 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 μM 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 minutes before either 200 μM tolbutamide or 20 μM gliclazide was applied via bath perfusion. We found that addition of 5 μM 8-pCPT-2'-O-Me-cAMP to the pipette solution did not significantly alter the extent of membrane potential depolarization induced by either sulfonylurea (Figure 7A). The effect of 5 μM 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 μM 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 minutes or >10 minutes after break-in compared to control cells (Figure 7B&C). However, an approximately -15 mV shift in the reversal potential of voltage-dependent Ba^{2+} current was observed after 10 minutes with 5 μM 8-pCPT-2'-O-Me-cAMP in the pipette (Figure 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, since equalizing the NMDG concentration in the intracellular and extracellular solutions abolished it (Figure 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 μM 8-pCPT-2'-O-Me-cAMP as illustrated in Figure 7D which shows Ba^{2+} current measured at 0 mV from a holding potential of -70 mV, in a

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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 μM tolbutamide or 20 μM gliclazide, or on the amplitude and voltage-dependence of voltage-gated Ca^{2+} channel activity in INS-1 cells. However, 5 μM 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- Since activation of EPAC2 leads to activation of PLC-ε 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 μM carbachol stimulated an increase in IP_1 accumulation in INS-1 cells that was completely blocked by 100 μM atropine (Figure 8A). Concentrations of 8-pCPT-2'-O-Me-cAMP-AM from 1-20 μM did not stimulate IP_1 accumulation above the basal level. However, 50 μM 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 μM carbachol (Figure 8A). Since 5 μM 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 μM 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 μM 8-pCPT-2'-O-Me-cAMP-AM, we reasoned that a small rise in intracellular Ca^{2+} stimulated by low levels of phospholipase C activation might be detectable. Therefore, we applieded increasing concentrations of 8-pCPT-2'-O-Me-cAMP-AM to fura2-loaded INS-1 cells and measured the change in 340nm/380nm ratio over 8-10 minutes. This longer time scale was used since 8-pCPT-2'-O-Me-cAMP-AM must be de-esterified intracellularly before it becomes active. We found that 8-pCPT-2'-O-Me-cAMP-AM dose-dependently increased intracellular

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Ca^{2+} concentrations, and that a significant increase over basal was observed at concentrations as low as 2 and 5 μM (Figure 8B). The rise in intracellular Ca^{2+} stimulated by 5 μM 8-pCPT-2'-O-Me-cAMP-AM was significantly blocked by pre-incubation of cells with 25 μM 2-aminoethoxydiphenylborate (2-APB) (Figure 8C). Since 2-APB is able to block both IP_3 receptors and TRP channels (Bootman et al., 2002), we examined the ability of 5 μM 8-pCPT-2'-O-Me-cAMP-AM to stimulate Ca^{2+} transients in the absence of extracellular Ca^{2+}. We found that 5 μM 8-pCPT-2'-O-Me-cAMP-AM stimulation of Ca^{2+} transients in INS-1 cells was abolished by removal of Ca^{2+} from the extracellular solution (Figure 8C). Thus, even though we were unable to detect an increase in IP_1 levels stimulated by 5 μM 8-pCPT-2'-O-Me-cAMP-AM, it does stimulate a small, but detectable increase in intracellular Ca^{2+} that could mediate the potentiation of sulfonylurea action.

Since 2-APB inhibits the 8-pCPT-2'-O-Me-cAMP-AM-stimulated increase in intracellular 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 K_{ATP} channel-independent activity of gliclazide. As shown in Figure 8D, 5 μM 8-pCPT-2'-O-Me-cAMP-AM increased insulin secretion stimulated by 200 μM tolbutamide by approximately 2-fold, while addition of 25 μM 2-APB alone did not significantly affect tolbutamide-stimulated insulin secretion . However, when INS-1 cells were treated with 25 μM 2-APB + 5 μM 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 μM 2-APB significantly blocked both the increase in intracellular Ca^{2+} concentration and the potentiation of tolbutamide-stimulated insulin secretion stimulated by 5 μM 8-pCPT-2'-O-Me-cAMP-AM in INS-1 cells.

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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 Ca^{2+} stimulated by low concentrations of 8-pCPT-2'-O-Me-cAMP-AM. PKC is activated by Ca^{2+} and is reported to phosphorylate RYR2 and enhance Ca^{2+}-induced 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 (Figure 9). As shown in Figure 9A, pre-incubation with 1 μM BIS significantly increased the amplitude of the Ca^{2+} transient stimulated by 200 μM tolbutamide to a similar extent as 5 μM 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 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 Ca^{2+} transients, 1 μM BIS also potentiated the insulin secretion stimulated by 200 μM tolbutamide (Figure 9C). In contrast to the experiments measuring Ca^{2+} transients, the combination of 1 μM BIS + 5 μM 8-pCPT-2'-O-Me-cAMP-AM potentiated insulin secretion stimulated by tolbutamide to a greater extent than 1 μM BIS alone (Figure 9D). Since inhibition of PKC potentiates both tolbutamide-stimulated Ca^{2+} transients and insulin secretion, it's unlikely that activation of PKC by EPAC2/Rap1 stimulation of phospholipase C activity plays a role in the potentiation of sulfonylurea-stimulated Ca^{2+} transients or insulin secretion.

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Discussion

Potentiation of tolbutamide or gliclazide-stimulated insulin secretion and 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 β-cell line INS-1 using a pharmacological approach. If tolbutamide activates EPAC2 in INS-1 cells, we 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 (Figure 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 μM; Figure 3A). In addition, the Ca^{2+} transient stimulated by either sulfonylurea was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM at 5 μM, but not at 1 μM (Figure 4). These findings are inconsistent with a direct activation of EPAC2 by 200 μM tolbutamide, but not 20 μM 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 since even 1 mM 8-pCPT-2'-O-Me-cAMP activated <25% of PKA activity at physiological concentrations of the enzyme (Christensen et al., 2003). However, sulfonylurea stimulation of pancreatic β- 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 since 100 μM Rp-cAMPs had no effect (Figure 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., 2009). This discrepancy may result from differences between glucose stimulation of β-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 and 50 μM 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 μM 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 (Figure 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 (Figure 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. (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.

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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 (Figure 2) stimulated by tolbutamide and gliclazide were both blocked by the L-type Ca^{2+} channel blocker nicardipine. Moreover, insulin secretion stimulated by both sulfonylureas was substantially blocked by unloading intracellular stores of Ca^{2+} with thapsigargin (Figure 2). Thapsigargin selectively inhibited the early peak of the Ca^{2+} transient stimulated by either tolbutamide or gliclazide (Figure 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 (Figure 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 since 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 was able to directly activate EPAC2 and Rap1 in INS-1 cells, it may have a unique ability to activate phospholipase C-ε and mobilize Ca^{2+} from internal stores via an RYR2-indepenedent, phospholipase C/IP_3 receptor-dependent mechanism. We found, however, that both tolbutamide and gliclazide markedly stimulated phospholipase C activity as assessed by accumulation of IP_1. 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 (Figure 5). The mechanism accounting for

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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_1 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 physiological concentrations of extracellular Ca^{2+} (Figure 6), suggest that gliclazide may activate a GPCR that stimulates phospholipase C (See Figure 10A). Attractive possibilities include cannabinoid or purinergic receptors. CB_1 and CB_2 receptors are present and coupled to activation of phospholipase C and Ca^{2+} mobilization in the rat pancreatic β-cell line Rinm5F (De Petrocellis et al., 2007) and mouse pancreatic β-cells (Li et al., 2010). The CB_1 receptor inverse agonists rimonabant and ibipinabant are reported to act as K_{ATP} channel openers (Lynch et al., 2011) at low μM concentrations, suggesting some similarity in the structural requirements for K_{ATP} channel and CB_1 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 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 type 2 diabetics compared to the sulfonylurea glibenclamide (Satoh et al., 2005). In addition, two large clinical studies have reported a reduced risk of death by cardiovascular disease in type 2 diabetes patients taking gliclazide compared to 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 LDL in human endothelial cells (Li and Renier, 2009), and to reduce adhesion of monocytes to endothelial cells (Renier et al., 2003) - early steps in

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atherosclerosis. Determining whether or not 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 μM) and the first phase of the Ca^{2+} transient stimulated by sulfonylureas (5 μM) 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 μM). A previous study reported activation of phospholipase C activity in INS-1 cells by 10 μM 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 μM 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 to the IP_1 HTRF assay in detecting the activation of phospholipase C has been 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 (See Figure 10B). Indeed, inclusion of 5 μM 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 (Figure 7C). Interestingly, several members of the TRP superfamily of cation channels are reported to conduct NMDG, including TRPC (Hillyard et al.), TRPA (Banke et al., 2010), and TRPV (Chung et al., 2008). TRPC channel subtypes are attractive candidates for this activity since they are blocked by 2-APB (Birnbaumer, 2009), and many are positively regulated by PLC activity (Beech, 2012). Interestingly, TRPC1 and TRPC4 are expressed in INS-1 cells and rat β-cells (Li and Zhang, 2009), and TRPC4 is expressed in βTC3 cells (Qian et al., 2002).

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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 since 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 that conducts it.

Authorship Contributions

Participated in research design: Hockerman, Jarrard, Wang, and Guerra

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References

Babenko AP, Aguilar-Bryan L and Bryan J (1998) A view of sur/KIR6.X, KATP channels. [Review] [93 refs]. *Annu Rev Physiol* **60**:667-87.

Banke TG, Chaplan SR and Wickenden AD (2010) Dynamic changes in the TRPA1 selectivity filter lead to progressive but reversible pore dilation. *Am J Physiol Cell Physiol* **298**:C1457-1468.

Barg S, Ma XS, Eliasson L, Galvanovskis J, Gopel SO, Obermuller S, Platzer J, Renstrom E, Trus M, Atlas D, Striessnig J and Rorsman P (2001) Fast Exocytosis With Few Ca^{2+} Channels in Insulin-Secretingmouse Pancreatic B Cells. *Biophys J* **81**(6):3308-3323.

Beech DJ (2012) Integration of transient receptor potential canonical channels with lipids. *Acta Physiol (Oxf)* **204**(2):227-237.

Birnbaumer L (2009) The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca(2+) concentrations. *Annu Rev Pharmacol Toxicol* **49**:395-426.

Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ and Peppiatt CM (2002) 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. *FASEB J* **16**:1145-1150.

Chepurny OG, Kelley GG, Dzhura I, Leech CA, Roe MW, Dzhura E, Li X, Schwede F, Genieser HG and Holz GG (2009) PKA-dependent potentiation of glucose-stimulated insulin secretion by Epac activator 8-pCPT-2'-O-Me-cAMP-AM in human islets of Langerhans. *Am J Physiol Endocrinol Metab* **298**:E622-633.

Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG and Doskeland SO (2003) cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem* **278**:35394-35402.

Chung MK, Guler AD and Caterina MJ (2008) TRPV1 shows dynamic ionic selectivity during agonist stimulation. *Nat Neurosci* **11**:555-564.

Cook DL and Hales CN (1984) Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature* **311**:271-273.

De Petrocellis L, Marini P, Matias I, Moriello AS, Starowicz K, Cristino L, Nigam S and Di Marzo V (2007) Mechanisms for the coupling of cannabinoid receptors to intracellular calcium mobilization in rat insulinoma beta-cells. *Exp Cell Res* **313**:2993-3004.

Ding WG and Gromada J (1997) Protein kinase A-dependent stimulation of exocytosis in mouse pancreatic beta-cells by glucose-dependent insulinotropic polypeptide. *Diabetes* **46**:615-621.

Dunne MJ and Petersen OH (1986) Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett* **208**:59-62.

Dzhura I, Chepurny OG, Kelley GG, Leech CA, Roe MW, Dzhura E, Afshari P, Malik S, Rindler MJ, Xu X, Lu Y, Smrcka AV and Holz GG (2010) Epac2-dependent mobilization of intracellular Ca(2)+ by glucagon-like peptide-1 receptor agonist exendin-4 is disrupted in beta-cells of phospholipase C-epsilon knockout mice. *J Physiol* **588**:4871-4889.

Eliasson L, Ma X, Renstrom E, Barg S, Berggren PO, Galvanovskis J, Gromada J, Jing X, Lundquist I, Salehi A, Sewing S and Rorsman P (2003) SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic B-cells. *J Gen Physiol* **121**:181-197.

Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B, Garner AM, Kaempf U, Kindler S, Gundelfinger ED and Garner CC (2000) Piccolo, a presynaptic zinc finger protein structurally related to bassoon. *Neuron* **25**:203-214.

Fujimoto K, Shibasaki T, Yokoi N, Kashima Y, Matsumoto M, Sasaki T, Tajima N, Iwanaga T and Seino S (2002) Piccolo, a Ca^{2+} sensor in pancreatic beta-cells. Involvement of cAMP-GEFII.Rim2.Piccolo complex in cAMP-dependent exocytosis. *J Biol Chem* **277**:50497-50502.

Grill V and Cerasi E (1978) Interacting effects of sulfonylureas and glucose on cyclic AMP metabolism and insulin release in pancreatic islets of the rat. *J Clin Invest* **61**(5):1346-1354.

Groop LC (1992) Sulfonylureas in NIDDM. *Diabetes Care* **15**(6):737-754.

Hallcher LM and Sherman WR (1980) The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *J Biol Chem* **255**(22):10896-10901.

Hillyard SD, Willumsen NJ and Marrero MB Stretch-activated cation channel from larval bullfrog skin. *J Exp Biol* **213**:1782-1787.

Horvath A, Szabadkai G, Varnai P, Aranyi T, Wollheim CB, Spat A and Enyedi P (1998) Voltage dependent calcium channels in adrenal glomerulosa cells and in insulin producing cells. *Cell Calcium* **23**:33-42.

Jacobo SM, Guerra ML and Hockerman GH (2009) $Ca_v1.2$ and $Ca_v1.3$ are differentially coupled to glucagon-like peptide-1 potentiation of glucose-stimulated insulin secretion in the pancreatic beta-cell line INS-1. *J Pharmacol Exp Ther* **331**:724-732.

Jorgensen CH, Gislason GH, Andersson C, Ahlehoff O, Charlot M, Schramm TK, Vaag A, Abildstrom SZ, Torp-Pedersen C and Hansen PR (2010) Effects of oral glucose-lowering drugs on long term outcomes in patients with diabetes mellitus following myocardial infarction not treated with emergent percutaneous coronary intervention--a retrospective nationwide cohort study. *Cardiovasc Diabetol* **9**:54.

Kang G, Joseph JW, Chepurny OG, Monaco M, Wheeler MB, Bos JL, Schwede F, Genieser HG and Holz GG (2003) Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca^{2+}-induced Ca^{2+} release and exocytosis in pancreatic beta-cells. *J Biol Chem* **278**:8279-8285.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE and Graybiel AM (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**:2275-2279.

Leech CA, Chepurny OG and Holz GG (2010a) Epac2-dependent rap1 activation and the control of islet insulin secretion by glucagon-like peptide-1. *Vitam Horm* **84**:279-302.

Leech CA, Dzhura I, Chepurny OG, Schwede F, Genieser HG and Holz GG (2010b) Facilitation of ss-cell K(ATP) channel sulfonylurea sensitivity by a cAMP analog selective for the cAMP-regulated guanine nucleotide exchange factor Epac. *Islets* **2**:72-81.

Li C, Bowe JE, Jones PM and Persaud SJ (2010) Expression and function of cannabinoid receptors in mouse islets. *Islets* **2**:293-302.

Li F and Zhang ZM (2009) Comparative identification of Ca^{2+} channel expression in INS-1 and rat pancreatic beta cells. *World J Gastroenterol* **15**:3046-3050.

Li L and Renier G (2009) The oral anti-diabetic agent, gliclazide, inhibits oxidized LDL-mediated LOX-1 expression, metalloproteinase-9 secretion and apoptosis in human aortic endothelial cells. *Atherosclerosis* **204**:40-46.

Lin M, Aladejebi O and Hockerman GH (2011) Distinct properties of amlodipine and nicardipine block of the voltage-dependent Ca^{2+} channels $Ca_v1.2$ and $Ca_v2.1$ and the mutant channels $Ca_v1.2$/Dihydropyridine insensitive and $Ca_v2.1$/Dihydropyridine sensitive. *Eur J Pharmacol* **670**:105-113.

Liu G, Jacobo SM, Hilliard N and Hockerman GH (2006) Differential modulation of $Ca_v1.2$ and $Ca_v1.3$-mediated glucose-stimulated insulin secretion by cAMP in INS-1 cells: distinct roles for exchange protein directly activated by cAMP 2 (Epac2) and protein kinase A. *J Pharmacol Exp Ther* **318**:152-160.

Liu K, Titus S, Southall N, Zhu P, Inglese J, Austin CP and Zheng W (2008) Comparison on functional assays for Gq-coupled GPCRs by measuring inositol monophospate-1 and intracellular calcium in 1536-well plate format. *Curr Chem Genomics* **1**:70-78.

Lynch CJ, Zhou Q, Shyng SL, Heal DJ, Cheetham SC, Dickinson K, Gregory P, Firnges M, Nordheim U, Goshorn S, Reiche D, Turski L and Antel J (2011) Some cannabinoid receptor ligands and their distomers are direct-acting openers of SUR1 K(ATP) channels. *Am J Physiol Endocrinol Metab* **302**:E540-551.

Mogami H, Zhang H, Suzuki Y, Urano T, Saito N, Kojima I and Petersen OH (2003) Decoding of Short-lived Ca2+ Influx Signals into Long Term Substrate Phosphorylation through Activation of Two Distinct Classes of Protein Kinase C. *J Biol Chem* **278**:9896-9904.

Oestreich EA, Malik S, Goonasekera SA, Blaxall BC, Kelley GG, Dirksen RT and Smrcka AV (2009) Epac and phospholipase Cepsilon regulate Ca^{2+} release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase II. *J Biol Chem* **284**:1514-1522.

Qian F, Huang P, Ma L, Kuznetsov A, Tamarina N and Philipson LH (2002) TRP genes: candidates for nonselective cation channels and store-operated channels in insulin-secreting cells. *Diabetes* **51 Suppl 1**:S183-189.

Renier G, Mamputu JC and Serri O (2003) Benefits of gliclazide in the atherosclerotic process: decrease in monocyte adhesion to endothelial cells. *Metabolism* **52**(8 Suppl 1):13-18.

Rorsman P and Trube G (1985) Glucose dependent K+-channels in pancreatic beta-cells are regulated by intracellular ATP. *Pflugers Arch* **405**:305-309.

Satoh J, Takahashi K, Takizawa Y, Ishihara H, Hirai M, Katagiri H, Hinokio Y, Suzuki S, Tsuji I and Oka Y (2005) Secondary sulfonylurea failure: comparison of period until insulin treatment between diabetic patients treated with gliclazide and glibenclamide. *Diabetes Res Clin Pract* **70**:291-297.

Schmid-Antomarchi H, De Weille J, Fosset M and Lazdunski M (1987) The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. *J Biol Chem* **262**:15840-15844.

Schramm TK, Gislason GH, Vaag A, Rasmussen JN, Folke F, Hansen ML, Fosbol EL, Kober L, Norgaard ML, Madsen M, Hansen PR and Torp-Pedersen C (2011) Mortality and cardiovascular risk associated with different insulin secretagogues compared with metformin in type 2 diabetes, with or without a previous myocardial infarction: a nationwide study. *Eur Heart J* **32**:1900-1908.

Seino S, Chen L, Seino M, Blondel O, Takeda J, Johnson JH and Bell GI (1992) Cloning of the alpha 1 subunit of a voltage-dependent calcium channel expressed in pancreatic beta cells. *Proc Natl Acad Sci USA* **89**:584-588.

Shibasaki T, Sunaga Y, Fujimoto K, Kashima Y and Seino S (2004) Interaction of ATP sensor, cAMP sensor, Ca2+ sensor, and voltage-dependent Ca^{2+} channel in insulin granule exocytosis. *J Biol Chem* **279**:7956-7961

Shibasaki T, Takahashi H, Miki T, Sunaga Y, Matsumura K, Yamanaka M, Zhang C, Tamamoto A, Satoh T, Miyazaki J and Seino S (2007) Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. *Proc Natl Acad Sci U S A* **104**:19333-19338.

Thore S, Dyachok O and Tengholm A (2004) Oscillations of Phospholipase C Activity Triggered by Depolarization and Ca^{2+} Influx in Insulin-secreting Cells. *J Biol Chem* **279**:19396-19400.

Van Obberghen E, Somers G, Devis G, Ravazzola M, Malaisse-Lagae F, Orci L and Malaisse WJ (1975) Dynamics of insulin release and microtubular-microfilamentous system. VII. Do microfilaments provide the motive force for the translocation and extrusion of beta granules? *Diabetes* **24**:892-901.

Wang Y, Sugita S and Sudhof TC (2000) The RIM/NIM family of neuronal C2 domain proteins. Interactions with Rab3 and a new class of Src homology 3 domain proteins. *J Biol Chem* **275**:20033-20044.

Wollheim CB, Blondel B, Trueheart PA, Renold AE and Sharp GW (1975) Calcium-induced insulin release in monolayer culture of the endocrine pancreas. Studies with ionophore A23187. *J Biol Chem* **250**:1354-1360.

Yada T, Hamakawa N and Yaekura K (1995) Two distinct modes of Ca^{2+} signalling by ACh in rat pancreatic beta-cells: concentration, glucose dependence and Ca^{2+} origin. *J Physiol* **488**:13-24.

Zhang CL, Katoh M, Shibasaki T, Minami K, Sunaga Y, Takahashi H, Yokoi N, Iwasaki M, Miki T and Seino S (2009) The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. *Science* **325**:607-610.

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Footnotes

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Figure Legends

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

Figure 2. ***Modulation of tolbutamide and gliclazide-stimulated insulin secretion and Ca^{2+} transients by nicardipine and thapsigargin- A***, Insulin secretion stimulated by either 20 μM gliclazide (white bars) or 200 μM tolbutamide (black bars) is completely inhibited by 2 μM nicardipine. ***B***, Insulin secretion stimulated by 20 μM gliclazide (white bars) is completely inhibited, and secretion stimulated by 200 μM tolbutamide (black bars) is partially inhibited by 1 μM thapsigargin. ***, $P < 0.001$, **, $P < 0.01$, * $P < 0.05$ compared to basal; †††, $P < 0.001$, ††, $P < 0.01$, † $P < 0.05$ compared to nicardipine or thapsigargin. **C-F**, Increases in intracellular Ca^{2+} concentration are shown as increases in the ratio of intensity of fluorescence at 510 nm excited at 340nm or 380nm (340nm/380nm ratio). Black circles represent the response to 200 μM tolbutamide (***C&E)*** or 20 μM gliclazide (***D&F)***. Data shown were corrected by subtracting the increase in 340nm/380nm ratio observed upon addition of KRBH buffer alone. White circles in

(***C&E***) and (***D&F***) represent the portion of the response to tolbutamide or gliclazide, respectively, inhibited by the indicated agent. 2 μM nicardipine completely inhibited the Ca^{2+} transient stimulated by either tolbutamide (***C***) or gliclazide (***E***). Pre-incubation with 2 μM thapsigargin (1 μM final concentration) selectively inhibited the rapid peak of the response stimulated by either tolbutamide (***D***) or gliclazide (***F***). Data shown are the mean ± SE of at least 3 representative experiments performed in quadruplicate.

Figure 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 μM tolbutamide and 20 μM gliclazide both stimulated insulin secretion that was significantly potentiated by 8-pCPT-2'-O-Me-cAMP-AM (ESCA) at concentrations $\geq$ 2 μM. ††, $P < 0.01$, †, $P < 0.05$ compared to sulfonylurea and sulfonylurea + all concentrations of 8-pCPT-2'-O-Me-cAMP-AM; ***, $P < 0.001$, *, $P < 0.05$ compared to 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 μM). ***, $P < 0.001$ compared to sulfonylurea alone; Sulfonylurea and ESCA +/- 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 μM 8-pCPT-2'-O-Me-cAMP-AM is completely blocked by the L-type Ca^{2+} channel blocker nicardipine (2 μM) ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ compared to basal; †††, $P < 0.001$ compared to 8-pCPT-2'-O-Me-cAMP-AM + sulfonylurea. (n = 9 in 3 independent experiments)

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Figure 4. ***Potentiation of Ca^{2+} transients by the EPAC-selective cAMP analog (ESCA) 8-pCPT-2'-O-Me-cAMP-AM- A-B***, Increase in fura2 340nm/380nm ratio in response to application of 200 μM tolbutamide (***A***) or 20 μM gliclazide (***B***) to INS-1 cells pre-incubated with KRBH buffer only (black circles), 1 μM (white circles), or 5 μM (black triangles) 8-pCPT-2'-O-Me-cAMP-AM (ESCA). Data shown are mean ± SE from a representative experiment done in quadruplicate, and were corrected by subtracting the change in 340nm/380nm ratio stimulated by injection of KRBH buffer only on to cells given the same pre-treatment. ***C-D***, Area under the curve analysis of the Ca^{2+} transients stimulated by tolbutamide or gliclazide alone, or in the presence of 1 or 5 μM 8-pCPT-2'-O-Me-cAMP-AM. The Ca^{2+} integrals for the entire post-stimulation period with pre-incubations of 1 μM or 5 μM 8-pCPT-2'-O-Me-cAMP-AM for three separate experiments were normalized to the Ca^{2+} integral for tolbutamide (***C***) or gliclazide (***D***) in cells pre-incubated with KRBH buffer alone. The Ca^{2+} integral for 5μM 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).

Figure 5. ***Stimulation of phospholipase C activity by carbachol and the sulfonylureas tolbutamide and gliclazide-*** ***A***, 500 μM carbachol stimulated significant accumulation of IP_1 over basal levels in INS-1 cells that was completely blocked by either 100 μM atropine or 10 μM U71322, but not 10 μM U73343. ***B-C***, 200 μM tolbutamide (***B***) or 20 μM gliclazide (***C***) stimulated significant IP_1 accumulation over basal levels in INS-1 cells that was completely blocked by 10 μM U71322, but not 10 μM U73343. ***D-F***, IP_1 accumulation stimulated by 500 μM carbachol (***D***), 200 μM tolbutamide (***E***), or 20 μM gliclazide (***F***) in the presence (white bars) or absence (black bars) of 300 μM diazoxide. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ compared to basal; †††, $P < 0.001$, ††, $P < 0.01$, †, $P < 0.05$ compared to indicated stimulus in the absence of

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diazoxide; ###, $P < 0.001$, #, $P < 0.05$ compared to carbachol alone. **G**, Representative whole-cell current clamp traces of tolbutamide (200 μM) or gliclazide (20 μM)-induced membrane depolarization and reversal by diazoxide. Solid lines represent the time during which the indicated sulfonylurea was applied to the cell. Bold dashed lines represent the time during which 300 μM diazoxide was applied to the cell. The thin dashed line represents membrane potential of the cell before perfusion of sulfonylureas. ***H***, Summarized data comparing the resting membrane potential (Con), the membrane potential in the presence of the indicated sulfonylurea (SU), and membrane potential in the presence of sulfonylurea and 300 μM diazoxide (SU+Diaz). Tolbutamide: Con = -67.9 ± 2 mV; SU = -34.3 ± 5 mV; DU + Diaz = -67.3 ± 2 mV (*n*=5-6). Gliclazide: Con = -67.4 ± 3 mV; SU = -31.5 ± 5 mV; DU + Diaz = -64.8 ± 2 mV (*n* = 5-6). ****P< 0.001* compared to control or SU + Diaz.

Figure 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 μM gliclazide (Glz) significantly stimulates IP_1 accumulation in the presence of 300 μM diazoxide (Diaz). In cells pre-treated 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 to control cells. Data shown are mean ± S.E. from four separate experiments done in triplicate. (***, $P < 0.001$ compared to the corresponding control; †, $P < 0.05$ compared to Glz + Diaz without PTx pre-treatment). ***B***, Representative experiment illustrating the transient rise in intracellular Ca^{2+} concentration in INS-1 cells stimulated by 20 μM Glz in the absence (black circles) or presence (white circles) of 300 μM 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

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experiments). ***C-D***, Increase in fura2 340nm/380nm ratio in response to application of 200 μM tolbutamide (***C***) or 20 μM gliclazide (***D***) to INS-1 cells pre-incubated with KRBH only (black circles). White circles in (***C***) and (***D***) represent the portion of the response to Tolb or Glz, respectively, inhibited by pre-incubation with 20 μM ryanodine (Ryn). Pre-incubation with Ryn completely inhibited the early peak of the transient stimulated by Tolb (***C***) but not Glz (***D***). Data shown are mean ± SE of representative experiments performed in quadruplicate. ***E,*** Removal of extracellular Ca^{2+} reduces, but does not eliminate, the Ca^{2+} transient stimulated by 20 μM Glz or 20 μM Glz + 20 μM Ryn. In experiments labeled 0 Ca^{2+}, KRBH with no added Ca^{2+} was used. Data shown are mean ± SE 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 340nm/380nm ratio in response to application of 20 μM gliclazide to INS-1 cells pre-incubated with KRBH only (black circles), 20 μM Ryn (white circles) or 20 μM Ryn after an 18 hour pre-treatment with PTx (gray diamonds). Data from cells pre-treated with PTx only was omitted for clarity. Data shown are mean ± SE, and are representative of 6 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).

Figure 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

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using whole-cell current clamp during application of tolbutamide or gliclazide in the presence (black bars) or absence (gray bars) of 5 μM 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 minutes 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 I_{Ba} density (pA/pF) measured at 0 mV from a holding potential of -70 mV using whole-cell voltage clamp. 5 μM 8-pCPT-2'-O-Me-cAMP 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 I_{Ba} 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 μM 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 minutes 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 I_{Ba} measured at 0 mV, form a holding potential of -70 mV, with 5 μM of 8-pCPT-2'-O-Me-cAMP at 3 minutes after break-in (control) or 10 minutes after break-in in the same INS-1 cell.

Figure 8. The EPAC-selective cAMP analog *(ESCA) 8-pCPT-2'-O-Me-cAMP stimulates phospholipase C activity and a slow, persistent rise in intracellular Ca^{2+} in INS-1 cells- A*, 8-pCPT-2'-O-Me-cAMP-AM significantly stimulates IP_1 accumulation at 50 μM, but not at 1,5,10,

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or 20 μM in INS-1 cells (black bars). A positive control for phospholipase C activation, carbachol (500 μM), stimulates IP_1 accumulation that is completely blocked by 100 μM atropine. ***, $P < 0.001$ compared to basal (One-way ANOVA with Tukey's post-hoc test). ***B***, Representative experiments showing fura2 340nm/380nm ratio over a ten minute interval following application of 2,5,25 or 50 μM 8-pCPT-2'-O-Me-cAMP-AM (n=2-7 in 7 independent experiments). Concentrations as low as 2 μM 8-pCPT-2'-O-Me-cAMP-AM displayed a sustained rise in intracellular Ca^{2+}. At 7minutes post injection, the Δ340nm/380nm ratio was significantly different from KRBH injection for all concentrations of 8-pCPT-2'-O-Me-cAMP-AM (2 μM, $P < 0.05$; 5 μM, $P < 0.05$; 25 μM, $P < 0.001$; 50 μM, $P < 0.001$). ***C***, Area under the curve analysis of Ca^{2+} transients stimulated by 5 μM 8-pCPT-2'-O-Me-cAMP-AM alone, in the presence of 25 μM 2-APB, or in the absence of extracellular Ca^{2+}. Data from 3-4 separate experiments performed in quadruplicate are shown as mean ± SE. ***, $P < 0.001$ compared to 8-pCPT-2'-O-Me-cAMP-AM stimulation alone. ***D,*** 25 μM 2-APB markedly reduces potentiation of tolbutamide-stimulated insulin secretion by 5 μM 8-pCPT-2'-O-Me-cAMP-AM. Data were normalized to secretion stimulated by 200 μM tolbutamide alone. Data shown are mean ± SE of three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, compared to tolbutamide + 2-APB; $^{###}$, $P<0.001$, compared to tolbutamide alone; †††, $P<0.001$ compared to tolbutamide + ESCA + 2-APB.

Figure 9. ***Tolbutamide action is enhanced by the PKC inhibitor bisindolylmaleimide I. A-B,*** Increase in fura2 340nm/380nm ratio in response to application of 200 μM tolbutamide (***A***) to INS-1 cells pre-incubated with KRBH buffer only (white diamonds), 5 μM 8-pCPT-2'-O-Me-cAMP-AM (grey squares), 1 μM bisindolylmaleimide (BIS)(black triangles) or both 5 μM 8-pCPT-2'-O-Me-cAMP-AM (ESCA) and 1 μM BIS (white circles)***.*** Data shown are mean ± SE from a representative experiment done in quadruplicate (**A**). Area under the curve analysis of Ca^{2+} transients (**B**) that were normalized to the Ca^{2+} integrals for tolbutamide in the absence of

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any treatment. The Ca^{2+} integrals for 200 μM tolbutamide plus 5 μM 8-pCPT-2'-O-Me-cAMP-AM, or 1 μM bisindolylmaleimide, or both 5 μM 8-pCPT-2'-O-Me-cAMP-AM and 1 μM bisindolylmaleimide were each significantly greater than tolbutamide alone. Data shown are mean ± SE for three separate experiments (n= 9). ***, $P < 0.001$, **, $P < 0.01$. ***C***, Potentiation of tolbutamide-stimulated insulin secretion by 1 μM BIS. Insulin secretion stimulated by 200 μM tolbutamide in the presence of 1 μM BIS was significantly greater than that stimulated by 200 μM tolbutamide alone. Insulin secretion in the presence of 1 μM BIS alone was not different from basal. Data shown are mean ± SE for three separate experiments (n= 9). ***, $P < 0.001$, **, $P < 0.01$ compared to basal; †††, $P < 0.001$, ††, $P < 0.01$ compared to BIS alone; $^{\#\#}$, $P < 0.01$ compared to tolbutamide alone. ***D***, Insulin secretion stimulated by 200 μM tolbutamide + 1 μM BIS is further increased by 5 μM 8-pCPT-2'-O-Me-cAMP-AM. Data shown are mean ± SE for three independent experiments. ***, $P < 0.001$, compared to tolbutamide alone; ††, $P < 0.01$ compared to tolbutamide + BIS.

Figure 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-ε 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.

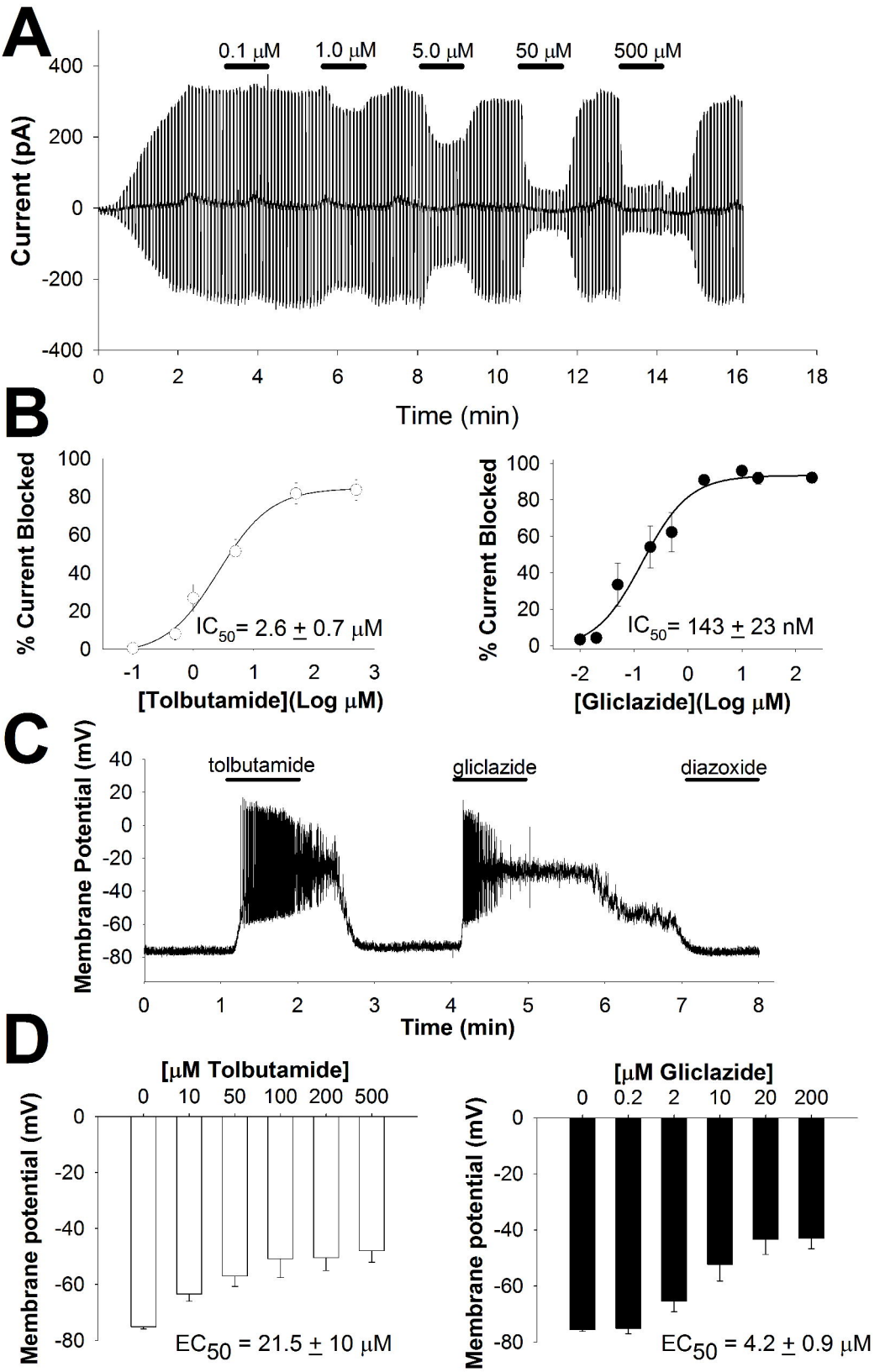
A
0.1 μM
1.0 μM
5.0 μM
50 μM
500 μM
Current (pA)
Time (min)
B
% Current Blocked
IC50= 2.6 ± 0.7 μM
[Tolbutamide](Log μM)
% Current Blocked
IC50= 143 ± 23 nM
[Gliclazide](Log μM)
C
tolbutamide
gliclazide
diazoxide
Membrane Potential (mV)
Time (min)
D
[μM Tolbutamide]
Membrane potential (mV)
EC50 = 21.5 ± 10 μM
[μM Gliclazide]
Membrane potential (mV)
EC50 = 4.2 ± 0.9 μM

Figure 1

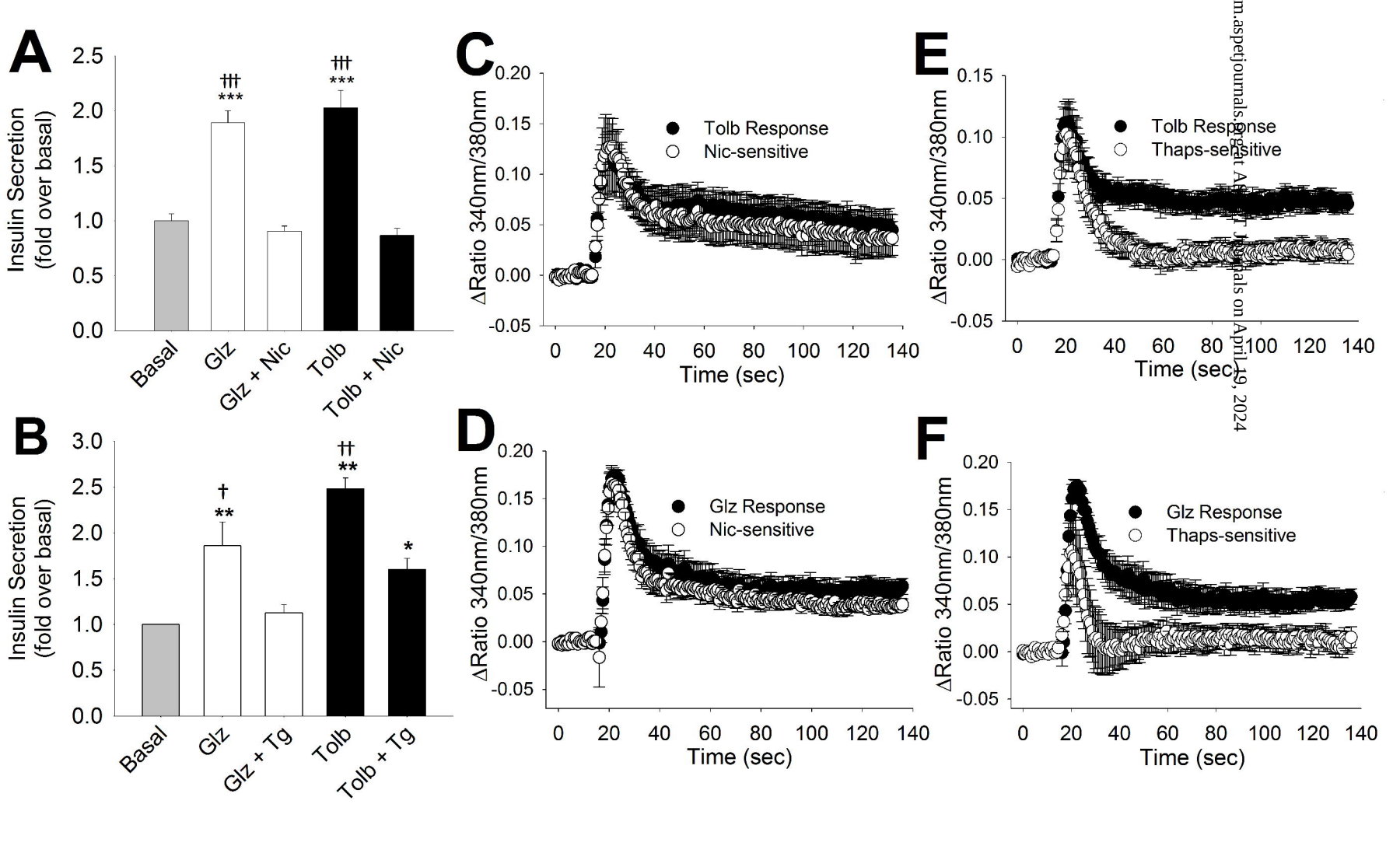

Figure 2

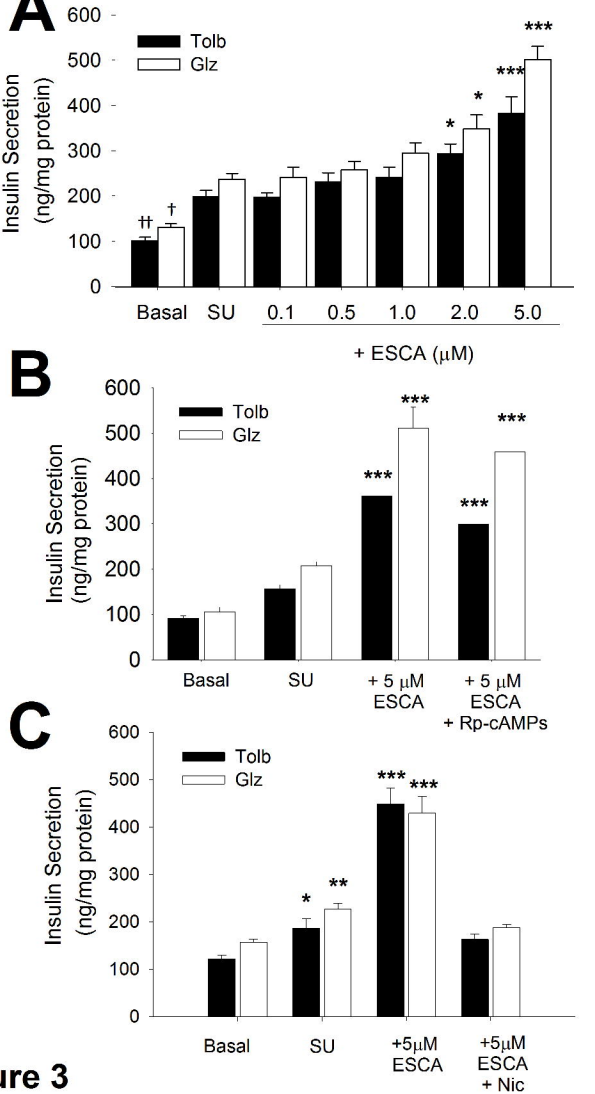
A
Insulin Secretion (ng/mg protein)
600
500
400
300
200
100
0
Tolb
Glz
††
†
*
*
***
***
Basal
SU
0.1
0.5
1.0
2.0
5.0
+ ESCA (μM)
B
Insulin Secretion (ng/mg protein)
Tolb
Glz
***
***
***
***
Basal
SU
+ 5 μM ESCA
+ 5 μM ESCA + Rp-cAMPs
C
Insulin Secretion (ng/mg protein)
Tolb
Glz
*
**
***
***
Basal
SU
+5μM ESCA
+5μM ESCA + Nic

Figure 3

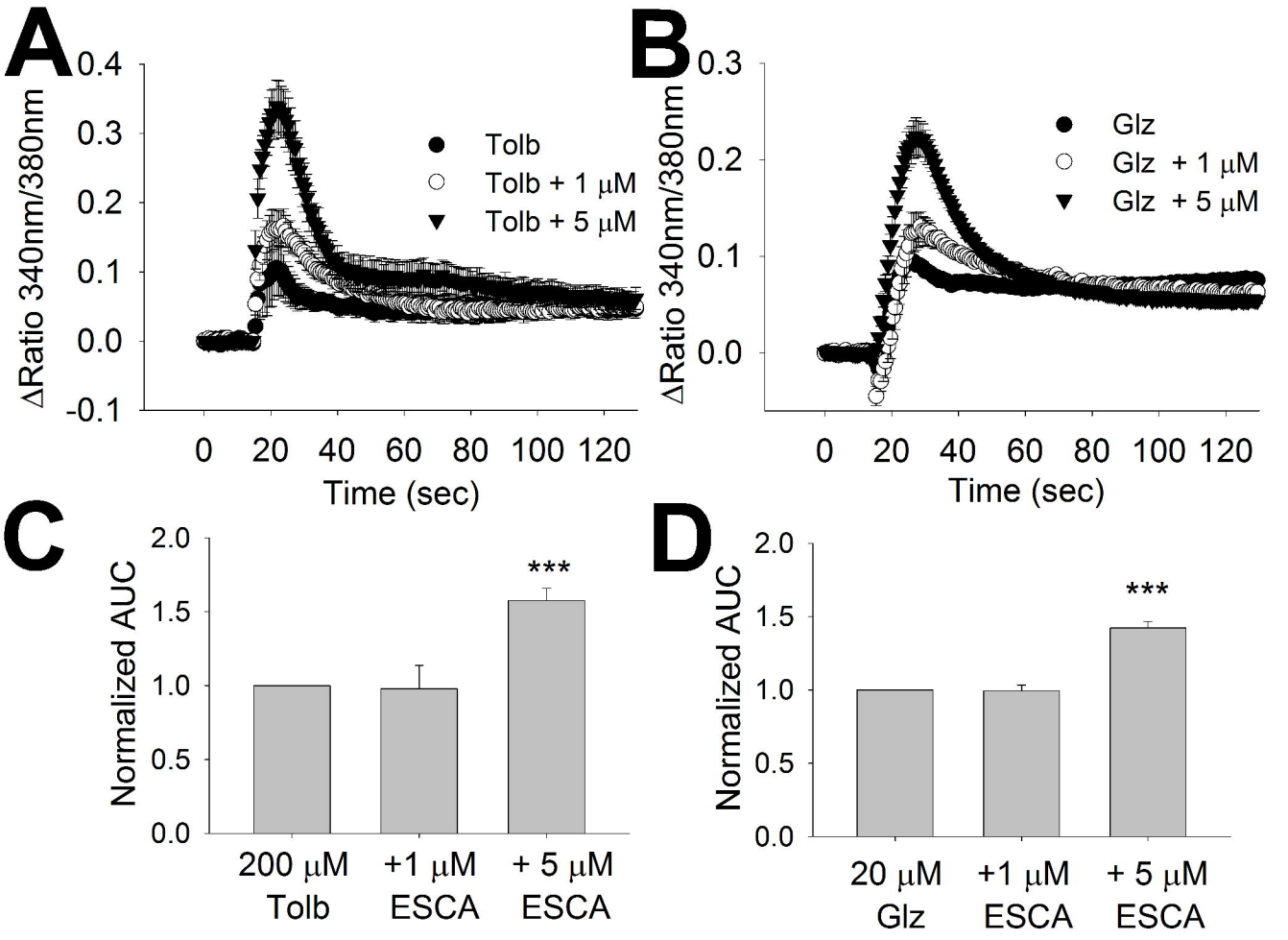
A
ΔRatio 340nm/380nm
0.4
0.3
0.2
0.1
0.0
-0.1
Tolb
Tolb + 1 μM
Tolb + 5 μM
0 20 40 60 80 100 120
Time (sec)
B
ΔRatio 340nm/380nm
0.3
0.2
0.1
0.0
Glz
Glz + 1 μM
Glz + 5 μM
0 20 40 60 80 100 120
Time (sec)
C
Normalized AUC
2.0
1.5
1.0
0.5
0.0
***
200 μM Tolb
+1 μM ESCA
+ 5 μM ESCA
D
Normalized AUC
2.0
1.5
1.0
0.5
0.0
***
20 μM Glz
+1 μM ESCA
+ 5 μM ESCA

Figure 4

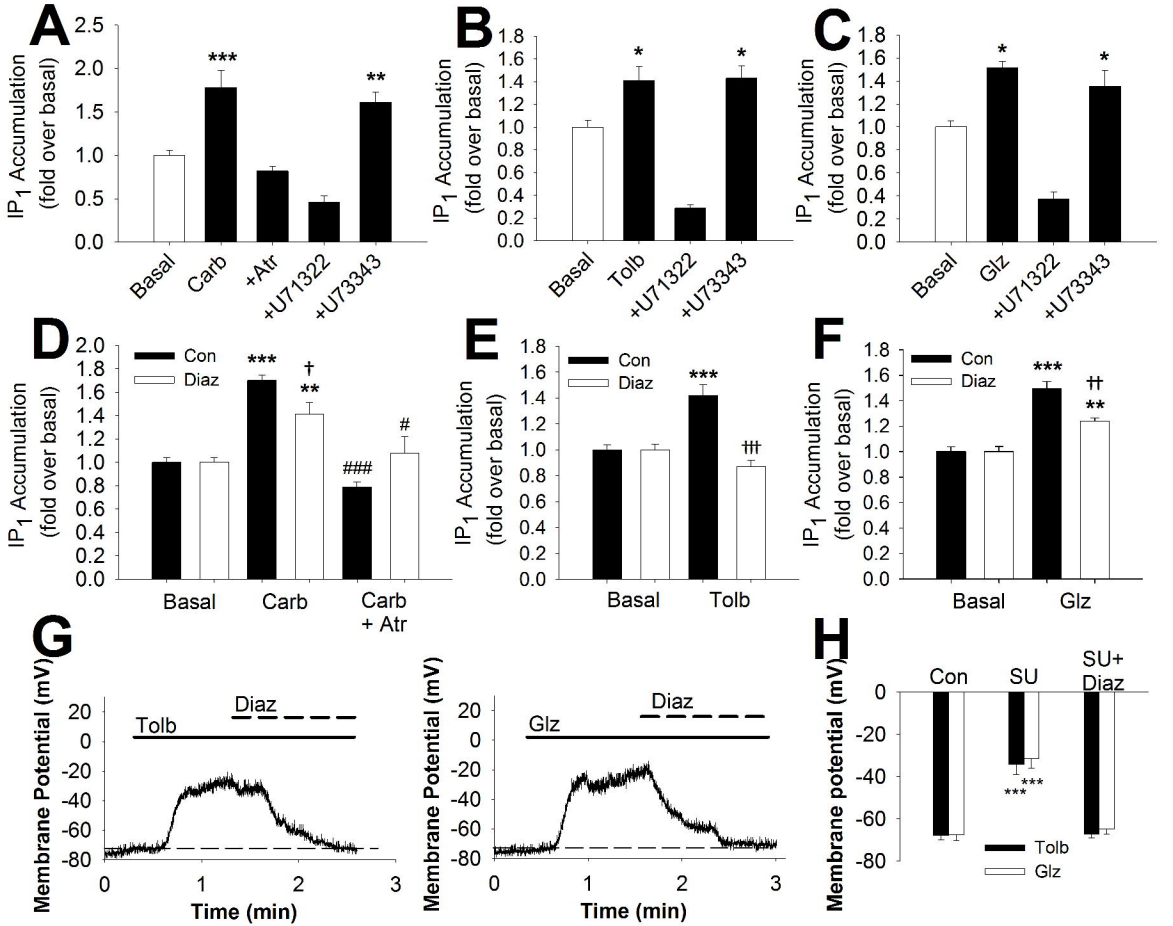
A
B
C
D
E
F
G
H
IP1 Accumulation (fold over basal)
Basal
Carb
+Atr
+U71322
+U73343
Tolb
Glz
Con
Diaz
Carb + Atr
Membrane Potential (mV)
Time (min)
Diaz
Tolb
Glz
Con
SU
SU+ Diaz
Membrane potential (mV)

Figure 5

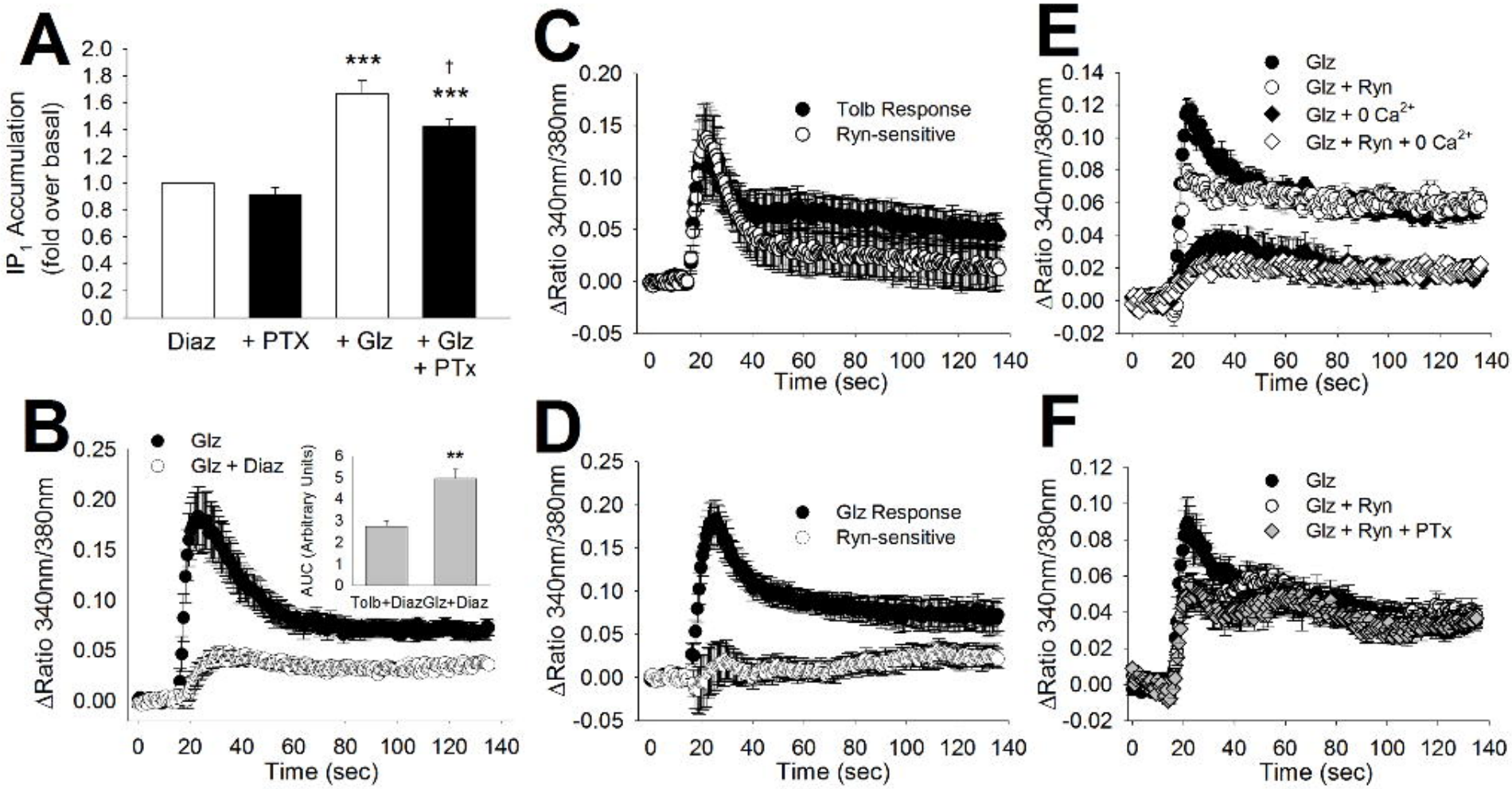
A
IP1 Accumulation (fold over basal)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
†
***
Diaz
+ PTX
+ Glz
+ Glz + PTx
B
Glz
Glz + Diaz
ΔRatio 340nm/380nm
0.25
0.20
0.15
0.10
0.05
0.00
AUC (Arbitrary Units)
6
5
4
3
2
1
0
**
Tolb+Diaz
Glz+Diaz
0 20 40 60 80 100 120 140
Time (sec)
C
Tolb Response
Ryn-sensitive
ΔRatio 340nm/380nm
0.20
0.15
0.10
0.05
0.00
-0.05
0 20 40 60 80 100 120 140
Time (sec)
D
Glz Response
Ryn-sensitive
ΔRatio 340nm/380nm
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0 20 40 60 80 100 120 140
Time (sec)
E
Glz
Glz + Ryn
Glz + 0 Ca2+
Glz + Ryn + 0 Ca2+
ΔRatio 340nm/380nm
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
0 20 40 60 80 100 120 140
Time (sec)
F
Glz
Glz + Ryn
Glz + Ryn + PTx
ΔRatio 340nm/380nm
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
0 20 40 60 80 100 120 140
Time (sec)

Figure 6

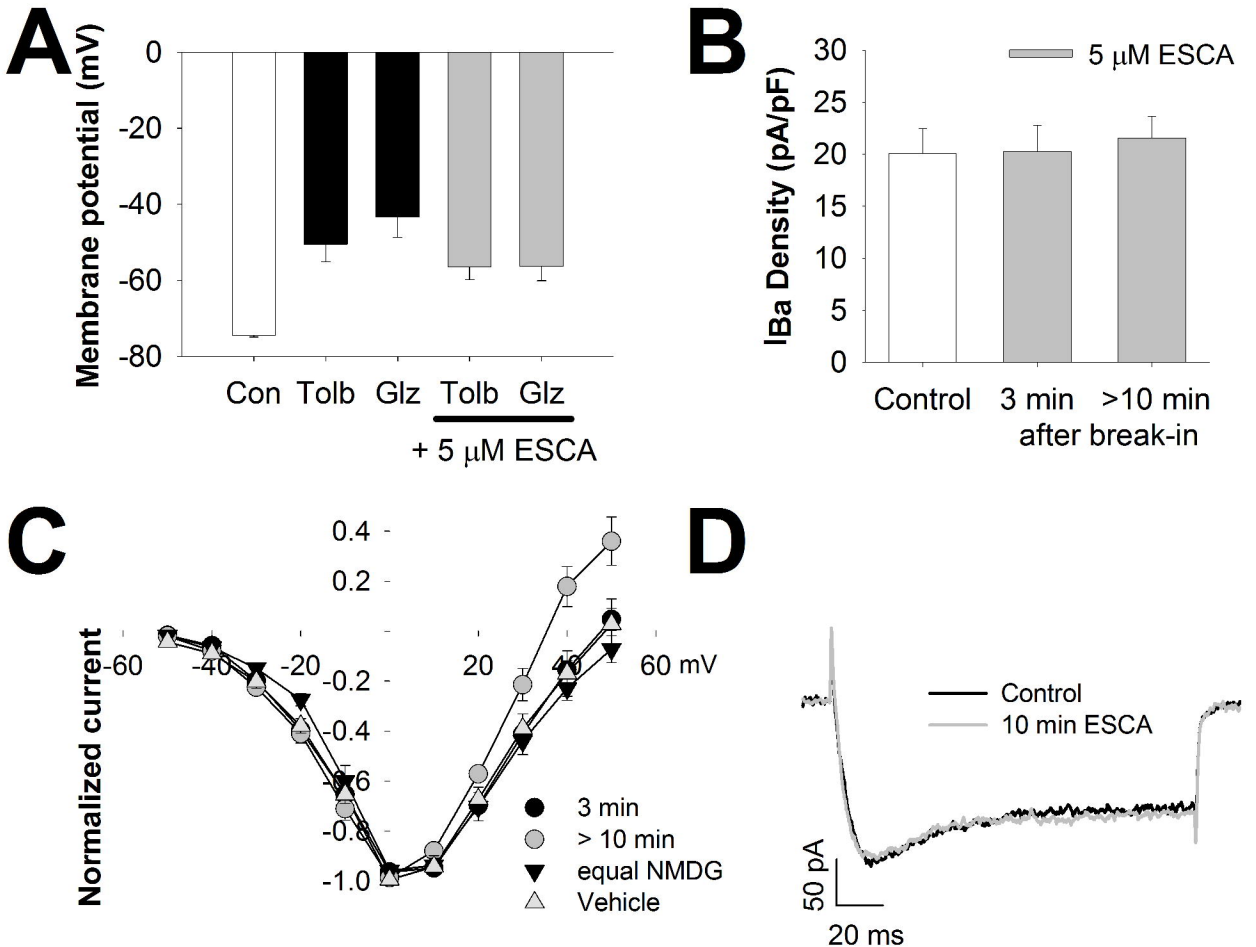
A
Membrane potential (mV)
0
-20
-40
-60
-80
Con
Tolb
Glz
Tolb
Glz
+ 5 μM ESCA
B
5 μM ESCA
IBa Density (pA/pF)
30
25
20
15
10
5
0
Control
3 min
>10 min
after break-in
C
Normalized current
0.4
0.2
-0.2
-0.4
-1.0
-60
-40
-20
20
60 mV
3 min
> 10 min
equal NMDG
Vehicle
D
Control
10 min ESCA
50 pA
20 ms

Figure 7

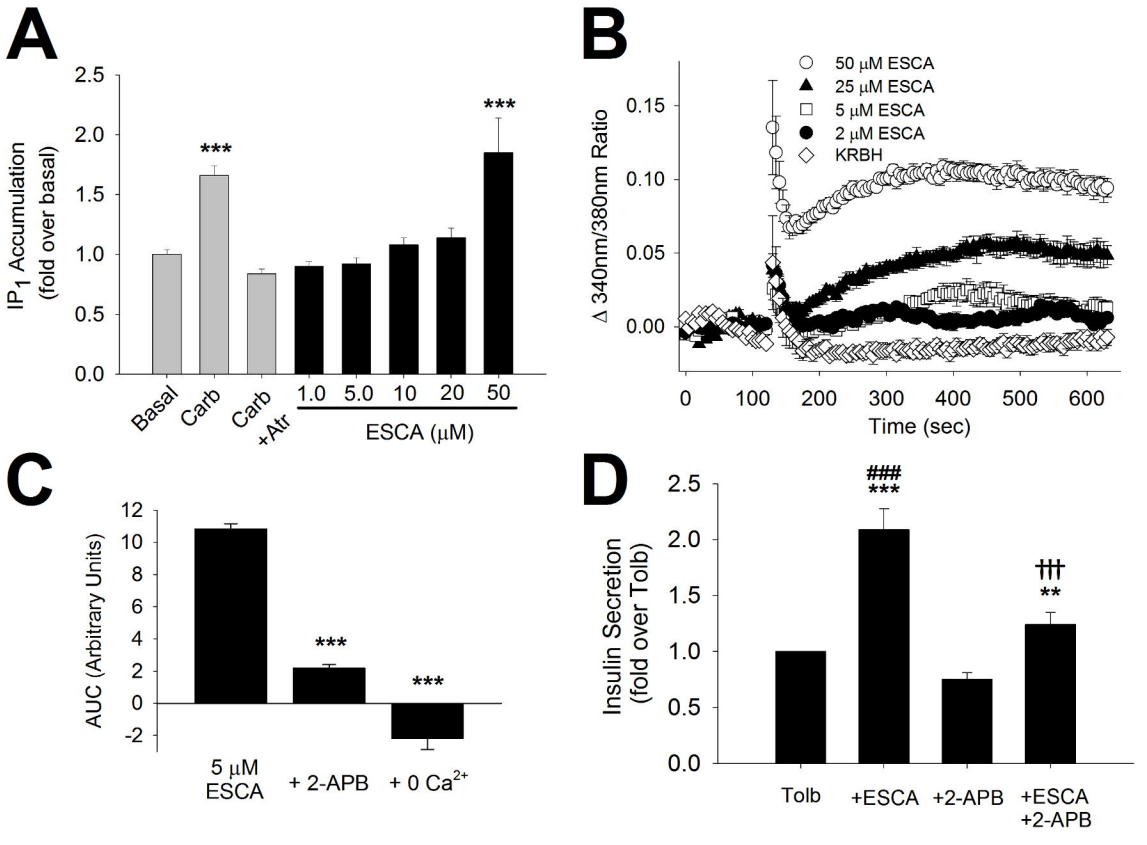
A
IP1 Accumulation (fold over basal)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Basal
Carb
Carb +Atr
1.0
5.0
10
20
50
ESCA (μM)
B
50 μM ESCA
25 μM ESCA
5 μM ESCA
2 μM ESCA
KRBH
Δ 340nm/380nm Ratio
0.15
0.10
0.05
0.00
0
100
200
300
400
500
600
Time (sec)
C
AUC (Arbitrary Units)
12
10
8
6
4
2
0
-2
***
***
5 μM ESCA
+ 2-APB
+ 0 Ca2+
D
Insulin Secretion (fold over Tolb)
2.5
2.0
1.5
1.0
0.5
0.0
###
***
††
**
Tolb
+ESCA
+2-APB
+ESCA +2-APB

Figure 8

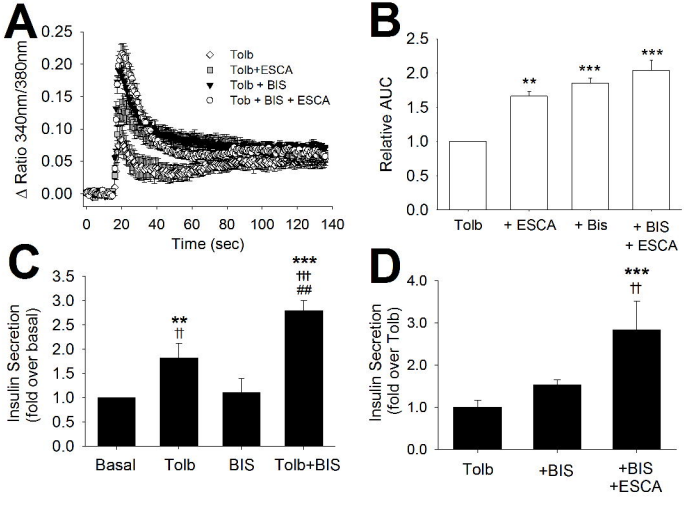
A
Δ Ratio 340nm/380nm
Time (sec)
Tolb
Tolb+ESCA
Tolb + BIS
Tob + BIS + ESCA
B
Relative AUC
Tolb
+ ESCA
+ Bis
+ BIS
+ ESCA
C
Insulin Secretion
(fold over basal)
Basal
Tolb
BIS
Tolb+BIS
D
Insulin Secretion
(fold over Tolb)
Tolb
+BIS
+BIS
+ESCA

Figure 9

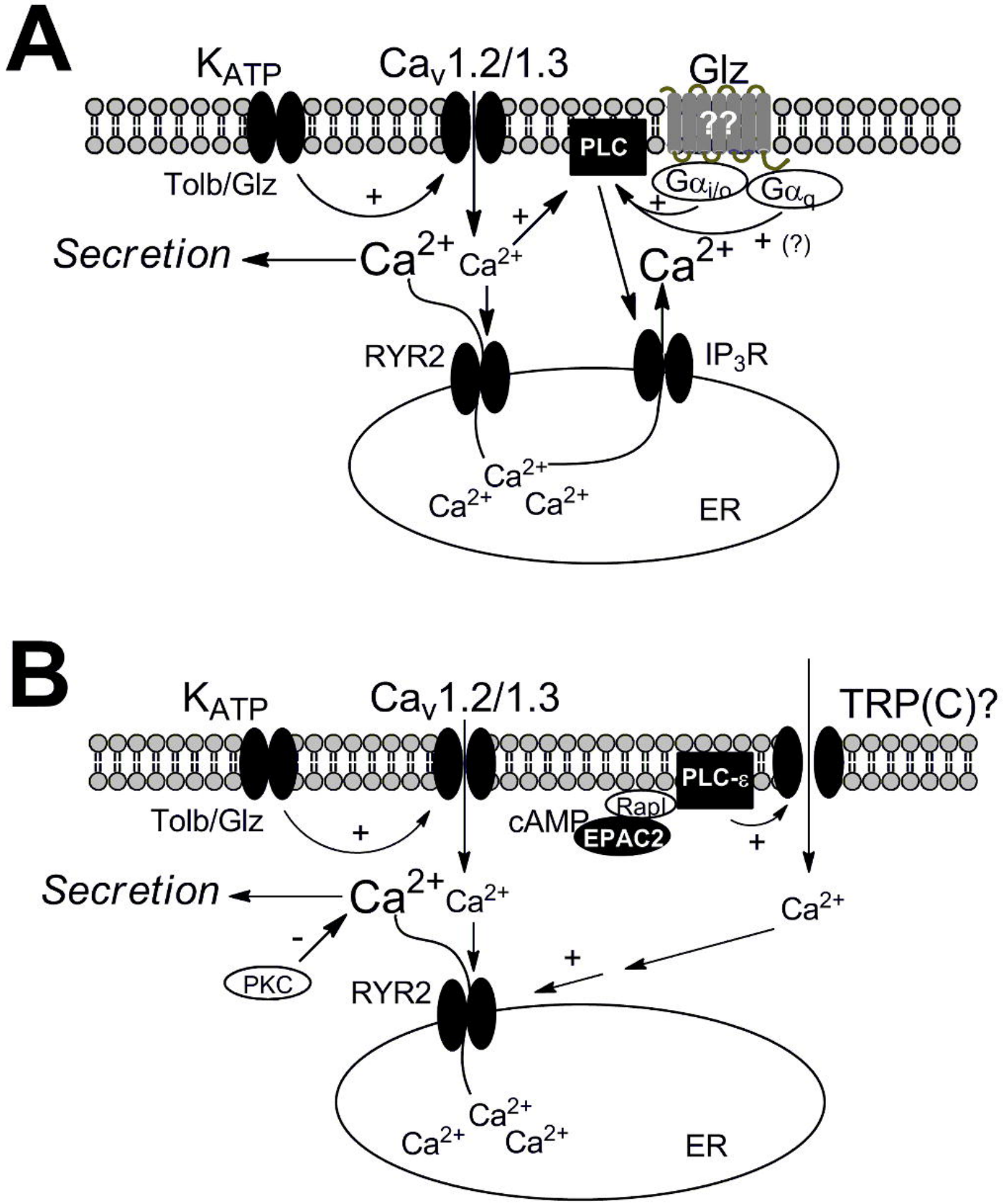
A
K_{ATP}
$Ca_V1.2/1.3$
Glz
??
PLC
Tolb/Glz
+
$G\alpha_{i/o}$
$G\alpha_q$
+
+ (?)
Secretion
Ca^{2+}
Ca^{2+}
Ca^{2+}
RYR2
IP_3R
Ca^{2+}
Ca^{2+}
Ca^{2+}
ER
B
K_{ATP}
$Ca_V1.2/1.3$
TRP(C)?
PLC-ε
Rapl
Tolb/Glz
+
cAMP
EPAC2
+
Secretion
Ca^{2+}
Ca^{2+}
Ca^{2+}
-
+
PKC
RYR2
Ca^{2+}
Ca^{2+}
Ca^{2+}
ER

Figure 10