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Facilitation of β-cell K_{ATP} channel sulfonylurea sensitivity by a cAMP analog selective for the cAMP-regulated guanine nucleotide exchange factor Epac

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Keywords: K-ATP channel, Epac, cAMP, sulfonylurea, GLP-1, drug interaction

Abbreviations: GLP-1, glucagon-like peptide-1; K_{ATP} channel, ATP-sensitive K^+ channel; PKA, protein kinase A; Epac, exchange protein directly activated by cAMP; ESCA, Epac-selective cAMP analog; PIP_2, phosphatidylinositol 4,5-bisphosphate; SES, standard extracellular solution; PLC, phospholipase C; FRET, Förster resonance energy transfer

Clinical studies demonstrate that combined administration of sulfonylureas with exenatide can induce hypoglycemia in type 2 diabetic subjects. Whereas sulfonylureas inhibit β-cell K_{ATP} channels by binding to the sulfonylurea receptor-1 (SUR1), exenatide binds to the GLP-1 receptor, stimulates β-cell cAMP production and activates both PKA and Epac. In this study, we hypothesized that the adverse in vivo interaction of sulfonylureas and exenatide to produce hypoglycemia might be explained by Epac-mediated facilitation of K_{ATP} channel sulfonylurea sensitivity. We now report that the inhibitory action of a sulfonylurea (tolbutamide) at K_{ATP} channels was facilitated by 2'-*O*-Me-cAMP, a selective activator of Epac. Thus, under conditions of excised patch recording, the dose-response relationship describing the inhibitory action of tolbutamide at human β-cell or rat INS-1 cell K_{ATP} channels was left-shifted in the presence of 2'-*O*-Me-cAMP, and this effect was abolished in INS-1 cells expressing a dominant-negative Epac2. Using an acetoxymethyl ester prodrug of an Epac-selective cAMP analog (8-pCPT-2'-*O*-Me-cAMP-AM), the synergistic interaction of an Epac activator and tolbutamide to depolarize INS-1 cells and to raise $[Ca^{2+}]_i$ was also measured. This effect of 8-pCPT-2'-*O*-Me-cAMP-AM correlated with its ability to stimulate phosphatidylinositol 4,5-bisphosphate hydrolysis that might contribute to the changes in K_{ATP} channel sulfonylurea-sensitivity reported here. On the basis of such findings, we propose that the adverse interaction of sulfonylureas and exenatide to induce hypoglycemia involves at least in part, a functional interaction of these two compounds to close K_{ATP} channels, to depolarize β-cells and to promote insulin secretion.

Introduction

Sulfonylureas such as tolbutamide and glyburide are orally-administrable insulin secretagogues prescribed for the treatment of type 2 diabetes. Both are known to require careful dosing in order to reduce the risk of hypoglycemia in vivo.[1] Exenatide, a synthetic version of exendin-4, is an agonist at the glucagon-like peptide-1 (GLP-1) receptor,[2] and it is a safer means by which to lower levels of blood glucose since it has a reduced ability to induce hypoglycemia.[3] Clinical studies in which sulfonylureas were paired with GLP-1 or exenatide revealed a surprising increase in the incidence of hypoglycemia.[4] The objective of the present study was to investigate a potential molecular mechanism of ATP-sensitive K^+ channel (K_{ATP} channel) regulation that might explain this adverse drug interaction.

The sulfonylurea receptor-1 (SUR1) expressed in pancreatic β-cells[5] oligomerizes with a pore-forming subunit (Kir6.2) to assemble functional K_{ATP} channels.[6] K_{ATP} channels are the predominant regulators of the β-cell resting membrane potential, and a reduced activity of K_{ATP} channels leads to depolarization, Ca^{2+} influx, and insulin secretion.[7] In the β-cells of healthy individuals, K_{ATP} channel activity is inhibited by glucose metabolism which increases the cytosolic [ATP]/[ADP] ratio. However, in type 2 diabetes the coupling of glucose metabolism to K_{ATP} channel inhibition is defective and a loss of glucose-stimulated insulin secretion (GSIS) is observed.[8] Under such conditions, the therapeutic utility of sulfonylureas derives from their ability to inhibit K_{ATP} channel activity independently of glucose metabolism.[9]

Stimulation of the GLP-1 receptor (GLP-1R) by GLP-1 or exenatide increases cAMP production in β-cells,[10, 11] thereby

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activating protein kinase A (PKA) and the exchange proteins directly activated by cAMP (Epac).[12] Although PKA was previously implicated in the regulation of K_{ATP} channels [13] and also voltage-dependent K^+ channels[14] in β-cells, new findings demonstrate that Epac proteins, including the isoform known as Epac2, couple cAMP production to K_{ATP} channel inhibition and the potentiation of GSIS from β-cells. For example, a cAMP analog that is a selective activator of Epac potentiates GSIS from mouse islets,[15] and this effect is attributable, at least in part, to its ability to inhibit the activity of β-cell K_{ATP} channels that are also inhibited by GLP-1.[16-18] The cAMP analogs that produce these effects in β-cells are designated as Epac-selective cAMP analogs (ESCAs). They include 2'-*O*-Me-cAMP, 8-pCPT-2'-*O*-Me-cAMP, and the acetoxymethyl ester "prodrug" of 8-pCPT-2'-*O*-Me-cAMP that is highly effective as an insulin secretagogue.[15]

The ability of ESCAs to inhibit K_{ATP} channel activity can be explained by a model in which these compounds activate Epac2 that is in association with nucleotide-binding fold-1 (NBF-1) of the K_{ATP} channel's SUR1 subunit.[6,17,19] In the model we have proposed,[20] activated Epac proteins promote Rap GTPase-dependent stimulation of phospholipase C-epsilon (PLCε),[21] with concomitant depletion of plasma membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP_2) located at the K_{ATP} channels. Since PIP_2 stimulates K_{ATP} channel activity,[22,23] ESCAs may act through Epac2, Rap, and PLCε to counteract this effect. Given that PIP_2 also decreases the apparent affinity of K_{ATP} channels for ATP,[22, 23] this model is fully consistent with a recent study demonstrating that Epac activators increase the K_{ATP} channel's apparent affinity for ATP in human β-cells and rat INS-1 cells.[18] Interestingly, one prediction of this model is that Epac2 activation should also increase K_{ATP} channel sulfonylurea sensitivity, and this prediction is tested in the present study. Our findings lead us to conclude that Epac2 activation does in fact increase the sulfonylurea sensitivity of K_{ATP} channels, thereby offering one simple explanation for how a cAMP-elevating agent such as exenatide interacts in vivo with sulfonylureas to depolarize β-cells, to induce excessive insulin secretion, and to promote hypoglycemia.[24]

Results

2'-O-Me-cAMP sensitizes K_{ATP} channels to the inhibitory action of tolbutamide in rat INS-1 cells. K_{ATP} channel activity was monitored in inside-out patches excised from INS-1 cells under conditions in which the bath solution contained Mg^{2+} but no ATP or ADP. Application of tolbutamide to the cytosolic face of a patch produced a dose-dependent and reversible inhibition of channel activity. After washout of tolbutamide, application of 10 µM 2'-*O*-Me-cAMP (labeled as ESCA) did not significantly alter channel activity (**Fig. 1A**). Such findings are consistent with our prior report that a low concentration of 2'-*O*-Me-cAMP inhibited K_{ATP} channel activity in an ATP-dependent manner.[18] However, when both tolbutamide and 2'-*O*-Me-cAMP were administered simultaneously, the action of tolbutamide was potentiated (**Fig. 1A**). The expanded traces of **Fig.1A** illustrate the actions of tolbutamide in the absence (i, ii) or presence (iii, iv) of 2'-*O*-Me-cAMP.

Population studies of multiple patches were then performed in order to quantify the interaction of tolbutamide and 2'-*O*-Me-cAMP to inhibit K_{ATP} channels (**Fig. 1B**). For this analysis, the normalized K_{ATP} current was defined as the current measured in the presence of tolbutamide relative to the current measured in the absence of tolbutamide (Relative Current). A relative current value of 1.0 was assigned to the K_{ATP} channel activity measured at the start of each experiment under control conditions in which no tolbutamide or 2'-*O*-Me-cAMP was present (left-hand histogram of **Fig. 1B**). After the tolbutamide dose-response relationship was determined in the absence of 2'-*O*-Me-cAMP, a test solution containing 10 µM 2'-*O*-Me-cAMP was administered. The steady-state K_{ATP} channel activity was then reassigned a relative current value of 1.0 and the tolbutamide dose-response was repeated in the presence of this ESCA (right-hand histogram of **Fig. 1B**). For 3 µM tolbutamide, the channel activity measured in the absence or presence of 2'-*O*-Me-cAMP was 57.6 ± 4.4% and 32.5 ± 5.5% of that measured under control conditions in which tolbutamide was not present, respectively (**Fig. 1B**; n = 7 patches). For 10 µM tolbutamide, the channel activity measured in the absence or presence of 2'-*O*-Me-cAMP was 34.6 ± 4.9% and 14.5 ± 2.4% of that measured under control conditions, respectively (**Fig. 1B**). This ability of 2'-*O*-Me-cAMP to facilitate K_{ATP} channel sulfonylurea-sensitivity was most likely mediated by Epac2 because no such action of the ESCA was measured in INS-1 cells expressing a dominant-negative Epac2 in which inactivating G114E and G422D mutations were introduced into the exchange factor's two cAMP-binding domains (**Fig. 1C**).[25, 26] Thus, the sensitization of K_{ATP} channel sulfonylurea sensitivity reported here cannot be explained by K_{ATP} channel rundown or non-specific channel block by 2'-*O*-Me-cAMP.

Cumulative dose-response relationships describing the action of tolbutamide (0.1 – 100 µM) in the absence or presence of a fixed concentration of 2'-*O*-Me-cAMP (50 µM) were then constructed by averaging data obtained from multiple excised patches using the experimental design described for **Fig. 1A** in which no ATP or ADP was present in the test solutions. Again, the normalized K_{ATP} current was defined as the current measured in the presence of tolbutamide relative to the current measured in the absence of tolbutamide (Relative Current). Tolbutamide inhibited INS-1 cell K_{ATP} channel activity with an IC_{50} value of 32.2 µM and this value shifted to 10.7 µM in the presence of 50 µM 2'-*O*-Me-cAMP (**Fig. 2A**). Facilitation of K_{ATP} channel sulfonylurea sensitivity by 2'-*O*-Me-cAMP was reversible and repeatable, and under these conditions of short-term exposure to test substances, 2'-*O*-Me-cAMP (50 µM) had no significant effect on K_{ATP} channel activity measured in the absence of tolbutamide.

2'-*O*-Me-cAMP sensitizes K_{ATP} channels to the inhibitory action of tolbutamide in human β-cells. The interaction of tolbutamide and 2'-*O*-Me-cAMP to inhibit K_{ATP} channels was also observed in excised patches obtained from human β-cells (raw data not shown). Under conditions identical to that described for INS-1 cells, the K_{ATP} channel activity decreased as the tolbutamide concentration was raised, and this channel activity recovered upon washout of tolbutamide. Subsequent

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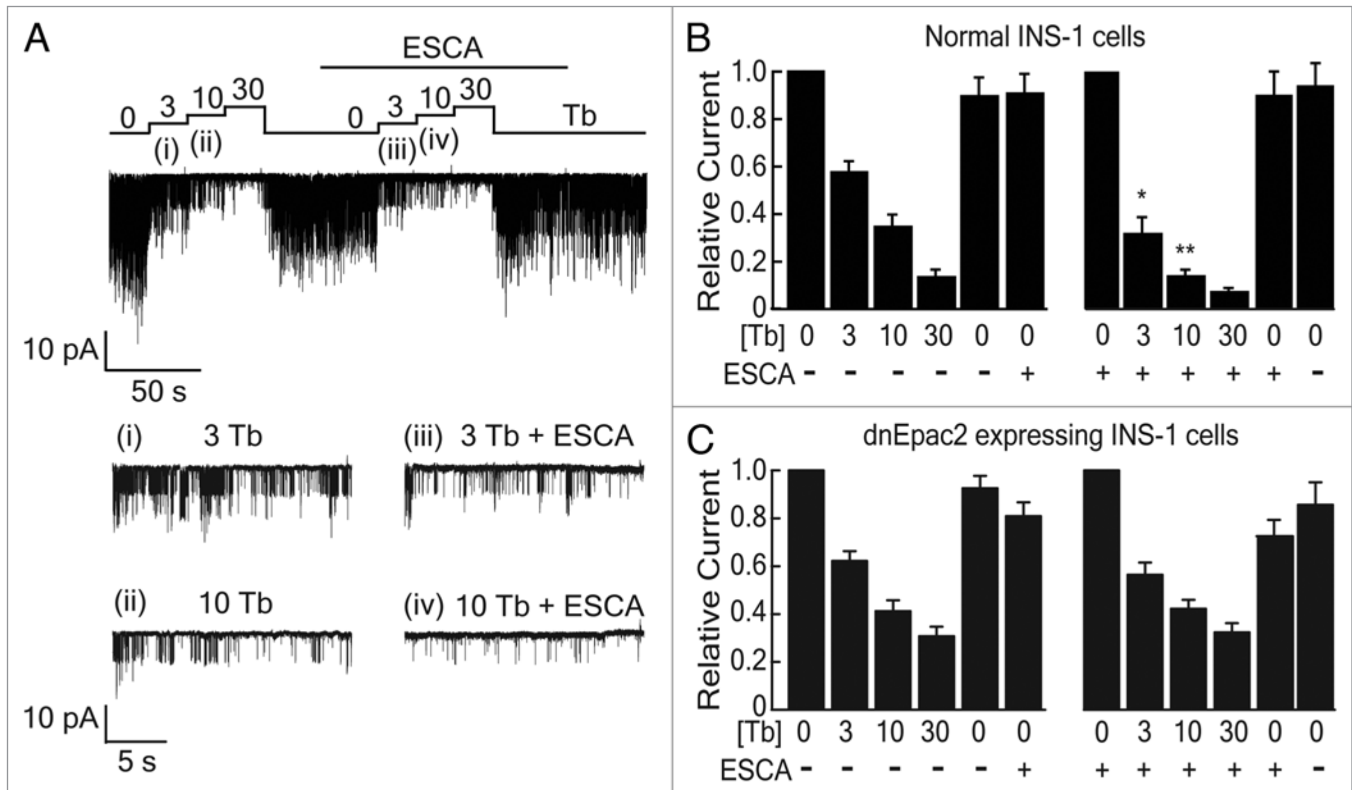

Figure 1. Inhibition of INS-1 cell K_{ATP} channel activity by tolbutamide in the absence or presence of 2’-O-Me-cAMP. (A) The tolbutamide (Tb) concentration was raised sequentially (0, 3, 10, 30 μM) and the action of tolbutamide was determined in the absence or presence of 10 μM 2'-O-Me-cAMP (ESCA). The lower traces in A illustrate channel activity on an expanded time scale for the periods marked (i-iv) in the top trace. Comparisons of channel activity in traces (i) and (iii) for 3 μM tolbutamide or (ii) and (iv) for 10 μM tolbutamide demonstrated that 2'-O-Me-cAMP sensitized K_{ATP} channels to the inhibitory effect of the sulfonylurea. (B) Averaged values for normalized K_{ATP} currents from 7 patches, as obtained using untransfected INS-1 cells and the experimental design illustrated in A. For the left panel of B, K_{ATP} current measured in the presence of tolbutamide was normalized relative to the current measured in the absence of tolbutamide and which was assigned a value of 1.0. For the right panel of B, the K_{ATP} channel activity measured in the presence of 2'-O-Me-cAMP alone was assigned a value of 1.0 and the action of tolbutamide was re-evaluated in presence of this ESCA. Note that channel inhibition at 3 μM (* p = 0.011) and 10 μM (** p = 0.007) tolbutamide was significantly greater in the presence of 2'-O-Me-cAMP. (C) 2'-O-Me-cAMP (10 μM) failed to sensitize K_{ATP} channels to the inhibitory effect of tolbutamide in patches obtained from INS-1 cells stably trnsfected with dominant negative (dn) Epac2. Data for C are the average of 15 patches. The experimental design and methods of normalization for panel C were identical to that described in panels A and B.

exposure of patches to 2'-*O*-Me-cAMP (50 μM) did not significantly alter channel activity, and in the presence of 2’-*O*-Me-cAMP, the inhibitory action of tolbutamide was significantly enhanced. For the K_{ATP} channels of human β-cells, the IC_{50} values measured in the absence or presence of 2'-*O*-Me-cAMP were 4.1 μM and 2.0 μM, respectively (**Fig. 2B**).

2'-O-Me-cAMP sensitizes K_{ATP} channels to the inhibitory action of gliclazide. It was recently reported that tolbutamide directly activates Epac2. [27] This finding raises the possibility that the binding of tolbutamide to Epac2 rather than SUR1 explains its increased potency to inhibit K_{ATP} channel activity when patches are treated with 2'-*O*-Me-cAMP. Thus, tolbutamide and 2'-*O*-Me-cAMP might synergistically activate Epac2 in order to inhibit K_{ATP} channel activity. Although this hypothetical scenario can not be ruled out at the present time, we found that gliclazide, a sulfonylurea that does not directly activate Epac2,[27] inhibited K_{ATP} channel activity in excised patches, and that this action of gliclazide was facilitated by treatment of the patches with 2'-*O*-Me-cAMP. For such experiments, it was first determined that 2'-*O*-Me-cAMP (10 μM) potentiated the inhibitory action of tolbutamide (10 μM) at K_{ATP} channels in excised patches of INS-1 cells (**Fig. 3A**). It was then demonstrated that gliclazide (50 nM) also inhibited K_{ATP} channel activity in the same patches, and that this inhibitory effect was facilitated by 2'-*O*-Me-cAMP (**Fig. 3B**; paired t-test). Since gliclazide binds with high affinity to SUR1, yet fails to activate Epac2,[27] it may be concluded that 2'-*O*-Me-cAMP is capable of facilitating K_{ATP} channel sulfonylurea sensitivity in a manner that is independent of any potential ability of sulfonylureas to activate Epac2 directly. To substantiate this conclusion, a Rap1 activation assay was performed using INS-1 cells expressing recombinant Epac2 and Rap1 (**Fig. 3C**). In this assay, neither tolbutamide nor gliclazide activated Rap1 when tested at 3-300 μM or 50-300 nM, respectively. However a significant increase of activated Rap1 was measured in response to combined administration of forskolin (2 μM) and IBMX (100 μM), or after administration of 8-pCPT-2'-*O*-Me-cAMP-AM (ESCA-AM; 3 μM). These data indicate that in INS-1

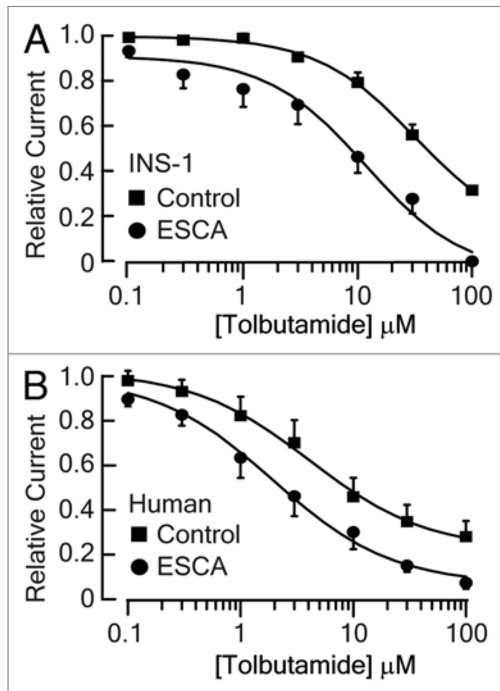

Figure 2. 2'-O-Me-cAMP left-shifts the dose-response relationship for K_{ATP} channel inhibition by tolbutamide. (A) Tolbutamide dose-response relationships for normalized K_{ATP} channel currents from INS-1 cells. Each data point is the mean ± SEM for 6 patches. Solid squares correspond to values of normalized K_{ATP} currents measured in the absence of 2'-O-Me-cAMP, whereas solid circles correspond to currents measured when 50 µM of the ESCA was present. (B) 2'-O-Me-cAMP left-shifts the dose-response relationship for K_{ATP} channel inhibition by tolbutamide in human b-cells. Each data point is the mean ± SEM for 7 patches. Solid squares correspond to normalized K_{ATP} channel currents measured in the absence of 2'-O-Me-cAMP, whereas solid circles correspond to currents measured when 50 µM of the ESCA was present.

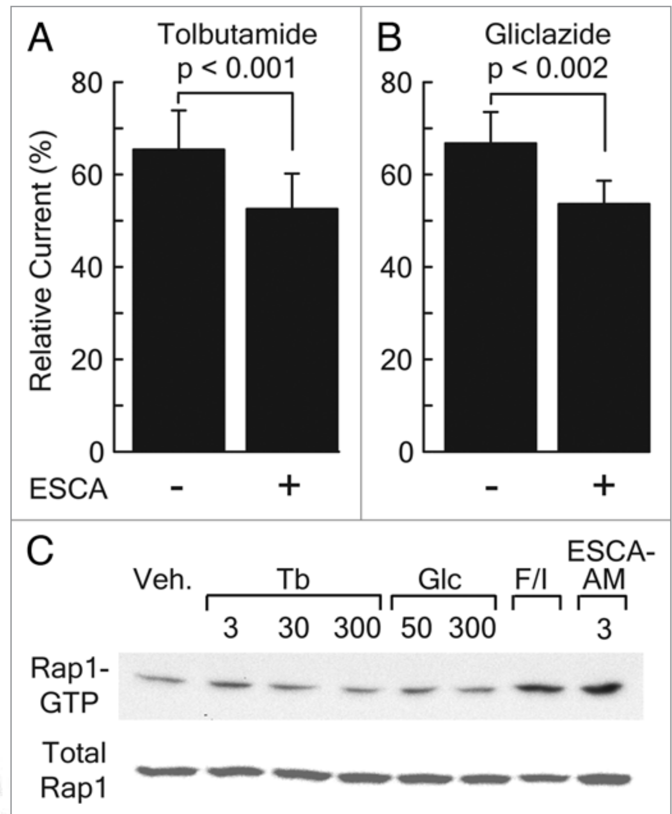

Figure 3. Direct activation of Epac2 by sulfonylureas may not explain the synergistic interaction of sulfonylureas and 2'-O-Me-cAMP to inhibit K_{ATP} channels. 2'-O-Me-cAMP (ESCA, 10 µM) sensitized K_{ATP} channels to the inhibitory actions of 10 µM tolbutamide (A) and 50 nM gliclazide (B). Data are for 6 (tolbutamide) and 8 (gliclazide) patches of INS-1 cells. (C) Neither tolbutamide (Tb, 3, 30 or 300 µM) nor gliclazide (Glc, 50 or 300 nM) activated Rap1 in INS-1 cells expressing recombinant Epac2, whereas levels of activated Rap1 (Rap1-GTP) were increased by combined administration of forskolin and IBMX (F/I, 2 µM + 100 µM) or 8-pCPT-2'-O-Me-cAMP-AM (ESCA-AM; 3 µM). Similar data were obtained in three independent experiments.

cells, neither tolbutamide nor gliclazide activated Rap1 via a direct effect at Epac2.

Epac2 is the predominant isoform of Epac expressed in INS-1 cells. Although the expression of Epac2 in INS-1 cells has been reported previously,[28] the relative levels of expression of Epac isoforms in this cell line have not been documented. This is a matter of significance since both Epac1 and Epac2 might play a role in K_{ATP} channel regulation. To address this uncertainty, we used RT-QPCR to determine the relative levels of expression of mRNAs corresponding to both isoforms of Epac proteins in INS-1 cells (**Fig. 4A**). For Epac1 and Epac2 the mean threshold crossing values relative to S18 mRNA were 9.13 ± 0.13 (n = 6) and 2.16 ± 0.18 (n = 6), respectively. Using the $\Delta\Delta C_t$ method,[29] Epac2 mRNA abundance was calculated to be approximately 125-fold higher than that of Epac1 ($p < 0.001$). Thus, Epac2 most likely constitutes the predominant effector protein activated by Epac-selective cAMP analogs in INS-1 cells.

Membrane depolarizing properties of an Epac activator. Although ESCAs inhibit K_{ATP} channel activity, it has yet to be demonstrated that these cAMP analogs produce membrane depolarization when applied extracellularly to intact β-cells. This situation exists due to the fact that the original Epac activator (8-pCPT-2'-*O*-Me-cAMP) has a low membrane permeability in β-cells.[30] We now demonstrate that the membrane-permeant acetoxymethyl ester of 8-pCPT-2'-*O*-Me-cAMP[30, 31] depolarized INS-1 cells and raised levels of $[Ca^{2+}]_i$ (**Fig. 5A1 and A2**). This action of 8-pCPT-2'-*O*-Me-cAMP-AM was measurable using low concentrations (1-10 µM) of the analog that allow selective activation of Epac but not PKA.[30] The Epac-selectivity with which 8-pCPT-2'-*O*-Me-cAMP-AM acted was verified through the use of a PepTag assay in which fluorescent Kemptide served as a substrate for PKA. It was demonstrated that 8-pCPT-2'-*O*-Me-cAMP-AM failed to activate PKA in INS-1 cells (**Fig. 5B**). However, the acetoxymethyl ester of a PKA-selective cAMP analog (6-Bnz-cAMP-AM)[32] was fully active (**Fig. 5C**).

Synergy of tolbutamide and Epac activators to depolarize INS-1 cells and to increase $[Ca^{2+}]_i$. If Epac activators facilitate the action of sulfonylureas to inhibit K_{ATP} channels, this interaction should be observable in assays of membrane potential and

$[Ca^{2+}]_i$ since K_{ATP} channel closure leads to depolarization and Ca^{2+} influx in INS-1 cells. As expected, we found that depolarization induced by tolbutamide was potentiated by 8-pCPT-2'-*O*-Me-cAMP-AM, as measured using INS-1 cells loaded with the voltage-sensitive FRET probes CC2-DMPE and DiSBAC2 (**Fig. 6A**). Interactions of tolbutamide and Epac activators to increase $[Ca^{2+}]_i$ were then assessed using INS-1 cells loaded with fura-2. When tolbutamide was paired with the cAMP-elevating hormone GLP-1, an increase of $[Ca^{2+}]_i$ was measured, and the magnitude of this response clearly exceeded the arithmetic sum of the individual responses measured when each compound was tested alone (**Fig. 6B**). Such a supra-additive response is expected if GLP-1 promotes the activation of Epac2,[33] thereby facilitating the inhibitory action of tolbutamide at K_{ATP} channels. Consistent with this concept, tolbutamide and 8-pCPT-2'-*O*-Me-cAMP-AM also interacted in a supra-additive manner to increase $[Ca^{2+}]_i$ (**Fig. 6C**). However, no such supra-additive response was measured when tolbutamide was paired with the PKA-selective cAMP analog 6-Bnz-cAMP-AM (**Fig. 6D**).

Epac activation stimulates PIP_2 hydrolysis in INS-1 cells. We recently proposed that inhibitory effects of Epac activators at K_{ATP} channels are explained by their ability to promote the hydrolysis of plasma membrane PIP_2.[20] We have now assessed the capacity of Epac activators to promote PIP_2 hydrolysis by performing live-cell imaging of INS-1 cells virally transduced with a biosensor incorporating the pleckstrin homology domain of PLCδ fused to GFP (PHD-GFP).[34] This biosensor is sequestered at the plasma membrane by virtue of its ability to bind PIP_2, and it is released into the cytosol in response to PIP_2 hydrolysis.[34] When INS-1 cells expressing the PHD-GFP biosensor were stimulated with 8-pCPT-2'-*O*-Me-cAMP-AM, there occurred a reversible redistribution of the biosensor away from the plasma membrane and into the cytosol (**Fig. 7A,B**). This effect of the ESCA was reproduced upon stimulation of INS-1 cells with GLP-1 (10 nM; data not shown; n = 8 cells). To obtain independent confirmation that redistribution of the PHD-GFP reporter measured in this manner did in fact correspond to PIP_2 hydrolysis, it was further demonstrated that cytosolic redistribution of the biosensor was measured in response to carbachol (250 μM; n = 18 cells, data not shown), an activator of cholinergic receptors positively coupled to PLC activation in INS-1 cells.

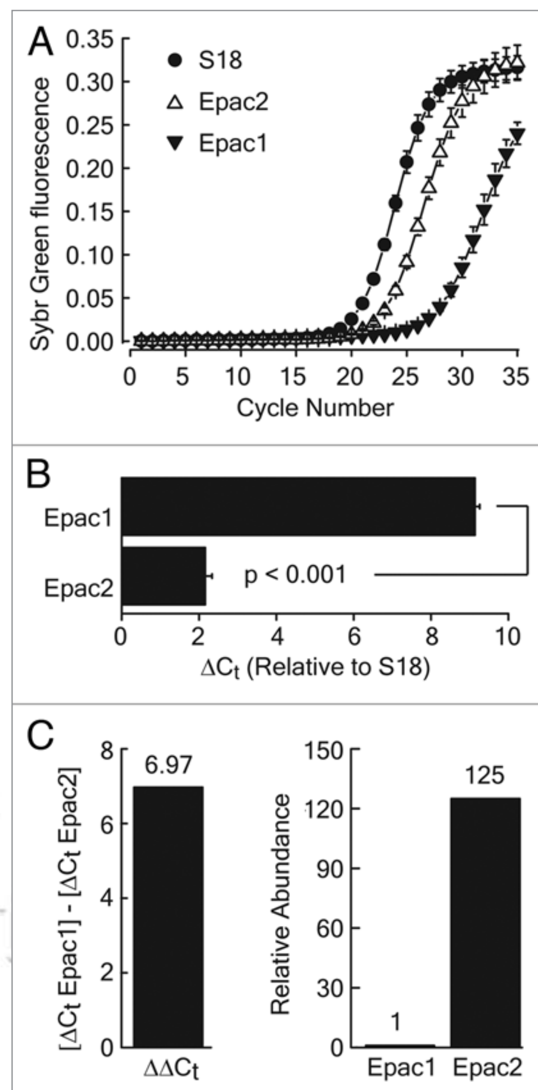

Figure 4. Expression of Epac isoforms in INS-1 cells. (A) RT-QPCR growth curves for S18 mRNA (reference gene, filled circles), Epac1 mRNA (filled inverted triangles) and Epac2 mRNA (open triangles) using RNA from p82 INS-1 cells. (B) The difference in the threshold crossing values for Epac isoforms relative to S18 (ΔC_t) was 2.16 ± 0.18 (n = 6) for Epac2 and 9.13 ± 0.13 (n = 6) for Epac1. These data indicate that Epac2 mRNA is expressed at a level significantly higher than that of Epac1 ($p < 0.001$). (C) The difference ($\Delta\Delta C_t$) in the mean ΔC_t values for Epac isoforms ([ΔC_t Epac1] – [ΔC_t Epac2]) was 6.97 and the relative abundance of Epac2 to Epac1 mRNAs was calculated to be 125:1.

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Discussion

Presented here are findings that demonstrate a previously unrecognized ability of an Epac-selective cAMP analog (2'-*O*-Me-cAMP) to render human β-cell and rat INS-1 cell K_{ATP} channels more sensitive to the inhibitory action of tolbutamide. Similarly, a cAMP-elevating hormone (GLP-1) or a membrane-permeant Epac activator (8-pCPT-2'-*O*-Me-cAMP-AM), are shown to functionally interact with tolbutamide in a supra-additive manner to produce membrane depolarization and to increase $[Ca^{2+}]_i$ in INS-1 cells. We propose that such findings have important implications for our understanding of how exenatide, an incretin mimetic, interacts with sulfonylureas to lower levels of blood glucose. It would appear that depending on the doses at which sulfonylureas and exenatide are administered, a K_{ATP} channel-dependent process may exist by which these two substances functionally interact to induce excessive pancreatic insulin secretion with attendant hypoglycemia.

Attention has recently focused on the potential roles of Rap and PLCε as intermediaries linking Epac activation to the inhibition of K_{ATP} channels.[20] This concept was advanced because Epac acts via Rap to stimulate PLCε, thereby hydrolyzing PIP_2 in various cell types.[21] Since PIP_2 is an activator of K_{ATP} channels, and because PIP_2 reduces these channel's sensitivities to both ATP

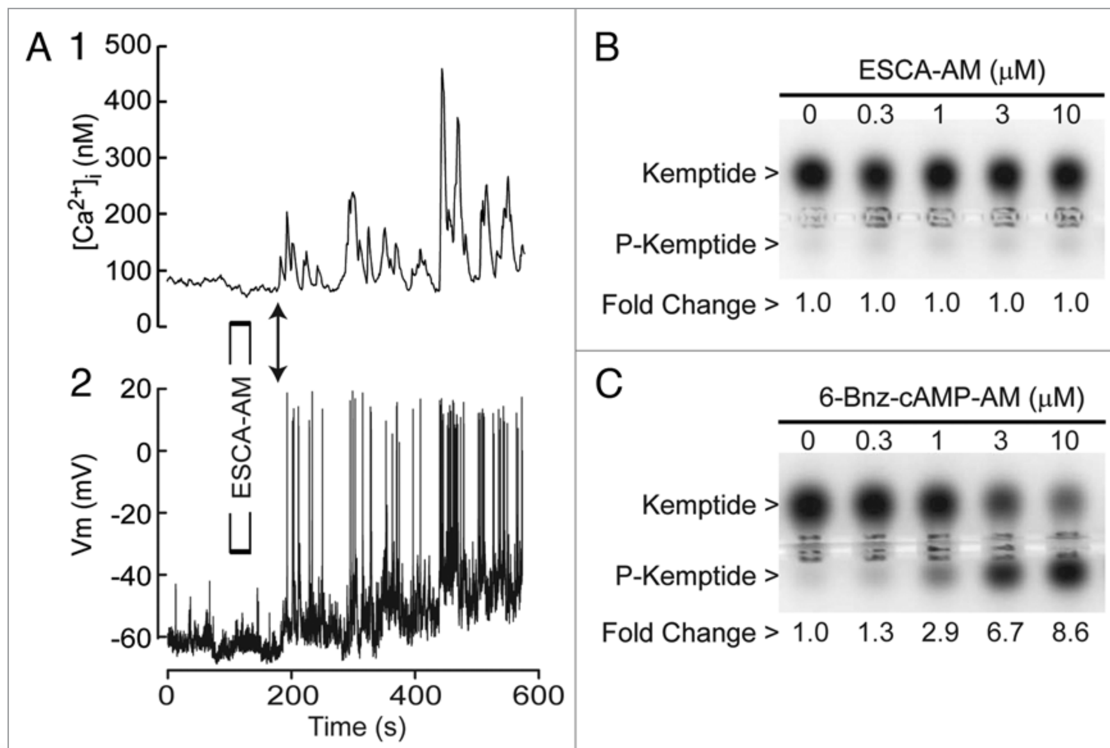

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Figure 5. Studies with 8-pCPT-2'-O-Me-cAMP-AM. (A) $[Ca^{2+}]_i$ elevating (A1) and membrane depolarizing actions (A2) of 8-pCPT-2'-O-Me-cAMP-AM in INS-1 cells. Application of 8-pCPT-2'-O-Me-cAMP-AM (ESCA-AM, 10 μM, indicated by bracket) induced action potentials and concurrent increases of $[Ca^{2+}]_i$ (double ended arrows). (B) A 30 min treatment of INS-1 cells with 8-pCPT-2'-O-Me-cAMP-AM did not induce phosphorylation of the PKA substrate Kemptide, as measured in lysates of INS-1 cells. (C) A PKA-selective cAMP analog (6-Bnz-cAMP-AM) produced a dose-dependent increase in Kemptide phosphorylation. The phosphorylated and non-phosphorylated forms of the substrate peptide are designated P-Kemptide and Kemptide, respectively.

and sulfonylureas,[22,23,35] the action of 2'-*O*-Me-cAMP reported here might be a consequence of its ability to deplete PIP_2 in the plasma membrane of INS-1 cells and human β-cells. Our data obtained through the use of live-cell imaging indicate that Epac activators do stimulate hydrolysis of PIP_2 in INS-1 cells. However, it remains to be determined if this is explained by the activation of PLCε by Epac2, or is instead explained by the activation of a Ca^{2+}-sensitive PLC that hydrolyze PIP_2 in response to the rise of $[Ca^{2+}]_i$ that occurs secondary to membrane depolarization.[36, 37] Regardless of which isoform of PLC is involved, the depletion of plasma membrane PIP_2 we observed allows two predictions to be made regarding the action of 2'-*O*-Me-cAMP. First, 2'-*O*-Me-cAMP should enhance the sensitivity of K_{ATP} channels to ATP, as was recently established.[18] Secondly, 2'-*O*-Me-cAMP should also enhance K_{ATP} channel sulfonylurea sensitivity. This second prediction is validated in the present study of human β-cells and rat INS-1 cells.

An additional possibility concerns whether or not PKC activation secondary to PLC stimulation contributes to the inhibitory effect of Epac activators at K_{ATP} channels. This may not be the case since we found that 2'-*O*-Me-cAMP sensitized K_{ATP} channels to the inhibitory action of sulfonylureas under ATP-free conditions. However, GLP-1 was previously reported to activate PKC in INS-1 cells,[38] and activators of PKC were found to inhibit K_{ATP} channels in an insulin-secreting cell line.[39] Thus, under true physiological conditions, PKC might play some role in K_{ATP} channel regulation by Epac activators such as GLP-1.

The ability of Epac to activate protein phosphatases, including the phosphatases PP-2A [40] and PP-2B, [41] suggests an additional explanation for how GLP-1 alters K_{ATP} channel function in β-cells. More specifically, Epac activation may result in phosphatase-catalyzed dephosphorylation of K_{ATP} channels, thereby increasing the channel's apparent affinity for sulfonylureas. This model is based on the findings of one prior study in which Epac activator 8-pCPT-2'-*O*-Me-cAMP acted via protein phosphatases to inhibit K_{ATP} channel function in vascular smooth muscle.[42] Given that certain phosphatases are known to be under the control of Ca^{2+}/calmodulin, this model is fully consistent with a prior report that calmodulin antagonists prevented the inhibitory action of GLP-1 at mouse β-cell K_{ATP} channels,[43] and that GLP-1 acted independently of PKA to inhibit K_{ATP} channels in rat β-cells.[44]

Perhaps more intriguing, it may be that Epac2 itself is a direct target of both 2'-*O*-Me-cAMP and tolbutamide, a concept recently advanced by Seino and co-workers.[27] In the model of Seino, cAMP and certain sulfonylureas directly bind to and activate Epac2. Since Epac2 interacts with NBF-1 of SUR1,[6,19,26] inhibitory actions of sulfonylureas at K_{ATP} channels might be explained by their ability to bind to a conventional sulfonylurea receptor (SUR1) as well as a non-conventional receptor (Epac2).

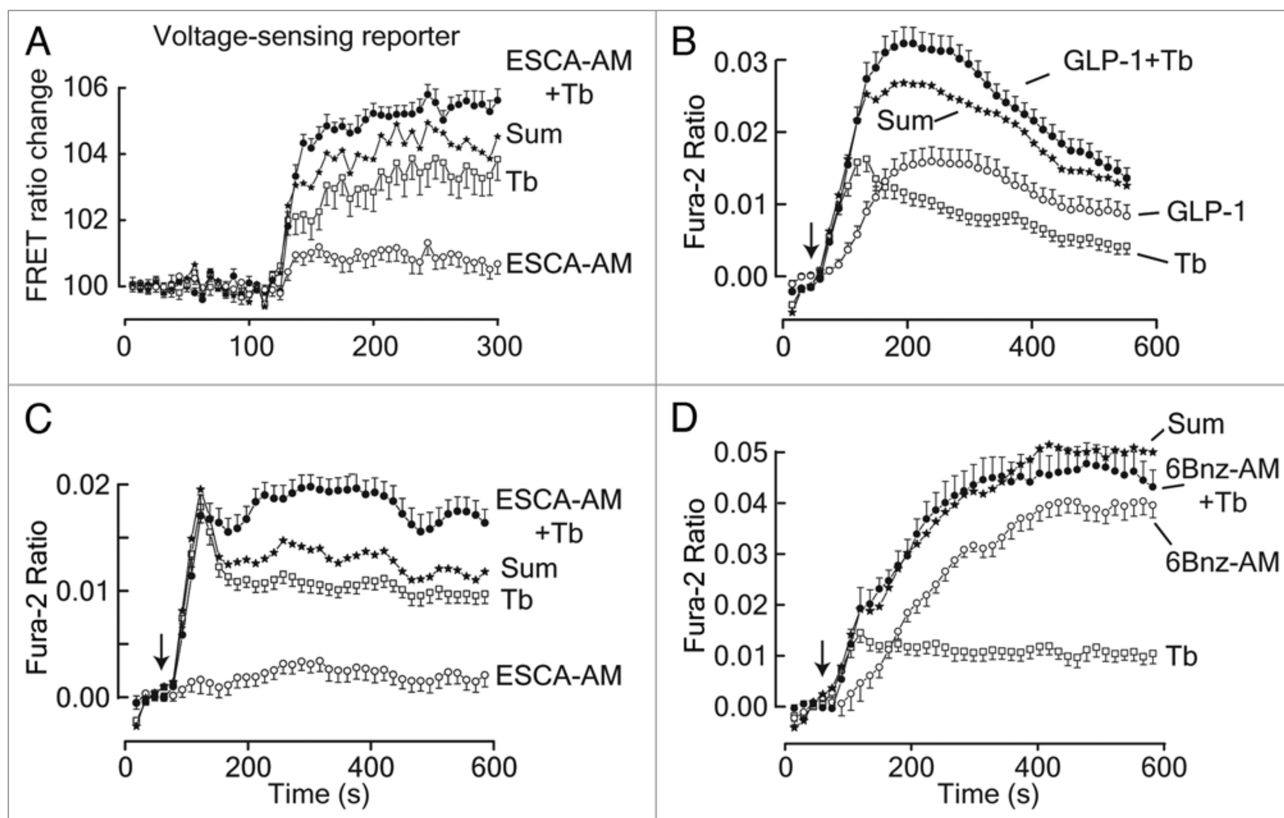

Figure 6. Interactions of tolbutamide and cAMP-elevating agents to produce membrane depolarization and to increase $[Ca^{2+}]_i$. All assays in this figure were performed on INS-1 cells in 96-well plates bathed in 1 mM glucose. (A) FRET recordings of membrane potential from INS-1 cells. Data averaged from 12 wells in 1 experiment shows that 8-pCPT-2'-O-Me-cAMP-AM (ESCA-AM, 1 μM) potentiated membrane depolarization induced by tolbutamide (Tb, 10 μM). (B) Supra-additive interaction of GLP-1 (1.5 nM) and tolbutamide (3 μM) to increase $[Ca^{2+}]_i$ in INS-1 cells. When both test substances were administered simultaneously, the increase of $[Ca^{2+}]_i$ exceeded the arithmetic sum (Sum) of the action of each substance when tested alone. (C) 8-pCPT-2'-O-Me-cAMP-AM (1 μM) exhibited a supra-additive interaction with tolbutamide (3 μM) to increase $[Ca^{2+}]_i$ in INS-1 cells. For panels B and C, data was averaged from 46 wells in 3 experiments. (D) Additive but not supra-additive interaction of tolbutamide (3 μM) and PKA-selective cAMP analog 6-Bnz-cAMP-AM (6Bnz-AM, 1 μM) to increase $[Ca^{2+}]_i$ in INS-1 cells. Data are from 24 wells in 2 experiments.

However, findings presented here seem to argue against a model in which Epac2 serves as a physiologically relevant sulfonylurea receptor. For example, we found that tolbutamide failed to activate Rap1 in INS-1 cells expressing Epac2, a finding we interpret to indicate that Epac2 is not directly activated by tolbutamide. Furthermore, in assays of K_{ATP} channel activity, we found that 2'-*O*-Me-cAMP potentiated the action of gliclazide, a sulfonylurea that failed to activate Epac2.[27] Thus, new findings presented here concerning gliclazide indicate that simultaneous activation of Epac2 by Epac-selective cAMP analogs and sulfonylureas is unlikely to constitute the primary mechanism by which these agents synergistically inhibit K_{ATP} channel function. However, such observations do not exclude the possibility that direct activation of Epac2 by sulfonylureas can occur under experimental conditions or in cell lines not used in our studies.

In conclusion, new findings presented here offer a novel "K_{ATP} dependent" mechanistic explanation for the adverse interaction of high dose exenatide and sulfonylureas to depolarize β-cells, to stimulate excessive insulin secretion, and to induce hypoglycemia in type 2 diabetic subjects.

Materials and Methods

Cell culture. Human islets provided by the NIH ICR centers were cultured[18] and β-cells were identified on the basis of rat insulin 2 gene promoter-directed expression of enhanced yellow fluorescent protein (EYFP).[45] INS-1 cells were passaged in culture as described previously.[46] Dominant-negative Epac2 [26] was stably expressed in INS-1 cells using methods described previously for Epac1.[18]

Measurement of K_{ATP} currents. For inside-out patch recording the pipette solution contained (in mM): 140 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 5 HEPES (pH 7.4). Patches were excised into a solution containing (in mM): 70 K_2SO_4, 2.0 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 0.2 GTP, 5 HEPES (pH 7.4). The patch was held at -100 mV and experiments were performed at 22–26 °C. Data were acquired using an EPC-9 amplifier controlled using Patchmaster software (Instrutech Corp., Mineola, NY), or an Axopatch 200B amplifier controlled with pCLAMP10 (Molecular Devices, Sunnyvale, CA). Currents were low-pass filtered (1 kHz), digitized at 10 kHz (Digidata 1440A), and analyzed using

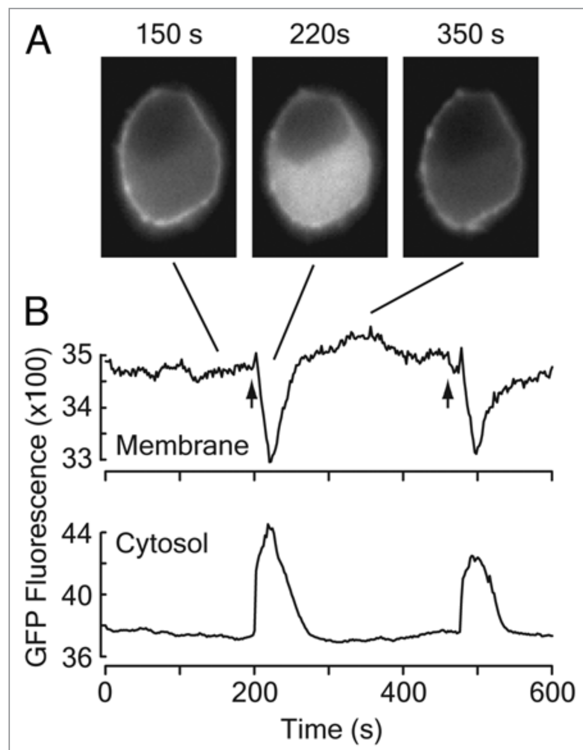

Figure 7. PIP_2 hydrolysis induced by 8-pCPT-2'-O-Me-cAMP-AM. INS-1 cells were virally transduced to express a PHD-GFP reporter that under basal conditions binds PIP_2 in the plasma membrane. Activation of PLC causes hydrolysis of PIP_2 and translocation of the reporter to the cytosol. Representative images of reporter-expressing cells are shown in A at the times indicated from the fluorescence traces in B. Two recording zones were set on cells, one encompassing the plasma membrane and one in the cell cytosol. Stimulation of the cell with 10 s pulses of 10 μM 8-pCPT-2'-O-Me-cAMP-AM (arrows) induced a decrease in plasma membrane fluorescence and an increase in cytosolic fluorescence. This translocation of the reporter is consistent with PLC activation.

pCLAMP10. Test substances were applied to inside-out patches using Dynaflow Pro II microfluidics chambers (Cellectricon Inc., Gaithersburg, MD). Statistical analysis and curve fitting was performed using Origin 8 (OriginLab, Northampton, MA). Values of channel activity are given as the mean ± SEM.

Measurement of $[Ca^{2+}]_i$. INS-1 cells were loaded with fura-2 in a standard extracellular solution (SES) containing (in mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES adjusted to pH 7.4 with NaOH and supplemented with 1 mM glucose, 20 μl ml^{-1} FBS, 1 μl ml^{-1} Pluronic F-127 and 1 μM fura-2 AM (Invitrogen Life Technologies, Carlsbad, CA). Single-cell measurements of $[Ca^{2+}]_i$ were obtained as described previously.[47] Measurements of $[Ca^{2+}]_i$ from monolayers of fura-2 loaded INS-1 cells grown on Costar 3904 plates were performed using a FlexStation 3 microplate reader using SoftMaxPro software (Molecular Devices). Spectrofluorimetry was performed using excitation light at 335/9 and 375/9 nm (center/bandpass) delivered using a 455 nm dichroic mirror. Emitted light was detected at 505/15 nm and the ratio of emission light intensities due to excitation at 335 and 375 nm was calculated.

Measurements of membrane potential. Perforated patch recording of the membrane potential was performed using the SES bath and pipette solutions described above, but with the pipette solution supplemented with nystatin.[16] For FRET-based determination of membrane potential, cells grown in 96-well plates were loaded with 3 μM CC2-DMPE and 3 μM $DiSBAC_2$ (Invitrogen) for 40 min at room temperature. Cells were excited using 400 nm excitation light and the emission light was measured at 460 nm and 580 nm using a FlexStation 3. An increase in the 460/580 emission ratio signifies membrane depolarization.[48]

Rap1 activation assay. Activation of Rap1 was assayed by Western blot using cell lysates from INS-1 cells transiently transfected with FLAG-epitope tagged Rap1 and Epac2. Transfection was performed using Lipofectamine Plus Reagent (Invitrogen). The methods used in this Ral-GDS-RBD-GST pull-down assay employing glutathione-conjugated agarose beads are described elsewhere.[30] The activated GTP-bound form and the total amount of Rap1 were detected using an anti-FLAG M2 monoclonal antibody conjugated to HRP (Sigma-Aldrich; Cat. No. A8592).

Quantitative PCR for Epac. RNA was isolated from INS-1 cells using RNEasy kits (Qiagen). RNA concentration and purity was assessed using a NanoDrop ND-1000 spectrofluorimeter. QPCR reactions were performed using QuantiTect Sybr-green one-step kits (Qiagen) with approximately 100 ng of template RNA. Reactions were performed using an MJ MiniOpticon cycler with 35 cycles of: 94°C for 15 s, 60°C for 30 s and 72°C for 30 s followed by a melting curve analysis from 60°C to 94°C. Reaction products were run on 2% agarose gels and bands were cut out and gel extracted using QIAquick kits (Qiagen). Product identity was confirmed by direct sequencing at an in-house core facility. The PCR primers were: Epac1 (sense) CATGGCAAGGGGCTGGTGAC, (antisense) GTCCTGCTTGTCCACACGCAG. Epac2 (sense) CGCCATGCAACCATCGTTACC, (antisense) GAGCCCGTTTCCATAACACC. Ribosomal S18 was used as the reference template with the following primers: (sense) GCCATCACTGCCATTAAGGG, (antisense) CCAGTCTGGGATCTTGTACTG. Primers were tested at different starting template concentrations to validate their equal efficiencies. Threshold crossing (C_t) values were set manually and the difference between the C_t value for S18 and Epac (ΔC_t) was calculated for each reaction. Relative expression levels for Epac1 and Epac2 were determined using the $\Delta\Delta C_t$ method.[29] ΔC_t values were entered into Origin 8 software for statistical analysis using ANOVA.

PKA activation assay. PKA activity in lysates of INS-1 cells was measured using a PepTag cAMP-Dependent Protein Kinase Assay (Promega). Prior to lysis, INS-1 cells were exposed to cAMP analogs for 30 min while equilibrated at 37°C in RPMI 1640 medium.

PIP_2 biosensor traslocation assay. INS-1 cells were split onto coverslips and placed in RPMI 1640 medium supplemented with 2% FBS and 10 mM HEPES. A viral vector expressing the pleckstrin homology domain of PLCδ fused to enhanced green fluorescent protein (PHD-GFP) [34] was introduced at a multiplicity of infection of 50 and the cells were transduced overnight before

washing with normal culture medium. After 48 h, the cells were placed in a recording chamber and washed with SES. To monitor GFP fluorescence, cells were excited at 480/40 nm, and emitted light was detected at 535/50 nm using a Cascade 512B camera controlled with Metafluor software (Molecular Devices). Individual cells were stimulated by pressure application of test substances from an automated micropipettor (Eppendorf).

Sources of reagents. 2'-*O*-Me-cAMP, N_6-Benzoyladenosine-3',5'-cyclic monophosphate-AM (6-Bnz-cAMP-AM), and 8-(4-Chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate-AM (8-pCPT-2'-*O*-Me-cAMP-AM) were synthesized by BIOLOG Life Science Institute (Bremen, Germany). Tolbutamide and Gliclazide were from Sigma-Aldrich. Epac1-camps in pcDNA3.1 was from Prof. M. Lohse (Würzburg, Germany). PHD-GFP was from Prof. C. Rhodes (Chicago, IL). Dominant-negative Epac2 was from Prof. S. Seino (Kobe, Japan).

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Note

C. A. Leech, I. Dzhura, O. G. Chepurny, and G. G. Holz have no conflicts to declare. H.-G. Genieser is CEO and F. Schwede is Head of R&D of BIOLOG Life Science Institute, which sells 8-pCPT-2'-*O*-cAMP-AM, 6-Bnz-cAMP-AM and 2'-*O*-Me-cAMP for research purposes.

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