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

Colin A. Leech,1* Igor Dzhura,1 Oleg G. Chepurny,1 Frank Schwede,2 Hans-G. Genieser,2 and George G. Holz1,3

Departments of 1Medicine and 3Pharmacology; State University of New York; Upstate Medical University; Syracuse, NY USA; 2 BIOLOG Life Science Institute; Bremen, Germany

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**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; PIP2, 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 \( K_{ATP} \) 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\(^{2+}\)], 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.

The sulfonylurea receptor-1 (SUR1) expressed in pancreatic β-cells5 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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*Correspondence to: Colin A. Leech; Email: leechc@upstate.edu
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activating protein kinase A (PKA) and the exchange proteins directly activated by cAMP (Epac). Although PKA was previously implicated in the regulation of K<sub>ATP</sub> channels and also voltage-dependent K<sup>+</sup> channels in β-cells, new findings demonstrate that Epac proteins, including the isoform known as Epac2, couple cAMP production to K<sub>ATP</sub> 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, and this effect is attributable, at least in part, to its ability to inhibit the activity of β-cell K<sub>ATP</sub> channels that are also inhibited by GLP-1. The cAMP analogs that produce these effects in β-cells are designated as Epac-selective cAMP analogs (ESCs). They include 2'-O-Me-cAMP, 8-pCPT-2'-O-Me-cAMP, and the acetoxyethyl ester “produg” of 8-pCPT-2'-O-Me-cAMP that is highly effective as an insulin secretagogue.

The ability of ESCs to inhibit K<sub>ATP</sub> 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<sub>ATP</sub> channel’s SUR1 subunit. In the model we have proposed, activated Epac proteins promote Rap GTPase-dependent stimulation of phospholipase C-epsilon (PLCε), with concomitant depletions of plasma membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) located at the K<sub>ATP</sub> channels. Since PIP<sub>2</sub> stimulates K<sub>ATP</sub> channel activity, ESCs may act through Epac2, Rap, and PLCε to counteract this effect. Given that PIP<sub>2</sub> also decreases the apparent affinity of K<sub>ATP</sub> channels for ATP, this model is fully consistent with a recent study demonstrating that Epac activators increase the K<sub>ATP</sub> channel’s apparent affinity for ATP in human β-cells and rat INS-1 cells. Interestingly, one prediction of this model is that Epac2 activation should also increase K<sub>ATP</sub> 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<sub>ATP</sub> 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.

**Results**

2'-O-Me-cAMP sensitizes K<sub>ATP</sub> channels to the inhibitory action of tolbutamide in rat INS-1 cells. K<sub>ATP</sub> channel activity was monitored in inside-out patches excised from INS-1 cells under conditions in which the bath solution contained Mg<sup>2+</sup> 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<sub>ATP</sub> channel activity in an ATP-dependent manner. 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<sub>ATP</sub> channels (Fig. 1B). For this analysis, the normalized K<sub>ATP</sub> current was defined as the current measured in the absence of tolbutamide relative to the current measured in the presence of tolbutamide (Relative Current). A relative current value of 1.0 was assigned to the K<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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).

Thus, the sensitization of K<sub>ATP</sub> channel sulfonylurea sensitivity reported here cannot be explained by K<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> channel activity with an IC<sub>50</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> channel activity measured in the absence of tolbutamide.

2'-O-Me-cAMP sensitizes K<sub>ATP</sub> channels to the inhibitory action of tolbutamide in human β-cells. The interaction of tolbutamide and 2'-O-Me-cAMP to inhibit K<sub>ATP</sub> 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<sub>ATP</sub> channel activity decreased as the tolbutamide concentration was raised, and this channel activity recovered upon washout of tolbutamide. Subsequent

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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<sub>ATP</sub> channels of human β-cells, the IC<sub>50</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> channels in excised patches of INS-1 cells (Fig. 3A). It was then demonstrated that gliclazide (50 nM) also inhibited K<sub>ATP</sub> 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<sub>ATP</sub> 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
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, 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 KATP 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, 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 KATP 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. We now demonstrate that the membrane-permeant acetoxymethyl ester of 8-pCPT-2′-O-Me-cAMP depolarized INS-1 cells and raised levels of $\left[Ca^{2+}\right]_i$ (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. 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 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.

Synergy of tolbutamide and Epac activators to depolarize INS-1 cells and to increase $\left[Ca^{2+}\right]_i$. If Epac activators facilitate the action of sulfonylureas to inhibit KATP channels, this interaction should be observable in assays of membrane potential and...
[Ca^{2+}], since K\textsubscript{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+}], 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+}], 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, thereby facilitating the inhibitory action of tolbutamide at K\textsubscript{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+}], (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\textsubscript{2} hydrolysis in INS-1 cells. We recently proposed that inhibitory effects of Epac activators at K\textsubscript{ATP} channels are explained by their ability to promote the hydrolysis of plasma membrane PIP\textsubscript{2}. We have now assessed the capacity of Epac activators to promote PIP\textsubscript{2} hydrolysis by performing live-cell imaging of INS-1 cells virally transduced with a biosensor incorporating the pleckstrin homology domain of PLC\textdelta fused to GFP (PHD-GFP). This biosensor is sequestered at the plasma membrane by virtue of its ability to bind PIP\textsubscript{2}, and it is released into the cytosol in response to PIP\textsubscript{2} hydrolysis. 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) and carbachol (250 μM; n = 18 cells, data not shown), an activator of cholinergic receptors positively coupled to PLC activation in INS-1 cells.

**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\textsubscript{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+}], 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\textsubscript{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 PLC\textepsilon as intermediaries linking Epac activation to the inhibition of K\textsubscript{ATP} channels. This concept was advanced because Epac acts via Rap to stimulate PLC\textepsilon, thereby hydrolyzing PIP\textsubscript{2} in various cell types. Since PIP\textsubscript{2} is an activator of K\textsubscript{ATP} channels, and because PIP\textsubscript{2} reduces these channel’s sensitivities to both ATP...
and sulfonylureas,\textsuperscript{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 PLC\(\epsilon\) 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.\textsuperscript{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.\textsuperscript{18} Secondly, 2'-O-Me-cAMP should also enhance K\(_{ATP}\) channel sulfonyurea 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,\textsuperscript{38} and activators of PKC were found to inhibit K\(_{ATP}\) channels in an insulin-secreting cell line.\textsuperscript{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\textsuperscript{40} and PP-2B,\textsuperscript{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.\textsuperscript{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,\textsuperscript{43} and that GLP-1 acted independently of PKA to inhibit K\(_{ATP}\) channels in rat β-cells.\textsuperscript{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.\textsuperscript{27} In the model of Seino, cAMP and certain sulfonylureas directly bind to and activate Epac2. Since Epac2 interacts with NBF-1 of SUR1,\textsuperscript{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\textsuperscript{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\textsuperscript{2+}]i in INS-1 cells. When both test substances were administered simultaneously, the increase of [Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{ATP} channel activity, we found that 2'-O-Me-cAMP potentiated the action of gliclazide, a sulfonylurea that failed to activate Epac2.\textsuperscript{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\textsubscript{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\textsubscript{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\textsuperscript{18} and β-cells were identified on the basis of rat insulin 2 gene promoter-directed expression of enhanced yellow fluorescent protein (EYFP).\textsuperscript{45} INS-1 cells were passaged in culture as described previously.\textsuperscript{46} Dominant-negative Epac2\textsuperscript{26} was stably expressed in INS-1 cells using methods described previously for Epac1.\textsuperscript{18}

Measurement of K\textsubscript{ATP} currents. For inside-out patch recording the pipette solution contained (in mM): 140 KCl, 1.0 MgCl\textsubscript{2}, 2.0 CaCl\textsubscript{2}, 5 HEPES (pH 7.4). Patches were excised into a solution containing (in mM): 70 K\textsubscript{2}SO\textsubscript{4}, 2.0 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{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
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. For FRET-based determination of membrane potential, cells grown in 96-well plates were loaded with 3 μM CC2-DMPE and 3 μM DiSBAC2 (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.

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 Rap1-GDS-RBD-GST pull-down assay employing glutathione-conjugated agarose beads are described elsewhere. 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 spectrophotometer. 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) GTCCCTGTCTGCAACACAGCAG. Epac2 (sense) CGCCATGCAACCATCGTTACC, (antisense) GAGCCCCGTTCATACGAC. Ribosomal S18 was used as the reference template with the following primers: (sense) GCCATCCTGCCATTAAGGG, (antisense) CCAGTCTGGGATCTTGTACTG. Primers were tested at different starting template concentrations to validate their equal efficiencies. Threshold crossing (Ct) values were set manually and the difference between the Ct value for S18 and Epac (ΔCt) was calculated for each reaction. Relative expression levels for Epac1 and Epac2 were determined using the ∆∆Ct method.

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 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) 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 1212 camera controlled with Metafluor software (Molecular Devices). Individual cells were stimulated by pressure application of test substances from an automated micropipettor.

**Sources of reagents.** 2′-O-Me-cAMP, N′,N′-Benzyoladenosine-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**


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**References**


