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Role of the cAMP sensor Epac as a determinant of K_{ATP} channel ATP sensitivity in human pancreatic β-cells and rat INS-1 cells

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Protein kinase A (PKA)-independent actions of adenosine 3′,5′-cyclic monophosphate (cAMP) are mediated by Epac, a cAMP sensor expressed in pancreatic β-cells. Evidence that Epac might mediate the cAMP-dependent inhibition of β-cell ATP-sensitive K^+ channels (K_{ATP}) was provided by one prior study of human β-cells and a rat insulin-secreting cell line (INS-1 cells) in which it was demonstrated that an Epac-selective cAMP analogue (ESCA) inhibited a sulphonylurea-sensitive K^+ current measured under conditions of whole-cell recording. Using excised patches of plasma membrane derived from human β-cells and rat INS-1 cells, we now report that 2′-*O*-Me-cAMP, an ESCA that activates Epac but not PKA, sensitizes single K_{ATP} channels to the inhibitory effect of ATP, thereby reducing channel activity. In the presence of 2′-*O*-Me-cAMP (50 μM), the dose–response relationship describing ATP-dependent inhibition of K_{ATP} channel activity (NP_o) is left-shifted such that the concentration of ATP producing 50% inhibition (IC_{50}) is reduced from 22 μM to 1 μM for human β-cells, and from 14 μM to 4 μM for rat INS-1 cells. Conversely, when patches are exposed to a fixed concentration of ATP (10 μM), the administration of 2′-*O*-Me-cAMP inhibits channel activity in a dose-dependent and reversible manner (IC_{50} 12 μM for both cell types). A cyclic nucleotide phosphodiesterase-resistant ESCA (Sp-8-pCPT-2′-*O*-Me-cAMPS) also inhibits K_{ATP} channel activity, thereby demonstrating that the inhibitory actions of ESCAs reported here are unlikely to arise as a consequence of their hydrolysis to bioactive derivatives of adenosine. On the basis of such findings it is concluded that there exists in human β-cells and rat INS-1 cells a novel form of ion channel modulation in which the ATP sensitivity of K_{ATP} channels is regulated by Epac.

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Although the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) activates protein kinase A (PKA), recent studies provide compelling evidence for an alternative mechanism of cAMP signal transduction, one that involves cAMP-regulated guanine nucleotide exchange factors (cAMP-GEF) known as Epacs (the *E*xchange *P*rotein directly *A*ctivated by *C*yclic AMP; de Rooij *et al.* 1998; Kawasaki *et al.* 1998). The analysis of Epac-mediated signal transduction has been facilitated by the development of Epac-selective cyclic AMP analogues (ESCAs). These synthetic analogues of cAMP incorporate a 2′-*O*-alkyl substitution on the ribose ring of cAMP, as in 2′-*O*-Me-cAMP, a modification that impairs their ability to activate PKA, while leaving intact their ability to activate Epac (Christensen *et al.* 2003). An ESCA that also incorporates a hydrophobic parachlorophenylthio (pCPT) substitution on the adenine moiety of cAMP is 8-pCPT-2′-*O*-Me-cAMP (Enserink *et al.* 2002). It is cell permeant and when it is applied to living cells, it stimulates Ca^{2+} signalling and exocytosis in multiple cell types (Holz *et al.* 2006, 2008*a*). We recently reported that 2′-*O*-Me-cAMP and 8-pCPT-2′-*O*-Me-cAMP inhibit a sulphonylurea-sensitive K^+ current in pancreatic β-cells through an as yet to be determined mechanism (Kang *et al.* 2006). In the present study we applied the methods of single channel analysis to human pancreatic β-cells and a rat β-cell line (INS-1 cells; a cell line that expresses Epac and K_{ATP} channels; Leech *et al.* 2000; Moritz *et al.* 2001) in order to investigate the nature of this effect.

ATP-sensitive K^+ channels (K_{ATP} channels) expressed in β-cells are hetero-octamers comprising Kir6.2 and sulphonylurea receptor-1 (SUR1) subunits (Ashcroft, 2005). Kir6.2 constitutes the K^+-selective pore-forming subunit, whereas SUR1 acts as an allosteric regulator of Kir6.2 gating (Nichols, 2006). Although ATP interacts directly with Kir6.2 to close the channel, the binding of

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Mg-ADP to SUR1 opens the channel. Intriguingly, new studies demonstrate direct interactions of SUR1 with Epac proteins, a finding first reported by Seino and coworkers (Ozaki *et al.* 2000). For example, when expressed in HEK cells, both Epac1 and Epac2 interact with full-length SUR1 (Kang *et al.* 2006), and for Epac2 this interaction appears to be mediated by nucleotide-binding fold-1 (NBF-1) of SUR1 (Shibasaki *et al.* 2004*a*,*b*; Bryan *et al.* 2007). Since cAMP-elevating agents inhibit β-cell K_{ATP} channel activity (Holz & Habener, 1992; Holz *et al.* 1993; Barnett *et al.* 1994; Gromada *et al.* 1998; He *et al.* 1998; Suga *et al.* 2000; Ding *et al.* 2001; Light *et al.* 2002; Kang *et al.* 2006), we have proposed that the interaction of Epac with SUR1 confers cAMP-dependent regulation of this channel's function (Holz *et al.* 2006; Kang *et al.* 2006).

In the present study we sought to determine if the interaction of cAMP with Epac might regulate the sensitivity of K_{ATP} channels to ATP. Consistent with this concept, we now demonstrate that in human β-cells and rat INS-1 cells, 2′-*O*-Me-cAMP sensitizes K_{ATP} channels to the inhibitory effect of ATP, thereby producing a left-shift of the concentration–response relationship describing ATP-dependent inhibition of K_{ATP} channel activity. We also report that Sp-8-pCPT-2′-*O*-Me-cAMPS, a cyclic nucleotide phosphodiesterase (PDE) resistant ESCA, produces a decrease of K_{ATP} channel activity, thereby demonstrating that the inhibitory actions of ESCAs reported here are unlikely to be a consequence of their hydrolysis to bioactive derivatives of adenosine, a confounding effect observed in other cell types (Laxman *et al.* 2006). In summary, the new findings presented here demonstrate that there exists in β-cells a novel form of ion channel modulation in which the ATP sensitivity of K_{ATP} channels is regulated by Epac.

Methods

Cell culture

With informed consent for tissue use for research, human islets of Langerhans were obtained from cadaver donors. They were provided, with safeguards for donor anonymity, by the Islet Cell Resource Service of the National Institutes of Health, National Center for Research Resources. (http://icr.coh.org/). Approval for the use of human islets in the laboratory of G. G. Holz was granted by the Institutional Review Board of New York University School of Medicine. Single cell suspensions of human islet cells were prepared by digestion of islets with trypsin-EDTA, and single cells were plated onto glass coverslips (25CIR-1; Fisher Scientific) coated with 1 mg ml^{-1} concanavalin A (type V; Sigma-Aldrich, St Louis, MO, USA). Cell cultures were maintained in a humidified incubator (95% air, 5% CO_2) at 37°C in CMRL-1066 modified culture medium (Mediatech, Inc., Herndon, VA, USA; cat. no. 99–603-CV) containing 10% (v/v) fetal bovine serum (FBS). β-Cells were identified by fluorescence microscopy after infection of the cultures with adenovirus directing expression of enhanced yellow fluorescent protein (EYFP) under the control of the rat insulin 2 gene promoter (Kang *et al.* 2003). INS-1 cells (passages 70–90) were obtained from Dr C. B. Wollheim (University Medical Center, Geneva, Switzerland). INS-1 cells are an immortalized cell line derived from a radiation-induced insulinoma of rat pancreas (Asfari *et al.* 1992). INS-1 cells were maintained in RPMI 1640 medium containing 10 mM Hepes, 11.1 mM glucose, 10% FBS, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (Asfari *et al.* 1992; Chepurny & Holz, 2007). INS-1 cells were passaged by trypsinization and subcultured once a week. Reagents for INS-1 cell culture were obtained from Invitrogen Life Technologies (Rockville, MD, USA).

Measurement of single channel K_{ATP} currents

For the detection of single K_{ATP} channel currents in inside-out patches, pipettes pulled from borosilicate glass were fire-polished and back-filled with a solution containing (mM): 140 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 5 Hepes (300 mosmol l^{-1}; pH 7.3). Patches were excised into a bath solution containing (mM): 70 K_2SO_4, 2.0 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 0.2 GTP, 5 Hepes, and the indicated [ATP]. The free [Ca^{2+}] of this solution was 160 nM, the pH was 7.3, and the osmolarity was 300 mosmol l^{-1}. The patch potential was maintained at −100 mV in order to measure inward K^+ currents through K_{ATP} channels. Data were acquired using an Axopatch 200B amplifier under the control of pCLAMP v.9.2 (Molecular Devices, Sunnyvale, CA, USA). Channel currents were low-pass filtered at 1 kHz (8-pole Bessel filter) and digitized at 5 or 10 kHz. All experiments were performed at 22–26°C on the stage of an inverted microscope.

Analysis of single channel K_{ATP} currents

K_{ATP} channel activity was determined prior to, during and following recovery from the application of test substances. Channel activity was calculated as the value NP_o from 20 to 40 s intervals of data, where N is the number of channels active in the patch, and P is the probability that a channel is open. Values of NP_o were determined by constructing all-points amplitude histograms of channel activity (bin width 0.5 pA) using pCLAMP v.9.2. Gaussian curves were superimposed on the histograms using Origin 7.5 (OriginLab Corp., Northampton, MA, USA). For studies in which values of NP_o were compared amongst groups of patches exposed to different test solutions, statistical significance of the results was evaluated using SigmaStat

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software (Systat Software, San Jose, CA, USA) in order to perform one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test for comparing control and test groups. Differences amongst the groups were considered significant at $P < 0.05$. Best-fits of the dose–response relationships describing the actions of 2′-*O*-Me-cAMP or ATP to inhibit K_{ATP} channel activity were obtained using the nonlinear regression curve-fitting module of SigmaPlot v.10.0 (Systat Software). The data were fitted to the Hill equation:

$$X/X_c = 1/[1 + ([\text{Test substance}]/\text{IC}_{50})^h], \qquad (1)$$

where IC_{50} is the concentration of 2′-*O*-Me-cAMP or ATP producing 50% inhibition, h is the Hill coefficient, X is the is the value of NP_o in the presence of 2′-*O*-Me-cAMP or ATP, and X_c is the NP_o value in the absence of 2′-*O*-Me-cAMP or ATP.

Application of test solutions and sources of reagents

cAMP analogues were applied to the inner surfaces of inside-out patches using a RSC-160 rapid solution changer (Molecular Kinetics Inc., Indianapolis, IN, USA) or a Dynaflow Pro II microfluidics drug delivery system using 8- or 16-well chambers (Cellectricon Inc., Gaithersburg, MD, USA). Rapid exchange of test solutions was performed under computer control. 2′-*O*-Me-cAMP, Sp-8-pCPT-2′-*O*-Me-cAMPS, Rp-cAMPS, 6-Bnz-cAMP, cAMP and 2′-*O*-Me-cGMP were obtained from BIOLOG (Bremen, Germany). ATP and GTP were from Sigma-Aldrich.

Results

2′-*O*-Me-cAMP inhibits K_{ATP} channel activity in INS-1 cells

When the inner surfaces of excised patches were exposed to a fixed concentration of ATP (10 μM), the Epac-selective cAMP analogue 2′-*O*-Me-cAMP (50 μM) inhibited INS-1 cell K_{ATP} channel activity in 16 of 20 patches (Fig. 1*A*). Measurement of NP_o values deduced from an all-points amplitude histogram (Fig. 1*B*) demonstrated that 2′-*O*-Me-cAMP reduced channel activity by $94 \pm 5\%$ in patches in which an effect of the ESCA was observed (Fig. 1*C*). This action of the ESCA was unlikely to be a consequence of a direct channel block because in 4 of 20 patches exhibiting K_{ATP} channel activity, 2′-*O*-Me-cAMP was without action whereas 1 mM ATP remained effective (data not shown). The inhibitory action of 2′-*O*-Me-cAMP developed over 5–10 s, longer than that measured for the onset of inhibition by ATP, despite the fact that the test solution containing 2′-*O*-Me-cAMP was delivered to the patch within 200 ms. With continual application of 2′-*O*-Me-cAMP, little or no desensitization was evident. Upon washout of 2′-*O*-Me-cAMP, partial recovery of channel activity was observed within 10–30 s, after which the effect of 2′-*O*-Me-cAMP was repeatable.

Determination of the cumulative dose–response relationship demonstrated the potency of 2′-*O*-Me-cAMP in this assay (Fig. 2*A* and *B*). In the presence of 10 μM ATP, the concentration of 2′-*O*-Me-cAMP producing a 50% inhibition of channel activity (IC_{50}) was 12 μM, a value in agreement with the reported affinity of 2′-*O*-Me-cAMP for Epac1 (Christensen *et al.* 2003). The action of 2′-*O*-Me-cAMP was mimicked by a high concentration (300 μM) of cAMP (Fig. 3*A*–*C*), whereas channel activity was unaffected by 100 μM 2′-*O*-Me-cGMP (Fig. 3*A* and *C*; note that 2′-*O*-Me-cGMP fails to activate Epac; Kang *et al.* 2006). It may be concluded that the inhibitory action of 2′-*O*-Me-cAMP reported here results from a selective interaction of this ESCA with a cAMP-binding protein that most likely corresponds to Epac.

2′-*O*-Me-cAMP acts independently of PKA to inhibit K_{ATP} channel activity in INS-1 cells

Although ESCAs activate Epac in a selective manner, they have a limited ability to activate PKA when tested at concentrations in excess of 100 μM (Enserink *et al.* 2002). However, such a PKA-mediated action of 2′-*O*-Me-cAMP is unlikely to explain the findings reported here. This conclusion is reached because 50 μM 2′-*O*-Me-cAMP inhibited K_{ATP} channel activity under conditions in which the inner surfaces of excised patches were exposed to 100 μM Rp-cAMPS (Fig. 4*A* and *C*). Rp-cAMPS is a cAMP antagonist that blocks the activation of PKA by cAMP (Dostmann *et al.* 1990), but which fails to prevent the activation of Epac by ESCAs (Rangarajan *et al.* 2003; Holz *et al.* 2008*a*). Interestingly, Rp-cAMPS, alone, inhibited INS-1 cell K_{ATP} channel activity when patches were exposed to a fixed concentration (10 μM) of ATP (Fig. 4*A* and *C*). In contrast, 6-Bnz-cAMP (100 μM), a cAMP analogue that activates PKA but not Epac (Christensen *et al.* 2003), stimulated channel activity (Fig. 4*B* and *C*). Such findings suggest a dual action of cAMP, one in which channel activity is stimulated in a PKA-mediated manner, whereas channel activity is inhibited in an Epac-mediated manner. Consistent with this concept, we found that when patches were exposed to 10 μM ATP, a low concentration of cAMP (1 μM) increased the value of NP_o by $20 \pm 5\%$ (mean $\pm$ S.E.M., $n = 5$ patches), whereas a high concentration of cAMP (300 μM) decreased the value of NP_o by $88 \pm 4\%$ ($n = 6$ patches).

2′-*O*-Me-cAMP interacts cooperatively with ATP to inhibit K_{ATP} channels in INS-1 cells

We next sought to determine to what extent 2′-*O*-Me-cAMP and ATP interact to inhibit K_{ATP}

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channel activity. To this end, the action of 2′-*O*-Me-cAMP was assessed under conditions in which test solutions contained either no ATP or a fixed concentration of ATP. When patches were exposed to a test solution containing no ATP, 2′-*O*-Me-cAMP (50 μM) inhibited K_{ATP} channel activity, but this action of the ESCA was slow in onset (Fig. 5*A*). Thus, in the absence of 2′-*O*-Me-cAMP, the value of NP_o measured in an ATP-free solution was 0.18 ± 0.03 and this value decreased to 0.06 ± 0.02 during a 40 s application of 2′-*O*-Me-cAMP. Subsequently, the value of NP_o recovered to 0.13 ± 0.02 after washout of 2′-*O*-Me-cAMP (mean ± S.E.M., $n = 5$ patches). It may be concluded that the inhibitory action of 2′-*O*-Me-cAMP reported here is not strictly ATP-dependent. Despite this fact, when excised patches were exposed to a test solution containing both 2′-*O*-Me-cAMP and 30 μM ATP, a faster and stronger inhibition of K_{ATP} channel activity was measured (Fig. 5*B*). This interaction of 2′-*O*-Me-cAMP

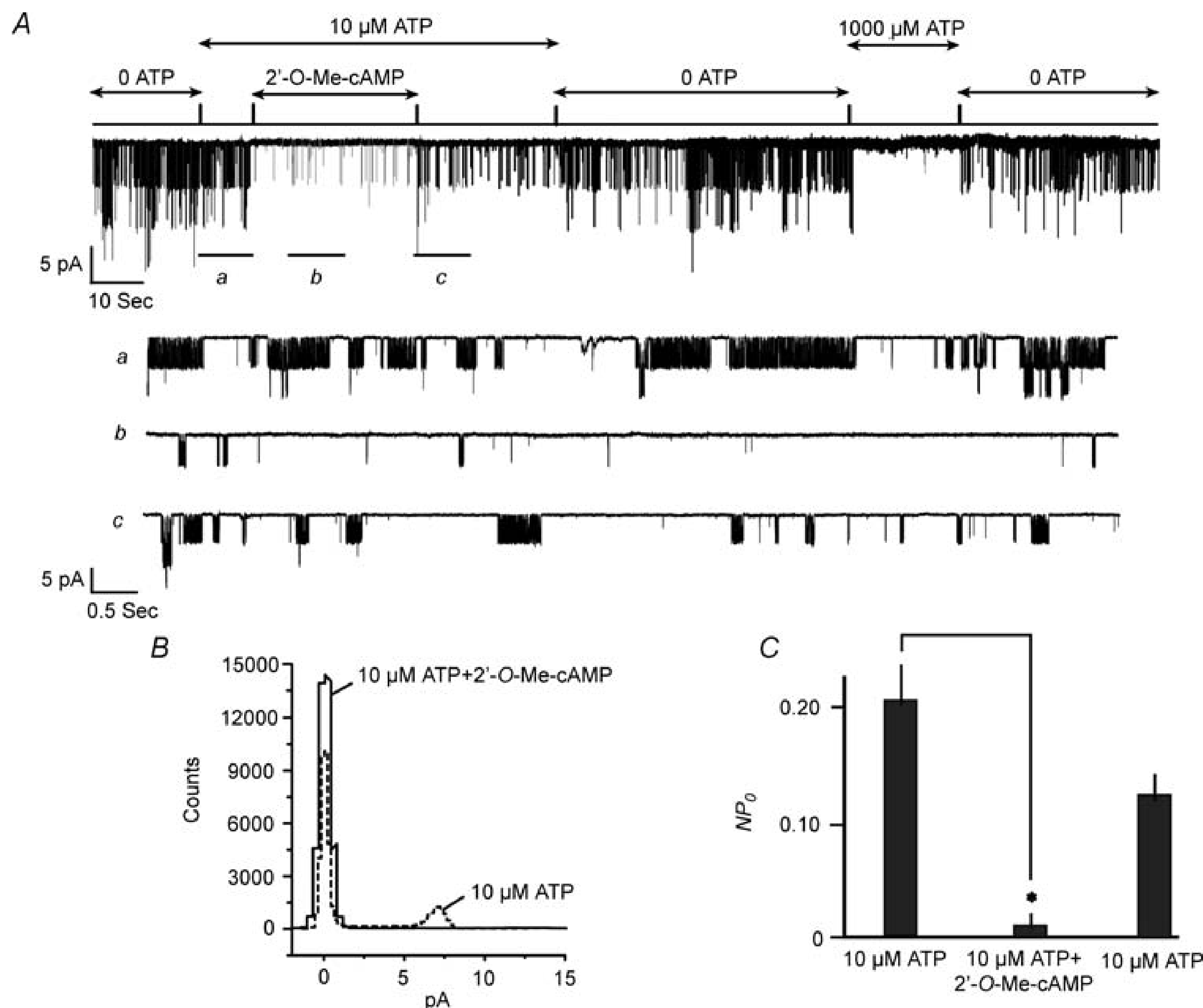

Figure 1. Inhibition of K_{ATP} channel activity by 2′-*O*-Me-cAMP in INS-1 cells

A, 2′-*O*-Me-cAMP was applied at a concentration of 50 μM to an inside-out patch. The inhibition of channel activity by 2′-*O*-Me-cAMP is illustrated on a compressed time scale (top trace) or on an expanded time scale (traces *a*, *b*, and *c*) in order to depict channel activity prior to (*a*), during (*b*), and following washout (*c*) of 2′-*O*-Me-cAMP. Unitary currents measured in this patch resulted from the activity of K_{ATP} channels because they were abolished during application of 1 mM ATP (top trace). Arrows at the top indicate when the patch was exposed to each test solution. Patch potential was −100 mV here and in subsequent figures. *B*, all-points amplitude histogram depicting K_{ATP} channel activity prior to (dotted line) or during (continuous line) application of 50 μM 2′-*O*-Me-cAMP to the same patch depicted in panel *A*. *C*, population study summarizing the action of 50 μM 2′-*O*-Me-cAMP to decrease values of NP_o in excised patches under conditions in which the ATP concentration was fixed at 10 μM. Channel activity prior to and following washout of 2′-*O*-Me-cAMP is depicted by the left-most and right-most bars, respectively. Values of NP_o for each bar are the mean ± S.E.M. for 16 patches and were not subjected to normalization. *The value of NP_o was different ($P < 0.05$ here and in subsequent figures).

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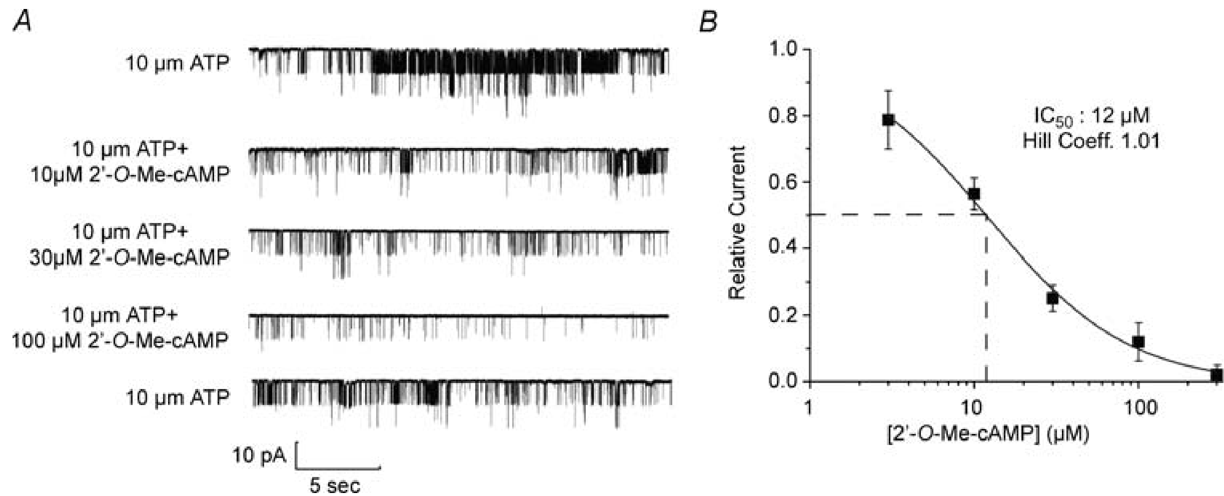

Figure 2. Dose–response relationship for INS-1 cell K_{ATP} channel inhibition by 2′-*O*-Me-cAMP
A, K_{ATP} channel activity was measured under conditions in which a progressively higher concentration of 2′-*O*-Me-cAMP was administered while maintaining the ATP concentration at 10 μM. Illustrated are continuous data subdivided into five traces. The top trace indicates control activity in the absence of 2′-*O*-Me-cAMP. The bottom trace indicates recovery of channel activity after wash out of 2′-*O*-Me-cAMP. *B*, cumulative dose–response relationship describing the inhibition of K_{ATP} channel activity, as generated using the experimental design illustrated in panel *A*. For each concentration of 2′-*O*-Me-cAMP the value of NP_o was normalized relative to a value of 1.0, which represents the relative current measured when patches were exposed to 10 μM ATP in the absence of 2′-*O*-Me-cAMP. Each data point (squares) is the mean ± S.E.M. for 5 patches. Dashed lines indicate the IC_{50} concentration of 2′-*O*-Me-cAMP.

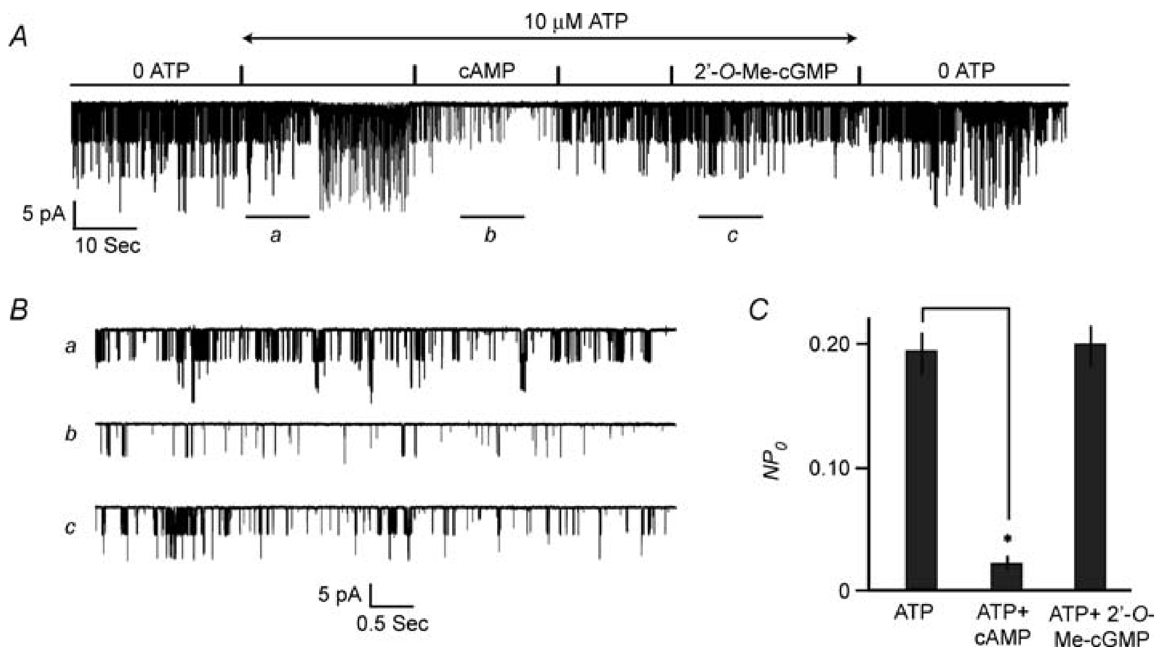

Figure 3. INS-1 cell K_{ATP} channel activity is inhibited by cAMP but not 2′-*O*-Me-cGMP
A, cAMP (300 μM) or 2′-*O*-Me-cGMP (100 μM) was applied under conditions in which an excised patch was continuously exposed to 10 μM ATP. *B*, the same experiment illustrated in *A* but depicted on an expanded time scale. Traces *a*, *b* and *c* correspond to the time periods indicated by the horizontal bars in the trace illustrated in *A*. *C*, population study summarizing the action of cAMP (300 μM) and the lack of action of 2′-*O*-Me-cGMP (100 μM) to alter values of NP_o in excised patches exposed to 10 μM ATP. Values of NP_o for each bar are the mean ± S.E.M. for 6 patches and were not normalized.

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Inhibition of K_{ATP} channel activity by 2′-*O*-Me-cAMP in human pancreatic β-cells

The physiological relevance of Epac-mediated K_{ATP} channel regulation was evaluated using human pancreatic β-cells maintained in primary cell culture. β-Cells were identified on the basis of rat insulin 2 gene promoter-directed expression of EYFP (Kang *et al.* 2003) and K_{ATP} channel activity was monitored in excised inside-out patches. Similar to what was observed for INS-1 cells, 2′-*O*-Me-cAMP exerted a dose-dependent inhibitory action (IC_{50} 12 μM; data not shown) to reduce channel activity under conditions in which the concentration of ATP was maintained at 10 μM. Furthermore, a fixed concentration of 2′-*O*-Me-cAMP (50 μM) facilitated the inhibitory action of ATP at the channels (Fig. 7). To evaluate the action of 2′-*O*-Me-cAMP in greater detail, the dose–response relationship describing ATP-dependent inhibition of K_{ATP} channel activity was determined in the absence or presence of 2′-*O*-Me-cAMP. In the absence of 2′-*O*-Me-cAMP, the concentration of ATP producing a 50% inhibition of channel activity was 21 μM. However, in the presence of 50 μM 2′-*O*-Me-cAMP, a left-shift of the ATP dose–response relationship was observed, and under these conditions the concentration of ATP producing a 50% inhibition of channel activity was 1 μM ($n = 6$ patches).

PDE-resistant Sp-8-pCPT-2′-O-Me-cAMPS inhibits K_{ATP} channel activity

PDE-catalysed hydrolysis of ESCAs can generate bioactive derivatives of adenosine, substances that alter cellular

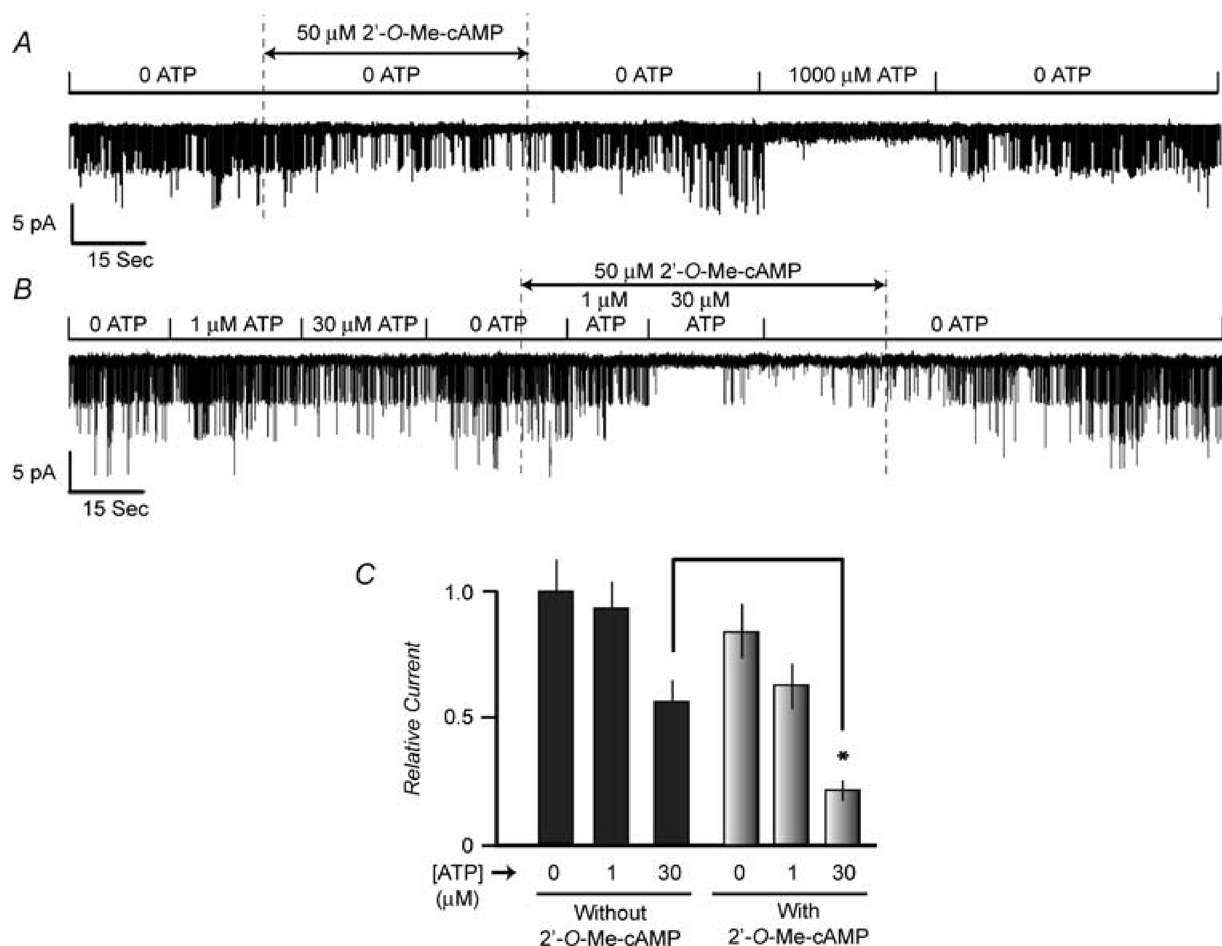

Figure 5. Interaction of ATP and 2′-*O*-Me-cAMP to inhibit K_{ATP} channels in INS-1 cells
A, K_{ATP} channel activity was inhibited by 2′-*O*-Me-cAMP (50 μM) in the absence of ATP. Note that the inhibition of channel activity developed slowly. Channel activity recovered upon wash out of 2′-*O*-Me-cAMP. *B*, in the presence of ATP (30 μM), the inhibitory action of 2′-*O*-Me-cAMP (50 μM) was faster in onset and of greater magnitude. *C*, population study summarizing the interaction of ATP and 2′-*O*-Me-cAMP to decrease values of NP_o. For each patch, ATP was tested at a concentration of 0, 1 and 30 μM in the absence or presence of 50 μM 2′-*O*-Me-cAMP. Values of NP_o for each bar in *C* are the mean ± S.E.M. for 7 patches and were normalized relative to the channel activity measured in an ATP-free solution, which was assigned a value of 1.0.

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function independently of Epac (Laxman *et al.* 2006). Such a confounding effect of cyclic nucleotide metabolism can complicate the interpretation of studies using ESCAs (Holz *et al.* 2008*a*). This complication may be circumvented by validating that a PDE-resistant ESCA such as Sp-8-pCPT-2′-*O*-Me-cAMPS mimics the action of a PDE-sensitive ESCA (Laxman *et al.* 2006). To validate that the inhibitory action of 2′-*O*-Me-cAMP reported here did not result from its hydrolysis to bioactive derivatives of adenosine, it was confirmed that Sp-8-pCPT-2′-*O*-Me-cAMPS inhibited K_{ATP} channel activity in INS-1 cells under conditions in which excised patches were exposed to a fixed concentration (10 μM) of ATP (Fig. 8*A* and *B*). Values of NP_o deduced from an all-points amplitude histogram (not shown) demonstrated that on average, 100 μM Sp-8-pCPT-2′-*O*-Me-cAMPS decreased K_{ATP} channel activity by 72 ± 3% (Fig. 8*C*).

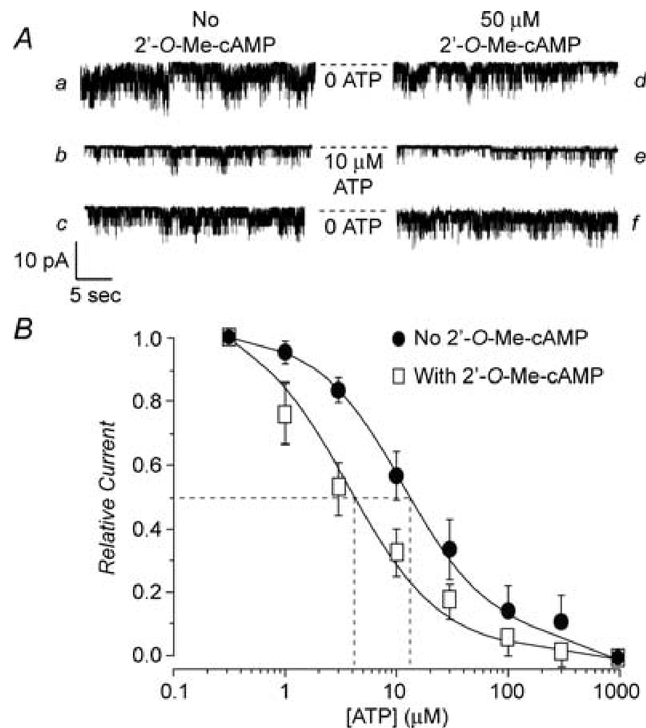

Figure 6. 2′-*O*-Me-cAMP increases the ATP sensitivity of INS-1 cell K_{ATP} channels
A, experimental design for establishment of the dose–response relationship describing the interaction of ATP and 2′-*O*-Me-cAMP to inhibit K_{ATP} channels. The K_{ATP} channel activity is illustrated for a single excised patch under conditions in which no 2′-*O*-Me-cAMP was present (traces labelled as *a*, *b* and *c*) or during administration of 50 μM 2′-*O*-Me-cAMP (right series of traces labelled as *d*, *e* and *f*). Traces *a* and *c* as well as *d* and *f* illustrate channel activity in an ATP-free solution. Traces *b* and *e* illustrate channel activity during exposure of the patch to 10 μM ATP. Each test solution was administered in the order *a* through *e* and the duration of exposure to each test solution was 45 s. Note that channel activity was inhibited by ATP in a reversible manner, and that the inhibitory effect of ATP was stronger under conditions in which the patch was also exposed to 2′-*O*-Me-cAMP. Dashed lines indicate the pipette current corresponding to the closed state of the channels. *B*, dose–response relationship describing the action of ATP to inhibit K_{ATP} channel activity under conditions in which excised patches were not exposed to 2′-*O*-Me-cAMP (●) or when patches were exposed to 50 μM 2′-*O*-Me-cAMP (□). ● indicate values of NP_o normalized relative to a value of 1.0, which represents the relative current measured in the presence of 0.3 μM ATP alone. □ indicate values of NP_o normalized relative to a value of 1.0, which represents the relative current measured in the combined presence of 0.3 μM ATP and 50 μM 2′-*O*-Me-cAMP. Each data point is the mean ± S.E.M. for 5 patches. Dashed lines indicate the method by which the IC_{50} concentration of ATP was estimated. For patches exposed to 2′-*O*-Me-cAMP, it was confirmed that similar values of channel activity existed prior to and following washout of the ESCA.

Discussion

Evidence for Epac-mediated regulation of K_{ATP} channel activity

Here the novel pharmacological properties of Epac-selective cAMP analogues (ESCAs) have been exploited in order to assess the potential role of cAMP sensor Epac as a determinant of K_{ATP} channel activity in human pancreatic β-cells and rat INS-1 cells. Using two structurally related ESCAs, 2′-*O*-Me-cAMP and Sp-8-pCPT-2′-*O*-Me-cAMPS, we document an inhibitory effect of ESCAs on the activity of K_{ATP} channels at the single channel level. Such findings extend on our prior study of human β-cells and rat INS-1 cells in which the ESCA 8-pCPT-2′-*O*-Me-cAMP was demonstrated to inhibit a sulphonylurea-sensitive K^+ current measured under conditions of whole-cell recording (Kang *et al.* 2006). The main finding of the present study is that 2′-*O*-Me-cAMP sensitized single K_{ATP} channels to the inhibitory effect of ATP, thereby reducing channel activity. This sensitizing action of the ESCA produced a left-shift of the dose–response relationship describing ATP-dependent inhibition of channel activity so that the concentration of ATP producing a 50% inhibition (IC_{50}) was reduced from approximately 22 μM to 1 μM for human β-cells, and from 14 to 4 μM for INS-1 cells. Conversely, under conditions in which the ATP concentration was held constant at 10 μM, 2′-*O*-Me-cAMP exerted a dose-dependent action to inhibit K_{ATP} channel activity, an effect that was both reversible and repeatable. On the basis of such findings it is concluded that there exists a previously unrecognized form of ion channel modulation in which the ATP sensitivity of β-cell K_{ATP} channels is regulated by Epac.

Interpretation of findings obtained using Sp-8-pCPT-2′-*O*-Me-cAMPS

Findings presented here are the first to demonstrate the efficacy of Sp-8-pCPT-2′-*O*-Me-cAMPS as an inhibitor of K_{ATP} channel function. An assessment of the action of Sp-8-pCPT-2′-*O*-Me-cAMPS was deemed necessary

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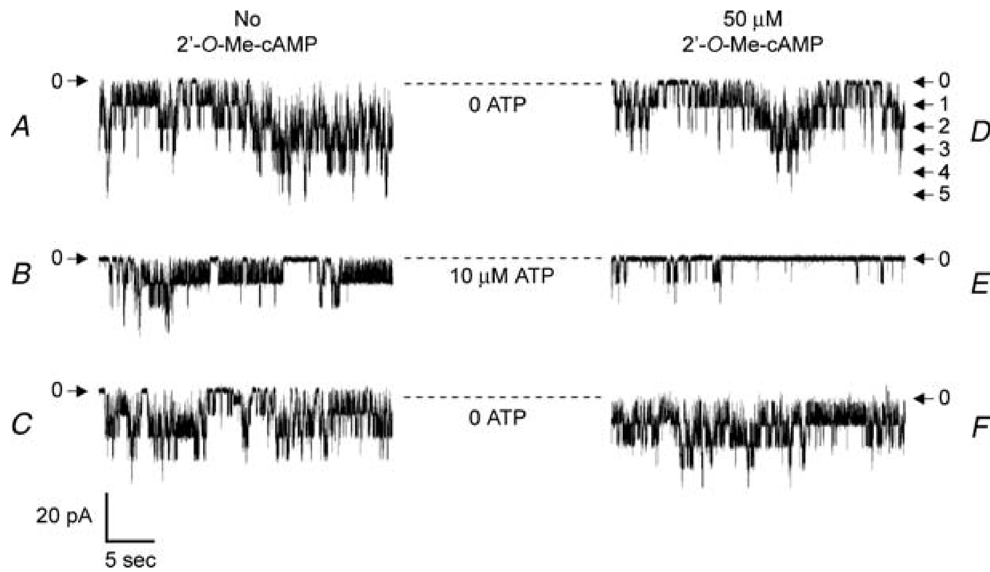

Figure 7. Interaction of ATP and 2′-*O*-Me-cAMP to inhibit K_{ATP} channels in human pancreatic β-cells
K_{ATP} channel activity is illustrated for an excised patch under conditions in which no 2′-*O*-Me-cAMP was present (traces *a*, *b* and *c*) or during administration of 50 μM 2′-*O*-Me-cAMP (right series of traces *d*, *e* and *f*). Arrows indicate the current levels at which channels are closed (0) or when one or more channels are open (1–5). Dashed lines indicate the pipette current corresponding to the closed state of the channels. Traces *a* and *c* as well as *d* and *f* illustrate channel activity in an ATP-free solution. Traces *b* and *e* illustrate channel activity during exposure of the patch to 10 μM ATP. Each solution was administered in the order *a* through *e* and the duration of exposure to each solution was 45 s. Note that channel activity was inhibited by ATP in a reversible manner, and that the inhibitory effect of ATP was stronger under conditions in which the patch was exposed to 2′-*O*-Me-cAMP.

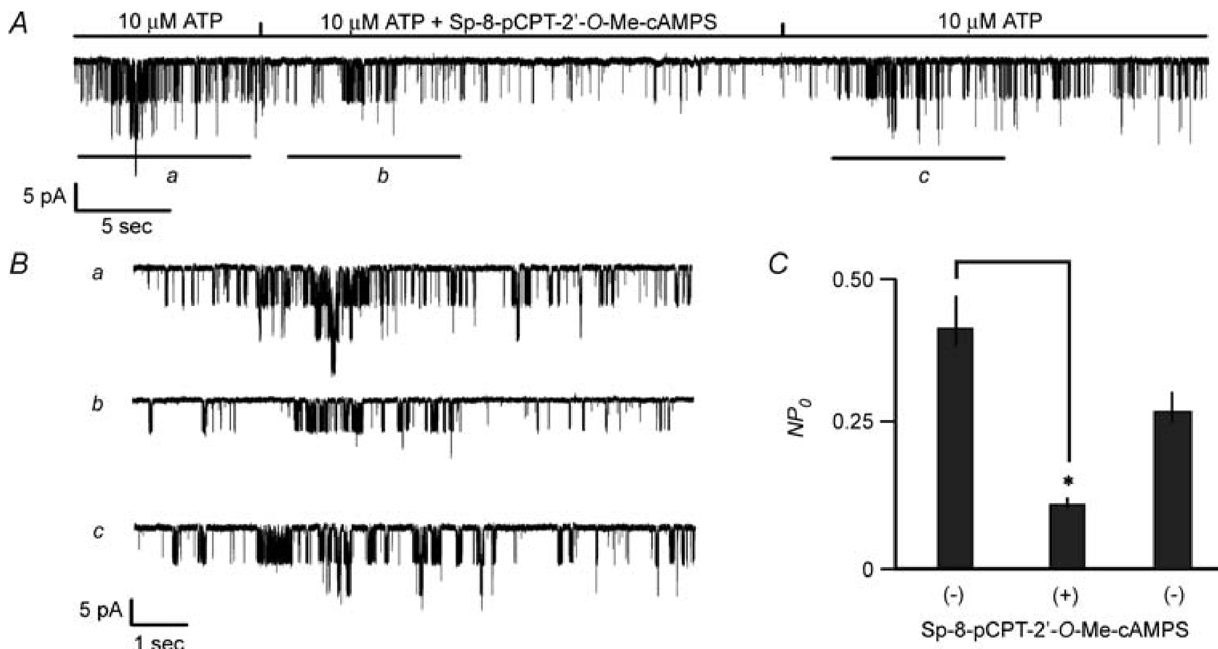

Figure 8. Inhibition of K_{ATP} channels by PDE-resistant Sp-8-pCPT-2′-O-Me-cAMPS
A, Sp-8-pCPT-2′-*O*-Me-cAMPS (100 μM) was applied under conditions in which a patch was continuously exposed to 10 μM ATP. *B*, the same experiment illustrated in *A* but illustrated on an expanded time scale. Traces a, *b* and *c* correspond to the time periods indicated by the horizontal bars in the trace illustrated in *A*. *C*, population study summarizing the action of Sp-8-pCPT-2′-*O*-Me-cAMPS (100 μM) to decrease values of NP_o in excised patches exposed to 10 μM ATP. Values of NP_o for each histogram bar are the mean ± S.E.M. for 8 patches and were not normalized.

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because in studies of the protozoan *Trypanosoma*, Laxman *et al.* (2006) reported that 8-pCPT-2′-*O*-Me-cAMP (a PDE-sensitive ESCA) exerted an antiproliferative effect not mediated by Epac. Evidently, PDE converts 8-pCPT-2′-*O*-Me-cAMP to 8-pCPT-2′-*O*-Me-adenosine, a bioactive metabolite that exerts Epac-independent actions to alter cellular function. Thus, if the PDEs of β-cells or INS-1 cells were to also hydrolyse ESCAs, a PDE-sensitive ESCA such as 2′-*O*-Me-cAMP might act independently of Epac to inhibit K_{ATP} channel function. However, such an Epac-independent mechanism of ESCA action is unlikely to explain the findings presented here. This conclusion is reached because we found that in INS-1 cells, PDE-resistant Sp-8-pCPT-2′-*O*-Me-cAMPS inhibited K_{ATP} channel activity in a manner analogous to that of 2′-*O*-Me-cAMP.

Laxman *et al.* (2006) also reported the surprising finding that the PDEs of *Trypanosoma* are inhibited by 8-pCPT-2′-*O*-Me-cAMP. Thus, if ESCAs were to inhibit the PDEs expressed in β-cells or INS-1 cells, the net effect might be increased levels of cAMP, activation of PKA, and the Epac-independent inhibition of K_{ATP} channel function. This confounding scenario is unlikely for three reasons. First, Rp-cAMPS, a selective inhibitor of PKA activation, failed to antagonize the inhibitory action of 2′-*O*-Me-cAMP at the K_{ATP} channels of INS-1 cells. Secondly, 6-Bnz-cAMP, a selective activator of PKA, failed to reproduce the action of 2′-*O*-Me-cAMP, and in fact 6-Bnz-cAMP stimulated rather than inhibited K_{ATP} channel function. Third, in our prior study (Kang *et al.* 2006) we reported that 8-pCPT-2′-*O*-Me-cAMP failed to inhibit the macroscopic K_{ATP} current measured under conditions in which INS-1 cells were transfected with a dominant negative Epac1 that fails to bind cAMP. Thus, available evidence indicates that it is Epac rather than PKA that mediates the inhibitory action of ESCAs at K_{ATP} channels.

A potential mechanistic explanation for K_{ATP} channel regulation by Epac

The exact molecular mechanism by which cAMP, acting through Epac, alters K_{ATP} channel ATP sensitivity remains to be determined. Epac is a cAMP-GEF, and it activates the Ras-related GTPases Rap1 and Rap2, thereby influencing multiple cellular functions (reviewed by Bos, 2003, 2006; Holz, 2004*a*, 2006, 2008*a*). Notably, Epac is reported to act via Rap GTPase to stimulate a novel membrane-associated phospholipase C-ε that is expressed in INS-1 cells and which was first discovered by Kelley and coworkers (Kelley *et al.* 2001; G. G. Kelley, personal communication). Phospholipase C-ε catalyses the breakdown of polyphosphoinositides and it has been implicated in the cAMP-dependent regulation of intracellular Ca^{2+} handling in cardiac myocytes (Oestreich *et al.* 2007; Pereira *et al.* 2007) and in mammalian cell lines (Schmidt *et al.* 2001; Evellin *et al.* 2002). Given the established role of phosphatidylinositol bisphosphate (PIP_2) as a regulator of K_{ATP} channel function (Nichols, 2006), and given that PIP_2 supports K_{ATP} channel activity at least in part by reducing the channel's sensitivity to ATP (Baukrowitz *et al.* 1998; Shyng & Nichols, 1998), a cAMP signalling mechanism that uses Epac, Rap, and phospholipase C-ε to reduce levels of PIP_2 in the plasma membrane would be expected to increase the sensitivity of K_{ATP} channels to ATP. Thus, the SUR1 subunit of K_{ATP} channels might be viewed as a scaffold protein at which a signalling complex comprising Rap and phospholipase C-ε forms as a consequence of the interaction of Epac with NBF-1 of SUR1. It will be of particular interest to ascertain in future studies to what extent the targeted gene deletion of phospholipase C-ε alters cAMP signalling and K_{ATP} channel function in β-cells.

Physiological significance of Epac-mediated K_{ATP} channel regulation

Numerous reports have recently appeared in which Epac is proposed to mediate stimulatory actions of cAMP on insulin secretion from β-cells (Seino & Shibasaki, 2005; Holz *et al.* 2006; Shibasaki *et al.* 2007). This Epac-mediated action of cAMP does not exist in isolation, but is instead complemented by a stimulatory effect of cAMP that is mediated by PKA (Holz, 2004*a*). Importantly, both the Epac and PKA-mediated pro-secretagogue actions of cAMP are known to be contingent on exposure of β-cells to elevated concentrations of glucose. This observation has led to the conclusion that cAMP exerts its secretagogue action by potentiating glucose-stimulated insulin secretion (GSIS). From a theoretical standpoint, cAMP could potentiate GSIS at either a late or an early step in stimulus–secretion coupling. In fact, a late step does exist in which cAMP, acting through Epac and PKA, facilitates the fusion of secretory granules with the plasma membrane (Renstrom *et al.* 1997; Takahashi *et al.* 1999; Kashima *et al.* 2001; Fujimoto *et al.* 2002; Eliasson *et al.* 2003; Hatakeyama *et al.* 2006, 2007). In marked contrast, evidence also exists that cAMP potentiates GSIS at an earlier step, one that involves the regulated closure of K_{ATP} channels. In one proposed model, cAMP acts through Epac and PKA to render K_{ATP} channels more sensitive to the increase of cytosolic ATP/ADP concentration ratio that occurs when β-cells are exposed to high levels of glucose (Holz, 2004*b*). This effect is achieved because cAMP promotes K_{ATP} channel closure by modulating the ATP and/or ADP sensitivity of K_{ATP} channels. The model is supported by published findings demonstrating inhibitory actions of cAMP-elevating agents at K_{ATP} channels, effects that are reinforced by glucose metabolism, and which are either ATP or ADP dependent (Holz & Habener, 1992;

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Holz *et al.* 1993; Barnett *et al.* 1994; Gromada *et al.* 1998; He *et al.* 1998; Suga *et al.* 2000; Ding *et al.* 2001; Light *et al.* 2002; Kang *et al.* 2006).

With these points in mind, the primary physiological significance of the findings presented here is that an Epac-mediated mechanism is shown to exist by which cAMP regulates the ATP sensitivity of K_{ATP} channels in human β-cells and rat INS-1 cells. Whether such findings are applicable to rat or mouse β-cells remains to be determined. This might be the case because in a prior study of rat β-cells, Suga *et al.* (2000) found that the cAMP-elevating hormone glucagon-like peptide-1 (GLP-1) increased the sensitivity of single K_{ATP} channels to ATP, an effect analogous to that reported here for 2′-*O*-Me-cAMP. This action of GLP-1, which resulted in a decrease of membrane conductance accompanied by membrane depolarization, was not blocked by Rp-cAMPS, as expected if it is Epac rather than PKA that regulates K_{ATP} channel ATP sensitivity.

In the study of Suga and coworkers the IC_{50} value for inhibition of K_{ATP} channel activity by ATP was 11.6 μM in the absence of GLP-1, and 5.6 μM in the presence of GLP-1 (Suga *et al.* 2000). Thus, in the presence of GLP-1, the IC_{50} value describing the inhibitory action of ATP at rat β-cell K_{ATP} channels is nearly identical to the IC_{50} value (4 μM) we measured under conditions in which the K_{ATP} channels of rat INS-1 cells were exposed to 2′-*O*-Me-cAMP. Although these IC_{50} concentrations of ATP are well below the millimolar concentrations of ATP known to exist in the cytosol of intact cells, it is noteworthy that our findings, as well as those of Suga and coworkers, were obtained under conditions in which the inner surfaces of excised patches were exposed to a bath solution to which no Mg-ADP was added. Since Mg-ADP stimulates K_{ATP} channel function, and since Mg-ADP is present in the cytosol of intact cells, it might be that in the presence of Mg-ADP, agents such as GLP-1 or 2′-*O*-Me-cAMP increase K_{ATP} channel ATP sensitivity at concentrations of ATP that are within the millimolar range and which are considered to be physiological.

Although not examined here, cAMP-elevating agents may also regulate the ADP sensitivity of K_{ATP} channels. In a mammalian cell line expressing recombinant Kir6.2 and SUR1, Light and coworkers reported that application of the catalytic subunit of PKA (cPKA) to inside-out patches bathed in a low concentration of Mg-ADP (0.2 mM) resulted in a decrease of K_{ATP} channel activity (Light *et al.* 2002). In that study, the ATP sensitivity of K_{ATP} channels was not affected by cPKA, whereas the stimulatory effect of 0.2 mM Mg-ADP at the channel was diminished by cPKA. It was suggested that cAMP-elevating agents such as GLP-1 close K_{ATP} channels by reducing the channel's sensitivity to Mg-ADP. Taken together, the findings of Light and coworkers, as well as the findings presented here, provide evidence for an action of cAMP to decrease K_{ATP} channel activation by ADP while simultaneously increasing the channel's inhibition by ATP. Such an effect would be expected to render K_{ATP} channels highly sensitive to the increase of cytosolic ATP/ADP concentration ratio that occurs upon exposure of β-cells to elevated levels of glucose. In this manner, GLP-1 and glucose metabolism would interact to promote membrane depolarization and Ca^{2+} influx, two key events in β-cell stimulus–secretion coupling (Holz *et al.* 2008*b*).

Conclusion

The emerging role of the cAMP sensor Epac as a regulator of ion channel function is an unexpected and exciting development. The potential significance of this newly recognized mechanism of cAMP signal transduction is emphasized by the fact that Epac influences not only K_{ATP} channel function, as reported here, but also the function of voltage-dependent Ca^{2+} channels, Cl^- channels, Na^+ channels, and Ca^{2+}-dependent K^+ channels in a variety of cell types (Novara *et al.* 2004; Aromataris *et al.* 2006; Helms *et al.* 2006; Ster *et al.* 2007). There is also evidence for the Epac-mediated regulation of intracellular Ca^{2+} release channels, including IP_3 receptors and ryanodine receptors (Kang *et al.* 2001, 2003, 2005; Schmidt *et al.* 2001; Tsuboi *et al.* 2003; Morel *et al.* 2005; Yip, 2006; Oestreich *et al.* 2007; Pereira *et al.* 2007). Thus, it would seem that a new paradigm of ion channel modulation has been established in which the second messenger cAMP exerts its effects via Epac.

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