cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic β cells and rat INS-1 cells

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The Epac family of cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs, also known as Epac1 and Epac2) mediate stimulatory actions of the second messenger cAMP on insulin secretion from pancreatic β cells. Because Epac2 is reported to interact *in vitro* with the isolated nucleotide-binding fold-1 (NBF-1) of the β-cell sulphonylurea receptor-1 (SUR1), we hypothesized that cAMP might act via Epac1 and/or Epac2 to inhibit β-cell ATP-sensitive K^+ channels (K_{ATP} channels; a hetero-octomer of SUR1 and Kir6.2). If so, Epac-mediated inhibition of K_{ATP} channels might explain prior reports that cAMP-elevating agents promote β-cell depolarization, Ca^{2+} influx and insulin secretion. Here we report that Epac-selective cAMP analogues (2′-*O*-Me-cAMP; 8-pCPT-2′-*O*-Me-cAMP; 8-pMeOPT-2′-*O*-Me-cAMP), but not a cGMP analogue (2′-*O*-Me-cGMP), inhibit the function of K_{ATP} channels in human β cells and rat INS-1 insulin-secreting cells. Inhibition of K_{ATP} channels is also observed when cAMP, itself, is administered intracellularly, whereas no such effect is observed upon administration N^6-Bnz-cAMP, a cAMP analogue that activates protein kinase A (PKA) but not Epac. The inhibitory actions of Epac-selective cAMP analogues at K_{ATP} channels are mimicked by a cAMP agonist (8-Bromoadenosine-3′, 5′-cyclic monophosphorothioate, Sp-isomer, Sp-8-Br-cAMPS), but not a cAMP antagonist (8-Bromoadenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer, Rp-8-Br-cAMPS), and are abrogated following transfection of INS-1 cells with a dominant-negative Epac1 that fails to bind cAMP. Because both Epac1 and Epac2 coimmunoprecipitate with full-length SUR1 in HEK cell lysates, such findings delineate a novel mechanism of second messenger signal transduction in which cAMP acts via Epac to modulate ion channel function, an effect measurable as the inhibition of K_{ATP} channel activity in pancreatic β cells.

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The regulation of ion channel function by cyclic adenosine 3′,5′-monophosphate (cAMP) is achieved via protein kinase A (PKA)-mediated phosphorylation of the channels, or by the direct binding of cAMP to the channels (Hille, 2001). These established actions of cAMP are likely to be complemented by a novel signalling mechanism that utilizes cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs) designated as Epac (the exchange proteins activated by cyclic AMP) (de Rooij *et al.* 1998; Kawasaki *et al.* 1998). In mammalian cells, two variants of Epac are expressed (Epac1 and Epac2), each of which binds cAMP with micromolar affinity (K_d 1–3 μM) (de Rooij *et al.* 2000; Christensen *et al.* 2003). By binding cAMP, Epac couples cAMP production to the activation of Rap1 and Rap2, two small molecular weight GTPases of the Ras family (Bos, 2003; Rehmann *et al.* 2003*a*,*b*,*c*). Cellular processes stimulated by Epac include integrin-mediated cell adhesion (Rangarajan *et al.* 2003), gap junction formation (Somekawa *et al.* 2005), neurite outgrowth (Kiermayer *et al.* 2005), and phospholipase C-epsilon (PLC-ε) activation (Schmidt *et al.* 2001). An emerging body of evidence indicates that Epac may also link cAMP production to the regulation of ion channel function, Ca^{2+} signalling, and exocytosis in excitable cells (Renstrom *et al.*

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1997; Kang *et al.* 2001, 2003, 2005; Eliasson *et al.* 2003; Kang & Holz, 2003; Holz & Chepurny, 2003, 2005; Miura & Matsui, 2003; Tsuboi *et al.* 2003; Holz, 2004*a*,*b*; Morel *et al.* 2005; Landa *et al.* 2005; Seino & Shibasaki, 2005; Hashiguchi *et al.* 2006). For these reasons, we have chosen to investigate the potential role of Epac as a determinant of ATP-sensitive K^+ channel (K_{ATP} channel) activity in pancreatic β cells of the islets of Langerhans.

A role for Epac in the control of K_{ATP} channel function is indicated because earlier studies demonstrated an *in vitro* interaction of Epac2 with the isolated nucleotide-binding fold-1 (NBF-1) of the β-cell sulphonylurea receptor-1 (SUR1, an ATP-binding cassette protein). This interaction was studied within the context of a yeast two-hybrid screen using NBF-1 as bait (Ozaki *et al.* 2000), or in an immunoprecipitation assay utilizing bacterially expressed Epac2 and NBF-1 (Shibasaki *et al.* 2004*a*,*b*). Given that SUR1 oligomerizes with Kir6.2 to form inwardly rectifying K_{ATP} channels (Inagaki *et al.* 1995), such findings suggest that Epac2 might function as an accessory subunit of K_{ATP} channels. If this were to be the case, an interaction of Epac2 with SUR1 might explain earlier reports that cAMP-elevating agents including forskolin, isobutylmethylxanthine, glucagon, and the blood-glucose-lowering hormone glucagon-like peptide-1-(7–36-amide) (GLP-1) inhibit K_{ATP} channel function in β cells (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).

Since the closure of K_{ATP} channels is established to be a stimulus for β-cell depolarization, Ca^{2+} influx and insulin secretion (Holz & Habener, 1992; Henquin, 2000; Ashcroft, 2005), any Epac-mediated action of cAMP to inhibit K_{ATP} channels would be of considerable interest. Despite this possibility, it has yet to be determined what effect, if any, Epac exerts at K_{ATP} channels. Similarly, it is not certain whether Epac2 interacts with full-length SUR1, nor has it been determined if Epac1 also interacts with SUR1. With these points in mind, we sought to determine if it is Epac that mediates the cAMP-dependent inhibition of K_{ATP} channel function in human β cells or rat INS-1 insulin-secreting cells. Our studies were facilitated by the availability of cAMP analogues 2′-*O*-Methyladenosine-3′,5′-cyclic monophosphate, (2′-*O*-Me-cAMP); 8-(4-Chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate, (8-pCPT-2′-*O*-Me-cAMP); 8-(4-Methoxyphenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate, (8-pMeOPT-2′-*O*-Me-cAMP) that activate Epac selectively (Enserink *et al.* 2002; Christensen *et al.* 2003; Rehmann *et al.* 2003*a*). We now demonstrate that Epac-selective cAMP analogues, but not a PKA-selective analogue (N^6-Benzoyladenosine-3′,5′-cyclic monophosphate (N^6-Bnz-cAMP), or a cGMP analogue 2′-*O*-Methylguanosine-3′, 5′-cyclic monophosphate (2′-*O*-Me-cGMP), inhibit K_{ATP} channel activity measured under conditions of whole-cell dialysis in which a low concentration of ATP is administered intracellularly. This inhibitory action of cAMP analogues is not observed in INS-1 cells transfected with a dominant negative Epac1 that fails to bind cAMP. Since both Epac1 and Epac2 are demonstrated to coimmunoprecipitate with full-length SUR1, it is concluded that in pancreatic β cells, the cAMP-dependent inhibition of K_{ATP} channels is Epacmediated.

Methods

Cell culture

Human islets of Langerhans were provided under the auspices of the National Institutes of Health, National Center for Research Resources, Islet Cell Resource Service. Single-cell suspensions of human islet cells were prepared by digestion of islets with trypsin-EDTA, and the single β 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) (Holz *et al.* 1995, 1999). 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; catalogue 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 maintained in RPMI 1640 culture 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; Skoglund *et al.* 2000; Chepurny *et al.* 2002; Chepurny & Holz, 2002). INS-1 cells were passaged by trypsinization and subcultured once a week. All reagents for INS-1 cell culture were obtained from Invitrogen LifeTechnologies (Rockville, MD, USA).

Patch-clamp electrophysiology

Cells were bathed in a standard extracellular saline solution (SES) containing (mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 11.1 glucose, and 10 Hepes (295 mOsm pH adjusted to 7.4 with NaOH). Experiments were performed at 32°C using an inverted microscope (TE300; Nikon, Melville, NY, USA) equipped with a temperature-controlled stage (Medical Systems Corp., Greenvale, NY, USA), and fitted with a video imaging system (IonOptix Corp., Milton, MA, USA) for detection of EYFP epifluorescence. The K_{ATP} current was measured using the whole-cell, tight-seal configuration of the patch-clamp technique. Patch pipettes pulled from borosilicate glass (Kimax-51, tip resistance 2–3 MΩ) were

fire-polished and back-filled with an intracellular solution containing (mM): 90 K_2SO_4, 10 NaCl, 1 $MgCl_2$, 1.1 EGTA, 0.1 $CaCl_2$, 0.3 ATP, 0.2 GTP, 5 Hepes (300 mOsm pH adjusted to 7.4 with NaOH). The free Ca^{2+} concentration of this solution was determined to be 160 nM. The patch pipette was connected to a Heka Electronik EPC-9 patch-clamp amplifier (Instrutech Corporation, Mineola, NY, USA) interfaced with a Macintosh G3 computer running Pulse version 8.31 software (Instrutech Corporation). Series resistance (R_S) and membrane capacitance (C_M) were updated on a continuous basis using the computer-controlled automated compensation features of the EPC-9 (Holz *et al.* 1993, 1995, 1999). The signal corresponding to the pipette current was low-pass filtered (0.5 kHz) and digitized (1 kHz). Raw data were analysed and statistical analyses were performed using IgorPro software version 5.03 (WaveMetrics Inc., Lake Oswego, OR, USA). Test solutions dissolved in SES were applied to individual cells from glass 'puffer' micropipettes (catalogue no. 1B150-6; World Precision Instruments, Sarasota, FL, USA), using a pressure ejection system (PicoSpritzer II; General Valve Corporation, NJ, USA) as described (Holz *et al.* 1993).

Generation and characterization of stably transfected cell lines

Human wild-type Epac1 (GenBank accession no. AAF103905) (de Rooij *et al.* 1998), and dominant-negative Epac1 (R279E) in pcDNA3.1 were obtained from Dr X. Cheng (Galveston, TX, USA) (Qiao *et al.* 2002). Epac cDNAs were subcloned into pCMV4-FLAG (Sigma-Aldrich) to insert the FLAG epitope at the N-terminus of the exchange factor. All Epac constructs were introduced into INS-1 cells using LipofectAMINE Plus (Invitrogen) (Chepurny *et al.* 2000; Chepurny & Holz 2002). Individual clones of stably transfected cells were selected for by treatment with geneticin (150 μg ml^{-1}; Invitrogen). Expression of FLAG Epac1 or Epac2 was confirmed by immunoblot analysis (Kang *et al.* 2005), or by indirect immunofluorescence cytochemistry (ICC) using fixed and permeabilized INS-1 cells. The primary antiserum for ICC was mouse anti-FLAG M2 monoclonal antiserum (Sigma-Aldrich; catalogue no. F3165). A goat anti-mouse polyclonal antiserum conjugated to AlexaFluor 488 (Molecular Probes, Inc., Eugene, OR, USA; 1:1000 dilution) served as the secondary antiserum. Cells were imaged using a CARV2 spinning-disk confocal microscope (BD Biosciences, San Jose, CA, USA) or a Zeiss LSM510 confocal microscope equipped with a $\times$100 objective. Expression of endogenous Epac2 in INS-1 cells was confirmed by immunoblot analysis using an Epac2-specific monoclonal antiserum developed in the laboratory of J. L. Bos.

RT-PCR for detection of Epac mRNA

Detailed methods for RT-PCR of Epac have been described (Leech *et al.* 2000; Kang *et al.* 2001). For detection of rat Epac1 (accession no. U78167), RT-PCR was performed using primer sets: 1A (forward 5′-CGTCCCCGGTGCTGCTCTTAC-3′; reverse 5′-GTCCCCCTGGCTGAACAACACA3′), 1B (forward 5′-GGCCCGGAATGCACCTGTTTG-3′; reverse 5′-CTGGCCATCATTCGCATCTTCTCA3′), and 1C (forward 5′-TCTGGCCGGGAGCTAGTGGATGG-3′; reverse 5′-GGGTCGGAGGGCGGGAAGG-3′). Primer sets A, B and C for Epac1 generate PCR products that span nucleotides 22–708, 1479–2423 and 265–1015 of the coding sequence of U78167 (Kawasaki *et al.* 1998). RT-PCR for rat Epac2 (U78517) was performed using primer set 2 (forward 5′-GTGGGGACGTTTGAACTGATGAGC-3′; reverse 5′-AGCCTGTACGCCTTGTGATTTCTG3′). Primer set 2 generates a PCR product which spans nucleotides 568–1007 of U78517 within the coding sequence of Epac2 (Kawasaki *et al.* 1998).

Co-immunoprecipitation and detection of SUR1, Kir6.2 and Epac

HEK293T cells cultured in DMEM (Invitrogen; supplemented with 10% FBS) were transfected at 30–40% confluency using FuGENE6 (Roche Diagnostics, Alameda, CA, USA) and the following cDNAs: (1) mouse Kir6.2-HA (accession no. NM_010602; Inagaki *et al.* 1995) in pcDNA3.0 (from Dr S. Seino, Kobe University Graduate School of Medicine, Kobe, Japan) but C-terminally tagged with an HA epitope as described by Pountney *et al.* 2001); (2) hamster FLAG-SUR1 (accession no. L40623; Aguilar-Bryan *et al.* 1995) in pECE (from Dr S.-L. Shyng, Portland, OR, USA, but subcloned into pcDNA3.1 and N-terminally tagged with the FLAG epitope by Dr Pilyali Dhar Chowdhury, New York University School of Medicine, NY, USA); (3) human Epac1 (from Dr X. Cheng, (The University of Texas Medical Branch, Galveston, Texas, USA) but subcloned into pCMV2 (Sigma-Aldrich) and N-terminally tagged with a c-myc epitope); (4) mouse Epac2 (from Dr S. Seino, but subcloned into pCMV2 and N-terminally tagged with a c-myc epitope); and (5) pGFP-N1 (Clontech) which was used as a marker of transfection. Forty-eight hours post-transfection, cells washed in ice-cold PBS were lysed in ice-cold buffer containing (mM): 25 Tris-HCl (pH 7.4), 150 NaCl, 5 EDTA, and supplemented with Triton X-100 (1% v/v), phenylmethylsulphonylfluoride (1 mM) and a protease inhibitor cocktail (Sigma-Aldrich). Whole-cell lysates containing 800 μg of protein were incubated overnight at 4°C with a rabbit polyclonal anti-FLAG antiserum (Sigma-Aldrich; catalogue no. F7425). FLAG-SUR1 complexed to anti-FLAG antiserum was then

immunoprecipitated with protein A/G Sepharose beads (Pierce, Rockford, IL, USA). Control samples of whole-cell lysates were incubated with non-specific rabbit serum IgG for immunoprecipitation using protein A/G Sepharose beads. Protein–antibody–bead complexes were washed three times in ice-cold buffer (same composition as above, but containing 0.1% Triton X-100), and resuspended in Laemmli SDS sample buffer (Bio-Rad, Hercules, CA, USA) without boiling. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes (Bio-Rad), and immunoblotted with mouse monoclonal anti-FLAG M2 antiserum (Sigma-Aldrich; 1:500 dilution), or mouse monoclonal anti-c-myc antiserum (Sigma-Aldrich; catalogue no. M5546; 1:500 dilution). The secondary antiserum was HRP-conjugated donkey antimouse IgG (Amersham-Pharmacia, Piscataway, NJ, USA). Immunodetection was performed using enhanced chemiluminescence (Pierce).

In vitro Rap1 activation assay

Rap1B (200 nM) loaded with the fluorescent GDP analogue 2′-/3′-*O*-(*N*′-methylanthraniloyl)-guanosine-diphosphate (mantGDP) was incubated in the presence of 20 μM GDP and 100 nM Epac1. Then 2′-*O*-Me-cAMP or 2′-*O*-Me-cGMP was added as indicated for the individual experiments. Epac-mediated nucleotide exchange on Rap1B was measured in real time as the decay in fluorescence using a spectrofluorometer (Rehmann *et al.* 2003*a*). The decay in fluorescence initiated by cAMP is caused by the release of Rap1B-bound mantGDP, which shows a higher fluorescence intensity in the hydrophobic environment of Rap1B than in the buffer solution. All data analysis, fitting, and plotting were done with Grafit 3.0 (Erithacus Software Ltd, Surrey, UK).

Sources of reagents

8-pCPT-2′-*O*-Me-cAMP, 8-pMeOPT-2′-*O*-Me-cAMP, 2′-*O*-Me-cAMP, 2′-*O*-Me-cGMP, 8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS), Rp-8-Br-cAMPS, and N^6-Bnz-cAMP were obtained from Biolog Life Science Institute (Bremen, Germany). ATP, GTP, cAMP and glyburide were from Sigma. H-89, myr-PKI and the anti-alpha-tubulin mouse monoclonal antibody were from Calbiochem (San Diego, CA, USA). Anti-Epac2 mouse monoclonal antibody was generated in the laboratory of J. L. Bos by F. J. T. Zwartkruis and J. Zhao.

Results

Inhibition of the K_{ATP} current of human β cells by an Epac-selective cAMP analogue

Primary cultures of human islet cells were prepared, and individual β cells were identified on the basis of their expression of EYFP, the synthesis of which was placed under the control of the rat insulin 2 gene promoter. Prior studies demonstrate that this method of selection is an accurate means by which to identify fluorescent β cells in a mixed population of islet cells (Kang *et al.* 2003). The macroscopic K_{ATP} current was measured under conditions of voltage clamp using the whole-cell, tight-seal configuration of the patch-clamp technique. β cells were equilibrated in a standard extracellular saline solution containing 11.1 mM glucose, and the cells were dialysed with a pipette solution that included 0.3 mM ATP, 0.2 mM GTP and 160 nM Ca^{2+}. This pipette solution was chosen because prior reports demonstrate that the inhibitory action of cAMP at K_{ATP} channels is facilitated by Ca^{2+} (He *et al.* 1998; Ding *et al.* 2001).

The membrane conductance was measured by determining the amplitude of the whole-cell current evoked by a $\pm$20 mV command potential from a holding potential of $-$70 mV. Upon rupture of the patch, the membrane conductance and the associated whole-cell current increased gradually (Fig. 1*A*). This increase signifies the opening of K_{ATP} channels, and it is a consequence of the intracellular dialysis of cells with a pipette solution containing a low concentration of ATP (Trube *et al.* 1986; Williams *et al.* 1993). Extracellular application of the cell-permeant Epac-selective cAMP analogue 8-pCPT-2′-*O*-Me-cAMP (100 μM; three 30 s applications indicated by vertical arrows) inhibited the whole-cell K_{ATP} current of human β cells measured under these conditions ($n = 5$ cells). This action of 8-pCPT-2′-*O*-Me-cAMP is depicted on a time scale that is compressed (Fig. 1*A*) or expanded (Fig. 1*B*). The whole-cell current measured in this manner corresponded to K_{ATP} current because it was abolished by the sulphonylurea glyburide applied extracellularly (10 nM; $n = 5$ cells; data not shown).

Epac-selective cAMP analogues inhibit the K_{ATP} current of rat INS-1 cells

The K_{ATP} current of INS-1 cells was also inhibited by Epac-selective cAMP analogues. This was the case for 8-pCPT-2′-*O*-Me-cAMP (100–300 μM; $n = 5$ cells; data not shown) and cell-permeant 8-pMeOPT-2′-*O*-Me-cAMP (300 μM; $n = 6$ cells) (Fig. 1*C*). Unlike human β cells, the inhibitory actions of both cAMP analogues in INS-1 cells were not reversible within 5–10 min. Why this is the case was not investigated, but it may reflect a difference in the level of cAMP phosphodiesterase (PDE) activity in the two cell types. 8-pCPT-2′-*O*-Me-cAMP and 8-pMeOPT-2′-*O*-Me-cAMP are both susceptible to hydrolysis by PDE, so the duration of their effect may be extended in INS-1 cells exhibiting a low level of PDE activity.

Current-clamp measurements of the membrane potential obtained under conditions of whole-cell dialysis

demonstrated that 100 μM 8-pCPT-2′-*O*-Me-cAMP depolarized INS-1 cells and generated action potentials ($n = 4$ cells; data not shown). Although not investigated, this depolarizing action of 8-pCPT-2′-*O*-Me-cAMP may explain its ability to produce a sustained increase of $[Ca^{2+}]_i$ and to stimulate exocytosis in INS-1 cells (see Fig. 6*D* of Kang *et al.* 2003). Consistent with the findings obtained using human β cells, the K_{ATP} current of INS-1 cells was inhibited by glyburide applied extracellularly (10 nM; $n = 5$ cells) (Fig. 1*D*).

Inhibition of the K_{ATP} current by intracellularly applied cAMP

Although the 8-pCPT and 8-pMeOPT substitutions of cAMP analogues confer membrane permeability, they could in theory also confer a direct channel-blocking activity. Therefore, we sought to determine what effect cAMP, itself, exerted when it was administered intracellularly. To this end, experiments were repeated under conditions in which cAMP was included in the patch pipette solution. Under control conditions in which cAMP was not present, the whole-cell K_{ATP} current of INS-1 cells increased gradually upon rupture of the patch (Fig. 2*A*). The amplitude of this current reached a plateau, after which a slow decrease ('run-down') occurred (Fig. 2*A*). When cAMP (300 μM) was included in the pipette solution, the initial increase of whole-cell K_{ATP} current was followed by a prompt decrease not measured under control conditions (Fig. 2*B*). This action of cAMP to inhibit the K_{ATP} current was clearly distinguishable from the process of run-down, and it did not result from a re-sealing of the membrane within the patch since no increase of series resistance was measurable. Thus, cAMP, itself, inhibits the activity of K_{ATP} channels in INS-1 cells.

Inhibition of the K_{ATP} current by Sp-8-Br-cAMPS but not Rp-8-Br-cAMPS

We next compared the efficacy of Sp- and Rp- isomers of 8-Br-subsituted cAMPS in order to determine if these

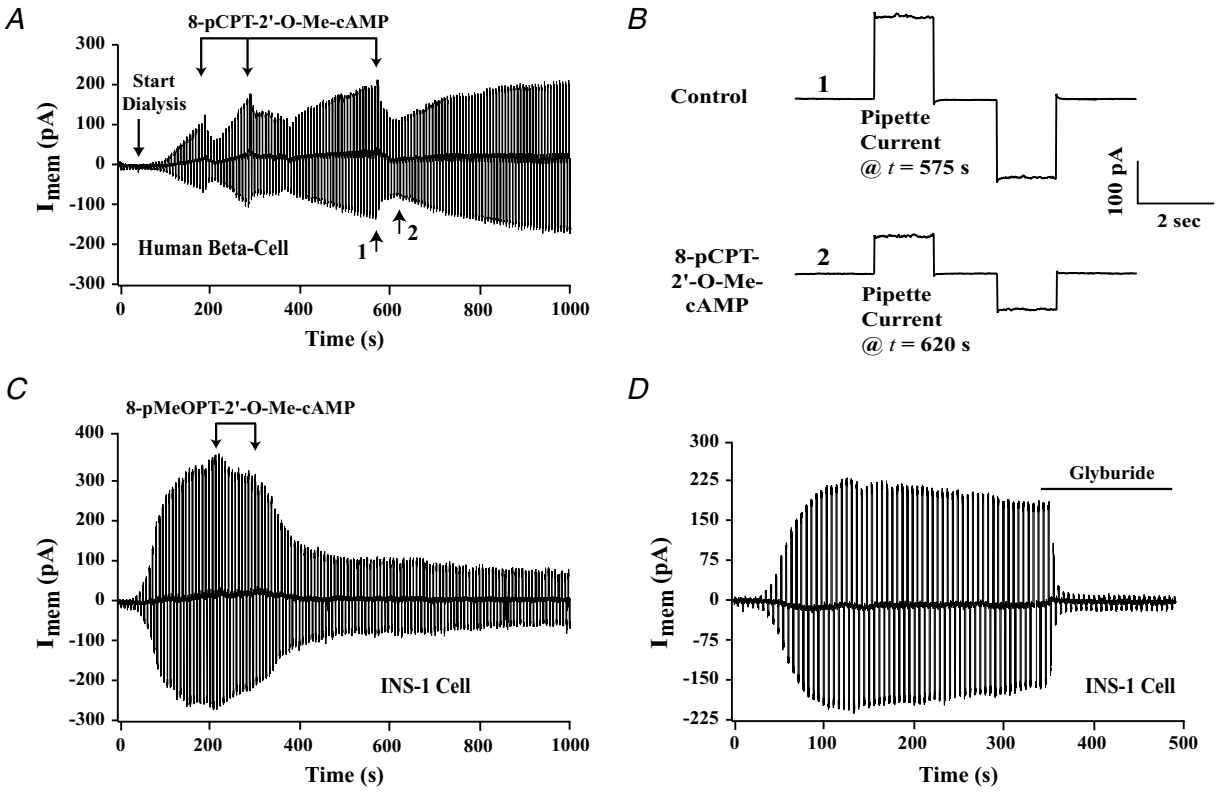

Figure 1. Inhibition of the whole-cell K_{ATP} current by Epac-selective cAMP analogues
A and *B*, 8-pCPT-2′-*O*-Me-cAMP (100 μM; individual 30 s applications indicated by vertical arrows) inhibited the K_{ATP} current of a human β cell, as depicted on a compressed (*A*) and an expanded (*B*) time scale. Time points 1 and 2 in *A* are illustrated in *B* as the pipette currents measured at $t = 575$ and $t = 620$ s. Outward currents are indicated by upward current deflections. The membrane conductances at $t = 575$ and $t = 620$ s, respectively, were 10.0 and 4.7 nS. *C*, the K_{ATP} current of an INS-1 cell was inhibited by 8-pMeOPT-2′-*O*-Me-cAMP (300 μM; individual 30 s applications indicated by arrows). *D*, glyburide (10 nM, continual application indicated by the horizontal line) abolished the K_{ATP} current of an INS-1 cell. All test solutions were applied extracellularly to individual cells using a 'puffer' pipette.

cAMP analogues act in a stereospecific manner when administered intracellularly. Consistent with prior studies in which the Sp- but not Rp- isomers of cAMPS were demonstrated to activate Epac1 (Christensen *et al.* 2003), we found that the K_{ATP} current of INS-1 cells was inhibited by 100 μM Sp-8-Br-cAMPS but not Rp-8-Br-cAMPS (Fig. 2*C* and *D*). Thus, these cAMP analogues interact with a binding site that discriminates between Sp- and Rp-isomers, and which might correspond to the cAMP-binding pocket of Epac (Rehmann *et al.* 2003*b*,*c*).

K_{ATP} channels are inhibited by 2′-*O*-Me-cAMP but not 2′-*O*-Me-cGMP

Given that Epac is activated by cAMP but not cGMP (Rehmann *et al.* 2003*a*), we sought to determine whether an appropriate cyclic-3′,5′-adenosine monophosphate specificity existed for the inhibitory actions of cyclic nucleotides described here. For this purpose, we examined the effects of cyclic nucleotides that are not 8-substituted, but which contain the 2′-*O*-Me substitution necessary for selective activation of Epac. We found that the K_{ATP} current

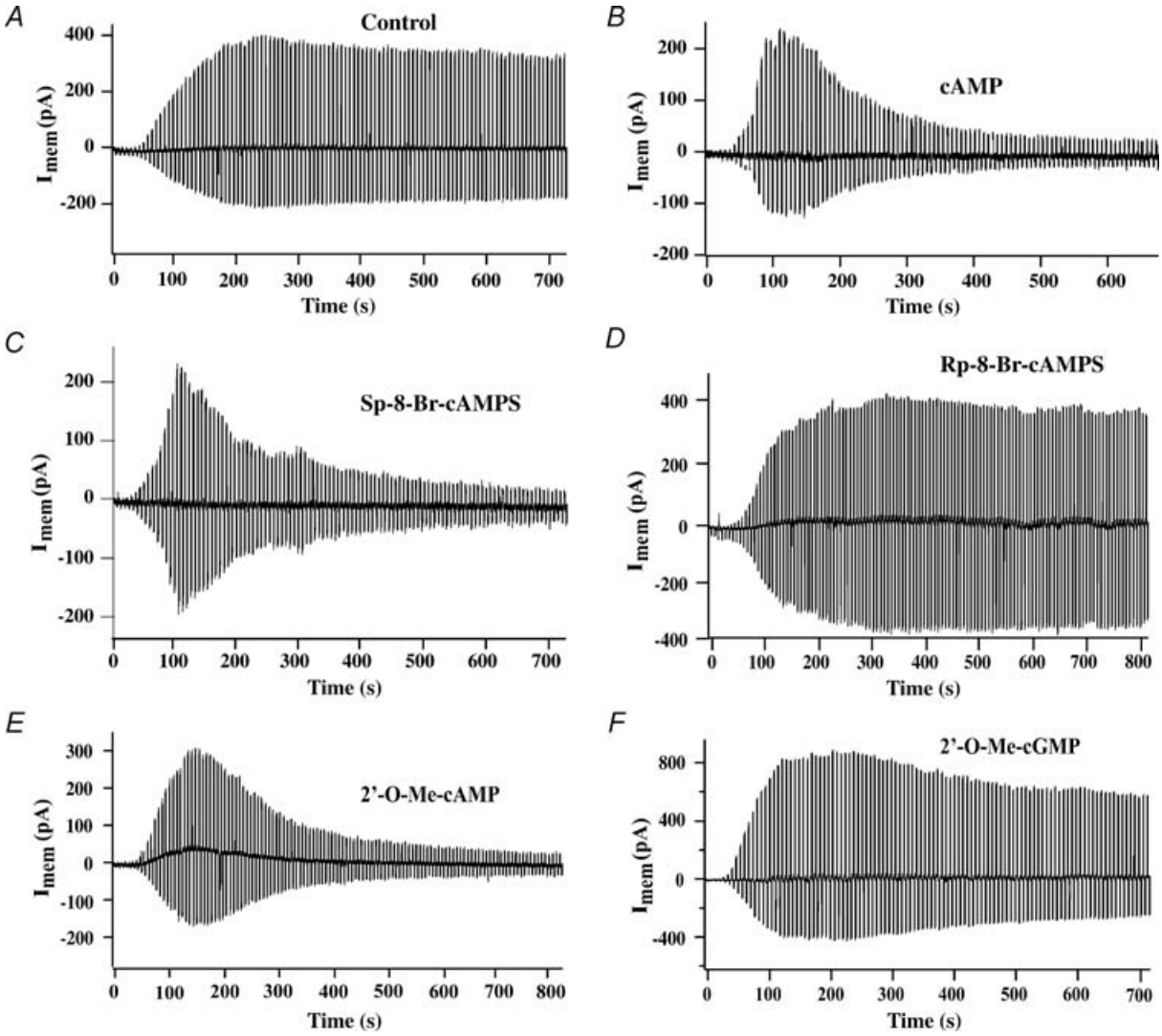

Figure 2. Inhibition of the K_{ATP} current of INS-1 cells by intracellularly applied cyclic nucleotide analogues
A, dialysis with a pipette solution containing no cyclic nucleotide resulted in the appearance of the whole-cell K_{ATP} current under control conditions. *B*, inclusion of cAMP (300 μM) in the patch pipette did not prevent the appearance of the K_{ATP} current once dialysis had commenced at $t = 0$, but it resulted in a prompt decrease of the K_{ATP} current measured at later time points. *C*–*F*, the action of cAMP was reproduced by 100 μM each of Sp-8-Br-cAMPS (*C*) and 2′-*O*-Me-cAMP (*E*), but not Rp-8-Br-cAMPS (*D*) or 2′-*O*-Me-cGMP (*F*).

of INS-1 cells was inhibited by intracellular administration of 2′-*O*-Me-cAMP (Fig. 2*E*), but not 2′-*O*-Me-cGMP (Fig. 2*F*). Because the relative efficacies of 2′-*O*-Me-cAMP and 2′-*O*-Me-cGMP as activators of Epac has not been reported, we validated that 2′-*O*-Me-cAMP activates both Epac1 and Epac2, whereas 2′-*O*-Me-cGMP is ineffective, as demonstrated in an *in vitro* assay of Epac-mediated guanyl nucleotide exchange on Rap1 (Fig. 3*A* and *B*). Therefore, it may be concluded that the K_{ATP} channels of INS-1 cells are inhibited in an adenine- but not guanine-nucleotide-specific manner.

Comparison of the average rates of decay of whole-cell K_{ATP} currents

The effects of cyclic nucleotide analogues administered via the patch pipette were analysed by performing population studies of INS-1 cells so that the average rates of decay of the K_{ATP} currents could be determined (Fig. 4*Aa*, *Ba* and *Ca*). Simultaneously, the average amplitudes of the currents were measured 350 s after the currents had reached their initial maxima (Fig. 4*Ab*, *Bb* and *Cb*). These analyses demonstrated inhibitory actions of cAMP (Fig. 4*Aa* and *b*), Sp-8-Br-cAMPS (Fig. 4*Ba* and *b*), and 2′-*O*-Me-cAMP (Fig. 4*Ca* and *b*). However, Rp-8-Br-cAMPS (Fig. 4*Ba* and *b*) and 2′-*O*-Me-cGMP (Fig. 4*Ca* and *b*) exerted no such inhibitory effect. To quantify these effects in greater detail, the time constant of K_{ATP} current decay was determined. Under control conditions in which the pipette solution contained no cAMP, the time constant of K_{ATP} current decay was 318 s. This value decreased to 129, 142 and 130 s when the pipette solution contained cAMP, Sp-8-Br-cAMPS or 2′-*O*-Me-cAMP, respectively. In contrast, the time constant of K_{ATP} current decay did not differ from the control value when evaluating the actions of Rp-8-Br-cAMPS and 2′-*O*-Me-cGMP (see online Supplemental material Fig. 1). Such findings validate that cAMP analogues exhibit a stereospecificity (Sp-8-Br-cAMPS *versus* Rp-8-Br-cAMPS) and adenine nucleotide specificity (2′-*O*-Me-cAMP *versus* 2′-*O*-Me-cGMP) consistent with an action of cAMP mediated by Epac.

N^6-Bnz-cAMP fails to inhibit the K_{ATP} current

Prior studies of insulin-secreting cells demonstrate a role for PKA as an inhibitor of K_{ATP} channel activity (Light *et al.* 2002). In contrast, studies of non-insulin-secreting cells that express Kir6.2 and SUR1 seem to indicate that PKA stimulates rather than inhibits K_{ATP} channel function (Beguin *et al.* 1999; Lin *et al.* 2000). Thus, we examined whether the K_{ATP} current of INS-1 cells is affected by N^6-Bnz-cAMP, a cAMP analogue that activates PKA but not Epac (Christensen *et al.* 2003). We found that N^6-Bnz-cAMP (100 μM) failed to alter the K_{ATP} current when it was included in the patch pipette solution (Fig. 5*Aa*

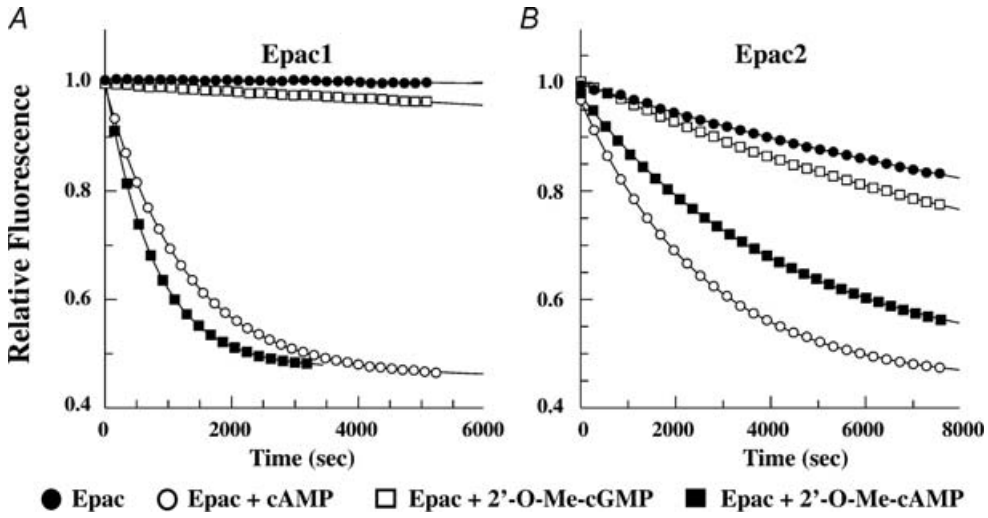

Figure 3. cAMP and 2′-*O*-Me-cAMP but not 2′-*O*-Me-cGMP act via Epac to stimulate guanyl nucleotide exchange on Rap1
Rap1B loaded with a fluorescent GDP analogue was incubated in the presence of non-fluorescent GDP and either Epac1 (*A*) or Epac2 (*B*). cAMP, 2′-*O*-Me-cAMP or 2′-*O*-Me-cGMP was then added at a final concentration of 500 μM. Exchange of fluorescent GDP for non-fluorescent GDP was measured spectrophotometrically in real time. When bound to Rap1B, fluorescent GDP exhibits greater fluorescence than when it is simply dissolved in buffer solution. Thus, the decay of fluorescence measured in this assay reflects Epac-mediated stimulation of guanyl nucleotide exchange on Rap1B. Note that when tested at a saturating concentration (500 μM), the Epac1-mediated action of 2′-*O*-Me-cAMP proceeds at a faster rate than that mediated by Epac2.

and *b*; $n = 10$ cells). For this reason, a role for PKA as an intermediary linking cAMP to the regulation of K_{ATP} channel activity was not substantiated in the study of INS-1 cells reported here.

Rp-8-Br-cAMPS fails to block the inhibitory action of 8-pCPT-2′-*O*-Me-cAMP

Rp-8-Br-cAMPS is a cAMP analogue that prevents activation of PKA by cAMP (Dostmann *et al.* 1990). In marked contrast, recent studies demonstrate that Rp-8-Br-cAMPS fails to block activation of Epac by cAMP (Christensen *et al.* 2003; Branham *et al.* 2006). In the present study of INS-1 cells, we found that the action of 8-pCPT-2′-*O*-Me-cAMP (100 μM) to inhibit K_{ATP} channels was unaffected by inclusion of Rp-8-Br-cAMPS (100 μM) in the patch pipette solution (Fig. 5*B*, $n = 5$ cells). Thus, available information indicates that it is Epac that is targeted by cAMP in this assay of whole-cell K_{ATP} current.

The action of 8-pCPT-2′-*O*-Me-cAMP is blocked by dominant-negative Epac1

If cAMP acts via Epac to inhibit K_{ATP} channels, such an effect should persist after exposure of cells to compounds that suppress the catalytic activity of PKA. To evaluate this possibility, INS-1 cells were treated with H-89 (1 μM) or

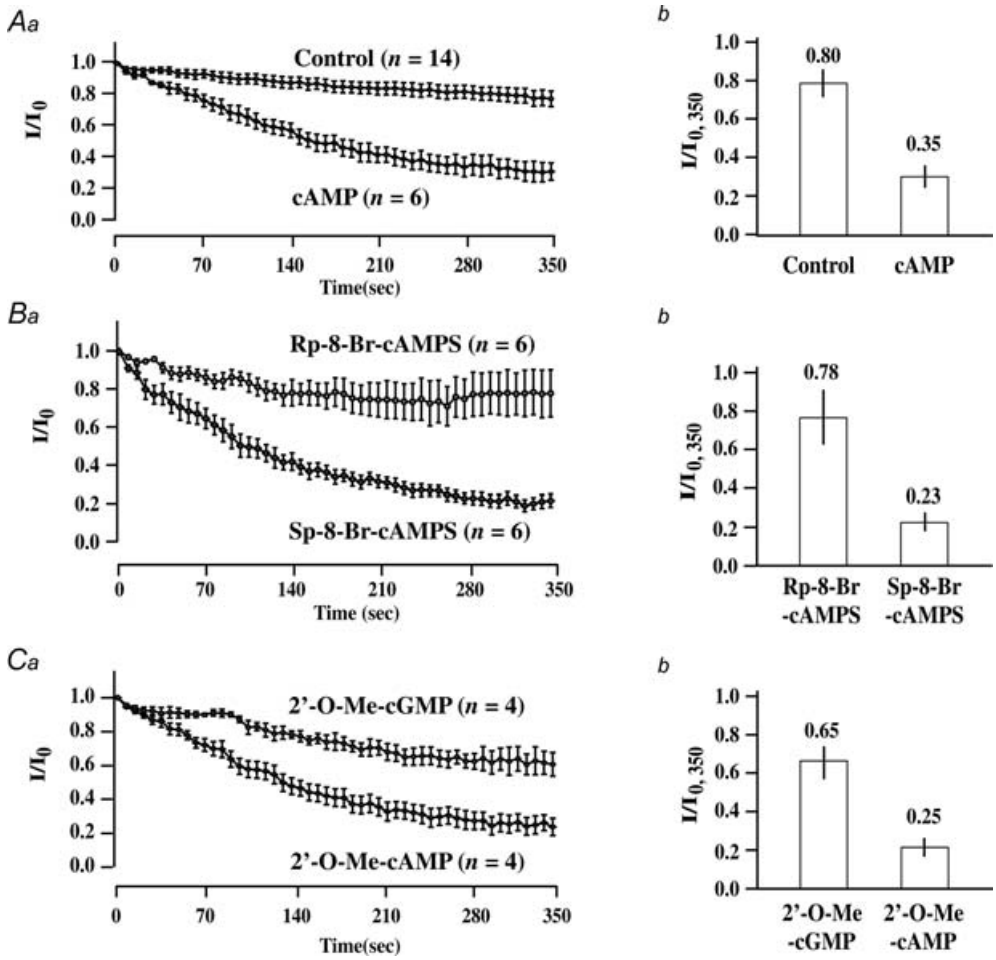

Figure 4. Comparison of the rates of K_{ATP} current decay measured under conditions in which INS-1 cells were dialysed with a pipette solution containing cyclic nucleotides
Aa, *Ba* and *Ca*, the time course of whole-cell K_{ATP} current decay is illustrated under control conditions, or conditions in which the pipette solution contained cyclic nucleotides. The current measured at $t = 0$ is the maximal current (I_o) achieved following the initiation of dialysis. The current (I) measured at subsequent time points is normalized relative to I_o. Values of n correspond to the number of cells studied under each experimental condition. *Ab*, *Bb* and *Cb*, the amplitude of the normalized K_{ATP} current measured at $t = 350$ s ($I/I_{o,350}$) is illustrated under control conditions, or conditions in which the pipette solution contained cyclic nucleotides. All cyclic nucleotides were administered at a concentration of 100 μM, except for cAMP (300 μM). Error bars indicate means ± S.E.M.

myr-PKI (10 μM), two inhibitors of PKA catalytic activity (Hidaka & Kobayashi, 1992). Unfortunately, we found that PKA inhibitors, alone, inhibited the K_{ATP} current measured under conditions of whole-cell dialysis (data not shown). A similar finding has been reported in studies of K_{ATP} channel activity in excised patches of plasma membrane derived from RINm5F insulin-secreting cells (Ribalet *et al.* 1989; RINm5F cells are the parental cell line of INS-1 cells). To circumvent this problem, we adopted a molecular biological approach in which INS-1 cells were stably transfected with wild-type (WT) or dominant-negative (DN) FLAG epitope-tagged Epac1. DN FLAG-Epac1 incorporates an amino acid substitution (R279E) that prevents the binding of cAMP, and it is reported to block Epac-mediated mobilization of intracellular Ca^{2+} in INS-1 cells (Kang *et al.* 2005). Using this approach, it was demonstrated that transfection with DN but not WT FLAG-Epac1 nearly abolished the inhibitory actions of 8-pCPT-2′-*O*-Me-cAMP (100 μM) and cAMP (300 μM) at K_{ATP} channels (Fig. 6*A*). Immunoblot analysis confirmed the expression of WT and DN FLAG-Epac1 in these stably transfected INS-1 cells, as detected using a specific anti-FLAG antiserum (Fig. 6*B*). Indirect immunofluorescence cytochemistry also demonstrated FLAG immunoreactivity corresponding to WT and DN FLAG-Epac1 expressed in these cells, as detected by confocal microscopy. This punctate immunoreactivity was detected at or near the plasma membrane and in the cytoplasm, but not within the nucleus (Fig. 6*C–F*).

Expression of endogenous Epac in INS-1 cells and human β cells

Semi-quantitative RT-PCR analysis confirmed the expression of endogenous Epac1 and Epac2 mRNA in INS-1 cells (Fig. 7*A*). Nearly identical findings were obtained using human islets (see Fig. 2 of Supplemental material). Although prior studies have focused on the role of Epac2 as a determinant of β cell stimulus–secretion coupling (Kang *et al.* 2001; Kashima *et al.* 2001; Eliasson *et al.* 2003), the presence of Epac1 mRNA in INS-1 cells is not surprising. In fact, mRNA corresponding to Epac1 can also be detected in rat islets (Leech *et al.* 2000). However, it should be noted that the predominant PCR product detected in INS-1 cells corresponded to Epac2 (Fig. 7*A*). Furthermore, Western blot analysis using a specific anti-Epac2 monoclonal antiserum demonstrated

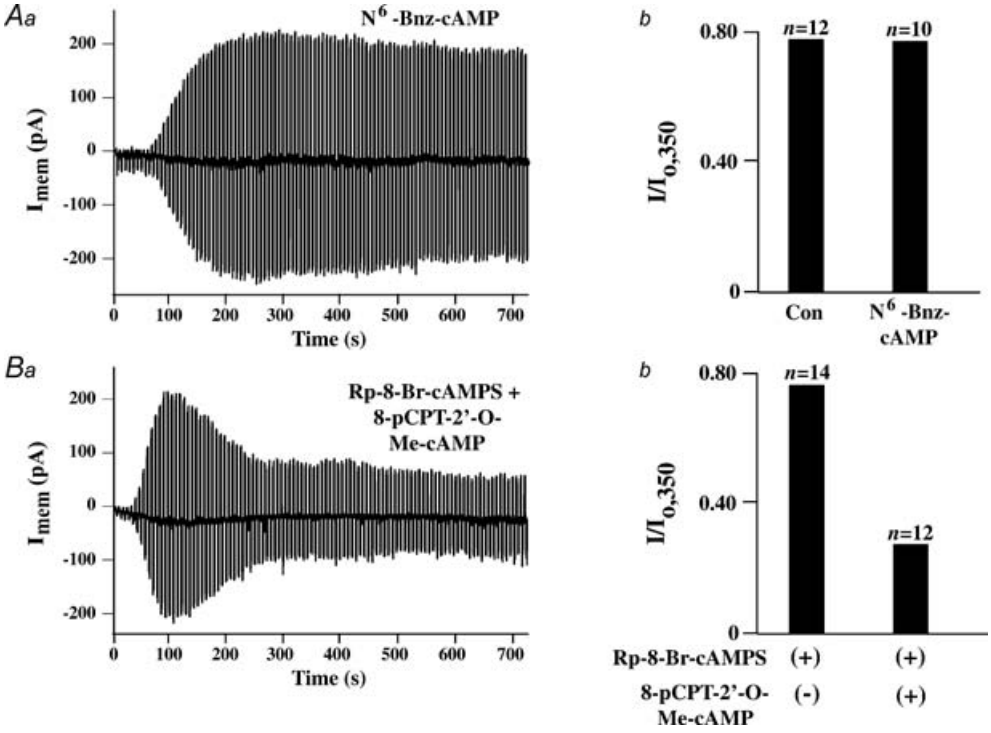

Figure 5. Assessment of the actions of N^6-Bnz-cAMP, Rp-8-Br-cAMPS and 8-pCPT-2′-*O*-Me-cAMP in INS-1 cells

A, inclusion of N^6-Bnz-cAMP (100 μM) in the patch pipette solution was without effect on the rate of decay (*Aa*) or absolute magnitude (*Ab*) of the K_{ATP} current measured under conditions of whole-cell dialysis. See the legend of Fig. 4 for an explanation of how the value of $I/I_{0,350}$ was calculated. *B*, inclusion of Rp-8-Br-cAMPS (100 μM) in the patch pipette solution failed to influence the action of 8-pCPT-2′-*O*-Me-cAMP (100 μM) to increase the rate of decay of the K_{ATP} current (*Ba*), and to decrease its absolute magnitude (*Bb*).

significant Epac2 immunoreactivity in INS-1 cell lysates (Fig. 7*B*). No such immunoreactivity was detected in OVCAR cells, an ovarian carcinoma cell line serving as a negative control (Fig. 7*B*). Although not shown, Epac1 immunoreactivity recognized by an Epac1 monoclonal antiserum was present only in trace amounts in INS-1 cells (data not shown). Thus, available evidence indicates that it is Epac2 that is expressed at the highest levels in INS-1 cells.

Epac1 and Epac2 interact with full-length SUR1

To assess whether Epac interacts with SUR1 in living cells, immunoprecipitation (IP) assays were performed using the supernatant fractions of lysates obtained from HEK cells transfected with FLAG-SUR1, and either myc-epitope-tagged Epac1 or Epac2. These studies demonstrated that both myc-Epac1 and myc-Epac2 interacted with FLAG-SUR1 (Fig. 8*A* and *B*). Interestingly, a complex of FLAG-SUR1 and either myc-Epac1 or myc-Epac2 was detected in lysates obtained from HEK cells transfected, or not transfected, with haemagglutinin (HA)-tagged Kir6.2 (Fig. 8*A* and *B*). Given that SUR1 is known to be confined to the endoplasmic reticulum in the absence of Kir6.2 (Zerangue *et al.* 1999), such findings indicate that both forms of Epac may associate with SUR1 at a step prior to the translocation of K_{ATP} channels to the plasma membrane.

Discussion

A role for Epac in β cell K_{ATP} channel regulation

Although cAMP-elevating agents such as forskolin, isobutylmethylxanthine, glucagon and GLP-1 inhibit K_{ATP} channels in β cells, it has remained uncertain until now whether such an effect is achieved via cAMP-dependent activation of PKA and/or Epac. Here we present evidence that Epac does in fact contribute to

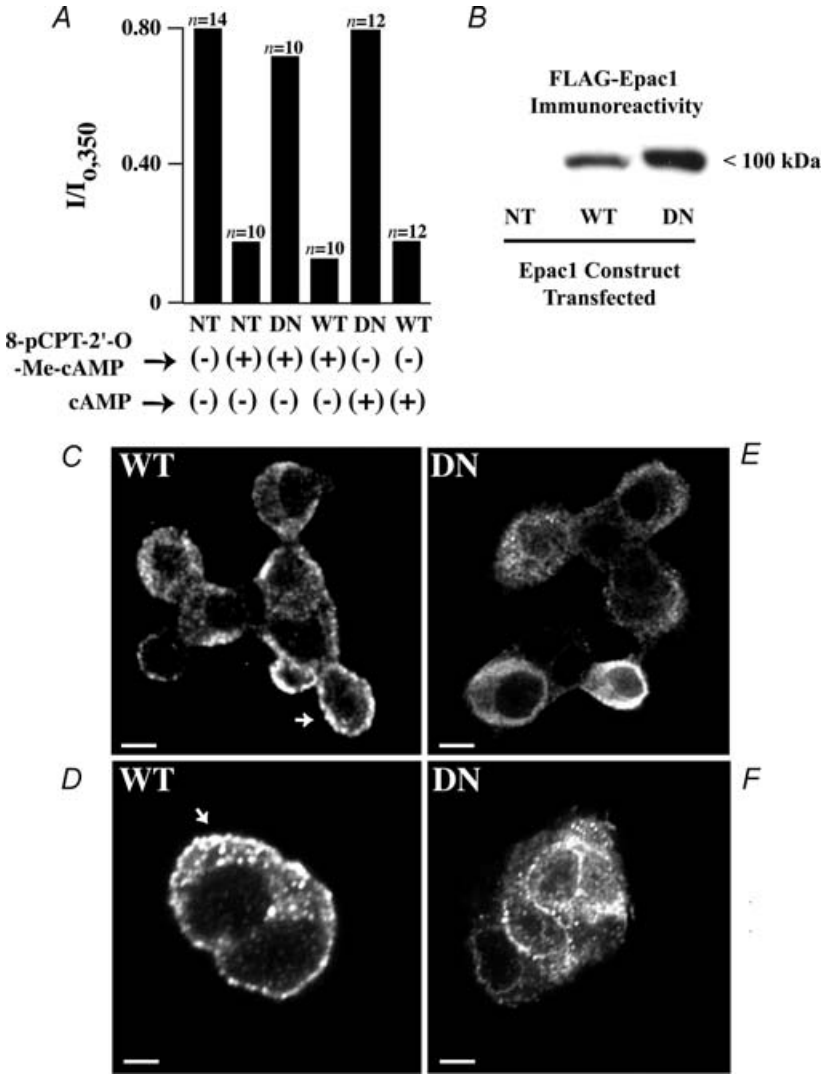

Figure 6. Dominant-negative Epac1 diminishes the action of 8-pCPT-2′-*O*-Me-cAMP
A, the amplitude of the normalized whole-cell K_{ATP} current of INS-1 cells measured at $t = 350$ s ($I/I_{o,350}$) is illustrated for INS-1 cells not transfected (NT) or stably transfected with either wild-type (WT) FLAG-Epac1 or dominant-negative (DN) FLAG-Epac1. Cells dialysed with 8-pCPT-2′-*O*-Me-cAMP (100 μM) or cAMP (300 μM) are indicated as (+), whereas cells not dialysed with 8-pCPT-2′-*O*-Me-cAMP or cAMP are indicated as (−). *B*, expression of WT and DN FLAG-tagged Epac1 in lysates of stably transfected INS-1 cells was confirmed by immunoblot analysis, whereas no such immunoreactivity was detected in INS-1 cells not transfected (NT). *C–F*, immunofluorescence cytochemistry for detection of WT and DN FLAG-Epac1 in stably transfected INS-1 cells. Punctate immunoreactivity (arrows) corresponding to WT or DN FLAG-Epac1 was apparent at the plasma membrane and in the cytoplasm of a cluster of cells (*C, E* and *F*) or a pair of cells (*D*). No such immunoreactivity was detected in cells not transfected with FLAG-Epac1. Calibration bars: 3.4 μm for *C*, *E* and *F*; 2.5 μm for *D*.

this process. We find that the activity of K_{ATP} channels is inhibited by Epac-selective cAMP analogues, whereas no such effect is observed after transfection with a dominant-negative Epac1. The analogues we tested inhibit K_{ATP} channels in a stereospecific and cyclic adenosine monophosphate-specific manner, as expected for a specific interaction of cAMP with the cyclic nucleotide-binding domain of Epac. Furthermore, both Epac1 and Epac2 are shown to interact with full-length SUR1. On the basis of these findings, we propose that Epac mediates a major inhibitory effect of cAMP at K_{ATP} channels.

It remains to be established which isoform of Epac subserves inhibitory actions of cAMP at K_{ATP} channels. Although the dominant-negative Epac1 we tested is likely to interfere with actions of cAMP mediated by Epac1, it may also disrupt signalling mediated by Epac2. This possibility exists due to the structurally conserved nature of the DEP, cAMP-binding, REM and GEF domains of Epac1 and Epac2 (Holz, 2004*a*). Thus, a contribution of both Epac1 and Epac2 to K_{ATP} channel regulation is not excluded. In fact, we find that INS-1 cells express Epac1 and Epac2 mRNA. Since both Epac1 and Epac2 coimmunoprecipitate with SUR1, it is seems likely that these two cAMPGEFs subserve similar functions at K_{ATP} channels.

Epac activators are effective inhibitors of K_{ATP} channels

It is perhaps surprising that we find that cAMP analogues, alone, exert a strong inhibitory effect at K_{ATP} channels. Given that the conditions of whole-cell dialysis used in the present study disrupt β-cell glucose metabolism, and given that cAMP-elevating agents inhibit K_{ATP} channel activity in a glucose-dependent manner (Holz *et al.* 1993), why is the efficacy of cAMP preserved? We believe a simple explanation for this finding is that the experimental design reported here allows us to study effects of cAMP under conditions that recapitulate the glucose-dependent increase of cytosolic ATP/ADP concentration ratio. This metabolic signal is known to support K_{ATP} channel inhibition by cAMP (Holz & Habener, 1992). Because our pipette solution contains 300 μM ATP but no added ADP, whole-cell dialysis with this solution results in an ATP/ADP concentration ratio that is elevated and which

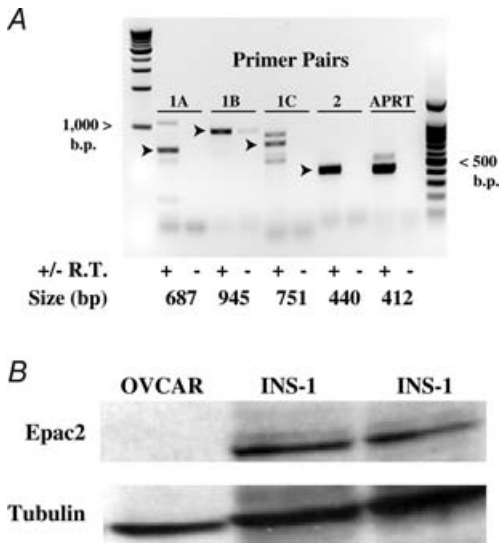

Figure 7. Expression of endogenous Epac in INS-1 cells
A, RT-PCR was performed using three sets of Epac1 primer pairs (designated as 1A, 1B and 1C) and a single Epac2 primer pair (designated as 2). PCR product size is indicated in base pairs. Arrowheads indicate PCR products of the expected sizes. Abbreviations: +/− R.T., template generated with or without reverse transcriptase added to the cDNA synthesis reaction; APRT, adenine phosphoribosyltransferase control. A PCR product corresponding to APRT can be derived by PCR of cDNA derived from mRNA but not genomic DNA when using the rat APRT forward (5′-TCCGAATCTGAGTTGCAGC-3′) and reverse primers (5′-CTGCACACATGGTTC-CTCC-3′). *B*, Epac2 was detected in INS-1 cells (two different platings) by Western blot analysis using an anti-Epac2 monoclonal antiserum. No such immunoreactivity was detected in an ovarian carcinoma cell line (OVCAR). Anti-tubulin antiserum was used to verify loading of the wells with equal amounts of proteins derived from whole-cell lysates.

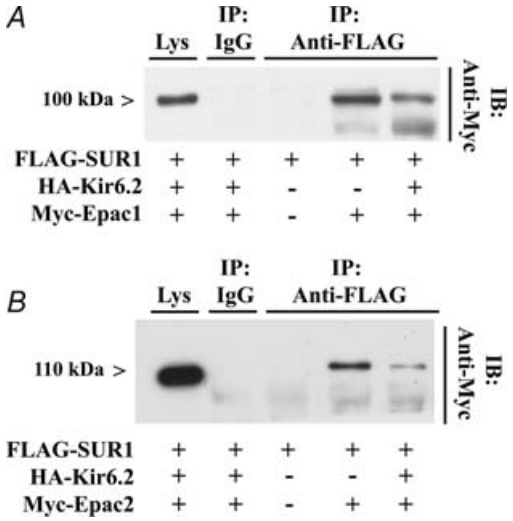

Figure 8. Epac1 and Epac2 interact with SUR1
A, anti-FLAG antiserum affixed to protein A/G Sepharose beads was used to immunoprecipitate FLAG-SUR1 from lysates of HEK cells transfected with FLAG-SUR1, HA-Kir6.2 and myc-Epac1. The immunoprecipitate (IP) was subjected to immunoblot (IB) analysis using anti-myc antiserum. A 100 kDa immunoreactivity corresponding to myc-Epac1 was detected in lysates of cells transfected with FLAG-SUR1 and myc-Epac1, but not in lysates obtained from cells transfected with FLAG-SUR1 only. Control experiments demonstrated that myc-Epac1 was expressed in whole-cell lysates (Lys), whereas no immunoreactivity was detected in lysates subjected to immunoprecipitation with non-specific rabbit IgG. *B*, identical findings to those presented in *A* were obtained when HEK cells were transfected with myc-Epac2 appearing as 115 kDa immunoreactivity. Lower bands correspond to non-specific myc immunoreactivity.

may approximate or even exceed the ratio achieved when β cells are bathed in a high concentration of glucose. For this reason, the strong inhibitory effect of cAMP analogues we report is not at odds with the established action of cAMP as a potentiator of glucose-dependent insulin secretion (Holz, 2004*b*). It will be of interest to determine in future studies exactly how alterations of intracellular [ATP] or [ADP] influence the potency and/or efficacy of Epac-selective cAMP analogues as inhibitors of K_{ATP} channels.

On the potential importance of PKA to K_{ATP} channel regulation

A prior report of Light and coworkers offers a competing hypothesis to explain the inhibition of β-cell K_{ATP} channels by cAMP. It was proposed that PKA-mediated phosphorylation of SUR1 decreases the channel's sensitivity to Mg^{2+}-ADP, thereby closing the channel (Light *et al.* 2002). In that study, the catalytic subunit of PKA (cPKA) inhibited K_{ATP} channel activity under conditions in which inside-out patches of plasma membrane were exposed to an intracellular solution containing 0.2 mM ADP. In marked contrast, channel activity was stimulated by cPKA in the presence of 0.5 mM ADP. Since glucose metabolism lowers cytosolic levels of ADP, it was suggested that the inhibitory action of cAMP at K_{ATP} channels might be most prominent under conditions in which β cells are exposed to elevated concentrations of glucose.

We believe that the study of Light and coworkers is not necessarily at odds with the new findings presented here. Although we find that the K_{ATP} current of INS-1 cells is not affected by N^6-Bnz-cAMP, an activator of PKA, it is possible that our use of whole-cell dialysis results in 'wash-out' of PKA. If so, no action of N^6-Bnz-cAMP is expected. Similarly, the wash-out of cAMP might preclude our ability to detect actions of endogenous cAMP that are PKA-mediated and which should be blocked by cAMP antagonist Rp-8-Br-cAMPS. In this regard, it is interesting to note that no such wash-out phenomenon exists when assessing the inhibitory action of Epac-selective cAMP analogues. Thus, it would appear that Epac is tightly associated with K_{ATP} channels, a conclusion that is supported by our observation that both Epac1 and Epac2 co-immunoprecipitate with SUR1 in HEK cell lysates.

Interpretation of findings obtained using PKA inhibitors

In the present study we found that H-89, an inhibitor of PKA, nearly abolished the K_{ATP} current of INS-1 cells. It remains to be determined if this effect of H-89 results from its selective inhibitory action at PKA, or a non-specific action unrelated to PKA. Perhaps a more meaningful finding obtained using PKA inhibitors was our observation that Rp-8-Br-cAMPS failed to block the inhibition of K_{ATP} channels by 8-pCPT-2′-*O*-Me-cAMP. This is a key finding because Rp-8-Br-cAMPS is poor antagonist of the cAMP-dependent activation of Epac (Christensen *et al.* 2003; Branham *et al.* 2006). Thus, the maintained efficacy of 8-pCPT-2′-*O*-Me-cAMP under conditions of Rp-8-Br-cAMPS treatment is not surprising, and in fact it is exactly what one would expect if it is Epac that mediates the cAMP-dependent inhibition of K_{ATP} channel function.

Signal transduction properties of Epac relevant to K_{ATP} channel regulation

Given that K_{ATP} channels are inhibited by ATP, and stimulated by Mg^{2+}-ADP, it is not unreasonable to propose that cAMP acts via Epac to influence the adenine-nucleotide sensitivity of K_{ATP} channels. Such an effect of cAMP might be conferred by a direct interaction of Epac with NBF-1 of SUR1 (Shibasaki *et al.* 2004*a*,*b*). For example, Epac bound to SUR1 might influence allosteric interactions of NBF-1 with its partner NBF-2. Such interactions dictate Mg^{2+}-ADP-dependent stimulation of K_{ATP} channel activity (Gribble *et al.* 1997). Simultaneously, SUR1 might act as a scaffold protein, recruiting Epac to the plasma membrane where it interacts with the Rap GTPases. In this regard, cAMP is reported to activate a signalling complex comprised of Epac1, Rap2B and PLC-ε in HEK cells (Schmidt *et al.* 2001). Since PLC-ε catalyses the hydrolysis of membrane-bound polyphosphatidylinositol 4,5-bisphosphate (PIP_2), and because PIP_2 stimulates the activity of K_{ATP} channels by reducing the channel's sensitivity to ATP (Baukrowitz *et al.* 1998; Shyng & Nichols, 1998), an ability of Epac to promote PIP_2 hydrolysis in β cells might explain the inhibitory action of cAMP reported here. Indeed, expression of PLC-ε (Kelley *et al.* 2001) has been confirmed in INS-1 cells (G. Kelley, personal communication). Thus, it will be of interest to assess whether there is a loss of Epac-mediated signal transduction in β cells derived from PLC-ε knockout mice (Wang *et al.* 2005).

Potential physiological significance

It is of interest to relate the findings presented here to current concepts regarding GLP-1 receptor-mediated signal transduction in pancreatic β cells. GLP-1 is a cAMP-elevating hormone and it stimulates insulin secretion, an action downregulated following exposure of islets to Epac2 antisense deoxyoligonucleotides (Kashima *et al.* 2001). Thus, speculation has centred on the possibility that cAMP-dependent and Epac-mediated inhibition of K_{ATP} channels might explain, at least in part, the ability of GLP-1 to act as an insulin secretagogue (Holz, 2004*a*,*b*). One report that might seem to contradict

this hypothesis is the finding that the inhibitory action of GLP-1 at K_{ATP} channels is not blocked by cAMP antagonist Rp-cAMPS (Suga *et al.* 2000). However, it should be noted that although Rp-cAMPS will block activation of PKA by cAMP, it is not a particularly effective inhibitor of Epac. For example, Rp-cAMPS fails to block Epac-mediated activation of Rap1 by cAMP in living cells (Christensen *et al.* 2003). Similarly, Rp-cAMPS fails to block the Epac-mediated stimulation of exocytosis by cAMP in sperm (Branham *et al.* 2006). Thus, an Rp-cAMPS-insensitive action of GLP-1 at K_{ATP} channels is expected if it is Epac that is the primary transducer of GLP-1 action.

With these concluding points in mind, it will be of special interest to determine if the previously reported PKA-independent insulin secretagogue action of GLP-1 in isolated islets of Langerhans (Kashima *et al.* 2001; Nakazaki *et al.* 2002) results, at least in part, from Epac-mediated inhibition of K_{ATP} channels. This possibility is advanced because GLP-1 is reported to be a less effective insulin secretagogue in knockout mice where expression of SUR1 has been disrupted (Nakazaki *et al.* 2002; Shiota *et al.* 2002; Eliasson *et al.* 2003; Doliba *et al.* 2004). Similarly, studies of Kir6.2 knockout mice demonstrate that the insulin secretagogue action of GLP-1, while being present, is markedly reduced relative to that which is measurable in wild-type mice (see Fig. 5*D* of Miki *et al.* 2005).

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Supplemental material

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and contains supplemental material consisting of two figures:

Figure 1. Time course of K_{ATP} current decay

Figure 2. RT-PCR of Epac from human islets and INS 1 cells

This material can also be found as part of the full-text HTML version available from http://www.blackwell-synergy.com