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Exchange protein activated by cAMP (Epac) mediates cAMP-dependent but protein kinase A-insensitive modulation of vascular ATP-sensitive potassium channels

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Exchange proteins directly activated by cyclic AMP (Epacs or cAMP-GEF) represent a family of novel cAMP-binding effector proteins. The identification of Epacs and the recent development of pharmacological tools that discriminate between cAMP-mediated pathways have revealed previously unrecognized roles for cAMP that are independent of its traditional target cAMP-dependent protein kinase (PKA). Here we show that Epac exists in a complex with vascular ATP-sensitive potassium (K_{ATP}) channel subunits and that cAMP-mediated activation of Epac modulates K_{ATP} channel activity via a Ca^{2+}-dependent mechanism involving the activation of Ca^{2+}-sensitive protein phosphatase 2B (PP-2B, calcineurin). Application of the Epac-specific cAMP analogue 8-pCPT-2′-*O*-Me-cAMP, at concentrations that activate Epac but not PKA, caused a 41.6 ± 4.7% inhibition (mean ± S.E.M.; $n = 7$) of pinacidil-evoked whole-cell K_{ATP} currents recorded in isolated rat aortic smooth muscle cells. Importantly, similar results were obtained when cAMP was elevated by addition of the adenylyl cyclase activator forskolin in the presence of the structurally distinct PKA inhibitors, Rp-cAMPS or KT5720. Activation of Epac by 8-pCPT-2′-*O*-Me-cAMP caused a transient 171.0 ± 18.0 nM ($n = 5$) increase in intracellular Ca^{2+} in Fura-2-loaded aortic myocytes, which persisted in the absence of extracellular Ca^{2+}. Inclusion of the Ca^{2+}-specific chelator BAPTA in the pipette-filling solution or preincubation with the calcineurin inhibitors, cyclosporin A or ascomycin, significantly reduced the ability of 8-pCPT-2′-*O*-Me-cAMP to inhibit whole-cell K_{ATP} currents. These results highlight a previously undescribed cAMP-dependent regulatory mechanism that may be essential for understanding the physiological and pathophysiological roles ascribed to arterial K_{ATP} channels in the control of vascular tone and blood flow.

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Abbreviations AbDF, antibody diluting fluid; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; CNG, cyclic nucleotide-gated; CREB, cAMP-responsive element binding protein; DAPI, 4′,6-diamidino-2-phenylindole; Epac, exchange protein directly activated by cyclic AMP; GEF, guanine-nucleotide exchange factor; K_{ATP}, ATP-sensitive K^+ channel; 8-pCPT-2′-*O*-Me-cAMP, 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′,5-cyclic monophosphate; PKA, protein kinase A; PLC_ε, phospholipase C_ε; PLP, paraformaldehyde/lysine/periodate; PP-2B, protein phosphatase 2B; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SUR, sulfonylurea receptor.

Exchange proteins directly activated by cAMP, or Epacs, are a recently discovered family of signalling proteins that bind cAMP and activate the small Ras-related monomeric G proteins Rap1 and Rap2 (de Rooij *et al.* 1998; Kawasaki *et al.* 1998; Bos, 2006). They represent the third major class of cAMP-binding effector proteins to be described after cyclic nucleotide-gated (CNG) ion channels and cAMP-dependent protein kinase (PKA) (Beavo & Brunton, 2002). Two isoforms of Epac are known to exist: the ubiquitously expressed Epac1 (also known as cAMP-GEFI) and the closely related Epac2 (cAMP-GEFII) (de Rooij *et al.* 1998; Kawasaki *et al.* 1998). These proteins contain an N-terminal cAMP-binding site (one on Epac1 and two on Epac2) and a C-terminal guanine-nucleotide exchange factor (GEF) domain that promotes GDP/GTP exchange on Rap1/2. Appreciation of the potential importance of Epac has come with the recent development of cell-permeant, Epac-specific

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cAMP analogues that allow discrimination between the different cAMP-dependent pathways (Enserink *et al.* 2002; Christensen *et al.* 2003). These synthetic analogues exploit small but significant structural differences between the conserved cAMP binding pockets found in PKA and CNG ion channels and those found in Epacs (Yagura & Miller, 1981; Enserink *et al.* 2002; Dao *et al.* 2006). The use of such compounds in the selective activation of Epac has revealed previously unrecognized roles in a range of cellular processes including exocytosis, Ca^{2+} mobilization and the regulation of ion channel function (reviewed in Holz *et al.* (2006)). This latter point is of particular interest given that until recently, cAMP was known to influence channel behaviour by only two mechanisms; direct binding, as in the case of CNG channels, or through PKA-mediated phosphorylation of channel subunits. Here we investigate whether vascular ATP-sensitive potassium (K_{ATP}) channels, which have a particularly high dependence on cAMP for their normal physiological function, are regulated by the activation of this novel cAMP effector.

K_{ATP} channels are sensitive to intracellular levels of adenosine nucleotides and thus link changes in cellular metabolism to membrane excitability (Nichols, 2006). They are expressed in pancreatic β-cells, certain types of neurones, cardiac, skeletal and smooth muscle and their physiological roles include regulation of insulin secretion, glucose-sensing in the hypothalamus, ischaemic cardioprotection and the control of blood flow (Quayle *et al.* 1997; Yokoshiki *et al.* 1998; Miki & Seino, 2005). Vascular K_{ATP} channels provide a background K^+ conductance important in the regulation of membrane potential and so smooth muscle contractility and blood flow (Quayle *et al.* 1997; Clapp & Tinker, 1998; Yokoshiki *et al.* 1998). Pharmacological inhibition of K_{ATP} channels has been shown to increase vascular resistance in the systemic and coronary circulations (Samaha *et al.* 1992; Duncker *et al.* 2001) and drugs that open vascular K_{ATP} channels are used to treat angina and hypertension. Genetically engineered mice that lack vascular K_{ATP} channel subunits develop hypertension and die prematurely from coronary vasospasm, a phenotype resembling vasospastic (Prinzmetal or variant) angina in humans (Chutkow *et al.* 2002; Miki *et al.* 2002). A substantial part of the physiological regulation of vascular K_{ATP} channels occurs via vasoactive transmitters. Endogenous vasodilators, including calcitonin gene-related peptide (CGRP), β-adrenoceptor agonists and adenosine, increase K_{ATP} channel activity by acting at Gs-coupled receptors to stimulate adenylyl cyclase and elevate intracellular levels of cAMP (Miyoshi & Nakaya, 1993; Quayle *et al.* 1994; Kleppisch & Nelson, 1995; Wellman *et al.* 1998). These cAMP-initiated effects are attributed to the activation of PKA, with experiments on cloned K_{ATP} channels suggesting that channel activity is increased by PKA-dependent phosphorylation at sites on both its pore-forming and regulatory subunits (Quinn *et al.* 2004). Even in the absence of vasodilators arterial K_{ATP} channels are subject to a tonic PKA-dependent activation, which arises from sustained cAMP production originating from basal adenylyl cyclase turnover (Hayabuchi *et al.* 2001*b*; Sampson *et al.* 2004). To date no comparable data exist on the role of Epac in the regulation of vascular K_{ATP} channel activity.

Here, using the well-characterized, Epac-specific cAMP analogue 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′,5-cyclic monophosphate (8-pCPT-2′-*O*-Me-cAMP) at concentrations that activate Epac but not PKA (Enserink *et al.* 2002; Christensen *et al.* 2003), we show that cAMP also modulates vascular K_{ATP} channel activity by a mechanism independent of PKA. We show that cAMP-mediated activation of Epac inhibits rat aortic K_{ATP} channels via a Ca^{2+}-dependent mechanism involving the activation of Ca^{2+}-sensitive phosphatase 2B (PP-2B, calcineurin). Since vasodilator-induced elevation of intracellular cAMP levels and activation of PKA is associated with K_{ATP} channel activation (Miyoshi & Nakaya, 1993; Quayle *et al.* 1994; Kleppisch & Nelson, 1995; Wellman *et al.* 1998), these data suggest that under certain conditions cAMP conveys opposite, inhibitory information to the channel. While *in vitro* cAMP affinity between Epac and cAMP is similar (Dao *et al.* 2006), the concentration of cAMP required for half-maximal activation of Epac1 is reported to be considerably higher than that required to activate PKA (de Rooij *et al.* 2000; Enserink *et al.* 2002; Rehmann *et al.* 2003). We discuss the possibility that Epac and PKA are differentially activated by different concentrations of cAMP and that Epac acts physiologically as a feedback regulator of K_{ATP} channel function. We also discuss an alternative pathophysiological role for Epac in the development of vascular hypertrophy.

Methods

Antibodies, polyacrylamide gel electrophoresis and immunoblotting

The following antibodies were used: anti-Epac (sc-28366), anti-Epac2 (sc-28326) and anti-SUR2B (sc-5793) (Santa Cruz Biotechnology), horseradish peroxidase (HRP)-conjugated anti-goat and anti-mouse (Jackson Immunochemical Laboratories), Alexa Fluor 488-conjugated anti-mouse (Invitrogen). Protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide-Tris gels and transferred electrophoretically onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Immunoblotting was performed as previously described (Sampson *et al.* 2003).

Animals

Tissues were obtained from adult male Wistar rats (175–200 g; Charles River Laboratories) which were killed by a rising concentration of CO_2 followed by exsanguination. The care and killing of animals conformed to the requirements of the UK Animals (Scientific Procedures) Act 1986.

Co-immunoprecipitation

Rat brain, aorta or mesenteric artery was homogenized in ice-cold lysis buffer (mmol l^{-1}: 20 Tris-HCl; 250 NaCl; 3 EDTA; 3 EGTA; pH 7.6) containing 1% Triton X-100 and protease inhibitors (1:100 dilution, Sigma-Aldrich Protease Inhibitor Cocktail). Insoluble material was pelleted by centrifugation and the lysate pre-cleared by incubation with immobilized protein A/G agarose beads (Thermo Fisher Scientific). Cleared lysate was incubated with 5 μg of anti-Epac antibody or 5 μg of mouse IgG non-immune control serum (Sigma-Aldrich). Antigen–antibody complexes were captured by incubating lysate/antibody mixture with pre-washed immobilized protein A/G agarose beads. Agarose beads were then pelleted and bound proteins released by boiling for 10 min in Laemmli (x2) sample buffer (Sigma-Aldrich). Samples were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and analysed by immunoblotting.

Immunocytochemistry

Rat aorta smooth muscle myocytes were isolated, plated onto poly-lysine-coated coverslips, fixed and permeabilized as previously described (Hayabuchi *et al.* 2001*a*). Cells were subsequently incubated with mouse monoclonal anti-Epac antibody, washed in PBS and then incubated with Alexa Fluor 488-conjugated donkey anti-mouse antibody. Coverslips were mounted onto microscope slides using DAKO fluorescent mounting medium (Dako Ltd) before being viewed using a Leica (SP2 AOBS) microscope system.

Immunohistochemistry

Rat aorta was fixed in paraformaldehyde/lysine/periodate (PLP) and incubated overnight in 30% sucrose. Vessels were then embedded in OCT compound (RA Lamb Ltd, UK) and cryostat-cut thin transverse sections (6–8 μm) were thaw-mounted on silinated glass slides. Tissue sections were blocked in antibody dilution fluid (AbDF; PBS containing 10 mg ml^{-1} bovine serum albumin (BSA) and 5% (v/v) goat serum). Antibody incubation and imaging was carried out as above.

Electrophysiology

Whole-cell K^+ currents were recorded from freshly dissociated rat aortic smooth muscle cells using an Axopatch 200B amplifier (Axon Instruments) as previously described (Sampson *et al.* 2004). For perforated patch experiments, amphotericin B (Sigma-Aldrich; stock concentration 30 mg ml^{-1} in dimethylsulphoxide (DMSO)) was diluted to a working concentration of 210 μg ml^{-1} in pipette-filling solution. Pinacidil, glibenclamide, cAMP, forskolin, KT5720, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) and amphotericin B were purchased from Sigma-Aldrich. Adenosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)-2′-*O*-methyl-, sodium salt (8-pCPT-2′-*O*-Me-cAMP), cyclosporin A, ascomycin and Rp-cAMPS (the Rp isomer of adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt) were purchased from Calbiochem. All experiments were performed at room temperature (18–22°C), and the results are expressed as the mean ± S.E.M. Statistical significance was evaluated using Student's *t* test (ANOVA for multicomparisons); the level of significance was taken as $P < 0.05$.

Results

Epac1 localizes to smooth muscle sarcolemmal membranes

To determine the predominant isoform of Epac expressed in vascular smooth muscle, proteins within rat aortic and mesenteric artery homogenates were separated by SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted with monoclonal antibodies against Epac1 and Epac2 (Fig. 1*A*). Rat brain, which was used as a positive control, produced single immunoreactive bands for each antibody at approximately 105 kDa (Epac1) and 125 kDa (Epac2), consistent with the expected molecular weights of the Epac proteins. Aortic and mesenteric homogenates produced a single band at 105 kDa, consistent with the expression of Epac1, but produced no significant staining for Epac2. Thin transverse sections of rat femoral artery labelled with anti-Epac1 and Epac2 and visualized by addition of secondary antibodies conjugated with Alexa Fluor 488 showed expression of Epac1 exclusively in the smooth muscle cell layer (Fig. 1*B*). No significant labelling above control levels (secondary antibody alone shown in lower inset) could be seen for Epac2. Similar results were obtained from confocal images of transverse sections of rat aorta labelled with anti-Epac1 and 4′,6-diamidino-2-phenylindole (DAPI) to show the characteristic 'cigar-shaped' nucleus within the smooth muscle cells (Fig. 1*C*). Confocal images of isolated aorta smooth muscle cells stained with anti-Epac1 and

visualized with Alexa Fluor 488-conjugated secondaries showed Epac1 localized predominantly to the cell periphery (Fig. 1*D*). Consistent with Western blot analyses and whole tissue sections, no staining was detected for Epac2 in aorta smooth muscle myocytes (data not shown). Similarly cells incubated with the secondary antibody alone showed no unspecific staining.

Antibodies against Epac1 co-immunoprecipitate vascular K_{ATP} channel subunits

Yeast two-hybrid analysis of the mouse insulin-secreting cell line MIN6 suggests that Epac2 is directly coupled to the regulatory SUR1 subunit of the pancreatic K_{ATP} channel (Ozaki *et al.* 2000). More recent work has supported this initial finding of Epac1 and 2 interacting

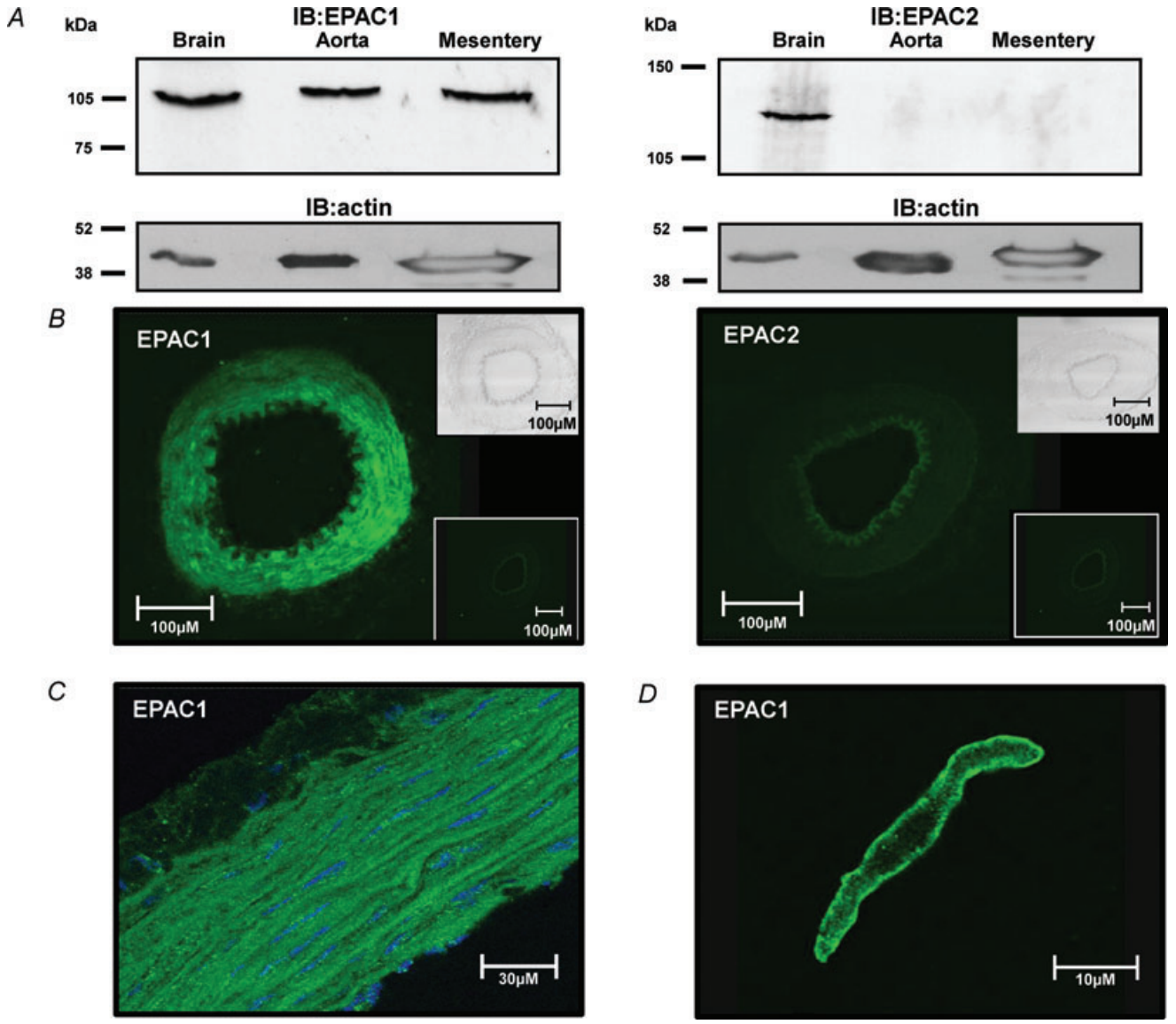

Figure 1. Epac1 is the predominant isoform found in vascular smooth muscle
A, Western blot analyses of rat brain, aorta and mesenteric artery homogenates immunoblotted with monoclonal antibodies against Epac1 (left panel) and Epac2 (right panel). Loading controls immunoblotted with antibodies against smooth muscle actin are shown in the lower panels. *B*, confocal images of thin transverse sections of rat femoral artery labelled with anti-Epac1 (left panel) and anti-Epac2 (right panel) and visualized by addition of secondary antibodies conjugated with Alexa Fluor 488. Respective bright-field images are shown in top inset of both panels. Non-specific, background staining (secondary antibodies applied alone) is shown in lower inset of both panels. *C*, confocal image of a thin transverse section of rat aorta labelled with anti-Epac1 and Alexa Fluor 488 (green), and DAPI (blue). *D*, confocal image of an isolated arterial smooth muscle cell stained with anti-Epac1. Cells incubated with the Alexa Fluor 488-conjugated secondary antibody alone showed no unspecific staining.

with full length SUR1 when coexpressed in HEK 293 cells (Kang *et al.* 2006). The localization of Epac1 at or near the plasma membrane in aortic myocytes raises the possibility that it may also interact with vascular K_{ATP} channel proteins. Basing our investigation on the interactions demonstrated in the Ozaki *et al.* (2000) and Kang *et al.* (2006) studies, we used antibodies against Epac1 to co-immunoprecipitate K_{ATP} channel subunits. Figure 2 shows that mouse monoclonal antibodies directed against Epac1 were able to co-immunoprecipitate the vascular-type, regulatory SUR2B subunit from rat aortic homogenates, indicating that these two proteins may exist in a complex at the sarcolemmal membrane. We were unable to test for the involvement of additional subunits as goat polyclonal antibodies raised against the pore-forming subunit ($K_{ir}6.1$) were ineffective in immunoprecipitation (data not shown).

Activation of Epac inhibits pinacidil-evoked whole-cell K_{ATP} currents

To determine whether selective activation of Epac modulates vascular K_{ATP} channel activity, we tested the effects of the Epac-specific cAMP analogue 8-pCPT-2′-*O*-Me-cAMP on whole-cell K_{ATP} currents recorded from cells isolated from rat aorta. *In vitro* 8-pCPT-2′-*O*-Me-cAMP has more than 100-fold lower affinity than cAMP for PKA. *In vivo*, 10 μmol l^{-1} 8-pCPT-2′-*O*-Me-cAMP induces near-maximal activation of Rap1 (a measure of Epac activity), but does not induce phosphorylation of the PKA substrate CREB (cAMP-responsive element binding protein) even at concentrations as high as 100 μmol l^{-1} (Enserink *et al.* 2002).

Application of 5 μmol l^{-1} 8-pCPT-2′-*O*-Me-cAMP, a concentration which has been shown to activate Epac but not PKA (Enserink *et al.* 2002; Christensen *et al.* 2003), caused a 41.6 ± 4.7% inhibition ($n = 7$) of pinacidil-evoked whole-cell K_{ATP} currents recorded from isolated rat aortic smooth muscle cells using the perforated patch technique (Fig. 3*A* and *C*). This suggests that elevation of cAMP within vascular cells can affect channel activity by a mechanism that is independent of PKA activation. While selective for Epac at the concentration used (Enserink *et al.* 2002; Christensen *et al.* 2003), recent reports suggest that hydrolysis of 8-pCPT-2′-*O*-Me-cAMP to bioactive metabolites may complicate interpretation of its action (Laxman *et al.* 2006). We therefore confirmed that the effects on channel function were cAMP-induced and independent of PKA by increasing intracellular levels of cAMP while inhibiting PKA. To inhibit PKA we chose two structurally and functionally distinct compounds, Rp-cAMPS and KT5720. Rp-cAMPS is a competitive inhibitor of the activation of PKA by cAMP and thus acts at the cAMP-binding regulatory subunits of the holoenzyme. While a particularly specific and potent blocker of activation of PKA by cAMP, it fails to inhibit activation of Epac (Christensen *et al.* 2003; Rangarajan *et al.* 2003; Branham *et al.* 2006; Holz *et al.* 2008). KT5720 in contrast acts at the catalytic subunit of PKA, blocking the ATP-binding site and thus directly inhibiting PKA-mediated phosphorylation. Elevation of cAMP by addition of the adenylyl cyclase activator forskolin in the presence of either KT5720 (1 μmol l^{-1}; Fig. 3*B* and *C*) or Rp-cAMPS (100 μmol l^{-1}; Fig. 3*C*) caused a 32.7 ± 1.3% ($n = 3$) and 38.9 ± 6.7% ($n = 4$) inhibition, respectively, of whole-cell K_{ATP} current. Application of forskolin alone had no significant effect, following pinacidil-evoked activation of the K_{ATP} current (Fig. 3*C*).

Since there are currently no Epac-selective antagonists, we tested the effects of the Epac-selective activator 8-pCPT-2′-*O*-Me-cAMP on rat aortic smooth muscle myocytes that had been dialysed with 100 μmol l^{-1} cAMP. Under these conditions, Epac (which binds cAMP with an affinity (K_d) of 2.8 μmol l^{-1}) should be fully activated and application of 8-pCPT-2′-*O*-Me-cAMP should have no additional effect upon whole-cell K_{ATP} currents. In support of this, application of 8-pCPT-2′-*O*-Me-cAMP in the presence of elevated intracellular cAMP (100 μmol l^{-1} cAMP included in the pipette-filling solution) caused only a 2.5 ± 0.5% inhibition of whole-cell K_{ATP} currents compared to 39.8 ± 4.7% under control (conventional whole-cell) conditions ($n = 3,\ 3$; $P < 0.01$) (Fig. 4*A* and *B*).

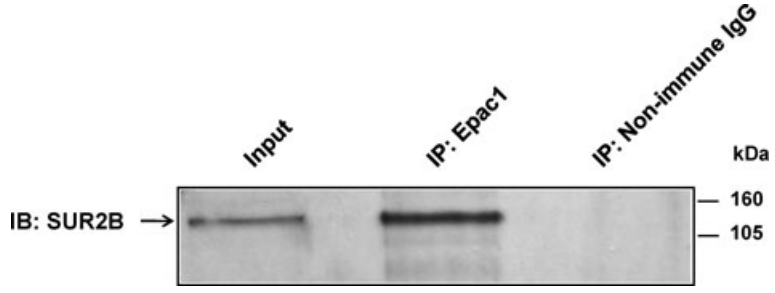

Figure 2. Epac1 co-immunoprecipitates with SUR2B from rat aortic homogenates
Antibodies directed against Epac1 were able to co-immunoprecipitate the regulatory SUR2B subunit of vascular K_{ATP} channels from rat aortic homogenates. Precipitated proteins were immunoblotted with antibodies against SUR2B, which runs as a single immunoreactive band of molecular mass 150 kDa. Ten per cent of the pre-cleared homogenate was run in the input lane. Non-immune IgG used was from mouse.

Epac-mediated inhibition of K_{ATP} channel activity is Ca^{2+} dependent

Since activation of Epac has been consistently linked to Ca^{2+} mobilization within cells (Kang *et al.* 2001, 2003, 2005;Tsuboi *et al.* 2003; Morel *et al.* 2005; Yip, 2006; Ster *et al.* 2007), we examined the Ca^{2+} sensitivity of the Epac-mediated inhibition of K_{ATP} current. Inclusion of the Ca^{2+}-specific chelator BAPTA (20 μmol l^{-1}) in the pipette-filling solution significantly reduced the ability of 8-pCPT-2′-*O*-Me-cAMP (5 μmol l^{-1}) to inhibit whole-cell aortic K_{ATP} currents. In the presence of BAPTA, the addition of 8-pCPT-2′-*O*-Me-cAMP caused only a $8.7 \pm 4.4\%$ reduction ($n = 4$) of pinacidil-evoked K_{ATP} currents compared with $39.8 \pm 4.7\%$ under control (conventional whole-cell) conditions ($n = 3$) (Fig. 5*A* and *B*) ($P < 0.01$). This inability of 8-pCPT-2′-*O*-Me-cAMP to affect K_{ATP} channel activity in the presence of BAPTA suggests that Epac-mediated channel inhibition relies upon an increase in intracellular Ca^{2+}. We thus investigated whether activation of Epac leads to an elevation in intracellular Ca^{2+} in vascular smooth muscle cells. Figure 5*C* shows a Ca^{2+} transient recorded from a single Fura-2-loaded aortic myocyte in response to application of the Epac-specific cAMP analogue 8-pCPT-2′-*O*-Me-cAMP. At a constant membrane potential of −60 mV and 0 mmol l^{-1} extracellular Ca^{2+}, rapid application of 5 μmol l^{-1} 8-pCPT-2′-*O*-Me-cAMP evoked a transient (386 ± 153 nmol l^{-1}; $n = 4$; Fig. 5*C*) increase in $[Ca^{2+}]_i$. The fact that the 8-pCPT-2′-*O*-Me-cAMP-induced elevation of intracellular Ca^{2+} occurred in the absence of extracellular Ca^{2+} suggests that it originates from the release of Ca^{2+} from intracellular stores and not from an influx of Ca^{2+} through sarcolemmal channels.

Epac-mediated regulation of K_{ATP} channels depends upon the Ca^{2+}-sensitive phosphatase 2B, calcineurin

While the application of 8-pCPT-2′-*O*-Me-cAMP caused a transient elevation in $[Ca^{2+}]_i$ (Fig. 5*C*) the inhibition of

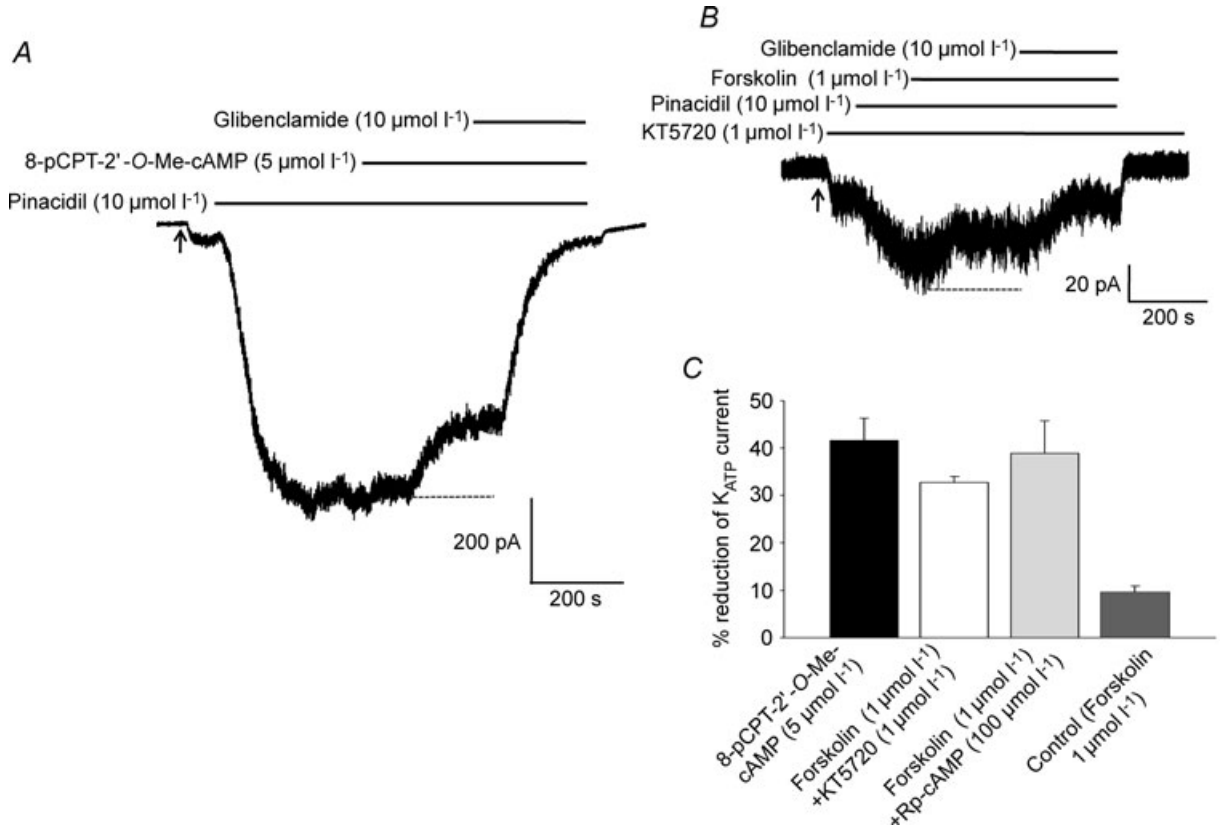

Figure 3. Specific activation of Epac inhibits pinacidil-evoked whole-cell K_{ATP} currents
Whole-cell current recorded from single isolated rat aortic smooth muscle cells using the perforated patch technique. Cells were held at −60 mV and at point indicated by arrow the extracellular solution was changed from 6 mmol l^{-1} to 140 mmol l^{-1} K^+ to increase the inward driving force for K^+. Application of the Epac-specific cAMP analogue 8-pCPT-2′-*O*-Me-cAMP (5 μmol l^{-1}) caused an inhibition of pinacidil-evoked, glibenclamide-sensitive whole-cell current (*A*). Similarly, in the presence of the protein kinase A inhibitor KT5720 (1 μmol l^{-1}) (*B*), elevation of intracellular cAMP by application of the adenylyl cyclase activator forskolin (1 μmol l^{-1}) caused an inhibition of pinacidil-evoked current. *C*, mean inhibition of pinacidil-evoked, glibenclamide-sensitive current in experiments like those in *A* and *B* ($n = 7, 3, 4, 4$ cells; ***$P < 0.001$).

K_{ATP} current was sustained (Fig. 3*A*), suggesting that Ca^{2+} *per se* does not affect channel activity and implicating the involvement of Ca^{2+}-activated enzymes. K_{ATP} channels have previously been shown to respond to fluctuations in intracellular Ca^{2+} through activation of the Ca^{2+}-sensitive protein phosphatase 2B (PP-2B, calcineurin) (Wilson *et al.* 2000). Activation of PP-2B inhibits K_{ATP} channel activity, possibly through dephosphorylation of a PKA-dependent phosphorylation site. To test the involvement of PP-2B in Epac-mediated responses, the effect of structurally distinct PP-2B inhibitors cyclosporin A and ascomycin (FK-520) was examined. Preincubation with cyclosporin A or ascomycin, significantly reduced the ability of 8-pCPT-2′-*O*-Me-cAMP to inhibit whole-cell K_{ATP} currents. Inhibition was $10.8 \pm 2.8\%$ ($n = 9$; $P < 0.001$; Fig. 6*A* and *C*) and $7.3 \pm 1.6\%$ ($n = 8$; $P < 0.001$; Fig. 6*B* and *C*), respectively.

Discussion

The identification of Epacs as an additional class of cAMP effector and the recent development of new pharmacological tools that discriminate between cAMP-mediated pathways allows the contribution of other cAMP-dependent but PKA-insensitive pathways to be examined. While their mode of action is for the most part unclear, Epacs have the ability to affect the function of a range of ion channels including amiloride-sensitive (ENaC) Na^+ channels, Ca^{2+}-dependent K^+ channels, pancreatic ATP-sensitive K^+ (K_{ATP}) channels, voltage-gated Ca^{2+} channels, Cl^- channels and intracellular Ca^{2+} release channels (IP_3 and ryanodine receptors) (Schmidt *et al.* 2001; Tsuboi *et al.* 2003; Novara *et al.* 2004; Kang *et al.* 2005, 2006, 2008; Aromataris *et al.* 2006; Helms *et al.* 2006; Ster *et al.* 2007). Here, we show that application of the Epac-specific cAMP analogue, 8-pCPT-2′-*O*-Me-cAMP at concentrations that activate Epac but not PKA, or the elevation of cAMP under conditions where PKA is blocked, inhibits pinacidil-evoked whole-cell K_{ATP} currents via a Ca^{2+}-dependent mechanism involving release of Ca^{2+} from internal stores and the activation of the Ca^{2+}-sensitive phosphatase calcineurin.

The essential link between Epac activation and the modulation of K_{ATP} channel activity appears to be an elevation in intracellular Ca^{2+}. Application of 8-pCPT-2′-*O*-Me-cAMP evoked a transient increase in intracellular Ca^{2+} in rat aortic myocytes that was unaffected by the removal of extracellular Ca^{2+}. Similarly, if the cell is dialysed with the Ca^{2+}-specific chelator BAPTA (by inclusion in the pipette-filling solution) the ability of 8-pCPT-2′-*O*-Me-cAMP to inhibit whole-cell aortic K_{ATP} currents is essentially abolished. Activation of Epac has been consistently linked to Ca^{2+} mobilization from intracellular stores within a variety of different cell types (Kang *et al.* 2001, 2003, 2005; Tsuboi *et al.* 2003; Morel *et al.* 2005; Yip, 2006; Ster *et al.* 2007), presumably through the Epac-mediated activation of intracellular Ca^{2+} release

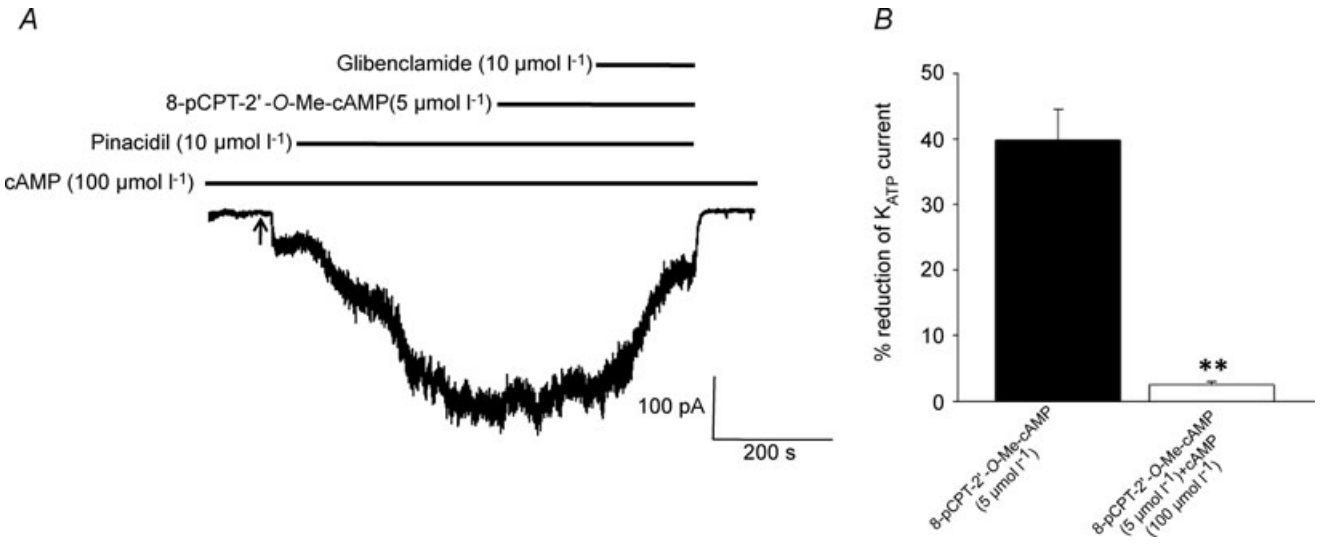

Figure 4. In the presence of elevated intracellular cAMP, specific activation of Epac has no effect on pinacidil-evoked whole-cell K_{ATP} currents
Whole-cell current recorded from single isolated rat aortic smooth muscle cells at −60 mV using the conventional patch configuration in the presence of 100 μmol l^{-1} cAMP (*A*). cAMP was included in the pipette-filling solution and allowed to dialyse into the cell prior to the start of recording. The arrow indicates the extracellular solution was changed from 6 mmol l^{-1} to 140 mmol l^{-1} K^+ to increase the inward driving force for K^+. *B*, mean inhibition of pinacidil-evoked, glibenclamide-sensitive current in experiments like those in *A* ($n = 3$, 3 cells; $^{**}P < 0.01$).

channels. In several cell types evidence for the activation of ryanodine receptors (RyRs) is strong given that the Ca^{2+}-mobilizing actions of 8-pCPT-2′-*O*-Me-cAMP are sensitive to ryanodine (Kang *et al.* 2001, 2005), and a large cAMP-regulated macromolecular complex containing both Epac and RyRs has been reported in cardiac myocytes (Dodge-Kafka *et al.* 2005). The mode of action of Epac on RyRs is unclear but could potentially involve either a direct interaction of Epac, or its immediate downstream target Rap, with the channel protein, or an indirect effect with Rap acting via intermediate kinases to phosphorylate and increase the Ca^{2+} sensitivity of RyRs. Ca^{2+} may, alternatively, be mobilized through an inositol trisphosphate (IP_3)-dependent pathway. Although Rap1 is the most studied member of the Rap family, Rap2B has recently been shown to be responsible for the modulation of phospholipase Cε (PLCε), initiating phosphoinositol hydrolysis and IP_3-dependent release of Ca^{2+} from intracellular stores (Schmidt *et al.* 2001). It is perhaps worth noting in the context of this second mechanism that while Rap1 is robustly expressed, we find no significant expression of Rap2 in arterial homogenates (data not shown).

While the mechanism by which Epac mobilizes intracellular Ca^{2+} remains unclear, the effect of changes in intracellular Ca^{2+} on K_{ATP} channel activity is more defined. Activation of Epac causes only a brief increase in cytosolic Ca^{2+}, with Ca^{2+} typically returning to basal values within 60 s of the start of the transient. In contrast the Epac-mediated inhibition of whole-cell K_{ATP} current is sustained, at least during our recording period (current inhibition typically monitored for a period of 2–3 min). This suggests that Ca^{2+} itself is unlikely to have a direct effect on channel activity and implicates the involvement of Ca^{2+}-activated signalling intermediaries. It has previously been shown that activation of the

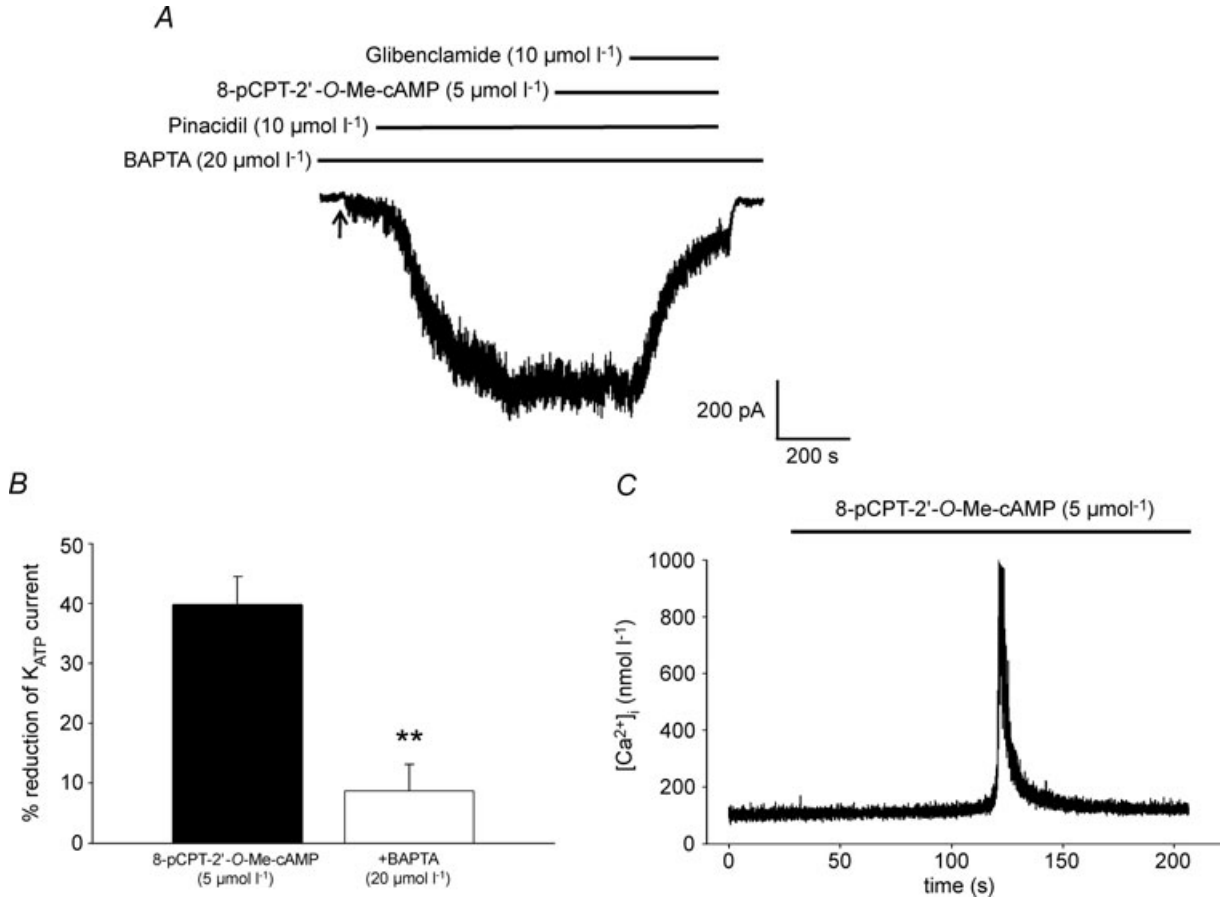

Figure 5. Epac-mediated regulation of vascular K_{ATP} channels is Ca^{2+} dependent
Whole-cell currents recorded at −60 mV from single isolated rat aortic smooth muscle myocytes using the conventional patch configuration in the presence of 20 μmol l^{-1} of the Ca^{2+}-specific chelator BAPTA (*A*). BAPTA was included in the pipette-filling solution and allowed to dialyse into the cell before the start of recording. *B*, mean inhibition of pinacidil-evoked, glibenclamide-sensitive current in experiments like those in *A* ($n = 7$, 4 cells; $^{**}P < 0.01$). *C*, representative Ca^{2+} transients induced by 8-pCPT-2′-*O*-Me-cAMP (5 μmol l^{-1}) in freshly isolated single rat aortic smooth muscle myocytes in the presence of 0 mmol l^{-1} extracellular Ca^{2+} ($n = 4$).

Ca^{2+}-sensitive protein phosphatase calcineurin inhibits K_{ATP} channel activity, possibly through dephosphorylation of a PKA-dependent phosphorylation site (Wilson *et al.* 2000). Consistent with the involvement of calcineurin in the Epac-mediated channel modulation, we find that application of the structurally distinct calcineurin inhibitors, cyclosporin A or ascomycin significantly reduces the ability of 8-pCPT-2′-*O*-Me-cAMP to inhibit whole-cell K_{ATP} currents.

Activation of Epac has recently been shown to suppress the activity of pancreatic K_{ATP} channels by a mechanism involving an increase in the channel's sensitivity to its physiological inhibitor ATP (Kang *et al.* 2008). Closure of K_{ATP} channels is well-established as the trigger for pancreatic β-cell depolarization, increased Ca^{2+} influx through voltage-gated Ca^{2+} channels and insulin secretion (Ashcroft, 2006). Thus Epac-mediated inhibition of pancreatic K_{ATP} channels provides a possible explanation for cAMP-induced, Epac-dependent increases in insulin release (Seino & Shibasaki, 2005; Shibasaki *et al.* 2007). Physiologically, elevation of intracellular cAMP occurs in vascular smooth muscle via the activation of Gs-coupled receptors by endogenous vasodilators such as adenosine and CGRP (Miyoshi & Nakaya, 1993; Quayle *et al.* 1994; Kleppisch & Nelson, 1995; Wellman *et al.* 1998). This is associated with activation of PKA, which phosphorylates the K_{ATP} channel on both the pore-forming and regulatory subunits resulting in the up-regulation of channel activity (Quinn *et al.* 2004; Shi *et al.* 2007, 2008). Activation of K_{ATP} channels leads to membrane hyperpolarization, decreased Ca^{2+} entry via voltage-gated Ca^{2+} channels and, ultimately, vasorelaxation (Nelson *et al.* 1990). This raises the question as to under what conditions a cAMP-mediated Epac-dependent inhibition of vascular K_{ATP} channel activity would occur. The functional cAMP binding site on Epac1 and Epac2 bind cAMP *in vitro* with a similar affinity to that of the PKA holoenzyme (K_d 2.8 μmol l^{-1} for Epac1; 1.2 μmol l^{-1} for Epac2 compared with 2.9 μmol l^{-1} for PKA) (de Rooij *et al.* 2000; Christensen *et al.* 2003; Dao *et al.* 2006). Epac2 has an additional low affinity cAMP-binding domain at its N-terminus (K_d 87 μmol l^{-1}), which has an uncertain function since it is not required for cAMP-induced activation of Rap (de Rooij *et al.* 2000). While *in vitro* cAMP affinity between Epac and PKA are virtually identical, the concentration of cAMP required

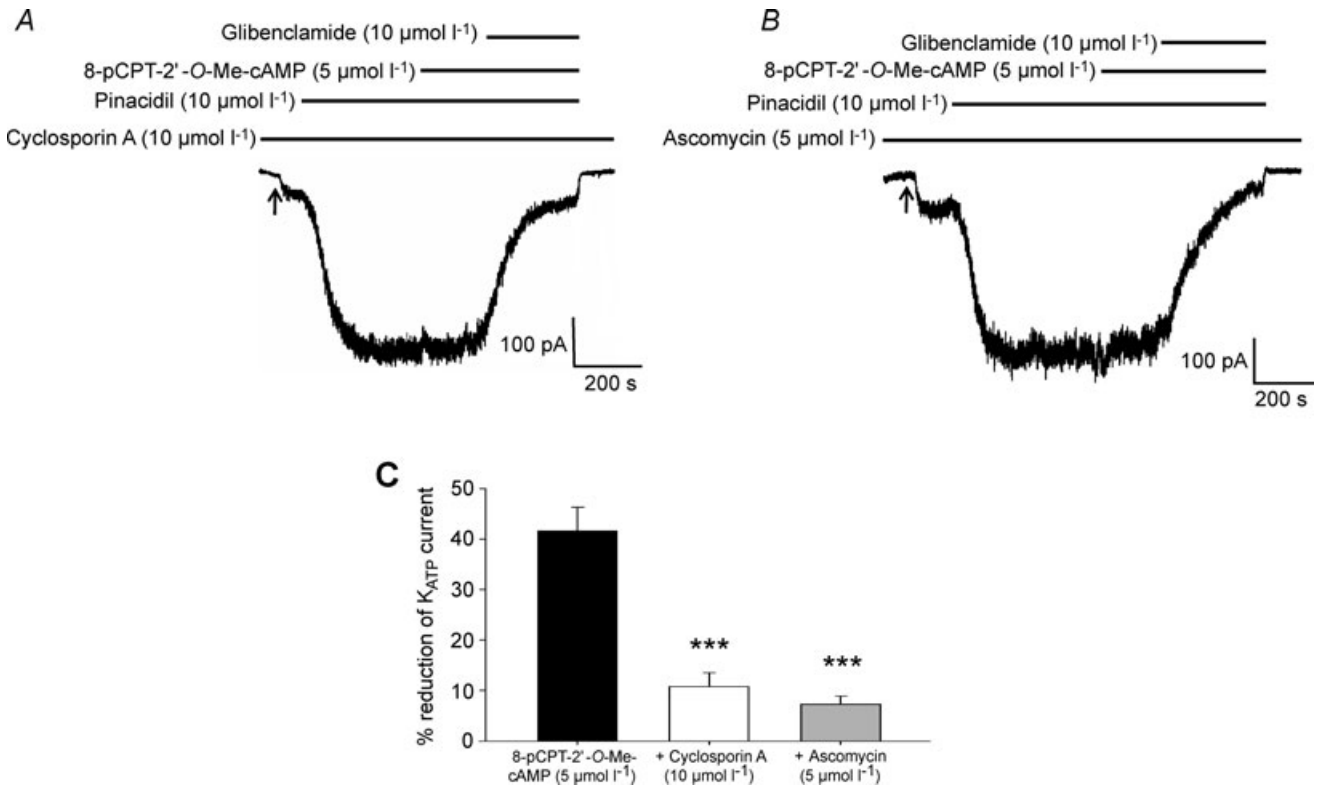

Figure 6. Epac regulation depends upon the Ca^{2+}-sensitive phosphatase, calcineurin
Whole-cell current recorded at −60 mV from single isolated rat aortic smooth muscle cells using the perforated patch technique. Preincubation with calcineurin inhibitors, cyclosporin A (10 μmol l^{-1}) (*A*) or ascomycin (FK−520; 5 μmol l^{-1}) (*B*) significantly reduced the ability of 5 μmol l^{-1} 8-pCPT-2′-*O*-Me-cAMP to inhibit whole-cell K_{ATP} currents. *C*, mean inhibition of pinacidil-evoked, glibenclamide-sensitive current in control experiments and experiments like those in *A* and *B* ($n = 7, 8, 8$ cells; ***$P < 0.001$).

for half-maximal activation of Epac1 is reported to be around 30 μmol l^{-1} compared to $\sim$1 μmol l^{-1} for PKA. This difference may reflect the positive co-operativity that exists between the two cAMP sites on PKA, which is lacking for the single cAMP-binding site on Epac1 (Dao *et al.* 2006). The implication of this is that PKA may be preferentially activated by small elevations in cAMP, while much higher levels of the second messenger are required to activate the Epac-mediated transduction pathway. It is thus tempting to speculate that Epac activation acts as a feedback regulator of K_{ATP} channel function following large fluctuations of cAMP. *In vivo* support for the idea that PKA is more responsive to changes in cAMP than Epac is currently lacking. Interestingly though cAMP biosensors based on cAMP-binding sites of PKA are more sensitive than those based on Epac (Ponsioen *et al.* 2004). There is also some evidence *in vivo* that PKA-mediated responses to certain growth factors occur at lower concentrations of cAMP than is required to activate Rap1 (Zwartkruis *et al.* 1998). Whether localized fluctuations of cAMP large enough to activate Epac occur physiologically within smooth muscle cells remains to be determined. Ultimately, the subcellular location of Epac and PKA, in relation to each other and to sites of cAMP production and degradation, may be crucial in determining which cAMP effector is preferentially activated. In this context it is interesting that Epac appears to complex with the regulatory SUR2B subunit of the channel. This initially seems surprising given the indirect Ca^{2+}-dependent route by which Epac inhibits channel activity. However, it is possible that the Epac/K_{ATP} channel complex is in very close proximity to the sarcoplasmic reticulum (SR) Ca^{2+} release channels, possibly even in a supramolecular complex somewhat resembling the voltage-gated Ca^{2+} channel/ryanodine receptor complexes involved in excitation–contraction coupling.

An alternative hypothesis is that the activation of Epac signalling pathways may not be involved in the normal regulation of channel activity, but are largely of pathophysiological significance. This is a potentially important point since Epac-mediated activation of calcineurin and subsequently of the Ca^{2+}–calcineurin-dependent transcription factor, nuclear factor of activated T cells, has recently been linked to the induction of hypertrophic gene expression and the development of hypertrophy in cardiac muscle (Morel *et al.* 2005). The role of Epac activation and the development of vascular disease states may be an area that warrants additional investigation.

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G. I. Purves: all experiments except for Ca^{2+} transient recordings, conception and design of experiments, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be published. T. Kamishima: Ca^{2+} transient recordings, conception and design of experiments, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published. L. M. Davies: analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be published. J. M. Quayle: conception and design of experiments, revising the article critically for important intellectual content, and final approval of the version to be published. C. Dart: conception and design of experiments, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be published. Experiments were undertaken in the School of Biological Sciences and the Department of Human Anatomy and Cell Biology at the University of Liverpool.

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