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Themed Section: Novel cAMP Signalling Paradigms

REVIEW

# Regulation of the inflammatory response of vascular endothelial cells by EPAC1

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Life-threatening diseases of the cardiovascular system, like atherosclerosis, are exacerbated by unwanted inflammation within the structures of large blood vessels. This inflammation involves increased permeability of the vascular endothelial cells (VECs) that form the lining of blood vessels, leading to exaggerated extravasation of blood components and accumulation of fluid in the extravascular space. This results in tissue dysfunction and increased secretion of chemokines that attract leukocytes and monocytes to the inflamed endothelium. Cyclic AMP is synthesized in VECs in response to endogenous Gs-coupled receptors and is known to limit cytokine action and reduce endothelial hyperpermeability induced by multiple pro-inflammatory stimuli. The mechanisms underlying this anti-inflammatory action of cyclic AMP are now being elucidated and it is becoming clear that the cyclic AMP sensor, exchange protein activated by cyclic AMP (EPAC1), appears to play a key role in suppressing unwanted inflammation. EPAC1 mediates at least three anti-inflammatory pathways in VECs by down-regulating inflammatory signalling through the induction of the suppressors of cytokine signalling 3 (*SOCS-3*) gene, limiting integrin-dependent vascular permeability and enhancing endothelial barrier function through the stabilization of VE-cadherin junctions. Given that manipulation of cellular cyclic AMP levels currently forms the basis of many effective pharmaceuticals and that EPAC1 is involved in multiple anti-inflammatory protective processes in VECs, does this make EPAC1 an attractive target for the development of activators capable of eliciting a coordinated programme of 'protection' against the development of endothelial dysfunction? Here we discuss whether EPAC1 represents an attractive therapeutic target for limiting endothelial dysfunction associated with cardiovascular diseases like atherosclerosis.

**LINKED ARTICLES**
This article is part of a themed section on Novel cAMP Signalling Paradigms. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.166.issue-2

**Abbreviations**
8-pCPT-2'OMe–cAMP, - (4- chlorophenylthio)- 2'- O- methyladenosine- 3', 5'- cyclic monophosphate; AKAP, A-kinase anchoring protein; EPAC, exchange protein activated by cyclic AMP; MAP; microtubule-associated protein

## Introducton

Inflammation is the normal body response to microbial, chemical, physical injury and aging, and is associated with increased levels of pro-inflammatory cytokines in the circulation, including IL-1, IL-6 and TNF-α (Hobbs and Ernst, 1997). Unresolved chronic pro-inflammatory signalling in vascular endothelial cells (VECs) is a common feature in the progression of cardiovascular diseases (CVDs) including atherosclerosis, hypertension, congestive heart failure, primary pulmonary hypertension and other inflammatory syndromes. Atherosclerosis arises from chronic localized inflammation at coronary and carotid arterial branch points, and remains the principle cause of death in the developed world despite changes in lifestyle and the widespread use of anti-hypertensive and lipid-lowering drugs (Bruunsgaard *et al.*, 2001; Calabro *et al.*, 2008). Compounding matters are associated problems of drug resistance and side effects, together

with the failure of individuals to change their lifestyle habits to minimize risk. Atherogenesis involves a diet-induced propagation of pro-inflammatory responses leading to the formation of plaques characterized by cholesterol deposition, fibrosis, remodelling and switching of VECs from an anti-coagulant/anti-inflammatory to a pro-thrombotic/pro-inflammatory phenotype. If untreated, these lesions either occlude vessels or trigger their rupture, resulting in the formation of thrombi that cause myocardial infarction or stroke. Despite recent declines, these conditions still account for some 187 000 premature deaths per year in the UK alone (http://www.bhf.org.uk/heart-health/statistics.aspx), while over 17 million are affected worldwide with numbers predicted to increase dramatically over the next 20 years (http://www.who.int/).

Endothelial dysfunction in atherosclerosis is associated with, and is partly driven by, elevated levels of adipocyte-derived pro-inflammatory cytokines such as TNFα, IL-6 and leptin, which propagate a chronic low-grade vascular inflammation triggered by hypercholesterolaemia and hypertension (Bruunsgaard *et al.*, 2001; Calabro *et al.*, 2008). Endothelial dysfunction is also associated with increased endothelial permeability, leading to exaggerated extravasation of blood components and accumulation of fluid in the extravascular space. This results in tissue dysfunction and increased secretion of chemokines that attract leukocytes and monocytes to the inflamed endothelium (Bruunsgaard *et al.*, 2001). As others have shown, strategies designed to 'switch off' the enhanced cytokine signalling that propagates the inflammatory response in VECs can block the development of CVD (Charo and Taub, 2011).

## Cyclic AMP and the inhibition of endothelial inflammation

During the course of the studies examining the molecular basis of cyclic AMP-mediated protective mechanisms during inflammation, we have found a new mechanism by which the intracellular second messenger, cyclic AMP, can suppress inflammatory responses in VECs via activation of the enzyme exchange protein activated by cylic AMP 1 (EPAC1). Cyclic AMP is synthesized in VECs in response to endogenous Gs-coupled receptors for adenosine, prostaglandins and epinephrine, and is known to limit cytokine action and reduce endothelial hyperpermeability induced by multiple pro-inflammatory stimuli (Fukuhara *et al.*, 2005). In addition, our recent work has demonstrated that elevation of intracellular cyclic AMP levels in VECs limits the pro-inflammatory actions of an IL-6 trans-signalling complex, via activation of EPAC1, which leads to the induction of the suppressors of cytokine signalling 3 (*SOCS-3*) gene (Sands *et al.*, 2006). Indeed, accumulation of SOCS-3 protein during inflammation has been shown to occur in VECs and within atherosclerotic plaques *in vivo*, highlighting the importance of this protein in regulating pro-inflammatory cytokine action (White *et al.*, 2011).

EPAC proteins are specific guanine nucleotide exchange factors (GEFs) for the Ras GTPase homologues, Rap1 and Rap2, which they activate independently of the classical protein kinase A (PKA)-dependent route of cyclic AMP signal transduction (Bos, 2006; Borland *et al.*, 2009b). Binding of cyclic AMP to a specific domain in EPAC proteins induces a conformational change that relieves the auto-inhibition of the catalytic GEF domain (Bos, 2006; Borland *et al.*, 2009b). EPAC1 and EPAC2 are expressed in most tissues: EPAC1 shows high expression in blood vessels, kidney, adipose, CNS, ovary and uterus (Kawasaki *et al.*, 1998; de Rooij *et al.*, 1998). EPAC2 shows high expression in CNS, adrenal glands and pancreas (Kawasaki *et al.*, 1998; de Rooij *et al.*, 1998). As such, both EPAC1 and EPAC2 play a role in numerous physiological functions and disease states (Borland *et al.*, 2009b; Grandoch *et al.*, 2009). Although the molecular mechanisms underlying the activation of EPAC1 and EPAC2 are conserved (Rehmann *et al.*, 2007), each isoform has distinct roles in controlling cell function in VECs and pancreatic β-cells, for example. EPAC1 mediates at least three pathways in VECs to down-regulate inflammatory signalling, namely:

- Down-regulation of IL-6-mediated inflammatory processes (Sands *et al.*, 2006) that occurs through CAAT/enhancer-binding protein (C/EBP) transcription factor-dependent SOCS-3 induction (Yarwood *et al.*, 2008),
- Limitation of vascular permeability through EPAC1-mediated activation of integrins involved in the adhesion of VECs to the basement membrane (Netherton *et al.*, 2007),
- Promotion of endothelial barrier function through actin- (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005; Birukova *et al.*, 2007; Baumer *et al.*, 2008) and microtubule-dependent (Sehrawat *et al.*, 2008) cell–cell junction formation through stabilization of VE-cadherin-mediated adhesion (Schmidt *et al.*, 2007).

Overall, the involvement of EPAC1 in multiple anti-inflammatory processes in VECs presents an intriguing model in which to study how distinct cellular processes may interact to present a coordinated programme of 'protection' against inflammatory stimuli. Here we will examine the role of EPAC1 in each of these anti-inflammatory responses in VECs

## EPAC1 and the regulation of the *SOCS-3* gene in VECs

One of the most important inhibitory pathways for limiting sustained elevation of cytokine-induced inflammatory pathways is that involving a family of eight related proteins called cytokine-inducible Src homology 2-containing proteins and SOCS-1–SOCS-7 (Krebs and Hilton, 2001). Of this protein family, only SOCS-1 and SOCS-3 have been intensely studied. Both proteins function in a classical negative feedback loop whereby activation of the signal transducers and activators of transcription (STAT) family of transcription factors promotes the induction of SOCS proteins, which, in turn, bind to and terminate signalling from activated cytokine receptors (Kubo *et al.*, 2003). SOCS-3 exerts its negative feedback through at least three different mechanisms:

- by virtue of an inherent Src-homology 2 (SH2) domain SOCS-3 is able to bind to Janus kinase (JAK)-phosphorylated receptors, thereby inhibiting further JAK activity and, consequently, promoting pro-inflammatory

signalling through the activation of STATs 1 and 3 (Sasaki *et al.*, 1999).
- SOCS-3 competitively inhibits interaction of JAK and STAT proteins to phosphotyrosine residues on activated cytokine receptors.
- SOCS-3 targets SH2-bound proteins for ubiquitination and proteolytic degradation by the proteosome (Sasaki *et al.*, 1999).

Through these mechanisms, the SOCS-3 protein is able to inhibit signal transduction from a wide variety of cell surface receptors, including the IL-6Rα, IFN-γR, colony stimulating factor 1 receptor, IL-12 receptor β2, IL-27Rα, IL-31Rα, G-CSF, and erythropoietin and leptin receptors (Dalpke *et al.*, 2008; Dimitriou *et al.*, 2008). Accordingly, and consistent with its role as a negative regulator of inflammatory signalling, SOCS-3 protein expression is dramatically increased at sites of acute and chronic inflammation *in vivo*, including VECs (White *et al.*, 2011). Moreover, conditional deletion of the *SOCS-3* gene in haematopoietic and endothelial cells of transgenic mice results in death caused by severe inflammatory lesions in the peritoneal and pleural cavities (Croker *et al.*, 2008). Consequently, cell permeable, recombinant forms of SOCS-3 protein have been used *in vivo* to suppress pathogen-induced acute inflammation by attenuating liver apoptosis and limiting haemorrhagic necrosis through inhibition of inflammatory cytokine production (Jo *et al.*, 2005).

The prototypical second messenger cyclic AMP has also been identified as a signal for the induction of the *SOCS-3* gene in a variety of cell types, including macrophages (Qin *et al.*, 2007), human umbilical vein VECs (HUVECs) (Sands *et al.*, 2006), mouse embryonic fibroblasts (MEFs; Yarwood *et al.*, 2008), pituitary and breast cancer cells (Bousquet *et al.*, 2001; Barclay *et al.*, 2007) and COS1 (CV-1 (simian) in origin, and carrying the SV40 genetic material) cells (Borland *et al.*, 2009a). While investigating the role of adenosine and prostaglandin receptors in regulating the signalling of pro-inflammatory cytokines in HUVECs we found that elevation of intracellular cyclic AMP levels limits pro-inflammatory signalling through the induction of the *SOCS-3* gene in response to activation of EPAC1 (Sands *et al.*, 2006). Specific studies on EPAC1 signalling in human vasculature have been carried out in primary endothelial cells subjected to EPAC1-specific siRNA depletion (Sands *et al.*, 2006; Birukova *et al.*, 2007). In this case, *SOCS-3* induction has been shown to inhibit IL-6/soluble IL-6Rα *trans*-signalling complex signalling to STAT3 phosphorylation (Sands *et al.*, 2006). Additional studies have also shown that expression of a constitutively active form of the EPAC effector Rap1, Val12Rap1, is sufficient to mimic the effect of EPAC1 on SOCS-3 protein induction in VECs (Sands *et al.*, 2006). Studies using transgenic mice expressing the human EPAC1 gene under the control of α-cardiac myosin heavy chain promoter have also revealed that EPAC1 protects the heart from the cytokine-induced cardiac dysfunction, at least in part, through the inhibition of the JAK-STAT pathway (Okumura *et al.*, 2007), suggesting the beneficial role played by sympathetic signals to antagonize pro-inflammatory cytokine signalling in heart failure. This mirrors our observations that EPAC1 inhibits JAK-STAT signalling in human umbilical vein and aortic endothelial cells (HUVECs) through the induction of the *SOCS-3* gene (Sands *et al.*, 2006). Stimulation of VECs with the EPAC-specific cyclic AMP analogue, 8CPT-2Me-cyclic AMP, leads to a SOCS-3-dependent blockade of cytokine signalling (Sands *et al.*, 2006) and potentiation of barrier function *in vitro* and *in vivo*, in normal mice (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005; Birukova *et al.*, 2008). Although EPAC1 knockout mice have been generated (Suzuki *et al.*, 2010) their complete phenotype has not been reported and it is therefore unclear as to whether there is a resulting impact on VEC function, although primary cortical neurons prepared from mice lacking EPAC1 seemed to be protected from apoptosis (Suzuki *et al.*, 2010).

The transcriptional regulation of the *SOCS-3* gene has been studied in a number of cell systems in response to various stimuli, including oestrogen (MacDougald and Lane, 1995), lipopolysaccharide (Qin *et al.*, 2007), leukaemia inhibitory factor (Auernhammer *et al.*, 1999; He *et al.*, 2003), pituitary adenylate cyclase activating polypeptide (Bousquet *et al.*, 2001), epinephrine (Bousquet *et al.*, 2001), prostaglandin E2 (Barclay *et al.*, 2007) and IL-6 (Ehlting *et al.*, 2005; Yang *et al.*, 2010). Deletion analysis of the murine gene has been used to isolate the minimal functional SOCS-3 promoter, which contains an activator protein 1 (AP-1) site between nucleotides 105 and 99 (Bousquet *et al.*, 2001; Barclay *et al.*, 2007) that is reported to bind c-Fos and JunB (Bousquet *et al.*, 2001), a GC-rich region (–58 to 52) that has the potential to bind either specificity protein 1 (SP1) (Barclay *et al.*, 2007) or SP3 (Ehlting *et al.*, 2005) transcription factors and two binding sites for STAT transcription factors (Auernhammer *et al.*, 1999; Ehlting *et al.*, 2005), one proximal to the transcription start site (pSTAT; –72 to –64) and one distal (dSTAT; –95 to –87). Activation of the promoter following cytokine stimulation involves the pSTAT site, which appears to be able to bind STATs 1, 3 or 5, depending on cell context (Emanuelli *et al.*, 2000; He *et al.*, 2003; Yang *et al.*, 2010). The function of the dSTAT site remains to be determined. *SOCS-3* responsiveness to IL-6 is also thought to require SP3 transcription factor binding to the GC-rich region, in addition to STAT3 interaction with the pSTAT site (Ehlting *et al.*, 2005; Yang *et al.*, 2010). In contrast, activation of the *SOCS-3* promoter by cyclic AMP appears to be independent of STAT binding, but rather relies on the AP-1 site (Bousquet *et al.*, 2001), or SP-1 interaction with the GC-rich region (Barclay *et al.*, 2007), again depending on the cell type studied.

We have found that *SOCS-3* induction in primary HUVECs requires activation of the microtubule-associated protein (MAP) kinase, ERK, by both PKCη and PKCε, and that the minimal ERK-responsive element of the *SOCS-3* promoter is contained within a region spanning nucleotides –107 to the transcription start site (SJ Yarwood, unpubl. obs.). Within this minimal promoter are conserved binding sites for AP-1 and SP1/SP3 transcription factors, as well as the pSTAT and dSTAT binding elements, all of which can be activated in an ERK-dependent manner by both cyclic AMP and the PKC-activator, PMA. Moreover, representative protein components of each of these transcription factor binding sites, namely c-Jun, STAT3 and SP3, undergo ERK-dependent phosphorylation within their respective transactivation domains (SJ Yarwood, in preparation). Together, these results describe novel, ERK-dependent regulation of transcriptional activity that requires codependent activation of multiple transcrip-

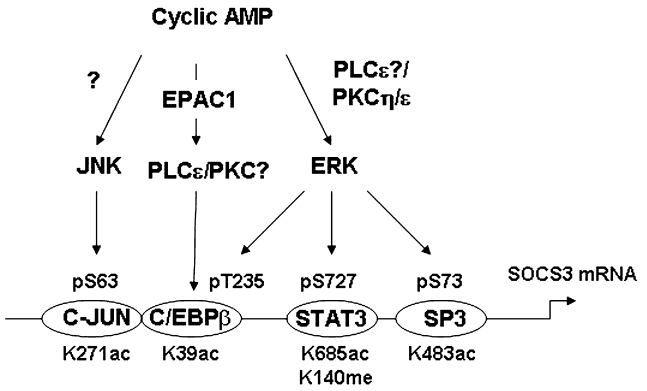



**Figure 1**

Cyclic AMP elevation leads to induction of the SOCS-3 gene in VECs. Cyclic AMP elevation following stimulation of adenosine or prostaglandin receptors in VECs leads to activation of the ERK and JNK MAP kinase pathways independently of the classical PKA route of cyclic AMP signalling. Activation of the ERK cascade leads to the phosphorylation and activation of C/EBPβ, STAT3 and SP3 transcription factors whereas activation of the JNK pathway leads to the phosphorylation and activation of c-Jun (SJ Yarwood, unpubl. obs.). Full activation of C/EBPβ requires activation of the cyclic AMP GEF, EPAC1, and is dependent on phospholipase Cε (PLCε) protein kinase Cα (PKCα) (Yarwood *et al.*, 2008; Borland *et al.*, 2009a). Activation of ERK by cyclic AMP requires PKC isoforms, η and ε. It is currently not known how cyclic AMP elevation leads to the activation of JNK MAP kinase. All the transcription factors indicated are required for full induction of the *SOCS-3* gene by cyclic AMP and are targets for either acetylation (ac), which promotes activation, or methylation (me), which inhibits activation, on the indicated lysine residues (Vries *et al.*, 2001; Ammanamanchi *et al.*, 2003; Yuan *et al.*, 2005; Yang *et al.*, 2010).

tion factors within the same region of the *SOCS-3* gene promoter. Downstream signalling from EPAC1 to the *SOCS-3* gene also appears to involve mobilization of the C/EBP family transcription factors, C/EBPβ and C/EBPδ, which directly interact with one or more C/EBP consensus binding sites within the *SOCS-3* promoter and are necessary and sufficient for the induction of the *SOCS-3* gene (Yarwood *et al.*, 2008). However, efficient induction of the *SOCS-3* gene by cyclic AMP in VECs also appears to require coordinate activation of the ERK MAP kinase cascade (Sands *et al.*, 2006; Woolson *et al.*, 2009) in particular, ERK-dependent phosphorylation of C/EBPβ on threonine 235 appears to be a prerequisite for efficient *SOCS-3* induction (Borland *et al.*, 2009a). In this case, the pathway leading from cyclic AMP to ERK is not known (Woolson *et al.*, 2009); however, it does appear to be independent of both PKA and EPAC1 activation. Studies such as these have now permitted us to put forward a hypothetical model detailing how cyclic AMP regulates the expression of the anti-inflammatory *SOCS-3* gene in VECs (Figure 1).

## EPAC1 and the regulation of cell adhesion in VECs

VECs are vital physical barriers between the circulatory system and smooth muscle cells and organs. To maintain cardiovascular health, a delicate balance of endothelial permeability is required to allow the passage of circulating signals to tissues through the endothelial monolayer, a process that is dynamic and varied in different areas of the vasculature. This semi-selective permeability to liquid, hormones and macromolecules arises as a result of EC-specific responses, inducing rearrangement of various cell structures and of cell shape. These cellular structures are tight junctions (Zona Occludens) (Morita *et al.*, 1999) and adherens junctions (Dejana *et al.*, 1999) that are maintained through transcellular interactions, cytoskeletal attachment and integrin linkages between the cytoskeleton and the extracellular matrix (Lampugnani *et al.*, 1993). Disruption of these structures can lead to disruption of basal membrane barrier function and result in oedema and inflammatory responses. Accordingly, the maintenance of permeability is a tightly regulated process, incorporating cell–cell interactions, cytoskeletal rearrangement and homeostatic regulation.

### *EPAC and barrier function*

The first observations of a cyclic AMP-dependent role in regulating endothelial barrier function correlated a decrease in the activity of thrombin and other inflammatory agents with increased levels of cyclic AMP (Moy *et al.*, 1998; Essler *et al.*, 2000; Patterson *et al.*, 2000). Later work demonstrated that thrombin-induced permeability was inhibited by the EPAC-specific, cyclic AMP analogue 8CPT-2Me-cyclic AMP, implicating EPAC specifically in the maintenance of vascular permeability (Kooistra *et al.*, 2005). PKA and EPAC signals appear to converge to mediate barrier function regulation; however, PKA targets actinomyosin contractility, whereas EPAC1 functions in the regulation of junction structures and adhesion, through the activation of integrins. Although PKA was shown to contribute ~60% of cyclic AMPs inhibitor effects on thrombin-induced permeability in this system, EPAC activation proved to provide a significant component (Kooistra *et al.*, 2005). It was also shown that basal barrier function could be enhanced by EPAC activation in a PKA-independent manner (Cullere *et al.*, 2005), confirmed by siRNA knockdown of EPAC1 in 8CPT-2Me-cyclic AMP-treated cells, resulting in a loss of barrier function (Kooistra *et al.*, 2005). EPAC1 and PKA were also shown to work alongside each other to mediate barrier regulation in HUVECs in response to calcitonin-receptor-like-receptor activation (Aslam *et al.*, 2011), although this cooperativity appears to be produced, again, through separate pathways, with PKA antagonizing thrombin through inactivation of myosin-light-chain phosphatase (Hartel *et al.*, 2007; Aslam *et al.*, 2010) and EPAC1 doing so through adherens junction regulation (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005).

### *EPAC and integrins*

Integrin-mediated adhesion to the basement matrix is another vital cellular function that impacts on barrier function in the VEC monolayer and EPAC1-mediated Rap1 activation is a well-characterized mediator of this phenomenon (Bos *et al.*, 2003). Regulation of adhesion through integrins occurs as a result of signalling that triggers integrin clustering (Bos, 2005), increased affinity for integrin targets and

increased integrin stability through cytoskeletal anchoring (Hughes and Pfaff, 1998). Various pathways mediate Rap1 effects on these integrin functions, including activation of the effector proteins Rap1-GTP-interacting adaptor molecule, Krev interaction trapped protein 1 and PDZ-containing protein afadin, to produce adhesive responses (Boettner and Van Aelst, 2009). In addition to integrin activation, rearrangements to the actin and microtubule cytoskeleton are essential for mediating the actions of EPAC1-activated Rap1 on cell adhesion, suggesting a complex interplay between cytoskeletal elements in VEC permeability. For example, disassembly of the actin cytoskeleton with cytochalasin D increases VEC permeability (Wojciak-Stothard and Ridley, 2002). Interestingly, Kooistra *et al*. (2005) also showed that the actin filament formation observed during EPAC-Rap1 signalling in barrier function occurred independently of VE-cadherin rearrangement, suggesting a role for VE-cadherin regulation downstream of actin rearrangement. This is supported by observations that Rap1-induced formation of actin bundles stabilize VE-cadherin in adherens and tight junctions (Fukuhara *et al*., 2005).

## *EPAC, Rac and Rho*

The mechanisms linking EPAC1 and Rap1 to the regulation of cytoskeleton function appear to involve the Rho family of small GTPases (Wojciak-Stothard and Ridley, 2002). Rho GTPases have been shown to be intricately linked to actin rearrangement (Hall, 1998) and RhoA has been shown to increase actinomyosin contractility, facilitating breakdown of tight and adherens junctions (Wojciak-Stothard and Ridley, 2002). EPAC1-mediated Rap1 activation leads to Rac1 activation and an increase in VE-cadherin binding and barrier functions. This is thought to occur through the inhibition of RhoA and its actions on the actin cytoskeleton. Indeed, chemical inhibitors of the Rho effector, Rho kinase, block the permeabilizing effects of thrombin in VECs (Essler *et al*., 1998; Wojciak-Stothard and Ridley, 2002). EPAC1-mediated Rap1 activation is thought to lead to the sequestration of the Rac-GEFs, Tiam1 and Vav2, to the plasma membrane thereby promoting cortical cytoskeleton rearrangements (Arthur *et al*., 2004). The protein vasodilator-stimulated phosphoprotein (VASP) also appears to play an important role in Rap1 and Rac control, with the ability of Rap1 to promote barrier function being greatly diminished in VASP knockout cells (Schlegel *et al*., 2008). This effect of VASP was shown to be primarily through mediating Rac activation and integrin stabilization (Schlegel and Waschke, 2009). Rap1 is also known to regulate the RhoA-specific GAP, ARAP3 (Krugmann *et al*., 2004), although whether this occurs through a cyclic AMP or Rac1 responsive route remains to be determined especially in light of reports suggesting differential RAC–RHO crosstalk between cell types (Aslam *et al*., 2011). Interestingly, EPAC signalling in endothelial barrier function was completely ablated by inhibition of Rac (Baumer *et al*., 2009) suggesting EPACs' effects are mediated by this small GTPase alone.

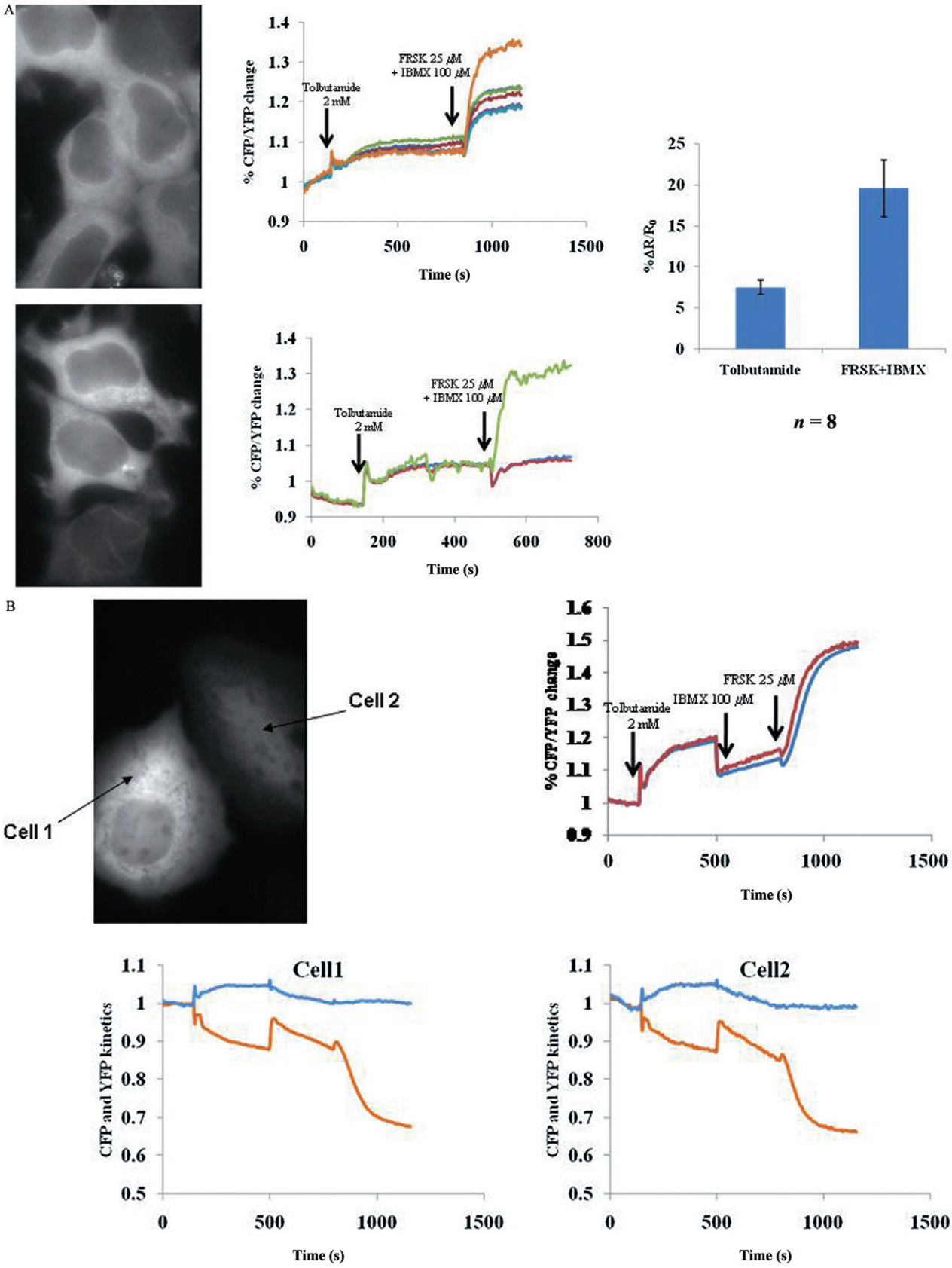
## *EPAC, and ezrin, radixin and moesin (ERM) proteins*

The interaction of all of the key components of endothelial cell barrier function, namely tight junctions, adherens junctions and integrin-mediated adhesion, with the actin and microtubule cytoskeletons highlights the importance of cellular filaments in controlling cell function. Of particular interest, therefore, is the emerging role of the ERM family of actin reorganizing proteins (Fehon *et al*., 2010) and their ability to bind to EPAC1 and effect cell adhesion and spreading (Gloerich *et al*., 2010). The discovery that ERM proteins interact with EPAC1 contributes to a growing list of EPAC-interacting proteins, including muscle-specific A-kinase anchoring protein (Dodge-Kafka *et al*., 2005), MAP light chains (Magiera *et al*., 2004; Yarwood, 2005; Borland *et al*., 2006), RanBP2 (Gloerich *et al*., 2011), Ras GTPase (Liu *et al*., 2008) and Ran GTPase (Liu *et al*., 2010). ERM proteins themselves represent a homologous group of actin-binding proteins, with a characteristic phospholipid- and protein-binding, N-terminal FERM domain, which have been implicated in the control of a wide range of cellular processes, most of which involve changes to cell structure and shape (Fehon *et al*., 2010). Some functional overlap in function has been observed among different ERM proteins but it is unlikely that all of the ERM protein functions are redundant. Firstly, it has been observed that all members display tissue-specific expression; with hepatocytes expressing radixin alone, ezrin is expressed in epithelial cells and endothelial cells express moesin, indicating specialized cell-specific roles (Fehon *et al*., 2010). Secondly, mouse knockouts of ERM proteins have different phenotypes, with ezrin knockout being lethal very early post-partum (Saotome *et al*., 2004), radixin knockouts suffer liver damage (Kikuchi *et al*., 2002), whereas the moesin knockout mouse does not show any obvious abnormalities (Doi *et al*., 1999).

Highlighting their importance as linkers between the actin cytoskeleton and the plasma membrane is their observed

## Figure 2

The sulphonyluea tolbutamide selectively activates EPAC1 in whole cells. The H30 FRET sensor, also called CFP-Epac (δDEP-CD)-YFP, is an EPAC1 based sensor in which the catalytically inactive EPAC1 (T781A/F782A double mutation on the GEF domain) has been sandwiched between the CFP and the YFP. Moreover the DEP domain has been deleted so that the sensor has a cytosolic localization(Ponsioen *et al*., 2004). In (A), HEK293 cells stably expressing H30 were seeded on uncoated cover slips. Cells were challenged with 2 mM tolbutamide, followed by the broad spectrum phosphodiesterase inhibitor, 100 μM IBMX, and the adenylyl cyclase inhibitor, 25 μM Forskolin, to check the saturation of the probe. Experiments were performed in saline solution (PBS with $Ca^{2+}$ and $Mg^{2+}$). Stimuli were diluted in saline solution also. During the time time-lapse cells were exposed for 200 ms every 5 s. Results from eight separate experiments are indicated in the histogram in the *left* hand panel. CHO cells were transiently transfected for 24 h with either the H30 sensor (B) or PKA-GFP (C) sensor [CNBD-CFP plus catalytic-YFP (Zaccolo and Pozzan, 2002)]. Cells were then challenged with 2 mM tolbutamide followed by 100 μM IBMX and 25 μM Forskolin to check the saturation of the probe. Experiments were performed in saline solution (PBS with $Ca^{2+}$ and $Mg^{2+}$), as described by Terrin *et al*. 2006 (Terrin *et al*., 2006), and stimuli diluted in the same solution. During the time time-lapse cells were exposed for 200 ms every 5 s. (D) Summarizes the results obtained from the indicated number (n) experiments carried out in (B) and (C).


A
% CFP/YFP change
Tolbutamide 2 mM
FRSK 25 μM + IBMX 100 μM
Time (s)
%ΔR/R0
Tolbutamide
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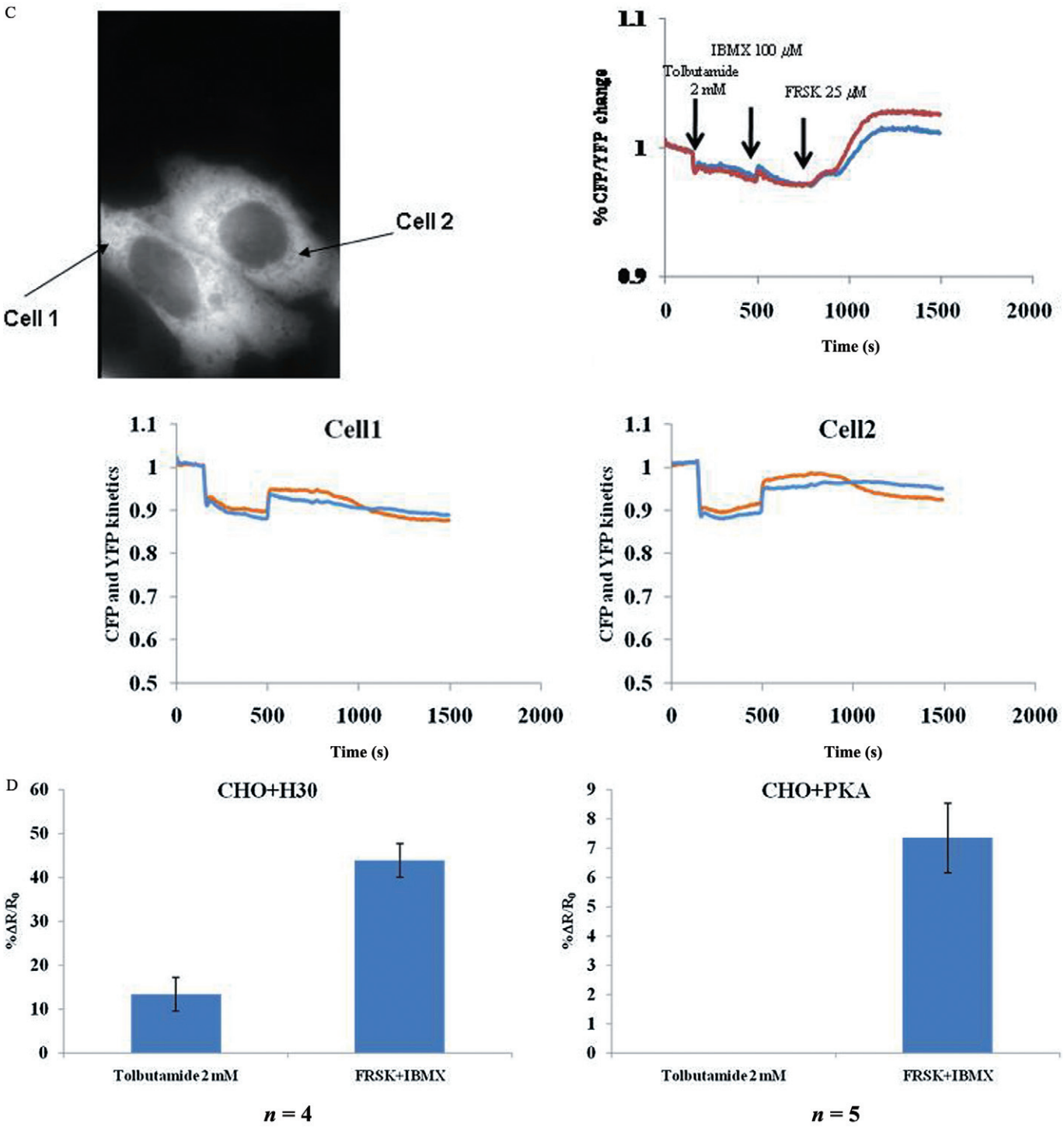



**Figure 2**
*Continued.*

functions, which include maintenance of oocyte polarity, roles in mitosis and metastasis, synapse formation between T-cells and the apical polarity complex (Fehon *et al.*, 2010), and the maintenance of epithelial junction integrity (Pilot *et al.*, 2006). Of particular interest, therefore, is whether or not ERMs, or moesin in particular, has the same junction-stabilizing effects in the vascular endothelium and whether this contributes to barrier function. Consistent with their role in adherens junction maintenance, ERM proteins do appear to be intimately linked with the function of EPAC1. For example, ezrin was isolated in a siRNA library screen of A549 cells treated with 8CPT-2Me-cyclic AMP aimed at identifying components

in the pathway leading from EPAC and Rap1 activation to cell spreading (Ross *et al*., 2011). Knockdown of Rap1A, in these cells ablated cell adhesion and spreading in response to 8CPT-2Me-cyclic AMP. EPAC1 is able to physically interact with all three ERMs, which appears to be a prerequisite for recruitment of EPAC1 to the plasma membrane and subsequent regulation of cell adhesion (Gloerich *et al*., 2010). Cell spreading in a variety of cell types, including VECs, was inhibited only by siRNA knockdown of ezrin and Radil though and neither radixin nor moesin could compensate for the lack of ezrin (Ross *et al*., 2011). However, interaction of EPAC1 with either ezrin, radixin or moesin may play a role in cell adhesion *per se*, because both deletion of the N-terminal portion of EPAC1, that is responsible for interaction with ERMs, or displacement of EPAC1 binding to ERMs through competition with the ABD domain of radixin, resulted in reduced EPAC1-promoted cell adhesion (Gloerich *et al*., 2010).

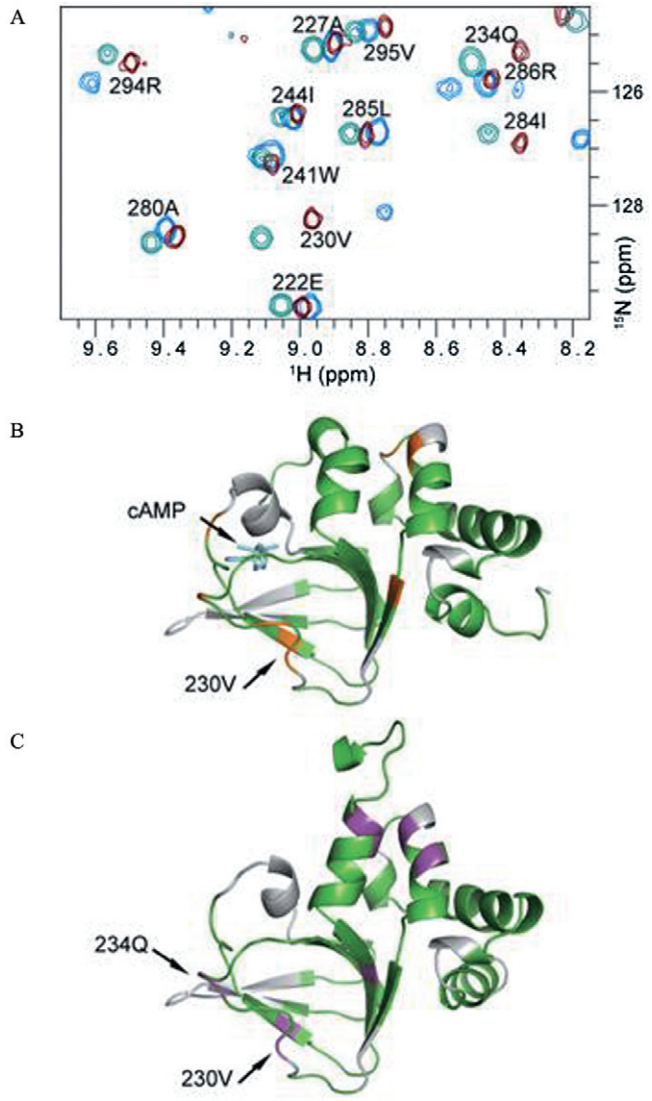
Further work is clearly necessary to fully delineate the importance of interactions between ERM proteins, EPAC1 and the actin cytoskeleton in the control of cell adhesion and barrier functions in VECs. Similarly, the role of the microtubule cytoskeleton in controlling EPAC1-regulated barrier function also requires further investigation. It has been known for some time now that EPAC interacts with tubulin and MAPs (Magiera *et al*., 2004; Gupta and Yarwood, 2005; Mei and Cheng, 2005; Borland *et al*., 2006) and that an intact microtubule network is required for EPAC1-mediated changes in cortical actin and barrier enhancement in VECs (Sehrawat *et al*., 2008). Moreover, recent work has shown that AKAP9, a protein involved in the sequesterization of PKA and EPAC1, is vital in mediating EPAC1 induced microtubule growth and barrier function in VECs (Sehrawat *et al*., 2010). These novel mechanisms demonstrate the importance of protein complex formation in determining functional cross-talk between the actin and microtubule cytoskeleton. The significance of this in controlling barrier function, places EPAC1 at the centre of a complex web of control vital to the maintenance of proper functioning of VECs.

## Is EPAC1 an effective drug target for the treatment of endothelial dysfunction? Lessons learned from EPAC2

The search for small molecules able to interact and modulate cellular EPAC activity for therapeutic intervention has largely been unsuccessful to date. Unlike PKA, no inhibitor of EPAC activity has yet been found, although Brefeldin A is being considered as a lead compound (Bala *et al*., 2011) despite being originally defined as an effective inhibitor of the small GTPase, ARF. Although the development of EPAC-specific cyclic AMP analogues has revolutionized cyclic AMP signalling research (Rehmann *et al*., 2003; Holz *et al*., 2008), these are unlikely to prove suitable as effective drugs as will be discussed here. Despite this, and given the central role that EPAC1 plays in mediating the anti-inflammatory actions of cyclic AMP in VECs, could it be envisaged that targeted activation of EPAC1 by small molecule therapeutics might be an effective treatment for endothelial dysfunction? In favour of this argument is work that suggests that EPAC2 may be a suitable target for therapeutic intervention in the area of type 2 diabetes and insulin-resistance syndromes. This is based principally on new research that demonstrates that EPAC2 mediates glucose- and incretin-enhanced insulin secretion from pancreatic β-cells through the activation of phospholipase Cε and subsequent calcium mobilization (Kashima *et al*., 2001; Holz, 2004; Dzhura *et al*., 2011). Further work has shown that activation of EPAC2, either orthosterically by cyclic AMP or allosterically by anti-diabetic sulphonylurea drugs (Zhang *et al*., 2009), enhances incretin-stimulated insulin secretion leading to lowered blood glucose levels.

The importance of EPAC2 for the regulation of insulin secretion stemmed from early observations that a PKA-independent component comprised roughly half of the effect of cyclic AMP on insulin secretion and that the PKA-independent action of insulin-secretagogues was absent following knockout of the classical sulphonylurea receptor (SUR1; Eliasson *et al*., 2003), a component of the ATP-dependant potassium channel (K-ATP channel) (Aguilar-Bryan *et al*., 1995). Classically, sulphonylurea drugs exert their therapeutic actions through interactions with the SUR1 receptor. By binding to the SUR1 receptor sulphonylureas, like tolbutamide, inhibit the flow of ions through the K-ATP channel, resulting in membrane depolarization, influx of Ca2+ and insulin granule fusion (Seino, 1999; Henquin, 2000). However, ongoing research has suggested that SUR1- and depolarization-independent actions of tolbutamide exist, suggesting that there may be multiple, functional targets for sulphonyureas in pancreatic β-cells (Eliasson *et al*., 1996). In this respect, Zhang *et al*. (2009) identified EPAC2 as a target of sulphonylureas and that Arginine 447 of EPAC2 contributes to their interaction (Herbst *et al*., 2011). Furthermore, binding assays indicated that sulphonylureas bind EPAC2 at a site distinct from the cyclic AMP-binding pocket and with a Kd higher than that of the SUR1 receptor (Zhang *et al*., 2009). This work was also supported by a set of studies carried out in EPAC2 knockout mouse demonstrating that maximum insulin release in response to sulphonylureas could not be achieved in the absence of EPAC2 (Zhang *et al*., 2009).

Despite this compelling evidence, the initial study by Zhang *et al*. (2009) remains controversial. For example, Tsalkova *et al*. (2011) argue strongly against EPAC2 being a direct target for sulphonylureas based on *in vitro* experiments involving isothermal titration calorimetry together with activation and binding assays using full-length, recombinant EPAC2. Despite this, nuclear magnetic resonance data from our laboratory supports a role for a low affinity tolbutamide interaction site within the EPAC1 cyclic nucleotide binding domain (CNBD) (Figure 3). Moreover, using a cell-based EPAC1 FRET sensor, we are able to show that high concentrations of tolbutamide are able to promote EPAC1 activation, or at least a conformational change, in cells, independently of PKA activation (Figure 2). Confusingly, however, experiments from the Zhang laboratory suggest that sulphonylureas selectively target EPAC2 and not EPAC1, although relatively low doses of the sulphonylurea, glibenclamide, were used in these experiments and tolbutamide was not tested (Herbst *et al*., 2011). This suggests that individual sulphonylureas may act through distinct mechanisms, a view




## Figure 3

Tolbutamide interacts with EPAC1 and appears to bind to, or produce conformational changes in, several residues important for cyclic AMP binding. (A) A region of the heteronuclear single quantum coherence spectra of EPAC1 cyclic nucleotide binding (CNB) domain (residues 169–316) alone (green) or in the presence of 1 mM cyclic AMP (blue) or 2 mM tolbutamide (red). Labelled amino acids undergo a change in chemical shift as a result of cyclic AMP and tolbutamide binding, indicative of direct interaction with the ligand or induced conformational change. (B) Homology model of EPAC1 in its open cyclic AMP bound state (based on PBD file 3CF6). The residues that undergo a significant chemical shift change upon cyclic AMP binding are coloured orange. (C) Homology model of EPAC1 in its autoinhibited state (based on PBD file 2BYV). The residues that undergo a significant chemical shift upon tolbutamide binding are coloured magenta. Unassigned residues are coloured grey. The locations of Valine 230 and Glutamine 234 are indicated, along with cyclic AMP bound in the phosphate binding cassette.

supported by the fact that gliaclazide, a sulphonylurea known to interact with SUR-1, was unable to elicit EPAC activation in the original study by Zhang *et al*. (Zhang *et al*., 2009), and is yet still capable of potentiating glucose-induced insulin secretion *in vivo*. In light of this, it may be worthwhile to assess the roles of different sulphonylureas on EPAC activation, because those reported to bind EPAC2 with high affinity may also interact with EPAC1 at mM levels as with tolbutamide (Figure 3).

Clearly, with EPAC1 being the sole EPAC isoform expressed in VECs, it would appear sensible to devise pharmaceuticals for endothelial dysfunction that specifically target this form of the enzyme to prevent side effects. The expression of EPAC2 is restricted to neural and pancreatic cells types whereas EPAC1 has a widespread tissue distribution (Kawasaki *et al*., 1998; de Rooij *et al*., 1998). Thus one would perhaps anticipate that chronic treatment with EPAC1/2 allosteric activators for management of diabetes or endothelial dysfunction may trigger adverse drug reactions. For example, EPAC1 is expressed in cardiac myocytes where it is involved in mediating excitation-contraction coupling and, as such, its activation can trigger ventricular tachycardia in murine heart (Hothi *et al*., 2008). Conversely, the effects of EPAC1 on cardiac hypertrophy are not clear cut: thus, while chronic 8CPT-2Me-cyclic AMP treatment of cardiac myocytes induces alterations in cell morphology and gene expression characteristic of hypertrophy (Morel *et al*., 2005), it can also suppress ERK5-mediated hypertrophy via activation of Rap1 (Dodge-Kafka *et al*., 2005). In addition, unlike PKA activators, 8CPT-2Me-cyclic AMP does not synergize with IL-1 to up-regulate cardiac IL-6 expression, which in turn leads to hypertrophy via JAK-STAT signalling (Gloerich and Bos, 2010). In terms of its effects on the nervous system, EPAC1 and 2 appear to have wide-ranging roles in acute and long-term regulation of neurotransmitter release, augmentation of pain responses, and control of neural differentiation. However, any on-target effects of EPAC activators in the CNS could be minimized by modifying drug design to block penetration of the blood–brain barrier.

On the issue of widespread EPAC expression potentially leading to adverse drug reactions, it is important to note that several drugs currently on the market exert their therapeutic effects by *globally* elevating cyclic AMP levels throughout the body. These include phosphodiesterase inhibitors such as pentoxifylline, ibudilast, drotaverine and (most recently) PDE4-selective inhibitor rofumilast. These drugs are efficacious but at least some of their side effects (typically nausea, emesis, diarrhoea and arrythmia) are due to the fact that the global elevation of cyclic AMP they achieve leads to the activation of both EPACs and PKA. We would argue therefore that selective EPAC1/2 activators will be better tolerated and thus would be particularly appropriate for conditions such as metabolic syndrome, which require long-term drug treatment. Moreover, the strategy of devising allosteric activators of EPAC1 and 2 further decreases the chance of off-target effects on PKA that would likely occur with orthosteric activators that would interact with the cyclic AMP-binding pocket of both EPAC1/2 and PKA.

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## Conflict of interest

The authors have no conflicts of interests associated with this manuscript.

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