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REVIEW

The role of Epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function

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Chronic degenerative inflammatory diseases, such as chronic obstructive pulmonary disease and Alzheimer's dementia, afflict millions of people around the world, causing death and debilitation. Despite the global impact of these diseases, there have been few innovative breakthroughs into their cause, treatment or cure. As with many debilitating disorders, chronic degenerative inflammatory diseases may be associated with defective or dysfunctional responses to second messengers, such as cyclic adenosinemonophosphate (cAMP). The identification of the cAMP-activated guanine nucleotide exchange factors for Ras-like GTPases, Epac1 (also known as cAMP-GEF-I) and Epac2 (also known as cAMP-GEF-II), profoundly altered the prevailing assumptions concerning cAMP signalling, which until then had been solely associated with protein kinase A (PKA). Studies of the molecular mechanisms of Epac-related signalling have demonstrated that these novel cAMP sensors regulate many physiological processes either alone and/or in concert with PKA. These include calcium handling, cardiac and smooth muscle contraction, learning and memory, cell proliferation and differentiation, apoptosis, and inflammation. The diverse signalling properties of cAMP might be explained by spatio-temporal compartmentalization, as well as A-kinase anchoring proteins, which seem to coordinate Epac signalling networks. Future research should focus on the Epac-regulated dynamics of cAMP, and, hopefully, the development of compounds that specifically interfere with the Epac signalling system in order to determine the precise significance of Epac proteins in chronic degenerative inflammatory disorders.
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Keywords: Epac; cAMP; AKAP; lung; neurons; inflammation

Abbreviations: 8-pCPT-2′-*O*-Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate; 8-pCPT-2′-*O*-Me-cAMP-AM, 8-(4-chlorophenylthio)-2′-*O*- methyladenosine-3′,5′-cyclic monophosphate acetoxymethyl; AKAP, A-kinase anchoring protein; ASM, airway smooth muscle; BCR, B cell antigen receptor; COPD, chronic obstructive pulmonary disease; C/EBP, CCAAT/enhancer-binding protein; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; Epac, guanine nucleotide exchange factors for Ras-like small GTPases; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase-3; PDE, phosphodiesterase; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, PKB, PKC, protein kinase A, B and C; PG, prostaglandin; PLC, phospholipase C; SOCS-3, suppressor of cytokine signalling-3; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor-α

Introduction

The most common and versatile second messenger, cyclic adenosinemonophosphate (cAMP), controls a range of diverse physiological processes, including metabolic events, calcium handling, cardiac and smooth muscle contraction, secretion, ion channel conductance, learning and memory, cell growth and differentiation, apoptosis, and inflammation (Beavo and Brunton, 2002). G protein-coupled receptors tightly control the cellular content of cAMP via both adenylyl cyclases and cAMP phosphodiesterases (PDEs), and subcellular localization to lipid rafts and caveolae seems to serve as organizing centres for such signalling (Hanoune and Defer, 2001; Lugnier, 2006; Conti and Beavo, 2007; Patel *et al.*, 2008a,b). Interaction of cAMP effectors with A-kinase anchoring proteins (AKAPs) facilitates subcellular compartmentalization and spatio-temporal cAMP dynamics, and likely reveals the distinct signal transduction events that are driven by cAMP (Wong and Scott, 2004; Gold *et al.*, 2006; Kinderman *et al.*,

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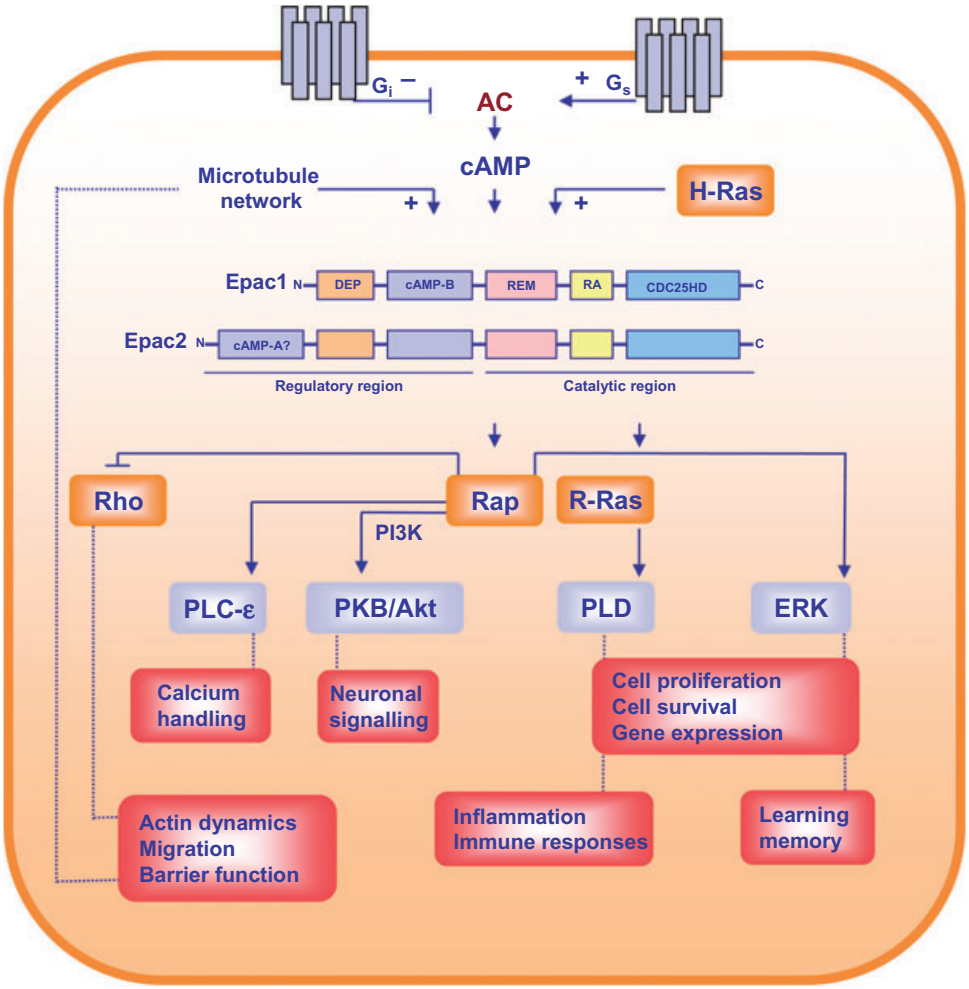


Figure 1 Epac: novel cyclic-adenosinemonophosphate (cAMP) mediators. Epac-driven signalling pathway. cAMP-modulating receptors are indicated in gray, small GTPases are presented in orange; other effectors are depicted in blue, and distinct cellular responses are shown in salmon. Epac2 is recruited by activated H-Ras to the plasma membrane, and Epac1 is regulated by the microtubule network. Inset, schematic representation of Epac1 and Epac1. AC, adenylyl cyclase; CDC25HD, guanine exchange factor domain; DEP, dishevelled, Egl-10, pleckstrin domain; ERK1/2, extracellular signal-regulated kinases 1 and 2; PKB/Akt, protein kinase B/Akt; PLC, phospholipase C; PLD, phospholipase D; RA, Ras-associating domain; REM, Ras-exchanger motif.

2006; Beene and Scott, 2008; Taylor *et al.*, 2008; Baillie, 2009).

The novel cAMP mediators Epac1 (cAMP-GEF-I) and Epac2 (cAMP-GEF-II) are guanine nucleotide exchange factors for Ras-like small GTPases, directly activated by cAMP. The discovery of Epac altered prevailing assumptions concerning cAMP signalling, which had previously been associated with protein kinase A (PKA) (Cohen, 2002; Bos, 2003; Zambon *et al.*, 2005; Bos, 2006; Schmidt *et al.*, 2007a; Roscioni *et al.*, 2008; Taylor *et al.*, 2008). Epac proteins are now assumed to control a range of diverse effectors and to regulate several pivotal processes, including calcium handling and ion transport, cell proliferation and differentiation, cell survival and apoptosis, gene transcription and chromosomal integrity, vesicle trafficking and secretion, and barrier function and neuronal responses. As illustrated (Figures 1–4), cAMP-regulated Epac proteins seem to contribute to DNA repair (Huston *et al.*, 2008), circadian pacemaker functioning (O'Neill *et al.*, 2008), learning and memory (Gekel and Neher, 2008; Gelinas *et al.*, 2008; Murray and Shewan, 2008; Ouyang *et al.*, 2008), axon growth and regeneration (Murray and Shewan, 2008), hypertrophy (Morel *et al.*, 2005; Oestreich *et al.*, 2007; Ulucan *et al.*, 2007; Métrich *et al.*, 2008), fibrosis (Huang *et al.*, 2007, 2008; Haag *et al.*, 2008; Yokoyama *et al.*, 2008c), and to inflammation (Aronoff *et al.*, 2005; Aronoff *et al.* 2006a; Lorenowicz *et al.*, 2006; Sands *et al.*, 2006; Yarwood *et al.* 2008; Scheibner *et al.* 2008; Serezani *et al.* 2008; Borland *et al.* 2009a,b).

It is interesting to note that Epac proteins exert their diverse biological functions either alone and/or in concert with PKA, and that distinct cAMP signalling complexes seem to be coordinated by AKAPs, which are known to interact with PKA and PDEs (Wong and Scott, 2004; Gold *et al.*, 2006; Kinderman *et al.*, 2006; Beene and Scott, 2008; Taylor *et al.*, 2008). In 2005, a cAMP-responsive multiprotein complex, maintained by nuclear envelope-associated mAKAP, PKA, PDE4D3 and Epac1, was first identified in neonatal rat cardiomyocytes (Dodge-Kafka *et al.*, 2005). Recent studies in our laboratory

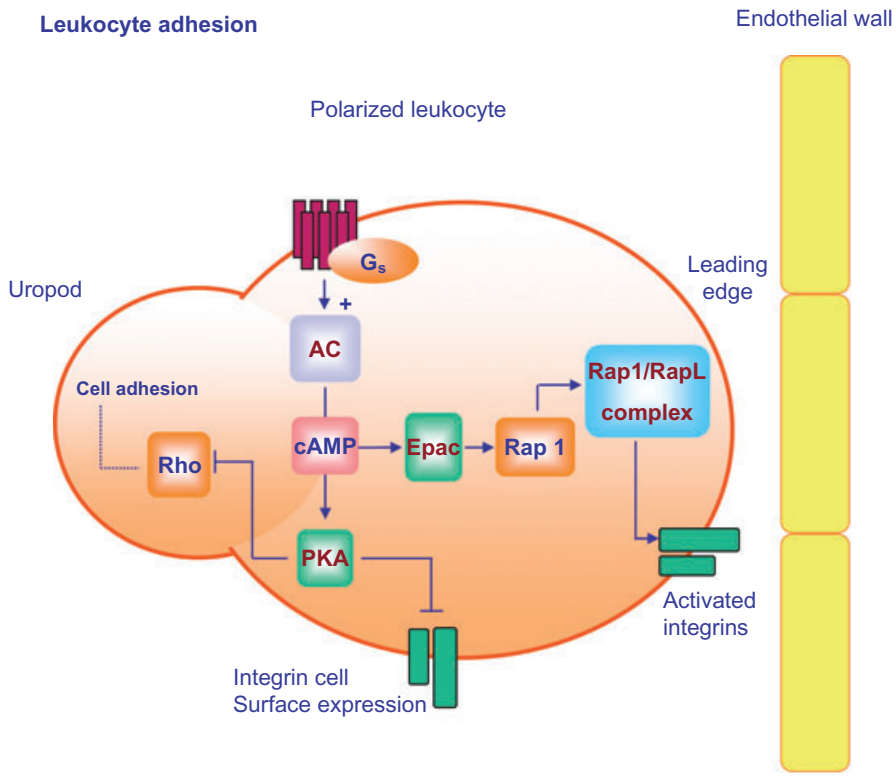


Figure 2 Epac: leukocyte migration and integrin-mediated adhesion. G_s-coupled receptors lead to adenylyl cyclase (AC)-dependent cellular cAMP elevations and subsequent activation of protein kinase A (PKA) and Epac. Whereas PKA inhibits expression of integrin receptors and Rho-dependent cell adhesion, Epac initiates Rap1 signalling to activate integrins via the Rap1 effector RapL and thereby promotes leukocyte polarization, migration and adhesion to endothelial cells.

have reported a novel link between neuronal plasma membrane-associated AKAP79/150, PKA and Epac2 (Nijholt *et al.*, 2008) (Figure 4). Such multiprotein complexes seem to alter the biological effects of cAMP (see later in the text).

Current insights into the molecular mechanisms of the regulation of multiple effectors and biological functions by Epac proteins can be found in several recent reviews on this topic (Bos, 2003; Holz *et al.*, 2006; Holz *et al.*, 2007; Roscioni *et al.*, 2008; Borland *et al.*, 2009b). Signalling of cAMP via cyclic nucleotide-gated channels, CNrasGEF and/or PSD-95/DlgA/ZO-1 (PDZ)-GEF has been outlined in prior reviews (Bos *et al.*, 2007; Biel and Michalakis, 2009; Biel, 2009; Pannekoek *et al.*, 2009; Raaijmakers and Bos, 2009). In this review, we will discuss our current understanding of Epac proteins as novel regulators of cAMP-dependent immune, lung and neuronal function. Some of this article was presented at *EPHAR, Manchester* 2008

Epac: novel cAMP mediators

Expression of Epac1 and Epac2

Epac1 (cAMP-GEF-1) and Epac2 (cAMP-GEF-II) are expressed in both mature and developing tissues. Recent studies have indicated that alterations in the cellular microenvironment are present in chronic degenerative inflammatory diseases and seem to affect the expression profile of Epac1 and Epac2. Initially, Epac1 mRNA expression was reported to be most abundant in the heart and kidney, although subsequently, it was found to be expressed in all human tissues being analysed (de Rooij *et al.*, 1998). Additionally, expression of Epac1 has been found in monocytes, macrophages, B and T lymphocytes, eosinophils, neutrophils, platelets, and in CD34-positive haematopoietic cells (Tiwari *et al.*, 2004; Bryn *et al.*, 2006; Gerlo *et al.*, 2006; Lorenowicz *et al.*, 2006). Recently, developmental changes of Epac1 mRNA expression have been reported in the heart, vasculature, brain, kidneys and lungs (Ulucan *et al.*, 2007; Yokoyama *et al.* 2008a,b; Murray and Shewan, 2008). Epac2 mRNA expression has been found to be prominent in the brain and adrenal glands (Kawasaki *et al.*, 1998), whereas Epac2 was undetectable in all hematopoietic cell types being studied (Tiwari *et al.*, 2004). It has also been reported that Epac2 mRNA expression in the heart, vasculature, brain, kidneys and lungs is subject to developmental changes (Ulucan *et al.*, 2007; Murray and Shewan, 2008; Yokoyama *et al.*, 2008a,b). Due to the recent identification of a novel splice variant of Epac2, designated Epac2B, the previously identified Epac2 has been renamed Epac2A; it has been reported that Epac2A mRNA is expressed in pancreatic islets and cerebral cortex, whereas Epac2B mRNA expression is restricted to adrenal glands (Niimura *et al.*, 2009).

There are distinct expression patterns of Epac1 and Epac2 proteins in rat brain, spinal cord and dorsal root ganglia at embryonic, neonatal and adult stages of development. Interestingly, it has been shown that the expression of Epac1 declines in adulthood, whereas Epac2 expression is dramatically up-regulated in adults. Such developmental regulation of Epac expression seems to promote axon growth and

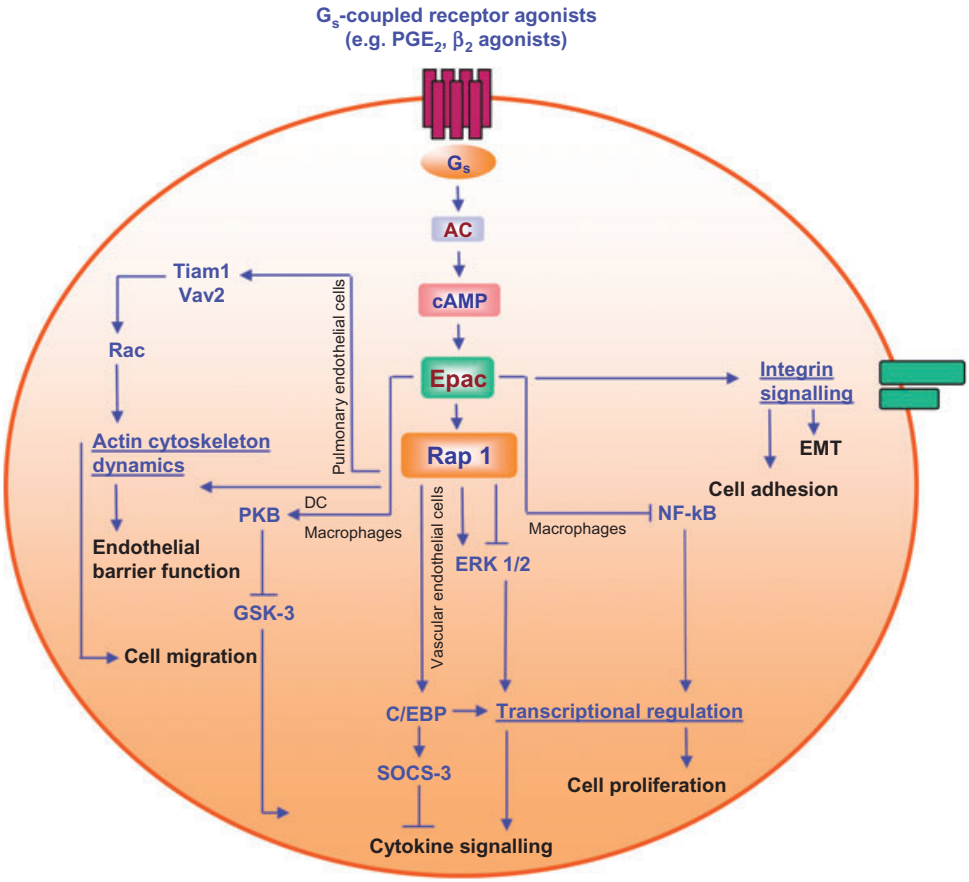


Figure 3 Epac and lung cell signalling. Activation of Epac proteins by G_s-coupled receptors profoundly alters actin dynamics, the microtubule network, gene expression and cytokine responsiveness and thereby modulates diverse processes, including barrier function, cell migration, cell division, cell adhesion and cell proliferation. See text for further details. AC, adenylyl cyclase; C/EBP, CCAAT/enhancer-binding protein; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase-3; PKB protein kinase B; PG, prostaglandin; SOCS-3, suppressor of cytokine signalling-3.

regeneration of the nervous system (Murray and Shewan, 2008). Expression of Epac1 mRNA, but not Epac2 mRNA, was found to be transiently increased in a mouse model of vascular injury, and up-regulation of Epac1 protein expression correlated with the progression of neointimal thickening (Yokoyama *et al.*, 2008a). Although the expression of Epac1 and Epac2 mRNAs was up-regulated in rat ductus arteriosus during the perinatal period, Epac1 activity correlated with the intimal cushion formation (Yokoyama *et al.*, 2008b). Studies on developmental changes of Epac1 and Epac2 mRNA expression have shown alterations of Epac1 and Epac2 in the heart, brain, kidneys and lungs of mice at different stages of development from foetus into adulthood. Interestingly, relative to Epac1, Epac2 became dominant in the adult brain and heart compared with fetal organs, whereas Epac1, relative to Epac2, became dominant in the adult kidney and lung compared with fetal organs (Ulucan *et al.*, 2007), suggesting that Epac1 and Epac2 differentially contribute to fetal and adult organ function. In mycocardial hypertrophy induced by chronic catecholamine infusion, Epac1 and Epac2 mRNA were up-regulated, whereas only Epac1 mRNA was increased in pressure overload-induced hypertrophy (Ulucan *et al.*, 2007). At present, it is not known whether Epac is a cause of the development of cardiac hypertrophy, or if its expression is a consequence of hypertrophy. Transforming growth factor β1 (TGF-β1) has been shown to reduce the expression of Epac1 mRNA in rat cardiac, lung and skin fibroblasts, and a decrease in Epac1 mRNA expression paralleled the increase in expression of TGF-β1 in a rat myocardial infarction model. Such a process may promote the synthesis of extracellular matrix at site of injury (Yokoyama *et al.*, 2008c). The reduction of Epac1 mRNA expression in U937 monocytic cells by TGF-β1 might protect against aberrant transendothelial migration of leukocytes during inflammation (Basoni *et al.*, 2005). Although the underlying molecular mechanisms have yet to be addressed, attenuation of Epac1 expression by (fibrogenic) agonists may represent a mode of adaptation of cAMP responses to pathophysiological alterations present in inflammatory diseases.

Localization of Epac1 and Epac2

Epac1 is localized in the plasma membrane, cytoplasm, perinuclear regions, nuclear membrane and mitochondria in various cell types, including HEK293, N1E-115, CHO, COS1(7), HeLa, PC12, PCCL3, primary hippocampal (cortical) neurons, peritoneal macrophages, rat cholangiocytes, primary rat neonatal ventriculocytes, and different tubular segmental cells of rat and human kidney (Qiao *et al.*, 2002;

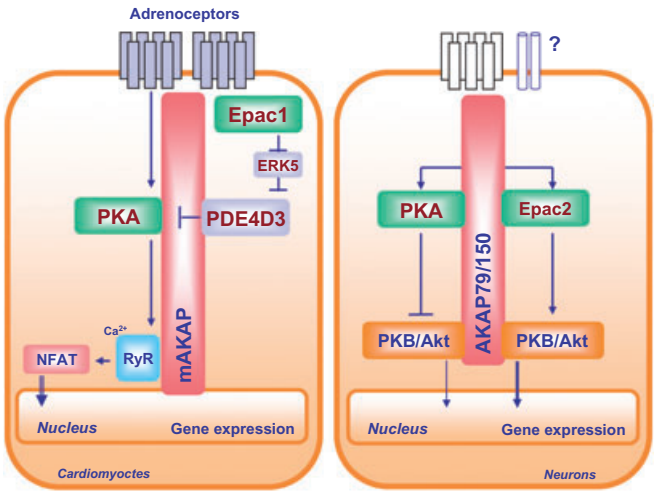


Figure 4 Coordination of protein kinase A (PKA)/Epac-driven cAMP dynamics by AKAPs. In neonatal rat cardiomyocytes, an AMP-responsive multiprotein complex is maintained by nuclear envelope-associated mAKAP, PKA, PDE4D3, ERK5 and Epac1, whereas in neuronal cells, such a functional cAMP-responsive multiprotein complex consists of plasma membrane-associated AKAP79/150, PKA, PKB/Akt and Epac2. Both cAMP organizing centers are most likely to facilitate distinct signal transduction events driven by cAMP. See text for further explanations. AKAP, A-kinase anchoring protein; ERK, extracellular signal-regulated kinase; NFAT, nuclear factor of activated T cells; PDE, phosphodiesterase.

DiPilato *et al.*, 2004; Nikolaev *et al.*, 2004; Ponsioen *et al.*, 2004; Dodge-Kafka *et al.* 2005; Morel *et al.*, 2005; Borland *et al.*, 2006; Hochbaum *et al.*, 2008; Li *et al.*, 2008; Métrich *et al.*, 2008; Di Benedetto *et al.* 2008; Banales *et al.*, 2009). In particular, studies in HEK293 and COS1(7) cells demonstrated that subcellular (re)distribution of Epac1 is subject to cell cycle- and cytoskeleton-dependent dynamics (Qiao *et al.*, 2002; Borland *et al.*, 2006; Huston *et al.*, 2008). Epac2 is localized to the (sub)plasma membrane, cytosolic fractions, actin cytoskeleton, meiotic midzone region and Golgi in several cells, such as MIN6, pancreatic islets, H1299, human microvascular endothelial cells, different tubular segmental cells of rat (human) kidney, rat cholangiocytes, adult rat ventricular myocytes and mouse spermatocytes (Ozaki *et al.*, 2000; Hong *et al.*, 2007; Shibasaki *et al.*, 2007; Leroy *et al.*, 2008; Li *et al.*, 2008; Aivatiadou *et al.*, 2009; Banales *et al.*, 2009; Niimura *et al.*, 2009). Clearly, Epac1 and Epac2 are localized to a number of subcellular regions, and their cell type-specific co-localization at the plasma membrane and cytoplasm indicates that Epacs might act in concert to modulate cellular responses.

Meanwhile, recent studies have reported on spatial and temporal regulation of cellular Epac signalling by using Epac-based fluorescence resonance energy transfer cAMP sensors or green fluorescent protein (GFP)/Flag-tagged Epacs (DiPilato *et al.*, 2004; Nikolaev *et al.*, 2004; Ponsioen *et al.*, 2004, 2009; Di Benedetto *et al.*, 2008; Leroy *et al.*, 2008; Liu *et al.*, 2008; Shafer *et al.*, 2008). The Epac-based cAMP sensor studies were important for the concept that the concentration of cellular cAMP rises to a level sufficient to activate Epac1 and Epac2. Initially, Nikolaev and colleagues demonstrated, by using single-chain cAMP sensors based on the cAMP-binding domains of Epac and PKA, that β-adrenoceptor-induced cAMP signals are rapidly propagated throughout the entire cell body of primary hippocampal neurons and peritoneal macrophages (Nikolaev *et al.*, 2004). Using Epac as an indicator of cAMP (either targeted to plasma membrane, mitchondria or nucleus), DiPilato and co-workers demonstrated differential dynamics of cAMP signaling in response to β-adrenoceptor or prostanoid receptor activation in HEK293, HeLa and in PC12 cells (DiPilato *et al.*, 2004). In contrast, Ponsioen *et al.* showed, by using a CFP-Epac-YFP construct, that Epac activation was not limited to membranes, but rather occurs throughout the cell (A431, HEK293, N1E-115 and MCF-7 cells (Ponsioen *et al.*, 2004). More recently, Ponsioen and colleagues reported, by using GFP-tagged Epac1, that activation of β- adrenoceptors induced a rapid translocation of Epac1 to the plasma membrane in A431, HEK293, OVCAR-3, ACHN, RCC10, MDCK, N1E-115, HeLa, Rat-1, GE11 and H1299 cells (Ponsioen *et al.*, 2009). Similarly, Liu *et al.* demonstrated, by using Flag-tagged Epac2, that Epac2 activation requires compartmentalization of Epac2 to Ras-containing membranes, a process that operates in COS, HEK293 and PC12 cells (Liu *et al.*, 2008). Although the concept of subcellular (re)distribution of Epac as a prerequisite for their activation remains controversial, Epac1 and Epac2 clearly represent novel cAMP sensors that contribute to the highly dynamic features of cAMP signalling.

Because the binding affinity of cAMP for PKA and for Epac has been found to be very similar (k_d ~2.9 μM), it has been proposed that Epac and/or PKA are activated in response to moderate increases of cellular cAMP, and that such activation depends upon cellular compartmentalization of cAMP formation and effector protein availability (Dao *et al.*, 2006). Indeed, studies in primary rat neonatal ventriculocytes have identified distinct intracellular cAMP signalling compartments composed of distinct PKA subtypes, G_s-coupled receptors and cAMP-hydrolyzing PDEs (Di Benedetto *et al.*, 2008). Recently, Leroy and colleagues reported on spatio-temporal dynamics of β-adrenoceptor-induced cAMP signalling in adult rat ventricular myocytes, and speculated that PDE3 regulates a 'constitutive' cAMP pool linked to cardiac contractility, whereas PDE4 regulates the cAMP microdomains driven by β-adrenoceptor stimulation (Leroy *et al.*, 2008). Intriguingly, very recent research suggests that different mechanisms contribute to the plasma membrane targeting of Epac1 and Epac2 (Liu *et al.*, 2008; Niimura *et al.*, 2009; Ponsioen *et al.*, 2009). Thus, an increasing weight of experimental data infers that cellular compartmentalization of cAMP signalling affects the net outcome of biological functions. Spatio-temporal cAMP signalling is believed to involve members of the AKAP family (Wong and Scott, 2004), and indeed cAMP-responsive multiprotein complexes, including Epac1 and Epac2, have been identified in the heart and neurones (Dodge-Kafka *et al.*, 2005; Nijholt *et al.*, 2008) (Figure 4). In neonatal rat cardiomyocytes, Dodge-Kafka and colleagues identified a cAMP-responsive multiprotein complex composed of nuclear envelope-associated mAKAP, PKA, PDE4D3 and Epac1 (Dodge-Kafka *et al.*, 2005). This cardiac-specific multiprotein complex was sensitive to differential cellular cAMP concentrations. Thus, at high cAMP concentrations, cardiac hypertrophy was attenuated upon Epac1-Rap1-dependent

inhibition of extracellular signal-regulated kinase5 (ERK5) and subsequent activation of PDE4D3, whereas at low cAMP concentrations, cardiac hypertrophy was enhanced upon ERK5-mediated inactivation of PDE4D3 and subsequent increased PKA signalling (Dodge-Kafka *et al.*, 2005) (Figure 4). Recent studies in our laboratory reported a novel link between neuronal plasma membrane-associated AKAP79/150, PKA, Epac2 and phosphatidylinositol 3-kinase (PI 3-kinase)-dependent protein kinase B (PKB)/Akt (Nijholt *et al.*, 2008) (Figure 4). Direct activation of PKA or Epac2 complexed to AKAP79/150 exerted opposing effects on neuronal PKB/Akt: direct activation of PKA inhibited PKB/Akt phosphorylation, whereas direct activation of Epac2 enhanced PKB/Akt phosphorylation (Nijholt *et al.*, 2008). At present, it is not known whether distinct PDEs are also tethered to the neuronal AKAP79/150 complex, and the input of distinct receptor-driven cAMP alterations has yet to be determined. Of particular interest, a recent study by Raymond and co-workers demonstrated the presence of distinct PKA- and Epac-based signalling complexes comprised of several PDEs and AKAPs (Raymond *et al.*, 2007). Because co-localization of PKA and Epacs to distinct AKAPs correlates with opposing biological effects in cardiomyocytes and neurones (Figure 4), the capacity of AKAPs to interact with additional signalling components might be crucial (Wong and Scott, 2004; Beene and Scott, 2008). Clearly, further studies are required to analyze AKAP-dependent cellular cAMP compartmentalization and its effect on cAMP-dependent biological functions.

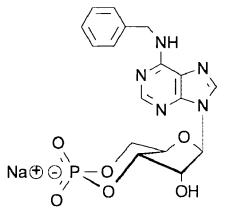
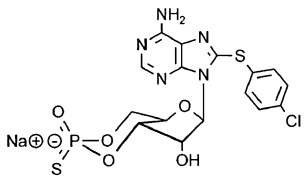
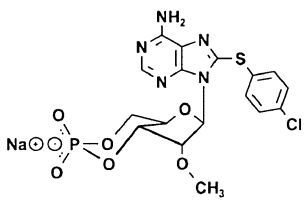
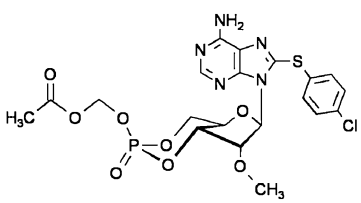
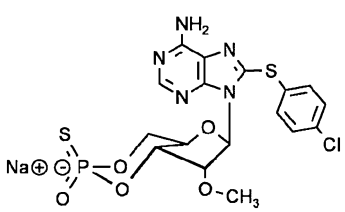
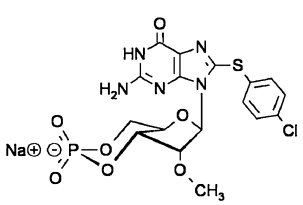
Structure of Epac1 and Epac2

Initial studies indicated that Epac1 and Epac2 consist of an auto-inhibitory N-terminal regulatory region that contains a DEP (dishevelled, Egl-10, pleckstrin) domain responsible for membrane association and a high-affinity cAMP-binding domain (cAMP-B), a C-terminal catalytic region bearing a CDC25 homology domain (CDC25HD) that exhibits GEF activity for Ras-like GTPases, a Ras-exchange motif (REM) domain believed to stabilize the GEF domain, and a Ras-associating (RA) domain present in several Ras-interacting proteins. Indeed, Epac2 has been shown to interact with GTP-bound Ras (Li *et al.*, 2006) (Figure 1). In addition, Liu *et al.* demonstrated that the interaction of Epac2 with Ras via its RA domain is required to redirect Epac2 to Ras-containing membranes and to subsequently induce cAMP-dependent activation of Rap proteins (Liu *et al.*, 2008). The authors proposed that coincident detection of both cAMP and Ras signals is required for Epac2 to activate Rap1 in a temporally and spatially controlled manner (Liu *et al.*, 2008). Similarly, Ponsioen and colleagues recently characterized the molecular mechanisms underlying the direct spatial control of Epac1 by cAMP (Ponsioen *et al.*, 2009). It has been reported that cAMP is required to release Epac1 from auto-inhibitory restraints, and that cAMP induces translocation of Epac1 to the plasma membrane, a process dependent on the DEP domain of Epac1 (Ponsioen *et al.*, 2009). Until very recently, it was believed that the second low-affinity cAMP-A domain of Epac2 exerted an as-yet undetermined biological function. However, Niimura and co-workers identified a novel splice variant of Epac2, designated Epac2B, and the initially identified Epac2 has now been renamed as Epac2A (Niimura *et al.*, 2009). Expression of Epac2A mRNA is restricted to pancreatic islets and cerebral cortex, and it has been shown that Epac2A-driven insulin secretion from pancreatic cells requires the cAMP-A domain, independent of its cAMP-binding capacity, to localize Epac2A near the plasma membrane (Niimura *et al.*, 2009). Clearly, translocation of Epac1, Epac2A and Epac2B to the plasma membrane is driven by rather different mechanisms, and it will be of interest to analyse the impact of AKAP proteins in these processes. Recent X-ray crystallography of Epac2 and NMR spectroscopy of Epac1 have provided novel insights into the dynamic equilibrium of critical conformational switches encompassing a closed, auto-inhibited state in which the N-terminal regulatory region sterically blocks the C-terminal catalytic region to a completely different, open and catalytically active state (Rehmann *et al.*, 2006, 2008; Mazhab-Jafari *et al.*, 2007; Das *et al.*, 2008; Harper *et al.*, 2008). It is to be hoped that development of Epac-specific cAMP analogues will arise as a result of these types of studies.

Methods to validate cAMP signalling via PKA and Epac

Membrane-permeable cyclic nucleotide analogues have been synthesized, and these pharmacological tools seem to differentiate between PKA and Epac signalling (Table 1). For example, N^6-benzyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP), 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp - 8 - CPT - cAMPS), 8 - (4 - chlorophenylthio) - 2′ - *O* - methyl - cAMP (8 - pCPT - 2′ - *O* - Me - cAMP), acetoxymethyl 8 - pCPT - 2′ - *O*-Me-cAMP (8 - pCPT - 2′ - *O*-Me-cAMP-AM), 8 - (4 - chlorophenylthio)-2′-*O* - methyladenosine - 3′,5′ - cyclic monophosphorothioate, Sp - isomer (Sp - 8 - pCPT - 2′ - *O* - Me - cAMPS) and 8 - (4 - chlorophenylthio) - 2′ - *O* - methyl - cGMP (8-pCPT-2′-*O*-Me-cGMP) are now used to activate and/or inhibit PKA and Epac, and 8-pCPT-2′-*O*-Me-cGMP is used as a negative control for 8-pCPT-2″-*O*-Me-cAMP (Enserink *et al.*, 2002; Christensen *et al.* 2003; Holz *et al.* 2006, 2007; Vliem *et al.* 2008; Haag *et al.* 2008) (Table 1). A 2′-*O*-methyl substitution on the ribose ring of cAMP of Epac-selective cAMP analogues confers specificity towards Epac, and such compounds are therefore used as pharmacological tools to analyse Epac-driven cAMP dynamics independent of PKA. However, currently available Epac activators do not differentiate between Epac1 and Epac2 (Table 1). As Epac1 and Epac2 are insensitive to inhibitors of PKA, such as Rp-8-CPT-cAMPS (Holz *et al.*, 2007) (Table 1), PKA inhibitors are used to demonstrate that Epac-specific analogues act via Epac independently of PKA. Studies in *Trypanosoma brucei* indicated, however, that 8-pCPT-2′-*O*-Me-cAMP might act via its 5′-AMP derivative upon inhibition of PDEs (Laxman *et al.*, 2006). In addition, recent studies in human platelets showed that various cyclic nucleotide analogues, including 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP might, in addition to their primary effects, also cause elevation of cAMP or cGMP upon inhibition of phosphodiesterases (Poppe *et al.*, 2008). Altogether, these studies suggest caution when interpreting data obtained with cyclic nucleotide analogues of dubious selectivity, especially when such agents are used to dissect PKA-dependent and -independent *versus* Epac-dependent and -independent signalling properties. At

Table 1 Cyclic nucleotide compounds

Compound	*Characteristics*	*References*
	N^6-benzyladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP), selective and membrane-permeable protein kinase A (PKA) activator	Christensen, Selheim *et al.*, 2003 Poppe *et al.*, 2008
	8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-CPT-cAMPS), competetive inhibitor of PKA	Christensen, Selheim *et al.*, 2003 Poppe *et al.*, 2008
	8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP (8-pCPT-2′-*O*-Me-cAMP), selective and membrane-permeable activator of Epac1 and Epac2	Enserink *et al.*, 2002 Christensen, Selheim *et al.*, 2003 Poppe *et al.*, 2008
	Acetoxymethyl 8-pCPT-2′-*O*-Me-cAMP (8-pCPT-2′-*O*-Me-cAMP-AM), see above	Vliem *et al.*, 2008
	8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-pCPT-2′-*O*-Me-cAMPS), selective and membrane-permeable Epac activator, insensitive to phosphodiesterases	Laxman *et al.*, 2006 Poppe *et al.*, 2008
	8-(4-chlorophenylthio)-2′-*O*-methyl-cGMP (8-pCPT-2′-*O*-Me-cGMP), negative control for 8-pCPT-2′-*O*-Me-cAMP	Haag *et al.*, 2008

present, highly specific pharmacological inhibitors of individual Epac isoforms, Epac1 and Epac2, are not available (Holz *et al.*, 2007; Poppe *et al.* 2008). However, the successful suppression of the endogenous expression of Epac1 and Epac2 by specific siRNAs has been reported (López de Jesús *et al.*, 2006; Haag *et al.* 2008, Huang *et al.* 2008; Yokoyama *et al.* 2008a,c). Recently, Seino *et al.* developed Epac2-deficient mice and demonstrated that Epac2/Rap1 signalling is essential in the regulation of insulin granule dynamics (Shibasaki *et al.*, 2007). Generation of additional Epac knockout mice and/or Epac reporter mice might help to gain further insights into Epac-related signalling.

Epac effectors and biological functions

Epac1 and Epac2 were initially characterized as cAMP-activated GEFs for Rap1 and Rap2 (de Rooij *et al.*, 1998; Kawasaki *et al.* 1998). Recent studies have indicated that Epac proteins also function as a molecular link between different Ras family members (Maillet *et al.*, 2003; Krugmann *et al.*,

2004; Morel *et al*., 2005; López de Jesús *et al*., 2006; Li *et al*. 2006; Métrich *et al*., 2008). Furthermore, our studies demonstrated that Epac1 binds to and activates R-Ras, a process that is likely to facilitate cytoskeleton dynamics and calcium handling driven by Epac (López de Jesús *et al*., 2006). Over the last 10 years, considerable progress has been made into the Epac-related cAMP dynamics associated with learning and memory (Gekel and Neher, 2008; Gelinas *et al*., 2008; Murray and Shewan, 2008; Ouyang *et al*., 2008), inflammation, fibrosis, and hypertrophy (Holz *et al*., 2006, 2007; Schmidt *et al*., 2007a; Roscioni *et al*., 2008; Schaafsma *et al*., 2008; Borland *et al*., 2009b). Epac1 and Epac2 seem to control these distinct cellular responses by signalling to a range of effectors. The number and diversity of these effectors is considerable. For example, phospholipase C-ε (Schmidt *et al*., 2001; Oestreich *et al*., 2007), phospholipase D (López de Jesús *et al*., 2006; Han *et al*., 2007) and ERK1/2 (Lin *et al*., 2003; Keiper *et al*., 2004; Kiermayer *et al*., 2005; Wang *et al*., 2006) have been implicated in the regulation of hypertrophic responses. ERKs (Ster *et al*., 2007, 2009; Gelinas *et al*., 2008; Ma *et al*., 2009) and PI 3-kinase-dependent PKB/Akt (Mei *et al*., 2002; Misra and Pizzo, 2005; Yano *et al*. 2007; Misra *et al*. 2008; Kwak *et al*., 2008; Nijholt *et al*. 2008) seem to modulate learning and memory. TGF-β1 receptor-regulated Smads are important for fibrogenic and inflammatory responses (Basoni *et al*., 2005; Conrotto *et al*., 2006; Yokoyama *et al*., 2008c). Finally, NF-κB (Fuld *et al*., 2005; Scheibner *et al*., 2008), the suppressor of cytokine signalling-3 (SOCS-3) (Sands *et al*., 2006; Yarwood *et al*., 2008; Borland *et al*., 2009a), the CCAAT/enhancer-binding protein C/EBP and glycogen synthase kinase-3 (GSK-3) (Jing *et al*., 2004; Xu *et al*., 2008) (Figure 1 and Figure 3), have been reported to regulate inflammation. Further details on the molecular mechanisms of the regulation of Epac-related effectors and their biological functions are outlined in recent excellent reviews on these topics (Cohen and Frame, 2001; Scheid and Woodgett, 2001; Kolch, 2005; Bunney and Katan, 2006; Oude Weernink *et al*., 2007; Perkins, 2007; Schmierer and Hill, 2007; Yoshimura *et al*., 2007; Borland *et al*., 2009b). Despite the novel insights into the molecular mechanisms linking Epac to a diversity of downstream effectors, it still remains to be determined whether co-localization of Epac to PKA-AKAP-based signalling complexes are of central importance to the net outcome of cAMP signalling.

Epac and immune cells

The immune system consists of diverse cells derived from pluripotent haematopoietic stem cells. The myeloid cell lineages leukocytes and monocytes/macrophages are believed to mediate adaptive and innate immunity, whereas lymphocytes are believed to be responsible for adaptive immunity, and together they represent the key effector cells of host defense mechanisms (Vivier and Malissen, 2005; Medzhitov, 2007). cAMP drives several signalling cascades in immune cells, and thereby also plays a pivotal role in multiple immune cell responses, including growth and differentiation, growth arrest and apoptosis, and the production of chemokines and cytokines (Kammer, 1988). Until recently, modulation of immune response by cAMP had been assigned to PKA and PKA-mediated changes in protein expression and function (Cohen, 2002; Zambon *et al*., 2005). However, studies have indicated that Epac1 and Epac2 seem to act as novel cAMP mediators in the regulation of innate and adaptive immune cell functions (Serezani *et al*., 2008).

Epac in monocytes and macrophages

Monocytes and macrophages are pivotal in host defense; after the entry of pathogens into the organism, they eliminate bacteria, viruses and parasites via phagocytosis. Significantly, most signalling pathways in monocytes have been described as cAMP dependent. In particular, cAMP has been reported to inhibit phagocytosis (Rossi *et al*., 1998; Aronoff *et al*., 2004) and the production of tumor necrosis factor-α (TNF-α) (Rowe *et al*., 1997). Traditionally, most inhibitory effects of cAMP were assigned to PKA; however, several earlier studies focused on mechanisms independent of PKA. In human monocytes, cAMP inhibits TNF-α secretion, phagocytosis and respiratory burst activity. Such actions of cAMP have been shown to be mediated via the classical PKA pathway (Bryn *et al*., 2006). Differentiation of monocytes to macrophages, however, was paralleled by increased expression of Epac1 and a contribution of Epac1 to macrophage responses (Bryn *et al*., 2006). Thus, the inhibitory effects of cAMP on macrophage activation and Fcγ receptor-mediated phagocytosis of pathogens have been reported to involve both PKA and Epac1 (Bryn *et al*., 2006). In contrast, cAMP-mediated inhibition of FcR-mediated phagocytosis in alveolar macrophages was independent of PKA via selective Epac1 activation, while synthesis of the inflammatory mediators leukotriene B_4 and TNF-α in alveolar macrophages was inhibited by cAMP in a PKA-dependent manner independent of Epac (Aronoff *et al*., 2005). However, chemokine and cytokine production in bone marrow-derived dendritic cells involved both Epac1 and PKA (Aronoff *et al*., 2006a). In addition, Xu *et al*. recently showed in the murine macrophage-like cell line J774A.1 that the cAMP-induced suppressive effects on interferon-β production were mediated via Epac (Xu *et al*., 2008). Recent studies in our laboratory have indicated that activation of Epac (most likely Epac1) inhibited apoptosis in human leukocytic cells (Grandoch *et al*., 2009a). As illustrated in Figures 1–3, both cAMP mediators (either alone and/or in concert) are important in the regulation of distinct immune responses and are likely to depend on the differentiation status of the cells. Regarding the specific functions of Epac in the immune system, Epac1 regulates the differentiation of monocytes to macrophages and subsequently controls cellular phagocytosis, as well as the production of inflammatory mediators, and apoptosis in human leukocytes. Future research will help to determine the molecular mechanism by which Epac proteins regulate the specific subset of immune functions.

Epac in integrin-mediated adhesion

Constant motion and recirculation of immune cells is pivotal for a proper function of the defense immune system. Immune cell trafficking is a highly regulated process that ensures appropriate cell migration and activation (Cahalan and

Gutman, 2006). As illustrated in Figure 2, during inflammation, there is an excessive extravasation of leukocytes from the blood into the surrounding tissue, a process that requires the production of chemokines to induce leukocyte migration, integrin-mediated adhesion of circulating leukocytes, and finally transendothelial migration (Springer, 1994; Lorenowicz *et al.*, 2007a, 2008). Again, cAMP plays a key role in these processes. However, the effect of cAMP on cell adhesion and chemotaxis of monocytes remains controversial (Zeidler *et al.* 2000; Fine *et al.*, 2001; Lorenowicz *et al.* 2006; Pannekoek *et al.* 2009). Zeidler *et al.* (2000) reported that an elevation of cellular cAMP levels by prostaglandin E_2 (PGE_2) inhibited monocyte adhesion to endothelial cells. In contrast, Lorenowicz *et al.* (2006) reported on the opposing effects of cAMP on the same cellular response. The molecular mechanisms behind the cAMP effects on monocyte adhesion to endothelial cells have been the focus of recent studies. The Epac-Rap1 signalling pathway has been shown to be involved in integrin-mediated cell adhesion in non-myeloid cell lines (Rangarajan *et al.*, 2003; Enserink *et al.*, 2004). Moreover, in sickle (red blood) cells, the Epac-dependent Rap1 activation promotes cell adhesion to the extracellular matrix protein laminin (Murphy *et al.*, 2005). Recent studies in monocytes reported that activation of Epac1 enhances monocyte adhesion to endothelial cells via fibronectin and promotes chemotaxis in the human promonocytic cell line U937 and primary monocytes (Lorenowicz *et al.*, 2006). As illustrated in Figure 2, Epac1 induced polarization of leukocytes (including human monocytic U937 cells), which is a crucial process for the directed migration of the cells by chemokines (Lorenowicz *et al.*, 2006, 2007a). Interestingly, Basoni *et al.* postulated that reduction of Epac1 mRNA expression by TGF-β1 and subsequent reduction of Rap1-driven leukocyte migration acts as an anti-inflammatory signal (Basoni *et al.*, 2005). In contrast, Wittchen *et al.* reported that the Epac effector Rap1 inhibits endothelial transmigration of leukocytes upon enhancement of endothelial barrier functioning (Wittchen *et al.*, 2005). In neutrophils, adhesion of the cells to endothelial surface has been shown to be inhibited by increased cAMP levels via inhibition of stimulus-induced L-selectin shedding and up-regulation of αMβ2 integrin (Berends *et al.*, 1997; Derian *et al.* (1995). These effects are mediated via PKA (Derian *et al.*, 1995), while the Epac-Rap pathway seems to promote leukocyte adhesion through activation of β1 integrins (Lorenowicz *et al.*, 2007a) (see Figure 2). These results further underline the complexity of cAMP signalling and the various outcomes of elevated levels of cAMP, which are most likely to be immune cell-dependent and may be predicted by the relative contributions of PKA and Epac. Certainly, Epac1 modulates integrin function (most probably by barrier function) and leukocyte transmigration. However, the net outcome of leukocyte transmigration by Epacs remains controversial, and the molecular mechanisms have yet to be determined.

Epac in B lymphocytes

Mature lymphocytes with their antigen-specific receptors are in permanent circulation from the blood stream to the lymphoid organs, and represent the main mediators of the adaptive immune response (Vivier and Malissen, 2005; Medzhitov, 2007). In B cells, signalling by the antigen-specific B cell antigen receptor (BCR) is a central step to maintain homeostasis and proper immune function, with cAMP functioning as an important physiological mediator (Kammer, 1988). Indeed, formation of cAMP has been detected in B cells after stimulation of the BCR, and has been implicated as a negative regulator of B cells (Wiener and Scarpa, 1989; Newell *et al.*, 1993). By serving as a mediator of BCR-induced growth arrest and apoptosis, cAMP thereby adapts pro- and anti-apoptotic cell responses, and it is generally believed that these positive and negative regulatory signals are crucially important for a balanced immune response and immune homeostasis (Goodnow, 1996; Monroe, 2000; Niiro and Clark, 2002). Historically, the inhibitory cAMP effects on lymphocytes were primarily assigned to the classical PKA pathway (Cohen, 2002; Zambon *et al.*, 2005), but recently, PKA-independent effectors have also been assumed to play a role. Indeed, expression of Epac1 (but not Epac2) has been reported in B cell chronic lymphotic leukaemia (Klein *et al.*, 2001; Tiwari *et al.*, 2004) (Table 2). It has been proposed that Epac acts as a mediator, and new cAMP target in BCR-induced growth arrest and apoptosis, although the underlying molecular mechanisms have not been addressed (Klein *et al.*, 2001; Tiwari *et al.*, 2004). Recently, we reported that the murine B lymphoma cell line WEHI-231 expresses both Epac1 and Epac2, and that Epac proteins signal to ERK1/2 and Akt upon activation of Rap1 and H-Ras, thus modulating BCR-induced growth arrest and apoptosis (Grandoch *et al.*, 2009b). The relative contribution of PKA and Epac to BCR-induced growth arrest and apoptosis remains controversial; however, recent research suggests a role of Epac.

Epac in T lymphocytes

In T lymphocytes, cAMP has been shown to inhibit cell proliferation and to reduce effector functions (Kammer, 1988; van Oirschot *et al.*, 2001). Initially, the inhibitory effects of cAMP had been assigned to PKA (van Oirschot *et al.*, 2001); however, upon identification of Epac1 and Epac2, the underlying molecular mechanisms have been re-evaluated (Table 2). Modulation of immune responses and inhibition of cellular growth by cAMP in human T lymphocytes has been reported to be PKA independent (Bryce *et al.*, 1999; Staples *et al.*, 2001). In addition, cAMP induces the expression of the immunomodulatory pituitary hormone prolactin in Jurkat T cells, as well as in human T lymphocytes. In Jurkat T cells, prolactin expression is mediated only by PKA, however, in human T lymphocytes, PKA-dependent and -independent mechanisms were observed, indicating a possible role of Epac (Gerlo *et al.*, 2006). Moreover, in human leukemic Jurkat T cells, regulation of cell cycle and cAMP-dependent growth arrest has been shown to be mediated by PKA, thereby reducing T cell production and activation (Fuld *et al.*, 2005). Intriguingly, Epac seems both to regulate and to suppress expression of a distinct subset of functional genes in the Jurkat T cells, including the NF-κB-related p53 phosphoprotein (Fuld *et al.*, 2005). In addition, the Epac effector Rap1A positively regulates T cell signalling, and these effects were reported to be mediated via the activation of integrins (Sebzda *et al.*, 2002; Bivona *et al.*, 2004; Dustin *et al.*, 2004). Indeed,

Table 2 Epac and immune cell responses

Cell system	*Cellular responses*	*References*
Sickle red blood cells	Adhesion to laminin via Epac-induced Rap1 activation	Murphy *et al.*, 2005
Monocyte-derived macrophages	FcR-mediated phagocytosis mediated via both protein kinase A (PKA) and Epac1-Rap1	Bryn *et al.*, 2006
Human primary monocytes	Adhesion to endothelial cells via Epac1 activation Up-regulation of monocyte migration toward the chemokine CCL2 (MCP-1) via Epac1 activation	Lorenowicz *et al.*, 2006
U937 (human monocytic cell line)	Epac activation promotes cell polarization Epac1 activation promotes adhesion to fibronectin by activation of β1-integrins Epac1 activation up-regulates monocyte migration toward the chemokine CCL2 (MCP-1)	
Human and rat (NR8383) alveolar macrophages	Activation of Epac1 suppresses FcR-mediated phagocytosis Stimulation of PKA or Epac1 inhibits bactericidal activity and H_2O_2 production	Aronoff *et al.*, 2005
J774A.1 (murine macrophages)	Suppression of endotoxin-induced interferon-β production via cAMP-induced Epac activation	Xu *et al.*, 2008
Human T lymphocytes	PKA-dependent and -independent prolactin gene expression	Gerlo *et al.*, 2006
Jurkat T cells (human leukaemic T cell line)	Down-regulation of c-Jun activity via Epac activation	Fuld *et al.*, 2005
WEHI231 (murine B lymphoma cell line)	B cell antigen receptor-induced cAMP-dependent growth arrest and apoptosis involve Epac	Grandoch *et al.*, 2009b
Human leukocytic cells (U937, HL-60, primary human mononuclear cells)	Epac activation inhibits apoptosis	Grandoch *et al.*, 2009a

CCL2, CCL identical to MCP-1; MCP-1, monocyte chemotactic protein-1.

PKA and the Epac-Rap pathway also seem to be important for the regulation of lymphocyte adhesion. While PKA-dependent signals promote T cell adhesion by different mechanisms, such as disassembly of the cytoskeleton (Rovere *et al.*, 1996), Rap1 activation has been shown to be required for cell adhesion via αLβ2 and αLβ4 integrins in Jurkat T cells (de Bruyn *et al.*, 2002). Again, it is reasonable to conclude that Epacs regulate integrin functioning, cell growth and gene expression in T lymphocytes. However, the precise regulation by Epacs of the molecular mechanisms in T lymphocyte functioning is still unclear.

Epac in immune cell responses

cAMP is a key mediator in the diverse cells of the immune system and acts as a pivotal modulator of different cell functions, such as migration, cell adhesion, gene transcription, cell proliferation and growth arrest (Table 2). For a long time, the diverse cAMP effects had been attributed solely to PKA; however, the identification of Epac proteins as novel cAMP mediators initiated studies that focused in more detail on the molecular mechanisms behind these diverse cAMP effects in order to discriminate between cellular responses driven by PKA and/or Epac. It is now known that distinct cellular responses require Epac, while others primarily require PKA. Thus, PKA and Epac seem to balance the diverse outcomes of cAMP effects in different cell systems in parallel pathways, and the net outcome depends on the respective distribution of both within the cell.

Epac and the lung

Chronic inflammatory lung diseases

Lung function is ensured by the cooperative action of diverse cells, and lung dysfunction may lead to lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Jeffery, 1998; Holgate, 2008), both of which are characterized by episodes of airway obstruction and structural changes in the bronchial wall, including increase in smooth muscle mass, epithelial cell damage and accumulation of the extracellular matrix (ECM) (Holgate, 2002). Unlike asthma, COPD is characterized by airway narrowing and progressive and irreversible decline in lung function (Holgate, 2002). Overall, these structural changes are known as airway remodelling, and they represent the hallmarks of both asthma and COPD (Jeffery, 2001). Structural alterations eventually impair lung function and sensitize the lung to react to external stimuli in ways that lead to cellular damage and inflammation (Bousquet *et al.*, 2000; Cockcroft and Davis, 2006). Indeed, asthma and COPD are characterized by chronic inflammation and the infiltration into the airways of a variety of activated immune cells, including neutrophils, eosinophils and lymphocytes (Azzawi *et al.*, 1990), which in turn produce inflammatory mediators and amplify the inflammatory response. Airway smooth muscle (ASM) cells are important because of their intrinsic capacity to migrate, to contract, to proliferate and to produce ECM components such as cytokines, growth factors and chemokines (Hirst 2000; Halayko and Amrani, 2003; Panettieri, 2003). Such observations have prompted researchers to assume that ASM cells play an important role in airway remodelling (Halayko *et al.*, 1996; Hirst, 2003). Pulmonary fibroblasts produce the main ECM component collagen I and enzymes involved in collagen I degradation, and therefore play a role in the homeostasis of ECM (Racke *et al.*, 2008). Although fibroblast recruitment, proliferation and collagen synthesis are important to regulate normal wound healing, excessive fibroblast activation can lead to pulmonary fibrosis, a common characteristic of many respiratory disorders (Huang *et al.*, 2007). In addition to ASM and fibroblasts, epithelial cells are also of importance for normal lung function. Epithelial cells form cell layers characterized by gap, adherens

Table 3 Epac and lung cell responses

Cell system	*Effect of Epac*	*References*
Airway smooth muscle cells	Inhibition of cell proliferation	Kassel *et al.*, 2008
Pulmonary fibroblasts	Inhibition of cell proliferation	Haag *et al.*, 2008;Huang *et al.*, 2008
Leukocytes	Adhesion and chemotaxis	Lorenowicz *et al.*, 2006
Human pulmonary artery endothelial cells	Endothelial barrier function	Birukova *et al.*, 2007; Birukova *et al.*, 2008
Mouse alveolar macrophage-like line	Modulation of hyaluronan-dependent inflammatory gene expression	Scheibner *et al.*, 2008
Vascular endothelial cells	Regulation of IL-6 signalling	Sands *et al.*, 2006
Alveolar macrophages	Suppression of macrophage phagocytosis	Aronoff *et al.*, 2005
Bone marrow-derived mouse dendritic cells	Suppression of LPS-induced MIP-1α /1β production	Jing *et al.*, 2004
Mouse macrophage line	Suppression of LPS-induced IFN-β production	Xu *et al.*, 2008
Alveolar macrophages	No effect on cytokine production	Aronoff *et al.*, 2006b
Mouse macrophage line	Production of IL-1-β and IL-6	Tan *et al.*, 2007a

IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein.

and tight junctions and adhesion molecules, such as cadherins and certain integrins, and may convert into mesenchymal cells through a process known as epithelial-mesenchymal transition (EMT) (Thiery and Sleeman, 2006). Whereas EMT is important for physiological functioning in adult organisms, fibrogenic properties of mesenchymal cells have also been shown to contribute to pulmonary fibrosis (Radisky, 2005). In the lung, when the first contact with foreign external particles occurs, resident immune effector cells, such as dendritic cells and alveolar macrophages, are activated in order to process antigenic material. However, dendritic cells and alveolar macrophages are also a rich source of cytokines and chemokines, and they may also amplify inflammation (Suarez *et al.*, 2008).

Expression of Epac in chronic inflammatory lung disease-associated cell types

Although chronic inflammatory lung disorders are a major cause of death and debilitation, there have been relatively few innovative breakthroughs into their cause, treatment or cure. Recently, research attention has been focused on new targets for therapeutic intervention (Barnes, 2007; Barnes, 2008; Chung and Adcock, 2008). Epacs represent a novel key effector in the lung due to their capacity to modulate airway inflammation and proliferation (Schaafsma *et al.*, 2008), and as part of the signalling cascade triggered by β_2-adrenoceptor agonists that target cAMP to alleviate the symptoms of asthma and COPD (Barnes and Hansel, 2004; Remington and DiGiovine, 2004; Balley and Tashkin, 2007). In the last few years, expression of Epac1 and Epac2 in several lung cell types has been successfully demonstrated (Figure 3 and Table 3). Epac1 and Epac2 mRNA were expressed in the lung epithelium and mesenchyme of mouse embryos at different developmental stages, and both cultured human ASM and bronchial epithelial cells express functional Epac1 and Epac2 (Schmidt *et al.*, 2007b). In human pulmonary fibroblasts, mRNA and protein expression of Epac1 was also recently detected, while expression of Epac2 remained under detection limits (Haag *et al.*, 2008; Huan *et al.* 2008). A few years ago, expression of Epac1 protein was identified in human and rat alveolar macrophages (Aronoff *et al.*, 2005) and mouse dendritic cells (Aronoff *et al.*, 2006b). Both cell types are believed to represent key effector cells in innate immunity. Studies on changes of Epac expression during development and under pathophysiological circumstances are still rather limited. Preliminary studies, however, reported on developmental changes of Epac1 and Epac2 in the heart, vasculature, brain, kidneys and lungs (Ulucan *et al.*, 2007; Murray and Shewan, 2008; Yokoyama *et al.*, 2008a,b) (see above), up-regulation of Epac1 protein expression in rat inflamed neurons (Wang *et al.*, 2007), up-regulation of Epac1 mRNA versus down-regulation Epac2 mRNA in Alzheimer's disease (McPhee *et al.*, 2005), and up-regulation Epac1 and Epac2 mRNAs in myocardial hypertrophy (Ulucan *et al.*, 2007) (see also above). Importantly, it has been recently discovered that the fibrogenic cytokine TGF-β1, a central mediator of inflammatory pulmonary diseases, induces conversion of resting fibroblasts to actively secrete myofibroblasts upon depletion of Epac1 (but not Epac2), which subsequently promotes the production of collagen and may increase pro-fibrotic signalling (Yokoyama *et al.*, 2008c). Intriguingly, in epithelial cells Epac1 also directly interacts with TGF-β1 and inhibits Smad-dependent TGF-β signalling (Conrotto *et al.*, 2006). Certainly, the expression of Epac (so far only reported for Epac1) is controlled by inflammatory mediators; however, the underlying molecular mechanisms have yet to be studied.

Epac in cell proliferation

The distinct expression profile and localization of Epac1 and Epac2 provide mechanisms for a more integrated and precise control of cAMP signalling, but also increase the signalling complexity. Cell-type specific functions of Epac are most likely to be the result of different cellular environments, cell-to-cell contacts, and the relative abundance, distribution and function of its diverse effectors. Paradoxically, Epac exerts opposing effects even on the same cellular process. For example, the role of Epac in the regulation of cell proliferation is known to be of central importance for the pathophysiology of asthma, COPD and pulmonary fibrotic disorders. Epac has been shown to drive pro-proliferative processes in endothelial cells (Fang and Olah, 2007), macrophages (Misra and Pizzo, 2005), thyroid cells (Hochbaum *et al.*, 2008) and osteoblasts (Fujita *et al.*, 2002), a cellular response believed to involve activation of extracellular signal-regulated kinases via

the Epac effector Rap. In contrast, recent studies, however, have reported on a novel anti-proliferative function of Epac in both ASM cells (Kassel *et al.*, 2008) and pulmonary fibroblasts (Haag *et al.*, 2008; Huang *et al.*, 2008) (Figure 3 and Table 3). The findings open important new dimensions in cAMP-related signalling in the lung, particularly because in these studies, cell proliferation of the lung cells was not altered by the classical cAMP effector, PKA. Kassel *et al.* (2008) showed that β_2-adrenoceptor agonists inhibit mitogen-stimulated cell proliferation of ASM cells, a process most likely driven by Epac via yet unknown molecular mechanisms. Haag *et al.* (2008) used specific Epac siRNA probes to diminish Epac expression in human pulmonary fibroblasts, and reported that Epac1 is responsible for anti-proliferative effects of PGE_2. Inhibition of cell growth by the Epac effector Rap1 had previously been attributed to B-Raf and ERK1/2 (Dugan *et al.*, 1999). Interestingly, inhibition of human fibroblast proliferation may not involve classical signalling routes, because PGE_2, PKA or Epac1 did not inhibit mitogen-stimulated ERK1/2 (Huang *et al.*, 2008). Direct interactions of Epac1/Rap1 with microtubules might be responsible for the inhibition of fibroblast proliferation (Huang *et al.*, 2008), and indeed Epac-cytoskeleton interactions might be of importance for cell cycle-dependent dynamics (Qiao *et al.*, 2002; Borland *et al.*, 2006, 2009b; Lorenowicz *et al.*, 2007b; Cheng *et al.*, 2008; Huston *et al.*, 2008) (Figure 3). Thus, Epac proteins (most likely Epac1) inhibit proliferation of human ASM and fibroblasts. However, future research should focus on unravelling the anti-proliferative signalling pathways driven by Epac.

Epac in cytoskeleton dynamics

The cAMP-elevating prostanoid PGE_2 also inhibits a range of lung cell functions, including migration of fibroblasts (White *et al.*, 2005) and differentiation of myofibroblasts (Kolodsick *et al.*, 2003). The cAMP-dependency of these processes has led to speculation on the potential and specific roles of Epacs. Recent studies have indicated that Epacs exert important pro- and anti-inflammatory signalling properties in the lung (Roscioni *et al.*, 2008; Schaafsma *et al.*, 2008). The complex inflammatory processes in the lung are driven and maintained by the immunomodulatory capacities of lung cells. In fact, several lung cells produce and release inflammatory chemokines and cytokines, and express surface receptors that are important for cell adhesion and activation of leukocytes. Indeed, Epac/Rap signalling regulates cell migration and integrin-mediated cell adhesion in a wide variety of cell types (Bos *et al.*, 2001, 2003; Lorenowicz *et al.*, 2006; Pannekoek *et al.*, 2009). Recently, Lyle *et al.* reported that cAMP-mediated Epac–Rap activation inhibits epithelial migration upon stabilization of focal adhesions and inhibition of membrane protrusions, possibly via regulation of cytoskeleton-integrin interactions (Lyle *et al.*, 2008). Expression of Epac1 in primary leukocytes and leukemic cell lines was found to correlate with pro-inflammatory effects, including chemotaxis, β_1-integrin dependent cell adhesion and Rap1-dependent cell polarization (Lorenowicz *et al.*, 2006) (Figure 2). Indeed, chronic inflammatory responses are typically characterized by uncontrolled extravasation of leukocytes from the peripheral blood into tissues, a process mainly governed by the permeability of the endothelial cell monolayer, and in part determined by their cell contact strength (Vestweber, 2002; Pannekoek *et al.* 2009). Intriguingly, recent evidence suggests that both Epac and Rap regulate the formation of endothelial cell junctions upon redistribution of E-cadherin and recruitment of the cell junction molecule β-catenin to areas of cell contacts, processes believed to enhance endothelial barrier function (Wittchen *et al.*, 2005). Recent studies on human pulmonary artery endothelial cells have indicated that enhanced endothelial barrier function involves Epac/Rap-induced membrane localization of the Rac-specific guanine nucleotide exchange factors Tiam1 and Vav2 and subsequent activation of Rac, and that such endothelial barrier-protecting mechanisms were shared by a variety of cAMP-elevating stimuli, including PGE_2, prostacyclin and atrial natriuretic peptides (Birukova *et al.*, 2007, 2008) (Figure 3 and Table 3). Certainly, inhibition of (inappropriate) leukocyte transmigration and enhancement of barrier function by Epac could serve to reduce inflammation.

Epac in inflammatory mediator production

Epac and Rap also regulate cell contact with the ECM proteins laminin (Enserink *et al.*, 2004) and fibronectin (Rangarajan *et al.*, 2003) in several cell lines, a process involving integrin-driven cytoskeleton dynamics and modulation of cellular adhesion and migration (reviewed in (Pannekoek *et al.*, 2009). Epac/Rap signalling to laminin-5 is of particular interest, because laminin-5 functions as a signal adaptor for growth factor-induced invasive growth in lung cancer (Kodama *et al.*, 2005). ECM might be envisioned not only as a target of inflammation, but also as a key effector in the activation of inflammatory cells. Indeed, fragments of the ECM component hyaluronan have been shown to modulate negatively mice lung inflammation and fibrosis (Jiang *et al.*, 2005). It has also been reported that during inflammation, hyaluronan is degraded to lower molecular weight fragments that subsequently stimulate macrophages and airway epithelial cells to produce mediators of tissue injury and repair, including TNF-α, interleukin (IL)-12, macrophage inflammatory protein (MIP)-1α, MIP-2, IL-8, tissue inhibitor of metalloproteinase-1 and inducible nitric oxide synthase (McKee *et al.*, 1996; Scheibner *et al.*, 2006). Scheibner *et al.* (2008) showed that adenosine, known to be up-regulated and released into extracellular space during inflammation, exerts anti-inflammatory effects by acting on the G_s-coupled A_{2A} adenosine receptor expressed in macrophages, dendritic cells, T and B lymphocytes, and epithelial cells. Importantly, it has been reported that the anti-inflammatory signalling properties of adenosine seem to involve Epac, which in turn inhibit NF-kB and modulated induction of inflammatory gene expression by lower molecular weight fragments of hyaluronan in *in vitro* and *in vivo* models (Scheibner *et al.*, 2008). Negative regulation of cytokine signalling by Epac seems not to be limited to these cells. In vascular endothelial cells, Epac/Rap signalling regulates expression of SOCS-3 and subsequently inhibits the IL-6 receptor trans-signalling complex (Sands *et al.*, 2006) (Figure 3). Recent studies in human umbilical vein endothelial cells indicate that Epac1 induces the C/EBP family of transcription factors, a process that seems to involve SOCS-3 induction by PKCα and ERK1/2 (Yarwood *et al.*, 2008; Borland *et al.* 2009a).

C/EBP proteins regulate cell differentiation and inflammation (Ramji and Foka, 2002), processes partly depending on cAMP (Pelletier *et al.*, 1998). The lack of PKA phosphorylation sites on cAMP-responsive C/EBP domains (Wilson and Roesler, 2002) further points to PKA-independent but Epac-dependent transcriptional events. Finally, Epacs exert functions in anti-inflammatory and immune responses mediated by alveolar macrophages and dendritic cells (Aronoff *et al.*, 2006b) (Table 3). Activation of Epac suppressed dose-dependent phagocytosis in alveolar macrophages, a process likely mediated by the Epac effector Rap (Aronoff *et al.*, 2006b). In bone marrow-derived mouse dendritic cells, Epac inhibited the release of the inflammatory chemokines, MIP-1α and MIP-1β, induced by lipopolysaccaride upon activation of PKB/Akt and the subsequent phosphorylation/inhibition of GSK-3, which promoted DNA binding of the transcriptional repressor CCAT displacement protein (Jing *et al.*, 2004). In the mouse macrophage cell line J774A.1, Epac also reduced the lipopolysaccaride-induced production of IFN-β via signalling to PKB/Akt and GSK-3 (Xu *et al.*, 2008). Epac did not alter the production of MIP-1α and MIP-1β in alveolar macrophages (Aronoff *et al.*, 2006b), whereas in the RAW264.7, mouse macrophage cell line activation of Epac increased the production of the pro-inflammatory mediators IL-1β and IL-6 (Tan *et al.*, 2007b). Together, these studies indicate that Epacs reduced and/or increased the production of inflammatory mediators in different phagocytotic cells. Clearly, the underlying molecular mechanisms of the regulation of inflammation by Epacs remain to be studied and might be strictly cell type-dependent.

Epac in lung cell responses

The subcellular localization of Epac and/or its effectors might differ in dendritic cells, alveolar macrophages and in other lung-related cells. Such mechanisms might also contribute to the distinct cellular responses and provide an explanation of opposing Epac-related responses (Figure 3 and Table 3). Epac-driven signals through activation of Rap might be also envisaged, but these have not yet been studied in the airways. In epithelial cells, down-regulation of EMT-related E-cadherin has been shown to signal to integrins via Rap1, the latter being activated upon E-cadherin endocytosis (Balzac *et al.*, 2005), suggesting that Epac/Rap signals might contribute to the complex network of EMT signalling. Rap1 clearly represents the main Epac effector in several systems (Roscioni *et al.*, 2008; Yarwood *et al.*, 2008; Pannekoek *et al.*, 2009). However, upon signalling to different additional key effectors, Epac might fulfil its complex and partly opposing functions in cells of lung origin. Future studies on Epac-related signalling in the lung may help to develop novel therapeutic strategies against inflammatory pulmonary diseases.

Epac and neurones

Epac in neuronal functions

Our current understanding of the molecular mechanisms underpinning the neuronal basis of learning and memory has progressed substantially. cAMP-dependent PKA is thought to be the major, if not the sole neuronal cAMP mediator, and its importance in memory consolidation is generally accepted (Abel and Nguyen, 2008). In the neuronal system, however, recent studies have reported that expression of Epac is subject to alteration during development and under pathophysiological conditions (McPhee *et al.*, 2005; Ulucan *et al.*, 2007; Murray and Shewan, 2008). It has been postulated that the developmental regulation of Epac expression promotes axon growth and regeneration in the nervous system (Murray and Shewan, 2008), and McPhee and co-workers reported up-regulation of Epac1 mRNA versus down-regulation of Epac2 mRNA in those regions of the human brain associated with Alzheimer's disease (McPhee *et al.*, 2005) (Figure 1, see above).

Studies on the functional role of Epac in the neuronal system have shown that Epac enhances neurotransmitter release in glutamatergic synapses (Sakaba and Neher, 2003; Zhong and Zucker, 2005; Gekel and Neher, 2008), and that Epac1 and Epac2 modulate neuronal excitability (Ster *et al.*, 2007). In dorsal root ganglions, Epac mediates the translocation and activation of protein kinase C (PKC), leading to the establishment of inflammatory pain (Hucho *et al.*, 2005), and promotes neurite outgrowth (Murray and Shewan, 2008). In spinal cord tissue, Epac advances neurite regeneration (Murray and Shewan, 2008). In suprachiasmatic nuclei of the hypothalamus, it has been very recently reported that Epac1 and Epac2 represent a novel core component of the circadian pacemaker (O'Neill *et al.*, 2008). These data suggest that Epacs may be key modulators in the regulation of neuronal functions as diverse as neurotransmitter release and neuronal excitability.

Epac and neuronal effectors

Epac seems to activate distinct molecular mechanisms in the neuronal system. In cultures from mouse hippocampus, Epac-induced elevation of neurotransmitter release seems to require functional PKC (Gekel and Neher, 2008). In cultured mouse cerebellar neurons, however, Epac-regulated neuronal excitability required Rap and ERKs (Ster *et al.*, 2007, 2009). Thus far, evidence for a role of Epac in the process of learning and memory is limited. Gelinas *et al.* (2008) reported that Epac activation enhances the maintenance of long-lasting synaptic potentiation in area CA1 of mouse hippocampal slices, a process that involves a transient increase in ERK1/2 immunoreactivity. Co-application of a selective PKA and a selective Epac activator has been shown to rescue the memory retrieval impairment observed in dopamine β-hydroxylase deficient mice, and the Epac effector Rap seemed to be important for these neuronal responses (Ouyang *et al.*, 2008). Although recent research efforts indicate that Epac regulates neuronal cAMP signalling, it remains controversial as to whether Epac requires input of PKA, localization to specific brain areas, and signalling to distinct effectors in a time- and space-limited manner.

Subcellular compartmentalization of Epac signalling

Increase in cellular cAMP can simultaneously induce activation of the two cAMP mediators PKA and Epac, and thus specificity and coordination of cAMP signalling requires tight regulation. Subcellular compartmentalization and spatio-temporal cAMP dynamics are believed to involve members of

the AKAP family (Wong and Scott, 2004; Beene and Scott, 2008). As outlined above, a functional cAMP-responsive multiprotein complex, maintained by nuclear envelope-associated mAKAP, PKA, PDE4D3 and Epac1, has been identified in neonatal rat cardiomyocytes (Dodge-Kafka *et al.*, 2005). Additionally, it has been reported that the AKAP-based signalling complex was of importance in sensing subtle alterations in cardiac cAMP levels and in regulating hypertrophic functions (Dodge-Kafka *et al.*, 2005). It is worth noting that both PKA anchored to neuronal AKAP79/150 and the Epac effector Rap have been reported to regulate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking during synaptic plasticity (Zhu *et al.*, 2002, 2005; Snyder *et al.* 2005). It is therefore reasonable to assume that AKAP79/150 might integrate cAMP signals in order to coordinate distinct signalling properties of PKA and Epac in neuronal cells. Indeed, research in our laboratory has recently identified a novel neuronal plasma membrane-associated AKAP79/150-based signalling complex comprised of PKA, Epac2 and PKB/Akt (Nijholt *et al.*, 2008) (Figure 4).

Epac in neuronal dysfunction

The dysfunction of signalling events driven by cAMP and the Epac effector PKB/Akt seems to be a key feature of inflammatory disease, including Alzheimer's dementia (Martinez *et al.*, 1999; Halliday *et al.*, 2000; Lin *et al.*, 2001; Griffin *et al.*, 2005; Rogers, 2008). To date, however, the exact nature of the connection of cAMP-dependent signalling with downstream PKB/Akt phosphorylation (activation) remains unknown. However, recent research in our laboratory suggests that this connection exists. In murine primary cortical neurons and HT-4 cells, activation of cAMP-dependent PKA reduced PKB/Akt phosphorylation, whereas activation of Epac enhanced PKB/Akt phosphorylation in a Rap-dependent manner. Studies with PKA-binding deficient neuronal AKAP79/150 and peptides-disrupting PKA anchoring to AKAPs have indicated that AKAP79/150 acts as a key regulator in the two cAMP pathways to control PKB/Akt phosphorylation (Nijholt *et al.*, 2008) (Figure 4). The novel link between neuronal AKAP79/150, PKA, PKB/Akt and Epac2 provides a molecular mechanism to exert a reciprocal effect on neuronal PKB/Akt phosphorylation. Importantly, the neuronal cAMP-responsive multiprotein complex exerted opposing effects on PKB/Akt signals (Nijholt *et al.*, 2008). Clearly, Epac2 (most likely complexed to or associated with AKAP79/150) enhances PKB/Akt phosphorylation, and most likely subsequent PKB/Akt signaling as well. Because Epac2 expression is reduced in those regions of the human brain associated with Alzheimer's disease (McPhee *et al.*, 2005), it is reasonable to assume that Epac2-induced PKB/Akt phosphorylation is reduced in patients suffering from such pathophysiological conditions. Such a mechanism might explain the dysfunction of PKB/Akt signals during inflammatory disorders, such as Alzheimer's dementia. Clearly, further research is required to unravel the molecular mechanisms driven by Epacs.

Future perspectives

Activation of GSK-3 by PKB/Akt might contribute to the regulation of many early and late neuronal functions, ranging from cell differentiation, proliferation, and survival to learning and memory (Peineau *et al.*, 2008), and the interaction of PKA and Epac with AKAPs might indicate distinct cAMP signalling properties. It has been reported that members of the AKAP family interact with muscarinic receptors and β_2-adrenoceptors (Hoshi *et al.*, 2003, 2005; Giembycz and Newton, 2005). Both receptor subtypes are coupled to cAMP driven signalling, and are important for proper lung function. Indeed, dysfunction of cholinergic transmission contributes to the development and progression of chronic inflammatory lung disorders (Belmonte, 2005; Barnes, 2008; Chung and Adcock, 2008; Schaafsma *et al.*, 2008). Recent research has reported that the existence of a GSK-3/β-catenin signalling axis in ASM cells is of key importance to mitogenic signalling (Gosens *et al.*, 2008; Nunes *et al.*, 2008). Interaction of AKAPs with muscarinic receptors and β_2-adrenoceptors might also coordinate compartmentalization and spatio-temporal cAMP dynamics in the lung, and subsequent signalling to PKB/Akt-regulated targets. Dysfunction of such mechanisms might contribute to the development and perpetuation of inflammatory disorders of the pulmonary and neuronal system.

Concluding remarks

Studies in diverse cell types, animal models and in genetically modified mice indicate that the novel cAMP mediators, Epac1 and Epac2, are important for several processes under physiological and pathophysiological circumstances. Further insights into the spatio-temporal dynamics of Epac-driven cAMP signalling will unravel the molecular mechanisms underlying the development and the progression of chronic degenerative inflammatory diseases, such as Alzheimer's dementia and COPD. The development of novel Epac-specific compounds for innovative therapeutic intervention for the causes, treatment or cure of these diseases definitely represents the primary challenge of the future.

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Conflicts of interest

None provided.

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