Epac: Defining a New Mechanism for cAMP Action

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Key Words

G protein, guanine nucleotide exchange factor, Rap, 8-pCPT-2′-O-Me-cAMP, cAMP-GEF

Abstract

cAMP is a second messenger that is essential for relaying hormonal responses in many biological processes. The discovery of the cAMP target Epac explained various effects of cAMP that could not be attributed to the established targets PKA and cyclic nucleotide–gated ion channels. Epac1 and Epac2 function as guanine nucleotide exchange factors for the small G protein Rap. cAMP analogs that selectively activate Epac have helped to reveal a role for Epac in processes ranging from insulin secretion to cardiac contraction and vascular permeability. Advances in the understanding of the activation mechanism of Epac and its regulation by diverse anchoring mechanisms have helped to elucidate the means by which cAMP fulfills these functions via Epac.

INTRODUCTION

Cyclic AMP (cAMP): 3′-5′-cyclic adenosine monophosphate

Adenylate cyclase: enzyme that catalyzes the synthesis of cAMP from ATP

PKA: protein kinase A

Epac: exchange protein directly activated by cAMP

Small G protein: protein typically 20 to 25 kDa in size that cycles between an inactive GDP-bound state and an active GTP-bound state

GEF: guanine nucleotide exchange factor

GAP: GTPase-activating protein

Rho family: family of small G proteins, including Rho, Rac, and Cdc42; essential in the regulation of actin reorganization

Hormones allow for communication between distant cells, which is essential for the homeostasis of multicellular organisms. Occupation of the hormonal receptor on the target cell is translated into intracellular signaling that results in the desired response, which is often initiated by second messengers. A great number of hormone receptors are linked to the production of the second messenger cyclic AMP (cAMP) via the activation of membrane-tethered adenylate cyclases. cAMP signaling mediates a wide range of cellular responses involved in the regulation of processes such as cardiac contraction, insulin secretion, and neurotransmitter release. Initially, the effects of cAMP were solely attributed to activation of protein kinase A (PKA) and cAMP-gated ion channels, but the contribution of the alternative cAMP target Epac (exchange protein directly activated by cAMP, also known as cAMP-GEF) has become more and more appreciated. Epac was identified in a database screen conducted to explain the PKA-independent activation of the small G protein Rap by cAMP (1). Independently, a screen for proteins containing cyclic nucleotide–binding (CNB) domains revealed the presence of mRNA for both Epac proteins, Epac1 and Epac2, which are enriched in the striatum (2). Another protein highly homologous to Epac with activity toward Rap, known as Repac, has been identified; however, this protein lacks a cAMP-binding domain (3). Epac1 and Epac2 are present in most tissues, albeit with different expression levels. Epac1 is highly abundant in blood vessels, kidney, adipose tissue, central nervous system, ovary, and uterus, whereas Epac2 is mostly expressed in the central nervous system, adrenal gland, and pancreas (1, 2, 4).

Epac proteins function as guanine nucleotide exchange factors (GEFs) for both Rap1 and Rap2 (3). Rap belongs to the Ras family of small G proteins, which cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. GEFs catalyze the exchange of GDP for the more abundant GTP and thereby the activation of the G protein, whereas GTPase-activating proteins (GAPs) enhance GTP hydrolysis. In addition to Epac, several other GEFs for Rap, which are all differentially regulated, have been identified. These GEFs connect different inputs to Rap activation and are linked to distinct functions of Rap. Rap1 was originally identified as a suppressor of Ras-induced transformation, and on the basis of its similarity in effector-binding region, it was proposed to compete with Ras for the same downstream proteins (5). We now know that Rap functions mostly independently of Ras, and knockout models for different Rap isoforms and their GEFs established that Rap signaling is pivotal for cell adhesion and cell-cell junction formation (6–11). Various effector proteins, including adaptor proteins implicated in modulation of the actin cytoskeleton, regulators of G proteins of the Rho family, and phospholipases (reviewed in Reference 12), relay signaling downstream from Rap.

MECHANISM OF Epac ACTIVATION

Epac1 and Epac2 are multidomain proteins (**Figure 1*a***) containing an N-terminal regulatory region that has one (Epac1) or two (Epac2) CNB domains and a DEP (Dishevelled, Egl-10, and Pleckstrin) domain. The C-terminal catalytic region harbors the CDC25-homology domain (CDC25-HD) for exchange activity, which is stabilized by a Ras exchange motif (REM) domain. Present between these domains is a Ras-association (RA) domain. In vitro experiments with the separate regions revealed an autoinhibitory function of the regulatory region that is relieved by binding of cAMP (3). The N-terminal CNB domain of Epac2 (CNB-A) binds cAMP with relatively low affinity and is dispensable for autoinhibition (3, 13); however, it may serve a modulatory role. Crystal structure analysis of the inactive Epac2 and active Epac2 proteins in complex with a cAMP analog and Rap1 revealed in atomic detail the mechanism of autoinhibition and activation of

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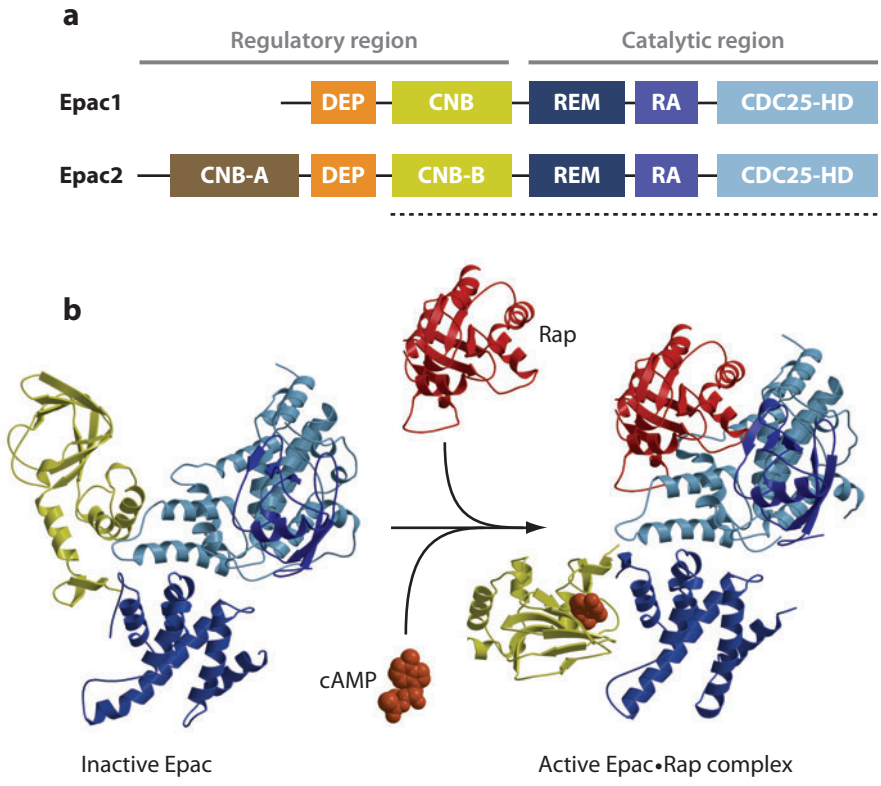

Figure 1

Mechanism of Epac activation by cyclic AMP (cAMP). (*a*) Domain architecture of the Epac proteins. The regulatory region contains one or two CNB (cyclic nucleotide–binding) domains and a DEP (Disheveled, Egl-10, and Pleckstrin) domain. The catalytical region harbors the enzymatically active CDC25-homology domain (CDC25-HD) that is stabilized by the Ras exchange motif (REM) domain, with a Ras-association (RA) domain between the two. (*b*) The crystal structure of inactive Epac2 and active Epac2 in complex with Rap1B and the cyclic nucleotide. For simplicity, only the catalytic region and the CNB-B domain (indicated with a dotted line in panel *a*) are shown. In the inactive conformation, the CNB-B domain sterically hinders binding of Rap to the CDC25-HD, which is relieved by a conformational change induced by binding of cAMP.

Epac (**Figure 1*b***) (13, 14). In the inactive conformation, the CNB domains sterically hinder Rap binding to the catalytic site. An ionic interaction between the C-terminal CNB (CNB-B) domain and residues in the CDC25-HD that are critical for Rap binding stabilizes this conformation. Upon binding of cAMP, a subtle change within the CNB-B domain allows the regulatory region to move away to the back side of the catalytic region. This position is stabilized by interactions among cAMP, the CNB-B domain, and the REM domain. No significant differences between the conformation of the CDC25-HD in the active and inactive conformations were found, supporting the idea that cAMP regulates the activity of Epac by lifting an autoinhibition, rather than by inducing an allosteric change in the Rap-binding site.

8-pCPT-2′-O-Me-cAMP: AN Epac-SELECTIVE AGONIST

cAMP is generated by hormonally regulated adenylate cyclases at the plasma membrane and by soluble adenylate cyclases, and it can subsequently be degraded by phosphodiesterase

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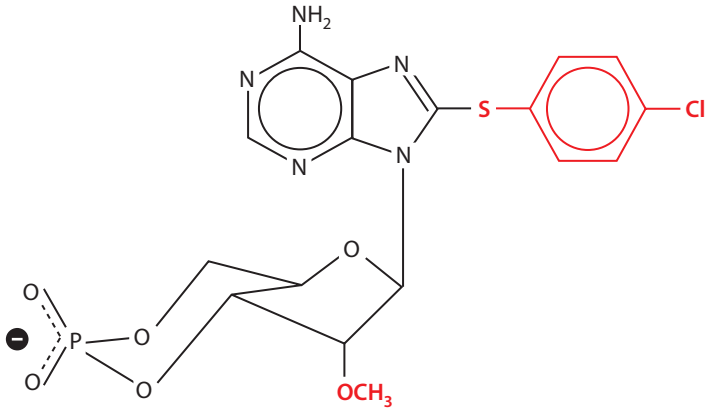

8-pCPT-2'-O-Me-cAMP

Figure 2

Chemical structure of the Epac-selective cyclic AMP (cAMP) analog 8-pCPT-2′-O-Me-cAMP (007). The proton of the 2′OH group of the ribose of cAMP has been replaced with a methyl (-CH_3) group, and the proton at the 8′ position of the base has been replaced with a 4-chlorophenylthio (pCPT) group (modifications are indictated in red) to mediate specificity for Epac proteins and increase affinity for Epac1.

(PDE) enzymes. Several compounds are available to modulate intracellular cAMP levels. Most commonly used are (*a*) forskolin, a natural compound from the Indian medical plant *Coleus forskohlii* that directly activates adenylate cyclases, and (*b*) a variety of inhibitors of PDE enzymes. In addition, several membrane-permeable cAMP analogs that nonspecifically target cAMP effectors, some of which are PDE insensitive, have been generated (see Reference 15). During the development of Epac-selective analogs it was noted that Epac proteins lack the glutamate that, in PKA and cAMP-gated ion channels, interacts with the 2′OH group of the ribose of cAMP. Analogs in which the 2′OH group has been replaced with 2′O-Me selectively interact with Epac1 and Epac2 (16). The addition of pCPT (4-chlorophenylthio) or related groups at the 8′ position of the base further increases affinity. As a consequence, 8-pCPT-2′-O-Me-cAMP (**Figure 2**), also known as 007, is more than tenfold as efficient as cAMP in activating Epac1 in vitro (K_d is 2.9 μM for 007 versus 45 μM for cAMP) (17). Furthermore, 007 results in a threefold-higher maximum activity of Epac1, making this analog a so-called superagonist for Epac1 (17). To resolve the relatively low membrane permeability of this compound, an AM (acetoxymethyl) -ester was introduced to mask the negatively charged phosphate group (18). This modification allowed exceptional cell permeability and is intracellularly removed by esterases to generate 007 (18). Although 007 is rather resistant to PDE action, it can bind and thereby inhibit PDE1, -2, and -6, and thus indirectly increase cAMP and 3′-5′-cyclic guanosine monophosphate (cGMP) levels (15). In addition, cellular effects induced by metabolites of 007 have been described (19). Therefore, it remains essential to prove that any effect of 007 is not due to the activation of targets other than Epac. Currently, no inhibitors for Epac proteins are available. The ARF-GEF inhibitor brefeldin A was shown to be an inhibitor of Epac function (20), but evidence that it directly attenuates Epac activity is lacking.

PDE: phosphodiesterase

007: 8-pCPT-2′-O-Me-cAMP

SPATIAL REGULATION OF Epac

Although cAMP can rapidly diffuse within the cytosol, cAMP-elevating hormones do not induce homogenous increases of cAMP within the cell. Instead, cAMP becomes unevenly distributed

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and concentrated in local microdomains (reviewed in Reference 21). At the foundation of this compartmentalization are various PDEs, which are confined to specific subcellular compartments. These PDEs mediate local cAMP degradation and the generation of cAMP gradients in the cell. In addition to the compartmentalization of cAMP, the cAMP effectors are also spatially regulated by binding to scaffolding proteins, as has been extensively studied for PKA (22). A-kinase anchoring proteins (AKAPs) target PKA to distinct subcellular locations and mediate the assembly of large signaling complexes, thereby linking PKA to specific cellular functions. Similarly, Epac proteins are spatially regulated by different anchoring mechanisms, which often discriminate between Epac1 and Epac2.

AKAP: A-kinase anchoring protein

Integrin: transmembrane receptor that mediates adhesion of cells to the extracellular matrix and to one another

In response to the cAMP-induced conformational change, Epac1 is targeted to the plasma membrane via its DEP domain (23). This is essential for its ability to induce Rap activation at the plasma membrane and for efficient integrin-mediated cell adhesion (23). Interestingly, activation of Epac1 in Rat1a cells predominantly activates Rap1 at the perinuclear region due to high RapGAP activity at the plasma membrane (24). This illustrates that not only GEFs but also GAPs mediate the spatial regulation of Rap activity. Epac2 binds via its RA domain to activated Ras proteins, independently of its conformational state, and is thereby targeted to the plasma membrane (25, 26). An alternative membrane targeting sequence resides within the N-terminus of Epac2; this sequence is absent in an adrenal gland–specific Epac2 isoform (27). Both mechanisms of membrane targeting of Epac2 have been associated with Rap-mediated processes at the plasma membrane (25–27).

Several other subcellular localizations of Epac1 that may link Epac1 to specific cellular processes have been described. Epac1 is present within the nucleus (23, 28), and nuclear Epac1 was found to regulate the DNA damage–responsive kinase DNA-PK (29). Epac1 is also targeted to microtubules in both interphase and mitotic cells (28, 30). This targeting may be mediated either by direct interactions with tubulin or by the microtubule-associated protein MAP1 (31, 32) and is probably required for the role of Epac in microtubule polymerization (30, 31). Other reported localizations of Epac1 that may be associated with distinct functions include centrosomes (28, 33), the nuclear pore complex (34), mitochondria (28), macrophagic phagosomes (33), and the apical membrane of renal epithelial cells (35, 36).

BIOLOGICAL FUNCTIONS OF Epac

The major catalytic function of Epac is the guanine nucleotide exchange of Rap1 and Rap2, and Epac thus controls the Rap-mediated processes downstream of cAMP. The Epac-selective cAMP analog 007 has helped to reveal a role for Epac and Rap in a wide range of biological processes, ranging from exocytosis of insulin in the beta cells of the pancreas to the regulation of calcium channels in cardiomyocytes and permeability of the vascular endothelium. Most of these processes are also modulated by signaling via the cAMP target PKA, demonstrating the interconnectivity between both cAMP pathways. Although it has not yet been employed in all studies, Epac protein depletion has confirmed the importance of Epac in these processes and has allowed for discrimination between Epac1 and Epac2. The mechanism of action of Epac and Rap and the downstream pathways are now beginning to be unraveled, as discussed further below.

Cardiac Function of Epac

For many years, cAMP has been known to be a critical regulator of heart function via both PKA and cAMP-gated ion channels. Dependent on the duration of signaling, cAMP can either induce acute alterations in cardiac contraction by modifying Ca^{2+} homeostasis or induce hypertrophy of the cardiomyocytes and eventually cardiac dysfunction (37).

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During cardiac contraction (**Figure 3**), depolarization of the cardiomyocyte membrane results in activation of Ca^{2+} channels (I_{Ca}), which mediates an inward Ca^{2+} current that triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR). This Ca2+-induced Ca2+ release (CICR) allows activation of actin myofilament proteins and thereby contraction of the cardiomyocyte. Dissociation of Ca^{2+} from the contractile machinery and clearance of Ca^{2+} from the cytosol result in the subsequent relaxation of the cell. The strength of cardiac contraction depends on both the amplitude and the duration of the Ca^{2+} transient and can be further regulated through modification of the sensitivity of the myofilaments to Ca^{2+} (38).

Activation of the beta-adrenergic receptor (βAR) mediates both potentiation of the developed contraction and accelerated relaxation of the cardiomyocytes, thereby affecting contractility and heart rate (37). This has mainly been attributed to the cAMP effector PKA, whose downstream targets include I_{Ca}; the SR Ca^{2+} release pump Ryr2; myofilament proteins; and phospholamban, an inhibitor of Ca^{2+} reuptake in the SR. Thereby, PKA signaling results in the increased release and accelerated reuptake of Ca^{2+}.

The initial indication for a role of Epac in βAR-mediated regulation of excitation-contraction coupling came from mice depleted of the Rap-effector phospholipase C epsilon (PLCε). These mice displayed decreased enhancement of electrically evoked Ca^{2+} transients and decreased left ventricular–developed pressure in response to βAR stimulation (39). Indeed, activation of Epac in murine ventricular myocytes resulted in an increase in amplitude of electrically evoked Ca^{2+} transients in a Rap- and PLCε-dependent manner (40). Together with PKA, Epac mediated the complete effect of βAR stimulation on CICR (40). PLCε requires phosphatidylinositol 4,5-bisphosphate (PIP_2)-hydrolytic activity for this effect on Ca^{2+} downstream of Epac and Rap, and it transduces its effects via protein kinase C epsilon (PKCε) and its substrate calcium/calmodulin-dependent protein kinase II (CaMKII) (**Figure 3**) (41). Accordingly, 007 stimulation of ventricular cardiomyocytes results in an increased phosphorylation of the CaMKII substrates Ryr2 and phospholamban (41, 42).

Numerous reports support a role for Epac in cardiac Ca^{2+} regulation, showing that Epac mainly acts on Ca^{2+} release from the SR via Ryr2 while leaving Ca^{2+} influx via I_{Ca} and Ca^{2+} clearance unaltered (40–43). However, the outcome of Ryr2 activation by Epac is unclear, as inhibition of Ca^{2+} release from the SR in response to Epac activation has also been demonstrated (42). This inhibition is explained by an increase in spontaneous Ca^{2+} leakage from the SR, which leads to depletion of this pool of Ca^{2+} (42). Further studies confirm the effect of Epac activation on spontaneous Ca^{2+} oscillations but show no alterations in the amplitude of electrically evoked Ca^{2+} transients by Epac (43, 44). Although Epac clearly participates in the regulation of Ca^{2+} release in the cardiomyocyte, the actual consequence of this participation requires further verification.

cAMP also affects heart contraction by enhancing the function of gap junctions, which mediate gating of ions and small molecules between cardiomyocytes and thus the coordinated excitation of the heart. The predominant gap junction protein in cardiomyocytes, connexin-43, is cooperatively regulated by PKA and Epac1. Whereas PKA increases the gating function of connexin-43-composed gap junctions, Epac1-Rap1 signaling enhances the accumulation of connexin-43 at cell-cell contacts (45). This may depend on the upregulation of N-cadherin at cell-cell contacts by Epac1 signaling, as the presence of adherens junctions is a prerequisite for the formation of gap junctions (45).

In contrast to the beneficial effects on heart function by acute βAR stimulation, prolonged βAR signaling in cultured cardiac myocytes and in animal models results in hypertrophy, which can progress into heart failure (37). Accordingly, failing hearts often show alterations in βAR expression and in levels of receptor agonists in the circulation (37). It remains unclear whether such alterations contribute to the pathogenesis of heart failure or reflect a compensatory mechanism.

Ca^{2+}-induced Ca^{2+} release (CICR): cellular process in which intracellular calcium mediates its own release from intracellular stores by binding to inositol 1,4,5-trisphosphate (IP_3) and ryanodine-sensitive receptors on the sarcoplasmic and endoplasmic reticula

Beta-adrenergic receptor (βAR): transmembrane receptor that, upon the binding of hormonal ligands, activates $G_{\alpha}s$ and subsequently adenylate cyclases

PLCε: member of the phospholipase C family that is activated by Rap and mediates the hydrolysis of phosphatidylinositol-4,5-bisphosphate into the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. In addition, this protein contains a CDC25-homology domain

Adherens junction: cell junction that is linked to the actin cytoskeleton and mediates cell-cell adhesion via transmembrane cadherin molecules

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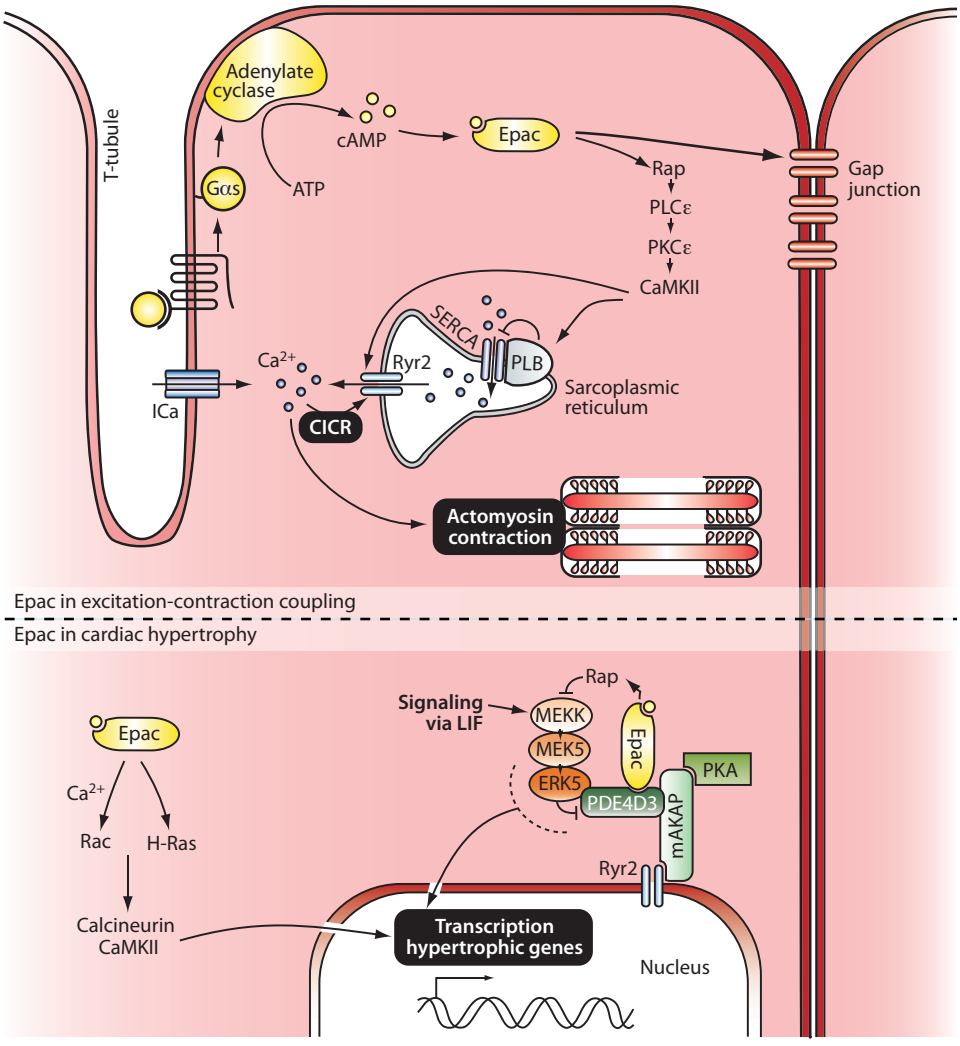

Figure 3

Proposed cardiac functions of Epac. Epac signaling can potentiate the contraction of cardiomyocytes (*top*) and induce cardiomyocyte hypertrophy (*bottom*). The role of Epac in excitation-contraction coupling involves the activation of the Rap effector phospholipase C epsilon (PLCε) and the downstream kinases protein kinase C epsilon (PKCε) and calcium/calmodulin-dependent protein kinase II (CaMKII). Epac signaling results in activation of the CaMKII substrate Ryr2 and thereby controls Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum. The CaMKII substrate phospholamban (PLB), which inhibits Ca^{2+} reuptake into the sarcoplasmic reticulum by sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), is also phosphorylated upon Epac activation, although the effects of Epac on Ca^{2+} reuptake have not yet been demonstrated. Cardiac hypertrophy induced by Epac signaling may be independent of Rap and has been linked to the Ca^{2+}-dependent activation of Rac and the activation of H-Ras, which result in activation of the Ca^{2+}-sensitive kinase CaMKII and phosphatase calcineurin. Epac1 is also part of a protein complex at the nuclear envelope that comprises muscle-specific A-kinase activating protein (mAKAP), protein kinase A (PKA), and phosphodiesterase 4D3 (PDE4D3). Activation of Epac in this complex results in Rap-dependent inhibition of extracellular signal–regulated kinase 5 (ERK5) and thereby relief of an inhibiting phosphorylation on PDE4D3 and inhibition of hypertrophic signaling via leukemia-induced factor (LIF). Abbreviations: MEK5, mitogen-activated protein/extracellular signal-regulated protein kinase kinase 5; MEKK, MEK kinase; T-tubule, transverse tubule.

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Signaling via Epac1 contributes to the induction of hypertrophy by βAR stimulation (**Figure 3**). Prolonged 007 stimulation induces alterations in cell morphology and gene expression characteristic of cardiomyocyte hypertrophy, which requires the Ca^{2+}-dependent phosphatase calcineurin and CaMKII (44, 46). Importantly, knockdown of Epac1 in neonatal rat ventricular myocytes impairs the induction of hypertrophic markers in response to βAR stimulation (46). Furthermore, Epac1 expression is upregulated in animal models of left ventricular hypertrophy and in the human failing heart, supporting a role for Epac1 in the progression of heart failure (46, 47).

Conversely, Epac1 may also have inhibitory effects on cardiac hypertrophy, as Epac1 activation inhibits cytokine-induced hypertrophy in neonatal cardiomyocytes (48). This inhibition involves the recruitment of Epac1 to a nuclear envelope–localized muscle-specific A-kinase activating protein (mAKAP) complex, which also harbors PKA, PDE4D, and extracellular signal–regulated kinase 5 (ERK5) (**Figure 3**) (48). Inhibition of ERK5 activity by Epac1-induced Rap activation enhances PDE4D-mediated cAMP degradation and negatively affects hypertrophic effects downstream of this kinase (48). Other proteins associated with mAKAP include the downstream Epac target Ryr2 (49), which potentially renders the mAKAP complex a broader platform for Epac1 signaling in cardiac cells.

Epac in Insulin Secretion

Glucose, the primary signal for insulin secretion by pancreatic beta cells (**Figure 4**), can be potentiated by cAMP signaling via the cooperative actions of PKA and Epac. Metabolism of glucose results in an influx of Ca^{2+} via voltage-dependent Ca^{2+}-channels, which triggers the release of a primed pool of insulin granules docked at the plasma membrane. This first phase of insulin secretion is followed by the mobilization of additional insulin granules, mediating a sustained second phase of insulin release (50).

Glucose and other nutrients also mediate the release of GLP-1 (glucagon-like peptide 1) and GIP (gastric inhibitory polypeptide) from gastrointestinal cells. These hormones act on receptors on the beta cell linked to cAMP production, which potentiates the glucose-induced secretion of insulin (51). Knockdown and dominant-negative approaches have shown that Epac2, the main Epac isoform expressed in beta cells (52), is required for the full action of GLP-1/GIP on insulin exocytosis (53, 54). Furthermore, specific activation of Epac by 007 induces exocytosis in human beta cells, which requires the presence of glucose (55, 56). In particular, Epac stimulation increases the number of exocytic sites on the plasma membrane of the beta cell (57). Primary cells from Epac2 knockout mice revealed that Epac2-Rap1 signaling plays an essential role in the potentiation of the first phase of insulin granule release, probably by controlling granule density near the plasma membrane (58).

Part of Epac2's role in the potentiation of insulin secretion is mediated by its effects on Ca^{2+} signaling. GLP-1 receptor stimulation results in a glucose-dependent increase in intracellular calcium levels in beta cell lines and primary cultures that is partially dependent on Epac2 (54, 55, 59). Similarly, 007 treatment of beta cells induces transient increases of Ca^{2+} associated with exocytosis (55, 56). Such increases may be mediated by direct effects of Epac2 on the function of K_{ATP} channels, as the Epac-selective agonist 007 inhibits the activity of these channels (60), and Epac directly interacts with the regulatory subunit of this channel, SUR1 (sulfonylurea receptor 1) (61). CICR from the endoplasmic reticulum also contributes to insulin secretion, and GLP-1 mediates CICR in beta cells in an Epac2-dependent manner (54, 55). Because the Epac agonist 007 fails to elevate inositol phosphate (IP) levels in beta cells and the Epac-mediated rise in intracellular Ca^{2+} and insulin secretion is inhibited by ryanodine but not by inhibitors of the IP_3 receptor, Epac probably targets the Ryr channel on the ER of the beta cell (54, 55, 62). As a consequence of this

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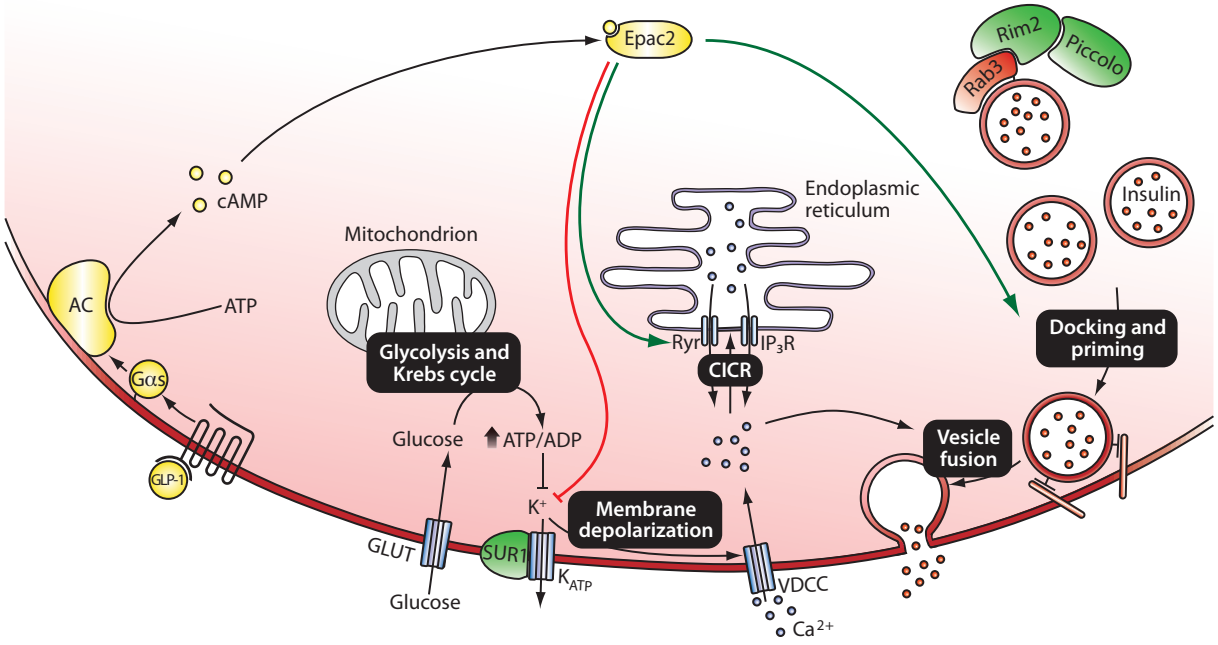

Figure 4

Proposed functions of Epac2 in insulin secretion. Glucose induces exocytosis of insulin by increasing the cytosolic ATP/ADP ratio and the subsequent closure of ATP-sensitive K^+ channels (K_{ATP} channels). This process results in membrane depolarization and opening of voltage-dependent Ca^{2+} channels (VDCCs), which trigger the release of insulin granules docked at the plasma membrane. Peptide hormones, such as glucagon-like peptide 1 (GLP-1), increase cAMP levels and thereby potentiate insulin secretion via the combined action of PKA and Epac2. Epac2 participates in several ways: by inhibiting K_{ATP} channels; by activating the ryanodine-sensitive Ca^{2+} channel, Ryr2, that is involved in Ca^{2+}-induced Ca^{2+} release (CICR) from the endoplasmic reticulum; and by recruiting insulin granules to the plasma membrane. The Epac2 interaction partners Sur1, Rim2, and Piccolo are implicated in this process. Rim2 and Piccolo also interact with the VDCC (interaction not shown), and SUR1 may also localize at the insulin granule membrane. Abbreviations: AC, adenylate cyclase; IP_3R, inositol 1,4,5-trisphosphate receptor.

increased mobilization of Ca^{2+} from intracellular stores, Epac2 may also indirectly induce the closure of K_{ATP} channels by increasing ATP production in the mitochondria (59).

Epac2 associates with various components of the beta cell exocytic machinery and may thereby regulate vesicle docking and fusion independently of its effects on intracellular Ca^{2+}. Epac directly binds to Rim2, an effector of Rab3 that localizes to the cytosolic face of insulin granules and the VDCCs (61, 63). Disruption of the interaction between Epac and Rim2 inhibits cAMP-induced exocytosis in beta cells (53). Similarly, Epac2 binding to the Rim-related protein Piccolo, which can form heterodimers with Rim2 in a Ca^{2+}-dependent fashion, is required for this effect (64). Furthermore, the Epac-interaction partner SUR1 is essential for the PKA-independent effect of cAMP on exocytosis (65). SUR1's interaction with Epac may affect not only K_{ATP} channel function but also Cl^- influx into insulin granules involved in acidification and therefore priming of the granule (65). Recently, it has been reported that sulfonylureas, which potentiate insulin release by binding to SUR1, may also directly activate Epac2, although this needs to be confirmed with in vitro experiments (66). In conclusion, numerous Epac-interacting proteins have been implicated in the Epac2-mediated potentiation of insulin secretion, but how these proteins link Epac2 to its downstream effects requires further study.

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Neuronal Function of Epac

The mechanism of neurotransmitter release at the neuronal synapse, in response to action potential–induced Ca^{2+} influx, bears a strong resemblance to that of insulin secretion. Both Epac1 and Epac2 are expressed in most parts of the nervous system (2), and numerous neurohormones modulate the release of synaptic vesicles by elevation of cAMP, in part via the action of Epac. An initial indication for this function of Epac came from a study on gamma-aminobutyric acid (GABA) receptor signaling (67). GABA inhibits synaptic transmission via reduction of cAMP levels, an effect that is counteracted by 007 (67). Further studies in the calyx of Held, hippocampal neurons, and the neuromuscular junction confirmed that Epac activation potentiates the excitation-induced postsynaptic current (20, 68–70). Similarly, Epac enhances exocytosis in neuroendocrine cells such as melanotrophs (71).

Expanding its role in neurotransmitter release, Epac activation also produces long-lasting effects on synaptic transmission that underlie synaptic plasticity and thus learning and memory (20, 68, 72, 73). Indeed, stimulation with 007 shows beneficial effects on memory functions in fear-conditioning experiments in mice (74, 75). Additional studies in mice revealed that Epac augments sensitivity to mechanical pain stimuli by sensitizing pain receptors (76–78). This feature is linked to the activation of PKCε and the downstream purinergic receptor P2X3 in dorsal root ganglion neurons, which are involved in transduction of the pain signal (77, 78). The role of Epac in augmenting the pain response may be pronounced in inflammation, during which Epac1 expression and signaling are upregulated in the dorsal root ganglions (78).

Together with PKA, Epac also contributes to the regulation of neuronal differentiation, neurite outgrowth, and axon regeneration, implicating a role for Epac in the development and maintenance of the nervous system (79–83). Furthermore, via activation of Rac, Epac-Rap signaling enhances alpha-secretase activity and therefore secretion of soluble amyloid precursor protein alpha (APPα), which harbors neurotrophic activities and memory-enhancing effects (84, 85). Finally, Epac takes part in the regulation of the circadian cycle downstream of oscillating cAMP levels in the suprachiasmatic nuclei (SCN) of the hypothalamus, and 007 activates circadian gene expression synchronously in individual SCN cells (86).

Vascular Function of Epac

The vascular endothelium constitutes the inner lining of blood vessels, thereby forming a barrier that controls the exchange of solutes, macromolecules, and cells between the blood and the surrounding tissues (87). Extravasation occurs by transport both through and between the cells. Extravasation between the cells is regulated by cell-cell junctions, which comprise both tight junctions and adherens junctions. The latter are formed by homophilic interactions between transcellular cadherin molecules that are linked to the underlying actin cytoskeleton (87). Permeability of the endothelial barrier is essential for extravasation of leukocytes during inflammation, although extensive endothelial leakage may result in pathologies such as edema and chronic inflammation (88). It has been well established that hormonal signaling via cAMP strengthens the barrier and can thereby counteract the increased permeability elicited by inflammatory mediators. This cAMP effect has been linked to the inhibition of actin dynamics and actin-myosin-based contractility by PKA, in part via its effects on G proteins of the Rho family (89).

Genetic studies in *Drosophila* have revealed a role for Rap1 in the regulation of cell-cell adhesion (7). Clones of mutant Rap1 wing cells display nonuniformal distribution of junctional proteins, and mutant cells disperse into the surrounding wild-type tissue (7). In mammalian epithelial cells, inhibition of Rap activity prevents the recruitment of E-cadherin to newly formed cell-cell contacts

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(90) and suppresses E-cadherin mediated adhesion (91). Conversely, activation of Rap antagonizes the disruption of adherens junctions during cellular transformation (91).

Indeed, signaling to Rap via Epac1, the main Epac isoform expressed in vascular endothelial cells (30, 92), contributes to the effects of cAMP-elevating hormones on endothelial function. 007 stimulation reduces permeability of cultured vascular endothelial cells, as measured by fluorescent dextran leakage and transendothelial electrical resistance, in an Epac1- and Rap-dependent manner (92–95). Similarly, the enhanced permeability induced by inflammatory mediators such as thrombin is counteracted by Epac1 activation (92, 93). Concurrently, activation of Epac results in accumulation of junctional proteins at cell-cell contacts, increased VE-cadherin-dependent cell adhesion, and linearization of cell-cell junctions (92–95). Consequently, Epac affects transendothelial cell migration, although this effect has been shown only for differentiated HL60 cells (95) and was not observed with neutrophils (93). The role of Epac1 in the regulation of vascular permeability was confirmed in vivo, as administration of 007 inhibits vascular endothelial growth factor–induced dye leakage from murine dermal blood vessels (94). Similarly, 007 counteracts VE-cadherin redistribution and hyperpermeability in response to platelet-activating factor in perfused rat mesentery microvessels (96).

The actin cytoskeleton, which is connected to the junctional adhesion molecules and allows for the generation of contractile force, is crucial for the regulation of the integrity of the endothelium (97). Actin rearrangements may underlie the effects of Epac1 on barrier function, as actin depolymerization by cytochalasin D blocks the effect of Epac activation on VE-cadherin-mediated cell adhesion and permeability (30, 94). Furthermore, Epac1-Rap signaling induces the accumulation of polymerized actin at the cell-cell contacts (92–94). The enrichment of cortical actin by 007 persists in the absence of cell-cell contacts, implying that Epac1 directly mediates actin remodeling (92, 93). This may require signaling to G proteins of the Rho family, as cAMP-elevating hormones activate Rac in an Epac1-dependent manner, and both Rac and its GEFs Tiam1 and Vav2 are required for cAMP-induced actin rearrangements and barrier strengthening (98, 99). Indeed, stimulation of endothelial cells with 007 results in increased Rac1 activity and enrichment of this G protein at cell-cell contacts (100). Although 007 does not affect basal RhoA activity, thrombin-induced RhoA activation is attenuated by Epac1, which may contribute to the opposing action of Epac1 on thrombin-induced permeability (93, 98). Recently, Krit1 (Krev1 interaction trapped gene 1, also known as CCM1), a protein linked to vascular abnormalities because of its association with cerebral cavernous malformations (101) and vascular phenotype of knockout animal models (102–106), was implicated in the regulation of barrier function downstream of Epac1 and Rap (107). Krit1 was identified as a direct interactor of active Rap1A (108), and Rap1 activity enhances the binding of Krit1 to adherens junction proteins such as beta catenin (107). Reduction of Krit1 levels in endothelial cells results in actin rearrangements, disruption of beta catenin at the cell-cell junctions, and decreased barrier function (107). Importantly, Krit1 depletion completely abolishes the inhibitory effect of 007 on thrombin-induced hyperpermeability (107).

The endothelial barrier and its regulation by Epac1 also depend on the integrity of the microtubule network, which functionally interacts with the actin cytoskeleton (30, 109). 007-induced actin remodeling and elevation of transendothelial resistance are blocked by inhibition of microtubule polymerization, whereas they are enhanced by the microtubule-stabilizing agent taxol (30). Epac has been implicated in microtubule polymerization in vitro (31), and exogenous Epac1 localizes to the microtubule network in endothelial cells (30). Indeed, 007 induces the elongation of microtubules toward the cell periphery in these cells, independently of the presence of cell-cell junctions and downstream Rap activation (30). These findings suggest that Rap-independent effects on microtubule dynamics may contribute to the role of Epac1 in endothelium permeability, potentially by mediating the delivery of junctional or Rap effector proteins.

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PGE2: prostaglandin E2

Epac1 signaling may also control intracellular responses in the vascular endothelium independently of its effect on endothelial permeability. For example, Epac1 inhibits activation of the JAK-STAT pathway by interleukin-6 receptor signaling in endothelial cells via upregulation of suppressor of cytokine signaling 3 (SOCS3) expression (110). In addition, Epac1 interconnects with PKA in vascular remodeling, as it enhances extracellular matrix adhesion and migration of endothelial and vascular smooth muscle cells (111–113). Finally, 007 induces secretion of von Willebrand factor–containing Weibel-Palade bodies, which may further contribute to the regulation of vasculature homeostasis by Epac1 (114).

Epac in Inflammation

In addition to affecting the vascular endothelium, cAMP signaling also directly controls inflammation by regulating the immune response of leukocytes. Most leukocytes express the Epac1 protein (115), which links part of the cAMP signal to the inflammatory response.

Phagocytic cells participate in innate immunity by phagocytosis of the pathogen and its subsequent killing, in part via increased phagosomal production of reactive oxygen species. Furthermore, these cells secrete a variety of inflammatory mediators. Prostaglandin E2 (PGE2), a lipid metabolite generated at sites of inflammation, negatively regulates these processes by increasing cAMP levels in phagocytes (116). The contribution of PKA and Epac downstream of cAMP in the inhibition of phagocyte activation varies among the different leukocytes. In alveolar macrophages, Epac1 inhibits FcγR-mediated phagocytosis, which may involve inhibition of PTEN activity, whereas the PKA-selective cAMP analog 6-Bnz-cAMP has no effect (117, 118). In contrast, in peripheral blood monocytes only PKA is involved (119), whereas in microglia and peritoneal macrophages both cAMP effectors attenuate myelin phagocytosis (120). In liver macrophages, specific Epac1 activation also suppresses the pathogen-induced production of reactive oxygen species, in which PKA activity has no role (121). This control of the respiratory burst and bacterial killing is mediated by both cAMP effectors, or solely by PKA, in alveolar macrophages and circulating monocytes, respectively (119, 122). This function of Epac1 may require its physical association with the phagosomes (as shown in alveolar macrophages), which is enhanced by PGE2 stimulation (33). Finally, although initial studies only implicated PKA in the cAMP-induced attenuation of chemokine production (117), Epac1 activation does modulate the pathogen-induced production of numerous inflammatory mediators in various leukocytes (122–126).

Epac1 may also function as a proinflammatory mediator by enhancing leukocyte adhesion and migration. At the site of inflammation, chemokines trigger the binding of circulating leukocytes to the endothelium, followed by their migration across the endothelium and to the site of infection. A role for Rap1 in integrin-mediated cell adhesion has been established in numerous cell lines including lymphocytes (reviewed in Reference 127), and Rap1 activity is essential for chemokine-induced lymphocyte adhesion, polarization, and transmigration (128, 129). Activation of Epac1 in the monocytic cell line U937 results in beta 1 integrin activation and adhesion to both the extracellular matrix component fibronectin and the vascular endothelium (115). In addition, 007 enhances polarization and directed migration of U937 cells (115), supporting a role for Epac1 in the regulation of leukocyte recruitment.

Renal Function of Epac

Epac1 is highly expressed throughout the various segments of the kidney tubules (1, 2, 36, 130). Although a polyclonal antibody directed against Epac2 also showed staining along the rat kidney epithelium (36), the specificity of this antibody requires further verification, as this observation

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seems to contradict evidence of Epac2 mRNA expression in adult human and mice kidney (2, 47). In the renal epithelium, Epac1 is enriched at the brush border membrane (35, 36), which is mediated by binding to the actin-linking protein Ezrin (J. Zhao and J.L. Bos, unpublished results). Its localization at the brush borders suggests a role for Epac1 in cAMP-mediated regulation of apical processes, such as ion and water absorption. Indeed, in the opossum and murine proximal tubule, activation of both PKA and Epac results in downregulation of Na^+/H^+ exchanger 3 (NHE3) activity (35, 131). Na^+/H^+ exchanger regulatory factor 1 (NHERF1) knockout mice revealed that this effect of 007 on renal NHE3 activity requires the presence of the adaptor protein NHERF1, which recruits Ezrin to NHE3 (131). Although the Epac1 protein is present in the intestinal ileum, the cAMP-mediated regulation of NHE3 in these cells is solely mediated by PKA, demonstrating that cAMP utilizes different signaling pathways to regulate ion transport in different tissues (131, 132).

In the cortical collecting duct, diverse intermingled cell types regulate the reabsorption of ions and water in response to cAMP elevation. Distinct hormones mediate this cAMP increase in the different cells and act on the beta-adrenergic, calcitonin, or vasopressin receptor. Epac1 activation was shown to be responsible for the calcitonin-induced stimulation of H,K-ATPase activity in the alpha-intercalated cells of the rat renal collecting duct (130). In contrast, H,K-ATPase activation in response to βAR stimulation in the beta-intercalated cells is completely PKA dependent (130). Finally, 007 stimulation mimics vasopressin-induced intracellular Ca^{2+} mobilization and, thus, apical exocytosis of the water channel aquaporin-2 in isolated rat inner medullary collecting ducts (133). The role of Epac in the regulation of membrane channels extends from its function in the renal epithelium, as Epac affects the activity of sodium channels in lung epithelium (134), Ca^{2+} and Cl^- channels in hepatocytes (135), and K^+ and Ca^{2+} channels in pancreatic beta cells and cardiomyocytes.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Since their discovery in 1998, Epac proteins have been implicated in many cAMP-regulated processes, ranging from insulin secretion to cardiac contraction and vascular permeability. The Epac-selective cAMP analog 007 (16) contributed to the recognition of many of these functions, but in some cases further verification may be required to confirm that the observed effects are indeed mediated by Epac. Conversely, due to the limited membrane permeability of 007, some functions of Epac may have been overlooked and may be demonstrated with the improved Epac agonist 007-AM (18).

In addition to identifying and describing Epac's biological functions, attaining a complete understanding of its temporal and spatial regulation remains a challenge. Doing so will offer an explanation for the various functions that Epac may fulfill even within the context of a single cell. It is likely that in this regard Epac resembles PKA, whose multiple cellular functions are regulated by its spatial environment established by distinct anchoring mechanisms. The multidomain structure of Epac indicates that it may have multiple binding partners, and indeed numerous interacting proteins have been described for both Epac1 and Epac2. Alternative splicing may further add to the complexity of the spatial regulation of Epac, and tissue-specific Epac2 splice variants that are differentially localized have been described (27).

Rap1 and Rap2 relay most of the effects downstream of Epac, although Rap-independent effects have been reported as well (30, 46, 136). In order for us to fully understand the mechanism of Epac-dependent cAMP signaling, it will be crucial to identify the effector proteins that link Epac and Rap to downstream biological effects. Some of the previously identified Rap effectors are clearly implicated in Epac-mediated effects. For instance, PLCε is essential in the Epac-mediated regulation of intracellular Ca^{2+} in cardiomyocytes (40), as is Krit1 in the regulation of

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the endothelial barrier function (107). Frequently, multiple downstream proteins are implicated in the same processes, indicating that Epac and Rap may activate several distinct pathways to coordinate one process.

Another major challenge lies in understanding the interconnectivity of Epac with other signaling pathways, which may include diverse feedback mechanisms. Most notable is the link between Epac and PKA. Both cAMP targets are often associated with the same biological process, in which they fulfill either opposite or synergistic effects. The interaction between the two signaling pathways has been studied in detail in cardiomyocytes, where Epac1 and PKA are targeted to the same molecular complex (48). This dual control may enhance the dynamic range of cAMP signaling, as PKA-mediated events are proposed to occur at much lower cAMP levels than the activation of Epac (137).

Finally, the role of Epac in disease is an important aspect of study. Increased levels of Epac1 expression have been observed in the brains of Alzheimer patients and in cardiac tissue of individuals with failing hearts (46, 138). It is unclear whether Epac contributes to the pathogenesis of these diseases, and beneficial effects of 007 on memory function in mice (74) instead suggest a compensatory role of Epac in Alzheimer disease. 007 has been further applied to mice to inhibit vessel leakage (94), implying that Epac1-selective agonists may be useful in the treatment of diseases with extensive vascular leakage such as septic shock and chronic inflammation. In addition, the role of Epac in integrin-mediated cell adhesion has been exploited to enhance homing of endothelial precursor cells to ischemic tissue and thereby to promote neovascularization (139). Similarly, induction of Epac-mediated cell adhesion by selective agonists may prove to be useful in inhibiting tumor metastasis. Finally, considering its role in insulin secretion, Epac2 selective agonists may be helpful in the treatment of diabetes.

SUMMARY POINTS

1. Epac1 and Epac2 are GEFs for the Rap small G proteins and are directly activated by cAMP.
2. In the autoinhibited state, binding of Rap to the catalytic region of Epac is sterically hindered by the regulatory region, which is released upon cAMP binding.
3. Compartmentalization of cAMP and of the Epac proteins underlies the spatial regulation of Epac signaling.
4. Replacement of the 2′OH group of the cAMP ribose, which interacts with a glutamate that is conserved in PKA and cAMP-gated ion channels but is absent in Epac, results in cAMP analogs that selectively target Epac.
5. Epac-selective cAMP analogs have helped to reveal cAMP-regulated processes that are mediated via the action of Epac. These include cardiac contraction, insulin secretion, and vascular permeability.
6. In most of these processes, Epac interconnects with signaling via PKA. Both cAMP effectors can also be targeted to the same molecular complex.

FUTURE ISSUES

1. The role of Epac in physiological processes and perhaps in pathogenesis must be further elucidated, and Epac should be validated as a therapeutic target.

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2. Further investigation of the different anchoring mechanisms of Epac, their joined regulation, and their contribution to specific cellular effects is needed.
3. The previously identified downstream proteins should be verified, and the Rap effector proteins and Rap-independent pathways that link Epac to its biological functions should be further identified.
4. The interconnectivity between Epac and other signaling pathways, in particular with the other cAMP target PKA, must be elucidated.

DISCLOSURE STATEMENT

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