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cAMP Sensor EPAC Proteins and Energy Homeostasis

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Abstract

The pleiotropic second messenger cAMP plays a critical role in mediating the effects of various hormones on metabolism. The major intracellular functions of cAMP are transduced by protein kinase A (PKA) and exchange proteins directly activated by cAMP (EPACs). The latter act as guanine nucleotide exchange factors for the RAS-like small G-proteins Rap1 and Rap2. While the role of PKA in regulating energy balance has been extensively studied, EPACs' impact remains relatively enigmatic. This review summarizes recent genetic and pharmacological studies concerning EPACs' involvement in glucose homeostasis and energy balance, through regulation of leptin and insulin signaling pathways. Additionally, the development of small molecule EPAC-specific modulators and their therapeutic potential for the treatment of diabetes and obesity are discussed.

Keywords

Exchange protein directly activated by cAMP (Epac); cAMP; energy balance; insulin secretion; leptin resistance

EPAC proteins, new kids on the block

Adenosine 3',5'-cyclic monophosphate (cAMP) is the prototypic second messenger discovered by Earl Sutherland and colleagues to play a critical role in mediating the intracellular effects of hormones [1]. The deciphering of the cAMP signaling pathway is of major historical significance in biology and has led to scientific breakthroughs including the discoveries of cAMP-dependent protein kinase/protein kinase A (PKA), G-proteins, and G-protein coupled receptors (GPCRs), for which Robert Lefkowitz and Brian Kobilka shared the latest Nobel Prize in Chemistry [2]. Since its initial discovery more than a half-century ago, cAMP has been shown to play important roles in almost all aspects of biological functions, including metabolic regulation [3]. In mammals, with the exceptions of cyclic nucleotide-gated (CNG) ion channels in photoreceptor cells, olfactory sensory neurons, and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels in cardiac sinoatrial node cells and neurons [4], the major cellular functions of cAMP are mediated by two families of ubiquitously expressed intracellular sensors: the classic PKAs, and the more recently discovered exchange proteins directly activated by cAMP (EPACs) [5, 6] (Box 1). The presence of two functionally different and highly coordinated sensors enables a more

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precise and cohesive control of intracellular cAMP signaling. Depending upon cellular context and their relative abundance, distribution, and localization, these two intracellular receptors may act independently, synergistically, or antagonistically in regulating a specific cellular function [7, 8].

EPACs exist in mammals as two isoforms, EPAC1 and EPAC2, produced by independent genes (Box 1). While EPAC1 is ubiquitously expressed, EPAC2 is predominantly expressed in the brain, liver, pancreas and adrenal gland [5, 6]. EPAC1 and EPAC2 act on the same immediate down-stream effectors, small GTPases Rap1 and Rap2, however, their cellular functions are different due to their distinct tissue distribution and their abilities to form signalsomes at various cellular loci through interaction with specific cellular partners. For details, please see review [9]. It is now well established that EPAC proteins are involved in numerous cAMP-mediated functions, and their roles in various diseases are increasingly appreciated [9, 10]. To date, most functional analyses of EPAC1 and EPAC2 have been performed *in vitro*. But the recent generation and use of EPAC1 and EPAC2 knockout mice has allowed for the study of these proteins *in vivo*. These *in vivo* analyses confirm some of the prior *in vitro* conclusions, but also reveal additional complexity and potential controversy. In this review, we will focus on the role of EPAC proteins in energy homeostasis and the development of obesity and diabetes, and discuss the potential of using small molecule modulators targeting these proteins, as an effective multi-mechanistic approach for the treatment of these chronic conditions.

EPAC and leptin resistance

Obesity is the result of a prolonged imbalance between energy intake and expenditure [11]. The identification of the leptin gene (*Ob*) provided a breakthrough in our understanding of obesity at the molecular level [12]. Leptin, an appetite suppressing hormone derived from adipose tissue, plays a key role in the central regulation of satiety and energy expenditure [13]. Leptin binds to and activates the “long form” of leptin receptor (OB-Rb or *LepRb*), a single-transmembrane-domain cytokine receptor expressed in the central nervous system (CNS) and particularly the hypothalamus [14]. In the arcuate nucleus (*Arc*), OB-Rb is expressed on the agouti-related protein (*AgRP*) and proopiomelanocortin (*POMC*) neurons, which upon activation express and release orexigenic and anorexigenic neuropeptides, respectively. Leptin binding to OB-Rb and activates intracellular signaling cascades including the JAK/signal transducer and activators of transcription (STAT) 3 and PI3K pathways [15-17]. A hallmark of increased adiposity and obesity is high circulating leptin levels that results in compromised leptin signaling in the hypothalamus and leptin resistance. Leptin resistance manifests itself as lack of a reduction in food intake, defective leptin receptor signaling, and a reduction in the phosphorylation levels of STAT3, as well as changes in the release of orexigenic and anorexigenic peptides from *AgRP* and *POMC* neurons [18, 19].

cAMP mediated signaling pathways are important for maintaining metabolic homeostasis and the effects of the glucagon/catecholamine-cAMP-PKA axis on energy balance have been well documented [20]. For example, deletion of the PKA 2 beta regulatory subunit (*RiIβ*) subunit in mice leads to increased expression of uncoupling proteins in brown adipose tissue, which results in an elevated metabolic rate and body temperature, and a lean phenotype with diminished white adipose tissue. These mice are also resistant to diet-induced obesity (DIO) and fatty liver [21]. On the other hand, the involvement of EPAC protein in leptin signaling and energy balance was only revealed recently. Elmquist and colleagues demonstrate that leptin mediated STAT3 phosphorylation is inhibited by cAMP elevating agents, such as forskolin and inhibitors of phosphodiesterases (PDE), enzymes that degrade cAMP [22]. This inhibitory effect of cAMP is not affected by the non-selective

PKA inhibitor H89, suggesting that it might be PKA independent. On the other hand, activation of EPAC using an EPAC selective cAMP analog, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP aka 007), blunts leptin signaling in the hypothalamus and impairs leptin-evoked depolarization of POMC neurons. Moreover, intracerebroventricular (ICV) infusion of 007 in mice blocked leptin-induced reduction of food intake [22]. These results suggest that pharmacological activation of the cAMP-EPAC pathway in the hypothalamus leads to leptin resistance.

Yan et al. combined both genetic and pharmacological approaches to determine which EPAC isoform modulates leptin action, and showed that EPAC1 plays a role in energy balance and leptin signaling *in vivo* [23]. C57BL/6 EPAC1 knockout (EPAC1^{-/-}) mice are more resistant to high-fat diet (HFD)-induced obesity, due to reduced food intake, and thus have reduced white adipose tissue and plasma leptin levels. However, they display heightened central leptin sensitivity as measured by STAT3 phosphorylation in the hypothalamus, and EPAC1^{-/-} mice on a HFD are more glucose tolerant than wt mice. Furthermore, pharmacological inhibition of EPAC1 by ESI-09, resulted in reduced plasma leptin *in vivo* and enhanced leptin signaling in organotypic wt hypothalamic slices from wild type mice [23]. Collectively, these results are in agreement with the previous study using an EPAC selective activator [22], and suggest that EPAC1 plays an important role in regulating adiposity and energy balance by modulating leptin signaling. While mice on HFD have increased Rap-GTP levels, an indication of elevation of EPAC signaling, in the hypothalamus [22], at this time studies that directly link EPAC hyper-activation in the hypothalamus to states of obesity or leptin resistance in humans are lacking.

Cross-talk of signaling pathways

To date, the contribution of EPAC1 to leptin signaling has been largely focused on the JAK/STAT3 pathway [22, 23]. *In vitro* studies in cell culture have revealed that EPAC1 activation leads to the induction of suppressor of cytokine signaling-3 (SOCS-3), a negative regulator of STAT3 and leptin receptors signaling [24, 25]. Indeed, suppression of leptin-mediated STAT3 phosphorylation by the EPAC selective activator 007 is associated with concomitant induction of SOCS3 [22], suggesting that EPAC1 induces leptin resistance through upregulation of SOCS3 and inhibition of STAT3 action. However, the cellular levels of SOCS3 in mouse hypothalamus are not affected by genetic deletion of EPAC1 or pharmacological inhibition [23]. The precise reason for this discrepancy between *in vitro* and *in vivo* studies is not known at this time. Considering the complexity of SOCS family of proteins, it is possible that instead of SOCS3, another member of the SOCS family is involved, or a compensatory effect has occurred in the EPAC1^{-/-} mice. On the other hand, the level of the SH2-containing phosphatase 2 (SHP2), another important regulator of leptin-JAK/STAT3 signaling [26], are elevated in the EPAC1^{-/-} hypothalamus [23]. While SHP2 can act both as a positive and negative regulator of leptin signaling *in vitro*, studies using tissue-specific SHP2^{-/-} mouse models demonstrate that SHP2 functions as a positive regulator sensitizing leptin signaling in the brain [27, 28]. Therefore, elevation of SHP2 is expected to increase leptin sensitivity in the hypothalamus as observed in EPAC null mice [23]. To date, most studies link leptin resistance to the defect of JAK2-STAT3 pathway, it has also been reported that the PI3K-PDE3B-cAMP, but not the JAK2-STAT3, pathway is impaired in DIO, as well as during the development of leptin resistance in hypothalamic neurons following chronic central infusion of leptin [29]. While no direct link between EPAC1 and PI3K-PDE3B has been established in hypothalamic neurons, it has been demonstrated that in human arterial endothelial cells, PDE3B binds directly to EPAC1 and modulates its ligand binding [30]. In addition, PDE3B recruits PI3K γ to the PDE3B-EPAC1 complex by directly binding to p84, a PI3K γ regulatory subunit. The formation of a PDE3B-based signalosome in which PDE3B integrates EPAC1 and PI3K signals, provides a

mechanism for EPAC1 to regulate PI3K activity [30]. Therefore, it is plausible that EPAC1 may also be involved in PI3K-PDE3B-cAMP associated leptin resistance if a similar signalosome is conserved in hypothalamic neurons (Figure 1).

Tissue-specific EPAC1^{-/-} mouse models are necessary to further determine if the apparent phenotypes of EPAC1 null mice are due to the loss of EPAC1 function specifically in peripheral tissues, the CNS, or both. For instance, some *in vitro* studies suggest that EPAC1 plays an important role in differentiation and lipolysis in adipocytes [31, 32], as well as regulation of skeletal muscle metabolic function [33-35] (Box3). Hence, tissue-specific EPAC1 deletion in these peripheral tissues is needed to better understand EPAC1's role in the overall regulation of energy homeostasis. However, it is worth noting that while genetic manipulations, such as knockout or transgenic mouse models, have been invaluable for unveiling physiological functions of a target gene through phenotypic characterization, several known limitations, such as compensation and/or adaptation, may mask the role of the gene in question. Additionally, there are now many examples where genetic and pharmacologic manipulations produce different phenotypes for the same target protein [36]. Considering that most therapeutics are small molecules, coupling new animal model studies and small molecule inhibitors, will not only provide additional insights into the function of EPAC1, but also may lead to the development of novel mechanism-based therapeutic strategies for leptin resistance and obesity. Interestingly, a recent study using an EPAC1^{-/-} mouse model with mixed genetic background showed that these mice develop β -cell dysfunction; they have defects in glucose stimulated insulin secretion (GSIS), elevated plasma triglyceride, severe DIO, and insulin resistance [37]. Thus, EPAC1 appears to play a role also in β -cell homeostasis and insulin secretion. However, it cannot rule out that the role of EPAC1 in glucose homeostasis in the β -cell is mediated through peripheral insulin target tissues such as the adipose, muscle, and liver. Follow-up studies aimed at defining the mechanistic role of each EPAC isoform in adiposity and glucose homeostasis, are needed. Some of these studies are discussed below.

EPAC and glucose homeostasis

EPAC2 in insulin secretion

GSIS is the fundamental pathway of insulin release from pancreatic β -cells [38] (Figure 2A). After transport into β -cells by facilitated diffusion, glucose undergoes oxidative phosphorylation, yielding an increase in the ATP/ADP ratio. The rise in ATP levels in turn leads to the closure of ATP sensitive potassium channels (K_{ATP}), triggering membrane depolarization and the opening of L-type voltage depended Ca^{2+} channels (VDCCs), which allow the influx of extracellular Ca^{2+} (Figure 2C). The cytosolic Ca^{2+} concentration is further elevated by Ca^{2+} induced Ca^{2+} release (CICR) and mobilization of internal Ca^{2+} stores (Figure 2E). The increase in Ca^{2+} is ultimately the stimulus of release for insulin containing vesicles. The secretion process is biphasic with a rapid high amplitude initial phase, followed by a weaker, but more sustained one [38].

GSIS is strongly potentiated by glucagon-like peptide-1 (GLP-1), an incretin hormone secreted by the intestinal endocrine L-cells in response to ingestion of food (Figure 2B) [39]. GLP-1 binds to its receptor GLP-1R, a GPCR on the surface of pancreatic β -cells, and stimulates the production of cAMP [39]. Soon after the discovery of EPAC, pharmacologic and genetic loss of function studies pointed to the involvement of EPAC2 in mediating GLP-1's insulin sensitizing effects, reviewed in [39]. The role of EPAC2 in GSIS potentiation has now been further elucidated by *in vitro* studies using a recently developed ester form of 007 (007-AM), which readily crosses the plasma membrane [40]. In mouse, rat, and human pancreatic β -cells, the 007-AM strongly potentiated first and second phases GSIS [41-43].

The generation and study of a mouse model lacking the expression of EPAC2A, the EPAC2 isoform expressed in the pancreas (Box1), provided great insight into the role EPAC2 in potentiating GSIS [44]. GSIS potentiation by the GLP-1 analog E4 was significantly reduced in pancreatic β -cells isolated from EPAC2A^{-/-} mice. The reduction affected both early and late phase secretion, although it was more drastic in the early phase. However, under glucose stimulation by itself, even under conditions of high glucose concentration, lack of EPAC2A had no impact on insulin secretion. The latter finding is corroborated by the fact that after an intraperitoneal glucose tolerance test (ipGTT), which normally does not lead to the production of intestinal incretin hormones [45, 46], EPAC2A^{-/-} mice fed chow diet were able to reduce plasma glucose to basal levels at the same rate as wt mice. On the other hand, concurrent administration of E4 during ipGTT did not accelerate glucose clearance in the EPAC2A^{-/-} mice as it did in wt mice. These results suggest that EPAC2A mainly facilitate the effects of GLP-1 on GSIS and does not significantly contribute to GSIS alone in lean mice. In light of the *in vitro* and *in vivo* findings showing EPAC2A's importance to incretin's secretagogue action however, it is surprising that after an oGTT, EPAC2A^{-/-} mice were as glucose tolerant as their wt counterparts [44], in concordance with an earlier study [47]. Oral administration of glucose at the utilized doses, is known to elicit production of intestinal incretins such as GLP-1 [46]. Hence, it seems contradictory that lack of EPAC2A blunts E4's effect on pancreatic islets and *in vivo*, but does not affect glucose tolerance in an oGTT. One potential explanation for this discrepancy is that EPAC2A^{-/-} mice exhibit markedly improved insulin sensitivity [44], which might enable these mice to compensate for impaired insulin potentiation by incretins in the absence of EPAC2A. The observed improvement in insulin sensitivity remains unexplained, but it suggests that EPAC2A might down-regulate insulin's effects in its target tissue. Glucose tolerance is multifactorial and in addition to insulin and incretin secretion, depends on insulin action in adipocytes, skeletal muscle, liver, and the hypothalamus. EPAC2A is not expressed in adipocytes, skeletal muscle, or liver tissue, but it is possible that suppression of EPAC2A in the CNS, where this isoform is expressed [48], leads to improved glucose tolerance. However, CNS-specific EPAC2A knockout models are required to test this theory. Additionally, insulin, through its receptor on β -cells of the pancreas is known to enhance GLP-1's effect on these cells [49], and therefore, the improved insulin sensitivity might have enhanced GLP-1's action on these cells independently of EPAC2A. Another plausible explanation is that the incretin levels released after an oGTT in mice are usually much lower [46] than those employed in the *in vitro* and *in vivo* experiments in this study [44]. It is possible that at physiological concentrations of GLP-1 in lean mice with normal insulin sensitivity and glucose homeostasis, lack of EPAC2A does not significantly reduce GLP-1's effect on GSIS. But when exogenous incretins are administered, their concentration is high enough to trigger the EPAC2A pathway. This possibility is supported by the fact that GLP-1 increases cAMP levels in a dose dependent manner [50].

While the absence of EPAC2A had little impact on GSIS in lean mice, it significantly inhibited glucose sensitivity in mice with HFD induced obesity, with the difference occurring mainly in the initial phase of insulin secretion. Furthermore, mice carrying a deletion of the PKA RI α subunit (Δ prkar1 α), which exhibit elevated PKA catalytic activity, are highly glucose sensitive and secrete drastically elevated levels of insulin in response to an ipGTT. However, crossing these mice with the EPAC2A^{-/-} mice reduced glucose sensitivity. β -cells isolated from the double KO mice had a significantly lower initial rise in GSIS, compared to those cells isolated from the Δ prkar1 α strain, although GSIS in both mouse lines was much higher than in wt mice. Collectively, these data show that EPAC2 is needed for optimal GSIS under conditions of increased demand for insulin secretion, such as during obesity or strong activation of the GLP-1 pathway [44]. EPAC2A's apparent facilitation of GLP-1 secretagogue action has potentially significant clinical implications as it has been shown that in the obese mice, incretin's contribution to GSIS and role in meeting

the increased insulin demand are drastically augmented [46], and in fact, administration of exogenous GLP-1 mimetics to obese diabetic patients improves glycemic control significantly [51]. In summary, EPAC2 might act by facilitating the increase in insulin secretion needed to combat obesity-induced insulin resistance. However, how obesity induces EPAC2 activity in pancreatic β -cells remains unclear. EPAC2 can mediate GSIS by several mechanisms involving the regulation of intracellular calcium dynamics and direct interactions with proteins that directly facilitate secretion of insulin granules (Box 4).

EPAC1's role in the pancreas?

EPAC2 is considered to be the main isoform mediating the function of EPAC in insulin secretion as its expression in mouse and human pancreatic islets is significantly higher than that of EPAC1 [41, 43]. Additionally, suppression of EPAC1 by shRNA affected neither basal insulin secretion nor GSIS or its potentiation by the GLP-1 analog E4 in mouse islets even when EPAC2A is simultaneously suppressed [44]; suggesting that EPAC1 plays no role in GSIS and any function it may have in pancreatic islets is non-redundant with EPAC2A. It is possible that EPAC1 in the pancreas serves to facilitate leptin signaling in this tissue as discussed below. Contrary to this study [44], a recent report showed that islets isolated from EPAC1^{-/-} mice had normal basal insulin secretion, but impaired GSIS [37]. However, in these global KO mouse models, there was a significant reduction in the expression of the glucose transporter Glut2 and the transcription factor PDX1, which is critical for β -cell function and insulin expression. As such, it is possible that islets from these mice could not adequately “sense” and respond to an increase in glucose levels.

Beyond insulin secretion?

cAMP is known to regulate the expression of the proglucagon gene (*Gcg*), which is processed into the hormones glucagon in pancreatic α -cells, where EPAC2 is also expressed, and GLP-1 and GLP-2 in enteroendocrine cells and certain brain neuroendocrine cells [52]. While PKA plays an important role in controlling the expression of this gene [52], a few recent *in vitro* studies also suggest the involvement of EPAC2 [53-56]. In rat primary pancreatic islets, cAMP, through EPAC2, enhances the phosphorylation and nuclear exclusion of the transcriptional regulator Oct-1, thus allowing stimulation of *Gcg* expression [56]. Furthermore, expression of a dominant negative EPAC2 reduced *Gcg* in the glucagonoma cell line InR1-G9, suggesting that suppression of EPAC2 inhibits *Gcg* expression [54]. Hence, it appears that EPAC2 plays an important role in glucose homeostasis on multiple levels, including positive regulation of the levels of GLP-1 and potentiation of its effect on GSIS. Furthermore, EPAC2 can potentially participate in the hypoglycemic response by promoting the production of glucagon, which is secreted by α -cells in the pancreatic islets in conditions of low plasma glucose in order to induce glycogenolysis and gluconeogenesis in the liver.

This multimodal model of EPAC2's role in glucose homeostasis has been challenged by a recent study showing that 007-AM failed to promote *Gcg* expression in GLUTag; a murine enteroendocrine L-cell line that expresses *Gcg* and secretes GLP-1 [57]. Furthermore, using a recently discovered EPAC2-specific inhibitor [58], the study also showed that inhibition of EPAC2 did not affect the induction of *Gcg* expression by cAMP. Additionally, over-expression of either a dominant negative or constitutively active form of EPAC2, led to a decrease in *Gcg* expression, indicating the previous finding that a dominant negative EPAC2 reduced *Gcg* expression [54], is probably a consequence of non-specific suppression by supra-physiological levels of EPAC2 [57]. However, EPAC-specific activators still significantly increased *Gcg* mRNA and the EPAC inhibitor ESI-05 reduced basal *Gcg* expression. Taken together, the results presented here cast some doubt on EPAC2's involvement in stimulating *Gcg* expression, but show that EPAC2 still plays a role in basal

expression of *Gcg* and promotes stability of *Gcg* mRNA [57]. It is also worth noting that this contradictory study was done in an immortalized cell line, GLUTag, whereas the results showing EPAC2's role in the nuclear exclusion of Oct1 were obtained in primary rat islets.

Targeting EPAC for therapeutic intervention

The studies reviewed here suggest that EPAC1 and EPAC2 play important roles in regulating leptin and insulin signaling, and represent attractive drug targets for the treatment of obesity and type 2 diabetes mellitus (T2DM). Small molecule EPAC-selective modulators have been developed (Box 2) and can be further explored for their therapeutic potential [58-61]. Considering that EPAC1 and EPAC2 exert diverse and distinct functions, isoform specific EPAC modulators, particularly EPAC1 specific inhibitors and EPAC2 specific activators are most desirable for therapeutic purposes. Obesity and T2DM are chronic conditions that are intimately linked and caused by dysfunction and dysregulation in the crosstalk among multiple systems, including the hypothalamus, liver, endocrine pancreas, skeletal muscle, and adipocytes (Figure 3). Hence, an evaluation of the therapeutic potential and possible drawbacks of specific small molecule EPAC modulators must take into account their effect on individual organs, as well as potential impact on the feedback loops and pathways linking the aforementioned systems.

Several studies have shown that independently of body weight control, the central actions of leptin improve glycemic control in obese diabetic rat and mouse models [62]. For instance, restoration of OB-Rb expression in OB-Rb-deficient mice, and in particular in POMC neurons, was sufficient to normalize blood glucose levels while having little impact on body weight [63, 64]. The mechanisms by which leptin signaling in the CNS improves glucose homeostasis are not completely understood, but include sensitization to insulin signaling in the hypothalamus [65], and in liver through the vagus nerve [66], in addition to insulin independent pathways [67]. These findings combined with previous reports showing that suppression of EPAC1 improves hypothalamic leptin sensitivity [22, 23], suggest that EPAC1 inhibitors might improve glycemic control in the context of obesity/diabetes and leptin/insulin resistance. Additionally, as EPAC1^{-/-} are resistant to DIO, pharmacologic inhibition of EPAC1 might lead to weight loss, which further enhances insulin sensitivity. Another regulatory pathway that needs to be considered in the evaluation of EPAC's therapeutic potential is the bi-directional insulin-leptin feedback loop (adipo-insular axis). Insulin increases leptin plasma levels by promoting its expression and production, while leptin decreases insulin expression and secretion [68]. It is thought that this bi-directional loop establishes a set point to regulate insulin levels in relation to adiposity, as the rising leptin levels with increased fat stores serve to diminish insulin's plasma concentrations, and subsequently its lipogenic effects [68]. Data from animal models and humans strongly suggest that dysregulation of the adipo-insular axis is a key factor in the development of T2DM. In obesity, hyperinsulinemia often precedes the development of insulin resistance; a phenomenon thought to occur because of leptin resistance in pancreatic β -cells; and thus ensues a self-perpetuating cycle in which hyperinsulinemia contributes to insulin resistance and further increases leptin levels, which in turn exacerbate leptin resistance [68]. Since leptin exerts its effects through the same pathways in the brain and pancreas, and EPAC1 is expressed in β -cells of the pancreatic islets [41, 43], albeit at low levels, it is conceivable that EPAC1 inhibitors might help in restoring normalcy to the adipo-insular axis. In fact, administration of leptin to lipoatrophic diabetes patients, who normally exhibit insulin resistance and low levels of leptin, dramatically improved glycemic control [69]. On the other hand, leptin had minimal efficacy in T2DM patients who exhibit both insulin and leptin resistance [70]. Together these clinical results support the notion that sensitization to leptin signaling, which can be achieved by inhibition of EPAC1, rather than administration of leptin, is an efficacious strategy to control hyperglycemia in obese patients.

Given that EPAC2 functions down-stream of GLP-1 and modulates K_{ATP} channels in pancreatic islets, activators of this protein may serve as effective anti-diabetic drugs. These drugs might be particularly efficacious since EPAC2 mainly facilitate the early phase of GSIS, which becomes defective in the early stages of T2DM and plays a major role the development of glucose intolerance in most patients [71]. Additionally, EPAC2 activators can potentially be used in combination with sulfonylureas (SFUs), which are a widely used class of anti-T2DM drugs that bind with high affinity to the SUR1 subunit of the K_{ATP} channel, leading to its closure, and subsequently Ca^{2+} influx and insulin secretion. In fact, several recent studies have shown that EPAC2 potentiates the secretagogue action of SFUs, as the former is activated by the latter [72]. While it is clear that SFUs activate EPAC2, the activation is most likely indirect and the exact mechanism by which SFUs activate EPAC2 remain undetermined [72].

One possible pitfall of using EPAC2 activators in the treatment of T2DM is their potential to increase glucagon levels. As discussed previously, although EPAC2's ability to increase glucagon expression and production is controversial, several studies show that this protein increases *Gcg* mRNA levels in pancreatic islets. Hyperglucagonemia is a key problem usually seen in diabetic patients and at least partially results from α -cell's inability to respond appropriately to hyperglycemia [73]. Hence therapies that elevate glucagon levels are obviously undesirable in the treatment of diabetes. However, this potential side effect of EPAC2 activators might be mitigated in two ways: increased insulin secretion by these drugs; as insulin inhibits glucagon secretion, and the addition of EPAC1 inhibitors. We speculate the latter class of molecules will combat hyperglucagonemia as it restores leptin sensitivity, which decreases glucagon levels [67].

Concluding remarks

In summary, the roles of EPAC1 and EPAC2 in energy balance are complex. In spite of recent major advances, our understanding remains limited and controversy persists in certain cases (Box 5). The generation of additional tissue-specific and conditional EPAC knockout animal models will help to further define the physiological functions of EPAC. At the molecular and cellular levels, the dissection of EPAC signalosomes within the context of compartmentalized cAMP signaling, will lead to further elucidation of EPAC specific signaling pathways. In addition, it is necessary to expand EPAC pharmacological tool kits by developing novel isoform specific small molecule modulators, particularly viable *in vivo* pharmacological probes. At present, there are no isoform specific EPAC agonists or EPAC1 specific antagonists available. These pharmacological tool kits will be invaluable for interrogating the physiological functions of EPAC isoforms and for developing potential mechanism based therapeutics targeting diseases where EPAC proteins are implicated.

Box 1

EPAC structure, isoforms and intracellular cAMP signaling

There are two isoforms of EPAC, EPAC1 and EPAC2, which are products of independent genes in mammals. EPAC1, which is ubiquitously expressed, was discovered by Bos and colleagues, by sequence-homology search of an expressed sequence tag database using the cAMP-binding domain (CBD) of PKA [5]. EPAC1 was found to mediate the PKA-independent Rap1 activation in response to cAMP. At the same time, EPAC1 and 2 were identified by Graybiel and colleagues, using a differential display method [6]. They named the proteins cAMP- regulated guanine nucleotide exchange factors (cAMP-GEFs). EPAC2 was also independently discovered by Seino and colleagues as a cAMP sensor coupled to the sulphonylurea receptor (SUR1), by yeast two-hybrid screening [74]. EPAC2 has three isoforms that differ only at the N-terminus

and have distinct expression patterns: EPAC2A is mainly expressed in CNS and pancreas, while EPAC2B and EPAC2C are restricted to the adrenal gland and liver, respectively [48]. EPAC1 and EPAC2 share extensive sequence conservation. Both proteins contain an N-terminal regulatory region and a C-terminal catalytic region (Figure I). The catalytic region of EPAC consists of a RAS exchange motif (REM), a RAS association (RA) domain, and a classic CDC25-homology domain (CDC25HD) responsible for nucleotide exchange activity. Whereas the regulatory regions of EPAC1 and EPAC2 share a Dishevelled/Egl-10/pleckstrin (DEP) domain followed by a CBD, an additional CBD N-terminal to the DEP domain is presented in EPAC2. The two CBDs of EPAC2 directly face each other in close contact, to form a continuous structural entity and each blocks the access of cAMP to the other binding site in the closed apo-EPAC2 crystal structure [75]. While this second CBD exerts much lower affinity towards cAMP and is not required for EPAC2 biochemical activity *in vitro*, it plays an important role for proper EPAC2 cellular targeting and physiological functions *in vivo* [76]. In addition, the interface between the CBDs in EPAC2 provides an allosteric regulation site for EPAC2 activation that can be explored pharmacologically [58]. X-ray crystal structure determinations of the apo-EPAC2 full-length protein [75] and the ternary complex of a deletion of EPAC2 in complex with a cAMP analog and Rap1B [77] have provided a “before and after” snapshot of the cAMP-induced activation process. Binding of cAMP triggers major structural reorganizations manifested by a localized “hinge” motion, which rotates the catalytic lobe about 90° sideways, frees the CDC25HD domain from the autoinhibitory regulatory lobe, and allows the binding of down-stream effector, the Ras superfamily small GTPases Rap1 and Rap2. This general model of EPAC activation based on X-ray crystallographic analyses is further supported by studies using various solution structural and molecular biophysical techniques [78-82].

Box 2

The discoveries of EPAC specific modulators

The discovery of EPAC-specific pharmacological probes has been a major endeavor in the research field. Initial efforts were focused on exploring the sequence divergence between EPAC and PKA. The realization of the absence of a highly conserved glutamate residue in EPAC proteins led to the development of a cAMP analog, 8- (4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (007) [83] (Figure I, Box 2). This critical glutamate residue forms hydrogen bonds with the 2'-hydroxyl of the ribose moiety of cAMP, which is required for high affinity binding of cAMP to PKA holoenzymes. By replacing the 2'-hydroxyl with a 2'-O-methyl group and the addition of 8-CPT moiety, 007 exerts about 100-fold selectivity towards EPAC1 over PKA and has become a widely used tool in EPAC-related research [84]. Nevertheless, limitations of the 007 class of compounds include low membrane permeability and poor cellular potency. Subsequently, a pro-drug, 007-AM, with enhanced membrane permeability has been developed [40, 42]. Despite this significant improvement, the biological applications of 007 related compounds are restricted by their off-target effects on inhibiting phosphodiesterases (PDEs) [85]. In addition, a series of recent studies have demonstrated that 8-CPT-conjugated cAMP analogs and their metabolites can exert diverse biological functions *via* cAMP-independent mechanisms [86-90]. These findings raise cautions over the specificity of 8-CPT-cAMP analogs as cAMP mimetics and stress the necessity of verifying data based on applications of 007 class compounds using an independent approach before making conclusions. The search of *de novo*, non-cyclic nucleotide EPAC selective inhibitors (ESI) bore fruit recently. Taking advantage of the large fluorescence signal changes associated with the binding of 8-NBD-cAMP to

EPAC2, a robust high throughput screening (HTS) assay was developed for the search of EPAC specific inhibitors [59]. A screen of a 14,400 drug-like diversity compound library leads to the discovery of several ESIs [58, 60]. While majority of the ESIs act as competitive inhibitors and block both EPAC1 and 2 by directly competing with cAMP binding, Some of the ESIs are isoform specific antagonists that exclusively inhibit EPAC2 but not EPAC1 presumably by binding allosterically to the interface formed by the two CBDs in EPAC2 [58]. Medicinal chemistry efforts in optimizing the potency and specificity of these ESIs have been also reported [91-93]. An independent screen of 640 diversity compounds using the $\Delta(1-148)$ EPAC1 led to the identification of a tetrahydroquinoline analog, CE3F4 that is capable of blocking EPAC1 activation. It is not clear if CE3F4 inhibits EPAC2 [61].

Box 3

EPAC1 in skeletal muscle

Skeletal muscle is a crucial organ in energy homeostasis as it is the main site of glucose and lipid utilization in the body [94]. Skeletal muscle fibers express several GPCRs that generate cAMP, like β -adrenergic receptors (β -AR), which play an important role in mediating metabolic functions in this tissue [95]. Two recent *in vitro* studies have implicated EPAC1 in regulating adrenergic signaling in skeletal muscle [33, 34]. It seems that EPAC1 links adrenaline signaling in muscle tissue to insulin-induced activation of the PI3K/AKT pathway, which is a major signaling node that has a wide ranging impact on muscle metabolism, including the inhibition of proteolysis and induction of protein and glycogen synthesis [33]. Treatment of rat soleus muscle with adrenaline alone did not affect PKB activation (phosphorylation), but potentiated insulin induced phosphorylation of this kinase [33]. The same potentiating effect was observed with the addition of the EPAC-specific agonist 007, while addition of the PKA inhibitor H89 did not attenuate adrenaline's ability to enhance insulin-induced activation of AKT. In fact, H89 increased insulin mediated AKT activation. The PI3K inhibitor wortmannin completely blocked AKT activation in the presence of adrenaline and insulin. Thus EPAC1 might regulate the cross talk between adrenergic and insulin signaling by mediating the former's upregulation of the latter's induction of the PI3K/AKT pathway, while PKA seems to inhibit AKT phosphorylation in this context. This conclusion was confirmed by a similar study in which EPAC1 potentiated insulin-induced AKT activation and the subsequent inhibition of muscle proteolysis in digitorum longus rat muscle [33]. In this study however, the cAMP/PI3K/AKT pathway also increased AKT phosphorylation and decreased proteolysis independently of insulin. In addition to its potential role in regulating protein break down, EPAC1 might have a significant impact on skeletal muscle metabolism. In a recent study, treatment of C2C12 myotubes with 007 increased mitochondrial biogenesis and activation of AMP-activated protein kinase [35], which positively regulates contraction induced glucose uptake, mitochondrial biogenesis, and fatty acid oxidation [96]. In summary, the studies suggest that EPAC1 is potentially involved in the inhibition of proteolysis and induction of mitochondrial biogenesis and AMPK. However, it is important to keep in mind that EPAC1's role in muscle metabolism has not been extensively studied thus far. The available data is based on *in vitro* experiments, which did not use EPAC-specific inhibitors and only utilized EPAC-specific agonists and PKA antagonists. Hence, it is not yet possible to draw definitive conclusions on this GEF's role in skeletal muscle metabolism.

Box 4**Mechanisms underlying EPAC2's role in insulin secretion**

EPAC2 potentiates GSIS most notably through the regulation of intracellular Ca^{2+} levels. It is now well established that EPAC2, through direct interaction and facilitation of K_{ATP} closure, mediates influx of extracellular Ca^{2+} and CICR to mobilize intracellular Ca^{2+} reserves [43, 97]. In addition to facilitating extracellular Ca^{2+} influx, EPAC2 plays a key role in mediating CICR [43, 98-100]. Recent studies have revealed that EPAC2 is primarily involved in the release of Ca^{2+} from endoplasmic reticulum (ER) reserves [99, 101]. Moreover, it has been shown that EPAC2 acts through the activation of a novel phosphoinositide (PI) phospholipase C isoform (PLC- ϵ), protein kinase C (PKC), and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), in a Rap1 dependent manner. Together, these results point to an intricate model in which EPAC2 controls the release of ER Ca^{2+} by activation of inositol trisphosphate (IP_3) receptors and PKC/CaMKII [101-103]. Furthermore, given EPAC2's interaction with the Kir6.2/SUR1 K_{ATP} channel, it is thought that EPAC2-stimulated phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis may occur in close proximity to K_{ATP} . Since PIP_2 increases K_{ATP} channel activity while also reducing the inhibitory action of ATP at the channel, EPAC2 activators are expected to deplete PIP_2 , thereby favoring channel closure in response to ATP [104] (Figure 2C).

In addition to regulating calcium dynamics, studies have implicated EPAC2 in insulin granule acidification / priming [105], by promoting plasma membrane exocytic and granule-to-granule fusions. EPAC2 might also directly interact with the fusion proteins Rim2 and Piccolo (a Ca^{2+} sensor) to mediate vesicle fusion [106] and facilitate the assembly of the SNAP (Soluble NSF Attachment Protein) REceptor (SNARE) proteins, vesicle associated membrane proteins (VAMP) and synaptosomal-associated protein 25 (SNAP-25). Both VAMP and SNAP-25 also mediate vesicle fusion [44] (Figure 2F). EPAC2 also enhances the trafficking of a readily releasable pool of insulin granules that is not pre-docked to the plasma membrane, by a yet unknown mechanism [107] (Figure 2D).

Box 5**Outstanding questions**

- Are the expression levels and/or activities of EPAC1 and EPAC2 altered in response to obesity and insulin resistance?
- What is the role of EPAC2 in leptin signaling in hypothalamus?
- Does EPAC2 affect *Gcg* expression and GLP-1 and/or glucagon production *in vivo*?
- Does EPAC1 play a significant role in insulin secretion? If so, what is the relationship with EPAC2 in this process?
- Does EPAC1 sensitize leptin signaling in the pancreas?
- How does the EPAC2/Rap1 pathway facilitate the trafficking of insulin vesicles?
- How do sulfonylurea drugs activate EPAC2?

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Highlights

- EPAC1 desensitizes leptin signaling in hypothalamus
- EPAC2 facilitates GSIS in response to GLP-1 and obesity induced insulin resistance
- Small molecule EPAC modulators have therapeutic potential for obesity and T2DM

Figure 1. Leptin and insulin signaling pathways in the hypothalamus

Leptin binding to its receptor (OB-Rb) induces receptor dimerization and JAK2 activation. Phosphorylation of the intracellular domain of OB-Rb by JAK2 leads to the recruitment and phosphorylation of STAT3. Phosphorylated STAT3 dimerizes and translocates to the nucleus where it activates target genes under the control of *pomc* promoter and suppresses *argp* promoter. Leptin can also control hypothalamic neuron functions by activating the PI3K/Akt pathway in a manner similar to insulin. EPAC1 may desensitizes leptin signaling by suppressing STAT3 activation as it has been shown that EPAC1 induces the expression of SOCS3, a STAT3 negative regulator, by recruiting CCAAT/enhancer-binding protein (C/EBP) transcription factors to the SOCS-3 promoter in endothelial cells.

Figure 2. GSIS potentiation by EPAC2

- Glucose enters pancreatic β -cells by facilitated diffusion and undergoes glycolysis and oxidative phosphorylation, leading to an increase in the ATP/ADP ratio.
- GLP-1 binds to its GLP-1R, which activates adenylyl cyclase (AC), which converts ATP to cAMP.
- The increase in ATP levels results in the closure of K_{ATP} , membrane depolarization, and influx of extracellular Ca^{2+} through VDCC. EPAC2 is activated by cAMP and directly interacts with K_{ATP} to facilitate the channel's closure. EPAC2, through Rap1, also activates PLC ϵ , which hydrolyzes PIP $_2$ in the vicinity of K_{ATP} , increasing the likelihood of channel closure.
- Through activation of Rap1, EPAC2 mediates the trafficking of a pool of insulin vesicles called "resting newcomers (RNC)", to replenish the "readily releasable pool (RRP)" located at the plasma membrane
- Activation of PLC ϵ /CaMKII and IP $_3$ release after PIP $_2$ hydrolysis, facilitates CICR as the former enhances RyRs' activity and the latter IP $_3$ R's.
- EPAC2 is involved directly in the fusion of insulin granules through formation of an EPAC2/Piccolo/Rim2/Rab3A complex and facilitation of SNARE complex assembly.

Figure 3. Therapeutic potential of EPAC small molecule modulators

EPAC1 inhibitors can potentially enhance hypothalamic leptin sensitivity, leading to an increase in satiety and energy expenditure, and augmentation of central and peripheral (liver) effects of insulin. EPAC2 activators can increase insulin secretion from pancreatic β -cells.

Figure I, Box 1.

Intracellular cAMP sensors and signaling pathways. (a) Generation of second messenger cAMP in response to the activation of the G-protein coupled receptor cascade at the cell membrane and subsequent activation of intracellular cAMP receptors PKA and EPAC. (b) Domain architecture of EPAC and PKA.

Figure I, Box 2.

Small molecule EPAC selective modulators. (a) EPAC selective cAMP analog 007 and its membrane permeable pro-drug 007-AM. (b) EPAC specific antagonists.