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Epac as a tractable therapeutic target

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ABSTRACT

In 1957, cyclic adenosine monophosphate (cAMP) was identified as the first secondary messenger, and the first signaling cascade discovered was the cAMP-protein kinase A (PKA) pathway. Since then, cAMP has received increasing attention given its multitude of actions. Not long ago, a new cAMP effector named exchange protein directly activated by cAMP (Epac) emerged as a critical mediator of cAMP's actions. Epac mediates a plethora of pathophysiologic processes and contributes to the pathogenesis of several diseases such as cancer, cardiovascular disease, diabetes, lung fibrosis, neurological disorders, and others. These findings strongly underscore the potential of Epac as a tractable therapeutic target. In this context, Epac modulators seem to possess unique characteristics and advantages and hold the promise of providing more efficacious treatments for a wide array of diseases. This paper provides an in-depth dissection and analysis of Epac structure, distribution, subcellular compartmentalization, and signaling mechanisms. We elaborate on how these characteristics can be utilized to design specific, efficient, and safe Epac agonists and antagonists that can be incorporated into future pharmacotherapeutics. In addition, we provide a detailed portfolio for specific Epac modulators highlighting their discovery, advantages, potential concerns, and utilization in the context of clinical disease entities.

1. Introduction

When cyclic adenosine monophosphate (cAMP) was first discovered in 1957, it received prompt attention from several research laboratories around the world (Rall and Sutherland, 1958; Sutherland and Rall, 1958). Extensive investigations into the structure, interactions, and functions of this central molecule immediately ensued, and the birth of the "second messenger" theory which revolutionized the field of molecular and cellular biology then followed (Robichaux and Cheng, 2018). Indeed, this theory elegantly explained how several hydrophilic extracellular signaling molecules rely on membrane receptors and intracellular second messengers to elicit their functions (Yan et al., 2016).

cAMP is generated from the nucleotide adenosine triphosphate (ATP) by the action of the enzyme adenylyl cyclase (AC) (Fig. 1). Ten isoforms of this enzyme are identified; ACs 1–9 are membrane-bound, while AC 10 is soluble and lacks transmembrane domains (Dessauer et al., 2017). Although they all lead to the production of cAMP, these isoforms are associated with different functions depending on their characteristic compartmentalization, which is defined by their differential localization into specific cellular compartments (Dessauer et al., 2017). The membrane-bound forms are generally activated by the alpha subunit ($G\alpha_s$) of stimulatory G protein coupled receptors (Robichaux and Cheng, 2018) (Fig. 1). Meanwhile, the soluble form is activated by bicarbonate and calcium ions (Schmid et al., 2014; Wiggins et al., 2018).

The action of cAMP is terminated when it is hydrolyzed by cyclic

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nucleotide phosphodiesterases (PDEs) into 5′-AMP (Fig. 1). Of the 11 families of PDEs, three are cAMP-specific (PDE-4, -7, and -8), three are cGMP-specific (PDE-5, -6, and -9), and five act on both cAMP and cGMP (PDE-1, -2, -3, -10, −11) (Houslay, 2010). Intriguingly, PDEs also display compartmentalization within the cell (Robichaux and Cheng, 2018). For instance, PDEs contribute to the creation of spatially distinct microdomains and prevent the extensive diffusion of cAMP throughout the cells (Zaccolo and Pozzan, 2002). In addition to ACs and PDEs, A kinase anchoring proteins (AKAPs) contribute to the compartmentalization of cAMP signaling. These scaffolding proteins orchestrate the formation of specific signalosomes by clustering the appropriate effectors together and tethering them to a specific organelle or cellular location (Dodge-Kafka et al., 2005; Dodge et al., 2001).

The aforementioned formation of distinct signalosomes explains the manifestation of a wide array of cAMP effects. These effects are tissue-dependent and require spatio-temporal coordination of many signals (Chao et al., 2019). In this context, cAMP utilizes a myriad of downstream signal transducing effectors, such as protein kinase A (PKA) (Musheshe et al., 2018; Taylor et al., 2013), cyclic nucleotide-gated channels (Biel and Michalakis, 2009; Kaupp and Seifert, 2002), Popeye domain-containing proteins (Schindler and Brand, 2016), cyclic nucleotide receptor involved in sperm function (Krahling et al., 2013), and exchange protein directly activated by cAMP (Epac).

Epac is characterized as a guanine exchange factor (GEF) that acts on Ras-related protein (Rap), a member of the Ras subfamily of protein GTPases. In this sense, Epac induces the conversion of GDP-bound inactive Rap into the GTP-bound active form (Caron, 2003, 2003d; de Rooij et al., 1998) (Fig. 1). Since its discovery by two research groups working separately in 1998 (de Rooij et al., 1998; Kawasaki et al., 1998), Epac continues to attract attention as a potential tractable target. Thus, this paper aims at comprehensively reviewing and discussing the structure, localization, and signaling of Epac proteins. Furthermore, insights regarding its potential integration into disease therapy through the design of selective agonists and antagonists are considerably offered.

2. Architecture of Epac proteins

Extensive efforts have been invested to delineate the architecture and dynamic activation-inactivation processes of Epac proteins. As expected, these proteins display substantial sequence homology with other cAMP-binding effectors and proteins with GEF activity (Cheng et al., 2008). Epac proteins are single polypeptides encoded by the *RAPGEF3* gene on chromosome 12q13.11 for Epac1 and *RAPGEF4* gene located on chromosome 2q31.1 for Epac2 with several isoforms existing for each (Banerjee and Cheng, 2015).

Structurally, Epac proteins have regulatory and catalytic domains (Altschuler et al., 1995; Vossler et al., 1997) (Fig. 2). The N-terminus functions as a regulator for the catalytically active C-terminus (Kawasaki et al., 1998). The regulatory subunit contains a Dishevelled/Egl-10/pleckstrin (DEP) domain and a regulatory cyclic nucleotide binding domain (CNBD-B) which binds cAMP (Consonni et al., 2012; Ponsioen et al., 2004). On the other hand, the catalytic subunit is composed of a Ras-association (RA) domain, a Ras exchange motif (REM), and a CDC25 homology domain (CDC25-HD) (Li et al., 2011). In specific, the CDC25-HD is mainly responsible for the catalytic GDP-to-GTP exchange, while REM stabilizes the interaction with the substrate without having any direct interference in the activity (Rehmann et al., 2003b).

Compared with this general structure of Epac, Epac2A is the only isoform known to have an additional cAMP binding domain termed CNBD-A, in addition to the CNBD-B that is classically present in other isoforms (Fig. 2). These two CNBDs come into close proximity to block the catalytic domain of Epac2A (Rehmann et al., 2003a, 2006). Some significant differences in the affinity of these CNBDs are known to exist. For instance, CNBD-A has a 70-fold lower affinity to cAMP compared to CNBD-B (Robichaux and Cheng, 2018). Epac2C, on the other hand, lacks the DEP domain which imparts a significant effect on its subcellular localization (Lewis et al., 2016).

Epac proteins display a form of autoinhibition. This is specifically achieved by the regulatory domain, CNBD-B, which sterically blocks the catalytic domain (Rehmann et al., 2003b) (Fig. 1). The process is mediated via a conserved 5 amino acid sequence ^{321}Val-Leu-Val-Leu--Glu^{325}. This sequence plays a crucial role in preserving the switchboard functionality through establishing an ionic interaction between CNBD-B and CDC25-HD (Rehmann et al., 2006; Schmidt et al., 2013). In addition, a hinge region exists between the CNBD-B and REM motifs that

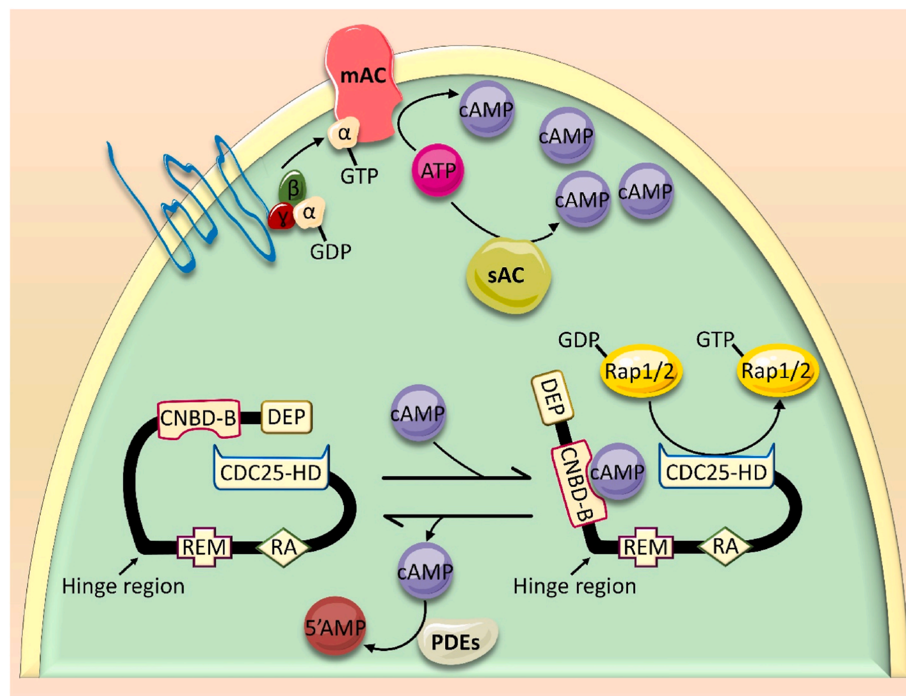


Fig. 1. The dynamics of activation and downstream signaling of Epac. The cascade begins with the activation of membrane-bound adenylate cyclase (mAC) or the soluble adenylate cyclase (sAC). These enzymes catalyze the conversion of adenosine monophosphate (ATP) into cyclic adenosine monophosphate (cAMP). The produced cAMP binds to cyclic nucleotide binding domain B (CNBD-B) of Epac, leading to the shift from the autoinhibited closed state to the open active state of the protein. In its active state, the CDC25 homology domain (CDC25-HD) domain of Epac is accessible to guanosine diphosphate-Rap (GDP-Rap), which is ultimately converted to guanosine triphosphate-Rap (GTP-Rap). The cascade is terminated by the hydrolyzation of cAMP back into 5′-AMP by the action of a phosphodiesterase (PDE).

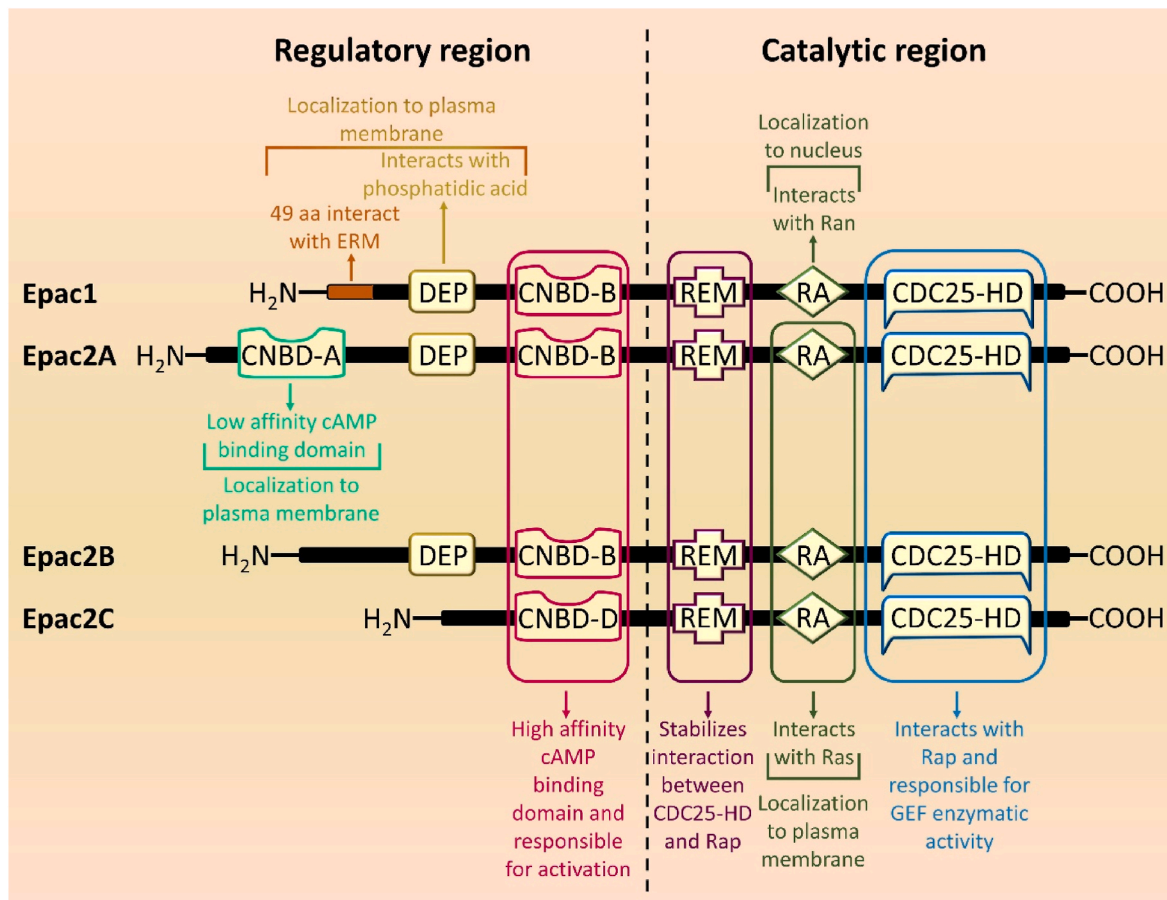


Fig. 2. The domains of Epac isoforms and the functions of each domain. All four isoforms share the high affinity cAMP binding domain (CNBD-B), which plays a crucial role in the transition from the inhibited to the activated state of the protein. The four isomers also share the domains CDC25 homology domain (CDC25-HD) and Ras exchange motif (REM), which are, respectively, responsible for the guanine exchange function (GEF) and for stabilizing the interaction with the downstream Rap protein. Although, the four isoforms share the same Ras-association (RA) domain, this domain plays different functions in Epac1 and Epac2. In the former, it orchestrates the localization to the nucleus via interacting with Ran, while in the latter it coordinates the localization to the plasma membrane via interacting with Ras. On the other hand, these isoforms have several structural differences. For instance, Epac2A has a unique additional cAMP binding domain, but with a lower binding affinity (CNBD-A). This domain participates in the autoinhibition/activation cycle of the enzyme and also plays a role in its localization to the plasma membrane. Moreover, Epac2C lacks a Dishevelled/Egl-10/pleckstrin (DEP) domain. Finally, the DEP domain of Epac1 and the first N-terminal 49 amino-acid sequence of this isoform mediate its localization to the plasma membrane via interaction with various effectors.

further contributes to the stability of the protein in its autoinhibited state (Tsalkova et al., 2012b). This self-regulation of Epac is relieved by the binding of cAMP. As such, cAMP reduces the ionic forces present within the hinge region favoring the active form of Epac rather than the inhibited one (Das et al., 2009) (Fig. 1).

3. Distribution, localization and downstream signaling

Epac1 and Epac2 mediate a plethora of physiologic functions in the different organ systems and tissues. These include, but are not limited to, neurotransmission (Sakaba and Neher, 2003), memory formation (Brandwein and Nguyen, 2019; Ster et al., 2009), pancreatic endocrine signaling (Tengholm and Gylfe, 2017), renal response to parathyroid hormone (Honegger et al., 2006), sperm motility (Kinukawa et al., 2006), bronchodilation (Robichaux and Cheng, 2018), and intestinal absorption (Coon et al., 2015). However, the two isoforms are not uniformly distributed throughout the body. Rather, they exhibit differential distribution in various tissue types. For instance, although the Epac1-encoding *RAPGEF3* gene appears to be ubiquitously transcribed in all tissues (Banerjee and Cheng, 2015), Epac1 proteins appear to be absent in ovarian tissue and abundantly present in the heart and kidneys (Robichaux and Cheng, 2018; Wehbe et al., 2020b). On the other hand, the three Epac2 variants (A, B, and C) are all products of the *RAPGEF4* gene and are the result of alternative splicing of mRNA. Each variant has its unique structure and exhibits a special expression profile. Epac2A appears to be highly expressed in the central nervous system, pituitary, and pancreas (Aumo et al., 2010; Wehbe et al., 2020a). Epac2B is differentially expressed in adrenal glands, whereas Epac2C is only identified in the liver (Hoivik et al., 2013; Ueno et al., 2001). This makes Epac more appealing as a drug target that allows tissue-specific modulation rather than disturbing universal body functions.

Epac proteins do not only exhibit a differential distribution in the various tissues and cell types, but they also display a differential spatial distribution within distinct compartments of the same cell, similar to or reminiscent of cAMP compartmentalization. Epac compartmentalization is supported and regulated by specialized domains of Epac proteins and several interactive effectors. Eventually, Epac1 and Epac2 can localize to the cell membrane, nuclear membrane, mitochondria, or cytosol guided by their molecular interactions (Huston et al., 2008; Magiera et al., 2004; Mei et al., 2002; Qiao et al., 2002). For instance, the DEP domain of Epac1 mediates its translocation to the plasma membrane (Ponsioen et al., 2009). Following cAMP binding to Epac, a conformational change in the DEP domain is induced priming its interaction with phosphatidic acid of the membrane (Hurley et al., 2002; Simons et al., 2009). Importantly, pharmacological interventions, which deplete phosphatidic acids, hinder this translocation (Consonni et al.,

2012). Another important mediator of the cell membrane localization is the ezrin-radixin-moesin (ERM) family of proteins (Gloerich et al., 2010). In specific, the interaction of the 49 N-terminal amino acids of Epac1 with ERM proteins contributes to the docking of Epac1 at the phospholipid bilayer (Gloerich et al., 2010; White et al., 2019). In contrast, targeting Epac1 to the nuclear membrane is dependent on the interaction of its RA domain with the small G protein Ran. When bound to GTP, Ran facilitates nuclear transport through its interaction with Ran-binding protein 2 (RanBP2), which is present in the vicinity of nuclear pores (Liu et al., 2010). In addition, evidence of Epac1 localization to the mitochondria has been documented; however, the exact domains and effectors involved are to be elucidated (Qiao et al., 2002). In this context, it has been shown that the mitochondrial targeting sequence is located among the first 73 N-terminal amino acids of the Epac1 protein (Qiao et al., 2002). It is worth mentioning that this portion shares a common feature, namely a net positive charge, with mitochondrial targeting pre-sequences (Maduke and Roise, 1996; Nakai and Kanehisa, 1992). On the other hand, Epac2A shows different mechanisms of localization. In fact, CNBD-A contributes to the plasma membrane localization of this isoform in pancreatic cells (Niimura et al., 2009). Moreover, in contrast to the RA domain of Epac1, the RA domain of Epac2 promotes its translocation to cell membranes via interacting with GTP-bound Ras (Li et al., 2006). The elicited differential localization of Epac isoforms to distinct cellular compartments contributes to signalosome formation and to the specificity of Epac signaling.

The wide array of Epac functions is mediated by interactions with several downstream effectors. Among these are the Rap proteins, which are small G-proteins of the Ras superfamily. Additionally, Epac also functions as a GEF for R-Ras protein that is, like Rap, a member of the Ras subfamily of protein GTPases (Lopez De Jesus et al., 2006; Wennerberg et al., 2005). Direct Epac-R-Ras interaction observed in HEK-293 cells ultimately leads to the activation of phospholipase D (Lopez De Jesus et al., 2006). Downstream targets of Epac2 have been best characterized following its regulation of insulin secretion from β-cells of the pancreas (Tengholm, 2012). This process seems to involve the direct interaction of Epac2 with at least three intracellular effectors namely, sulfonylurea receptor 1, Rim2, and Piccolo (Bos, 2006). Epac2 seems to interact directly with the sulfonylurea receptor 1, a subunit of ATP-dependent potassium (K^+) channels, inciting cell depolarization. Notably, cell depolarization is induced by closure of ATP-dependent K^+ channels and is essential for insulin secretion (Bos, 2006; Eliasson et al., 2003; Holz, 2004). Furthermore, Epac2 directly interacts with secretory granule-associated proteins involved in insulin exocytosis (Takeda et al., 2011). These include Rim2, a Rab-interacting protein, and Piccolo, a Rim2-associated protein (Fujimoto et al., 2002; Ozaki et al., 2000). Epac appears also to activate the small protein GTPase Rit involved in the differentiation of neurons (Shi et al., 2006). However, this activation is neither direct nor Rap-mediated suggesting that a different downstream effector acts as the missing link between Epac and Rit (Shi et al., 2006). Similarly, Epac is noted to activate the c-Jun N-terminal kinase in a Rap-independent manner (Fardoun et al., 2020; Hochbaum et al., 2003). This process is rather dependent on Epac's REM domain. One could speculate that this connection is either direct or mediated by a REM-interacting protein (Hochbaum et al., 2003). Undoubtedly, the application of proteomic analyses and in-silico studies on Epac can help fill the gaps in signaling pathways and uncover novel downstream effectors (Wehbe et al., 2020b).

4. Epac as a therapeutic target

Following more than two decades of extensive research since its discovery, the involvement of Epac in physiology and pathophysiology has been well-established and supported by vast evidence. Indeed, Epac is implicated in the pathogenesis and progression of cancer (Kumar et al., 2017; Misra and Pizzo, 2013; Wehbe et al., 2020b), cardiovascular disease (Fardoun et al., 2020; Jeyaraj et al., 2012; Lezoualc'h et al., 2016; Wehbe et al., 2020a; Yang et al., 2021), neurological disorders (Kelly et al., 2009; McPhee et al., 2005; Sarkar et al., 2009; Srivastava et al., 2012; Vina et al., 2021; Wagner et al., 2021), diabetes mellitus (Schmidt et al., 2013; Yang et al., 2022; Zummo et al., 2022), chronic obstructive pulmonary disease (COPD) (Laudette et al., 2018; Oldenburger et al., 2012), idiopathic pulmonary fibrosis (Cao et al., 2021), renal disease (El-Mokadem et al., 2021), and nicotine addiction (Liu et al., 2019). These findings strongly implicate the potential utility of Epac as a therapeutic target in the treatment of these pathological conditions. Not only that, but several characteristics of Epac make it more appealing for pharmacological modulation and a better alternative to certain existing drugs. Although Epac is a pivotal player and a promising molecular target in all the aforementioned diseases, here we dissect its specific implication in cancer and cardiovascular disease, which are, by far, the most significant contributors to global morbidity and mortality.

4.1. Involvement in cardiovascular disease

The role of Epac in the cardiovascular system has been extensively studied and established by many groups including ours (Eid, 2012; Eid et al., 2008; Fardoun et al., 2020, 2022; Garcia-Morales et al., 2014; Jeyaraj et al., 2012; Welter et al., 2020; Zieba et al., 2011). In the context of atherosclerosis, a recent study established a connection between Epac1 activation and the development of atherosclerotic plaques (Robichaux et al., 2020). Using Epac1$^{-/-}$ knockout mice, this study showed that Epac1 is a primary player in the uptake of oxidized low-density lipoproteins (ox-LDL), a crucial step in the formation of endovascular atherosclerotic plaques (Robichaux et al., 2020). The knockout mice were less prone to the formation of foam cells or atherosclerotic lesions (Robichaux et al., 2020). Moreover, it was observed that the Epac1 agonist, 007-AM, which will be discussed later in this paper, resulted in a significant increase in the accumulation of lipids in macrophages of wild-type mice, but not Epac1 knockouts (Robichaux et al., 2020). The same group had previously shown that inhibiting Epac suppresses injury-induced neointima formation (Wang et al., 2016). This is in line with other studies showing a role for Epac in attenuating vascular inflammation, suppressing migration of vascular smooth muscle cell, and reducing atheroma formation (Jiang et al., 2017; Kato et al., 2015; Lehrke et al., 2015). Based on these findings, it would be crucial to further explore the potential of Epac1 inhibitors as therapeutic drugs to prevent the development or progression of atherosclerotic plaques in patients at risk. If such drugs are found to have a clinically significant protective impact, they can be integrated as a standard of therapy in future regimens for primary or secondary prevention of cardiovascular disease. It will be tempting to explore this possibility through rigorous testing on higher animal models and, later, through the design and execution of clinical trials with long follow-up.

A role for Epac in other cardiovascular diseases such as arrhythmia has also been noted. In this context, Epac1 activated the L-type calcium channels, leading to prolongation of the action potential and ultimately precipitating atrial fibrillation (Zhang et al., 2019). Moreover, activation of Epac1 appears to decrease rapid delayed rectifier potassium (IKr) current and increase action potential duration, which could induce a pro-arrhythmic effect (Zhu et al., 2021). In line with these findings, another study showed that Epac1 inhibition exerted protective effects against atrial and ventricular arrhythmias (Prajapati et al., 2019). Taken together, this further cements the argument that Epac modulators can perhaps be absorbed more into management of CVD.

4.2. Involvement in cancer

Accumulating evidence shows that Epac plays a significant role in proliferation, apoptosis, migration and invasion of cancer cells. Importantly, the impact of Epac and its downstream targets can be cancer-promoting or cancer-attenuating depending on the cancer type. For

instance, Epac promotes the proliferation of prostate cancer, pancreatic neuroendocrine tumor, and rectal cancer cells via its modulation of cyclins B1, D1, and E1, respectively (Ahmed et al., 2022; Wehbe et al., 2020b). On the other hand, Epac was found to inhibit the proliferation of glioblastoma and renal cell carcinoma through pathways involving MAPK and PI3K, respectively (Sugimoto et al., 2013; Vacas et al., 2012). In a similar fashion, Epac can influence the migration and invasion potential of cancer cells in diverse patterns. For instance, Epac promotes migration or metastasis of breast, cervical, melanoma, pancreatic, fibrosarcoma and lung cancer cells, while it suppresses the metastatic potential of bladder and ovarian cancer cells (Almahariq et al., 2013; Baljinnyam et al., 2011; Harper et al., 2010; Krishnan et al., 2022; Wehbe et al., 2020b). More recently, it was shown that in breast cancer, Epac plays an important role in lipid droplets dynamics, a hallmark of carcinogenesis (Silva et al., 2023).

4.3. Why is Epac a promising potential target?

It is not surprising that drugs which modulate intracellular cAMP have been used to treat various pathological conditions. However, given that cAMP is a ubiquitous molecule and crucial for signaling across many tissues and systems, its therapeutic targeting would inevitably generate unfavorable systemic adverse effects (Almahariq et al., 2016). These can include gastrointestinal discomfort, coagulopathies, insomnia, depressive mood, palpitations, and arrhythmias (Chong et al., 2017, 2018; El-Hachem et al., 2020). Herein, as a basic tenant in pharmacotherapeutic approaches, the more specific a drug is, the less unwanted adverse effects would be expected. Hence, targeting effectors downstream of cAMP, namely PKA-cAMP response-binding protein (CREB) axis and Epac, can provide a rational vessel for future pharmacologic endeavors. In this context, several studies have focused on the discovery of potent PKA modulators and their potential roles in disease therapy, especially cancer (Ishimoto et al., 2015; Sapio et al., 2014). Similarly, CREB has also been an attractive and specific therapeutic target that gained significant attention when it comes to the design of targeted agents (Sapio et al., 2020; Steven et al., 2020). It is crucial to acknowledge the importance of these two molecules as promising targets; however, in this manuscript, we focus on the appealing characteristics of Epac as a tractable therapeutic target. In specific, Epac would plausibly offer several advantages as its isoforms show differential distribution in various tissues and as each isoform orchestrates specific functions. Thus, the development of selective modulators permits targeting one isoform while sparing the others (Wang et al., 2017a). In other words, Epac modulation is promising in terms of achieving tissue-specific effects rather than precipitating unwanted systemic ones. In this context, it was recently shown that in the setting of traumatic spinal cord injury treatment, Epac2 activators can be superior to cAMP modulators which precipitate ubiquitous side effects (Guijarro-Belmar et al., 2021). Likewise, Epac1-selective pharmacologic inhibition protects neuronal cells from ferroptosis, a cell death mechanisms associated with neurodegenerative disease (Musheshe et al., 2022). It is important to be cautious here that this relation between Epac1 and cell death is not universal. For instance, Epac1 can induce, rather than suppress, death of other neuronal cells, namely retinal ganglion cells (Liu et al., 2020).

One of the prominent notions about Epac's function is that its signalosome formation and intracellular localization strongly advocate the possibility of subcellular domain-specific targeting (Schmidt et al., 2013). This can be achieved indirectly by targeting specific AKAPs, ERM proteins, or other scaffolding players and disrupting certain signalosomes (Christian et al., 2011; Dekker et al., 2010; Patel et al., 2010). Alternatively, this can be accomplished by directly targeting specific localization sequences and domains in Epac itself using genetic or molecular tools. Consequently, blocking the subcellular trafficking of Epac into a certain compartment interrupts the protein's functions in that compartment while keeping its other cellular functions preserved. Utilizing compartmentalization and subcellular localization of Epac allows for selective targeting, which would allow for minimal unwanted cellular disturbances. For instance, thyroid stimulating hormone (TSH) signals via Epac1 and PKA to promote the proliferation of thyrocytes. For proper transduction, Epac1 and PKA co-localize to the plasma membrane through their interaction with ERM (Hochbaum et al., 2011). The disruption of this signalosome formation by silencing Radixin expression leads to preferential Epac localization to the nucleus rather than the plasma membrane and blocks the cascade (Hochbaum et al., 2011). By analogy, we speculate that genetic or molecular targeting of the Epac1 amino acid sequence that particularly interacts with Radixin will generate similar results. Notably, blockage of TSH signaling can be utilized in ameliorating TSH-induced thyroid hyperplastic conditions with minimal effects on other Epac signalosomes and isoforms.

Experiments on knockout mice have shown that certain physiological roles can be preserved if the expression of one Epac isoform is impaired due to compensation by the other isoform. In fact, when either Epac1 or Epac2 was knocked out in mice models, they did not exhibit deleterious phenotypes or a failure to thrive (Robichaux and Cheng, 2018). On the other hand, mice in which both Epac1 and Epac2 were knocked out displayed novel cognitive impairments not seen in single knockouts (Yang et al., 2012). This suggests that the two isoforms can play some interchangeable roles that will be preserved even if one isoform was knocked out (Robichaux and Cheng, 2018; Schmidt et al., 2013). We speculate that, similarly, the inhibition of one Epac isoform will not pose an irreversible impairment on certain physiologic functions.

As such, we expect Epac modulators to be effective drugs given the implication of Epac in a plethora of pathologies and, in several cases, the dependence of the pathologic phenotypes on a high level of expression and activity of the protein. This is well-documented in several pathologies such as cancer (Almahariq et al., 2013, 2015a; Wehbe et al., 2020b), cardiovascular disease (Fujita et al., 2017; Laudette et al., 2019), and neurodegenerative disorders (Suzuki et al., 2010). For instance, a cohort study of 141 gastric cancer patients shows an overexpression of Epac1 in gastric malignant cells. Moreover, the generated Kaplan-Meier curves reveal a significant correlation for overexpression of Epac1 with poorer prognosis and lower survival (Sun et al., 2017). Similarly, Epac1 is abundantly detected in breast cancer cells, where its blockade significantly attenuates the proliferation and invasiveness of these cancerous cells (Kumar et al., 2017). In addition, Epac modulators do not only appear to be promising as drugs for cancer chemotherapy in the classical sense, but they also carry the potential to potentiate the efficacy of cancer immunotherapy, which has been attracting a lot of interest from the scientific and medical community in the past two decades. In this context, Epac is known to mediate immunosuppressive roles in the tumor microenvironment as it participates in the function of regulatory T cells as attenuators of the immune response of effector T cells (Almahariq et al., 2015b). Therefore, the utilization of Epac inhibitors as adjuvants of immunotherapy is expected to enhance the efficacy of this therapeutic modality. Hence, it is worth directing the attention of the research community towards this possibility to further delve into its details and assess its clinical significance.

Apparently, Epac modulators represent promising drugs which could be integrated in many future treatment regimens as independently acting agents and adjunctive therapies as well. In fact, it has been shown that co-administration of Epac inhibitors synergistically enhances lithium anticancer effect against pancreatic tumors (Wang et al., 2017b). Additionally, Epac modulators can be used to potentiate the actions of other drugs and to offset their unwanted collateral effects. For instance, the therapeutic effects of PDE-4 inhibitors against asthma and COPD are frequently jeopardized by the pro-inflammatory effects that result from the activated cAMP-Epac pathway in macrophages (Hertz et al., 2009). Taken together, it is suggested that the concomitant use of Epac inhibitors with PDE-4 inhibitors may potentiate the latter's actions and counteract their unwanted pro-inflammatory effects (Hertz et al., 2009).

Nevertheless, it is important to acknowledge that Epac modulators

still have a long way of preclinical studies on higher animal models followed by clinical trials before they can be integrated into treatment regimens. Moreover, it would be important to investigate how combining Epac and PKA modulators could affect efficacy, or help reduce the unwanted signaling from other effectors downstream of cAMP.

5. The design of Epac modulators

5.1. Agonists

The mechanism and molecular interactions underlying Epac activation by cAMP dictate that Epac agonists be mainly derived from cAMP (Table 1 and Fig. 3). However, it is important to note that the CNBD domain of Epac shares several structural and molecular characteristics with the cAMP-binding domain of the regulatory subunit of PKA (Cheng et al., 2008). Therefore, this evolutionary conservation of cAMP-binding domains makes it challenging to activate either Epac or PKA without activating the other. Fortunately, this was made possible by the identification of a glutamate residue in the binding domain of PKA, known to be engaged in the formation of a hydrogen bond with the 2′-hydroxyl group (2′-OH) of cAMP (Christensen et al., 2003; Enserink et al., 2002). This hydrogen bonding is crucial for the affinity of cAMP or any cAMP-derived analogue to PKA. It was accordingly predicted that modifying this 2′-OH will result in cAMP analogues that preferentially bind Epac but not PKA (Christensen et al., 2003; Enserink et al., 2002). Notably, the 2′-alkylated analogues, including 2′-O-methyl-cAMP (2′-O-Me-cAMP), have been shown to be up to 100 times more selective for Epac than PKA (Wang et al., 2017a).

Table 1
Pharmacological characteristics of Epac agonists.

Name	Isoform	Binding Domain	Comments	References
8-pCPT-2′-O-Me-cAMP	Epac1/Epac2 (preferential towards Epac1)	CNBD-B	- Super agonist - Three times maximal activity for Epac1 compared to cAMP - Attenuates inflammatory responses in the lung and GI tract - Inhibits tubulointerstitial inflammation in mice with diabetic nephropathy	(Christensen et al., 2003; Enserink et al., 2002; Schwede et al., 2015; Song et al., 2022; Wang et al., 2017c; Yang et al., 2022)
pCPT-2′-O-Me-cAMP-AM	Epac1/Epac2 (preferential towards Epac1)	CNBD-B	- Membrane permeable - Hydrolyzed back into 8-pCPT-2′-O-Me-cAMP inside the cells - Promotes insulin secretion from pancreatic Beta cells	(Veluthakal et al., 2018; Vliem et al., 2008)
Sp-8-pCPT-2′-O-Me-cAMPS	Epac1/Epac2 (preferential towards Epac1)	CNBD-B	- PDE resistant	(Poppe et al., 2008; Schwede et al., 2015)
Sp-8-BnT-cAMPS	Epac2	CNBD-B	–	Schwede et al. (2015)
Sulfonylureas	Epac2	CNBD-A	- Used as anti-diabetic drugs - Controversy over whether they bind directly to Epac2	(Shibasaki et al., 2014; Tsalkova et al., 2011; Zhang et al., 2009)

It is important to mention that the selectivity of these agents can be further enhanced by the introduction of phenylthiol groups at position 8 of the adenine base (Christensen et al., 2003). In fact, the analogue 8-para-chlorophenylthio-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP), known as 007, confers three-fold greater selectivity for Epac than 2′-O-Me-cAMP. Of note, these aforementioned analogues are also considered super agonists of Epac1, as they show greater potency than cAMP itself (Christensen et al., 2003). In particular, the maximal activity achieved by 8-pCPT-2′-O-Me-cAMP activation of Epac1 is three times higher than that achieved by cAMP (Enserink et al., 2002)(Table 1 and Fig. 3). On the other hand, the increase in affinity and maximal activity is not observed towards Epac2 indicating that 8-pCPT-2′-O-Me-cAMP is a preferential agonist of Epac1 (Schwede et al., 2015). This is attributed to a single amino acid residue difference, which is Gln-270 in Epac1 versus Lys-405 in Epac2 (Schwede et al., 2015). Several *in vivo* experiments have employed 8-pCPT-2′-O-Me-cAMP as an Epac activator. These studies show a great potential for this drug in attenuating the inflammatory response and increased vascular permeability that mediate acute lung injury in response to lipopolysaccharides (Wang et al., 2017c). Furthermore, it appears to play a similar protective role against spontaneous colitis through maintaining endothelial barrier integrity in the gastrointestinal tract of IL-$10^{-/-}$ mice (Song et al., 2022).

However, one concern with the use of 8-pCPT-2′-O-Me-cAMP is its inability to cross the plasma membrane, which then leads to decreased bioavailability. The observed lipophobic characteristic is imparted by the negatively charged phosphate groups (Vliem et al., 2008). This fact has led to the development of a membrane-permeable esterified analogue through the addition of an acetoxymethyl group. This chemical modification creates a prodrug, 8-pCPT-2′-O-Me-cAMP-AM (007-AM), which readily crosses biological membranes and is converted back to the active form, 8-pCPT-2′-O-Me-cAMP, within the cells (Vliem et al., 2008)(Table 1 and Fig. 3). Prodrug conversion confers substantial entrapment of the active form inside the cells with an enhanced intracellular availability and efficacy. However, the reaction hydrolyzing the prodrug to the parent drug results in the release of formaldehyde and acetic acid (Vliem et al., 2008). Although concerns have been raised regarding the possible toxicity and side effects of these byproducts, they have faded by the virtue that similar metabolite releasing drugs, such as ampicillin and acetylsalicylic acid, are already in clinical use and elicit good safety profiles (Vliem et al., 2008). In fact, 8-pCPT-2′-O-Me-cAMP-AM has been shown to rescue the impaired insulin secretion in response to glucose by pancreatic islet cells obtained from diabetic human donors (Veluthakal et al., 2018).

Another challenge posed by 8-pCPT-2′-O-Me-cAMP is its potential to interact with PDEs, either as a substrate or as an inhibitor. The effects of 8-pCPT-2′-O-Me-cAMP are self-limiting as the molecule is hydrolyzed by PDEs 5 and 10 (Poppe et al., 2008). This issue has been rectified with the introduction of a phosphorothioate bond, which imparts resistance to PDEs hydrolysis. Phosphorothioate bond is formed by stereo-specific replacement of the axial oxygen in the cyclic phosphate group with a sulfate moiety, yielding the Sp-8-pCPT-2′-O-Me-cAMPS compound (Schwede et al., 2015)(Table 1 and Fig. 3). Although the creation of such bond offers better drug stability, it does not hamper the inhibition of PDEs 1, 2, and 6 by 8-pCPT-2′-O-Me-cAMP. On the contrary, Sp-8-pCPT-2′-O-Me-cAMPS exacerbates the inhibition of PDEs 1, 2, 3, 5, 6, and 10 (Poppe et al., 2008), resulting in the accumulation of cyclic nucleotides, cAMP and cGMP, and the subsequent activation of their respective downstream effectors, PKA and PKG (Poppe et al., 2008). Unquestionably, this aspect should be addressed prior the introduction of such agonists into treatment regimens.

Compared with Epac1-selective agonists, Epac2-selective agonists have been equivocally the focus of many researchers. It has been shown that Sp-cAMPS modifications create super-agonists that amplify Epac2 maximal activity without altering the affinity (Schwede et al., 2015) (Fig. 3). However, further addition of a benzylthio group (BnT) at the 8

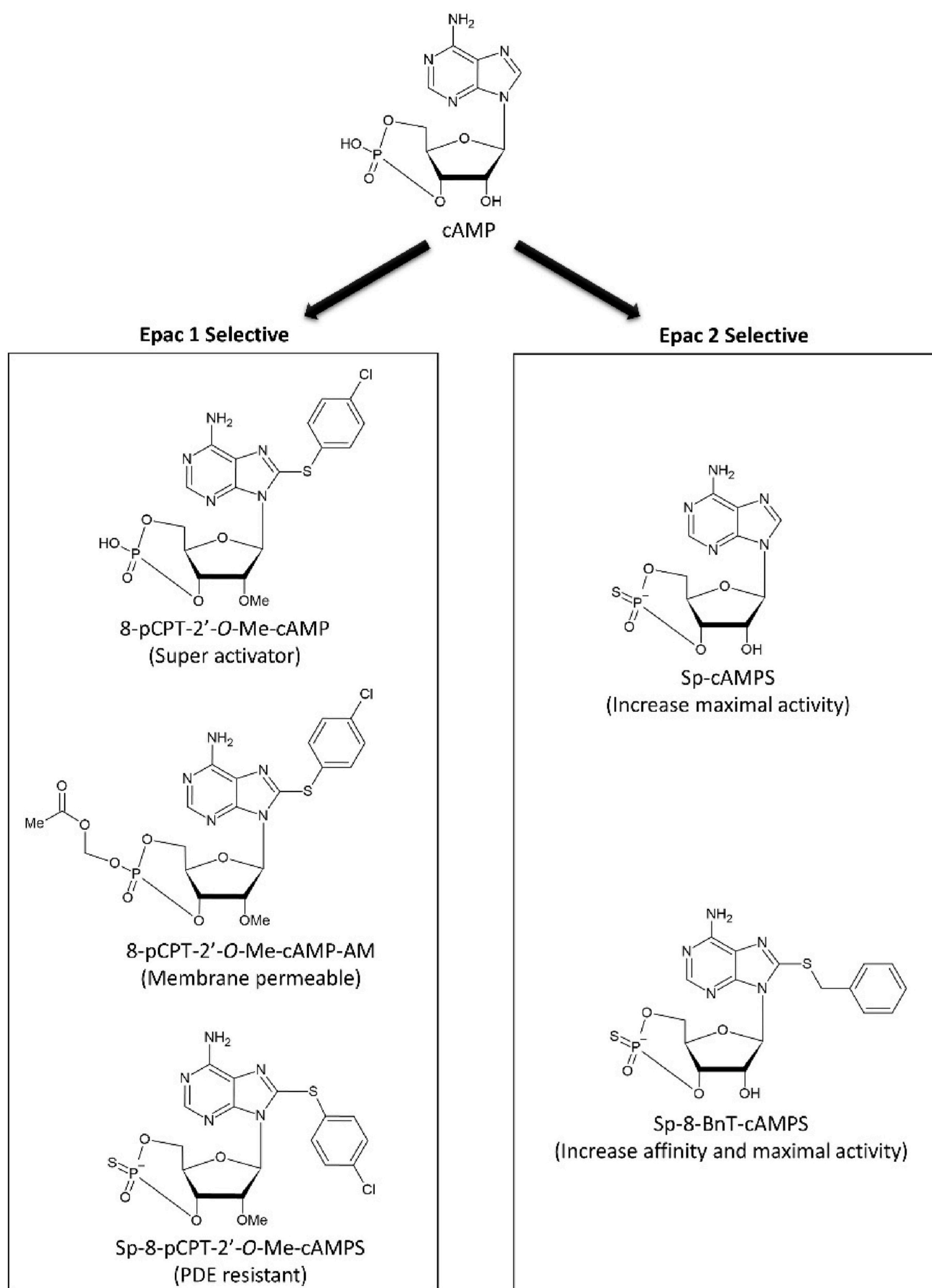


Fig. 3. The structures of cAMP, Epac1-selective agonists, and Epac2-selective agonists. As evident, all Epac agonists share an obvious structural homology with cAMP, however, slight modifications to each result in its selectivity and unique features. The 2′-O methylation of cAMP and the addition of 8-pCPT results in the creation of a super activator that has a higher affinity to Epac1 and higher potency in selectively activating it than cAMP. The addition of acetoxymethyl (AM) leads to a membrane permeable prodrug with a better bioavailability and that can achieve higher intracellular concentrations. Furthermore, the introduction of a phosphorothioate bond to these Epac1-selective agonists yields a compound (Sp-8-pCPT-2′-O-Me-cAMP) that is resistant to PDE hydrolysis. On the other hand, the addition of phosphorothioate bond to cAMP itself yields a compound that can achieve higher Epac2 maximal activity compared to cAMP alone. Moreover, an additional benzylthio group at position 8 leads to Sp-8-BnT-cAMPS which has a superior affinity and potency towards Epac2 vis a vis unmodified cAMP.

position of the adenine base results in increased affinity and maximal activity. In fact, the resulting compound, Sp-8-BnT-cAMPS, displays the most favorable potency profile for Epac2 activation (Schwede et al., 2015)(Table 1 and Fig. 3).

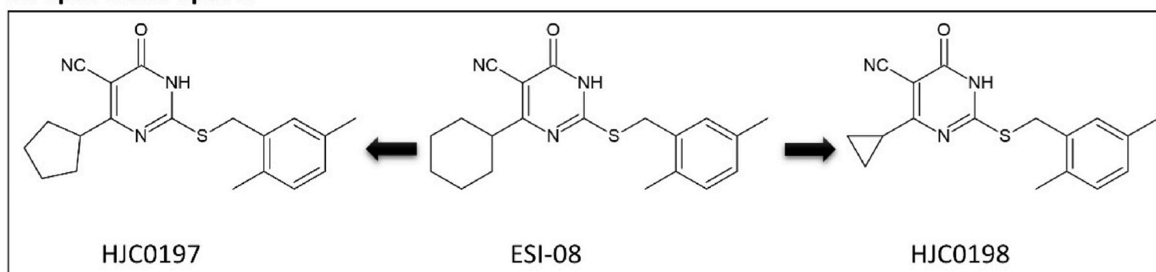
Interestingly, the widely used oral insulin secretagogues, sulfonylureas, have been proven to elicit an Epac2-activating role. Sulfonylurea-induced activation of Epac2 has been shown to potentiate insulin secretion mediated by closure of ATP-dependent potassium channels. In this context, studies have reported that *RAPGEF4* knockout mice are less responsive to sulfonylureas (Zhang et al., 2009). Contextually, fluorescence resonance energy transfer (FRET) analysis and binding experiments have shown a direct interaction between sulfonylureas and the allosteric site on Epac2 (Zhang et al., 2009). However, these findings could not be replicated by other research groups and their reliability remains questionable. Nevertheless, it was later elucidated that the nature of the biologic samples is critical for result validity and reproducibility. In contrast to those utilizing whole cell samples, experiments using purified full-length Epac2 and Rap1 have been able to show that sulfonylureas alone cannot bind and activate Epac2 (Tsalkova et al., 2011). This indicates that the activation seen within whole cell samples was not completely due to a direct Epac2-sulfonylurea interaction but rather due to other bridging players. A suggested model depicts cAMP as a crucial player (Shibasaki et al., 2014). In specific, Epac2A possesses a sulfonylurea binding region contained in CNBD-A. However, this region is not accessible to sulfonylureas if they are present alone due to the closed conformation that CNBD-A and CNBD-B form together (Shibasaki et al., 2014). Intriguingly, the presence of low to moderate amounts of cAMP breaks this conformation and allows the access of sulfonylureas to CNBD-A, suggesting a synergistic Epac2 activation by cAMP and sulfonylureas (Shibasaki et al., 2014). In contrast, high amounts of cAMP displace sulfonylureas due to the proximity of the binding regions of sulfonylureas and cAMP within CNBD-A (Shibasaki et al., 2014). Therefore, the hypoglycemic effects of sulfonylureas seem to be attenuated in the setting of high concentrations of cAMP. Intuitively, the impact of cAMP concentration on sulfonylureas-mediated activation of Epac may be one of the underlying causes for the discrepancy between studies. At any rate, the discussed implication of Epac2A in the augmentation of insulin secretion opens the door for further exploration into other Epac2A selective activators as a new generation of therapeutic drugs for type 2 diabetes mellitus. Another promising pharmaceutical investigation is the modification of existing sulfonylurea drugs to create analogues that have a greater avidity and potency in binding Epac2A, and thus a greater impact as insulin secretagogues.

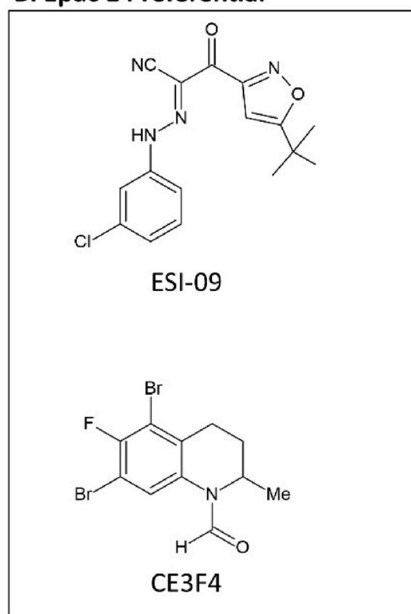
5.2. Antagonists

The implication of Epac in the pathogenesis of many diseases has provided a strong impetus for the development of antagonists with the ultimate goal of mitigating its actions (Table 2 and Fig. 4). Major milestones have been successfully achieved through the establishment of a high-throughput screening assay that allows the identification of potential Epac inhibitors. In specific, the assay is based on the detection of differences in intensity of emission from fluorescent 8-nitrobenzoxadiaole-cAMP (8-NBD-cAMP), before and after the introduction of the investigated drug (Tsalkova et al., 2012a). The intensity of fluorescence reflects the amount of labelled cAMP bound to Epac. Therefore, any significant drop in this intensity implies that cAMP has been displaced from the active site through direct competition or allosteric modulation by the tested drug (Tsalkova et al., 2012a). The application of this technique to a library of 14,400 compounds yielded the discovery of several Epac specific inhibitors (ESIs). Some of the subsequently established ESIs manifest isoform selectivity between Epac1 and Epac2. In specific, ESI-05 and ESI-07 were identified to be more selective inhibitors toward Epac2 than toward Epac1 (Tsalkova et al., 2012b) (Table 2 and Fig. 4). This selectivity is explained by the fact that these drugs inhibit Epac2 via binding to the interface between the two CNBDs,

Table 2
Pharmacological characteristics of Epac antagonists.

Name	Isoform	Binding Domain	Comments	References
ESI-05 ESI-07	Epac2	Interphase between CNBD-A and CNBD-B	- Locks Epac2 in auto-inhibited state - ESI-05 protects against neuronal cell death and brain edema - ESI-05 attenuates cocaine reinforcement in mice and might be a promising therapeutic agent in cocaine addiction	(Liu et al., 2022; Tsalkova et al., 2012b; Zhang et al., 2018)
ESI-08 HJC0197 HJC0198	Epac1/Epac2	CNBD-B (studied on Epac2)	- HJC0197 and HJC0198 are 5-cyano-6-oxo-1,6-dihydro-pyrimidine derivatives of ESI-08	Chen et al. (2012)
ESI-09	Epac1/Epac2 (preferential towards Epac1)	CNBD-B	- Exhibits good safety profile - Has a synergistic inhibitory effect with lithium on the proliferation of pancreatic cancer cells - Inhibits the proliferation and migration of human keloid fibroblast implicated in the pathogenesis of keloids - Reduces the morbidity and mortality associated with fatal rickettsiosis - Attenuates the replication of respiratory syncytial virus in epithelial cells and the associated inflammation	(Chen et al., 2013; Gong et al., 2013; Lezoualc'h et al., 2016; Lv et al., 2021; Singhmar et al., 2018; Sukhanova et al., 2017; Wang et al., 2017a; Wang et al., 2017b; Zhu et al., 2015)
CE3F4	Epac1	Tyr-242, Ile-243, Asp-267, Arg-294, and Gln-270 within CNBD-B	- Unconventional uncompetitive inhibitor - Locks cAMP-bound Epac in inactive state that is not accessible to Rap - Protects against atrial fibrillation - Attenuates chronic pain and hyperalgesia following spinal surgical procedures in a rat model (34988165)	(Ahmed et al., 2019; Boulton et al., 2018; Courilleau et al., 2012, 2013; Prajapati et al., 2019; She et al., 2021; Sonawane et al., 2017)
EPAC 5225554 **EPAC 5376753**	Epac1 (data suggests that they can inhibit Epac2 also)	Hinge region	- Noncompetitive inhibitors. - EPAC 5376753 has better membrane permeability and safety profiles	(Brown et al., 2014; Lezoualc'h et al., 2016)





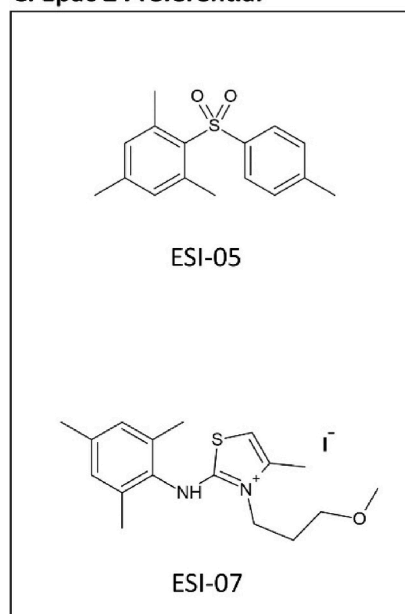


Fig. 4. The structure of Epac antagonists grouped according to their selectivity towards Epac1, Epac2, or both. ESI-08 and its structurally similar analogues HJC0197 and HJC0198 achieve a non-selective inhibition of both Epac1 and Epac2. Whereas ESI-09 and CE3F4 show a preferential antagonism towards Epac1. On the other hand, ESI-05 and ESI-07 show a preferential antagonism towards Epac2.

which does not exist in Epac1. Remarkably, these selective antagonists lock Epac2 in its autoinhibited state (Tsalkova et al., 2012b). In this context, the Epac2-selective inhibitor ESI-05 was used in rat model of traumatic brain injury, and it has shown promising protective effects against brain edema and neuronal cell death (Zhang et al., 2018). Furthermore, another confirmed Epac antagonist is ESI-08, which inhibits both Epac1 and Epac2 (Chen et al., 2012). However, 5-cyano-6-oxo-1,6-dihydro-pyrimidine derivatives of ESI-08, namely 6 h (HJC0197) and 6 g (HJC0198), display higher antagonistic activities against Epac2 (Chen et al., 2012)(Table 2 and Fig. 4).

A fourth identified Epac antagonist is ESI-09, which binds the common CNBD-B and inhibits both Epac1 and Epac2 (Chen et al., 2013; Lezoualc'h et al., 2016)(Table 2 and Fig. 4). However, it does have a slightly preferential antagonism towards Epac1 rather than Epac2 with IC_{50} values of 3.2 μM and 7.0 μM, respectively (Wang et al., 2017a). Distinctly, ESI-09 gained great attention and became widely used in Epac-related experiments. Nevertheless, one study raised a concern that ESI-09 might act as a non-specific-protein denaturant rather than as an Epac-selective inhibitor (Rehmann, 2013). This claim was refuted by other studies, which showed that ESI-09 protein-denaturing effects are not elicited at pharmacological effective doses (Zhu et al., 2015). Despite this, ESI-09 is still used in cell and animal models given its excellent pharmacological and toxicological profiles within its therapeutic window (Singhmar et al., 2018; Sukhanova et al., 2017). Moreover, ESI-09 has shown notable effects in preclinical studies highlighting its ability to inhibit the formation of keloids (Lv et al., 2021), decrease the morbidity and mortality of rickettsiosis (Gong et al., 2013), and reduce the replicability of respiratory syncytial virus (Gong et al., 2013).

The application of another fluorescence screening assay granted the discovery of CE3F4, an Epac1-specific inhibitor (Table 2 and Fig. 4). This compound is a tetrahydroquinoline derivative that acts as an unconventional uncompetitive inhibitor (Courilleau et al., 2012). As an enzyme, Epac1 has a catalytic substrate-binding site, CDC25-HD which binds Rap1, and an allosteric regulating site, CNBD-B which binds cAMP. Conventionally, classical uncompetitive inhibitors are compounds that show preferential binding to the enzyme-substrate complex, which is Epac1:Rap1 in this case (Ahmed et al., 2019; Robin et al., 2018). However, CE3F4, being an unconventional uncompetitive inhibitor, preferentially blocks the enzyme-allosteric activator complex, which is Epac1:cAMP in this context (Ahmed et al., 2019; Courilleau et al., 2013; Sonawane et al., 2017). The specific binding site of CE3F4, as identified by nuclear magnetic resonance (NMR) spectroscopy, is shown to fall within the CNBD-B of Epac1, but at a distinct subdomain other than the cAMP-binding one (Boulton et al., 2018). Indeed, this explains the lack of inhibitory effect for CE3F4 on Epac1 mutants devoid of CNBD-B (Ahmed et al., 2019). The specific amino acids crucial for this inhibition are Tyr-242, Ile-243, Asp-267, Arg-294, and Gln-270 (Ahmed et al., 2019). It is worth noting that CE3F4 selectivity towards Epac1 over Epac2 is dependent on the Gln-270 residue, whose equivalent is Lys-405 in Epac2 (Boulton et al., 2018). Once bound to its subdomain in

Epac1, CE3F4 locks the protein in the closed inactive form which is inaccessible to Rap1 (Boulton et al., 2018). This property warrants drug isoform selectivity which can be utilized purposefully in therapeutic interventions.

Finally, with the use of bioluminescence resonance emission transfer, two barbituric acid derivatives were identified as potential Epac-selective inhibitors (Brown et al., 2014)(Table 2). One of these is the compound 5225554, which was first identified and proven to inhibit the Epac-mediated activation of Rap1. However, several concerns, regarding cytotoxicity and limited membrane permeability, accompany the potential applicability of this drug (Brown et al., 2014). Accordingly, several modifications were introduced, which resulted in the thio-barbiturate derivative, 5376753. This compound has better membrane permeability and a better safety profile (Brown et al., 2014). Furthermore, 5376753 elicits greater inhibition of Epac owing to the π stacking between Trp-283 of Epac and the dichlorophenyl group, which is absent in 5225554 (Brown et al., 2014). Nevertheless, both compounds act as noncompetitive antagonists that bind Epac at its hinge region (Lezoualc'h et al., 2016).

It is worth mentioning that a novel quantitative structure–activity relationship model has been recently developed and validated (Mohamed et al., 2022). This model can pave the way towards a more efficient screening of Epac-specific agents that can be used in the pharmacologic targeting of this pivotal molecule.

6. Conclusion

Epac is a relatively recent discovery in the realm of molecular biology and pharmacology. Its structure, distribution, and localization, as well as its implication in several physiologic and pathologic processes make it a promising tractable target in the fight against many diseases. Its unique properties confer several advantages over other effectors that have been traditionally targeted. Nonetheless, pharmacological approaches along with design of selective agonists and antagonists, by the virtue of utilizing high-throughput screening and computer-assisted drug discovery, merit further attention into Epac's therapeutic potentials. To this end, further *in vitro* and *in vivo* experimentations are warranted to establish the safety, efficiency, and practicality of Epac modulators. Ultimately, well-designed studies are needed for data projection into humans in order to incorporate these agents in the future therapeutic regimens.

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CRediT authorship contribution statement

Hasan Slika: Writing – original draft, Data curation. **Hadi Mansour:** Writing – original draft, Data curation. **Suzanne A. Nasser:** Writing – original draft. **Abdullah Shaito:** Writing – original draft. **Firas Kobeissy:** Writing – review & editing. **Alexander N. Orekhov:** Writing – review & editing. **Gianfranco Pintus:** Writing – review & editing. **Ali H. Eid:** Conceptualization, Writing – review & editing, Formal analysis, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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N/A

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