EPAC2: A new and promising protein for glioma pathogenesis and therapy

Seidu A. Richard

Department of Medicine, Princefield University, Ho, Ghana, West Africa

Abstract

Gliomas are prime brain cancers which are initiated by malignant modification of neural stem cells, progenitor cells and differentiated glial cells such as astrocyte, oligodendrocyte as well as ependymal cells. Exchange proteins directly activated by cAMP (EPACs) are crucial cyclic adenosine 3',5'-monophosphate (cAMP)-determined signaling pathways. Cyclic AMP-intermediated signaling events were utilized to transduce protein kinase A (PKA) leading to the detection of EPACs or cAMP-guanine exchange factors (cAMP-GEFs). EPACs have been detected as crucial proteins associated with the pathogenesis of neurological disorders as well as numerous human diseases. EPAC proteins have two isoforms. These isoforms are EPAC1 and EPAC2. EPAC2 also known as Rap guanine nucleotide exchange factor 4 (RAPGEF4) is generally expression in all neurites. Higher EAPC2 levels was detected in the cortex, hippocampus as well as striatum of adult mouse brain. Activation as well as over-secretion of EPAC2 triggers apoptosis in neurons and EPAC-triggered apoptosis was intermediated via the modulation of Bcl-2 interacting member protein (BIM). EPAC2 secretory levels has proven to be more in low-grade clinical glioma than high-grade clinical glioma. This review therefore explores the effects of EPAC2/RAPGEF4 on the pathogenesis of glioma instead of EPAC1 because EPAC2 and not EPAC1 is predominately expressed in the brain. Therefore, EPAC2 is most likely to modulate glioma pathogenesis rather than EPAC1.

Correspondence: Seidu A. Richard, Department of Medicine, Princefield University, P.O. Box MA 128, Ho-Volta Region, Ghana, West Africa.
Tel.: +233508404595. E-mail: gbepoo@gmail.com

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Introduction

Gliomas are prime brain cancers which are initiated by malignant modification of neural stem cells, progenitor cells and differentiated glial cells such as astrocyte, oligodendrocyte as well as ependymal cells.[1-4] These cancers are histologically categorized into Grades I- IV based on the World Health Organization (WHO) criteria.[5,6] Most commonly, Grade I gliomas are found in children and they often have good outcomes.[1]

On the other hand, Grade II gliomas have 5-8-years average survival rate and are usually depicted with hypercellularity.[1,7,8] Nevertheless, Grade III comprises of astrocytoma or anaplastic astrocytoma according to histological classification. They are depicted with hypercellularity, nuclear atypia as well as mitotic figures. The anaplastic astrocytoma has 3-years average survival rate.[1,5,6,9-11] Glioblastoma multiforme (GBMs) constitutes Grade IV gliomas.[1]

However, the 2016 WHO classification of CNS tumors presents major restructuring of the diffuse gliomas, medulloblastomas as well as other embryonal tumors, and included new entities that are defined by both histology and molecular features, such as glioblastoma, IDH-wildtype and glioblastoma, IDH-mutant; diffuse midline glioma, H3 K27M-mutant; RELA fusion-positive ependymoma; medulloblastoma, WNT-activated as well as medulloblastoma, SHH-activated; and embryonal tumors with multilayered rosettes, C19MC-altered.[12] GBMs are often depicted with hypercellularity, nuclear atypia, mitotic figures as well as angiogenesis and/or necrosis. GBM patients with ages >60 years often have very short survival rate while patients with age <60 years have average survival rates of 12-18 months.[1,7,13-15] Also, patients' outcomes are often poor because tumor cells invade normal brain tissue around the tumor which make surgery resection incomplete and often necessitating adjuvant therapy with irradiation as well as chemotherapy to lessen the remaining tumor cells.[16,17]

Cyclic adenosine monophosphate (cAMP) is normally produced from adenosine 5'-triphosphate by adenylate cyclases.[4,18,19] It is a prototypic second messenger that partakes in cellular reactions. These reactions trigger several intermediates signaling pathways linked with many human diseases such as cancer, diabetes, and urinary dysfunction as well as immunological, central nervous and cardiac diseases.[4,18,20] Exchange proteins directly activated by cAMP (EPAC) is a new and promising protein that is expressed in almost all mammals' cells.[18,21] It is one of the prime cAMPs' effector proteins in mammals. EPAC proteins have two isoforms. These isoforms are EPAC1 and EPAC2.[18,21] EPAC2 is also referred to as Rap guanine nucleotide exchange factor 4 (RAPGEF4).[19,22] EPAC2 happens to be more regulated and restricted to the brain, pancreas, testes, as well as secretory cells.[19,21,23-25] This review therefore explores the effects of EPAC2 on the pathogenesis of glioma instead of EPAC1 because EPAC2 and not EPAC1 is predominately expressed in the brain. Therefore, EPAC2 is most likely to modulate glioma pathogenesis rather than EPAC1.

Origin of EPAC

EPAC protein was discovered in 1998 during a database exploration aimed at detecting the machinery via which cAMP-dependent activation of GTPase Rap1 that was uninhibited by protein kinase A (PKA).[21,26-28] EPAC protein is also referred to as cAMP-guanine exchange factor (cAMP-GEF).[26] Kawasaki *et al.* established that, EPAC1 also referred to as cAMPGEF-I and EPAC2 also referred to as cAMP-GEF-II where independently detected *via* a differential display screen for novel cyclic nucleotide binding domain-bearing proteins, which where augmented in the striatum.[25]

EPAC proteins were discovered in Metazoa within the evolutionary hierarchy as single polypeptide molecules.[21] De Rooij *et al.* established that EPAC1 is a novel cAMP sensor that intermediates the PKA-independent RAP1 stimulation in feedback reaction to cAMP[27,28] while Ozaki *et al.* established that, EPAC2 is a cAMP sensor linked to the sulfonylurea receptor (SUR1) in a yeast two-hybrid screen.[29]

EPAC protein is made-up of a C-terminal catalytic region and an N-terminal regulatory region.[19,26] The C-terminal catalytic region triggers Rap1 but not Ras, Ral, or R-ras.[21,27] This region contains the enzymatic GEF domain as well as the RAS exchange motif (REM), which are desired for stability of the GEF domain.[21,26] The N-terminal segment of EPAC houses the disheveled, Egl-10 and pleckstrin (DEP) domain and a cAMP binding domain. The function of DEP domain is uncertain but the cAMP binding domain is analogous to the cAMP binding domains at the regulatory subunit of PKA.[21] Also, the N-terminal region serves as an auto-inhibitory domain during activation of full-length EPAC *in vitro via* cAMP.[19,21]

Structure and function of EPAC2

Epac2, was coded by RAPGEF4 genes which comprised of 31 exons as well as 30 introns situated on chromosome 2q31.[19] EPAC2 is a multi-domain protein with a molecular weight of ~116 kDa, containing a regulatory as well as catalytic components.[4,26] NH_2-terminal forms the regulatory segment while COOH-terminal form the catalytic segment.[21,25] The amino terminal regulatory segment contains cNBD-A and cNBD-B cyclic nucleotide-binding domains as well as a DEP domain.[4,19,30] Furthermore, an extra CNB domain expressed NH2 terminal to the DEP domain is well-known in a complete EPAC2.[21]

It was affirmed that, EPAC2 CNB-A domain's affinity for cAMP is much punier than that of CNB-B domain.[18,23,26] Furthermore, isolated EPAC2 CNB-B domain was necessitous to inhibit GEF action of the EPAC2 catalytic part.[21,26] However, EPAC2 CNB-A and DEP domains are not requisite for upholding EPAC2 in an autoinhibitory state.[21,28] Also, EPAC2 catalytic segment was depicted with a Ras exchange motif (REM), a Ras-association (RA) domain, as well as a continuous CDC25 homology domain (CDC25-HD) which are conscientious to the nucleotide exchange activity of EPAC2.[18,19,21] The continuous CDC25 -HD is also known as the GEF for Ras-like small GTPases (RasGEF) domain.[21]

The main function of EPAC2 is a GEF for Rap1 and Rap2 with a small GTPases cycle involving an inactive GDP-bound form as well as an active GTP-bound form. Rap1 and Rap2 are strictly modulated by GEFs and GTPase-activating proteins (GAPs), which are liable for triggering of GTP loading and catalysis of GTP hydrolysis, correspondingly.[19,21,23,26] CDC25-HD of EPAC2 interrelates with GDP-bound Rap1. It is consequently stimulated by exchange of GDP for GTP resulting in down-regulated signaling via interface with its specific effector proteins. Studies have shown that, EPAC2 was more regulated and restricted to the brain, pancreas, testes, as well as secretory cells.[24,25] EPAC2 was therefore straightforwardly linked to the pathogenesis of Glioma and several neurological disorders.[4,19]

Seo and Lee demonstrated that, EPAC2-inhibition compromised pituitary adenylate cyclase-activating peptide (PACAP)-triggered astrocytic differentiation of neural precursor cells without affecting neuronal differentiation.[31] They stressed that, upsurge in intracellular calcium levels was critical in the PACAP-EPAC2 signaling pathway-triggered astrocytogenesis.[31]

EPAC and apoptosis

Cell survival as well as cell death are very crucial events in tissues with post-mitotic cells constitution.[32] It was obvious that, cAMP is able to wield a definite effect on cell predisposition to apoptosis thereby safeguarding neuronal cells.[32] Also, EPAC2 was triggered by 8-*p*-methoxyphenylthon-2-O-methyl-cAMP. Furthermore, over-secretion of EPAC2 expressively augmented DNA fragmentation as well as terminal deoxynucleotidyltransferase-mediated biotin nick end-labeling (TUNEL)-positive cell numbers in mouse cortical neurons. Thus, the effect of cAMP on cell death has been comprehensively studied.[32]

It was obvious that, EPAC2 triggers cAMP signals in neuronal cells leading to decrease in the rate of neuronal cell death. The experiment above were performed in variety of stresses like β-amyloid protein, sialoglycopeptide as well as low potassium-induced neurotoxicity.[32-35] Nevertheless, studies have demonstrated that, dopamine or prostanoid receptor-mediated cAMP generation stimulates neurotoxicity.[36,37] It was affirmed that, the influence of cAMP signaling on apoptosis focused principally on PKA, a classic target molecule of cAMP.[32]

EPAC2 has demonstrated to modulate a diverse cellular activity such as cell proliferation, migration, secretion, as well as differentiation.[32,38] Studies have shown that, either EPAC2 alone or with PKA provides protection to immune cells against apoptosis.[39,40] Suzuki *et al.* demonstrated that, activation as well as over-secretion of EPAC2 triggers apoptosis in neurons. Their study established that, EPAC-triggered apoptosis is intermediated *via* the modulation of Bcl-2 interacting member protein (BIM).[32] BIM acts on mitochondria as a pro-apoptotic factor resulting in the distraction of mitochondrial membrane potential.[32] Studies have demonstrated that, BIM binds to Bcl-2 and neutralizes its pro-survival role, leading to apoptosis in several cell types.[41,42] EPAC2 is therefore a crucial protein to avert apoptosis during gliomagenesis. Further studies on the effect of apoptosis on glioma cells are still warranted to determine whether EPAC2 can prevent the progressing of glioma.

EPAC2 expressive levels

It was affirmed that, the cortex, hippocampus as well as striatum of adult mouse brain express EAPC2[21,43] (Figure 1A). It was further affirmed that, EPAC2 was unanimously expressed in all neurites.[21] Analyses of EPAC2 protein levels in rat brain, spinal cord, as well as dorsal root ganglion (DRG) neurons at distinctive phases of growth revealed a developmental modulation of high EPAC2 in the rat nervous system[21,44] (Figure 1A). Also, northern-blot analyses revealed that full-length EPAC2 was highly

expressed in the pituitary gland during transcription.[21,44] EPAC2 mRNA was predominantly interconnected with the central nervous system (CNS) and adrenal gland with restricted quantities in heart, small intestine, as well as the testis.[25] Moreover, a truncated transcription revealed EPAC2 expressive levels in liver with low levels in the lungs, kidneys as well as pancreatic islets.[21,29,45]

It was affirmed that; mature neurons raise the concentrations of EPAC2 to regulate dendrite stability and over growth. It was further demonstrated that, elevated levels of EPAC2 were limited to growth cones and neurites.[21,46] It was also proven that, in the CNS, EPAC2 release was similar to neurotransmitter release. This form of EPAC2 expression is another form of exocytosis used by neurons to precipitously interconnect throughout the body.[21,45] Furthermore, mossy fiber CA3 synapses in the hippocampus demonstrated genuine basal activity as well as short sequences of synaptic transmission when EPAC2 was inhibited, but lasting action as well as forskolin-dependent potentiation was compromised.[21,43] Moreover, during EPAC2 inhibition, the quantity of existing vesicles to express EPAC2 after prolonged synaptic activity were reduced. This affirms that, EPAC2 was fundamental in the modulation of the spare pool of vesicles as well as vesicle release in reaction to augmented cAMP quantities.[21,43]

Nevertheless, EPAC2 inhibited synapses also exhibited destruction of NMDA receptor related long-standing depression.[21,47] In comparison, it was established that normal brain tissues expressed more EPAC2 levels than clinical glioma tissues[4] (Figure 1B). This therefore means that, the number of EPAC2-secreting cells may reduce during glioma pathogenesis.[4] Further studies are still warranted to determine the expressive levels of EPAC2 in glioma microenvironment.

EPAC2 inhibition

EPAC2 inhibition is very crucial during laboratory experiments. It was established that, all compounds produced through bioisosteric substitution of tert-butyl isoxazole ring with tert-butyl phenyl group reserved EPAC2 inhibitory activities.[48] Liu *et al.* formulated and synthesized novel series of 2-substituted phenyl-N-phenyl-2-oxoacetohydrazonoyl cyanides as EPAC2 inhibitors through ingenuous chemistry with low-cost maiden material as well as synthetic affluence appropriate for scale up.[48] ZL0524 was the utmost potent EPAC2 inhibitory (Figure 2) activities with IC50 values of 1.2 mM. Docking analyses of ZL0524 with triggered EPAC2 showed that it occupies the cAMP binding domain 2 (CBD2) hydrophobic domain, constitutes hydrogen bonds with Arg448 as well as stretched to the solvent region.[48]

Tsalkova et al demonstrated that ESI-05 as well as ESI-07 inhibited (Figure 2) cAMP-intermediated EPAC2 GEF activity with obvious IC50 of 0.43±0.05 as well as 0.7±0.1 μM, correspondingly.[49] It was affirmed that, the role of ESI-07 is to bind to the interfaces of two CBDs on EPAC2 as well as hairs the protein

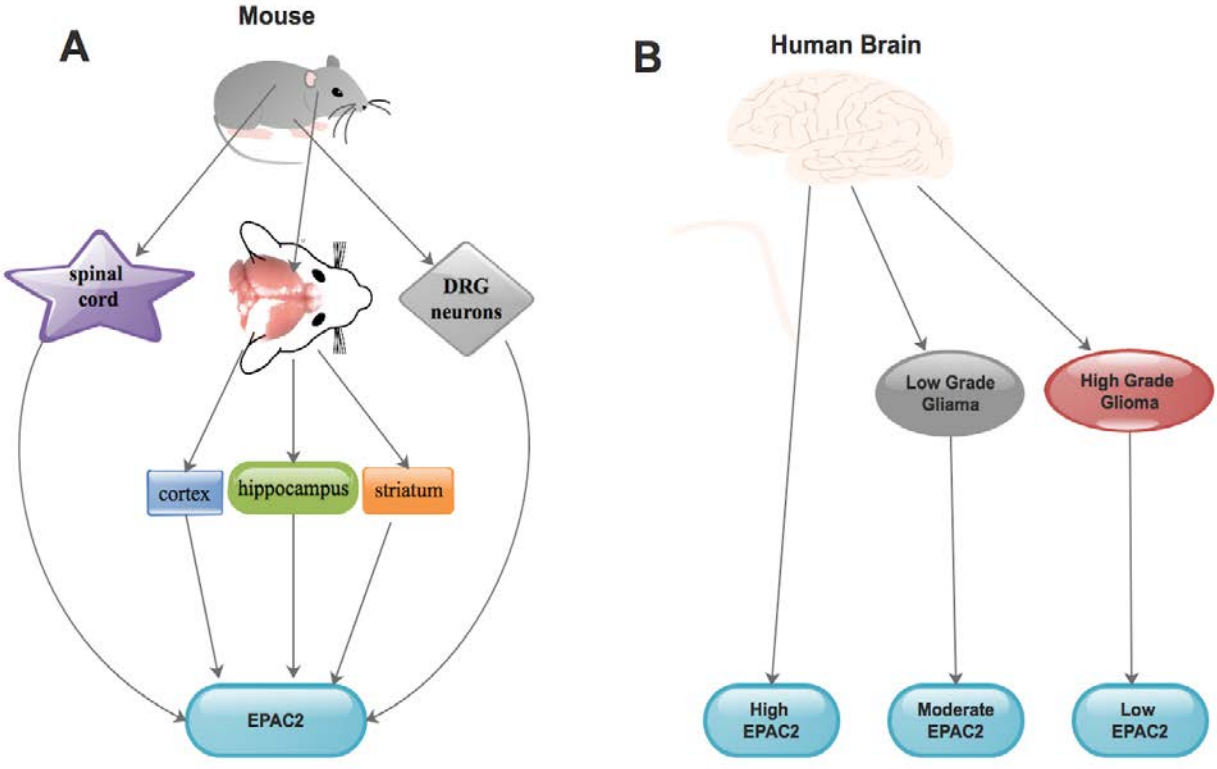

Figure 1. Expression of EPAC2 in adult mouse brain (A) and human normal brain/glioma tissues (B). A) The expressive levels of EPAC2 in brain (cortex, hippocampus and striatum), spinal cord as well as the dorsal root ganglion (DRG) neurons of adult mouse; B) the expressive levels of EPAC2 in normal brain tissues, low-grade glioma tissues as well as high-grade glioma tissues.

in its autoinhibitory conformation. This means that ESI-07 was completely specific for EPAC2.[49] Tsalkova *et al.* indicated that, the likelihood that ESI-07 binds to alternative unknown allosteric site on EPAC2 cannot be entirely exclude. This binding process could have prohibited the triggering of EPAC2 by stabilizing the inactive conformation.[49]

Rehmann indicated that, ESI-09 and HJC0197 do not function as selective inhibitors of EPAC2[50] (Figure 2). He indicated that, both compounds appear to have undefined protein denaturing influences. He further explained that, a weak influence of particular inhibition was covered by the typical protein denaturing influences, but both influences could occur in the uniform concentration range.[50] ESI-09 as well as HJC0197 affect EPAC2 devoid of the cAMP concentration and even inhibit the cAMP neutral intrinsic action.[50] It was established that, ESI-05 is a direct and selective inhibitor of EPAC2[51] (Figure 2). It was estimated that, ESI-05 binds EPAC2 with approximately 20-fold higher affinity than cAMP.[51] Chen *et al.* established that, numerous EPAC2 inhibitors could be coin out of ESI-05.[51] Also, ESI-05 as well as ESI-10 have been isolated as cAMP-mediated EPAC2 GEF inhibitors with IC50 values of 0.5 µM and 18 µM, correspondingly.[51] Nevertheless, ESI-05 shows a selective antagonist affinity for EPAC2 while ESI-10 is not completely specific for EPAC2.[49]

EPAC2 signaling pathways in the neurons

The development as well as conservation of dendrites are imperative to the routes of synaptic transformation or remodeling as well as plasticity of the brain.[21] EPAC2 stimulation of Rap triggers synapse destabilization of the dendritic spines during synaptic transformation or remodeling.[21] EPAC2 influences spine decline as well as internalization of α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) leading to decrease excitatory synaptic transmission. This process results in destabilization of the synapse but avoids synaptic eradication.[21] Also, this action may be synchronized *via* interface between EPAC2 and neuroligin, which recruits EPAC2 to the membrane thereby enhancing GEF activity.[21] Further studies on EPAC2 and neuroligin in the glioma microenvironment could lead to a novel therapeutic discovery.

The detection of a rare EPAC2 mutation in the RA domain in numerous autistic patients established that, the function of EPAC2 was maintenance of basal dendrites.[52] This rare EPAC2 mutation as well as other forms of mutation could further be exploited in the glioma microenvironment. This mutation has proven to efficiently reduced the quantity as well as length of basal dendrites that was associated with the activities of EPAC2 *via* a Ras-intermediated pathway in pyramidal neurons.[52] The ability of EPAC2 to astrocyte differentiation was evaluated in EPAC2 knockout mice. It was established that, EPAC2 needed PACAP to modulate external influx of calcium necessary for GFAP secretion as well as differentiation.[31]

It was well established that, growth arrest was needed to bestow comprehensive differentiation though axonal growth is essential for the maturation of neurons.[53] Therefore, a synchronized action between the cAMP-neutral nerve growth factor (NGF) triggering of extracellular-signal-regulated kinase (ERK) as well as EPAC2 stimulation of p38 mitogen activated protein kinase (MAPK) triggered growth arrest and neuritogenic consequences, correspondingly in neuroscreen-1 (NS-1) as well as PC12 cells.[53] It was established that, EPAC2 alone could decrease the significant Rap1B activity as well as configuration of axons in cultured hippocampal neurons.[21] It was also established that, triggering of EPAC2 augmented Rap and p38 stimulation to assemble intracellular calcium, which in turn triggers calcium-sensitive big potassium channels, ion channels normally linked with PKA stimulation in cerebellar granule cells.[54,55] It was affirmed that; activation of these ion channels generates slight membrane hyperpolarization weakening neuron firing.[54,55] Also, stimulation of EPAC2 triggered the phosphorylation of syntabulin, a microtubule-related as well as syntaxin-1-binding protein that binds syntaxin-filled vesicles to microtubules and kinesin I as well as intermediates anterograde transport of syntaxin-1 to neuronal processes in INS-1E cells.[56] Ras-binding happens to be obligatory for the cAMP-determined stimulation of Rap1 through EPAC2 though binding of EPAC2 to Ras-GTP is devoid of cAMP.[57] It was established that EPAC2 interrelates precisely with the nucleotide-binding fold-1

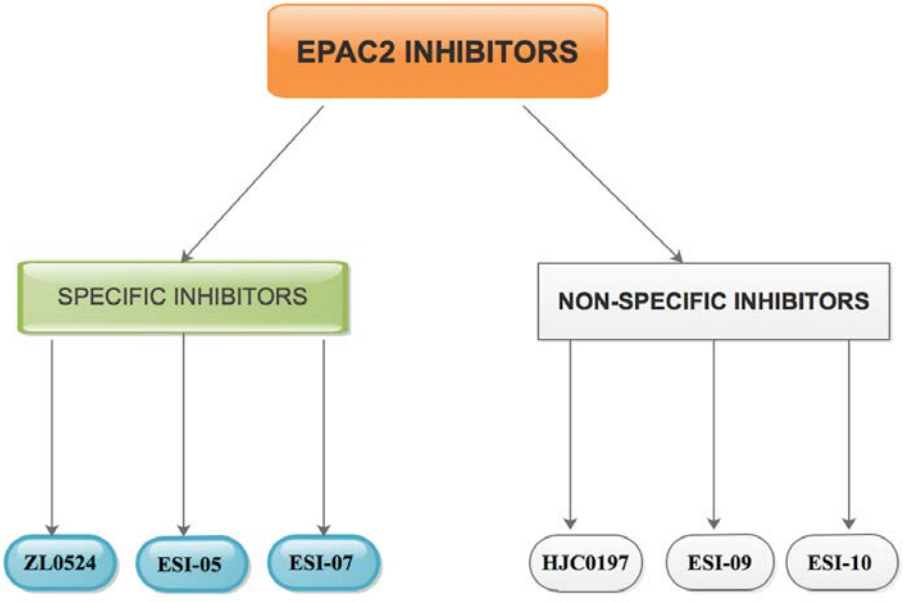

Figure 2. EPAC2 inhibitor: The inhibitors are classified into specific/selective and non-specific/non-selective.

(NBF-1) of sulfonylurea receptor SUR1, a subunit of the ATP-sensitive potassium channel (K_{ATP}).[58,59] This association in gliomagenesis is yet to be determined. Furthermore, EPAC2 binds Rim1α, resulting in its augmented linkage with Rab3 to expedient synaptic vesicles juxtaposition to the plasma membrane.[58-60] Additionally, EPAC2-intermediated production of DAG via the PLC pathway may be associated with the stimulation as well as translocation of Munc13-1 binding to Rim1α following SNARE-intermediated synaptic vesicle expression.[58,60] It was also perceived that, EPAC2's binding to SUR1 influenced exocytosis in dentate granule presynaptic terminals. Its interface weakens the action of the neuronal-type K_{ATP} but boosted voltage-dependent Ca^{2+} channel activityh.[61]

EPAC2 and glioma

It was obvious that, EPAC2 was universally secreted in all neurites and the expression of EPAC2 was higher in normal brain tissues.[21] We established that EPAC2 secretory levels were more in low-grade clinical glioma than high-grade clinical glioma. Which means that, high-grade clinical glioma tissues have lesser quantities of EPAC2 glands unlike low-grade clinical glioma tissues[4] (Figure 1B). Furthermore, using U251 and U87 cell lines, we demonstrated that, MMP-2 secretion was reduced subsequent to EPAC2 over-secretory plasmid transfection unlike normal control as well as plasmid control cells.[4] Our results indicated that EPAC2 over-secretion resulted in diminished MMP-2 protein levels through the EPAC2/MMP-2 signaling pathway. We therefore concluded that, in the glioma microenvironment, MMP-2 triggers EPAC2 glands, which resulted in a trigger of higher levels of EPAC2 during glioma pathogenesis[4] (Figure 3).

Seo and Lee established that, EPAC2 inhibition compromised proapoptotic caspase adaptor protein (PACAP)-triggered astrocytic differentiation of neural precursor cells devoid of neuronal differentiation.[31] They further proposed that an upsurge in intracellular calcium levels was essential in the PACAP/EPAC2 signaling pathway-triggered astrocytogenesis[31] (Figure 3). Studies have proven that astrocytogenesis was primarily linked with PACAP/EPAC signaling pathway.[4,62,63] We therefore advocated that further studies on signaling pathways through which EPAC2 secretion reduces in glioma specimens other than the EPAC2/MMP-2 signaling pathway[4]. It was further advocated that, EPAC2 could be of therapeutic valve in glioma since EPAC2 levels are drastically reduced in glioma microenvironment. Base on the hypothesis above, further studies on the up-regulatory effect of EPAC2 on glioma cells are still warranted.

EPAC2 and cAMP in glioma pathogenesis

It was established that, cAMP produced from adenosine triphosphate (ATP) by adenylyl cyclase, was a second messenger for intracellular signal transduction in numerous diverse organisms.[48,64] Several studies have demonstrated that, cAMP-intermediated signaling events were utilized to transduce PKA leading to the detection of EPACs or cAMP-GEF.[25,27,34,64,65] It was affirmed that, brain region-specific transformations in cAMP levels have been associated with the pattern of gliomagenesis.[66] Studies have proven that, low levels of cAMP triggered glioma configuration in neurofibromatosis-1 genetically engineered mouse models.[66-68]

Several studies have demonstrated that, cAMP is a ubiquitous modulator of inflammatory as well as immunological reactions.[69,70] Also, cAMP modifies several physiological activities through the stimulation PKA and EPAC2. Studies have shown that, PKA and EPAC2 are molecular competitors that are down-regulated by cAMP.[70,71] Sugimoto *et al.* evaluated the consequences of cAMP on Ras as well as Akt signaling pathways in U87MG human malignant glioma cells.[66] They indicated that cAMP inhibits p44/42 MAPK activity as well as proliferation in PTEN-depleted human glioblastoma cells *in vitro via* PKA/EPAC2 pathway.[66]

It was proven that cAMP suppress cell growth as well as p44/42 MAPK action via down-regulation of the Ras signaling pathway, but not Akt activity in a PKA and EPAC2-determined fashion.[66] Also, higher levels of cAMP in the cell resulted in the stimulation of diverse cAMP targets such as PKA and EPAC2.[66] As

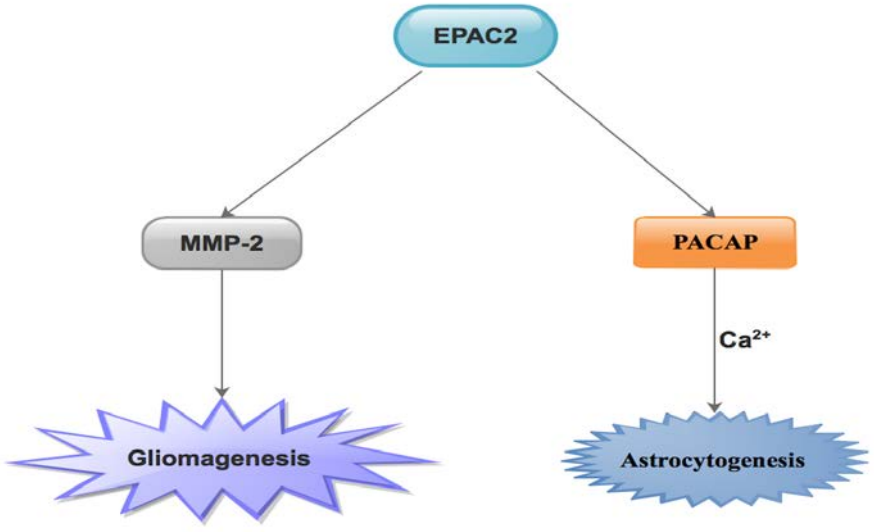

Figure 3. The known pathways via which EPAC2 participating in gliomagenesis as well as astrocytogenesis.

indicated earlier, PKA and EPAC2 are accountable for cAMP-dependent p44/42 MAPK dephosphorylation which affirms that cAMP inhibits cell growth *via* PKA and EPC2 stimulation in U172 as well as U87MG human glioblastoma cells.[72] Furthermore, cAMP exhibited inhibitory actions towards Akt *via* EPAC2-PTEN pathway stimulation in glial as well as osteosarcoma cells.[71,73]

Conclusions

Cyclic AMP-intermediated signaling events were utilized to transduce PKA leading to the detection of EPACs or cAMP-GEF. EPAC2 inhibition compromised proapoptotic caspase adaptor protein (PACAP)-triggered astrocytic differentiation of neural precursor cells devoid of neuronal differentiation. Furthermore, EPAC2 over-secretion resulted in diminished MMP-2 protein levels through the EPAC2/MMP-2 signaling pathway. Further studies on signaling pathways through which EPAC2 secretion reduces in glioma specimens other than the EPAC2/MMP-2 signaling pathway are warranted. Compounds like ZL0524, ESI-05 as well as ESI-07 are absolutely specific inhibitors for EPAC2 while ESI-09, ESI-10 and HJC0197 do not function as selective inhibitors of EPAC2 (Figure 2). As a hypothesis, up-regulatory effect of EPAC2 on glioma cells could lead to discovery on the therapy of gliomas.

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