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The Role of Cyclic 3'-5' Adenosine Monophosphate (cAMP) in Differentiated and Trans-Differentiated Vascular Smooth Muscle Cells

Martine Glorian and Isabelle Limon

Additional information is available at the end of the chapter

1. Introduction

Vascular Smooth Muscle Cells (VSMC) are highly specialized cells whose principal functions are contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. In healthy adult blood vessels, these cells proliferate at a very low rate, exhibit very low synthetic and migratory activity and express a unique repertoire of contractile proteins, ion channels, and signalling molecules required for the cell's contractile function. VSMC undergo significant phenotypic modulation following vascular injuries including hypoxia, oxidative stress and mechanical injury. This phenotypic transition is mainly characterized by the loss of contractility and the acquisition of a proliferative, migratory and synthetic phenotype. These drastic phenotypic alterations allow VSMCs to migrate from the media to the intima of the arterial wall where they proliferate and secrete an extracellular matrix and pro-inflammatory molecules. This phenotypic transition, also called the trans-differentiation process, plays a critical role in pathological vascular remodellings such as atherosclerosis, post-angioplasty restenosis, bypass vein graft failure, and cardiac allograft vasculopathy [1,2]. Hypoxia, mechanical stress and oxidative stress can induce VSMC trans-differentiation directly or indirectly by stimulating the release of pro-inflammatory molecules and growth factors from endothelium, macrophages, T lymphocytes or VSMC themselves. Signalling pathways involved in VSMC trans-differentiation are diverse. Among them, the 3'-5'-cyclic adenosine monophosphate (cAMP) signalling pathway stands out since cAMP is not only described to play important roles both in differentiated and transdifferentiated VSMCs, but can also have opposite effects in VSMCs with the same phenotype. Indeed, in trans-differentiated VSMCs, cAMP has dual opposite effects on migration and inflammation and stops cell proliferation. Alternatively, in differentiated VSMCs, cAMP induces relaxation, expression of

contractile proteins, maintenance of a low proliferation rate and can stimulate or inhibit apoptosis (Figure 1). The diversity of cAMP effects in VSMC (and in cells in general), is due to the ability of this second messenger to transduce extracellular signals in a compartmentalized manner, allowing individual stimuli to produce distinct pools of cAMP localized in discrete subcellular regions. These pools of cAMP are produced near a subset of cAMP effectors, themselves located near their substrates and engage specific cell responses according to the cellular context [3]. Adenylyl cyclases (AC), phosphodiesterases (PDE) and the scaffolding proteins A kinase anchored proteins (AKAPs) play a determinant role in cAMP compartmentalization. Final cAMP effect depends on which isoforms of these proteins are expressed. During the VSMC trans-differentiation process, important changes in the expressions of such proteins occur, allowing a re-organization of the cAMP signalling compartmentalization, therefore giving VSMC the ability to acquire properties specific to the trans-differentiated state. After a presentation of the cAMP signalling pathway, this chapter discusses data demonstrating the diversity of roles of cAMP in differentiated and transdifferentiated VSMCs.
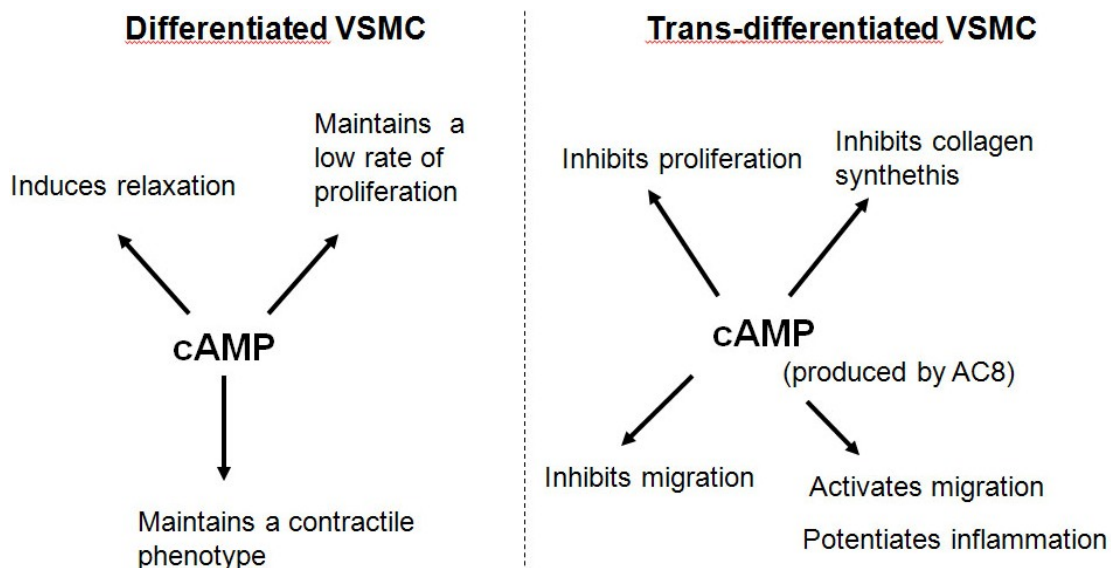

Differentiated VSMC

Induces relaxation

Maintains a low rate of proliferation

cAMP

Maintains a contractile phenotype

Trans-differentiated VSMC

Inhibits proliferation

Inhibits collagen synthethis

cAMP
(produced by AC8)

Inhibits migration

Activates migration

Potentiates inflammation

Figure 1. Roles of cAMP (3'-5' adenosine monophosphate) in differenciated and trans-differentiated vascular smooth muscle cells (VSMC); AC8: adenylyl cyclase 8.

2. the c-AMP signaling pathway

2.1. Overview

The c-AMP signalling pathway begins with the release of cAMP into the cell which is mostly initiated by the activation of G-protein coupled receptors (GPCRs) by several different hormones and neurotransmitters. The ligand-bound GPCR catalyzes the exchange of GDP for GTP on the α-subunit of the coupled heterotrimeric G protein, which results in the activation of the α-subunit and its dissociation from the βγ dimer. Both the α and the βγ subunits can

then activate or inhibit distinct intracellular signalling cascades. The αs of the Gs subtype activates adenylyl cyclases (AC) witch catalyzes the synthesis of cAMP from ATP. Increased levels of cAMP are translated into cellular responses by cAMP effectors. The best known is the c-AMP dependant protein kinase A (PKA), but also include cyclic-nucleotide gated ion channels (CNGCs) and the recently discovered Rap1-guanine nucleotide exchange factor (Epac), three effectors known to mediate a multitude of cAMP signalling pathways. (Figure 2). The end of cAMP signalling is achieved by its decomposition into AMP catalyzed by phosphodiesterases (PDEs) and its active efflux through transporters of the multidrug resistance-assocuated protein (MRP) family [4,5]. One particularity of the cAMP signalling pathway is its high degree of compartmentalization. Multiprotein complexes organize the location of the different cAMP effectors to specific subcellular locations and allow cAMP to propagate a plethora of cell responses in a spatio-temporal manner [3]. These multiprotein complexes are at the foundation of cAMP compartmentalization, they involve AC, the scaffolding proteins AKAPs and PDEs.
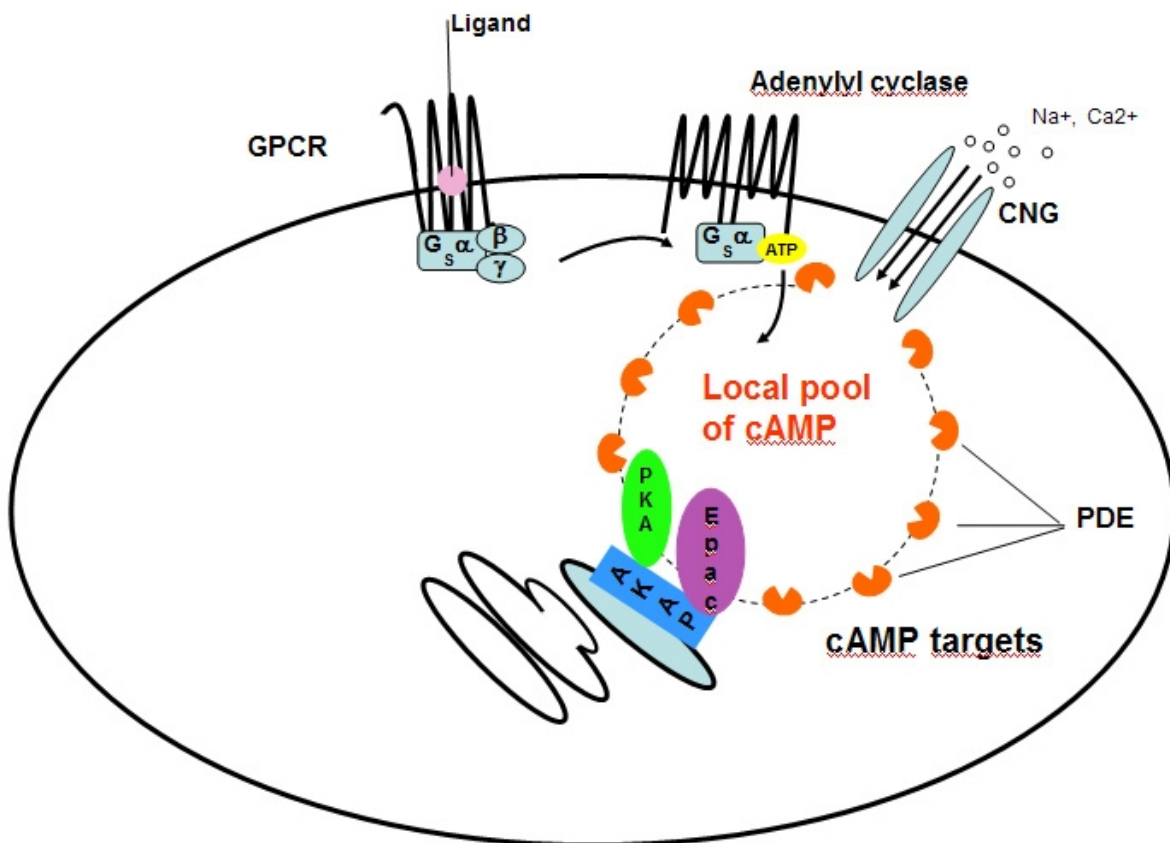

Figure 2. Cyclic adenosine 3', 5'-monophosphate (cAMP) is produced from ATP by adenylyl cyclase (AC) upon activation of Gs-protein coupled receptors. The local concentration and distribution of cAMP gradients is limited by phosphodiesterases (PDE) which generate localized pools of cAMP throughout the cell. The increase in cAMP is translated to cellular responses by the cAMP effectors protein kinase A (PKA), EPAC (exchange protein activated by cAMP) and cyclic nucleotide -gated ion channels (CNGCs). A kinase anchored proteins (AKAPs) target cAMP effectors to distinct cell compartments. They also intract with AC, PDE, cAMP effectors substrates and further scaffolding proteins, providing spatial and temporal specificity of the cAMP pathway.

2.2. Components of the c-AMP signalling pathway

2.2.1. Formation of c-AMP is regulated by adenylyl cyclases

In mammals, cAMP is synthesized from ATP by members of the Class-III AC (Adenylyl Cyclase)/ADCY family (E.C 4.6.1.1)[1] [6]. This class is comprised of nine trans-membrane (tm) AC enzymes and one soluble AC (sAC). tmAC are grouped into three major sub-families: group 1: AC1, AC3, AC8; group 2: AC2, AC4, AC7; and group 3: AC5, AC6. All nine tmAC can be activated by GTP-bound Gαs and, with the exception of AC9, by the plant diterpen forskolin. Nevertheless, each isoform has a specific pattern of regulation by G proteins, calcium/calmodulin, and proteine kinases [7-9]. For example, differences in patterns of regulation by G proteins have been associated with isoform-specific differences in AC activation. Whereas AC1, AC5, AC6 and AC8 are inhibited by Gαi, AC2, AC4, AC7 are not. Furthermore, whereas Gβγ subunits inhibit isoforms AC1 and AC8, they stimulate AC2, AC4 and AC7. GTP-bound Gαs, the activator of all tmAC, is the result of the exchange of GDP for GTP on the α-subunit of G protein and its subsequent dissociation from the βγ dimer. This activation can be a consequence of the binding of GPCR by several different hormones or neurotransmitters (e.g., β-adrenergic, H2-histamine, EP2-prostaglandin, α2a adrenergic and M2-muscarinic receptor), making GPCRs guanine nucleotide exchange factors (GEFs) for Gα subunits. The exchange of GDP for GTP can also be mediated independently from conventional GPCR/G protein signalling. This way involves entities called "non-GPCR GEFs", such as the recently identified cholinesterase -8a (Ric8a), a cytosolic protein reported to bind to and act as a GEF for numerous Gα in mammalian cells [10]. Signal de-activation is achieved by Gα-mediated GTP hydrolysis (endogenous GTPase activity) allowing return of the Gα subunit to the inactive GDP-bound and its association with Gβγ dimer to form a Gα βγ-heterotrimeric complex.

Beyond their synthase activity, ACs can function as scaffolds, and therefore contribute to the cAMP signalling compartmentalization. Indeed, several works have shown that specific AC isoforms have the capacity to interact with several proteins/enzymes on their N-terminus allowing an isoform selective coupling with specific downstream signalling cascades [11,12]. AC isoforms are themselves confined in several structural specific cellular compartments. The best characterized is their association with caveolar, lipid-rafts and the anchoring proteins AKAP [13,14]. Selective adenylyl cyclase isoform localization, regulation and coupling with specific downstream targets provide adenylyl cyclase isoform-selective patterns of signalling, that links specific AC isoforms to distinct cell processes [15,16]. For example, alteration of the AC population expressed in DDT1-MF2 cells (derived from hamster vas deferens smooth muscle) changes the processing of stimulatory and inhibitory input [17] and differential expression of AC isoforms in two VSMC models account for opposite effect of isoprenaline on cAMP production [18].

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1 Adenylyl cyclases (ACs) are currently grouped in six classes based on their primary amino acid sequences. Class I ACs have been found exclusively in γ -proteobacteria. Class II ACs are toxins secreted by Bacillus anthracis, Bordetella pertussis and Pseudomonas aeruginosa. Only few members of class IV, V and VI ACs have been described to date and consists in bacterial enzymes. Class III ACs is universal. Class III ACs is found in metazoa, protozoa, fungi, eubacteria, some archaebacteria and certain green algae. Neither class III ACs nor any other type of AC has ever been conclusively identified in higher plants (Embryophyta).

Differentiated VSMC have been shown to express different isoforms of AC [18,19]. AC3-5-6 are clearly the most highly expressed isoenzymes in VSMCs, while Type 8 AC (AC8) is undetectable in differentiated VSMCs and is strongly induced in trans-differentiated VSMC [20,21].

2.2.2. Degradation of cAMP is regulated by the cyclic nucleotide phosphodiesterases

Phosphodiesterases (PDE) comprise a large superfamily of enzymes; 11 families (PDE1-PDE11) have been characterized on the basis of their amino acid sequences, substrate specificity, allosteric regulatory characteristics and pharmacological properties [22,23]. In total, the superfamily of PDEs encompasses 25 genes in mammals giving rise to 200 reported distinct gene products corresponding to different splice variants that are often expressed in a tissue-specific manner. The substrate specificity of PDEs includes cAMP-specific, cGMP specific, and dual-specific PDE. PDE 4-7-8 are highly specific for the hydroysis of cAMP, PDE5, 6, 9 are cGMP specific and PDE1, -2, -3, -10, -11 hydrolyse both cAMP and cGMP. There are four major PDE families found in VSMCs: PDE1, PDE3, and PDE4 PDE5 [24]. PDE3 and PDE4 have been shown to account for the majority of cAMP hydrolysis, whereas PDE1 and PDE5 are mainly responsible for cGMP-hydrolysis [25,26]. PDE1A and -1B, are expressed in differentiated VSMC. PDE1A has the particularity to be localized in different cell compartments according to the VSMC phenotype; it is predominantly cytoplasmic in medial contractile VSMC and becomes nuclear in neointimal synthetic VSMC [27]. PDE1C is specifically induced in trans-differentiated VSMC [28]. PDE3A, the main isoform expressed in arterial tissue, platelets and cardiac tissue is found is VSMCs as well as PDE3B. The largest PDE family to date, the cAMP specific PDE4 family, is expressed in numerous tissues, notably in vascular tissue. Four genes (PDE4A/B/C/D) encode over 20 distinct PDE4 isoforms as a result of mRNA splicing and the use of distinct promoters [29]. It was reported that two PDE4 "long forms", PDE4D3 and PDE4D5 are expressed in rat and human VSMC [30,31] and that the two "short forms" PDE4D1 and PDE4D2 are specifically expressed in trans-differentiated VSMC [32]. PDE5A is the major cGMP hydrolyzing PDE expressed in arterial tissues[33,34].

2.2.3. Effectors of cAMP action

2.2.3.1. PKA

The first intracellular target of cAMP identified is the well characterized PKA holoenzyme. cAMP-PKA-mediated signalling is known to affect numerous intracellular targets in response to a wide variety of molecular signals. Numerous studies over the past 40 years have identified hundreds of PKA substrates in the plasma membrane, nucleus, and cytoplasm of cells. The PKA holoenzyme is a tetramere consisting of two catalytic subunits (C) that are maintained in an inactive conformation by a regulatory (R) subunit dimer [35]. Binding of two cAMP molecules on each R subunit leads to a conformational change and dissociation of two catalycally active C monomers, which phosphorylate serine and threonine residues on specific substrate proteins. Molecular cloning identified 4 R subunits and 4 C subunits called respectively RIα, RIβ, RIIα, RIIβ, Cα, Cβ, Cγ, and PRKX (the human X chromosome-encoded protein

kinase X, a cAMP dependent kinase that forms a catalytically inactive holoenzyme only with the RI subunit). The R subunits exhibit different cAMP binding affinities and can form both homo and heterodimers leading to a large number of combinations. The subcellular localization of PKA is determined by PKA binding to A kinase ankoring proteins, AKAPs. AKAPs act as scaffolds which give PKA access to substrates localized in specific compartments within the cell and participate to cAMP signalling compartmentalization as depicted below [36,37].

2.2.3.2. Epac family

Epac proteins are the most recent addition to the group of cAMP signalling effectors. Their discovery explains various effects of cAMP that could not be attributed to the established targets PKA and CNGs. Epac was identified in a database screen conducted to explain the independent activation of the small G protein Rap by cAMP [38]. At the same time, a screen for proteins containing cyclic-nucleotide-binding domains revealed the presence of two isoforms of Epac, Epac1 and Epac2 [39]. Epac proteins function as guanine nucleotide exchange factors (GEFs) both for Rap1 and Rap2. Rap1 and rap2 proteins belong to the Ras family of small G proteins, which cycle between an inactive GDP-bound state and an active GTP-bound state. The GTP-bound Rap mediates signalling by associating with and activating effector proteins. GEFs catalyze the exchange of GDP for GTP and thereby the activation of the small G protein (Figure 3). Herein, Epac1 and Epac2 proteins are also called cAMP-GEF I and II respectively. Their subcellular localizations are determined, like PKA, by binding to AKAPs. Epac1 and Epac2 are present in most tissues, though with different expression levels. Epac1 is highly abundant in blood vessels, kidney, adipose tissue, central nervous system, ovary and uterus, whereas Epac2 is mostly expressed in the central nervous system, adrenal gland, and pancreas. Epac proteins are implicated in many cAMP-regulated processes such as insulin secretion, cardiac contraction, vascular permeability, cell migration, neurotransmitter release and immunity [40,41].

2.2.3.3. CNG famly

Cyclic nucleotide-gated (CNG) channels are non-selective cation channels first identified in retinal photoreceptors and olfactory sensory neurons. They are opened by the direct binding of cAMP and cGMP. Although their activity shows very little voltage dependence, CNG channels belong to the super-family of voltage-gated ion channels.

CNG channels consists in heterotetrameric complexes resulting from the association of two or three subunits. Six different genes encoding CNG channels, four A subunits (A1 to A4) and two B subunits (B1 and B3), give rise to different channels. Their activity is modulated, at least in part, by Ca2+/calmodulin and by phosphorylation. The role of CNG channels has been established in retinal photoreceptors and in olfactory sensory neurons. Mutations in CNG channel genes give rise to retinal degeneration and color blindness [42].

CNG channels are widely expressed in vascular tissues across species and vascular beds [43,44]. Specifically, CNGA1 was found to be very expressed in the endothelium layer and, with a much lower extent, in VSMC [44]. In contrast, strong expression of CNGA2 has been

found in both the endothelium and media of human arteries [43]. Functionally, CNG channels play an important role in endothelium dependent vascular dilatation to a number of cAMP-elevating agents including adenosine, adrenaline and ATP [45-47]. Concerning the function of CNG in differentiated VSMC, to our knowledge, only one report demonstrates that CNG contributes to thromboxaneA2-induced contraction of rat small mesenteric arteries[48].
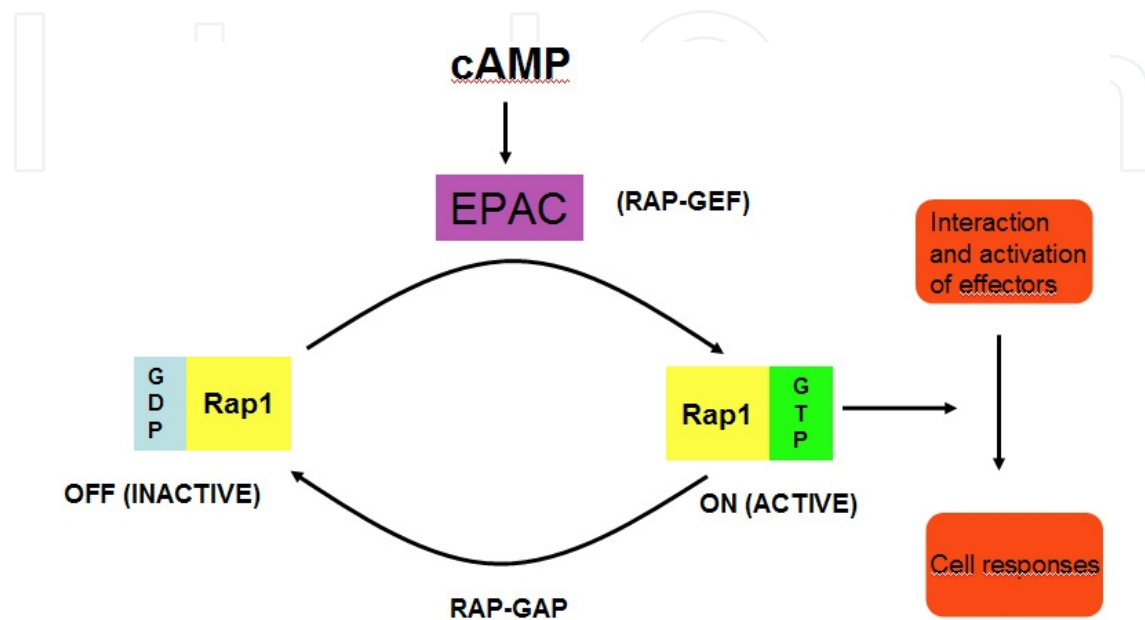
Adapted from Jeyaraj et al..Life Sciences, 2011

Figure 3. The Rap1 GTPases cycle between a GTP-bound (active state) and GDP- bound (inactive state). Cycling between the active and inactive states is facilitated by guanine nucleotide exchange factors (GEFs) that release GDP and allow binding of GTP, as well as GTPase activation proteins (GAPs) wich accelerate GTP hydrolysis.

2.3. ACs, PDEs and AKAPs are essential to cAMP signaling compartimentalization

The idea of compartimentalized pools of cAMP originated in 1979 when Brunton et al. showed that while both the β-adrenergic receptor agonist isoprotrenol and prostaglandin E1 increased cAMP concentration in perfused rat hearts, only isoproterenol increased glycogen metabolism and phosphorylation of troponin [49]. These results illustrated the fact that different hormones may act through the same messenger to generate different pools of cAMP and mediate distinct physiological responses. An increasing number of results support now the existence of distinct cAMP microdomains that control cAMP signalling. ACs, PDEs and the scaffolding proteins AKAPs are at the foundation of this cAMP signalling compartmentalization [50,51]. As mentioned, -ACs can orchestrate their own microenvironment by recruiting a variety of signalling and scaffolding molecules, - PDEs mediate local cAMP degradation and literally sculpt gradients of cAMP surrounding specific signalling complexes and therefore regulate

the availability of cAMP/cGMP to their effectors –AKAPs dynamically assemble the three different cAMP effectors to control the cellular actions of cAMP [37]. As their name implies, AKAPs were originally described to target PKA to distinct subcellular locations and confine activation to only a subset of potential targets. In reality, these proteins have the ability to form complexes with other signalling molecules including Epac proteins, protein kinases, phosphatases, phosphodiesterases, AC, as well as GPCR and ion channels. AKAPs are localized to numerous cellular sites, including the plasma membrane, Golgi, centrosome, nucleus, mitchondria and cytosol. The first AKAP to be characterized was microtubule associated protein-2 (MAP2), initially identified because of it co-purified with RII from brain extract [52]. The AKAP family has grown and includes more than 50 structurally diverse, but functionally similar members. Despite their diversity, AKAP orthologues have been identified in a range of species, including yeast, nematodes, mice and humans. All AKAPs share common properties: 1) they contain a PKA-anchoring domain 2) compartmentalization of individual AKAP-PKA units occur through specialized targeting domains that are present on each anchoring protein 3) they have the ability to form complexes with other signalling molecules including protein kinases, phosphatases, phosphodiesterases, AC, as well as GPCR and ion channels 4) AKAPs are recruited into much larger multiprotein complexes through the interactions with other adaptator molecules such as PDZ and SH3 domain containing proteins. These four properties of AKAPs allow these proteins to integrate multiple signalling pathways, allowing the convergence of signals to a common target [36,37].

3. Roles of cAMP in differentiated VSMCs

3.1. cAMP induces relaxation of differentiated VMCs

Elevation of intracellular cAMP after activation of Gs coupled receptors by vasorelaxing hormones such as adrenaline, noradrenaline and the endothelium-derived prostaglandine I2 (PGI2) induces a rapid and efficient relaxation of mature differentiated SMCs [53]. Moreover, the cAMP elevating agent forskolin induces a relaxant effect in VSMCs *in vivo* which is potentiated by inhibitors of PDE3 and PDE4, the two main PDE isoforms expressed in VSMCs [25,26,30] [54]. In SMCs, cAMP contributes to muscle relaxation through two different mechanisms; one through the stimulation of the Ca pump at the sarcolemmal membrane (Ca extrusion) and sarcoplasmic reticulum (Ca accumulation), and the other through the de-phosphorylation of myosin light chain kinase (MLCK). De-phosphorylation of MLCK is accomplished by the myosin light chain phosphatase (MLCP) which is well known to be activated upon phoshorylation by the cAMP target PKA or the cGMP dependent protein kinase G (PKG) [55,56]. Conversely, when phosphorylated by Rho-associated kinase (ROCK) or PKC, MLCP activity is inhibited, resulting in contraction. A new mechanism of cAMP-mediated relaxation has been recently described in airway and aortic smooth muscle cells involving Epac, the last cAMP effector identified. Activation of Epac by an Epac selective cAMP analog in pre-contracted aortic smooth muscle cells and airway smooth muscle cells results in the down regulation of RhoA activity and in the increase of Rap1 or Rac1 activities, leading to cell relaxation [57,58]. cAMP pools involved in SMC relaxation may be mainly generated by the

type 6 adenylyl cyclase (AC6). Indeed, overexpression of only AC6 (and not AC5, AC2, or AC1) in primary aortic VSMCs enhances smooth muscle relaxation [59]. Furthemore, a recent study using selective short interfering RNA sequences reveals that AC6 is the predominant isoenzyme involved in vasodilator-mediated cAMP accumulation in aortic VSMCs, accounting for 60% of the total response to β-adrenoceptor (β-AR) stimulation [60].

3.2. cAMP maintains a low rate of proliferation in differentiated VSMC

A cause to effect relationship between the decreased expression of some specific components of the cAMP signalling and proliferative capacity of VSMC has been demonstrated. Inversely, emergence of PDEs in trans-differentiated VSMC allows them to proliferate.

3.2.1. Role of CREB

The cAMP Response Element Binding Protein (CREB) is a transcription factor, well known to be phosphorylated and activated by PKA. CREB expression has been shown to be dramatically decreased in cultured trans-differentiated VSMCs and in the media of numerous rodent and porcine models of vascular diseases. Depletion of this transcription factor *in vivo* elicits changes consistent with those observed in SMCs from pathologically remodelled arteries whereas forced depletion of CREB with small interfering RNA in aortic SMCs is sufficient to induce their proliferation, hypertrophy, migration, de-differentiation, and ECM production. Furthemore, CREB is inactivated in VSMCs by several proliferative stimuli and overexpression of wild type or constitutively active CREB, in primary cultures of SMC arrests cell cycle progression induced by these stimuli [61-66]. Additionally, Transforming growth factor beta and thiazolidinediones activate CREB to oppose to aortic SMC proliferation induced by growth factors [62,67]. Nevertheless, some apparent contradictory studies show that CREB is involved in VSMC proliferation induced by ATP and thrombin [68,69].

3.2.2. Role of CREB AKAP12β and AKAP5

AKAP12β, a member of the AKAP family, is markedly decreased in human and rodent vascular lesions. Overexpression of AKAP12 β attenuates serum-induced SMC growth in *vitro* and a causal relationship exists between the induction of the expression of this protein and the inhibition of serum-induced VSMC proliferation by all trans retinoic acid [70]. An other AKAP shown to repress VSMC growth is AKAP5 (AKAP79/AKAP75/AKAP150 in human, bovine, rat respectively) since over-expression of this protein inhibits serum-induced VSMC proliferation and local delivery of AKAP5 to balloon-injured vessels wall reduced the extent of neointimal burden [71].

3.2.3. Role of PDE1-C

PDE1C, a PDE isoform hydrolyzing both cAMP and cGMP, is expressed in proliferating human VSMCs but is absent in quiescent cells. In *vivo*, PDE1C is expressed in human foetal aortas containing proliferating SMCs, but not in newborn aortas in which SMC proliferation has ceased. Moreover, a causal relationship has been established between the emergence of

PDE1-c in VSMCs and their capacity to proliferate, since specific inhibition of PDE1C in SMCs isolated from normal aorta or from lesions of atherosclerosis results in suppression of SMC proliferation [72].

3.3. Others roles of cAMP in differentiated VSMC

3.3.1. cAMP maintains the contractile phenotype of differentiated VSMCs

As mentioned above, CREB depletion elicits changes consistent with those observed in SMCs from pathologically remodelled arteries *in vivo.* These changes include modifications in the expression of SMC markers and contractile factors such as SM myosin, and strongly suggest that cAMP is important in maintaining the contractile phenotype of differentiated VSMCs [64]. The role of CREB in the maintenance of the contractile phenotype is reinforced by a recent publication showing that cAMP elevation by cilostazol, a potent type 3 phosphodiesterase inhibitor, promotes VSMC differentiation through CREB [73].

3.3.2. cAMP has dual opposite effects on apoptosis of differentiated VSMCs

Some studies demonstrate that cAMP is pro-apoptotic in SMCs whereas others present cAMP as an anti-apoptotic factor in these cells. The opposite effect of cAMP on apoptosis in the same type cell can be explained by the compartmentalization of cAMP signalling since these studies use different ways to elevate intracellular cAMP. Some studies use cAMP elevating agents, whereas others use hormones such as prostacyclin. In aortic VSMC, Torella et al. show that cAMP analogs inhibits apoptosis through Ser83 phosphorylation of p85αPI3K [77]. Additionally, in the same model, the AC activator forskolin reduces apoptosis in serum-deprived rat aortic VSMC at a site upstream of caspase 3 via activation of PKA [78]. In line with these studies, inhibition of CREB function in aortic VSMC induces apoptosis of rat aortic VSMC, possibly through downregulation of bcl2 expression [79]. Adversely, cAMP elevation in response to prostacyclin induces apoptosis in rat aortic VSMC through the inhibition of extracellular signal-regulated kinase activity [80].

4. Roles of cAMP in trans-differentiated VSMCs

4.1. cAMP inhibits proliferation of trans-differentiated VMCs

cAMP is well known to diminish cell growth and to promote cell-differentiation in general, it can even be antagonistic to the effect of growth factors [81]. The first clue that cAMP might have a role in controlling growth of cultured cells emerged from two studies. Burk observed that two drug inhibitors of cAMP phosphodiesterase activity, caffeine and theophylline, slowed the growth of normal and transformed baby hamster kidney (BHK) cells [82]. At the same time, Ryan and Heidrick reported that cAMP itself inhibited the growth of Hela cells [83]. The first demonstration that cAMP inhibits proliferation of VSMCs was done by Southgate and Newby showing the inhibitory effect of 8-Br-CAMP on serum-induced proliferation of

rabbit aortic smooth muscle cells [84]. This inhibitory effect of cAMP on VSMC growth was confirmed *in vitro* [85,86] and *in vivo* by Indolfi et al., demonstrating that local or oral administration of cell-permeable, cyclic AMP analog, 8-Br-cAMP and non-selective phosphodiesterase-inhibitor drugs to rats markedly inhibits neo-intimal formation after balloon injury *in vivo* and/or *in vitro* in SMC [87,88]. Selective inhibitors of PDE3A and PDE4D, the two main PDE isoforms expressed in VSMCs that account for cAMP hydrolysis [25,26,30] were also shown to inhibit proliferation of trans-differentiated VSMCs. PDE3 and PDE4 inhibitors markedly potentiate both the anti-proliferative effect and the increase in cAMP caused by forskolin and PGI2 and significantly inhibit PDGF-induced VSMC proliferation and migration [89,90]. [Of note, PDE4D is the first gene that has been linked to common forms of stroke such as cardiogenic and carotid strokes [91]. Moreover, PDE3 inhibitors administred orally are able to inhibit VSMC proliferation in a model of photochemically-induced vascular injury (Kondo et al., Atherosclerosis, 1999), and a recent publication clearly demonstrates that PDE3A depletion *in vitro* and *in vivo* inhibits mitogen-induced VSMC proliferation [61]. The AC isoform that could play a role in cAMP-mediated inhibition of VSMC growth is the type 3 adenylyl cyclase (AC3) since Wong et al. demonstrated that this protein mediates the inhibitory effect of prostaglandin E2 (PGE2) on basal and PDGF-BB-induced proliferation in murine and human arterial VSMC [51]. Various molecular mechanisms have been proposed to explain AMP-mediated inhibition of VSMCs. Such mechanisms include subsequent suppression of growth factor-mediated activation of mitogenic protein kinases in VSMCs. Indeed, cAMP can oppose to the mitogen-activated protein (MAP) kinases ERK1/2 [61,92], to JNK1 [93] as well as to the phosphatidylinositol 3-kinase effector S6K1 [92]. In addition, cAMP can regulate gene/protein expression which may contribute to its anti-proliferative action. For example, cAMP elevating agents restore expression of p53-p21 in response to PDGF [61,94], prevents serum-induced expression of cyclin-dependent kinases [95], inhibits basal and glucose-induced VSMC growth by a down-regulation of the transcription factor E2F [25] and can reduce the serum-induced expression of the S-Phase kinase-Associated Protein 2 (Skp2), an important factor for cell cycle progression in VSMCs [96]. Furthermore, prostacyclin-induced cAMP intracellular elevation inhibits the proliferation of arterial smooth muscle cells by inhibiting the smad1/5 driven expression of Id1 (inhibitor of DNA binding protein) gene [97]. Some of the genic effects of cAMP in VSMCs may be mediated by CREB since this transcription factor has been demonstrated to inhibit the expression of a number of cell-cycle and mitogenic genes in trans-differentiated VSMCs as well as genes encoding growth factors, growth factor receptors, and cytokines [61,64,98]. The cAMP effectors PKA and Epac both are involved in cAMP VSMC growth inhibition. Indeed, PKA inhibitors have been shown to reverse or, at least, inhibit the effect of cAMP elevating agents on VSMC proliferation [71,77,87,88,99]. Concerning the involvement of Epac in VSMC proliferation, Mayer and collaborators and Hewer and collaborators respectively demonstrated that Epac is involved in the adenosine-mediated decrease of cell proliferation in human VSMCs and acts synergically with PKA to mediate cAMP-dependent cell-cycle arrest and associated induction of a stellate- morphology in VSMCs [100,101].

4.2. cAMP has dual opposite effects on migration of trans- differentiated VMCs

4.2.1. cAMP inhibit migration of trans-differentiated VSMCs

A growing body of evidence emerged in the beginning of the 1990's implicating cAMP in the inhibition of trans-differentiated VSMC migration. These studies, using analogs of cAMP, activators of ACs and cAMP raising agents in VSMCs, have demonstrated that an increase in cAMP positively correlates with the inhibition of VSMC migration. Indeed, raising the intracellular concentration of cAMP either with dopamine, acting throught D1 receptors, adrenomedullin, or forskolin, inhibited migration of VSMCs stimulated with PDGF or serum [102-104]. Studies in rat aortic SMCs suggest that vasoactive agents that elevate intracellular cAMP inhibit cell movement by disassembling actin stress fibers of the cytoskeleton [105,106]. Furthermore, downregulation of PKA abrogates inhibition of VSMC chemotaxis by forskolin [89]. The inhibitory effect of cAMP on VSMC migration is re-inforced by the fact that inhibiting all together PDE3 and PDE4D, the two main PDE isoforms expressed in VSMCs that account for cAMP hydrolysis in VSMC [26,30,107] markedly potentiated both the anti-migratory effect and the increase in cAMP caused by forskolin and significantly inhibited PDGF-induced VSMC proliferation and migration [90,108,109]. In addition, Newman et al demonstrated that forskolin inhibits TNFα-induced interleukin 6 expression and migration in human vascular smooth muscle cells [110]. This effect could involve the transcription factor CREB since PDGF-induced migration was decreased by active CREB and augmented with dominant negative CREB [66,95] ; In addition, a negative correlation has been described between the CREB level and the PDGF-activated SMC migration [64]. Nonetheless, the role of CREB in SMC migration remains unclear since CREB has been demonstrated to be involved in UTP, arachidonic acid and TNF alpha-induced SMC migration of VSMCs [111,112]. Moreover, recent studies show that oxidized and non-oxidized fatty acids induce SMC motility through this transcription factor [113,114].

4.2.2. A specific endogenous pool of cAMP induces migration of trans- differentiated VSMCs

By demonstrating that differential expression of ACs isoforms in two VSMC models account for opposite effects of isoprenaline on cAMP production in VSMC, Webb and co-workers suggested for the first time that changes of AC isoform(s) expression in VSMCs could account for the manifestation of vascular diseases [18]. In line with this study, Limon's group recently demonstrated that the emergence of the calcium/calmodulin positively regulated AC isoform 8 (AC8) in trans-differentiated VSMCs is involved in VSMC migration. Type 8 AC is barely undetectable in differentiated VSMCs and is strongly induced in trans-differentiated VSMCs. A causal relationship between AC8 apparition and the migratory capacities of VSMCs has been established. Indeed, authors show that 10 days after balloon angioplasty[2], rat carotid artery displayed high AC8 immuno-labelling only in the neo-intima and was no longer detectable when it was analyzed after the re-endothelization phenomenon during which VSMC migration/proliferation halted. More-

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2 Balloon angioplasty in rat carotid artery serves as an in vivo model of VSMC migration and proliferation.

over, the forced expression of AC8 in primary rat VSMC cultures triggered the re-colonization of a wounded zone, whereas blocking it in IL-1β−cells stopped the IL-1β-induced migration [21] This finding was extending *in vivo*, on human samples, where only the neo-intimal VSMCs a high level of AC8. Of note, AC8 is well known for its role in stress adaptation, mood disorders and opiate dependence [115]. The involvement of this enzyme in VSMC trans-differentiation was therefore unexpected. Molecular mechanisms underlying AC8-mediated VSMC migration does not involve PKA but could involve Epac1 since it has been shown that Epac 1 expression is upregulated in the neointima after vascular injury of mouse arteries and induces VSMC migration. Moreover Rap1, one of the described targets of Epac, is well known for its involvement in cell migration [116].

4.3. cAMP has dual opposite effects on inflammation of trans-differentiated VMCs

A study from Adkins and coll. demonstrate that the elevation of intracellular cAMP by rapamycin inhibits the secretion of the pro-inflammatory molecule Tumor Necrosis Factor alpha (TNF-α) in lipopolyssacharide treated VSMCs from human saphenous vein segments [110]. Adversely, Clement and collaborators suggest that the production of cAMP specifically by AC8 is involved in the potentiator effect of prostaglandin E2 (PGE2) on the secretion of phospholipase A2 (sPLA2), a marker of inflammation, in response to interleukine 1 β (IL1β) in primary cultures of rat aortic smooth muscle cells [20]. In details, authors show that PGE2 i) induces the transition of CMLV towards a trans-differentiated/ inflammatory state through the activation of the subtype 4 Gs-linked PGE2 receptor EP4, ii) acts in synergy with IL1β to potentiate the secretion of phospholipase A2 and the disorganization of the alpha actin cytoskeleton. This potentiator effect is the result of a simultaneous activation of PGE2 receptors EP4 and EP$_3$: in differentiated VSMC, EP3 receptors inhibit cyclase activity induced by EP4 and become activator of this activity in trans-differentiated VSMC. This switch of regulation is the result of the emergence of AC8 in IL1β−treated VSMC.

4.4. cAMP inhibits collagen synthesis of trans-differentiated VSMCs

Synthetic VSMCs, in the atherosclerotic and neointimal lesions, produce an abundant exra-cellular matrix (ECM), rich in type I collagen (collagen I). This ECM plays an important role in vessel wall thickening and in the occlusion of the vessel lumen. In addition, collagen I in vascular lesions may also regulate VSMC proliferation/migration, platelet circulation, mono-cyte activation, lipid accumulation, calcification, and plaque stability [74]. cAMP elevating agents have been shown to inhibit collagen I synthesis induced by fetal calf serum- and TGF-β [75]. Emergence of PDE1-c in trans-differentiated SMCs from rat aortic and human saphe-nous vein explants opposes the inhibitory effect of cAMP on collagen 1 synthesis, and accounts, at least in part, for the increase of collagen 1 expression in trans-differentiated VSMCs. The use of specific pharmacological inhibitors and si-RNA reveal that the cAMP-mediated inhibitory effect on collagen 1 synthesis involves cyclic nucleotide gated channels but not PKA, nor Epac [76].

5. Conclusion

Depending on the relative abundance and localization of the components of the cAMP signalling pathways, cAMP effects on VSMC vary in differentiated and trans-differentiated VSMCs (Figure 4). Because trans-differentiated VSMCs play a crucial role in atherosclerosis and are solely responsible for post-angioplasty restenosis, understanding molecular mechanisms leading to VSMC trans-differentiation is crucial to develop novel therapeutic strategies. Reducing post-angioplasty restenosis which affects 20-25% of patients treated with bare metal stents, is one of the major challenges in cardiovascular medicine. At the beginning of 2000's, the apparition of stents locally releasing anti-proliferative drugs (ie drug-eluting stents (DES), have significantly changed interventional cardiology, due to their remarkable ability to reduce restenosis compared to bare metal stents, However, their overwhelming success has quickly decreased since is limited due to an increased risk of late stent thrombosis. Poor re-endothelialization remains the major important pathologic predictor of late stent thrombosis [117], therefore, it has been suggested that DES should ideally have a selective anti-migratory and/or proliferative effect on VSMCs, without affecting, or, even better, promoting re-endothelialization [77,118]. Identifying the specific components of the cAMP pathway specifically involved in VSMC trans-differentiation may be a novel concept for the development of new drugs for DES, therefore improving the treatment of pathological vascular remodellings.
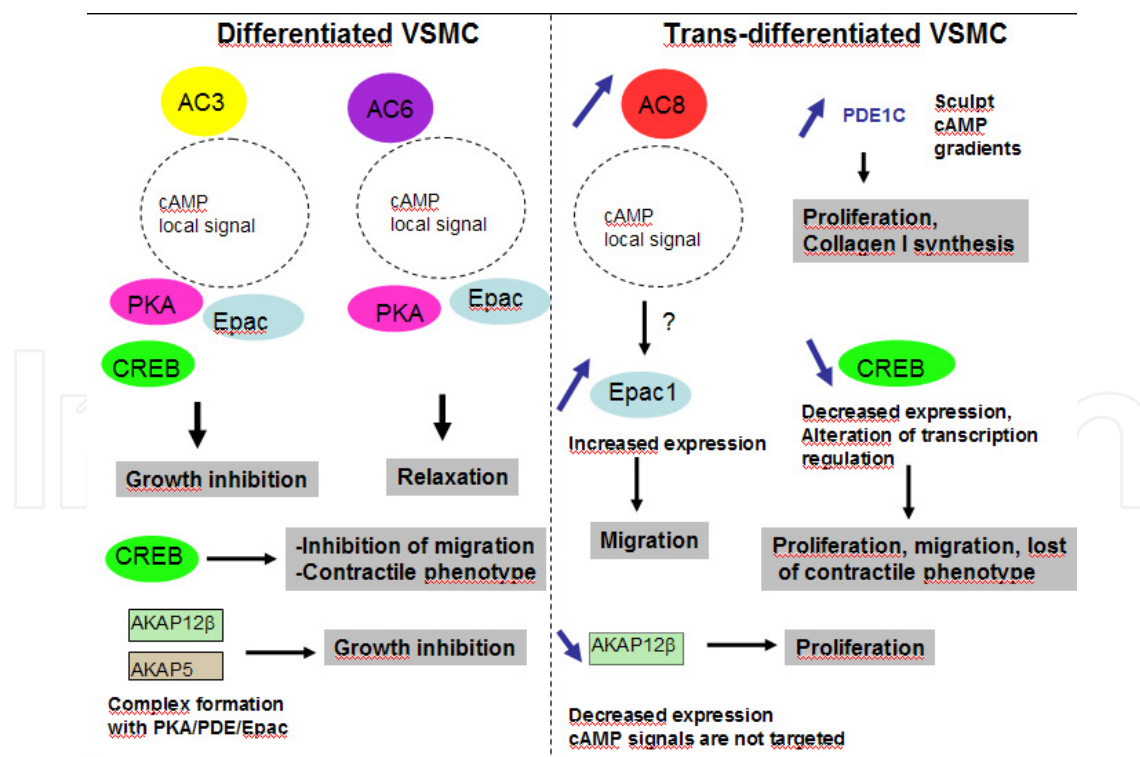

Figure 4. Expression of cAMP components in differentiated and trans-differentiated VSMC and consequences on VSMC functions. AC adenylyl cyclase; AKAP A-kinase anchoring proteins; Epac exchange proteins directly activated by cAMP; CREB cAMP response element binding protein; PDE phosphodiesterases.

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