Cyclic Nucleotides Regulate Vascular Tone

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Several mediators and drugs regulate blood flow and blood pressure. The cyclic nucleotides cAMP and cGMP are the key messengers mediating vasodilation under physiological conditions and are therefore involved in the physiological regulation of vascular tone. Their function is due to the existence of several mechanisms that include the reduction of [Ca2+]i and a decrease in the sensitivity of the contractile machinery; both mechanisms may occur together and decrease MLC phosphorylation. On the other hand, these mechanisms are regulated by several cellular effectors, including ion channels that regulate the membrane potential and Ca2+ influx. All these mechanisms promote vessel relaxation by reducing vascular contractility and tone.

compartmentation cAMP cGMP vascular smooth muscle cells cardiovascular diseases

1. cAMP Effector Proteins

cAMP is generated in response to the activation of a wide range of membrane receptors belonging to the GPCR superfamily. After binding a ligand, a G-protein is activated and promotes the activation of the AC that generates cAMP from ATP. This second intracellular messenger, in turn, regulates an infinity of physiological and pathological processes in different organs, including the CV system, and this is achieved by activating the so-called cAMP effectors. These effectors include cAMP-dependent protein kinase (protein kinase A, PKA), exchange protein directly activated by cAMP (EPAC), cyclic nucleotide-gated (CNG) ion channels, and the recently discovered Popeye domain-containing proteins (POPDC) [1].

The major effects of cAMP in eukaryotic cells result from the activation of PKA [2]. PKA was first discovered by Walsh et al. in 1968 [3]. Its catalytic subunit (C) was the first protein kinase to be crystallized. Since then, many studies have focused on it, and is currently one of the most studied protein kinases. However, and despite intense research, some important aspects such as its activation and inactivation in the complex cellular environment still need to be investigated [4].

Structurally, the PKA holoenzyme is a tetramer formed by two catalytic C subunits, and a dimer regulatory (R) subunit, where cAMP binds [5]. The three isoforms of the C subunit (α, β, γ) have virtually identical kinetic and physicochemical properties, while the four regulatory subunits (RIα, RIIα, RIβ, and RIIβ) exhibit distinct binding affinities for cAMP and are differentially located within cells [5][6][7][8][9]. PKA holoenzymes containing the RI subunits are PKA type I (RIα and RIβ) and are predominantly in the cytoplasm. In contrast, PKA holoenzymes containing type II subunits are called PKA type II (RIIα and RIIβ) and appear to be associated with cellular structures and organelles [5][9]. Initially it was thought that this could be due to the anchoring of A-kinase anchoring proteins

(AKAP) with greater affinity for the RII subunits [5]. Still, other studies have demonstrated that there are AKAP with dual specificity for the RI and RII subunits and AKAP that bind only to the RI subunits. The studies show both PKA type I and II may be anchored in subcellular compartments within the cells [9][10][11][12] and, therefore, the specificity of PKA signaling is achieved by binding to these anchoring proteins [13][14][15]. Overall, AKAP direct holoenzymes PKA to different subcellular sites near neighbouring proteins, optimizing signal transduction and allowing events responsive to local cAMP to occur within specific compartments of the cell (i.e., resulting in compartmentalized cAMP signaling, explained in more detail below) [15][16]. Thus, an imbalance in the expression or activity of some of the PKA subunits [17][18] or anchoring by AKAP [19] may lead to the development of cardiovascular diseases (e.g., myocardial infarction).

In the absence of cAMP, PKA is in an inactive state, with two regulatory subunits, R, and two catalytic subunits, C, forming a complex called a tetrameric holoenzyme (R2C2) [20]. In each regulatory subunit R, there are two binding sites (A and B) to which cAMP cooperatively binds during activation [9]. The inactive PKA holoenzyme exposes the available B site for cAMP binding. When occupied, the binding of cAMP to site A is increased, which leads to an intramolecular conformational change: the regulatory subunits dissociate from the R2C2 complex and generate two active C subunits, which phosphorylate their substrates in the cytosol and the nucleus [9][15][21][22][23][24]. Importantly, only the C subunit is in the nucleus [25]. In other words, the binding of the C subunit to the inhibitory sites of the respective R subunit renders the kinase-inactive, while the cAMP allosteric binding to two C-terminal tandem cAMP binding domains (CNB-A and CNB-B) of the R subunits triggers the catalytic activity of the holoenzyme [26][27]. However, it has been suggested that cAMP can also activate PKA without releasing the catalytic subunits C [4][28][29][30] and there are intact and active holoenzymes within the cytoplasm in the presence of cAMP. Other studies suggest that a more significant number of R subunits than C are essential for reducing the release of catalytic C subunits and increasing their recapture [25].

PKA may also play a critical role in the phenotypic modulation of the vascular SMCs, that mainly depend on intracellular ATP concentrations. Hogarth at al demonstrated that low micromolar ATP concentrations induce a negligible effect on DNA synthesis but induce higher serum response factor activity, and showed a higher gene expression of the markers of differentiated smooth muscle cells, such as SM-α-actin and SM22, indicating a contractile SMCs phenotype. On the contrary, high micromolar ATP concentration inhibits serum response factor activity and the markers genes expression and promotes cultured cell growth in a manner dependent on PKA activation. The change into the contractile phenotype by high ATP intracellular can be prevented, and even reversed, by inhibition of PKA activity [31].

Although PKA is the primary protein kinase activated by cAMP, several studies suggest that some of the biological effects of cAMP may, at least in part, be mediated by activation of PKG [32][33][34] which is classically activated by cGMP. The cyclic nucleotide-binding domain of PKG binds cGMP with greater affinity than cAMP. Moreover, intracellular concentrations of cAMP are generally higher than cGMP, which show that cAMP is also an agonist of PKG [35]. In this sense, Eckly-Michel et al. (1997) showed clearly that cAMP mediates PKG activation in vascular smooth muscle [36].

In addition to PKA, another important downstream effector of cAMP [37] is the EPAC family [3][38][39]. The EPAC proteins (1 and 2) act as guanine nucleotide exchange factors (GEFs) for small Ras-like GTPases (Rap1 and Rap2), and for this reason are also designated as cAMP-GEF proteins. The EPAC1 encoded by the RAPGEF3 gene is present in the human vasculature [37]. Although EPAC and PKA can act independently, they often act together in the same biological process in which they mediate synergistic or opposite effects. Discovery of EPAC discovery is relatively recent, but it modulates several different systems, including the CV system, which plays a critical role [37]. Normally, in vascular SMCs, activation of RhoA/Rho kinase (ROCK) signalling phosphorylates MLCP, inhibiting its phosphatase activity. Thus, phosphorylation of MLC by MLCK increases, activated by the Ca^{2+}-CaM complex, and contraction occurs. EPAC induces a vasorelaxation of vascular SMCs through inhibition of RhoA/ROCK signalling. The cAMP-mediated activation of EPAC/Rap1 releases the inhibitory effect of RhoA/ROCK on the MLCP. In turn, this leads to dephosphorylation of MLC and subsequent vasorelaxation of SMCs [37]. However, the role of EPAC in the vasculature is controversial: these proteins may promote or inhibit SMCs proliferation and migration. Moreover, if EPACs induce vasorelaxation of vascular SMCs extracted from large vessels, on the other hand they induce vasoconstriction of micro vascular SMCs. In other words, EPAC proteins may act as mediator of vascular SMCs phenotypic switching or, on the other hand, have a protective role [37]. This disagreement of the effects of EPACs seems to be based on the use of different cellular model systems, as it is known that the susceptibility of vascular SMCs to phenotypic switching is variable according to their origin, the vascular bed from which they were isolated, as well as differences in species, strain, age, or sex of the animal model. In this sense, there is no doubt that their role in the vasculature is crucial, and that anomalous EPAC signaling may be frequently implicated in pathological CV conditions [40]. Therefore, Rap1-independent EPAC signalling may be a useful target for drugs designed to treat atherosclerosis and hypertension.

Additionally, other effectors of cAMP are CNGs, expressed in vascular SMCs [41][42] and EC [42][43][44]. CNGs are nonselective cationic channels that open in response to direct binding of cAMP and cGMP [45], modulating the vascular tone.

Moreover, POPDC also play the role of cAMP effectors. The POPDC family of proteins (1-3) contain an extracellular N-terminal domain, three transmembrane domains, and a cytosolic Popeye domain [46]. This latter domain serves as the binding site for high-affinity cAMP. POPDC is expressed in vascular SMCs colocalized with α-smooth muscle actin and appears to be involved in the mechanisms of vasculogenesis [46][47]. However, its role as a cAMP effector in vascular SMCs remains unclear.

2. cGMP Effector Proteins

cGMP concentration is critical for the maintenance of CV homeostasis in several cell types. Moreover, the classical and significant physiological effect of an increase in the intracellular concentration of cGMP in vascular cells is vasodilation [2]. The cGMP pathway begins with the activation by one ligand (NO, ANP, BNP, and CNP) to guanylyl cyclase, either soluble or particulate, that induces the increase of the intracellular concentration of cGMP, which may activate three classes of cGMP effector proteins: (1) PKG, (2) CNG channels and (3) PDE, whose activity can be regulated by cGMP as previously described [48][49][50].

This protein kinase (also known as cGKs) alters the activity of target proteins, phosphorylating specific radicals of serine and threonine [51][52]. Moreover, PKG is considered the most important target of cGMP in the CV system. In mammals, two PKG genes have been identified, namely *PRKG1 AND PRKG2*, that encode PKG1 and PKG2, respectively [53][54][55]. The membrane PKG 2 is not expressed in the CV system. On the other hand, cytosolic PKG 1 has two different splice variants (PKG 1α and PKG 1β) that differ only in the first~100 amino acids. However, the one most expressed in vascular SMCs is PKG 1β [53][56][57][58]. In these cells, both PKG 1α and PKG 1β are expressed, while EC expresses only PKG 1β [48]. However, the expression of PKG is dependent of the cell density in culture, low density, or reduced expression of PKG [59]. Moreover, the increase in the expression of PKG contributes to change for the contractile phenotypic in cultured vascular SMCs, and the suppression of PKG expression during cultured growth in vitro may facilitate the modulation to a more synthetic, dedifferentiated phenotype [60]. The crystal structure of PKG 1 was elucidated only in 2016 [61]. PKG1 consists of a homodimer divided into a regulatory (R) and a catalytic (C) region. The regulatory region is composed of four functional domains, leucine zipper, auto-inhibitory (AI) (which engages the catalytic region of the PKG in the committed/inactive state), and two tandemly arranged low and high-affinity cGMP binding domains (CNB-A and CNB-B) [61][62]. Selective interaction of cGMP with the cyclic nucleotide-binding domain binding pockets leads to a 10-fold higher sensitivity for PKG 1α compared to PKG 1β [63]. Two domains constitute the C-terminal catalytic region, the kinase domain (where the Mg^{2+}/ATP binds) and the AGC-kinase C-terminal domain (where substrate/downstream proteins bind and phosphorylation occurs) [62]. This protein can bind or activate several signaling pathways, but the exact mechanisms underlying their interactions with PKG have not yet been elucidated. Briefly, PKG operates in parallel through several mechanisms to reduce vascular contractility: (1) PKG inhibits IP3 stimulated SR Ca^{2+} release by the IP3R by phosphorylating the IP3 receptor-associated PKG-substrate (IRAG) [64][65]; (2) PKG can inhibit by direct phosphorylation the IP3R [65]; (3) PKG phosphorylates phospholamban which removes the brake on SERCA activity, increasing Ca^{2+} import into the SR; (4) IP3 concentration due to phospholipase C activation is suppressed by PKG phosphorylation of the regulator of G-protein signalling 2 (RGS2) [66]; (5) PKG prevents the inactivation of MLCP by ROCK [67][68]; (6) PKG phosphorylate Ser-695 on the myosin phosphatase target subunit 1 (MYPT1) of the MLCP, which prevents ROCK from phosphorylating the adjacent Thr-696 that usually leads to enzyme inactivation [69][70]; (7) PKG phosphorylates the small heat shock protein HSP20 which induces vascular relaxation (by arresting the reorganization of the actin cytoskeleton required for vasoconstriction) and inhibits of agonist-induced constriction [71][72]; (8) PKG activates large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) [73]; (9) PKG activate Kv [73], and (10) PKG inhibits the voltage-dependent Ca^{2+} channels (see review [74]).

Concerning the CNG channels, these channels are nonselective cation channels activated by the binding of cGMP or cAMP [45], as previously mentioned for cAMP effector proteins. The CNG channels reveal a higher sensitivity for cGMP than for cAMP. In physiological conditions, CNG channels carry inward Na^+ and Ca^{2+} currents [75]. Even if the divalent cations can permeate the channel, higher concentrations induce these cations to bind to specific sites within the channel pore and block further ion flow. Knowledge of CNG channels is greater regarding the visual and olfactory systems while in other areas is less. Despite that, the CNG channels are used with the patch-clamp recordings to observe cyclic nucleotide changes [76].

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