Phosphodiesterase 6 generates intracellular cGMP microdomains in the native endothelium

BY

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Résumé

Les cellules endothéliales (CEs) participent au maintien de l'homéostasie vasculaire en générant et libérant de nombreux agents bioactifs, incluant l'oxyde nitrique (NO). Le NO module plusieurs fonctions vasculaires telles que le tonus et la perméabilité vasculaire via la stimulation de la guanylate cyclase soluble (GCs) provoquant la formation de GMPc. D'autre part, les phosphodiestérases (PDEs) sont des enzymes métabolisant les nucléotides cycliques (GMPc et AMPc) et participent donc à des étapes essentielles du contrôle des voies de signalisation du GMPc et de l'AMPc. Bien que les CEs soient la source principale de NO, la voie de signalisation NO-GMPc endothéliale et les répercussions fonctionnelles demeurent méconnues. Nous avons alors émis l'hypothèse qu'une population spécifique de PDEs ciblant le GMPc (PDEs-GMPc) permettrait aux CEs de maintenir des niveaux de GMPc faible malgré l'importante production de NO. L'expression des isoformes de PDEs-GMPc dans les artères mésentériques de souris fut initialement déterminée. PDE5 et PDE6 furent détectées tant sous la forme d'ARNm que de protéines dans les artères natives alors que PDE6 est absente de lignées de CEs en culture. La distribution intracellulaire des deux enzymes est distincte. Alors que PDE5 est distribué uniformément dans le cytoplasme des cellules endothéliales, les sous-unités catalytiques de PDE6 (α et β) sont préférentiellement présentes dans la région péri-nucléaire. Ces résultats suggèrent que PDE6 puisse être impliqué dans le contrôle de microdomaines de GMPc. Des simulations effectuées à l'aide d'un modèle mathématique développé sur la base de ces données sont en accords avec la notion selon laquelle l'expression et la distribution subcellulaire de PDE6 sont responsables de microdomaines de GMPc dans l'endothélium. En absence de PDE6, l'ajout de NO sous forme de bolus unique ou répétée mène à une augmentation homogène de la concentration cytoplasmique en GMPc malgré la présence de PDE5. Toutefois, la présence de PDE6 à la membrane péri-nucléaire crée un espace péri-nucléaire pauvre en GMPc. Les résultats de cette étude forment les premières évidences de l'expression et de la distribution intracellulaire hétérogène de PDE6 dans les cellules endothéliales natives et suggèrent leur implication dans la génération de microdomaines.

Keywords: cGMP, PDE5, PDE6, modèle mathématique, microdomaines.

Abstract

Endothelial cells (EC) are essential regulator of vascular homeostasis through the generation and release of various bioactive agents, including nitric oxide (NO). NO modulates several vascular functions such as vascular tone and permeability, through the stimulation of soluble guanylate cyclase (sGC) leading to the production of cGMP. Conversely, phosphodiesterases (PDEs) are enzymes metabolizing cyclic nucleotides (cGMP and cAMP) and are therefore major regulatory players for cGMP and cAMP signalling pathways. Although ECs are the main source of NO, little is known on the endothelial NO-cGMP signalling pathway and cellular outcomes. It was then hypothesized that a specific population of cGMP-phosphodiesterases allows ECs to stabilize cGMP levels despite the elevated production of NO. Expression of cGMP-phosphodiesterases was initially studied in resistance mesenteric arteries from mice. PDE5 and PDE6 were both found at mRNA and protein levels in native arteries but PDE6 is not found in cultured ECs. Interestingly, subcellular distributions of both enzymes were distinct. PDE5 appeared to be homogeneously distributed whilst PDE6 catalytic subunits (PDE6α and PDE6β) showed a preferential staining in the perinuclear region. These results suggest that PDE6 might be involved in the regulation of cGMP microdomains. Based on these findings, a mathematical model was developed. Simulations of dynamic cGMP levels in ECs support the notion of cGMP microdomains dependent on PDE6 expression and localization. In the absence of PDE6, application of NO either as a single bolus or repetitive pulses led to a homogeneous increase in cGMP levels in ECs despite PDE5 homogeneous distribution. However, PDE6 subcellular targeting to the perinuclear membrane generated a cGMP-depleted perinuclear space. The findings from this study provide the first evidence of the expression and specific intracellular distribution of PDE6 in native endothelial cells that strongly support their involvement in the generation of cGMP microdomains.

Keywords: cGMP, PDE5, PDE6, mathematical model, microdomains.

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List of abbreviations

AC: Adenylate cyclase

ACh: Acetylcholine

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

Ang II: Angiotensin II

Ang: Angiopoietins

ANP: Atrial natriuretic peptide

ATP: Adenosine-5'- triphosphate

BK: Bradykinin

Ca^{2+}: Calcium ion

CaM: Calmodulin

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: 3',5'-cyclic adenosine monophosphate

cDNA: complementary DNA

cGK: cGMP-dependent protein kinase

cGKI or PKG: Cyclic nucleotide-dependent protein kinase G

cGMP: cyclic Guanosine monophosphate

CO: Carbon monoxide

Cx: Connexins

DAPI: 4',6- diamidino-2-diphenylindole

EC: Endothelial cell

ECM: Extracellular matrix

EDHF: Endothelium-derived hyperpolarizing factor

EDRF: Endothelium-derived relaxing factor

eNOS: Endothelial nitric oxide synthase

ER: Endoplasmic reticulum

ET-1,2: Endothelin

GTP: Guanosine, 5'-triphosphate

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HUVEC: Human Umbilical Vein Endothelial Cells

IK_{Ca}: Intermediate conductance Ca^{2+} activated potassium channels

iNOS: Inducible nitric oxide synthase

IP_3: Inositol trisphosphate

IP_3R: Inositol triphosphate receptors

K^+: Potassium ion

$K_{Ca}2.1$: Calcium-activated potassium channels 2.1

$K_{Ca}3.1$: Calcium-activated potassium channels 3.1

MEGJ: Myoendothelial gap junction

MEP: Myoendothelial projection

MLC: Myosin light chain

MLCP: Myosin light chain phosphatase

MLCK: Myosin light chain kinase

mRNA: Messenger ribonucleic acid

nNOS, NOS I: Neuronal nitric oxide synthase

NO: Nitric oxide

NP: Natriuretic peptides

O_2: Oxygen

PAF: Platelet-activating factor

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PDE: Phosphodiesterase

PDE5: Phosphodiesterase 5

PDE6: Phosphodiesterase 6

pGCs: Particulate Guanylyl cyclase

PGI_2: Prostacyclin

PKG: Protein kinase G

RNA: Ribonucleic Acid

SDS: Sodium dodecyl sulfate

SERCA: Sarco/endoplasmic reticulum calcium ATPase

sGC: soluble guanylate cyclase

SMC: Smooth muscle cells

SR: Sarcoplasmic reticulum

VDCC: Voltage-dependent calcium channel

VEGF: Vascular endothelial growth factor

VSMCs: Vascular smooth muscle cells

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Chapter 1: Introduction

1. Blood vessels

The cardiovascular network can be divided into three main types of blood vessels: arteries, veins and capillaries. They are the conduits through which the blood is delivered to organs and allow an appropriate supply of oxygenated blood and nutrients. It further washes out the metabolic end products. As a closed system, the three blood vessel types are connected to each other in the following order, starting from the heart, arteries, capillaries and veins. Altogether, the different parts of the vascular network work in collaboration to ensure proper perfusion of the entire body. But the role of each type of vessel is different and is matched by a distinct structure that even varies within a vessel type.

1.1 Structure

The structure of blood vessel walls can be divided into three layers (from the outside to the lumen): tunica adventitia (or externa), tunica media and tunica intima. The tunica adventitia is constituted of connective tissue and collagen fibers conferring elasticity and compliance to the blood vessel. The tunica media is mainly populated by smooth muscle fibers that actively regulate blood vessel diameter. The tunica intima is a single sheet of endothelial cells covering the inside of the blood vessels. The endothelial cells are separated from the smooth muscle cells by an internal elastic membrane (**Fig. 1.1**). Although they share the same general structure as detailed above, arteries are different from the veins. For example, arteries have a thicker media layer which is due to their specific function.

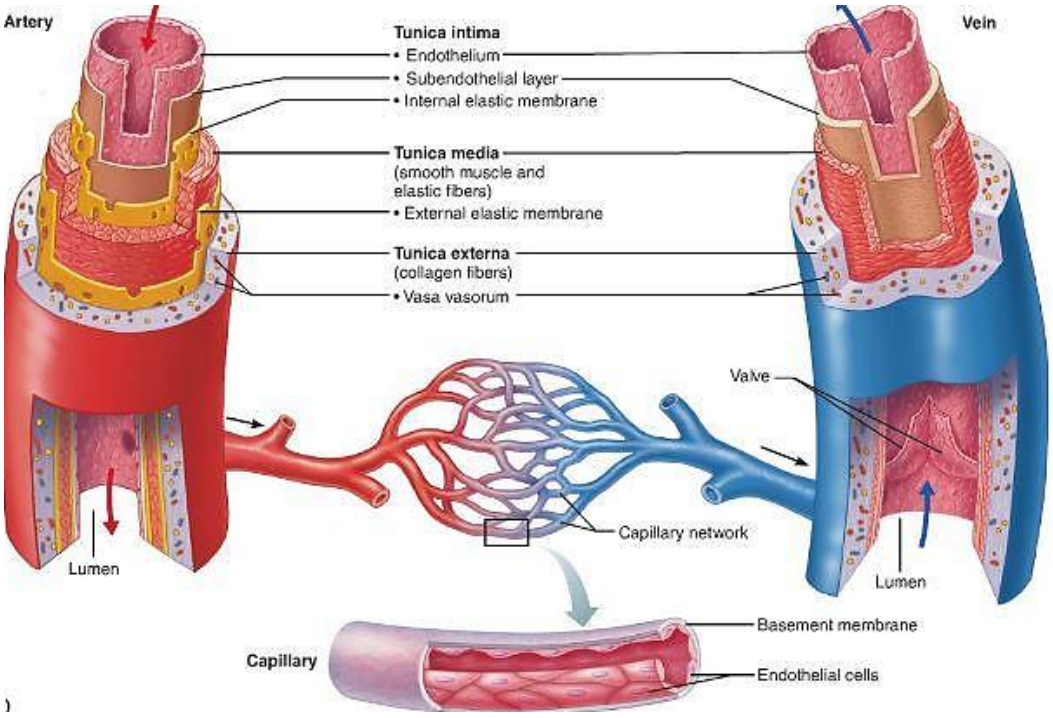

Figure.1.1 Structure of blood vessels. Blood vessels are constituted of three layers: tunica adventitia (externa), tunica media and tunica intima (adapted from https://classconnection.s3.amazonaws.com)

1.1.1 Smooth muscle cells

Vascular smooth muscle cells (VSMCs) are the main constituent of the arteries and are responsible for the tone and the active control of vessel diameter. They are aligned perpendicularly to the vessel orientation and surround the endothelial cells. Communication between endothelium and smooth muscle cells occurs through a structure called myoendothelial projection (MEP) which is important for ECs-VSMCs communication (Moore & Ruska, 1957).

1.1.1.1 Structure

VSMC is non-striated, spindle shape mononucleated myocyte with a diameter ranging from 2 to 10 μm (Panner & Honig, 1967). The contractile apparatus is made of three filament types: the long myosin filaments, actin filaments, and filaments of intermediate size that contain two specific proteins, desmin and vimentin (Panner & Honig, 1967). Unlike skeletal and cardiac muscles, smooth muscle cells contain no myofibrils or sarcomeres. Smooth muscle cells communicated with each other via gap junctions that electrically couple the cells. They make a low resistance pathway allowing rapid spread of electrical signals.

1.1.1.2 Function

The role of VSMCs is not limited to contraction since they also have the capacity to proliferate, migrate, synthesize materials composing extracellular matrix (ECM) components, and secrete cytokines and growth factors. These functions make VSMCs a regulator of vascular homeostasis that goes beyond the control of vessel diameter. For acute and appropriate blood redistribution within the body, the local blood perfusion is regulated by diameter of blood vessels and is dependent on relaxation and contraction of VSMCs.

Calcium required for contraction comes mostly from voltage-dependent calcium channels at the plasma membrane and to a lower extent from the sarcoplasmic reticulum (**Fig. 2**). When intracellular calcium level increases, Ca^{2+} ions bind to calmodulin (CaM) and the Ca^{2+}/CaM complex activates myosin light chain kinase (MLCK), which then phosphorylates myosin. Phosphorylated myosin binds actin filaments to initiate cross bridge cycling and contraction. Myosin dephosphorylation occurs when cytoplasmic Ca^{2+} levels lower and this result in detachment of myosin heads from actin (myocyte relaxation).

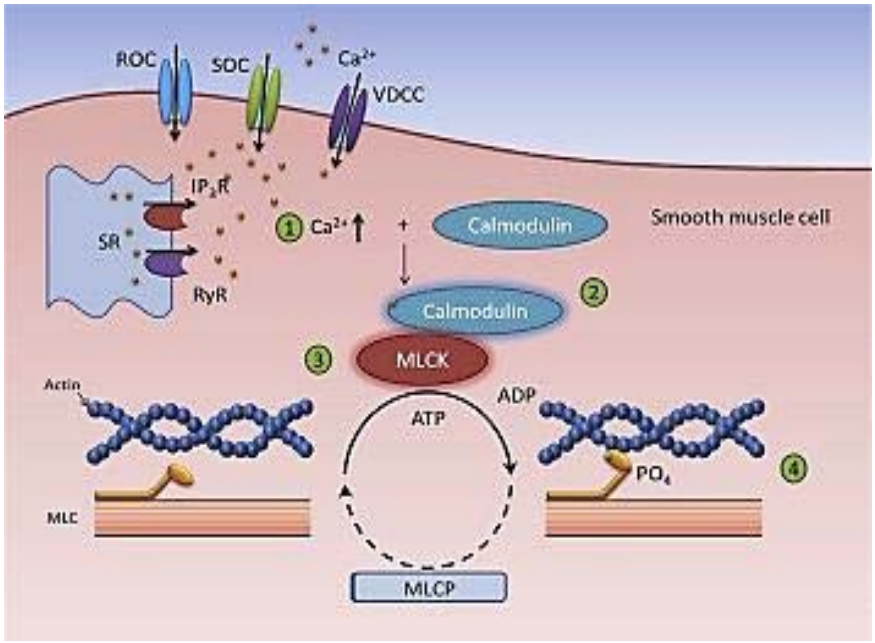

Figure.1.2 Smooth muscle contraction: Increase in intracellular Ca^{2+} levels will provoke smooth muscle contraction. The main source of Ca^{2+} contributing to contraction originates from extracellular space. ROC: receptor-operated channels, VOC: voltage-operated channels, VDCC: voltage-dependent calcium channels, SR: sarcoplasmic reticulum, IP_3P: Inositol trisphosphate receptor, RyR: Ryanodine receptors, ADP: Adenosine diphosphate, ATP: Adenosine triphosphate, MLC: Myosin light chain, MLCK: myosin light chain kinase, MLCP: Myosin light chain phosphatase (adapted from Zhao et al., 2015).

1.1.2 Endothelial cells

1.1.2.1 Structure

The endothelium was initially characterized as an inert cellophane-like membrane lining the cardiovascular system until the 1800s, when von Reckingausen determined that vessels are lined by a monolayer of cells called endothelial cells (ECs). ECs line the entire vascular system and they are connected to each other by various types of adhesive structures and cell-to-cell junctions (Cines et al., 1998).

1.1.2.2 Function

The primary function of ECs is to maintain vascular homeostasis through the generation and release of a wide range of factors in response to chemical and physical signals. These factors, molecules or mechanisms, allow the endothelium to regulate vascular tone, thrombosis, vascular inflammation, smooth muscle cell proliferation and cellular adhesion.

1) **Blood vessel formation**

Development of vascular networks is coordinated by ECs through the release of growth factors such as vascular endothelium growth factor (VEGF) that initiates vasculogenesis or angiogenic sprouting and leads to formation of immature vessels. Other families of growth factors like angiopoietins (Ang) and ephrin are important for blood vessels formation, remodelling and maturation. For example, Ang1 is an important molecule that maintains the stability of the mature vessels (Gale & Yancopoulos, 1999; Yancopoulos et al., 2000). Indeed, embryos lacking Ang1 or Tie2, the main receptor for Ang, develop a normal primary vasculature but it fails to undergo any further remodelling. On the other hand, mouse embryos lacking VEGF fail to develop a normal primary vasculature (Suri et al., 1996).

2) **Coagulation and fibrinolysis**

In physiological conditions, the endothelium is both antithrombotic and anticoagulant because it secretes several molecules needed for regulation of platelet functions and blood coagulation. Endothelial damage or pro-inflammatory stimuli would alter the capacity of the endothelium to maintain this antithrombotic capability (Cines et al., 1998).

Nitric oxide (NO) and prostacyclin (PGI_2) are the main antiplatelet agents secreted by ECs (Cines et al., 1998). They increase cyclic nucleotide levels in platelets, preventing their aggregation (de Graaf et al., 1992). Stimulation of ECs with bradykinin, thrombin or ATP will

results in an increased production of NO and PGI_2, and inhibition of thrombi formation. However, NO and PGI_2 are not the only mechanisms involved in endothelial regulation of coagulation. A more detailed review of the mechanisms have been documented elsewhere (Michiels, 2003).

3) **Endothelial cell barrier**

The endothelium is also a selective barrier allowing a discrete transport of molecules between tissues and blood (Pries et al., 2000). This barrier property is conferred by three types of junctions which are morphologically and functionally different: adherens junctions, tight junctions, and gap junctions (Dejana et al., 1995). Adherens junctions are made of cadherins anchored to a network of intracellular cytoplasmic proteins named catenins (Dejana, 1996). Tight junctions connecting ECs are responsible for tight sealing property of the endothelium. These junctions juxtaposes plasma membrane of attached cells through association of occludins that are connected to several cytosolic proteins such as ZO-1, ZO-2-and cingulin (Bazzoni et al., 1999).

4) **Vascular tone**

ECs are essentially recognized as the major regulator of vascular tone. They can finely tune vascular diameter via the generation and release of several vasodilatory or vasocontractile agents that command contractile status of VSMCs. The delicate equilibrium between these two classes of mechanisms is dependent on the endothelial health as endothelial dysfunction is associated with an impaired endothelial-dependent vasodilation and increased endothelium-associated vasoconstriction.

A) **Vasodilation**

The most potent endothelial-derived relaxing factor is nitric oxide (NO), a free radical gaseous molecule (Palmer et al., 1987), was also the first endothelial modulator identified. Several agonists such as acetylcholine, bradykinin, histamine or adenosine stimulate endothelial production of NO (Govers & Rabelink, 2001). Shear stress, the force evoked by blood flow on endothelium, can also increase endothelial NO production through upregulation of endothelial nitric oxide synthase (eNOS) gene expression and eNOS activation (Govers & Rabelink, 2001). In addition to its impact on vascular myocyte tone, NO inhibits coagulation and smooth muscle cell proliferation (Xiao et al ., 1997).

NO is not the only endothelial-derived vasorelaxant. PGI_2 is also an important vasodilator, which acts through the activation of smooth muscle adenylate cyclase and generation of cAMP. ECs also generate EDHF (Endothelium-dependent hyperpolarizing factor), a non-NO/PGI_2 factor that induces a smooth muscle hyperpolarization and lead to myocyte relaxation. EDHF consists of an amalgam of different molecules (EETs, H_2O_2) or mechanisms (K^+ clouds, $K_{Ca}2.x/K_{Ca}3.1$ channels) that evoke smooth muscle hyperpolarization (Félétou, 2011).

B) **Vasoconstriction**

ECs also generate and release vasoconstrictive factors such as endothelin-1, thromboxane A2 and angiotensin II (Furchgott & Vanhoutte, 1989). It is important to note that angiotensin II is not generated by endothelial cells per se but an enzyme, the Angiotensin converting enzyme (ACE) at the endothelial membrane cleaves circulating angiotensin I into angiotensin II.

5) **Inflammation**

Inflammation is a protective process involving a series of mechanisms where endothelial cells play a key role. However, inflammation can become deleterious if excessive or chronic as it

could lead to increased vascular permeability and thus results in exaggerated fluids exudation and leukocytes migration (Muller, 2003). Endothelial adhesion molecules play major roles in the different steps of leukocyte extravasation including activation, rolling, adhesion and trans-endothelial migration (Allport et al., 1997).

1.1.3. Endothelial-smooth muscle communication: Myoendothelial Junction (MEJ)

The endothelium is physically isolated from the underlying smooth muscle cells by the internal elastic laminae (IEL). This structure, made of collagen and elastin, is too thick to allow endothelium-myocyte contacts and intercellular interactions. However, the IEL is not continuous and fenestrations of the IEL allow formation of structures called myoendothelial projections (MEP). MEPs, found in resistance arteries, are cellular extensions through IEL fenestrations where close apposition of endothelial and smooth muscle membranes occur (myoendothelial junctions; MEJ) (Moore & Ruska, 1957; Heberlein et al., 2009). ECs-VSMCs communication through these MEPs allows bi-directional signaling to accurately modulate vessel diameter. Interestingly, the number of MEJs and gap junction channels increase in distal arteries, where the endothelial-derived hyperpolarizing factor (EDHF) is elevated, while it is less frequent in proximal arteries (Sandow & Hill, 2000; Straub et al., 2011). Indeed, MEPs are considered as a nexus of EC-VSMC communication pathways aiming to finely coordinate vascular tone. MEPs are enriched in endoplasmic reticulum (ER) (Isakson et al., 2007), caveolae (Straub et al., 2011; Saliez et al., 2008), cytosketetal components (Isakson et al., 2008) and ion channels (Ledoux et al., 2008). The presence of cytoskeletal components (actin and microtubules) within MEPs (Isakson et al., 2008) suggests that these structures are dynamic and could support protein trafficking (Straub et al., 2014).

Gap junctions are communication pathways between ECs and VSMCs where second messengers, ions, charges, and small metabolites can freely move between the two adjacent cells (Brisset et al., 2009; Straub et al., 2014). Electrical propagation secondary to changes in

membrane potential can occurs through gap junctions (Saez et al., 2003). Structurally, gap junction channels are made of six connexins (Cx) subunits connecting the cytoplasm of adjacent cells (Saez et al., 2003). Cx37, Cx40, Cx43, and Cx45 are the only connexins reported in the vascular wall (Yeh et al., 1997) and their expression varies according to the vascular bed and species studied. However, only Cx37, Cx40, and Cx43 were found in the MEJ (Isakson et al., 2008).

The presence of ER at the MEP is supportive of a unique and spatially restricted Ca^{2+} signalling pathway (Isakson et al., 2007). Indeed, calcium pulsars are repetitive Ca^{2+} release events that are restricted to endothelial MEP. Specific localization of Ca^{2+} pulsars is due to presence of IP_3R-dense ER stores and leads to specific activation of adjacent $K_{Ca}3.1$ channels within endothelial MEP. The hyperpolarization thus generated provokes myocyte relaxation (Ledoux et al., 2008) (**Fig. 1.3**).

1.1 Regulation of blood vessel diameter

Vascular tone and arterial diameter is essentially dependent on VSMCs contractile status. Ca^{2+} entry through voltage-dependent Ca^{2+} channels being the main source of Ca^{2+} to produce contraction, modulation of vascular tone will preferentially target opening of these Ca^{2+} channels via changes in membrane potential. Indeed, depolarization of myocyte membrane will provoke Ca^{2+} channel opening, allowing Ca^{2+} influx and increase in cytoplasmic Ca^{2+} levels and thereby ensure contraction. Conversely, hyperpolarization of VSMCs membrane will decrease the open probability of voltage-dependent Ca^{2+} channels and thus lead to relaxation. Cyclic nucleotides (cAMP and cGMP) are involved in signalling pathways regulating myocyte contraction.

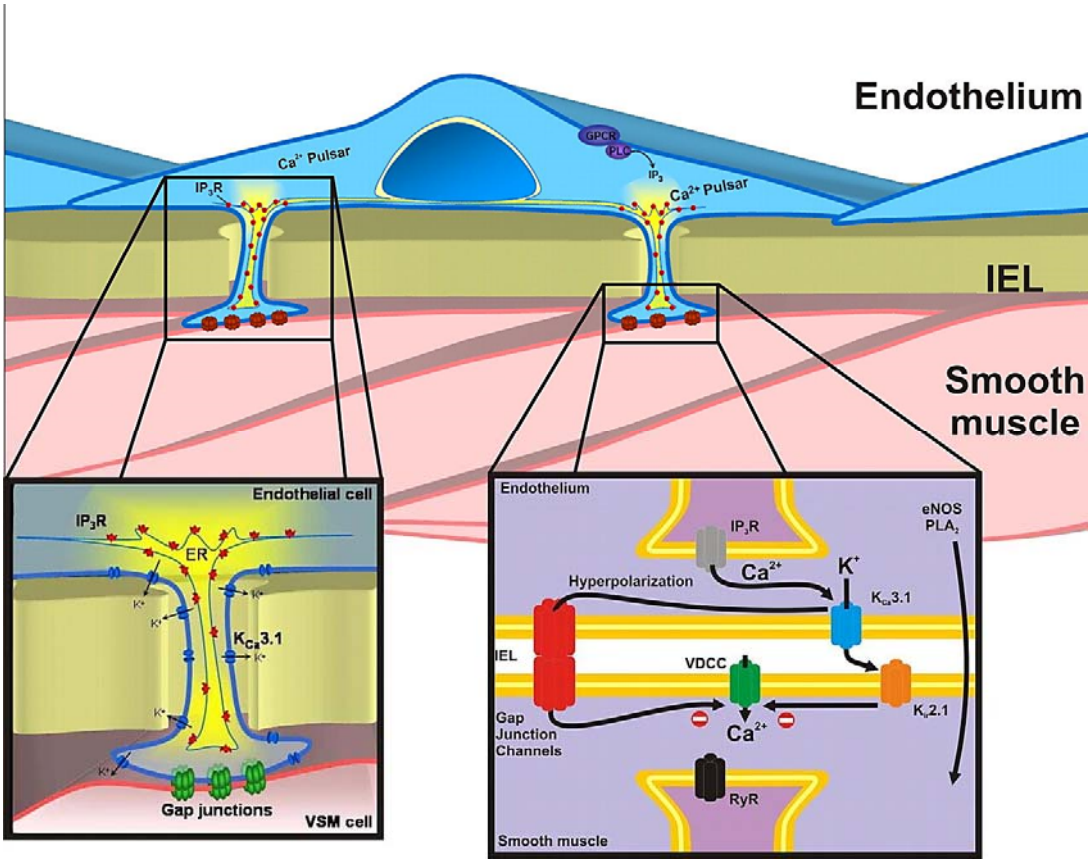

Figure 1.3 Endothelial calcium pulsars: Localized Ca^{2+} release mediated by IP_3 receptors within MEPs activates $K_{Ca}3.1$ channels and lead to smooth muscle hyperpolarization and relaxation. IEL: Internal elastic lamina, IP_3R: Inositol triphosphate receptors, VDCC: Voltage-dependent calcium channels, RyR: Ryanodine receptors, ER: endoplasmic reticulum (adapted from Ledoux et al., 2008)

1.2.1 cAMP-dependent vasodilation

Adenylate cyclase activation evoked by PGI_2 binding to its receptor will induce an increase in cAMP levels and activation of cAMP-dependent protein kinase (Protein Kinase A; PKA). PKA phosphorylation is associated with a decrease in cytoplasmic Ca^{2+} levels via several mechanisms including an increase in SERCA (Sarco/endoplasmic reticulum calcium ATPase) pump activity, a lower MLCK activity and hyperpolarization of VSMCs membrane (Morgado et al., 2012; Adelstein et al., 1978).

1.2.2 cGMP-dependent vasodilation

In parallel to the cAMP-PKA pathway, a cGMP-centric signalling pathway is also found in VSMCs. In this case, protein kinase G (PKG) is the main effector of the cGMP pathway. An increase in cGMP levels activates PKG, which in turn promotes VSMCs relaxtion through various mechanisms, including phosphorylation of K^+ channels and hyperpolarization of the myocyte. It also stimulates Ca^{2+} extrusion from the cytoplasm through enhanced pumping in the SR (Morgado et al., 2012).

1.3 NO-cGMP-PKG pathway

1.3.1 Nitric oxide synthase (NOS)

Initial compoenent of the NO-cGMP-PKG pathway, NOS are heme-containing enzymes that catalyze the NADPH- and O_2-dependent oxidation of L-arginine to nitric oxide (NO) and citrulline. Initially identified and described in 1989 (Bredt & Snyder, 1990; Alderton et al., 2001), homodimeric NOS are generally considered as the physiologically functional form of the enzyme (Hevel et al., 1991; Alderton et al., 2001).

1.3.1.1 Classification of Nitric oxide synthetase (NOS)

Three isoforms of NOS have been identified: NOS I, II and III. The neuronal isoform (nNOS, NOS I) is mainly found in neurons. NO produced in neurons acts as a neuronal messenger modulating synaptic neurotransmission (Michel & Feron, 1997; Prast & Philippu, 2001). On the other hand, the inducible NOS isoform (iNOS, NOS II) is expressed in cells in response to inflammatory mediators (Michel & Feron, 1997). The main source of NO within vessel walls is the endothelial isoform of NOS (eNOS, NOSIII), which is constitutively expressed in ECs (Palmer et al., 1988) (Sandoo et al., 2010). Although NOS isoforms differ in their function,

tissue localization and regulation, they share a common structure and catalytic mechanism (McMillan et al., 1992).

1.3.1.2 eNOS activation

When located in caveolae, eNOS is found as monomer and is inactive. Once intracellular Ca^{2+} increases, eNOS unbind caveolin-1 and homodimerizes to become active (Bucci et al., 2000). Endothelial agonists like acetylcholine, bradykinin, substance P and shear stress can induce intracellular Ca^{2+} increase and NO production through activated eNOS (Michel & Feron, 1997; Bae et al., 2003). Moreover, shear stress can also upregulate eNOS expression (Alderton et al., 2001). In addition to intracellular Ca^{2+}, phosphorylation of eNOS has been shown to modulate eNOS activity. For example, phosphorylation of Thr495 inhibits the enzyme while phosphorylation at Ser1177 and Ser633 stimulate NO production (Alderton et al., 2001). The important literature on eNOS illustrates the significant impact of the enzyme on vascular tone.

1.3.2 Nitric oxide (NO)

1.3.2.1 Nitric oxide as a signalling molecule

As expressed previously, eNOS is highly expressed in ECs and is the main source of NO found in plasma (Walter & Gambaryan, 2009). NO is a colorless, inorganic gas, dissolvable in water and lipids (Knowles & Moncada, 1994; Garthwaite & Boulton, 1995). This latter property is crucial to the signaling molecule as it conveys an elevated diffusibility and allows the molecule to act on neighboring VSMCs. However, the half-life of NO is very short (approximately 5-6 seconds) (Garthwaite & Boulton, 1995). The main target of NO is the soluble guanylate cyclase (sGC). Indeed, within the nanomolar range, NO binds a prosthetic heme on the β-subunit of sGC which results in a 100- to 200-fold increase in the enzyme activity (Ignarro et al., 1982).

However, despite the massive production of NO by ECs, NO-cGMP pathway in ECs remains poorly understood.

1.3.2.2 Cardiovascular effects of NO

As a multipotent molecule, NO modulates many cardiovascular processes such as vascular tone, myocardial functions, and platelet aggregation (Forstermann & Sessa, 2012 ; Bryan et al., 2009 ; Schafer et al., 1980). Alteration in NO bioavailability (either decreased production or increased degradation) would result in higher vascular tone, decreased vessel lumen and elevated blood pressure. Moreover, insufficient NO is also associated with VSMCs proliferation as NO, under normal conditions, supresses VSMC proliferation (Ignaro et al, 2001). NO also increases cGMP through activation of cGC, leading to PKG activation and phosphorylation of various targets, including ion channels resulting in smooth muscle relaxation (Bryan et al., 2009). Modulation of vascular tone is not the only process involving enhanced cGMP production as NO supresses thromboxane A2-induced platelets aggregation in a cGMP-dependent manner (Schafer et al., 1980; Wang et al., 1998).

Additionnally, NO decreases monocytes adhesion to endothelium and therein minimizes monocyte exudation and initiation of atherosclerotic plaque (Tsao et al., 1997). Moreover, antioxidant properties of NO make the endothelium-derived molecule an excellent atheroprotective agent. Indeed, NO inhibits oxidation of LDL cholesterol, the critical step in atherosclerosis (Hogg et al., 1993). Furthermore, NO stimulates growth hormone production (Rubinek et al., 2005)

The brief half-life of NO is not the only limiting factor to NO-dependent outcomes. NO rapidly bind to oxy- and deoxyhemoglobin (Hb) (Lancaster, 1994) that are abundant in erythrocytes, suggesting that a significant portion of endothelium-derived NO is scavenged in blood.

However, in physiological conditions, NO production is sufficient to adequately modulate smooth muscle cells function (Lancaster, 1994).

1.3.3 Guanylate cyclases

Two isoforms of guanylate cyclase, enzyme generating cGMP, have been characterized, the soluble (sGC) and the particulate guanylate cyclase (pGC). Essentially, pGC is a plasma membrane receptor activated by natriuretic peptides whilst sGC is a cytosolic enzyme activated by NO, but also by carbon monoxide (CO) (Sharina et al., 2012; Lukowski et al., 2014). Application of NO donors rapidly elevates cytoplasmic cGMP level. Actually, cGMP levels peaks within ~50s following application of NO donors to VSMCs (Wyatt et al., 1998).

Although sGC is highly expressed in VSMCs, the enzyme has also been found in ECs. However, limited information is currently available on sGC-cGMP functions in ECs. Recent work showed that cGMP levels modulate endothelial permeability and binding/activation of immune cells to endothelium (Ahluwalia et al., 2004).

1.3.4 cGMP

Upon NO binding, sGC converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (Guanosine 3', 5'-cyclic phosphate; cGMP), an essential activator of PKG (**Fig.1.4**). Conversely, cGMP is hydrolyzed in GMP by phosphodiesterases (PDEs), a class of enzymes that metabolizes cyclic nucleotides (cAMP and cGMP) by degradation of 3`-phosphodiester bond (**Fig.1.4**). In recent times, PDEs have gained interest as potential therapeutic avenues for treatment of pathologies associated with decreased cAMP/cGMP levels. Indeed, the balance between the activity of sGC and PDEs is the primary determinant of intracellular cGMP levels (Francis et., 2010).

Intracellular cGMP levels is probably not homogeneous throughout cellular cytoplasm since several players in cGMP synthesis/degradation are heterogeneously distributed and thus creating pools of cGMP (cGMP microdomains) in discrete cellular regions (Fischmeister et al., 2006). However, global arterial cGMP level is generally around ~0.1 μM in resting conditions (Francis et al., 2010).

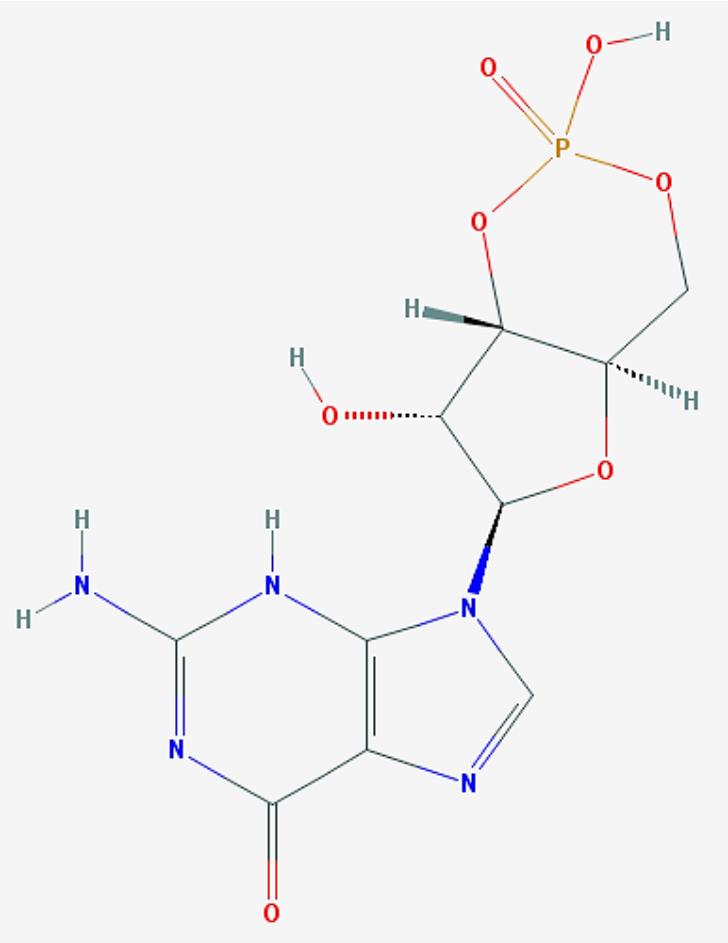

Figure 1.4 – Structure of guanosine 3', 5'-cyclic phosphate (cGMP) (adapted from http://pubchem.ncbi.nlm.nih.gov)

1.3.5 PKG

PKG is a serine-threonine kinase activated by cGMP. Binding of cGMP to the allosteric domain of PKG enhances phosphorylation activity by 3 to 10-Fold (Francis & Corbin, 1999; Schlossmann & Desch, 2009). Three mammalian PKG isoforms have been reported, PKGIα, PKGIβ and PKGIIα. PKGIα is the predominant isoform expressed in endothelial cells (Lukowski et al., 2014; Kalderon & Rubin, 1989).

1.3.6 Phosphodiesterases

PDEs play a critical role in intracellular signaling pathway involving cyclic nucleotides via hydrolyzation of cAMP and cGMP and thus negatively regulate associated proteins and transcription factors (Keravis & Lugnier, 2012). According to their high catalytic efficiency, they are indeed considered as the main determinant of cyclic nucleotide-dependent pathways.

Mammalian PDEs have been classified in eleven families (**Table 1**) mainly based on cAMP/cGMP affinity, regulation, structural similarities, tissue distribution and pharmacological properties (Bender & Beavo, 2006; Lugnier, 2006). Some PDEs, such as PDE3, show higher affinity to cAMP, while some, such as PDE5 and PDE6, have higher affinity to cGMP. Nevertheless, PDE1C, PDE2A and PDE11A have a similar affinity to both substrates (**Table 1**). From a structural perspective, PDEs are found in dimers. Each monomer has three distinct domains, a N-terminal regulatory domain, a catalytic domain, and a C-terminal domain (Keravis & Lugnier, 2012). PDEs regulation is not limited to binding of cyclic nucleotides, but is rather comprehensive including phosphorylation steps, anchoring proteins, subcellular targeting and translocation, etc. (Bender & Beavo, 2006; Lugnier, 2006; Francis et al., 2010).

The intracellular localization of PDE isoforms as well as their tissue distribution is different (**Table. 2**) and the physiological impact of PDEs varies accordingly. For example, PDE3B is highly expressed in hepatocytes where it modulates insulin signaling (Bender & Beavo, 2006).

As mentioned above, cyclic nucleotide affinity varies amongst PDE family members, with PDE1A and B, PDE5, PDE6 and PDE9 showing a higher cGMP/cAMP affinity ratio (**Table. 1**). PDE1 and PDE9 are predominantly expressed in neuronal and vascular smooth muscle cells but have been found in other cell types (**Table. 2**). However, PDE1 and PDE9 sensitivity to cGMP is lower than PDE5 and PDE6 (**Table. 1**). PDE6 expression is mostly restricted to retina while PDE5 is expressed in multiple tissues, including vascular cells (**Table 2**).

Cellular outcomes of PDE activity are also diversified (**Table. 3**) as evidenced by the multiple pathologies associated with altered PDE function. Bronchial asthma, erectile dysfunction, hypertension, heart failure, schizophrenia are example of such diseases (Barnett & Machado, 2006; Carson, 2007; Coward & Carson, 2008; Dorsey et al., 2010; Francis, Corbin, & Bischoff, 2009; Galie et al., 2009; Galie et al., 2005; Mebazaa et al., 2010; Rubio-Aurioles et al., 2009; Stehlik & Movsesian, 2009). Actually, therapeutic approaches targeting PDEs are already used in treatment of pathologies like autoimmune and inflammatory diseases (Francis et al., 2010). The most famous is undoubtedly the inhibition of PDE5 for the treatment of erectile dysfunction but PDE5 inhibitors are now also used to treat pulmonary hypertension (Francis et al., 2010).

Compartmentalization of the substrate, cGMP, has been reported in different types of cells. Such studies used natriuretic peptides to activate pGC or NO donors to activate sGC. However, studies on subcellular targeting of PDEs revealed that cGMP-specific PDEs play a fundamental role in creation of the specific intracellular patterns of cGMP. For example, in VSMC, cGMP synthesis induced by atrial natriuretic peptide (ANP) is spatially confined to the subplasma

membrane space, while this spatial localization is lost in the presence of a PDE5 inhibitor, (Nauch et al., 2008).

Table 1. PDE enzymes kinetic properties (adapted from Bender & Beavo, 2006).

Isoform	**Substrate specificity**	K_m		*Vmax*	
		cGMP	**cAMP**	**cGMP**	**cAMP**
		μM		*μmol / min / mg*	
PDE1A	cAMP<cGMP	2.6—3.5	72.7—124	50—300	70—450
PDE1B	cAMP<cGMP	1.2—5.9	10—24	30	10
PDE1C	cAMP=cGMP	0.6—2.2	0.3—1.1	—	—
PDE2A	cAMP=cGMP	10	30	123	120
PDE3A	cAMP>cGMP	0.02—0.15	0.18	0.34	3.0—6
PDE3B	cAMP>cGMP	0.28	0.38	2.0	.5
PDE4A	cAMP>cGMP	—	2.9—10	—	0.58
PDE4B	cAMP>cGMP	—	1.5—4.7	—	0.13
PDE4C	cAMP>cGMP	—	1.7	—	0.31
PDE4D	cAMP>cGMP	—	1.2—5.9	—	0.03—1.56
PDE5A	cGMP>cAMP	2.9—6.2	290	1.3	1.0
PDE6A/B	cGMP>cAMP	15	700	2300	—
PDE6C	cGMP>cAMP	17	610	1400	—
PDE7A	cAMP>cGMP	—	0.1—0.2	—	—
PDE7B	cAMP>cGMP	—	0.03—0.07	—	—
PDE8A	cAMP>cGMP	—	0.06	—	—
PDE8B	cAMP>cGMP	—	0.10	—	—
PDE9A	cGMP>cAMP	0.70—0.17	230	—	—
PDE10A	cAMP<cGMP	13—14	0.22—1.1	—	—
PDE11A	cAMP=cGMP	0.95—2.1	2.0—3.2	—	—

Table.2 The localization of PDE families (adapted from Bender and Beavo, 2006)

Isoform	Localization	
	Tissue/Cellular	**Intracellular**
PDE1A	Smooth muscle, sperm; PDE1A1 in heart and lung, PDE1A2 in brain	Predominantly cytosolic
PDE1B	PDE1B1 in smooth muscle cells, lymphocytes, and neurons; PDE1B2 in lymphocyte and macrophages.	Cytosolic
PDE1C	Brain, smooth muscle, spermatids; PDE1C2 in olfactory epithelium	Cytosolic
PDE2A	Brain, adrenal medulla heart, macrophage subsets, platelets, endothelial cell subsets.	PDE2A3 and PDE2A2 are membrane-bound, but PDE2A1 is cytosolic
PDE3A	Vascular smooth muscle, heart, platelets, kidney and oocyte.	Membrane-associated or cytosolic.
PDE3B	Vascular smooth muscle, hepatocyte, adipocytes, kidney, sperm, β cells, macrophages and T lymphocytes.	Predominantly membrane-associated; Localized to microsomal fractions and endoplasmic reticulum.
PDE4A	Olfactory system, brain, immune cells, and testis.	PDE4A5 restricted to membrane ruffles through its Src homology domain; PDE4A1 is membrane-associated; PDE4A4 associates with Src family kinases.
PDE4B	Variable tissues, notably in brain and immune cells.	PDE4s are recruited to interact with β –arrestin.
PDE4C	PDE4 isoform that has the least expression; found in lung, neurons and testis.	Predominantly cytosolic.
PDE4D	Widely expressed but mainly in brain tissue.	Cytosolic or particulate fractions.
PDE5A	Vascular smooth muscle, platelets, brain, heart, lungs and kidney; PDE5A1 and PDE5A2 are widely distributed, PDE5A3 is specific to vascular smooth muscle	Cytosolic
PDE6A/ PDE6B	Rod cells of the photoreceptors and pineal gland	Targeted to the membrane by isoprenylation; its association with the δ subunit results in cytosolic localization
PDE6C	Cone cells of the photoreceptor and pineal gland	Cytosolic by virtue of its association with the δ subunit
PDE7A	Skeletal muscle, endothelial cells; PDE7A1was found in immune cells, PDE7A2 in heart.	Cytosolic.
PDE7B	Heart, brain, liver, pancreas, skeletal muscle and testis.	Cytosolic
PDE8A	Testis, small intestine, spleen, colon, ovary and kidney.	Cytosolic and particulate fractions
PDE8B	PDE8B1 is expressed in thyroid, PDE8B3 and PDE8B2 are expressed in brain and thyroid	Cytosolic and particulate fractions
PDE9A	Kidney, spleen, brain, gastrointestinal tissues, and prostate.	PDE9A5 is cytosolic, whereas PDE9A1 is localized in nucleus
PDE10A	Brain, heart, testis, and thyroid; also found in pituitary gland and cardiac and skeletal muscle.	PDE10A1 and PDE10A3 are cytosolic, while PDE10A2 is particulate.
PDE11A	Skeletal muscle, testis, prostate, salivary gland, liver and thyroid gland.	Cytosolic

Table.3 The role of PDE families in physiological processes (adapted from Bender & Beavo, 2006).

Isoform	Role
PDE1	PDE1A regulate vascular smooth muscle contraction and sperm function; PDE1B is involved immune cells activation and in dopaminergic signaling; PDE1C is needed for the proliferation of vascular smooth muscle cell, neuronal signalling and sperm function.
PDE2	PDE2 regulates both cGMP and cAMP pathways; it controls aldosterone secretion, barrier role of endothelial cells in case of inflammation, cAMP and PKA phosphorylation of Ca^{+2}channels in the heart, long term memory and cGMP in neurons.
PDE3	PDE3A regulates platelet aggregation, cardiac contractility, vasoconstriction, renin release and oocyte maturation; PDE3B mediates insulin signaling and mediates the inhibitory action of leptin as well as regulates cell cycle/proliferation.
PDE4	PDE4 has role in brain function, activation of immune cells, neutrophil infiltration, and proliferation of vascular smooth muscle, cardiac contractility, vasodilation and fertility.
PDE5	PDE5 regulates vascular smooth muscle contraction, especially in lung and penis and lung ; it is a main component of NO-cGMP signaling in platelets to regulate its aggregation. It plays a role in regulation of cGMP signaling in the brain
PDE6	PDE6 is involved in the photoresponse (signal transduction) as well as melatonin release from the pineal gland
PDE7	PDE7 plays a role in activation of T-cell and other inflammatory cells
PDE8	PDE8 has a role in T cell activation, leydig cell, or sperm function
PDE9	The function of PDE9 is unknown, but it is thought to regulate NO-cGMP signaling in the brain
PDE10	PDE10A may regulate cGMP in the brain and thought to has a role in learning and memory
PDE11	PDE11 has a role in sperm function and development

1.3.6.1 PDE5

PDE5 is the most extensively studied cGMP-specific PDE in both animals and human because of its critical role in the cardiovascular system. PDE5 is therefore well characterized on a structural/function perspective but is also granted with an interesting pharmacological toolbox.

1.3.6.1.1 Structure

A single gene encodes PDE5A, with three splice variants reported so far (PDE5A1-A3). The variation lies in the length of N-termini but function does not seems to be altered (Loughney et al., 1998; Nagendran et al., 2007). PDE5A1 and PDE5A2 are expressed in several tissues such

as platelets, VSMCs, skeletal muscle, heart, kidney, lung, and brain, whilst PDE5A3 expression is restricted to VSMCs (Bender & Beavo, 2006).

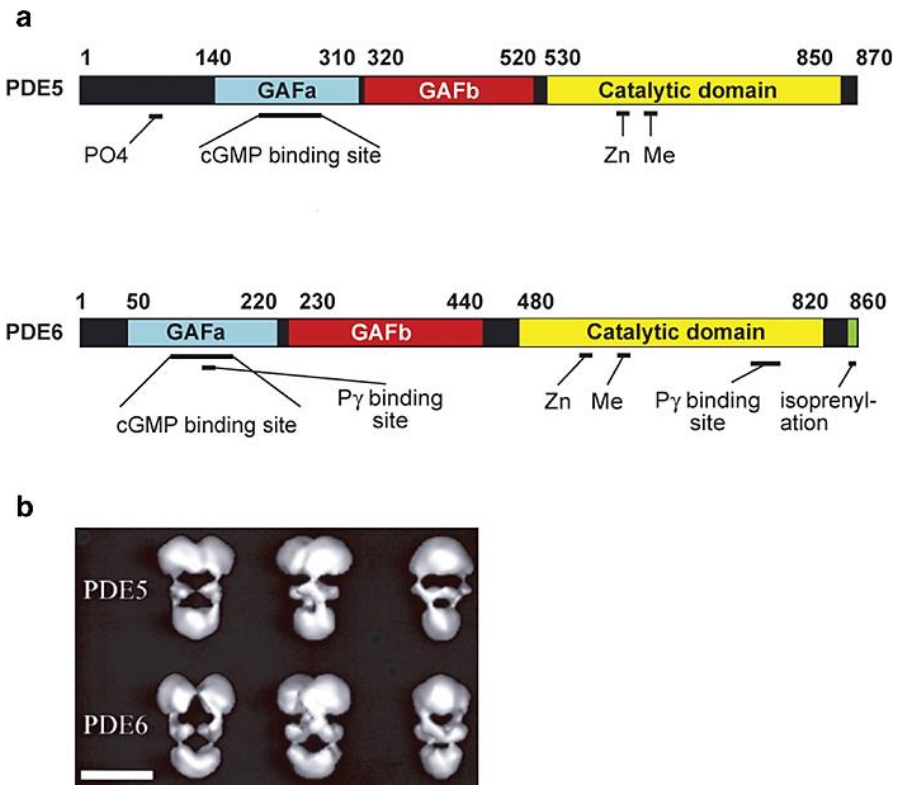

Figure 1.5 - Structural similarities between PDE5 and PDE6, **a**) Organization of domains in PDE5 and PDE6. **b**) 3-D surface representation of PDE5 and PDE6 holoenzymes (adapted from Cote, 2004)

PDE5 affinity for cGMP is essentially determined by two highly homologous GAF domains (GAF-A and GAF-B) with a 100-Fold higher selectivity for cGMP over cAMP (Zoraghi et al., 2005) (**Fig. 1.5**). High affinity binding of cGMP to GAF-A domain evokes PDE5 conformational change in the C-terminal tails, initiating the catalytic activity towards cGMP

(Castro et al., 2010). PDE5 affinity for cGMP can also be modulated by phosphorylation of the enzyme.

According to this peculiar property (cGMP hydrolyzation induced by cGMP activation of the enzyme), PDE5 is essentially playing a role as a negative feedback switch. cGMP signals are then generally transient despite continuous addition of NO, as with the use of NO donors (Koesling et al., 2005).

1.3.6.1.1 Pharmacology

The first selective PDE5 inhibitor developed was Zaprinast. However, the initial PDE5 inhibitor commercially available was Sildenafil, a false substrate for PDE5, with a significantly higher (10-Fold) binding affinity compared to cGMP (Kass, 2012). The main side-effect reported for the use of Sildenafil and other PDE5 inhibitors to treat erectile dysfunction is visual disturbance. This non-vascular effect is due to cross-reactivity with PDE6, a strongly homologous PDE expressed in retinal cells. Other PDE5 inhibitors were then developed with a stronger selectivity for PDE5 over PDE6, such as Tadalafil (1020-fold) (Y. Y. Huang et al., 2013). However, the molecular mechanism responsible for drug selectivity towards PDE5 vs. PDE6 remains to be established and might require the elucidation of PDE6 crystal structure.

1.3.6.2 PDE6

Also called the "photoreceptor PDE", PDE6 has been initially characterized as a critical step in light transduction in photoreceptors only. Later work reported expression of PDE6 in pineal gland (Morin et al., 2001). The role of PDE6 in retina has been evidenced by the identification of PDE6 mutation as causal to stationary night blindness, making PDE6 the first PDE known to cause a disease secondary to its mutation (Bender & Beavo, 2006).

1.3.6.2.1 Structure

In rod photoreceptors, PDE6 is found in dimer of PDE6α (PDE6A) and a PDE6β (PDE6B) subunit. Similarly to PDE5, PDE6 has a catalytic domain in the C-termini with an elevated cGMP-specificity. The N-termini contains two GAF domains (GAF-A and GAF-B) with a cGMP-binging site in GAF-A (**Fig. 1.5**). Catalytic efficiency of PDE6 is the highest of all known PDEs. When fully activated, PDE6 catalytic rate is impressive with >2000 μmol/min/mg of cGMP (Gillespie & Beavo, 1988; Cote, 2004; Bender & Beavo, 2006). Interestingly, PDE6 heterodimerization does not seem to be essential as heterodimers were not detected in avian pineal glands (Morin et al., 2001) or retina of chicken (Huang et al., 2004; Bender & Beavo, 2006).

In retina, PDE6 is basically responsible for fast hydrolysis of cGMP following light stimulation, causing closure of cGMP-gated channels and thus membrane hyperpolarization (Ames et al., 1986), therein triggering photoreceptor activation (Cote, 2004). PDE6A and PDE6B are expressed in rod cells, while PDE6C (also called PDE6α) are expressed in cone cells of the retina. On the other hand, PDE6 γ and δ isoforms have also been described but they both lack catalytic properties. Alternatively, they are considered as inhibitory subunits of catalytically active PDE6 isoforms. PDE6 that are expressed in rod cells is composed of two large subunits (≈99 kDa) which are the α and β catalytic subunits which are encoded by PDE6A and PDE6B genes respectively. It also has two small subunits; the first one (≈ 11 kDa) is encoded by PDE6G, the second one (≈ 17 kDa), is a δ regulatory subunit that is encoded by PDE6D. On the other hand, PDE6 in cones is composed of two α' catalytic subunits encoded by PDE6C and two inhibitory γ subunits encoded by PDE6H (Francis et al., 2011).

Until recently, PDE6 expression was considered to be restricted to retinal cone and rod cells (Miki et al., 1975) and pineal gland (Morin et al., 2001). However, PDE6 expression has

recently been reported in mouse lung (Tate et al., 2002; Nikolova et al., 2010) and in cultured cell lines such as HEK293 cells (Wan et al., 2001), melanoma cells lines (Bazhin et al., 2010).

1.3.6.2.1 Activation and pharmacology

In dark condition, retinal cGMP levels are extremely high (≈ 70 μM) as PDE6 is in an inactive form. cGMP activate cGMP-gated cation channels, inducing depolarization. When retina is exposed to light, PDE6 triggers a cascade following photon activation of Transducin, a G-protein. Rod and cone photopigments absorb photons leading to activation of Transducin. Activated transducin removes inhibitory influence of PDE6γ and allows catalytic PDE6 to hydrolyze cGMP, leading to deactivation of cGMP-gated cation channels at the photoreceptor membrane. The PDE6-evoked hyperpolarization then initiates neuronal photoreceptor response (Arshavsky et al., 2002).

Presence of PDE6 in tissues other than retina suggests potential alternative and photon-independent mechanisms for PDE6 activation. Unfortunately, there are currently no specific PDE6 inhibitors commercially available. PDE6 pharmacology mostly overlaps with PDE5. As indicated above, visual side effects reported in patients using PDE5 inhibitors for erectile dysfunction are due to PDE6 inhibition.

1.3.6.3 Comparison between PDE5 and PDE6

From a structural perspective, PDE5 and PDE6 share high degree of similarity in terms of pharmacological and substrate specificity. Indeed, the amino acid sequence of catalytic domain and three-dimensional structure of catalytic domains of both subunits are similar (**Fig.1. 5**).

However, PDE5 and PDE6 are distinct in respect to their enzymatic characteristics. For example, maximal cGMP-hydrolysis rate is significantly higher for PDE6 (6000 to 8000 cGMP/s) compared to PDE5 (three orders of magnitude lower) (D'Amours & Cote, 1999; Thomas et al., 1990). Also, activation of PDE6 essentially requires inhibition of the interaction with PDE6γ subunits and is therefore constitutively active unless inhibited by PDE6γ. On the other hand, PDE5 activation is dependent on binding of cGMP to GAF domains or phosphorylation of catalytic domains (Corbin et al., 2000; Cote, 2004).

1.4 Mathematical models

NO has been extensively studied over the last decades, and is a key signaling molecule in many physiological functions, including the microcirculatory tone. *In vivo*, NO-cGMP-PDE pathway is a complex process and the detailed pathway is yet to be fully quantified and analyzed. The use of mathematical models is an interesting approach that can provide valuable insights for the physiological role of NO. For example, mathematical models have highlighted the impact of the cellular components in the NO/cGMP pathway, and clarified NO signaling and transport (Tsoukias, 2008).

In 1994, two independent groups (Lancaster, 1994; Wood & Garthwaite, 1994) created the first mathematical models to study NO biotransport in VSMC. The model parameters were based on available data obtained from the published literature. To date, all the published models investigated the NO/cGMP pathway in VSMC despite the fact that the NO/cGMP pathway is also present in endothelial cells. In general, simulation models are usually considering experimental conditions with a temperature of 20°C, where NO is used to initiate the cGMP pathway, and with a homogeneous intracellular distribution of sGC as well as PDE5. sGC is included as a generator of cGMP in response to NO, and PDEs is included as a catalyst for cGMP hydrolysis.

Mathematical models showed that Hb scavenge significant amounts of NO, but vasodilation is maintained, suggesting that either some NO escapes from scavenging or a carrier molecule transports NO to VSMC and prevents its scavenging by Hb. More recent studies used different geometries and NO transport models based on a continuum approach and standard mass transport equations. (Butler et al., 1998; Vaughn et al., 1998). These approaches coupled to hydrodynamic modeling showing the existence of a layer free of red blood cells next to the endothelium indicated significant NO scavenging rate.

A recent study reports that NOS activity is the dominant parameter controlling NO release (Jiang & George, 2011). As such, modeling the impact of enhanced arginase activity showed little impact of l-arginine bioavailability for NOS activity in lung epithelial cells. More recently, intracellular signaling and interaction of sGC, NO and PDE5 was shown to modulate cGMP levels within the smooth muscle cells (Yang et al., 2005). The presence of multiple PDEs and their specific distribution within the endothelial cells could be integrated in such a modeling approach and might depict the actual cellular physiological system with an improved accuracy.

Chapter 2. Objectives of research

2.1 Rationale

NO pathway is a fundamental endothelial mechanism regulating smooth muscle contraction and vascular tone. In endothelial cells, Ca^{2+}-calmodulin complex activates NOS3 and the NO then generated diffuses to the VSMCs. In myocytes, activation of sGC by NO leads to an increase in cGMP levels and ultimately evokes muscle relaxation and vasodilation. ECs, as major modulator of vascular diameter, are generating colossal amount of NO to modulate smooth muscle tone. On the other hand, pharmacological inhibition of cGMP degradation by PDE5 is also an efficient way to induce vasodilation.

Although the NO-sGC-cGMP-PDE pathway has been extensively studied in VSMCs, the current understanding of this pathway is ECs is unfortunately limited, despite NO production occurring within these cells. The main hypothesis of this study was that phosphodiesterases in native endothelium allows a strict control of cGMP levels despite NO production.

2.2 Broader objectives

- To determine the expression of cGMP-dependent PDE6 isoforms and PDE5 in native endothelium.
- To establish the subcellular distribution of PDE6 isoforms and PDE5 in native endothelial cells.
- To elucidate the impact of heterogeneous intracellular distribution of PDE6 and PDE5 on cGMP levels with a mathematical model.

Chapter 3. Phosphodiesterase 6 generates intracellular cGMP microdomains in native endothelium

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3.1 Abstract

Endothelial cells regulate vascular tone partially through the production and release of nitric oxide (NO). The NO-cGMP pathway can be finely tuned through modulation of the cGMP degradation by phosphodiesterases (PDEs). Despite elevated production of NO by endothelial cells (ECs), desensitization of the NO-cGMP pathway does not occur in ECs. Moreover, NO-cGMP pathway is an important regulator of endothelial permeability. cGMP-specific PDEs might therefore be involved in the control of cGMP levels. PDE6, although originally thought to be restricted to retina and pineal gland, is the most efficient PDE and could be involved in this process, creating thereof cGMP microdomains. PDE6α, PDE6β and PDE5 were found at the transcription and protein level in mesenteric arteries from mice. Although PDE5 is homogeneously distributed in vascular cells, PDE6α and more prominently PDE6β showed a preferential localization in the perinuclear region. Interestingly, PDE6 subunits were not detected by *in situ* immunofluorescence in vascular smooth muscle or HUVEC cultured cells. Computer modeling revealed that, in the absence of PDE6 unitary or repetitive NO pulses would result in a global rise in cGMP levels ([cGMP]) that could not be abolished by PDE5. However, PDE6 presence in the perinuclear region created a [cGMP] gradient with the perinuclear region having less cGMP. This study provides the first evidence of the expression PDE6 isoforms in native endothelial cells. Moreover, mathematical modeling strongly suggests that the specific perinuclear localization of PDE6 subunits has a dramatic impact on cGMP levels, creating cGMP microdomains in native endothelium.

3.2 Introduction

Vascular tone is critically regulated by dynamic mechanisms originating from the endothelium. The well-described nitric oxide (NO) pathway is a major component of the endothelial mechanisms controlling vascular smooth muscle contractile state. Upon rise in endothelial

intracellular Ca^{2+}, the Ca^{2+}-calmodulin complex activates endothelial nitric oxide synthase (NOS3). The NO thus generated from l-arginine diffuses to the underlying vascular smooth muscle and activates the soluble guanylate cyclase (sGC) to produce cGMP. Protein kinase G activation by cGMP promotes relaxation of the smooth muscle through several pathways including activation of BK_{Ca} channels and inhibition of IP_3R. On the other hand, cGMP levels are also controlled through cyclic nucleotide hydrolysis by phosphodiesterases (PDEs). Therefore, inhibition of smooth muscle PDEs promotes vasodilation as evidenced with the development of drugs such as sildenafil.

Phosphodiesterases are enzymes that catalyze the hydrolysis of cyclic nucleotides such as cGMP and cAMP into GMP and AMP, respectively. Up to now, 11 families of PDEs have been identified in mammals***(2, 9)***. PDEs are sorted based on their specificity toward cGMP or cAMP. PDE1, PDE5, PDE6 and PDE9 are cGMP-specific phosphodiesterases. Interestingly, PDE6 is the most efficient cGMP-specific phosphodiesterase. Indeed, despite a slightly lower affinity for cGMP in comparison to PDE5, PDE6 has the highest catalytic efficiency of all PDEs, with a hydrolysis rate of 3000 cGMP/sec for each PDE6 enzyme. This characteristic is highly desired for the initiation of the photoreceptor activation in the retina and has been extensively studied***(3)***. Conversely, such prominent catalytic efficiency could also be beneficial in limiting desensitization of the signalling pathway in endothelial cells, which are producing important amounts of NO. However, PDE6 is generally considered as a retina-specific PDE since its expression was thought to be restricted to retina and pineal gland***(2)***. Recent evidences suggest that PDE6 expression might not be restricted to these tissues but might also be expressed in melanoma cells and in the lung***(1, 11)***.

Although the NO-cGMP-PDEs pathway has been extensively studied in vascular smooth muscle cells and despite the fact that endothelium constitutively generate significant amount of NO, the understanding of this pathway in endothelial cells is limited, Moreover,

investigations of endothelial NO signalling pathway generally focussed on the modulation of endothelial permeability ***(4, 14)***. With respect to the growing interest on cyclic nucleotide microdomains***(5, 6, 13)***, endothelial cGMP-specific PDEs and their potential impact on the generation of cGMP microdomains were investigated. PDE6 regulation of cGMP microdomains in endothelial cells was then dynamically studied in relation to endothelial NO production with a mathematical model.

3.3 Materials and methods

Tissue preparation:

Mesenteric arteries (3rd and 4th order) were harvested from C57BL/6 mice (3 - 4 months old) and cleaned from connective tissue in cooled HEPES solution. All animal manipulations were approved by the Montreal Heart Institute Research Center Animal Deontology Committee.

Immunohistochemistry:

Mesenteric arteries were cut open and pinned on a sylgard block with the endothelium facing up. Arteries were then fixed with paraformaldehyde 4% (20 min) then permeabilized with 0.2% triton x-100 and blocked with 4% normal donkey serum (60 min). The arteries were first incubated overnight at 4°C with primary antibodies (Table 1). Arteries were subsequently washed with PBS and incubated (60 min) with donkey anti-rabbit ALEXA 555 (dilution 1/1000) fluorescent antibody. Incubation with DAPI (5 min, 300 nM) was used to reveal the nucleus. Immunofluorescence was collected using a Zeiss LSM 510 system confocal microscopy (63x oil objective, numerical aperture 1.4, excitation laser wavelength: 488 nm, 543 nm and 405 nm). All images were deconvolved with Huygens Professional software and reconstructed with Zen 2009 light edition with experimental PSFs.

Table 3.1: Primary antibodies used for immunohistochemistry

Target Protein	Host	Company	Catalog number	Dilution
PDE5	Rabbit	Santa Cruz	SC-32884	1/100
PDE6α	Rabbit	Abcam	Ab5659	1/100
PDE6β	Rabbit	Abcam	Ab5663	1/100
PDE6γ	Rabbit	Abcam	Ab14627	1/100

RNA extraction and real time PCR:

RNA from whole mesenteric arteries (n=3, for each PDE) was extracted using Qiagen columns (RNEasy micro kit) and cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad). Real time PCR was made with iTaq fast SYBR Green with ROX (Bio-Rad) according to the manufacturer's protocol with a MX3005 thermocycler (Stratagen). Specific primers were designed for PDE5, PDE6α and PDE6β. Real time PCR end-products were migrated on a 3% agarose gel. The following primers sequence were used: PDE5: FWD 5'-AGTCATAGGCGTCTGCCAACTT-3', REV 5'-CTCTCCACCGCTTCATACATCT-3'; PDE6α: FWD 5'-CCACAGACCTTGCCTTGTAT-3', REV 5'-CTGCTCCAGCATCATGTACT-3'; PDE6β: FWD 5'-ACCTCCACAACTGTGAGACACGC-3', REV 5'-GATGTCTGTCAGCTCTTCAAACACCTT-3'.

Protein extraction:

Mesenteric arteries immersed in ice-cooled acetone (10% trichloroacetic acid, 10 mM DTT) were lyophilized and heated at 95°C for 10 minutes in SDS-gel sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 0.1 M DTT) ***(8, 15)*** The sample was then placed on a rotator at 4°C overnight. Proteins from the control tissue were isolated through enzymatic digestion with/without triton X-100.

PDE6a (control tissue, n=4), (mesentric artery, n=3), P=***0.001

PDE6b (control tissue, n=3), (mesentric artery, n=3), P=*0.05

PDE5 (control tissue, n=3), (mesentric artery, n=3), P=*0.05

Unpaired *t*-test was done to determine if there was a significant difference between control and mesenteric tissues.

Western blot:

Proteins were migrated on 10% polyacrylamide gel and then transferred to a nitrocellulose membrane (100 V, 90 minutes). Protein detection was observed after incubation overnight with primary antibody (see table 1) and a HRP-conjugated secondary antibody. Detection was obtained using western lightning Plus-ECL (Perkin Elmer).

Mathematical model:

The mathematical model is based on a reaction-diffusion equation for cGMP:

$$\frac{\partial[\mathrm{cGMP}]}{\partial t} = D\left(\frac{\partial^2[\mathrm{cGMP}]}{\partial x^2} + \frac{\partial^2[\mathrm{cGMP}]}{\partial y^2}\right) + R\left(E_{5c}, [\mathrm{cGMP}]\right)$$

where R is the chemical reaction for cGMP creation and D = 130 $\mu m^2/s$. As proposed in ***(17)***, the presence of GTP is assumed to be abundant for cGMP production such that the kinetics is mainly dependent on the guanylate cyclase concentration. The reaction term is thus given by:

$$R\left(E_{5c}, [\mathrm{cGMP}]\right) = V_{\max,GC} E_{5c} - \frac{v_{pde5}[\mathrm{cGMP}]^2}{K_{m,pde5} + [\mathrm{cGMP}]} - M(x,y)\frac{v_{pde6}[\mathrm{cGMP}]^2}{K_{m,pde6} + [\mathrm{cGMP}]}$$

where $V_{max,GC}$ = 1.26 μM/s, $K_{m,pde5}$ = 3.0 μM, v_{pde5} = 0.0695 s^{-1}, $K_{m,pde6}$ = 15.0 μM, v_{pde6} = 71.35 s^{-1} ***(17)***}. Parameters for PDE6 isoforms are derived from table I of ref. ***(2)*** and represents

the PDE6 α and β isoforms. *M(x,y)* is the perinuclear spatial distribution of PDE6 isoform and is set as a thin annulus with thickness of 0.356 μm. E_{5c} is the activated proportion of GC bound to NO, which is given by a simplified set of ordinary differential equations derived from ***(17)*** assuming:

$$\frac{dE_{6c}}{dt} = k_1[\mathrm{NO}] - \left((k_1 + k_3)[\mathrm{NO}] + k_{-1} + k_2\right)E_{6c} - k_1 E_{5c}[\mathrm{NO}]$$
$$\frac{dE_{5c}}{dt} = \left(k_3[\mathrm{NO}] + k_2\right)E_{6c} - k_4 E_{5c}$$

where $k_1 = 2.0\ \mathrm{nM^{-1}\ s^{-1}}$, $k_{-1} = 100\ \mathrm{s^{-1}}$, $k_2 = 0.2\ \mathrm{s^{-1}}$, $k_3 = 0.003\ \mathrm{nM^{-1}\ s^{-1}}$, and $k_4 = 0.4\ \mathrm{s^{-1}}$. The spatial 2D domain of the cell is based on a real endothelial cell morphology taken from a confocal image (see immunohistochemistry section).

Together with NO scavenging, the total NO consumption rate may be approximated as a first-order kinetics ***(17)*** such that the fast reaction diffusion equation for [NO] is given by:

$$\frac{\partial[\mathrm{NO}]}{\partial t} = D_{NO}\left(\frac{\partial^2[\mathrm{NO}]}{\partial x^2} + \frac{\partial^2[\mathrm{NO}]}{\partial y^2}\right) + J_{\mathrm{NO}} - k_{dno}[\mathrm{NO}]$$

where NO consumption rate constant $k_{dno} = 0.01\ \mathrm{s^{-1}}$. For the simulations presented here, J_{NO} = 0.015 nM/s was used to get a steady state [NO] = 1.5 nM yielding a constant [cGMP] = 1 μM. The diffusion coefficient for NO was set to $D_{NO} = 9600\ \mu\mathrm{m^2/s}$ ***(7)***.

Numerical method:

The reaction-diffusion is discretized in space using a centred finite difference method with Δx=Δy= 0.178 μm and no-flux boundary conditions at the cell and nucleus borders for both NO and cGMP PDEs. The explicit fourth-order Runge-Kutta integration scheme was used for time integration of the model. The method was implemented in Matlab v. 7.11.

Simulations:

Two sets of simulation have been calculated. The first set corresponds to a homogeneous addition of 220 nM of NO. The second set corresponds to pulsatile addition of NO localized to a sub-region of the cell. In both cases, simulations were done with and without perinuclear PDE6 to compare its impact on spatial-temporal [cGMP].

3.4 Results

Expression of PDE5 and PDE6 subunits in mesenteric arteries

Expression of PDE5, PDE6α and PDE6β was first sought by RT-PCR in mesenteric arteries from mice. Presence of PDE5, PDE6α and PDE6β were detected at the transcription levels as shown in **Figure 3.1A**. Moreover, transcripts for PDE5, PDE6α and PDE6β were detected in freshly isolated endothelial cells. Accordingly, Western blotting revealed the presence of PDE5 in mesenteric arteries from mice. Using specific antibodies, both PDE6 subunits were also detected at the protein level in mesenteric arteries from mouse (**Figure 3.1B**). PDE6γ protein, the inhibitory subunit was also detected in mesenteric arteries by immunofluorescence **(Figure 3.2 D)**. In agreement with the literature, these findings suggest that native endothelial cells express PDE5. This data also strongly support the hypothesis that PDE6 subunits are expressed in endothelial cells.

Intracellular distribution of PDE5 and PDE6 subunits

Microdomains result from a specific subcellular targeting of components of the signalling pathway involved. The intracellular distribution of PDE5 and PDE6 subunits was then studied to identify potential players in the control of cGMP microdomains. Using the specific

antibodies identified in Figure 3.1, a homogeneous distribution of PDE5 was found in *in situ* endothelium (**Figure 3.2A**). Figure 3.2A, B shows that PDE5 is also homogeneously distributed in the underlying smooth muscle cells. HUVEC cultured cells appears to express PDE5. On the other hand, PDE6α protein was not detected in smooth muscle layers and a faint signal was barely detectable in HUVEC. In native endothelial cells, PDE6α was found in aggregates, with preferential localization around the nucleus. Similarly, fluorescence associated with PDE6β was not detected in smooth muscle layers or in HUVEC cells. In panels i from Figure 3.2, endothelial cells border is evidenced by PECAM-targeted antibody (purple) and nuclear membrane is shown by anti-lamin antibody (blue). Perinuclear staining was stronger for PDE6β with little if any cytoplasmic staining in endothelial sheets. This heterogeneous distribution of PDE6α and β suggests that PDE6 might be involved in controlling cGMP microdomains. A mathematical model was then developed to dynamically assess the impact of this heterogeneous distribution of PDE6 on cGMP microdomains in endothelial cells.

Mathematical modeling of cGMP in a typical endothelial cell

A spatial 2D endothelial cell model was based on an image obtained in the immunohistochemistry experiments described above to increase the accuracy of the computer modelling. In order to reproduce dynamic control of cGMP levels in physiological conditions, NO was used as primary step in the cGMP pathway (**Figure 3.3A**). *In silico* model included sGC as the generator of cGMP in response to NO and PDEs as catalyst for cGMP hydrolysis. Distribution of sGC and PDE5 was established as homogeneous throughout the cell model and diffusion of NO and cGMP was also integrated in the model***(7)***. Application of a single NO bolus (200 nM) to the entire cell induced a gradual increase in cGMP (11 μM at 45s) throughout the cell. As shown in figure 3.3B, cGMP levels gradually decreased in the entire cell with no

apparent cGMP microdomains. However, addition of perinuclear PDE6 induced a significant modification in the spatial gradient of cGMP. Please note that since enzymatic properties of PDE6α and β are highly similar***(2)***, both were considered as one PDE6 to simplify the modelling. Beside a fast increase in cGMP levels (2 μM peak at 18 s), perinuclear cGMP never reached levels similar to the one found farther from the nucleus (**Figure 3.3C** and **G**). Actually, the PDE6-dependent difference in cGMP levels between the perinuclear region and distal regions is important as illustrated in figure 3.3D (85% lower in cGMP).

However, NO production is dynamic and may respond to spatially restricted oscillatory Ca^{2+} signals. Therefore, NO application was then modelled as spatially localized pulses (**Figure** 3.4). While NO rapidly increased and diffused, the ensuing cGMP cumulated in the endothelial cell. A slight gradient in [cGMP] was slowly established throughout the cell in the absence of PDE6. Again, inclusion of perinuclear PDE6 in the *in silico* endothelial cell resulted in a low increase in [cGMP] near the nucleus, which only reached 1 μM (**Figure 3.4C** and **F**). Interestingly, with NO pulses, the [cGMP] gradient was not restricted to the perinuclear region (**Figure 3.4D**). Therefore, [cGMP] modelling with NO pulses shows that despite NO distribution reflects normal diffusion pattern, cGMP is significantly lower in the perinuclear region in a PDE6-dependent manner.

3.5 Discussion

PDEs are important modulators of cellular functions, including vascular tone and endothelial permeability***(2, 12)***. Growing body of evidence suggests that cGMP microdomains are tightly regulated in several cell types and that these microdomains play essential roles in cellular functions***(2, 12)***.

Interestingly, in vascular smooth muscle cells, we have previously shown that PDE5 is sufficient to restrict ANP-induced increase in cGMP to the submembrane region***(10)***. Indeed,

application of ANP only increased cGMP near the membrane unless PDE5 was inhibited. In such condition, the entire cytoplasm of the vascular myocyte was filled with cGMP. Conversely, exposure of myocytes to a NO donor induced a significant increase in cytoplasmic cGMP levels despite a functional PDE5.

PDE6 has been well characterized in retina where it plays an essential role in vision adaptation to light***(3)***. Actually, expression of PDE6 was thought to be restricted to the retina and pineal gland. However, recent work from Nikolova and colleagues shed light to PDE6 expression in other cell types***(11)***. Indeed, their work in lung suggested the functional expression of PDE6 outside of the retina and pineal gland and its alteration in pathological conditions. Recent work also suggested expression of PDE6 in melanoma cell lines***(1)***.

However, the available pharmacology does not discriminate between PDE5 and PDE6 and therefore proscribes a pharmacological approach to study the role of PDE6 heterogeneous distribution in endothelial cells***(2)***. According to our immunofluorescence data, the use of culture cells is not an option either as HUVEC cells do not express PDE6 isoforms. Even the transfection of cultured cells with PDE6 would not be useful as they lack polarity found in native endothelial cell, which is essential to the heterogeneous distribution of proteins such as PDE6, is not conserved. Therefore, elucidation of the physiological impact of PDE6-dependent cGMP microdomains will require the development of compounds that will specifically target PDE6 over PDE5. Nevertheless, our *in silico* endothelial cell clearly establish that PDE6 has a strong influence on local cGMP levels (see **Figures 3.3** and **3.4**) and is most likely responsible for cGMP microdomains formation.

Several mathematical models have been developed studying nitric oxide pathway in smooth muscle cells over the years***(16)***. Our *in silico* model was therefore developed based on similar vascular smooth muscle models. However, these models obviously focussed on PDE5 control

of cGMP levels***(7)***. To our knowledge, this is the first computational model expressing PDE6 influence on cGMP microdomains. Moreover, it strongly suggests that PDE6 is a very important regulator of cGMP microdomains and may be an important regulator of endothelial function. However, the experimental elucidation of the impact of PDE6 in endothelial cells is the purpose of future studies. It remains to evaluate how the simplistic 2D representation influences the results close to the nucleus where cell height becomes more important. This point may interact with the perinuclei distribution of PDE6.

This study also brings the interesting question regarding the regulatory mechanisms of PDE6 in endothelial cells. In retina, photon-excitation of transducin results in the activation of PDE6 catalytic subunits through the relief of the inhibitory influence of PDE6γ. PDE6γ was detected in native endothelium and our immunohistochemistry data suggested a diffuse intracellular distribution. PDE6γ might therefore be involved in the negative regulation of cGMP hydrolysis by PDE6α and β. However, it seems obvious that other mechanisms than photon-activated transducin is necessary to regulate endothelial PDE6 and identification of such mechanisms will require further investigation.

3.6 Acknowledgement

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3.7 Figures

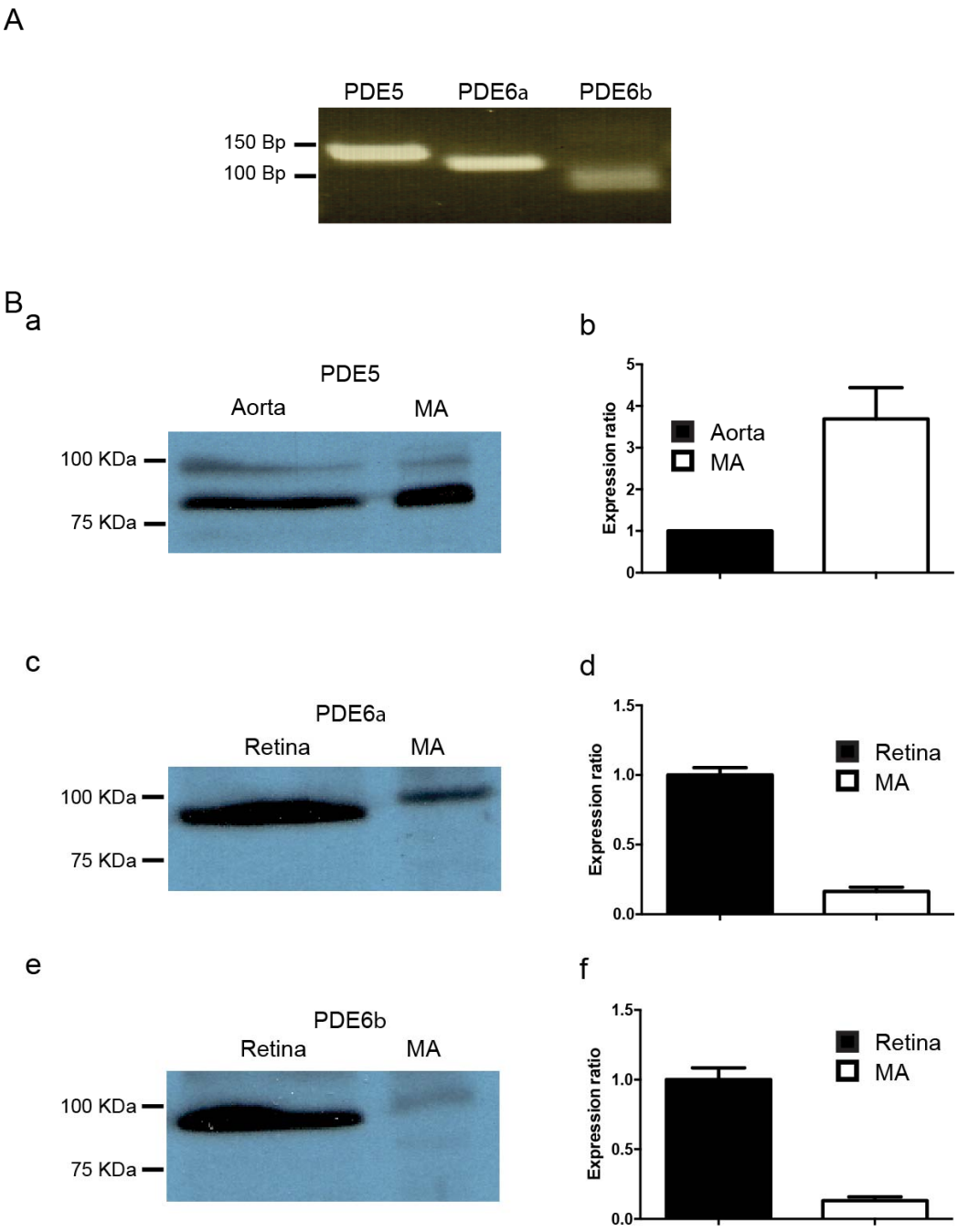

Figure 3.1. mRNA and protein expression of PDE5, PDE6α and PDE6β in mesenteric arteries from mice.

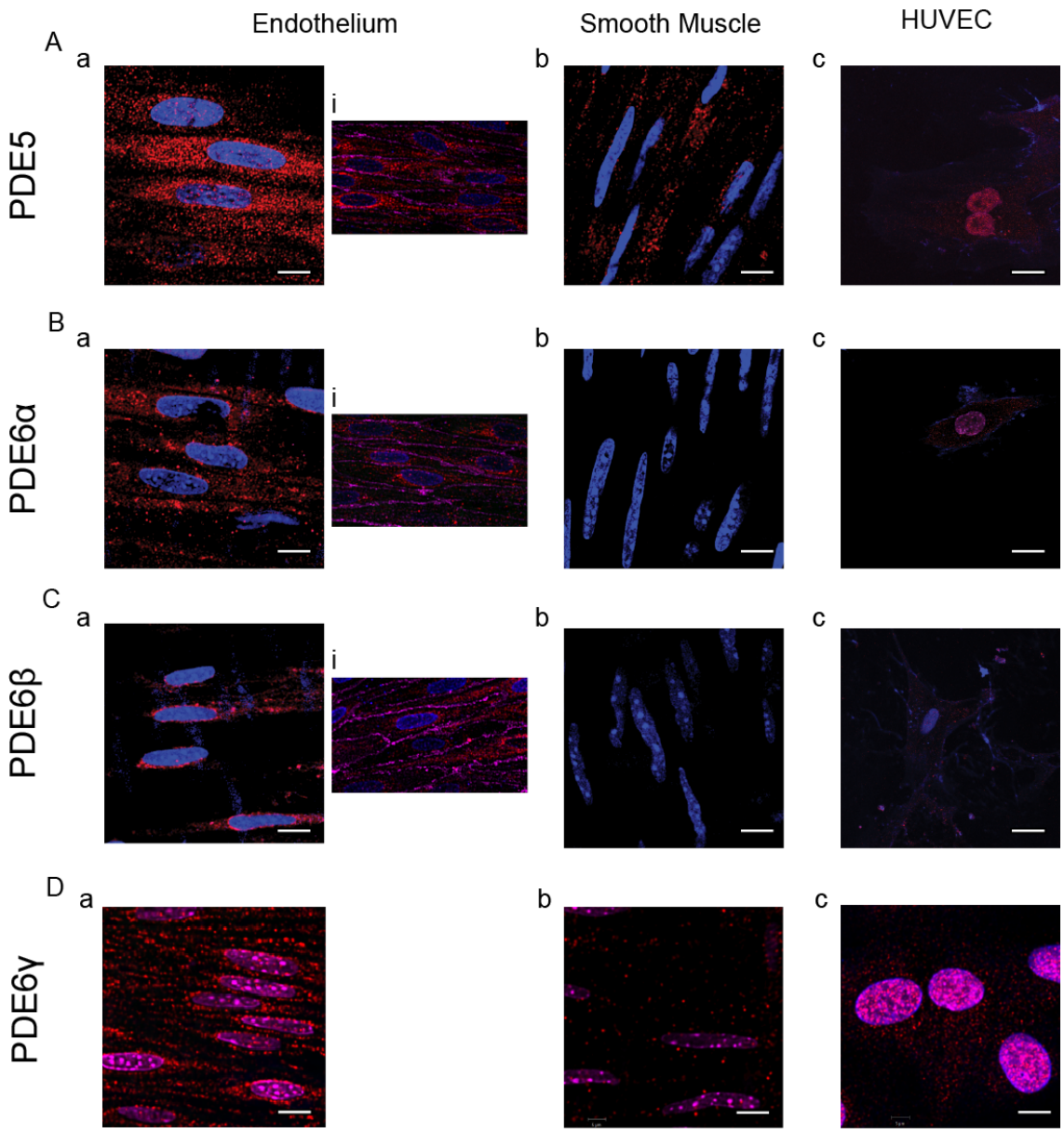

Figure 3.2. Intracellular distribution of PDE5, PDE6α and PDE6β in mesenteric arteries (MA) from mice and HUVEC cells.

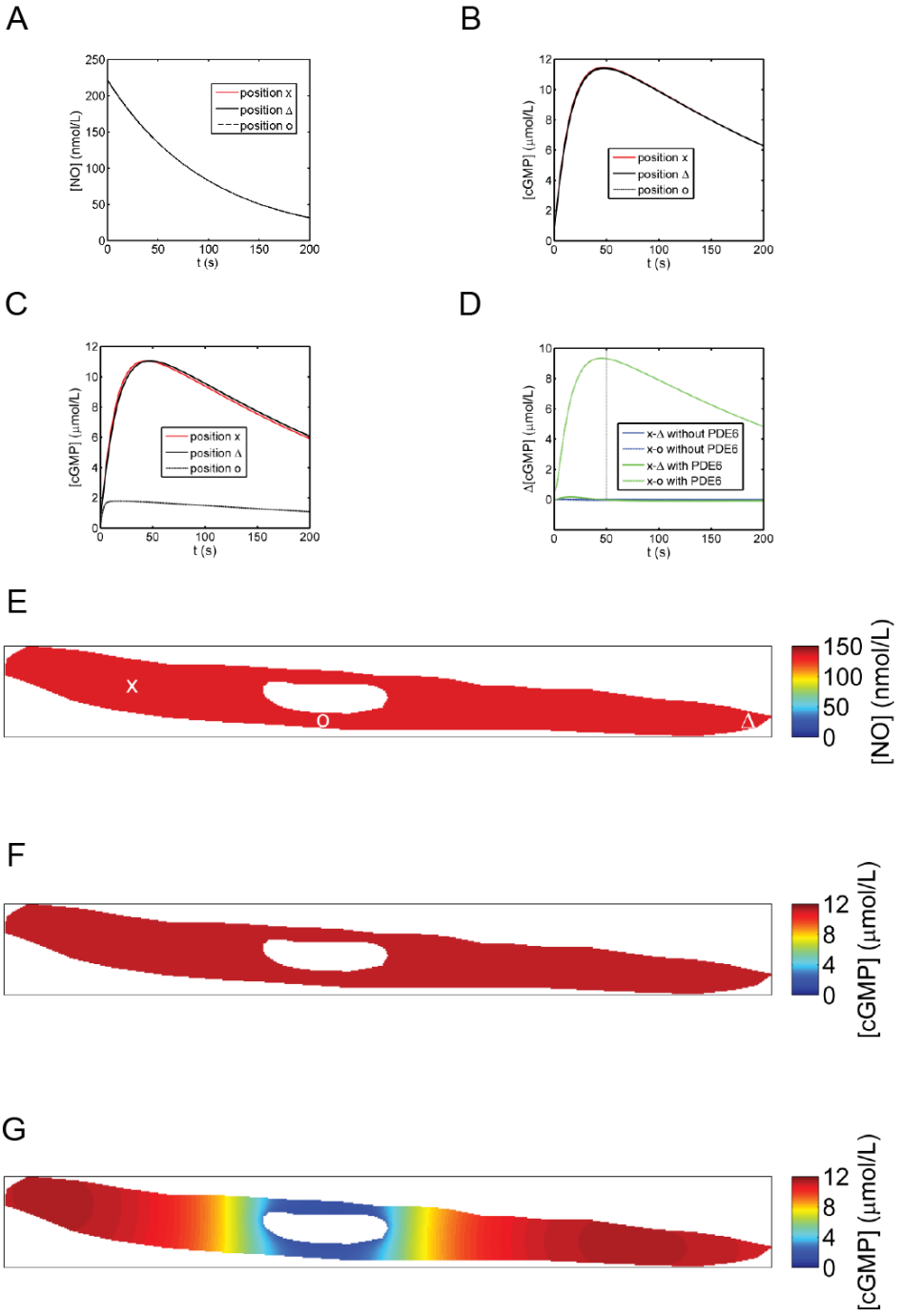

Figure 3.3. Simulation results of the cGMP distribution in a typical endothelial cell following application of a cell-wide bolus of NO (200 nM).

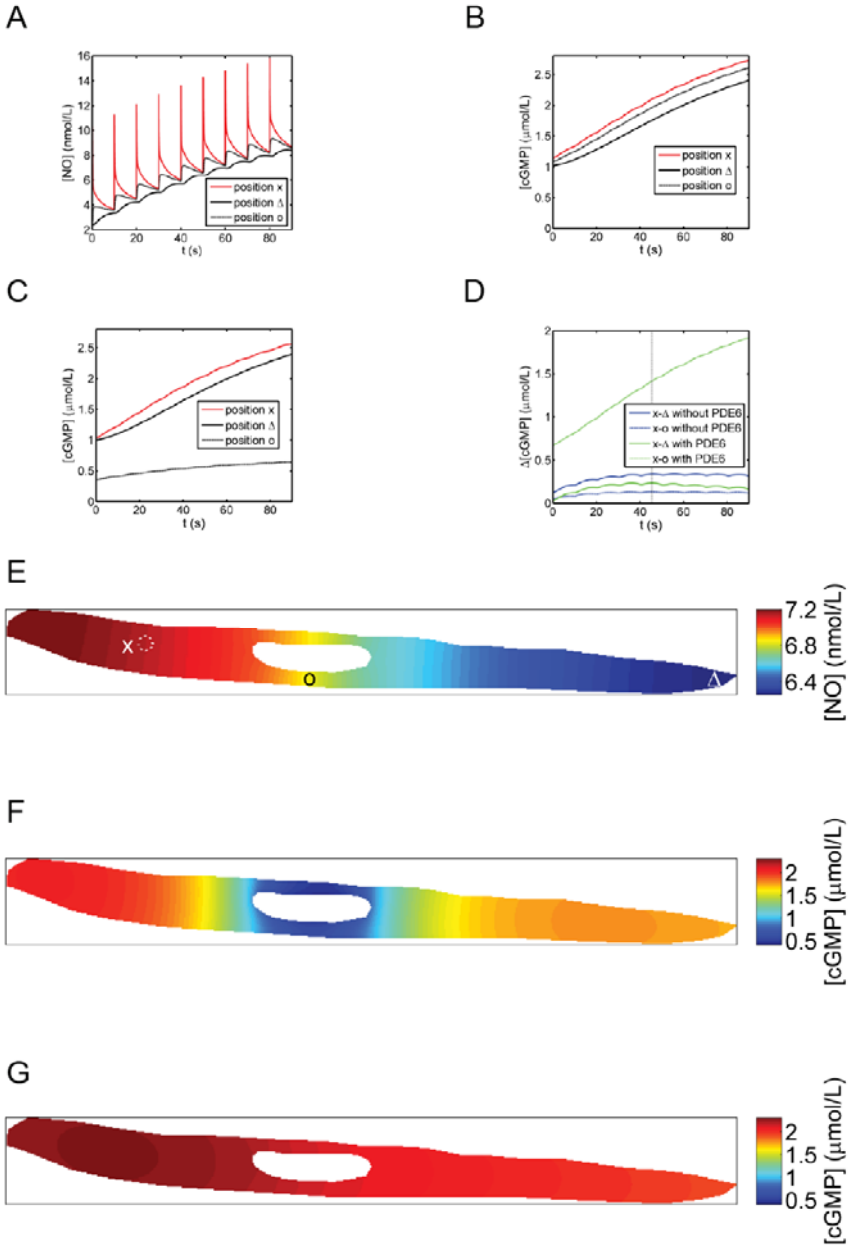

Figure 3.4. Simulation results of the cGMP distribution in a typical endothelial cell following spatially localized NO pulses.

3.8 Figure legends

Figure 3.1. mRNA and protein expression of PDE5, PDE6α and PDE6β in mesenteric arteries from mice. **A.** Amplicons were migrated on a 3% agarose gel. mRNA templates were isolated from mesenteric arteries of C57/BL6 mice. **B.** Protein migration on 10% gel in control tissue, which is aorta for PDE5 and retina for PDE6, (left lane and black column) and mesenteric arteries (right lane and white column) for PDE5 (**a, b**), PDE6α (**c, d**) and PDE6β (**e, f**). Expression of PDEs is normalized to actin expression levels.

Figure 3.2. Intracellular distribution of PDE5, PDE6α and PDE6β in mesenteric arteries (MA) from mice and HUVEC cells. Intracellular localization of PDE5 (**Aa**), PDE6α (**Ba**) and PDE6β (**Ca**) (red) in endothelium and smooth muscle (**Ab, Bb, Cb**) of cut-open mesenteric arteries. PDE5 (**Ac**), PDE6α (**Bc**) and PDE6β (**Cc**) distribution in HUVEC cells (right column). Staining of the nucleus with DAPI is shown in blue except in panels i. In panels i, antibodies targeting PECAM (purple) and lamin (blue) show endothelial cell plasma and nuclear membrane, respectively. Scale bar = 10 μm.

Figure 3.3. Simulation results of the cGMP distribution in a typical endothelial cell following application of a cell-wide bolus of NO (200 nM). **A.** Homogeneous decrease of [NO] over time as depicted by the superimposed curves obtained at three different positions in the cell (positions are shown by the X, o, and Δ markers in panel E). **B.** Simulation without PDE6: Intracellular [cGMP] for the same three positions exhibiting a biphasic variation with an initial increase in the concentration peaking at ~45 s followed by a decrease. **C.** Same as in B but with the presence of peri-nuclear PDE6 **D.** Difference in [cGMP] between positions x and Δ (x - Δ) and positions x and o (x - o) with (green curves) and without (blue curves) perinuclear PDE6. **E.** Uniform spatial [NO]≈134.9 nM at t=50 s. **F.** Uniform [cGMP] ≈11.3 μM at t=50 s in absence of PDE6. **G.** Spatial distribution of [cGMP] with perinuclear PDE6 obtained at t=50 s clearly showing the quenching effect of PDE6 on local [cGMP]. All simulations were carried out with a homogeneous distribution of PDE5.

Figure 3.4. Simulation results of the cGMP distribution in a typical endothelial cell following spatially localized NO pulses. **A.** Increase of [NO] by repetitive pulses (10 s periods) where 220 nM of NO is added in the region defined by the dashed circle (in panel E). NO diffusion within the cell dampens NO oscillations after each pulse with an incremental distance from the source as highlighted by the reduced variations from the x position (closest to the source, panel E) to the Δ position (furthest from the source). **B.** Traces of [cGMP] obtained without PDE6 at the same three positions showing higher [cGMP] close to the source (position x) and lower [cGMP] far from the NO source (position Δ). **C.** Same as in panel B but for a simulation obtained with perinuclear PDE6. **D.** Difference in [cGMP] between positions x and Δ (x - Δ) and positions x and o (x - o) with (green curves) and without (blue curves) perinuclear PDE6.

E. Spatial distribution of [NO] at t = 50 s showing a clear [NO] gradient within the cell with lower concentration far from the NO source. **F.** Spatial distribution of [cGMP] at t = 50 s obtained without perinuclear PDE6. **G.** Same as panel F but with perinuclear PDE6 quenching the [cGMP]. All simulations were carried out with a homogeneous distribution of PDE5.

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Chapter 4. Discussion

Endothelium is a major regulator of vascular homeostatsis, including blood pressure via modulation of arterial diameter. Amongst numerous mechanisms, ECs generate and release NO to evoke VSMCs relaxation and vasodilation. In VSMCs, NO triggers the sGC-cGMP pathway leading to a decreased muscular tone. However, despite the fact that NO is produced in ECs and most likely activates endothelial signalling, very little information is available on the sGC-cGMP pathway in ECs. The appropriate machinery for cGMP production and its effector (PKG) has been reported in ECs. One could then ask how this system remains functional and below saturation. The work from this thesis provides the first evidence of intracellular organization allowing generation of subcellular cGMP microdomains in native endothelial cells.

Dynamic microdomains of cGMP have been reported and seem to be involved in regulation of cellular functions in other cell types. Nausch and co-workers reported that ANP-induced increase in cGMP is regulated by PDE5 in VSMCs. ANP activation of pGC increases cGMP in VSMCs but this increase is restricted to sub membrane space. Indeed, unless PDE5 is inhibited, cytoplasmic cGMP levels will remain low except in close proximity of the plasma membrane. However, inhibition of PDE5 allows a global increase in cytoplasmic cGMP levels in response to ANP. Similar control of cGMP levels was not found when cGMP was increased with NO donors. This established PDE5 as a major regulator of cGMP microdomains in native VSMCs (Nausch et al., 2008). Our work is consistent with this study, suggesting that PDE6 in the near nuclear space would regulate local cGMP levels even in the presence of NO and functional sGC. However, our simulation did not provide evidence of PDE5-dependent cGMP microdomains in native ECs.

The elevated efficiency in cGMP hydrolysis by PDE6 is a coveted property for an endothelial PDE. Indeed, a highly efficient degradation of cGMP is required to maintain cytoplasmic levels in the low range despite the production of NO by ECs. However, the initial preliminary evidence of PDE6 expression in ECs were unexpected, especially since PDE6 expression was thought to be restricted to retina and pineal gland until recently (Nikolova et al., 2010; Bazhin et al., 2010; Morin et al., 2001). Our work provides the first evidence of endothelial expression of PDE6 and establishes the enzyme as a key regulator of endothelial cGMP. Interestingly, cultured endothelial cell lines are not expressing PDE6 isoforms, suggesting that loss of PDE6 expression is part of the phenotype alteration induced by cell culture processes. Moreover, PDE5 and PDE6 have distinct subcellular targeting in native endothelial cells. The relative catalytic properties of individual PDE isoforms, in addition to their intracellular distribution, would reflect distinct roles in modulation of endothelial functions. A current challenge in this field is the overlap of pharmacology targeting PDE5 and PDE6, therefore making it difficult to dissect the distinct roles of each isoenzyme.

In this work, we found the expression of PDE5, PDE6α and PDE6β at the transcription levels (using RT-PCR) as well as at translation levels (using Western blot) in mesenteric arteries from mice. PDE6γ protein was also detected in mesenteric arteries by immunofluorescence. These findings confirm the presence of PDE5 in native endothelium and sustain the hypothesis that PDE6 subunits are expressed in endothelial cells. As the intracellular distribution of PDE5 and PDE6 subunits is responsible for creation of cGMP microdomains at certain intracellular areas, immunofluorescence studies, by using specific antibodies, were used. A homogeneous distribution of PDE5 was seen in endothelium and in the underlying smooth muscle cells and in HUVEC cells, while PDE6α protein was detected in native endothelial cells, in aggregates around the nucleus, but the fluorescence associated with PDE6α was not detected in smooth muscle cells, and it was barely detectable in HUVEC cells. Likewise, fluorescence associated

with PDE6β was detected in native endothelial, but not in smooth muscle or HUVEC cells. The heterogeneous distribution of PDE6α and β support the hypothesis that PDE6 might be involved in controlling cGMP microdomains.

In silico studies that mimick *in vivo* NO diffusion and biodistribution have been developed over the years (Tsoukias, 2008). Even though, the mathematical representation used in our simulations is the first computational model studying the influence of combined PDE5/PDE6 on cGMP in endothelial cells, it has been developed based on an approach used to study the effect of PDE5 on cGMP in vascular smooth muscle cells (Yang et al., 2005). Our mathematical approach has been used to look at two different aspects of intracellular cGMP. Firstly, it is the first model studying the role of local NO in endothelial cGMP formation. Secondly, the models suggest how specific intracellular distribution of PDE6 can be an important regulator of cGMP microdomains. Further development of the model would involve the simulation of more physiological conditions, where NO production is linked to endothelial intracellular Ca^{2+} dynamics including time-dependent changes. Indeed, oscillatory Ca^{2+} signals could result in oscillatory NO production with correlating (but not identical) kinetics. Indeed, the timescale of processes leading to cGMP levels are relatively small and therefore imply frequency-dependent memory effects and variations in cGMP oscillation frequency and amplitude.

However, the actual function of these microdomains remains to be elucidated, but it can be hypothesized that low perinuclear cGMP levels might be involved in control of nuclear transport or receptor activity found at the nuclear membrane. Interestingly, expression of PDE6 appears to be absent from cultured endothelial cells. It is thus tempting to postulate that PDE6 expression could be involved in regulation of gene expression or even endothelial cell proliferation. This hypothesis will be evaluated in future studies. A contribution of these cGMP microdomains in regulation of endothelial permeability also remains to be established.

The activation mechanism of PDE6 in native endothelial cells is also unclear. Indeed, in retina, PDE6 is activated following photon activation of transducin, which removes PDE6γ inhibition. Since it appears unlikely that photons would be the initial activation step in arteries, different regulatory processes are probably involved and will necessitate further investigation.

Despite its significant relevance, physiological impact of PDE6 in endothelium has not been addressed in this study. The pharmacological tools currently available do not allow a clear specific assessment of the effect of PDE6 inhibition on cellular function. Moreover, our immunofluorescence data showed the lack of PDe6 isoforms in HUVEC cells. Therefore, investigation of endothelial PDE6 essentially relies on native tissue, limiting molecular biology approaches aiming at an alteration of PDE6 isoform expression. The use of whole arteries is also a limiting factor as smooth muscle cells are inevitably present, with a working NO-cGMP-PDE5 pathway.

The use of a mathematical model to simulate cGMP levels in endothelial cells is a very powerful approach. However, such approach unfortunately relies on assomptions that might affect simulations outcomes. Indeed, the current model consider a 2D endothelial cell although three dimensional cells could have a different propagation/localization pattern of the moleculaes considered. Also, for the sake of simplification, other partners in the NO-cGMP pathway were ignored in this first iteration, including other PDE isoforms.

Chapter 5. Conclusion

The NO-cGMP pathway is an essential modulator of several vascular functions. PDEs are crucial molecular regulators of this signalling pathway. However, the endothelial pendant of the NO-cGMP-PDE pathway is poorly understood. In addition to PDE5, this study established the expression of PDE6, a cGMP-specific PDE that was initially thought to be restricted to the retina, in native endothelial cells. All known PDE6 isoforms were detected both at mRNA and protein levels in native endothelial cells from resistance arteries. Interestingly, signals associated with PDE6 expression were not detected in cultured endothelial cell lines.
Moreover, peculiar PDE6α and β intracellular localization near the nuclear membrane suggests a potential involvment in the generation and sustainment of cGMP microdomains. Since PDE5 is found homogenously distributed in the native cells, roles for PDE5 and PDE6 is most likely distinct.

The computational model applied on endothelial cells showed that in the absence of PDE6, repetitive or single NO pulses cause a global intracellular increase in cGMP levels, which cannot be efficiently controlled by PDE5. However, the presence of PDE6 surrounding nuclei creates a perinuclear zone with lower cGMP levels (cGMP microdomains), suggesting a possible functional role for the enzyme within the perinuclear region in native endothelium.

Elucidated presence of the highly potent PDE6 in multifunctional cells such as endothelial cells, now warrants further investigations to explore the physiological role of cGMP microdomains. Future studies will evaluate the hypothesis that PDE6 expression could be involved in regulation of gene expression and endothelial cell proliferation as well as the possibility of its impact in mechanisms of permeability.. Moreover, further understanding of PDE6 regulatory mechanisms in endothelial cells would also be relevant to the development of potential pharmacological tools.

Chapter 6. Bibliography

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