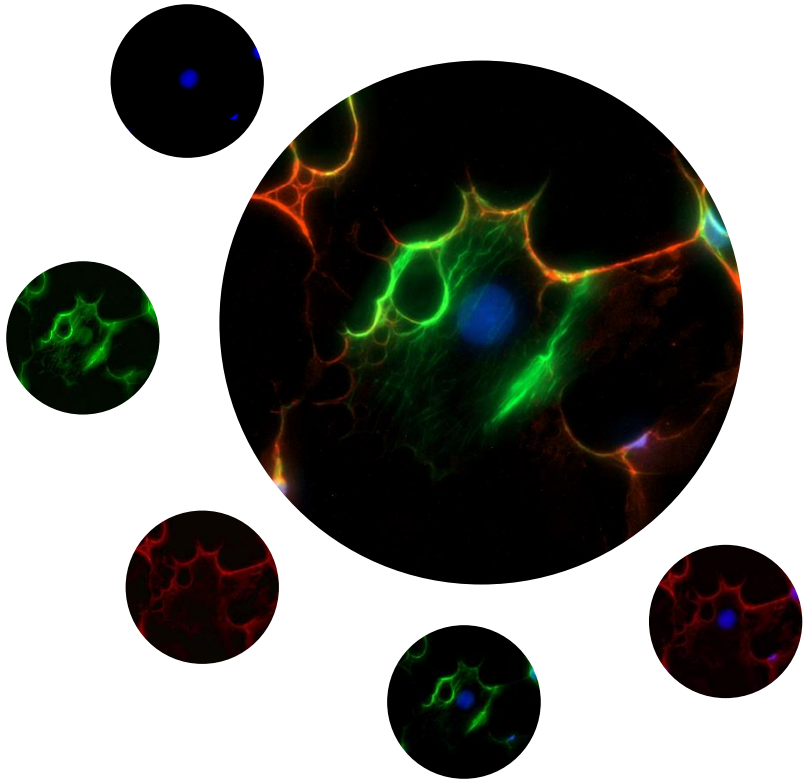

Adenosine receptors in mesenteric arteries adventitia: evidence for a nuclear location

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

A adenosina é um potente mediador de inúmeras atividades biológicas envolvendo, por exemplo, os sistemas nervoso, imune e cardiovascular, crescimento celular, proliferação e apoptose. Atualmente, são conhecidos quatro recetores de adenosina denominados A_1, A_{2A}, A_{2B} e A_3, através dos quais a adenosina desencadeia variadas respostas em células alvo. Estes recetores pertencem à diversificada família de recetores acoplados a proteínas G.

Nos últimos anos há uma crescente evidência de que a membrana plasmática já não pode ser considerada o *locus* da sinalização dos recetores acoplados a proteínas G, havendo dados na literatura que apoiam a presença de vários recetores acoplados a proteínas G funcionais (tais como os recetores da angiotensina II e da endotelina 1) em membranas intracelulares, particularmente no núcleo. Indo ao encontro desta ideia, e sem haver evidências na literatura em relação à localização de recetores de adenosina em estruturas intracelulares, estudos do nosso grupo, recorrendo à microscopia confocal, sugeriram um padrão de sobreposição entre o DAPI, um marcador nuclear, e a imunorreatividade dos anticorpos para os recetores da adenosina, compatível com a presença destes recetores no núcleo de células da camada adventícia de artérias mesentéricas. Desta forma, o objetivo deste trabalho foi avaliar a presença de recetores de adenosina no núcleo celular em artérias mesentéricas superiores. Para cumprir este objetivo foi desenvolvido um protocolo para criação de culturas celulares primárias a partir de artérias mesentéricas superiores de ratazana. As células foram submetidas a fracionamento subcelular e as frações nuclear e membranar foram analisadas por *western blot* utilizando anticorpos individuais seletivos para os recetores de adenosina. Foram ainda realizadas técnicas de imunocitoquímica, bem como imunomarcação de núcleos isolados para avaliar a distribuição dos subtipos de recetores de adenosina. Dados quantitativos demonstraram uma forte co-localização entre o marcador nuclear e a imunomarcação dos recetores de adenosina, bem como uma menor expressão e presença nuclear do subtipo A_{2B}. A presença dos recetores A_1, A_{2A} e A_{2B} foi sustentada pela análise feita por *western blot*.

Ensaios funcionais estão em curso para tentar esclarecer a eventual funcionalidade dos recetores nucleares de adenosina. Futuras investigações serão necessárias para aprofundar o papel fisiológico e possível fisiopatológico de cada um destes recetores.

Palavras-chave

Recetores de adenosina, Camada adventícia, Culturas celulares primárias, Recetores acoplados à proteína G, Artéria mesentérica, Núcleos

Abstract

Adenosine is a potent mediator of innumerous biological activities involving the cardiovascular, nervous and immune system, cell growth, proliferation and apoptosis, to name a few. The four known adenosine receptors (ARs) named A_1, A_{2A}, A_{2B} and A_3, from which adenosine elicit its various responses on target cells, are G-protein-coupled receptors (GPCRs).

Over the last few years it has become clear that the plasma membrane should no longer be considered the exclusive signaling *locus* of GPCRs, and growing evidence supports the presence of many functional GPCRs (such angiotensin II or endothelin 1 receptors) on intracellular membranes, particularly in the nucleus. In agreement with this idea, and despite the lack of evidence in literature concerning ARs location in intracellular structures, studies in our laboratory employing confocal microscopy suggested an overlay pattern of DAPI, a nuclei marker, with ARs antibody immunoreactivities, compatible with the presence of these receptors in the nuclei of adventitia layer cells from mesenteric arteries. In this way, the aim of this work is to evaluate the presence of ARs in the cell nucleus of rat adventitia mesenteric arteries. To accomplish that, a protocol to develop primary adventitia cell cultures from rat superior mesenteric arteries (SMAs) was created. Cells were submitted to subcellular fractionation and nuclear and membrane fractions were analyzed by western blot (WB) using individual AR selective antibodies. Immunocytochemistry as well as immunolabeling of isolated nuclei was performed to evaluate ARs subtypes distributions. Quantitative data showed a strong colocalization between the nuclear marker and ARs immunolabeling, and a less expression and nuclear presence of the A_{2B} subtype. A_1, A_{2A} and A_{2B} receptors presence in the nucleus was supported by WB analysis.

Ongoing functional assays are being performed as an attempt to clarify nuclear ARs functionality and future investigations will be necessary to deepen its physiological and even pathophysiological roles.

Keywords

Adenosine receptors, Adventitia layer, Primary cell cultures, G-protein coupled receptors, Mesenteric artery, Nuclei

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List of Abbreviations

AC - adenylate cyclase/adenylyl cyclase

ADA - adenosine deaminase

ADO - adenosine

ADP - adenosine diphosphate

AK - adenosine kinase

AMP - adenosine monophosphate

AMPK - adenosine monophosphate kinase

APS - ammonium persulfate

ARs - adenosine receptors

ATP - adenosine triphosphate

BAY 60-6583 - 2-[[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

Ca^{2+} - calcium

Caffeine - 1,3,7-Trimethylpurine-2,6-dione

CCPA - 2-Chloro-CPA

CGS 21680 - 2-p-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride

CPA - N6-Cyclopentyladenosine

CREB - cAMP response element binding protein

C-terminal - carboxyl-terminal

CTCF - Corrected Total Cell Fluorescence

cyto-5'NT - cytosolic-5´-nucleotidase

DAG - diacylglycerol

DPCPX - 1,3-Dipropyl-8-cyclopentylxanthine

ECL - extracellular loop

EDTA - Ethylenediaminetetraacetic acid

eNOS - endothelial NO synthase

ER - endoplasmic reticulum

ET-1 - endothelin 1

F-actin - filamentous actin

FBS – fetal bovine serum

FDA - Food and Drug Administration

GPCR - G-protein coupled receptors

G-protein - guanine nucleotide-binding protein

GRKs - G protein-coupled receptor kinases

H – hour

HRP - horseradish peroxidase

IB-MECA - 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide

ICL - intracellular loop

IF – immunofluorescence

IP3 - inositol 1,4,5-trisphosphate

IUPHAR - International Union of Pharmacology

K^+ - potassium

LSCM - laser scanning confocal microscopy

M - mitochondrial/membrane (fraction)

MAPKs - mitogen-activated protein kinases

Min – minutes

MRS 1220 - N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide

MRS 1523 - 2,3-Diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate

MRS 1754 - N-(4-Cyanophenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]acetamide

MRS 1706 - N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide

N - nuclear (fraction)

NC – negative control

NE - nuclear envelope

NECA - 5'-N-Ethylcarboxamidoadenosine

NLS - nuclear localization signal

NO - nitric oxide

N-terminal - amino-terminal

NTPDase1 - nucleotidase triphosphate diphosphohydrolase 1

NTs - nucleoside transporters

P – passage

PAGE - Polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PI3K - phosphatidylinositol 3-kinase

PKB - protein kinase B

PKC - protein kinase C

PLC - phospholipase C

PKA - protein kinase A

PVDF - polyvinylidene difluoride

ROI - region of interest

SAH - S-adenosyl-homocysteine

SCH 58261 - 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

SDS - sodium dodecyl sulphate

SEM - standard error of the mean

SMA - superior mesenteric artery

TEMED – tetramethylethylenediamine

Teophylline - 3,7-Dihydro-1,3-dimethyl-1H-purine-2,6-dione

TM – transmembrane

tM1 - Manders' split colocalization coefficient using threshold

WB - western blot

ZM 241385 - 4-(2-[7-Amino-2-[2-furyl]-[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol

2CI-IB-MECA - 2-Chloro-N6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine

I. Introduction

1. Adenosine

Adenosine is a purine nucleoside composed of a heterocyclic aromatic molecule of adenine attached to a ribofuranose *via* a β-glycosidic bond (Figure 1). Quoting from Eltzschig [1], purines are "among the oldest and most influential biochemical compounds in evolutionary history" playing a central role in the energy metabolism [2], also working as neurotransmitters/neuromodulators [3] and even autocrine and paracrine substances [4].

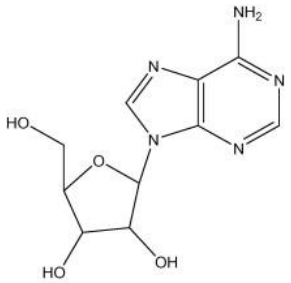

Figure 1 - Adenosine molecule. Source: [8].

In 1929, Drury and Györgyi introduced the idea that purines could function as extracellular signaling molecules, reporting the first physiological role for adenosine. They found out that adenosine infusion, isolated from different tissue extracts, caused a "definite and transient effect upon the mammalian heart" and had the ability to "lower general arterial pressure […] due in part to a general arterial dilation" [5]. Since then, adenosine has been implicated in numerous physiological processes including nervous system modulation, immune response, vascular function and metabolism [6,7].

Uncharged at physiological pH, adenosine is primarily associated to nucleic acids synthesis as well as a component of adenosine triphosphate (ATP), that is, an integral part of cellular energy system [8]. Nevertheless, adenosine, as a signaling molecule, works as an alarm, a warning signal that triggers cellular and tissue adaptation under metabolic stress, mainly hypoxia and ischemia. During several acute conditions, adenosine plays a major protective role that aim homeostasis reestablishment and repair promotion [9], triggering several effects that comprise increasing in oxygen delivery, decreasing cell energy consumption, attenuation of inflammatory process and angiogenesis promotion [10,11], which indicates vast potential therapeutic approaches based on adenosine molecule.

2. Adenosine synthesis and metabolism

Adenosine is always present both within and outside cells [4]. Under normal physiological conditions, its base line levels, both extracellular and intracellular, are in the nanomolar range (of 30 to 200 nM) [12,13].

Intracellular formation can be mediated by a cytosolic-5´-nucleotidase, which catalyzes the dephosphorylation of adenosine monophosphate (AMP), or can also be generated from the reversible hydrolysis of S-adenosyl-homocysteine (SAH) by SAH hydrolase [14].

In the extracellular fluid, adenosine formation comes from the breakdown of precursor nucleotides: ATP released into the extracellular space can be double dephosphorylated by nucleotidase triphosphate diphosphohydrolase 1 (NTPDase1) to adenosine diphosphate (ADP) and then to AMP, culminating in the formation of adenosine by a ecto-5'-nucleotidase (Figure 2) [15]. Another potential source of extracellular AMP is cyclic adenosine monophosphate (cAMP), which can be transported out of cells upon activation of adenylate cyclase (also known as adenylyl cyclase, AC) and converted by extracellular phosphodiesterases [11]. In the brain, extracellular adenosine levels seem also to be regulated by glutamatergic agonists [16,17] and nitric oxide [18]. More recently, in the nervous system and renal cells guanosine was proved to increase adenosine levels [19].

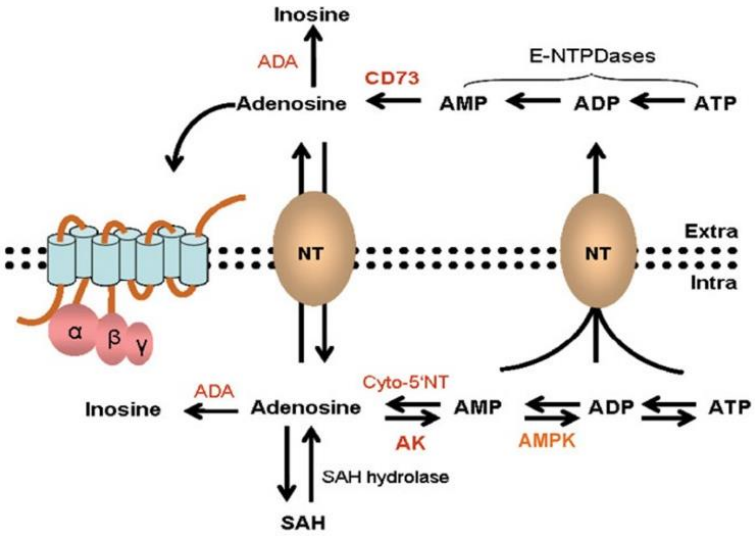

Figure 2 - Adenosine synthesis and partial schema of metabolic pathways inside and outside of a cell. Abbreviations: Adenosine deaminase (ADA), adenosine diphosphate (ADP), adenosine kinase (AK), adenosine monophosphate (AMP), adenosine monophosphate kinase (AMPK), adenosine triphosphate (ATP), Cytosolic-5´-nucleotidase (cyto-5'NT), nucleoside transporters (NT), S-adenosyl-homocysteine (SAH). Source: [185].

Adenosine has a short half-life due to its rapid and irreversible conversion into inosine by adenosine deaminase (ADA) and the quick re-uptake by reversible nucleoside transporters (NTs) into the cytoplasm [20]. Once inside the cells, adenosine is substrate of several enzymes such as ADA, SAH hydrolase and adenosine kinase (AK), which phosphorylates adenosine back into AMP (Figure 2) [12].

Adenosine can be released from intracellular stores [21–23]. However, under energy depletion or metabolically-stressful conditions such as ischemia, hypoxia, inflammation, trauma or tissue damage, interstitial levels of adenosine increase substantially reaching the millimolar range [24] mainly due to the release of ATP from injured cells *via* transmembrane protein channels including pannexins [25] or connexins [25,26] which then follows the above-mentioned adenosine formation pathway.

3. Adenosine receptors

3.1. Classification and characterization

As it has been exploited, adenosine is an important signaling molecule present in all tissues of a mammalian organism modulating a variety of processes comprising the cardiovascular, nervous, immune, digestive, renal and respiratory systems, to name a few [11]. It elicit various responses on target cells by engaging four purinoceptors - P1 - or adenosine receptors (ARs) both in physiological and pathological conditions, named as A_1, A_{2A}, A_{2B} and A_3 according to the International Union of Pharmacology (IUPHAR) nomenclature rules [11,27]. Initially, A_{2A} and A_{2B} receptors were considered as only one class, the A_2 receptors. The discrimination of A_2 ARs into two subtypes was later proposed based on substantial differences observed for adenosine binding and affinity as well as anatomical distribution [28,29].

The existence of ARs was initially suggested in the 1970s [30] but the idea was not widely accepted until the first adenosine receptor were cloned in 1990 [31]. At present, all AR subtypes have been cloned from rat, mouse and human and characterized using pharmacological e biochemical techniques [11]. Each AR subtype has a cellular/tissue specific distribution, distinct affinity for adenosine and different downstream signaling [32].

There is strong homology between respective ARs in different species with exception of the A_3 receptor that usually exhibits large differences in structure as well as tissue distribution and functional and pharmacological properties between species [33].

Compared to rats (Table 1), human ARs have a sequence identity of 94.8 %, 84.3 % and 86.1 % for A_1, A_{2A} and A_{2B} subtypes, respectively, prevailing a rather distant relationship of 73.9 % in case of A_3 receptor [33].

Table 1 - Brief characterization of rat adenosine receptors. Data from National Center for Biotechnology Information.

A_1 receptor [34]	**A_{2A} receptor** [35]	**A_{2B} receptor** [36]	**A_3 receptor** [37]
Receptor structure			
Heptahelical G-protein coupled receptor	Heptahelical G-protein coupled receptor	Heptahelical G-protein coupled receptor	Heptahelical G-protein coupled receptor
326 amino acids Mass = 36.7 kDa	410 amino acids Mass = 45.0 kDa	332 amino acids Mass = 36.4 kDa	320 amino acids Mass = 36.6 kDa
Gene structure			
Gene: *Adora1* Gene location: 13q13	Gene: *Adora2a* Gene location: 20p12	Gene: *Adora2b* Gene location: 10q23	Gene: *Adora3* Gene location: 2q34

ARs are integral membrane proteins consisting of a single polypeptide chain forming seven α-helical hydrophobic transmembrane (TM) domains connected by three extracellular and three intracellular hydrophilic loops of unequal size (Figure 3). The carboxyl-terminal (C-terminal) is facing the cytosol while the amino-terminal (N-terminal) lies on the extracellular side. The four AR subtypes are asparagine-linked glycoproteins and all but the A_{2A} possess sites for palmitoylation near the carboxyl terminus [38,39].

A_1, A_{2B} and A_3 ARs display identical protein mass. Though, A_{2A} subtype manifest a relatively large size corresponding to a much longer C-terminal tail (approximately more 80 amino acids than the other ARs) [40] that is required for receptor's interaction with several accessory proteins [41].

3.2. Signaling pathways

ARs belong to the diverse superfamily of guanine (G)-protein-coupled receptors (GPCRs), fitting into class 1 GPCRs according to IUPHAR nomenclature, defined by sequence homology to Rhodopsin receptor [42].

The physiological responses mediated by class 1 GPCRs result from interactions of both third intracellular loop and TM 2 of AR receptor with different heterotrimeric guanine (G) nucleotide-binding proteins (G-proteins) composed of α, β and γ subunits [43]. When the

GPCR is stimulated, the G-protein complex dissociates into two activated components: α-GTP and βγ, both capable of stimulating downstream effectors [43]. The specificity of interaction between the receptor and a G-protein predominantly dictate the subsequent cellular response triggered; and the array of potential connections is quite complex since there are inumerous isoforms and a large range of G-protein subunits, and hence, possible combinations. Besides that, one GPCR can couple several different G-proteins [44]. In this way, activation of adenosine receptors is involved in multiple cellular functions *via* multiple downstream signaling cascades, and only some of the major adenosine pathways will be discussed.

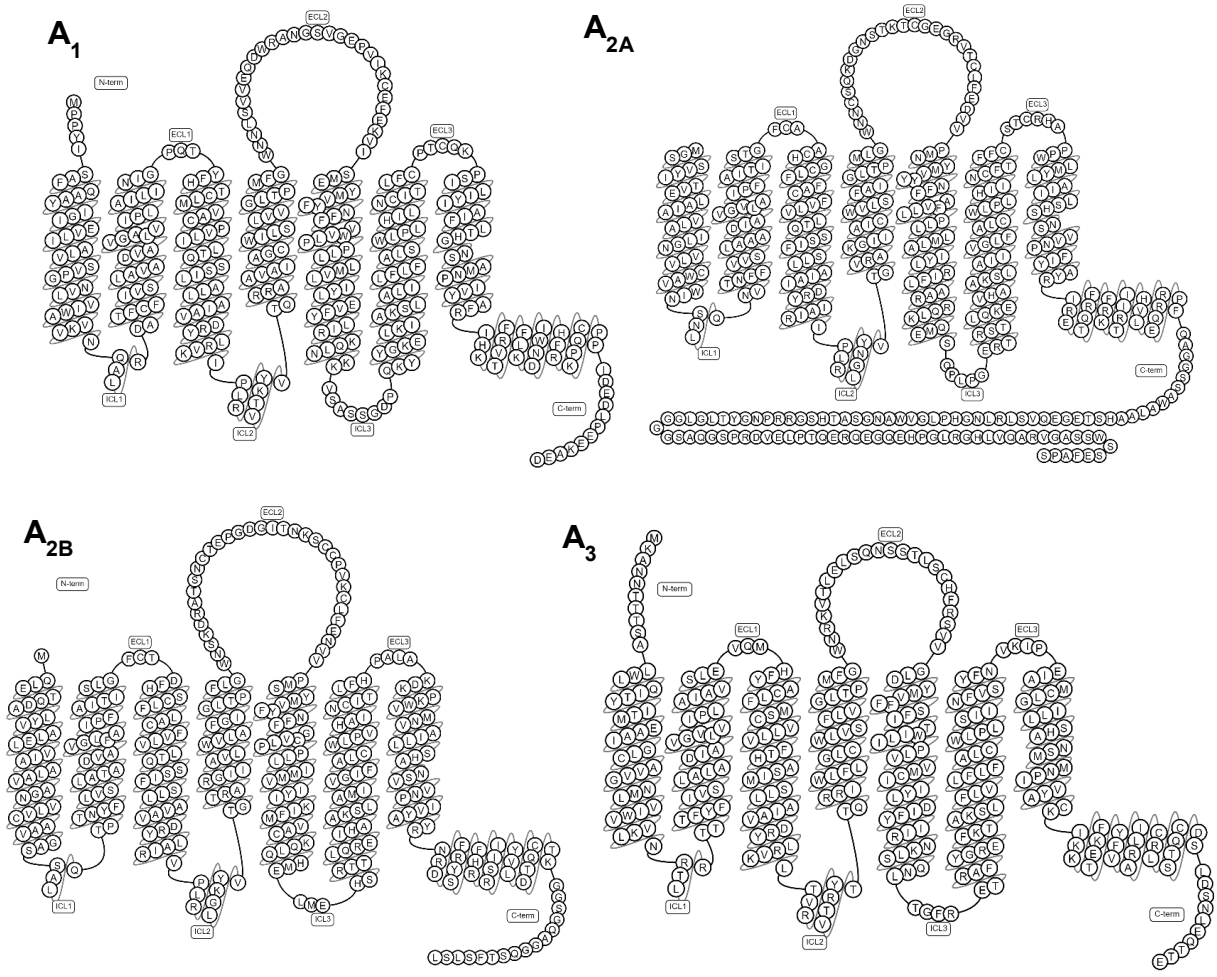

Figure 3 - Schematic rat ARs representation. Each transmembrane protein highlights the seven α-helical TM domains, N- and C-terminus (N-term, and C-term, respectively) as well as the three extracellular (ECL) and three intracellular (ICL) loops. Source_ GPCRdb (aa1r_rat, aa2ar_rat, aa2br_rat and aa3r_rat in http://gpcrdb.org).

ARs have traditionally been classified based on their differential effects on AC and accordingly its ability to regulate cAMP levels. A_1 and A_3 receptors preferably couple with

the inhibitory G-protein subunit $G\alpha_{i/0}$ and thereby inhibit AC, consequently lowering intracellular levels of the second messenger cAMP decreasing protein kinase A (PKA) activity. In contrast, A_{2A} and A_{2B} are commonly coupled to stimulatory $G\alpha_s$ protein, activating AC and, therefore, having opposite effects of those previously described for A_1 and A_3 ARs [45–47]. PKA can modulate a diversity of processes such as gene transcription *via* cAMP response element binding protein (CREB) or ion channels/pumps activation [48].

Many other G-protein interactions have been reported indicating that adenosine signaling pathways are "multiple, parallel and interrelated" [49]. In other words, ARs are pleiotropic, coupling with several transduction mechanisms depending on their degree of activation or cellular/subcellular localization [50].

A_{2B} and A_3 subtypes also couple to $G\alpha_q$ protein [11] leading to phospholipase C (PLC) β stimulation, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) followed by Ca^{2+} release from internal stores and protein kinase C (PKC) activation [51]. Through $G\alpha_i$ subunit, A_1 AR is also able to increase PLC activity [11]. A_{2A} AR was shown to activate the PLC-PKC pathway as well [52] and has also demonstrated to induce IP3 formation and PKC activation *via* $G\alpha_{15}$ and $G\alpha_{16}$ proteins [53].

The four ARs are engaged in mitogen-activated protein kinases (MAPKs) activation [54], even though mechanisms appear to differ substantially [54].

In some tissues, A_{2A} AR can mediate its effects predominantly through $G\alpha_{olf}$ activation, which is similar to $G\alpha_s$ coupling to AC [55]. Ras homolog gene family member A-phospholipase D 1 pathway has been implicated in some important A_3 AR functions [56]. A_1 and A_3 receptors can induce potassium (K^+) channels, as well as K_{ATP} channels opening [57] and voltage sensitive calcium (Ca^{2+}) channels types Q, N and P inhibition [11,58]. Moreover, A_2 receptor subtypes are also known to promote N-type Ca^{2+} channels activation [59].

3.3. Oligomerization

ARs have been described to be able to form homo- and/or heterodimers and also oligomeric structures [60,61]. Particularly, they can interact with each other and with other GPCRs.

As a component of a macromolecular complex, the receptors may not activate and signal simultaneously [62]. Additionally, they could display different receptor pharmacology, functional coupling, and intracellular signaling pathways from their monomeric counterparts [63]. This phenomenon has been studied using a variety of experimental techniques, usually in cell lines. Thus, its relevance at the physiological or pharmacological level *in vivo* is still to be clarified and further studies are required to fully understand the interplay between them [64].

3.4. ARs signaling regulation

GPCRs signaling is negatively regulated by Gα intrinsic GTPase activity, sequestration or metabolism of second messengers, desensitization, internalization and down-regulation of receptors by regulators of G protein signaling proteins, G protein-coupled receptor kinases (GRKs) and β-arrestins [43].

ARs appear to undergo desensitization, a regulation process commonly characterized by a diminished GPCR responsiveness upon continued or repeated exposure to an agonist [65]. This mechanism involves AR uncoupling from G-proteins and usually receptors' internalization that, depending on agonist time exposure, can be recycled or degraded [39]. It has been shown that distinct AR subtypes undergo different desensitization mechanisms [39]: while A_1 receptor is believed to desensitize slowly and incompletely, A_{2A} and A_{2B} subtypes demonstrated a quicker desensitization response and A_3 subtype an even faster one [11].

3.5. Agonists and antagonists

As previously mentioned, adenosine has been implicated in several processes both in health and disease and its outcome is triggered by its binding to ARs widely expressed. The development and discovery of agonists and antagonists has been a fundamental tool to elucidate how these receptors function, with special interest in compounds that display high-affinity binding and receptor-subtype selectivity (Table 2). Site-directed mutagenesis studies in parallel with molecular modeling approaches have been used as powerful strategy to design selective and potent ARs ligands [66].

Both TMs and extracellular regions of ARs have been implicated in the formation of the ligand-binding pocket [67], with exception of TM 4 whose mutations apparently do not affect the ligand binding [11]. Particularly, two histidine residues located in TMs 6 and 7 and conserved among AR subtypes (with the exception of the A_3 receptor that lacks the first one) proved to establish the most important interactions required for recognition of agonist and/or antagonist [68–70]. Nevertheless, there are other amino acids residues that also seem to be important for ligand recognition, affinity or binding [71–73]. Some residues are involved in species-related differences in ligands pharmacology, e.g. threonine 277 (TM7) residue in human A_1 AR [74]. It is also important to mention the distal region of the second extracellular loop of ARs that has also a role in the binding of both agonists and antagonists [75].

The lack of high-resolution information available for the ARs (with the exception of A_{2A} AR subtype) hinders molecular modelling techniques due to the lack of knowledge regarding the entrance and definitions of the binding pocket between ARs [76].

The discovery for ARs agonists is mainly achieved through modifications of adenosine itself [77]. Adenosine is a full agonist for all ARs. In 2001, Fredholm and colleges observed that inosine could weakly bound to A_1 and A_3 ARs in functional assays. However, they concluded that inosine cannot be considered a natural agonist of A_1 receptors but might be seen as a partial agonist of A_3 ARs [78]. In the case of the ARs antagonists, it has been accomplished by modifications of xanthines such as caffeine and theophylline [66]. Currently, there are selective agonists and antagonist available for all four subtypes [79–84].

The affinity of adenosine for ARs is exceedingly difficult to determine directly in binding assays due to its rapid metabolization and formation in biological preparations, thus, truly reliable binding data do not exist and, therefore, we must rely on functional assays [64]. A_1 and A_{2A} receptors have high affinity to adenosine, requiring lower concentrations to be activated than A_{2B} and A_3 ARs (Table 2). Thus, these last two receptors are thought to remain silent under physiological conditions and to be activated due to excess accumulation of extracellular adenosine as in metabolic and stressful conditions [85].

Table 2 - Ligands currently used for adenosine receptors classification.

Ligand type	Abbreviation	AR subtype	K_i value for rat AR (nM)[a]				References
			A_1	A_{2A}	A_{2B}	A_3	
Agonist	ADO	A_1, A_{2A}, A_{2B}, A_3	73[b]	150[b]	5100[b]	6500[b]	81
	NECA	A_1, A_{2A}, A_{2B}, A_3	5,1	9,7	1110	113	86–88
	CPA	A_1	0,12	845	–	213	6
	CCPA	A_1	1,3	950	6160	237	89,90
	CGS 21680	A_{2A}	1800	19	>10000	584	89
	BAY 60-6583	A_{2B}	514	>10000	100	2750	86
	IB-MECA	A_3	54	948	3340	1,1	86,91,92
	2CI-IB-MECA	A_3	820	470	1210	0,33	86,92
Antagonist	Teophylline	A_1, A_{2A}, A_{2B}, A_3	1400	22000	15100	85000	93–95
	Caffeine	A_1, A_{2A}, A_{2B}	44000	45000	30000	>100000	96–98
	DPCPX	A_1	1	500	186	43000	94,99
	SCH 58261	A_{2A}	121	2,3	–	>10000	100
	ZM 241385	A_{2A}	684	1,25	373	>10000	86
	MRS 1754	A_{2B}	16,8	612	13	570	101
	MRS 1706	A_{2B}	37,6	548	–	–	101
	MRS 1220 [c]	A_3	305	52	–	0,65	102
	MRS 1523 [d]	A_3	15600	2050	>10000	113,0	89

[a] most data from binding experiments
[b] Data are from adenylate cyclase assay
[c] extremely potent in binding to the human but not rat A_3AR receptors [11]
[d] broad applicability to various species [11]

Abbreviations: ADO – Adenosine; NECA - 5'-N-Ethylcarboxamidoadenosine; CPA - N6-Cyclopentyladenosine; CCPA - 2-Chloro-CPA; CGS 21680 - 2-p-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride; BAY 60-6583 - 2-[[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2- pyridinyl]thio]-acetamide; IB-MECA - 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide; 2CI-IB-MECA - 2-Chloro-N6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine; Teophylline - 3,7-Dihydro-1,3-dimethyl-1H-purine-2,6-dione; Caffeine - 1,3,7-Trimethylpurine-2,6-dione; DPCPX - 1,3-Dipropyl-8-cyclopentylxanthine; SCH 58261 - 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; ZM 241385 - 4-(2-[7-Amino-2-[2-furyl]-[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol; MRS 1754 - N-(4-Cyanophenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]acetamide; MRS 1706 - N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide; MRS 1220 - N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide; MRS 1523 - 2,3-Diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate.

4. Adenosine clinical utility

Based on the omnipresence and beneficial effects attributed to adenosine, it has long been considered that ARs regulation has substantial therapeutic potential such as neuro- and cardioprotection, anti-inflammatory properties and even sleep promotion (see review from Sachdeva & Gupta: [8]). Several pharmaceutical companies are developing innumerous ARs ligands and consider ARs as promising targets for diverse diseases (Figure 4), and a lot of them are currently in advanced clinical trials for different therapeutic applications (as reviewed in Sousa & Diniz: [103]). However, approvall faces a major challenge due to adenosine and its receptors widespread distribution and actions, hindering safety delivery and clinical effectiveness. Thence, practically only adenosine itself was approved for clinical use.

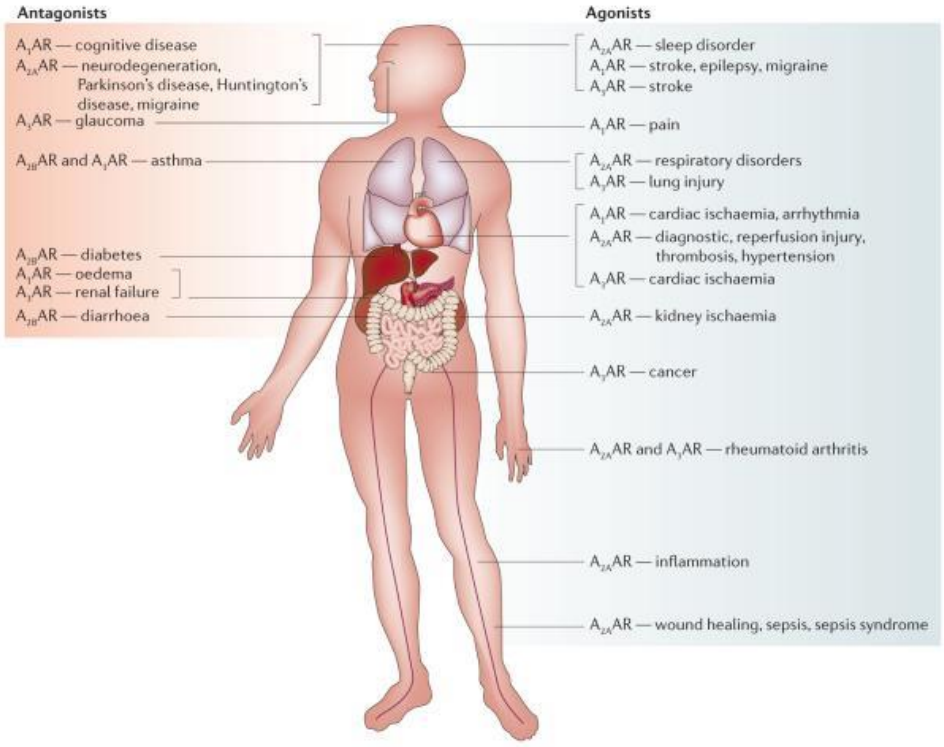

Figure 4 - Brief representation of promising disease targets for selective adenosine receptor ligands. Source: [6].

5. Adenosine in the vasculature

Hemodynamic effects of adenosine comprise direct actions on heart and vasculature as well as indirect neuroinhibitory activity [104]. Some examples of these actions will be

addressed, with primary focus on the vasculature, the selected tissue used for the entire scientific experiments discussed below.

The vascular walls of arteries and veins are similar basic structures. These structures comprise three major concentric layers: intima, media and adventitia (Figure 5). The intima layer includes the endothelium, the basement membrane (glycoproteins) overlying some connective tissue fibers, and the internal elastic lamina. The media layer primarily comprises smooth muscle cells and the external elastic lamina [105]. The adventitia layer is composed of connective tissue that contains nerve terminals and several cell types such as fibroblasts/fibrocytes, immune cells or mesenchymal stem cells [106].

The main difference between the two blood vessels types is that arteries have more smooth muscle cells than do comparably sized veins. Large arteries such as aorta are considered elastic arteries due to the numerous layers of elastin fibers between smooth muscle cells, providing the ability to expand and recoil according to hearts' pumping activity. In contrast, smaller arteries are named muscular arteries because of the thicker layer of smooth muscle and narrow lumina, providing greatest resistance to blood flow through the arterial system. The arterial system branches extensively in capillaries composed of just one cell layer, the endothelium. Veins contain most of the total blood volume, representative of high vascular capacitance [105].

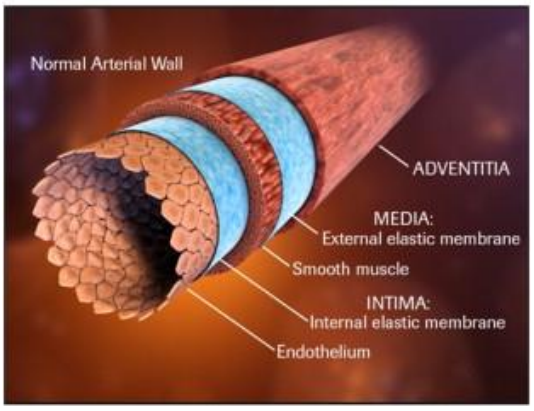

Figure 5 - Schematic representation of distinct layers of arterial wall. Source: [186].

It has been reported the presence of ARs at the three layers of different blood vessels such as coronary, mesenteric and pulmonary arteries, renal vasculature and aorta [67,75].

5.1. Vascular functional effects mediated by adenosine

Adenosine is a potent regulator of vascular tone and blood flow [107]. The functional responses mediated by adenosine in vasculature can be either dilation or contraction [108], depending on the location and basal blood vessel tone [109].

Adenosine exerts its effects either directly on vascular smooth muscle cells and endothelial cells or through indirect prejunctional modulation of perivascular sympathetic neurotransmission [107,110,111].

Generally, adenosine induces dilation of most vascular beds, with exception of renal and pulmonary circulation and some other vascular beds, which constrictor effect is transient and specie-dependent [104]. This biphasic response mediated by adenosine is due to its binding to A_1 or A_2 ARs leading to smooth muscle contraction or relaxation, respectively [109].

In smooth muscle cells, A_2 ARs activation increases cAMP levels with consequent PKA activation and K_{ATP} channels opening, which hyperpolarizes the smooth muscle culminating in muscle relaxation [112]. cAMP production might also inhibit myosin light chain kinase, which decreases muscle contractile force. Adenosine can inhibit voltage L-type calcium channels and, therefore, Ca^{2+} entry into the cell [113], an ion responsible for smooth muscle contraction, which presupposes that reduced intracellular Ca^{2+} levels causes relaxation. In arterioles, A_1 AR mediated vasodilation also seems to involve the activation of ATP-dependent K^+ channels in smooth muscle cells [114].

There is no direct evidence on A_3 AR vasorelaxation/constriction in physiological conditions, but it might be implicated in pathological conditions when adenosine levels are high, such as hypertension [115]. Indirectly, A_3 AR activation in mast cells might lead to arterioles vasoconstriction [116].

The A_{2A} receptor is the major AR subtype responsible for coronary blood flow regulation in endothelial or smooth muscle cell-dependent fashion [117]. Marketed as *Adenoscan,* adenosine is one of the most commonly used agents to induce coronary arterial vasodilation for myocardial perfusion imaging [118]. The undesirable side effects associated to this non ARs subtype selective agonist led to Food and Drug Administration (FDA) approval of *Regadenoson* (CVT-3146, Lexiscan), a selective and more potent A_{2A} receptor agonist [118].

A_1 and A_{2A} ARs on endothelial cells stimulates the vasodilator nitric oxide (NO) production through indirect endothelial NO synthase (eNOS) activation [119]. Additionally, *via* phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB), A_{2B} receptors also contributes to eNOS phosphorylation and further generates NO [120].

Neuromodulatory effects of adenosine are of major importance in vascular tonus regulation. At the adventitia layer of blood vessels, perivascular symphatetic nerves release vasocontrictor neurotransmitters such as noradrenaline and ATP. The four prejunctional AR subtypes were reported to modulate postganglionic sympathetic transmission in several vessels, including aorta [121], pulmonary [122], renal and tail [123] arteries and mesenteric vessels [124]. Adenosine can be generated from ATP co-released with noradrenaline [125] and trigger neuro-inhibitory (*via* A_1 and A_3 ARs) or facilitatory (A_2 ARs) effects. Additionally, endothelium-derived adenosine was found to activate prejunctional ARs, mainly A_1 and A_{2A} subtypes, influencing sympathetic neurotransmission [126]. Ultimately, NO predominantly derived from neuronal NO synthase also plays a modulatory role on sympathetic transmission *via* activation of inhibitory A_1 AR [127].

6. Mesenteric vessels

Mesenteric vasculature is linked to the “splanchnic circulation”, a term that describes the blood flow to the abdominal gastrointestinal organs, and which comprises three major unpaired branches of the abdominal aorta: the celiac trunk, the superior mesenteric artery (SMA) and the inferior mesenteric artery (Figure 6) [128].

The splanchnic circulation plays a prominent role in the regulation of systemic blood pressure and overall body hemodynamics [129,130]. It receives around 60 % of the cardiac output and contains about one third of the total blood volume [131], therefore being the mesenteric vessels frequently used in vascular experiments.

The mesenteric arteries and veins have significant resistance and capacitance functions in the systemic circulation [131]. The elasto-muscular SMA is the largest of the splanchnic arterial vessels delivering more than 10 % of the cardiac output [128]. When viewed in cross section, it is possible to discriminate the three layers: the intima, the media (combined by five to seven layers of smooth muscle cells separated by three to four medial elastic fibers) and the adventitia [106].

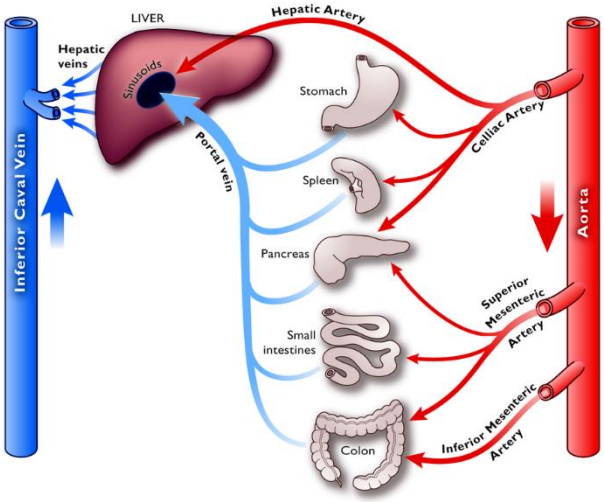

Figure 6 - Schematic representation of the splanchnic circulation. Source: [187].

The tone of SMA is mainly regulated by sympathetic nerves through the release of noradrenaline and the adrenergic cotransmitters neuropeptide Y and ATP, mediating vasoconstriction by engaging postjunctional receptors on smooth muscle cells [128,132]. The corelease of ATP from sympathetic nerves can also be judged as one source, but not the only, of adenosine, as previously mentioned. This adenosine might access to the four AR subtypes that have been identified in mesenteric arteries [133–136], with special interest in the ARs on sympathetic nerve fibers at adventitia [137–139], initiating specific signaling cascades modulating neurotransmitters release.

7. Nuclear GPCRs

The nucleus is typically the largest organelle within the cell and it is delimited by the nuclear envelope (NE) comprising two phospholipid bilayers: the inner nuclear membrane and outer nuclear membrane (contiguous with outer membrane of endoplasmic reticulum [ER]), separated by the intermembrane space [140,141]. Despite being continuous, these membranes are perforated by nuclear pore complexes which control trafficking of ions and macromolecules (greater than 40 kDa) [140]. Nevertheless, a concentration gradient for some ions does exist between cytoplasm and nucleoplasm [142,143]. Additionally, NE contains a complex branched network of invaginations that reach deep into the nucleoplasm and could even traverse the nucleus completely, which may facilitate intranuclear cargo transport, increasing membrane surface area [141].

It has long been suggested the presence of functional channels [144,145], receptors [146,147], pumps and exchangers [148] at the nuclear level. Since 1987 [149], more than 30 GPCRs have been detected by ultrastructural, immunohistochemistry, pharmacological and molecular techniques at the nucleus [43,150] in species from *Caenorhabditis elegans* to mammals, indicative of evolutionary preservation [151].

Traditional GPCRs signaling models believe these receptors are exclusively located on cell surface membranes when outside ligand binds initiating various intracellular signaling cascades, the so-called "outside-in model". However, as is becoming apparent with the growing evidence from the literature, GPCRs are also present on intracellular membranes, most notably the nucleus in a wide variety of different cell systems including cardiac myocytes, endothelial cells, vascular smooth muscle cells, neurons, hepatocytes, and kidney cells [43].

We may consider nuclear GPCRs as native resident nuclear membranes receptors or translocated from plasma membrane [152]. The first case can refer to receptors synthesized within the nucleus, since protein synthesis in this organelle accounts for approximately 10-15 % of total cell protein synthesis [153]. Alternatively, it could refer to lateral diffusion of newly rough ER synthesized proteins [154,155] or even from vesicular transport after ER and/or trans-Golgi network post-translational modifications [156]. The second case could occur as part of an agonist-mediated internalization event [157]. Trafficking to the nucleus could be attributed to the presence of a nuclear localization signal (NLS), a small peptide sequence consisting of basic aminoacid residues (frequently lysine-arginine or glycine-arginine repeats) commonly found in the C-terminal or in one intracellular loop [157–159].

Nuclear GPCRs were reported to be located at either NE membranes or, in some cases, within the nucleoplasm, possibly at the above mentioned invaginations, without excluding the possibility of GPCRs being incorporated in nonmembranous lipophilic domains and/or engulfed in lipid vesicular sheds within the nucleus [150,160]. Regarding GPCRs location at the NE, the receptor orientation remains a question largely unanswered, but it is most believed they are likely positioned in a way signals would be directed to the cytosol or nucleus.

Multiple functional members of class 1 GPCRs have been identified in the nucleus [43], such as α1-, β1- and β3-adrenergic receptors [161,162], angiotensin II [163] and endothelin 1 (ET-1) receptors [164]. These receptors may function in the same way as their cell surface counterparts or they could have distinct signaling pathways and therefore mediated

different biological responses [43,165]. These functions include modulation of nuclear ionic homeostasis which directly affects gene expression, apoptosis and/or the cell cycle [166]. Nuclear GPCRs functionality is supported by *in vivo* and *in vitro* experiments that demonstrate the presence in the nucleus of most downstream signal transduction components usually associated with cell surface GPCRs, such as various G protein subunits, effector molecules and second messengers related to AC-cAMP-PKA and PLC-PKC-IP3/DAG pathways or even MAPKs and PI3K-PKB signaling pathways, as well as regulatory proteins (β-arrestins and GRKs) [43].

II. Aim

The increased reported presence of several class 1 GPCRs in the cell nucleus, as well as all the necessary signal transduction and regulatory mechanisms involved in the main Gα pathways, has opened new research horizons for these receptors superfamily beyond the commonly signaling studied from the cell surface.

With respect to ARs, there is still no knowledge regarding its location in other intracellular structures besides the plasma membrane. In our research group, this hypothesis has gained a greater support after previous confocal studies focusing on ARs in rat adventitia SMAs. This inspired the begging of this work that first aim to investigate the presence of each ARs subtype in the nucleus of adventitia cells from mesenteric arteries. Secondly, regarding the receptor subtypes that demonstrate a plausible nuclear location, we also intended to evaluate if they display similar proportions between them, and with the respective counterparts in the cell surface. To accomplish that, different strategies have been implemented, namely the initiation of primary adventitia cell cultures; the use of intact culture cells and isolated nuclei to perform immunolabeling experiments; and also, the use of culture cells for subcellular fractionation and subsequent analysis of nuclear and plasma membrane fractions by western blot.

III. Materials and Methods

1. Animals

Adult (10-20 week-old) *Wistar Han* rats (Vivarium ICBAS, Porto, Portugal) were used regardless of gender. Animals were kept under light/dark cycles of 12/12 hours (h) at room temperature of 20-24 °C and had free access to food and water. Handling and care of animals were conducted according to the European guidelines (Directive 2010/63/EU) on the protection of animals used for scientific purposes in agreement with the NIH guidelines. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Rats were sacrificed and mesenteric vessels were dissected out and immediately placed on cold oxygenated sterile Krebs-Henseleit solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHPO_4$ 1.2, glucose 11, $NaHCO_3$ and ascorbic acid 0.3 (pH 7.4) in ultrapure water. SMAs were isolated and cleaned of fat.

2. Chemicals

Vectashield mounting medium was purchased from Vector Laboratories, United Kingdom; fetal bovine serum (FBS) from ThermoFisher Scientific (Lisbon, Portugal); glycerol from Himedia (Mumbai, India); coomassie blue from Bio-Rad (Lisbon, Portugal) and NZY Protein Marker II (MB09002) from NZYTech (Lisbon, Portugal). All other reagents were purchased from Sigma–Aldrich (Madrid, Spain).

Primary and secondary antibodies used and corresponding dilution are listed in table 3.

3. Experimental protocols

3.1. SMAs immunohistochemistry preparation for laser scanning confocal microscopy (LSCM)

Immunohistochemistry procedures were previously described [138]. Briefly, four tissue preparations were obtained from each SMA and immediately placed in cold phosphate buffer solution (PBS; in mM: NaCl 136.9, $Na_2HPO_4.2H_2O$ 4.3, KCl 2.7, KH_2PO_4 1.4 (pH

7.2). Each preparation was longitudinally opened and fixed (paraformaldehyde 4 % PBS; 50 min; room temperature). After two 15 min PBS washing cycles, artery segments were incubated with primary antibodies raised against rabbit polyclonal individual adenosine receptor subtypes. Thereafter, tissues were incubated with a species specific fluorescent secondary antibody. Negative controls (NC) were performed by omitting primary antibodies using serum instead and maintaining all other experimental conditions. After two PBS washing cycles, tissue preparations were mounted with antifading agent (Vectashield mounting medium with DAPI), with the adventitial side facing up.

Table 3 – Antibodies used for immunolabeling.

Protein	Catalog N^{er}	Brand	Dilution
Primary antibodies			
Rabbit polyclonal anti-Adenosine A1 Receptor	ab82477	Abcam	WB: 1µg/mL; IF: 5µg/mL
Rabbit polyclonal anti-Adenosine A2a Receptor	ab3461	Abcam	WB: 1:1000; IF/IH: 10µg/mL
Rabbit polyclonal anti-Adenosine A2b Receptor	ab229671	Abcam	WB: 1:2000; IF: 1:500
Rabbit polyclonal anti-Adenosine A3 Receptor	ab203298	Abcam	WB: 1:500; IF: 1:100
Rabbit polyclonal anti-A1 Adenosine Receptor	A-268	Sigma-Aldrich	WB: 1:500; IF/IH: 1:200
Rabbit polyclonal anti-Adenosine A2b Receptor	AB1589P	Millipore	WB: 1:1000; IF/IH: 1:200
Rabbit polyclonal anti-Adenosine A3 Receptor	AB1590P	Millipore	WB: 1:500; IF/IH: 1:200
Rabbit polyclonal anti-A1 Adenosine Receptor	AAR-006	Alomone	WB: 1:500; IF: 1:200
Rabbit polyclonal anti-A2A Adenosine Receptor	AAR-002	Alomone	WB: 1:500; IF: 1:200
Rabbit polyclonal anti-A2B Adenosine Receptor	AAR-003	Alomone	WB: 1:500; IF: 1:200
Rabbit polyclonal anti-A3 Adenosine Receptor	AAR-004	Alomone	WB: 1:500; IF: 1:200
Rabbit polyclonal anti-Histone H3	ab21054	Abcam	WB: 1:5000
Rabbit monoclonal anti-Lamin B1	ab133741	Abcam	WB: 1:5000
Rabbit polyclonal anti-Na+/K+ ATPase a-1	06-520	Millipore	WB: 1:750; IF: 1:500
Phalloidin-iFluor 488	ab176753	Abcam	IF: 1:1000
Hoechst 33342	14533	Sigma-Aldrich	IF: 1:200
Secondary antibodies			
Goat anti-rabbit IgG-HRP	sc-2004	Santa Cruz	WB: 1:5000
Goat anti-rabbit IgG Fc (Alexa Fluor 594)	ab150092	Abcam	IF: 1:1000
Goat anti-rabbit IgG H+L (Alexa Fluor 488)	A-11034	ThermoFisher	IF: 10µg/mL
Bovine anti-goat IgG-TR (Texas Red)	sc-2786	Santa Cruz	IF: 1:200

Abbreviations: HRP - horseradish peroxidase; IF – immunofluorescence; WB - western blot

Preparations were visualized with Leica SP5 laser scanning confocal microscopy system (Leica Microsystems, Germany) fitted with an inverted microscope (x63 oil immersion lens). Stacks of 1 µm thick serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei [167]. Image

acquisition was performed always under the same laser power, brightness and contrast conditions. Adventitia was scanned along each mesenteric artery and the resulting images were reconstructed separately for each wavelength: at 360 nm Ex/ 460 nm Em (for location of cell nuclei) and at 488 nm Ex/525 nm Em (for location of individual adenosine receptor subtypes containing cells).

3.2. Cell culture

A protocol was created to obtain primary adventitia cell cultures initiated from SMAs dissected and cleaned as mentioned above. Individual arteries were incubated with an enzymatic solution containing several types of collagenases in sterile Krebs-Henseleit during 25 min at 37 °C with constant oxygenation. To detach the adventitia layer, the artery was gently scraped and then removed. Adventitial cells suspension was centrifuged at 300 g, 15 min at 4 °C and the pellet re-suspended in RPMI 1640 medium supplemented with 20 % FBS and 1 % of penicillin/streptomycin. Centrifugation and re-suspension was repeated twice and ultimately placed in a 25 cm^2 culture flask. Cell preparations were kept at 37 °C in humidified atmosphere of 95 % air, 5 % CO_2. Medium was replaced twice a week and the primary cultures maintained until 80 % confluence (15-25 days). When the desired cell density was reached, adherent cells were enzymatically released with a 0.25 % trypsin-EDTA solution and distributed (10x) for new flasks maintained in the same conditions mentioned above, except FBS medium concentration that is decreased to 15 %.

3.3. Subcellular fractionation

Cells from the first subculture (P1) were submitted to a subcellular fractionation procedure using Cell Fractionation Kit – Standard (Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions with minor modifications to separate nuclear (N), cytosolic and mitochondrial/membrane (M) fractions. Briefly, filtered solution was centrifuged at 300 g, 10 min at 4 °C and the pellet re-suspended in kit buffer A supplemented with a protease inhibitor cocktail. Cell count was performed using trypan blue dye in order to ensure a minimal of 6.6 x 10^6 cells/mL. Cell suspension was incubated with Kit detergent I followed by two consecutive 1 min centrifuges at 5000 and 10000 g at 4 °C. The resulting supernatant was the cytosolic fraction. Pellets were re-suspended in kit detergent II and subsequently subjected to two more consecutive

centrifuges at the same previous conditions. The resulting supernatant was the M fraction and the pellets the N fraction. All fractions were stored at -80 °C until western blot analysis.

3.4. Nucleus isolation

Viable isolated nuclei were obtained as Jong and O'Malley [168] described with minor modifications for posterior immunolabelling performance. Briefly, cells from 80 % confluence P1-subcultures were centrifuged at 1000 g 10 min, 4 °C, resuspended in a hypotonic buffer and placed on ice. Cells were disrupted by repeated passages through a 25 gauge needle and cells homogenization were evaluated by trypan blue staining. The resultant suspension was submitted to sequential centrifugations using the same previous conditions, resulting in a pellet containing the purified nuclei that were stored at -80 °C until immunolabelling experiments.

3.5. Cell culture immunocytochemistry and isolated nuclei immunolabelling

Primary adventitia cells from early stages (P0) and after the first three subcultures (P1, P2 and P3) were seeded in a 96 multiwell plates at a density of $2x10^4$ cells/mL for 72 h. Cells were fixed at room temperature with 3.7 % paraformaldehyde solution for 15 min and permeabilized/blocked with a solution containing 1 % BSA, 10 % FBS, 300 mM glycine, and 0.1 % Triton X-100, 1 h at 37 °C. Washes were carried out using a PBS solution with the following composition (mM): $NaHPO_4$ 100, NaCl 50, pH 7.4 in ultrapure water.

Isolated nuclei were used for immunolabeling experiments performed in a poly-L-lysine coated 96 multiwell plate at a density of $2x10^6$ nuclei/mL. After a 10 min, 300 g at 4°C centrifugation, samples were incubated with primary antibodies. Washes were carried out by repeated centrifugations and resuspensions with a PBS solution supplemented with 2 % FBS. The same procedure was performed with intact cell for comparison.

Preparations (cells and isolated nuclei) were incubated overnight at 4˚C with the primary antibodies against the four adenosine receptor subtypes and species specific fluorescent secondary antibodies were next applied. Cell nuclei were stained, when required, with

5mg/mL Hoechst 33342 dye for 1 min incubation in dark. In a set of experiments in cultured cells, filamentous actin (F-actin) was labelled with phalloidin-iFluor 488 conjugated for 30 min at 37 °C in dark. Finally, mounting medium (glycerol/PBS) was added directly to the wells, and fluorescent imagens were immediately acquired under 20x objective on Lionheart™ FX Automated Microscope (Biotek, United Kingdom) using 469, 337 and 586 nm excitation wavelengths.

3.6. Western blot analysis

Protein concentration of the subcellular fractions and culture cell lysates was determined using the Bradford assay (Bio-Rad, Lisbon, Portugal). Molecular weight marker (NZY Protein Marker II) along with samples (containing different protein amounts) diluted in sodium dodecyl sulphate (SDS)-Polyacrylamide gel electrophoresis (PAGE) solution from the Cell Fractionation Kit (4:1) were loaded on a homemade polyacrylamide gel composed by a 12 or 8 % resolving gel (0.1 % SDS, 0.05 % ammonium persulfate – APS, 0.05 % tetramethylethylenediamine – TEMED, and 25 % Tris-HCl 1.5 M solution, pH 8.8 in ultrapure water) and a 4% stacking gel (0.1 % SDS, 0.05 % APS, 0.05 % TEMED, and 25 % Tris-HCl 0.5 M solution, pH 6.8 in ultrapure water). Proteins were separated by electrophoresis according to their molecular weight at the constant voltage value of 100 V during stacking and then resolved at 150 V for approximately 30 min. Proteins were subsequently electrotransferred onto polyvinylidene difluoride (PVDF) membranes (P1620174, Bio-Rad, Lisbon, Portugal) using different conditions according to protein molecular weight. Membranes were washed with 0.1 % bovine serum albumin (BSA) in PBST (PBS + 0.5 % Tween-20, pH 7.2), blocked with 5 % BSA at room temperature for 1 h and finally incubated at 4°C overnight with primary antibodies raised against individual AR subtypes. Thereafter, the membranes were washed and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. In negative controls, the primary antibodies were omitted. Immunocomplexes were detected using Novex ECL Chemiluminescent kit (Invitrogen, Life Technologies, Madrid, Spain) and ChemiDoc MP Imaging System (Bio-Rad, Lisbon, Portugal).

To access subcellular fractionation quality, primary antibodies against the nuclear markers Histone H3 and Lamin B1 and the plasma membrane marker Na+/K+ ATPase α-1 were also used complemented with the same previously applied secondary antibody.

4. Data Analysis

4.1. Fluorescent images quantification

Fluorescence quantitative analysis of confocal z-stacks and Lionheart images was performed using the image analysis software FIJI (ImageJ). Background were subtracted using BG subtraction from region of interest (ROI) plugin. Fluorescence was measured (in the green channel) in cells/nuclei labeled using the following formula: Corrected Total Cell Fluorescence (CTCF) = Integrated Density - Area of selected cell x Mean fluorescence of background readings.

A ROI proximal to the nucleus was defined by thresholding the Hoechst 33342 for colocalization analysis. Colocalization was obtained using the Colocalization Threshold plugin that applies Costes method for auto threshold determination [169]. The Manders' split colocalization coefficient using threshold (tM1) was calculated analyzing the proportion of signal from ARs immunoreactivity that colocalizes with Hoechst 33342 stain (values between 0-1, 1 indicating total colocalization).

4.2. Western blot quantitative analysis

Chemiluminescent protein signal and colorimetric signal of PVDF membranes stained with Coomassie blue were measured using the image analysis software FIJI (ImageJ). Quantification data reflects the relative amount as a ratio of areas of the immunolabeled protein band relative to the total proteins in that lane stained with Coomassie blue.

4.3. Statistics

Results are expressed as mean ± standard error of the mean (SEM), and n denotes the number of independent experiments. Differences of means were compared for significance using one-way ANOVA, followed by post-hoc Holm-Sidak's multicomparison *t* test. A P value lower than 0.05 was considered to denote statistically significant differences.

IV. Results and Discussion

1. ARs localization in the SMAs adventitia layer from LSCM studies

Previous confocal microscopy studies in our laboratory focusing on ARs distribution in rat SMAs adventitia layer raised the possibility that adenosine receptors could be located at the cell nucleus [138]. Indeed, A_1, A_{2A}, A_{2B} and A_3 receptor staining profiles (green) support the presence of the four AR subtypes in cells of SMA adventitia layer and further suggest a nuclear location (highlighted by white arrows in the squares evidencing light blue spots resulted from labeling overlay) for these receptors in addition to the well-known plasma membrane position (Figure 7A). Quantitative analysis of immunolabels revealed a lower A_{2B} receptors expression comparatively to that obtained for the other receptor subtypes (Figure 7B).

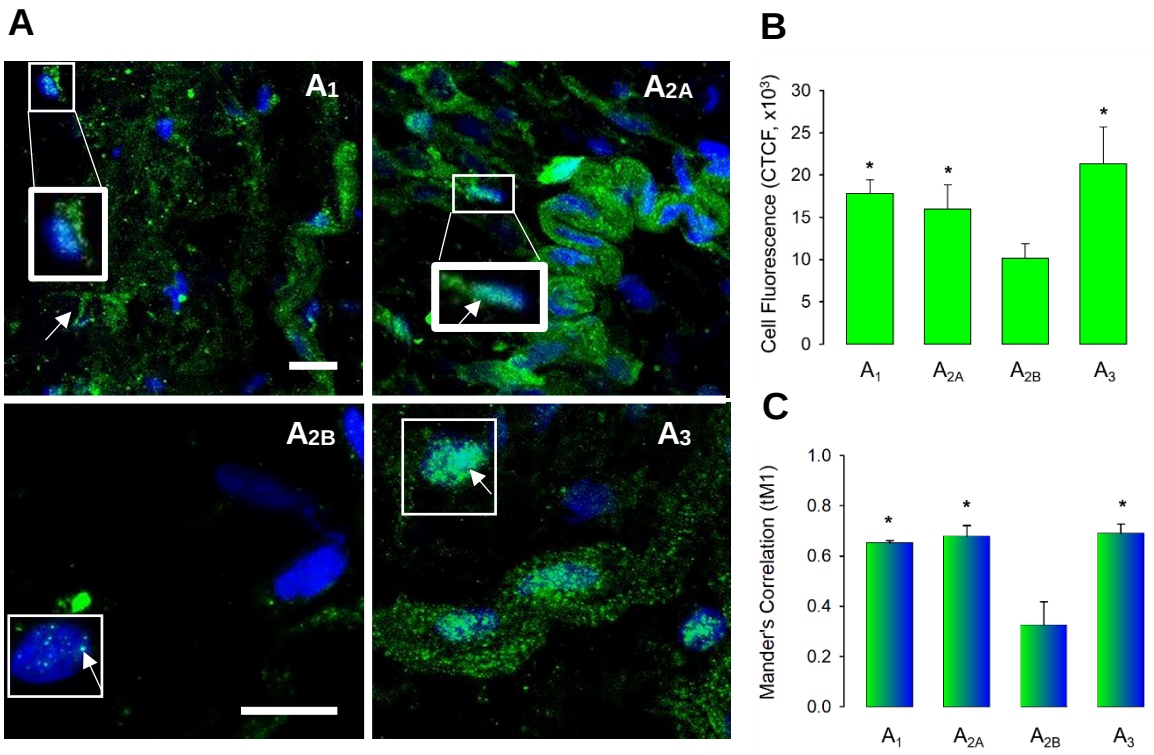

Figure 7 - Laser scanning confocal microscopy of Wistar Han SMAs adventitia layer labeling the four individual AR subtypes (green) and nuclei (blue). A - representative images exhibiting the overlay of immunoreactivities for adenosine receptors A_1, A_{2A}, A_{2B} and A_3 with the nuclear marker (DAPI, Blue). Images are reconstructions from 9–28 serial optical sections acquired under x63 oil immersion lens. Scale bar = 20 µm. B - Quantitative analysis of immunofluorescence elicited by immunolabeling of adenosine receptor subtypes, n = 3 rats per AR subtype, 3 cells per rat. C - Colocalization analysis: quantitative analysis of co-expression levels by Manders´ coefficient in SMA adventitia layer, analyzing the ratios between AR subtypes/DAPI. * $P < 0.05$. Differences from A_{2B} values.

Manders' split overlap coefficients were measured between ARs and nuclear fluorescence to evaluate signal colocalization between each AR subtype antibody and the nuclear marker in order to confirm a putative location at the cell nucleus (Figure 7C). High values obtained for A_1, A_{2A} and A_3 receptors point to a strong colocalization of these AR subtypes with the nuclear marker. Also, A_{2B} receptors revealed nuclear location, however, the colocalization of A_{2B} receptors in the nuclei was less extensive than that obtained with the other AR subtypes (tM1 value of 0.33 ± 0.09: Figure 7C).

These data reinforces the presence of the four ARs at adventitia layer cells from mesenteric arteries as reported in the past [137–139] and further reveals a minor expression of A_{2B} AR compared to that obtained for the other AR subtypes. Surprisingly, colocalization results corroborate the innovated hypothesis of the presence of ARs in the nucleus, suggesting a stronger presence of A_1, A_{2A} and A_3 ARs at the cell nucleus compared to A_{2B} receptors.

ARs have been detected in rat mesenteric adventitia layer: in sympathetic nerves, glial cells [139] and in other adventitia cells [138]. In vascular postganglionic sympathetic nerve endings, presynaptic ARs display a functional role in neurotransmission modulation: A_1 and A_3 ARs exert an inhibitory effect and A_{2A} and A_{2B} subtypes facilitate noradrenaline release [123,133]. Noteworthy, there are evidence in the literature indicating ARs presence on immune cells [170] and cultured cardiac fibroblasts [171], suggesting the possibility of other biological roles for ARs in the adventitia layer. The plausible ARs nuclear location in adventitia cells raises the possibility that biochemical events that are mediated by cell surface receptors may happen in a parallel manner at the nucleus, and in this way, we could somehow extrapolate some of these functions to the nucleus. However, we should not rule out the possibility of nuclear ARs evoke distinct responses and as such, the final physiological effect mediated by ARs in the cell would result from the integration of their actions trigged from the plasma membrane and the nucleus.

2. Primary adventitia cell cultures monitoring

New experiments were performed to complement and confirm the previous results since the possibility of an intracrine ARs signaling beyond the plasma membrane, namely the nucleus, would challenge the traditional way we see these receptors at present. An innovative approach was carried out: a protocol was established to obtain primary adventitia cell cultures initiated from rat SMAs, the same tissue used in the preceding

immunohistochemistry study. Early cell culture stages (P0) and the first three subcultures (P1, P2 and P3) were monitored. Culture changes over time were evaluated to select the appropriate subculture to proceed with experiences.

In an adventitia cell culture, different cell morphologies are expected since adventitia layer is composed by different cell types, such as fibroblasts, fibrocytes, macrophages, lymphocytes, Schwann cells and neurons (nerve endings). In figures 8 and 9 it is possible to observe cellular heterogeneity in the adventitia cultures with most cells presenting an oval speckled nucleus (e.g., fibroblasts, fibrocytes, Schwann cells) and with some presenting a central/rounded nucleus consistent with those of macrophages or lymphocytes. These cells morphology contrasts with typical nuclei morphology of smooth muscle cells, tunica media components, which exhibit a large fusiform shape [130]. The putative presence of smooth muscle cells in this culture was not evaluated by immunocytochemistry, since an observation based on the shape of the nuclei helps its identification and distinction from the adventitia cells, as previously established by others [167].

Cells from adventitia layer have also a particular feature which is the ability to produce matrix components that over time leads to collagen deposition and/or elastin formation granting an irregular brunched morphology to the cells with a more extensive cytoplasm [130] which can be seen with culture time progression revealed by increased confluence and passages (Figures 8 and 9).

F-actin, composed by monomeric actin that polymerizes in two long stable filaments that turn around each other [172], form actin filaments (also known as microfilaments) that are components of the cytoskeleton. They are particularly abundant beneath the plasma membrane where they form a network that determines cell shape [173]. Actin cytoskeleton is reorganized during mitosis to form rounded cells, following the cell cycle progression [174]. Moreover, in fibroblasts, actin filaments are of major relevance at focal adhesions, where they serve as anchors between the cytoskeleton and the extracellular matrix [173]. The toxin phalloidin binds to actin filaments [173] and when labeled with a fluorescent dye, provides an idea of the cell culture morphology, helps distinguish between fibroblasts and other cell types and further evidence cell proliferation (Figure 9).

Primary adventitia cell culture monitoring allowed to conclude that the protocol to initiate and maintain this primary culture was successfully established since it was obtained a cell types' mixture with morphologies and behavior compatible with the expected for adventitia cells.

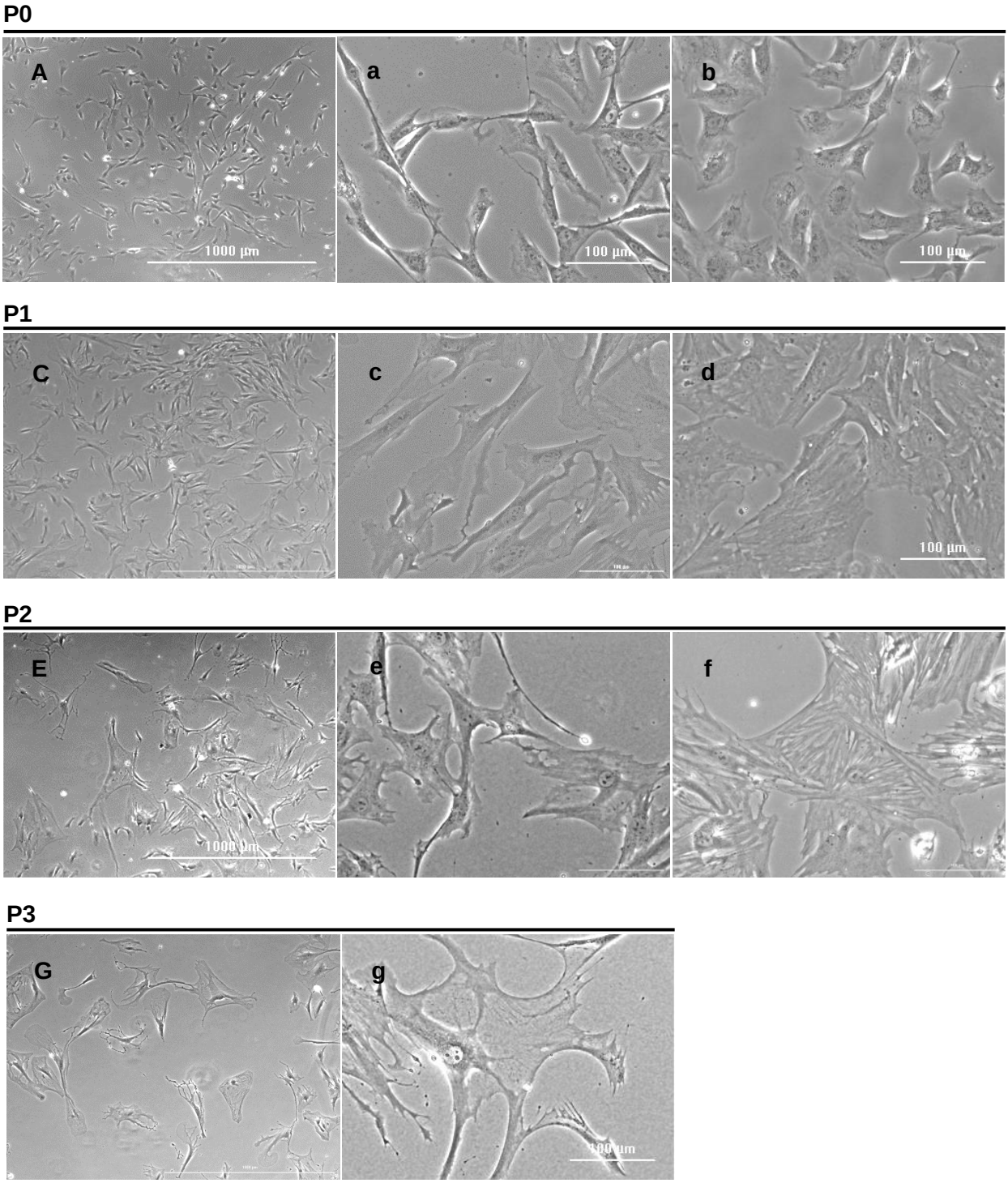

Figure 8 – Phase contrast images of early (P0) primary adventitia cell culture and of the first three subcultures (P1, P2 and P3). A, a, C, c, E, e G and g – cultures at approximately 50% confluence; b, d and f– cultures at near 80% confluence. n = 3. Images acquired under 4x (capital letters) and 20x (smaller letters) objectives and white scale bars represent 1000 µm and 100 µm, respectively.

P3 subcultures were only kept until about 50 % confluence (Figure 8G and 8g) since visible culture morphology changes has happened. Although no confirmation has been

made in the present work, in primary cell cultures of coronary artery adventitial fibroblasts, fibroblasts, crucial components of adventitia layer, have shown to differentiate to myofibroblasts from the third culture passage [175], a non-characteristic phenomenon in physiologic conditions [176]. Thus, we excluded immediately P3 subcultures as inappropriate for future experiments.

To choose the best subculture, besides similarity with the initial culture (P0), we also took into account that the extracellular matrix hinders subsequent work and thus, it was stipulated that the first subculture (P1) would be the one that would fit our experimental purposes.

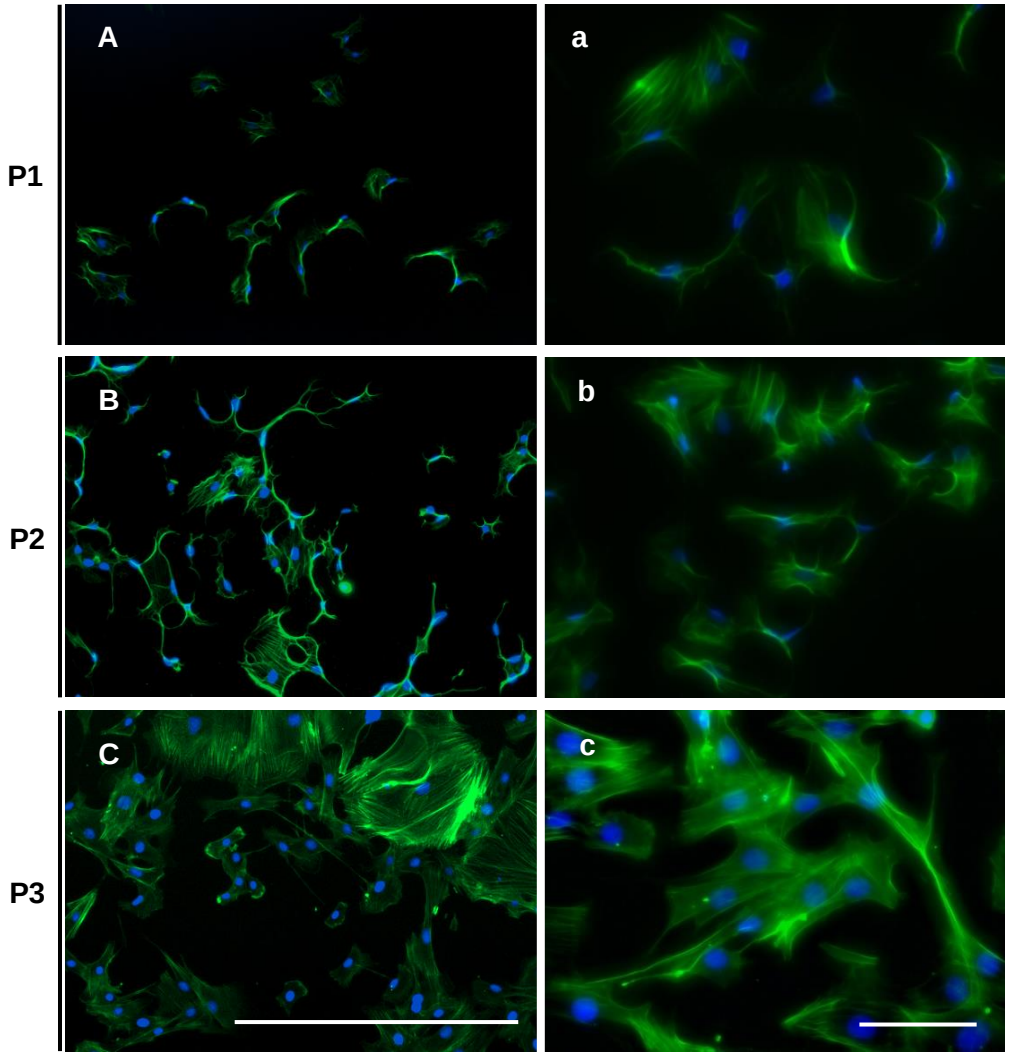

Figure 9 – Fluorescent images of actin (green) immunoactivity in primary adventitia culture cells of the first three subcultures (P1, P2 and P3). n = 3. Images acquired under 4x (capital letters) and 20x (smaller letters) objectives and white scale bars represent 500 µm and 100 µm, respectively.

3. ARs located in the primary SMAs adventitia cell cultures

3.1. ARs distribution within adventitia cells

Immunofluorescence labelling of the four AR subtypes was evaluated to assess its presence and distribution within the primary adventitia cell culture.

Independent antibody verification was applied to validate ARs antibodies [177]. Hence, differentially raised (non overlapping epitopes) primary antibodies recognizing the same protein target, the four individual ARs, were used revealing identical labeling (data not shown), supporting the specificity of immunolabeling.

At this point we accomplished similar results through immunohistochemistry analyzing rat SMA adventitia layer and immunocytochemistry performed in primary rat SMA adventitia cell cultures. All four ARs demonstrated to be expressed and, in both techniques, receptors labeling were not only restricted to the plasma membrane, but also suggested to be located at the cell nucleus. Indeed, all AR subtypes revealed to be present in adventitia cells cultured from rat SMAs (Figure 10). A closer observation of ARs immunofluorescence, evidenced by the arrows, suggests these receptors seem to be present in nuclear membranes since it is possible to visualize ARs immunoreactivity in the nuclear location stained with the nuclear marker: Hoechst 33342.

3.2. Nucleus isolation

Taking into account all the evidences already demonstrated, we considered as major relevance the isolation of P1-subculture cells' nuclei either to obtain a more accurate ARs labelling, or to carry out future functional assays. The emerging paradigm of nuclear GPCRs can be studied using high-resolution electron microscopy that permit the identification with great precision of receptors' subnuclear distribution [178]. However, to overcome experimental and mainly functional limitations of this technique, alternative methodologies, e.g. nuclei isolation, may be used to access receptors presence in this cellular organelle, as long as isolation is successfully reached [168]. Therefore, to ensure the correct isolation, a double immunolabeling for a plasma membrane marker, Na+/K+ATPase, and a nuclear marker, Hoechst 33342, was performed in isolated nuclei and intact cells (Figure 11).

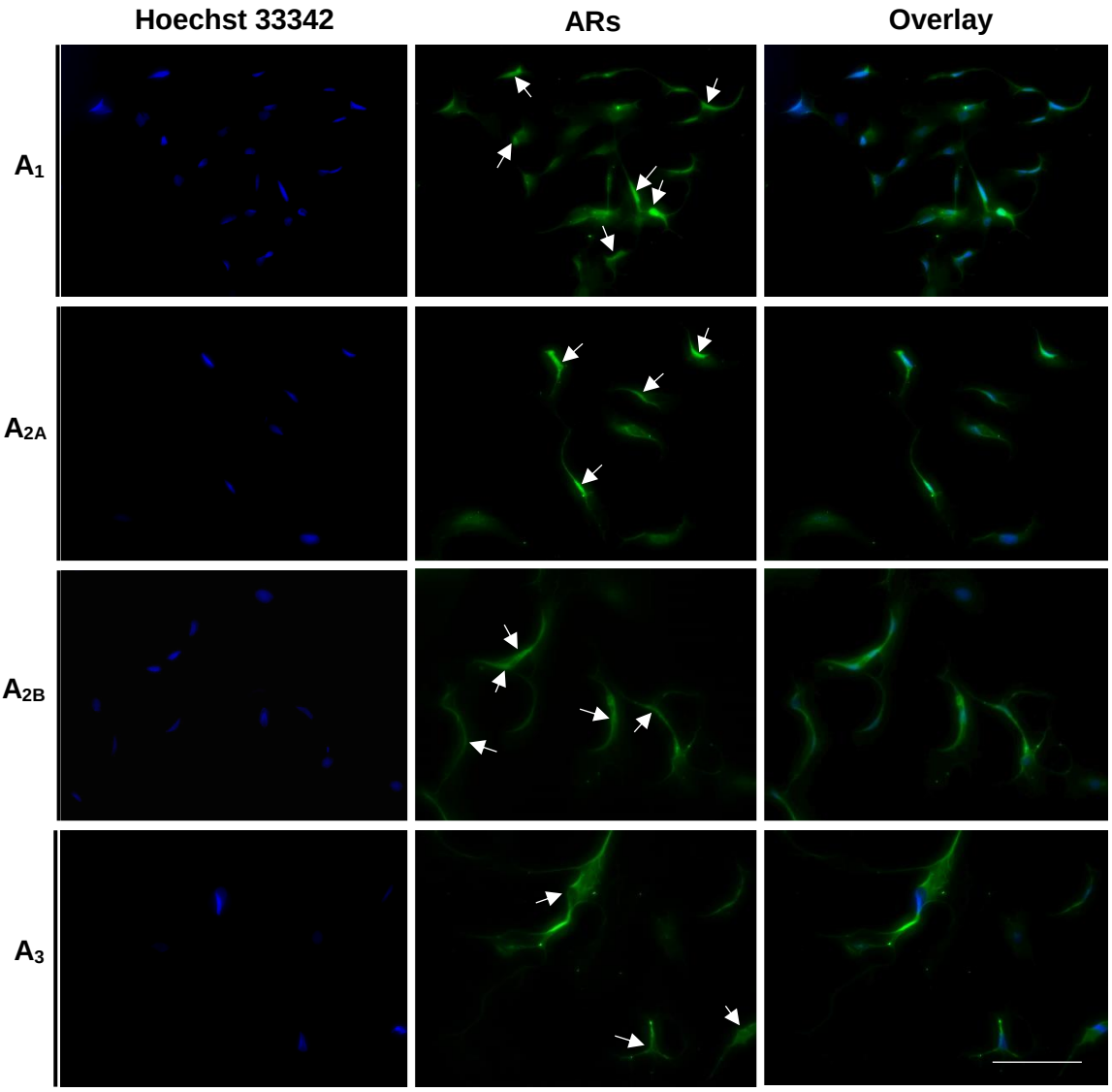

Figure 10 – Adenosine receptors in culture cells from rat adventitia SMAs. P1-subculture cells showing double immunolabeling for nuclear marker (Hoechst 33342 – blue) and for A_1, A_{2A}, A_{2B} and A_3 (primary rabbit polyclonal anti-A1, A2A, A2B or A3 and a species-specific secondary Alexa 488 antibody: green). Arrows evidencing ARs immunoreactivity coincident with nuclear location. n = 3. Images acquired under 20x objectives. Scale bar = 100 µm.

Results indicate the absence of staining for the membrane marker in isolated nuclei, which contrast with the immunolabeling for Na+/K+ATPase observed in intact cells (Figure 11). The lack of plasma membrane marker fluorescence suggests a remarkable isolation of nuclei that, from contrast images, only seems to might have attached some ER, which is acceptable since this intracellular structure is contiguous with nuclear envelope, as described in introduction section 6.

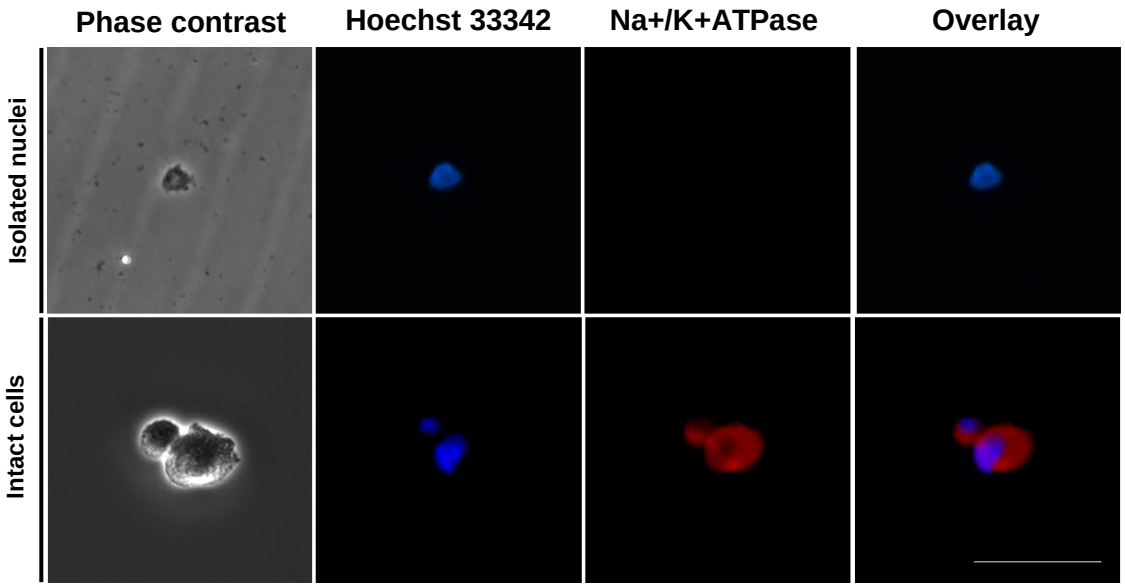

Figure 11 – Isolated nuclei of culture cells from mesenteric adventitia arteries. Isolated cell nuclei and intact cells from SMAs adventitia subculture showing immunolabeling for nuclear marker Hoechst 33342 (blue) and plasma membrane marker anti-Na+/K+ATPase (red). n = 3. Images acquired under 20x objectives. Scale bar = 100 µm.

3.3. ARs presence in isolated nucleus

The presence of the receptors in isolated nuclei might be seen as an intermediate point between protein synthesis and transport to the cell surface [179]. However, data from literature reported the presence of NLS sequences in some nuclear GPCRs (e.g. α1-, β1- and β3-adrenergic receptors, angiotensin II and ET-1 receptors [165]) which seems to indicate that the nucleus are an alternative or even the preferred location for the receptors. Concerning ARs, to my best knowledge, the occurrence of a NLS sequence still has to be explored.

In isolated nuclei from adventitia cultured cells, ARs immunolabeling is depicted (Figure 12A) confirming the occurrence of A_1, A_{2A}, A_{2B} and A_3 receptors in the nuclei. It is important to denote that approximately only two thirds of the nuclei were labeled (data not shown) and ARs fluorescence were measured for each labeled one and the values for the four AR subtypes are graphically represented in Figure 12B. ARs expression in the nucleus seems to follow a similar trend as immunohistochemistry results: all AR subtypes are expressed and A_{2B} receptors demonstrate the lowest presence.

In parallel, colocalization analysis of ARs immunoreactivity and the nucleus (stained with Hoechst 33342) was performed by manually delimiting a ROI around total ARs

fluorescence associated to each isolated nucleus. tM1 values demonstrate a similar colocalization profile for all ARs with the nuclear marker used (Figure 12C).

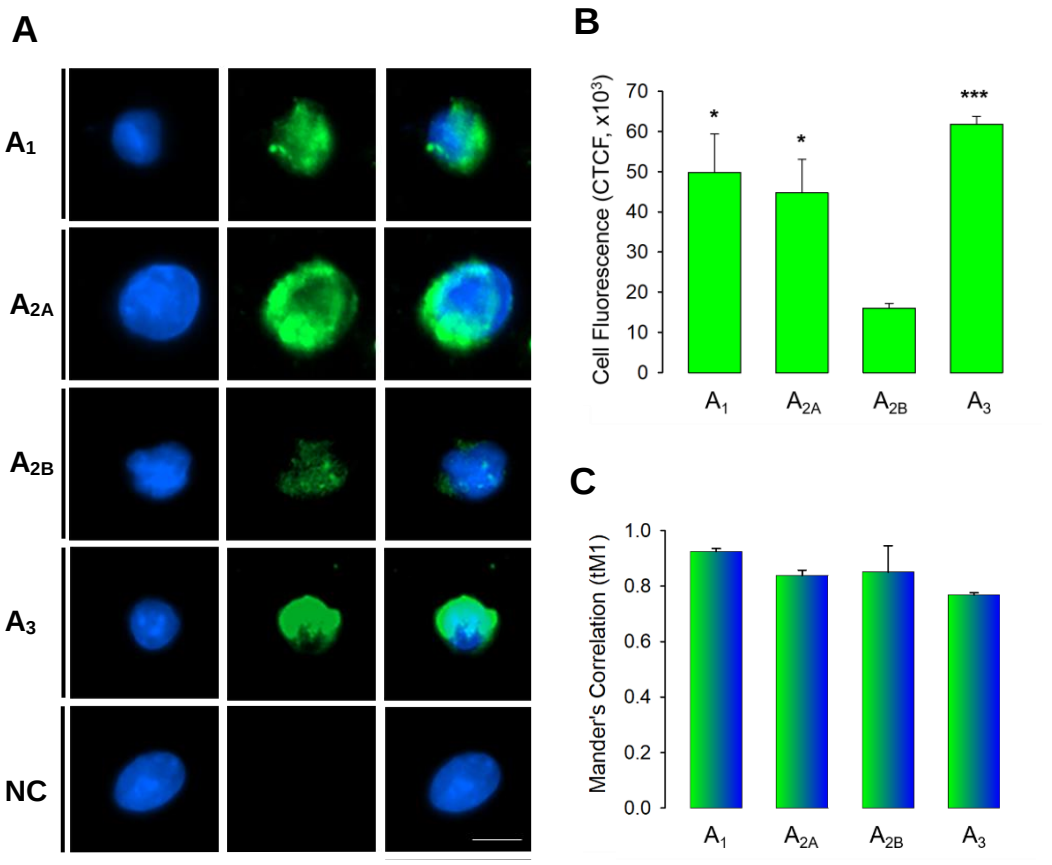

Figure 12 – Presence of the four AR subtypes in isolated nuclei from adventitia culture cells. A- colocalization of nuclear marker Hoechst 33342 (blue) and ARs (green). B – Quantitative analysis of immunofluorescence elicited by immunolabeling of AR subtypes. Scale bar = 10 µm. C - Colocalization analysis; quantitative analysis of co-expression levels by Manders' coefficient in the isolated nuclei, analyzing the ratios between AR subtypes/Hoechst 33342. n = 3, more than 20 nuclei evaluated for each AR. * $P < 0.05$, *** $P < 0.005$. Differences from A_{2B} values. NC = negative control.

Colocalization results differ slightly to the LSCM studies in intact cells regarding A_{2B} subtype. This could result from an easier permeabilization and access of antibodies to the receptors present in isolated cells/nuclei compared to immunohistochemistry that presents greater barriers in this regard.

The use of isolated nuclei, when compared to the prior immunolabelling techniques, allow more clarified results and quantitative analysis restricting substantially the receptors fluorescent signal to the nucleus, since isolation was successfully achieved, clearly reinforcing nuclear ARs finding. Thus, all the four ARs can be found in SMAs adventitia cells' nuclei and, despite A_{2B} receptors revealing minor expression, their

immunoreactivity colocalizes with the nucleus in the same proportion as the other AR subtypes.

4. Western Blot analysis of nuclear and membrane fractions

To reinforce the results obtained so far, ARs presence in the cell nucleus was ultimately evaluated through western blot analysis of N and M fractions derived from SMAs adventitia cultured cells.

Western blot optimization has only been completed for A_1, A_{2A} and A_{2B} ARs so far (Figure 13A). Relative quantifications of the target proteins revealed the expression of these AR subtypes in nuclear fractions, suggesting, once again, the presence of adenosine receptors in the nucleus of SMA adventitia cells (Figure 13B). Higher ARs levels were found in membrane fractions indicating that all receptor subtypes are more expressed at the plasma membrane than the nucleus, although ARs could also be present in the mitochondria, since there are other GPCRs that have been found in this organelle [180].

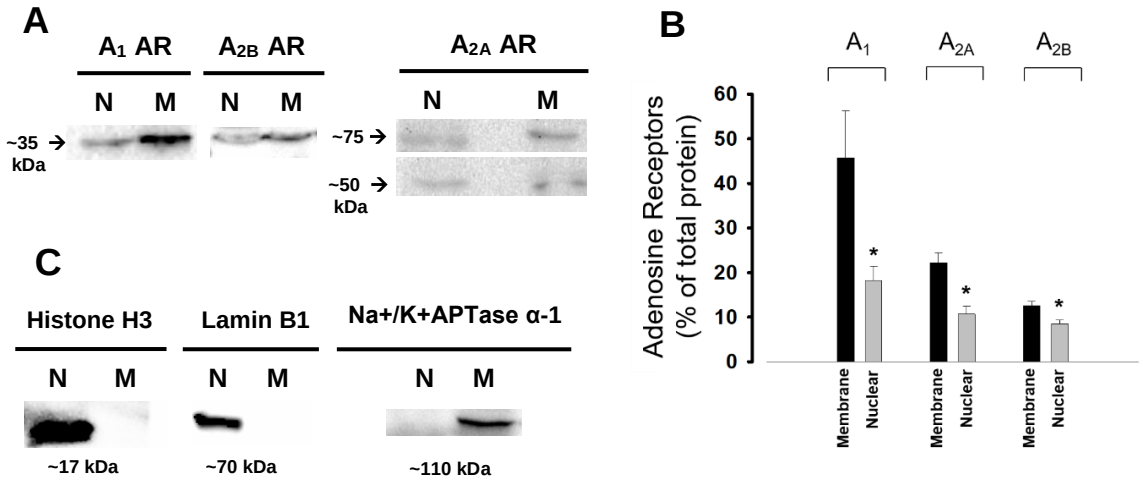

Figure 13 – Western blot analysis of nuclear and membrane fractions from adventitia cultured cells. A - Representative images of A_1, A_{2A} and A_{2B} ARs expression in nuclear and membrane fractions. (B) Relative quantifications of ARs expression. * $P < 0.05$. Differences from membrane fractions. C - Representative images of the of expressions the nuclear markers Histone H3 and Lamin B1 and the plasma membrane marker Na+/K+ATPase in nuclear (N) and membrane (M) fractions to access subcellular fractionation quality. n = 3.

Quality control of these fractions was performed by the evaluation of nuclear protein markers (Histone H3 [181] and Lamin B1 [182]) and the plasma membrane marker previously

used to access nuclei isolation quality (Na+/K+ATPase). Subcellular fractionation was successfully achieved (Figure 13C) validating these results.

5. Summary

In summary, we collect strong evidence suggesting, for the first time, ARs presence at the cell nucleus. Immunocytochemistry, isolated nuclei immunolabelling as well as western blot techniques were used to evaluate ARs expression at the nuclear level of SMAs adventitia cells. Data from this work support the presence of the four ARs at adventitia layer cells from mesenteric arteries [137–139] and further reveals a minor expression of A_{2B} receptor when compared to the other AR subtypes. Surprisingly, all ARs revealed a plausible nuclear location, and interestingly, as happened with the results in the whole cell, A_{2B} receptors displayed the lowest expression.

V. Conclusions and future perspectives

ARs display a broad distribution, in a way that there are no known tissue or organ unresponsive to adenosine [8,11,41]. To the best of our knowledge, ARs only have been described in cytoplasmic membranes so far. In fact, no evidence was found concerning ARs presence in other intracellular structures, including the nucleus. In this regard, this study is a pioneer for adenosine receptors.

The hypothesis emerged from LSCM studies in rat SMA adventitia layer, which colocalization analysis between each AR subtype immunoreactivity and the nuclear marker revealed a plausible nuclear location. To confirm and complement this hypothesis, a protocol was established to initiate and maintain primary adventitia cell cultures from rat SMAs. These cultures allowed the obtainment of a high number of adventitia cells for subsequent experimental purposes reducing the animal sacrifice.

Successful nucleus isolation from these cultured cells was performed as described by others [168]. ARs immunolabelling and nuclear staining in this isolated cellular structure as well as in intact cultured cells showed that ARs location should not only be restricted to the plasma membrane, but they might also be found in the nucleus of SMAs adventitia cells. Western blot analysis of nuclear fractions obtained from cultured cells reinforced the expression of A_1, A_{2A} and A_{2B} receptors at the nucleus. Therefore, in all the data gather in this work, from immunohistochemistry, immunocytochemistry, isolated nuclei immunolabelling as well as western blot analysis, we collect strong evidence suggesting ARs presence in the nucleus of SMA adventitia cells. Since it was possible to obtain similar evidence from the tissue, cultured cells and isolated nucleus, data seems to be consistent with the occurrence of a nuclear location of ARs, which should have a biological relevance that urges to study.

In recent years many functional GPCRs have been localized at intracellular membranes, particularly the nucleus [43], such as ET-1 type B receptor that belong to the same GPCR class as ARs, and has been reported to localize in nuclear membranes of several cardiovascular cell types, including human cardiac [183], vascular smooth muscle [147] and vascular endothelial cells [166] as revealed by studies of fluorescence microscopy including in isolated nuclei. Moreover, most downstream signal transduction components and regulatory proteins such as heterotrimeric G proteins, effector molecules, β-arrestins, GRKs and a variety of ion channels usually associated with cell surface GPCRs have also shown to be located in the nucleus [43]. Nuclear GPCRs functionality has been

demonstrated by its ability to increase nuclear calcium from perinuclear stores, cAMP and NO production and protein kinases and CREB activation, to name a few [165]. Taking this into account, the presence of ARs at the nucleus suggests a possible intracrine signaling beyond the plasma membrane.

In this regard, future investigations are needed for an in-depth knowledge of the biological role of nuclear ARs. Ongoing and future work comprise western blot optimization for A_3 AR. Furthermore, since isolated nuclei demonstrated to be viable (data not shown) and as has been done for other reported nuclear receptors [184], fluorescent calcium assays are now being performed, and future cAMP assays will also be made as an attempt to clarify if nuclear ARs are functional, and if so, which signaling pathways are activated and which effects could adenosine trigger from these intracellular receptors that will challenge novel pharmacological approaches.

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