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Abbreviations

7TM	Seven transmembrane α-helices
A_1/A_1R	A_1 adenosine receptors
A_{2A}/A_{2A}R	A_{2A} adenosine receptors
A_{2B}/A_{2B}R	A_{2B} adenosine receptors
A_3/A_3R	A_3 adenosine receptors
AD	Aldehyde dehydrogenase
ADH	Alcohol dehydrogenase
AR	Aldehyde reductase
ARs	Adenosine receptors
AUC	Area under curve
BBS	Low-calcium bicarbonate buffered saline solution
BSA	Bovine serum albumin
COMPT	Catecholamine-O-methyltransferase
DβH	Dopamine beta-hydroxylase
DA	Dopamine
DHMA	3, 4- dihydroxymandelic acid
DHPG	3, 4- dihydroxyphenylglycol
DOPA	Dihydroxyphenylalanine
DOPAL	3, 4-dihydroxyphenylacetaldehyde
DOPAC	3, 4- dihydroxyphenylacetic acid
DOPEGAL	3, 4-dihydroxyphenylglycolaldehyde
DOPET	3, 4-dihydrophenylethanol
EP	Epinephrine
GPCRs	G protein-coupled receptors
LDCVs	Large dense-core vesicles
nAChRs	Nicotinic acetylcholine receptors
MAO	Monoamine oxidase
MHPG	3-methoxy-4-hydroxyphenylglycol
MN	Metanephrine
MOPEGAL	3-methoxy-4-hydroxyphenylglycolaldehyde
NE	Norepinephrine
NMN	Normetanephrine

NPY	Neuropeptide Y
PMD	Piecemeal degranulation
PNMT	Phenylethanolamine-N-methyltransferase
TH	Tyrosine hydroxylase
VDCCs	Voltage-dependent calcium channels
VIP	Vasoactive intestinal peptide
VMA	Vanillylmandelic acid
VMT	Vesicular monoamine transporter

Resumo

As glândulas supra-renais estão localizadas em cima de cada rim e são compostas por duas zonas funcionalmente distintas: a medula, ocupando uma posição central, e o córtex, na periferia. A medula da glândula supra-renal é constituída maioritariamente por células cromafins que são responsáveis pela libertação de catecolaminas (dopamina, adrenalina e noradrenalina) por exocitose.

Os receptores de adenosina pertencem à superfamília de receptores acoplados à proteína G e são considerados quatro diferentes subtipos: A_1, A_{2A}, A_{2B}, e A_3, tendo em conta diferenças na estrutura molecular e perfil farmacológico. O papel dos subtipos de receptores de adenosina - A_1, A_{2A}, A_{2B}, A_3 – na glândula supra-renal e a sua distribuição são factos que ainda não são claros. Alguns estudos tentaram elucidar estes aspectos, mas muito continua ainda desconhecido.

No presente trabalho foi possível localizar os receptores de adenosina A_{2A} na medula da glândula supra-renal por imunohistoquímica. Foi realizada a optimização de um Sistema de Perfusão usando glândulas supra-renais de murganho possibilitando um estudo mais fisiológico da libertação de catecolaminas da glândula supra-renal de murganho. Utilizando este Sistema de Perfusão os resultados obtidos sugerem que o receptor de adenosina A_{2A} tem efeito estimulatório na libertação de catecolaminas (adrenalina e noradrenalina) induzida pela nicotina na glândula supra-renal de murganho uma vez que o CGS 2160, agonista dos receptores de adenosina A_{2A}, aumenta a libertação de catecolaminas mediada pela nicotina enquanto o SCH 58261, antagonista dos receptores de adenosina A_{2A} inibiu o efeito.

Em resumo, este estudo fornece uma nova ligação entre as células cromafins e os receptores de adenosina, usando o protocolo alternativo de Sistema de Perfusão.

Palavras-chave: *Glândula supra-renal, células cromafins, catecolaminas, Sistema de Perfusão, receptores de adenosina.*

Abstract

Adrenal glands are located above each kidney and are composed by two distinct functional zones, in a general classification: medulla, in a central position, and cortex, in the periphery. Adrenal medulla is mostly composed by chromaffin cells that are responsible for catecholamine release by exocytosis (dopamine, epinephrine and norepinephrine).

Adenosine receptors belong to the superfamily of G protein-coupled receptors and four different subtypes of receptors are considered: A_1, A_{2A}, A_{2B}, and A_3, according to differences in molecular structure and pharmacological profile. The role of adenosine receptors subtypes - A_1, A_{2A}, A_{2B}, A_3 - in adrenal gland and their distribution are still not clear. Some studies tried to elucidate these aspects but a lot remains unknown.

In the present work it was possible to localize the A_{2A} adenosine receptors in mouse adrenal medulla by immunohistochemistry. It was performed the optimization of a Perfusion System using mouse adrenal glands leading to a more physiological study of catecholamine release by mouse adrenal gland. Using this Perfusion System results suggest that A_{2A} adenosine receptor has a stimulatory effect on catecholamine release (epinephrine and norepinephrine) induced by nicotine from mouse adrenal gland since CGS 21680, adenosine receptors A_{2A} agonist, enhances catecholamine release mediated by nicotine whereas SCH 58261, adenosine receptor A_{2A} antagonist inhibited the effect.

In summary, this study provides a new link between chromaffin cells and adenosine receptors, using the Perfusion System alternative protocol.

Keywords: *Adrenal gland, chromaffin cells, catecholamines, Perfusion System, Adenosine receptors.*

Chapter I – Introduction

1.1 The adrenal glands and chromaffin cells

Adrenal glands own this name because of their location above each kidney. Two distinct functional zones compose these endocrine glands: medulla, in a central position, and cortex, in the periphery. It is also described two types of cell populations within the adrenal gland: adrenocortical cells that are responsible for steroid production and the chromaffin cells responsible for catecholamine production. Adrenal cortex and medulla are both involved in stress response and homeostasis maintenance [1].

Despite the adrenal cortex and the adrenal medulla are often considered separate units, scientific research on adrenal physiology has been largely focused on the individual understanding of each cell type. However, there is a coexistence of the two tissues in the same organ demonstrating their physiological interaction, suggesting a communication between medullary and adrenocortical cells in multiple contact zones; *reviewed in* [2], [3]**.** Furthermore there is no physical separation by connective tissue or interstitial membranes which is possibly contributing to an optimal function [4].

Adrenal cortex has a mesodermic origin and is responsible for production of mineralocorticoids and glucocorticoids. It is composed by three distinct zones: zone glomerulosa, the outer zone, composed of a thin region of columnar cells arranged in an accurate pattern, responsible for the production of the steroid hormone aldosterone; zone fasciculatas, the thickest zone (around 70 % of the cortex), composed of columns of secretory cells that produces glucocorticoids, and the zone reticularis which is also composed by polyhedral cells with a less linear arrangement, responsible for glucocorticoids production and in some species also produces small amounts of sex steroids like androgens, estrogens and progestins; *reviewed in* [5].

The adrenal medulla has structural and functional differences from adrenal cortex. In primer instance is derived embryologically from neural crest [6]. In the adrenal medulla, besides chromaffin cells, are also ganglion cells and sustentacular cells as fibroblasts and also Schwann cells [7, 8]**.** Chromaffin cells are responsible for catecholamine release (dopamine (DA), epinephrine (EP) and norepinephrine (NE) and also peptides including neuropeptide Y (NPY) [9, 10, 11]**.** This release of catecholamine and peptides occurs by a process of exocytosis from chromaffin granules described in section 1.1.4; *reviewed in* [2] **.**

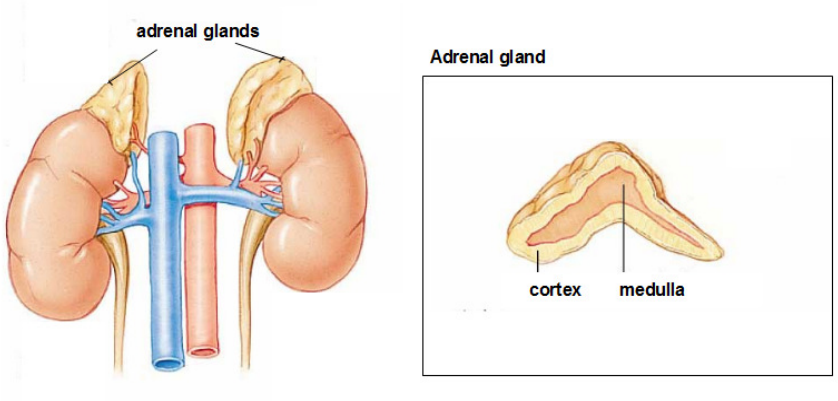


Figure 1.1 - Adrenal glands. The adrenal glands are located in the upper poles of each kidney and are composed by two distinct functional zones: medulla, in a central position, and cortex, in the periphery of the gland. Adapted from http://images.yourdictionary.com/adrenal-gland.

1.1.1 Chromaffin cells

The principal secretory cells in adrenal medulla are chromaffin cells. These cells are known as chromaffin cells because after exposure to oxidizing agents, such as chromate, the formation of colored polymers of catecholamines occurs [19]. Chromaffin cells are like postganglionic sympathetic neurons, derived from the neural crest and so, they also are excitable cells that generate action potentials [6]. Chromaffin cells are specialized on the synthesis and release of catecholamine. The medullary cells also produce other substances besides EP and NE. Some of those molecules are co-released from chromaffin granules with EP and NE; *reviewed in [5],* [6]. Some of these molecules are: leu-enkephalin and met-enkephalin [12, 13], NPY [13, 14], substance P [15], vasoactive intestinal peptide (VIP) [16, 17], inhibitors of endogenous proteases, chromogranins, secretogranins, glicoproteins and opioid peptides; *reviewed in [18].*

Splanchnic nerve activation, a cholinergic chemical synapse, stimulates chromafin cells to secrete EP or NE into the blood [20]; *reviewed in [21]***.** Acetylcholine binds to nicotinic and muscarinic receptors in chromaffin cells (*reviewed in [21]*), and the stimulation of nAChRs (nicotinic acetylcholine receptors) results in the entry of calcium (Ca^{2+}) and sodium (Na^{+}) from the extracellular medium through the ionic channel. This influx of ions causes a slight

membrane depolarization, which induces the opening of Na^+ channels sensitive to voltage inducing a large depolarization of the cytoplasmic membrane. This despolarization causes the opening of several types of VDCCs (voltage-dependent calcium channels), with the subsequent entry of Ca^{2+}, which leads to an increase of intracellular calcium that, subsequently promotes the translocation of chromaffin granules and consequent fusion with the plasma membrane for exocytosis. There is also mobilization of Ca^{2+} from intracellular reservoirs, favoring the process [22, 24]; *reviewed in* [21]. (Figure 1.2).

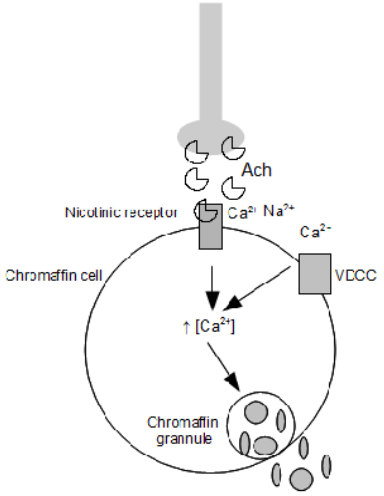


Figure 1.2 - Catecholamine release in chromaffin cells due to nicotinc receptor activation. Due to splanchnic nerve activation, acetylcholine binds to nicotinic receptors in chromaffin cells. That stimulation results in the entry of calcium (Ca^{2+}) and sodium (Na^+) causing a slight membrane depolarization which leads to the opening of VDCCs (voltage-dependent calcium channels). The increase of intracellular calcium promotes chromaffin granules exocytosis. *Based on [21, 22, 24]*

1.1.2 Catecholamine synthesis

The first and also rate-limiting step in the synthesis of catecholamines is the enzymatic conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), revealing the fundamental role of the amino acid tyrosine. DOPA is then converted by aromatic L-amino acid decarboxylase to dopamine. After these steps, dopamine enters the chromaffin granule, where it is converted to NE by dopamine beta-hydroxylase (DβH). NE leaves the granule to be converted into EP in the cytosol by phenylethanolamine-N-methyltransferase (PNMT) and then EP re-enters the granule for storage in the chromaffin granule *reviewed in* [25] (Figure 1.3).

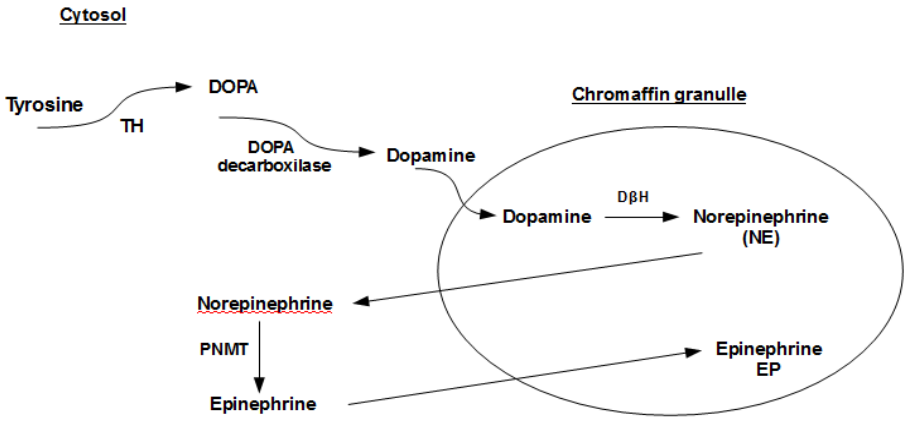


Figure 1.3 - Catecholamine's synthesis on chromaffin cell. After the enzymatic conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), DOPA is then converted to dopamine that enters the chromaffin granule. Inside the chromaffin granule dopamine suffers the action of beta-hydroxylase, and norepinephrine is formed. Norepinephrine leaves the granule to be converted into epinephrine by PNMT and then re-enters into the chromaffin granule. *Based on [25]*

1.1.3 Catecholamine metabolization

Catecholamine metabolization is described in a two-step reaction (Figure 1.4). The enzymes involved in the first step of this reaction are monoamine oxidase (MAO), which performs the oxidative deamination of catecholamines with deaminated aldehydes formation and catecholamine-O-methyltransferase (COMPT) that performs the addition of a metil group to catecholamine. There are two types of MAO enzymes, MAO-A and MAO-B [26] and have different functions and localizations [27, 28]. In adrenal gland, MAO-A has a higher enzymatic activity and it is localized in peripheral chromaffin cells in the medulla, while MAO-B is localized in chromaffin cells in a central position of medulla [29, 30]. DA is deaminated to DOPAL (3, 4-dihydroxyphenylacetaldehyde) and NE and EP both originate the same aldehyde intermediate: DOPEGAL (3, 4-dihydroxyphenylglycolaldehyde). This two deaminated aldehyde metabolits of cathecolamines are short-lived intermediates of the catecholamines metabolization process, and so, they are converted to more stable alcohol or acid metabolites in the second step. DOPAL is metabolized to DOPET (3, 4-dihydrophenylethanol) by aldehyde reductase (AR) and DOPAC (3, 4- dihydroxyphenylacetic acid) by aldehyde dehydrogenase (AD)

whereas DOPEGAL is metabolized to DHPG (3, 4- dihydroxyphenylglycol) by aldehyde reductase (AR) and DHMA (3, 4- dihydroxymandelic acid) by aldehyde dehydrogenase (AD) [30]. DA is preferentially metabolized to DOPAC and NE and EP to DHPG, the acid metabolites. This occurs because DA and DOPAL do not have a β-hydroxyl group which favors the oxidation by aldehyde dehydrogenase, and that β-hydroxyl group is present in NE, EP and DOPEGAL, favoring in this case the action of aldehyde or aldose reductase; *reviewed in [33]*.

Catecholamine metabolism and catecholamine synthesis occurs in the cytoplasm of the same cells because there is a passive leakage of catecholamine from the storage granules into cytoplasm in a highly dynamic state. The most considerable quantity (90 %) of catecholamine are sequestered by the vesicular monoamine transporter (VMT) back to storage vesicles while a small value is metabolized (10%), and so, under basal condition this process exceeds metabolization after exocytose. During the leaking of catecholamines from storage granules into the cytoplasm, they may suffer *O*-methylation by COMPT (catechol-*O*-methyltransferase), as referred below, which originates metanephrine (MN) and normetanephrine (NMN) from EP and NE, respectively. In humans, about 90 % of MN and 40 % of NMN results from the metabolism of catecholamine in chromaffin cells [31]. COMPT in these cells is mainly present in the membrane-bound form which is an isoform that has higher affinity to catecholamine, than the soluble form present in other tissues [32], *reviewed in [33]* . NMN and MN are deaminated by MAO into MOPEGAL (3-methoxy-4-hydroxyphenylglycolaldehyde). DHPG can also be converted by COMPT to MHPG (3-methoxy-4-hydroxyphenylglycol) and then to MOPEGAL by ADH (alcohol dehydrogenase). Because MOPEGAL is also a short-lived aldehyde intermediate, it can be converted to VMA (vanillylmandelic acid) in the liver that is the major end-product of norepinephrine and epinephrine metabolism in humans; *reviewed in [33]*.

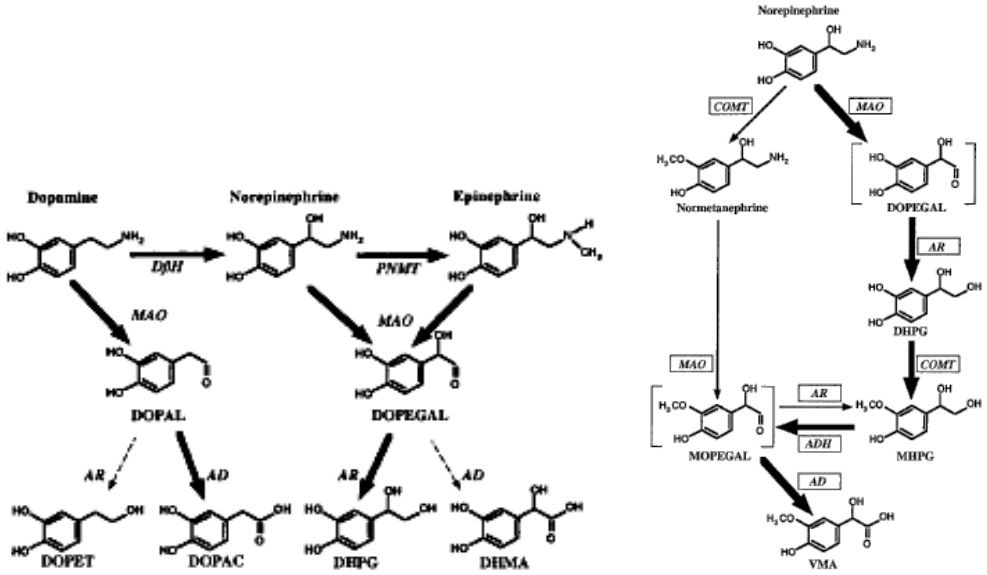


Figure 1.4 - Pathways of catecholamine metabolization. Oxidative deamination of catecholamines (DA, NE and EP) to their aldehyde intermediates (DOPAL and DOPEGAL). By the action of AR (aldose or aldehyde reductase) and AD (aldehyde dehydrogenase) a stabilization step occurs with the formation of alcohol or acid metabolites: DOPEC, DOPAC, and DHPG and DHMA. b) Metabolism of norepinephrine with VMA formation. *Adapted from* [33]

1.1.4 The release of Catecholamine by exocytosis

Chromaffin cells contain 10.000 - 20.000 chromaffin granules (large dense-core vesicles (LDCVs) [23]. After a stimulus catecholamines and peptides (as refereed in section 1.1.1) are released from chromaffin granules in a highly regulated process, so an excessive release of catecholamine does not occur [34, 35], *reviewed in* [36]. Chromaffin granules with NE have slight to moderate electron-dense content.

The conventional exocytosis process, also called complete fusion, occurs by the merging of chromaffin granule membrane with the plasma membrane and so, the liberation of contents of the vesicles occurs followed by collapse of the vesicle and integration of its membrane into the plasma membrane (complete membrane distention). This means a rapid granule content discharge, and is therefore a quick adaptive reaction (Figure 1.5) [38]

After a stimulus, there occur an intracellular Ca^{2+} rise and additionally a disorganization of the cytoskeleton of chromaffin cells, filamentous actin net, so the access of granules to exocytosis

sites is easier. When chromaffin granules are close to exocytosis sites occurs *docking* due to the formation of a complex called the soluble NSF attachment receptor (SNARE) [39]. SNARE proteins are central on the process of exocytosis and play a role in both docking and they are part of a docking complex. Synaptobrevin (v-SNARE, a vesicle-membrane associated SNARE protein), syntaxin, and SNAP-25 form a ternary complex, the SNARE complex that drives the fusion of plasma membrane and vesicle lipid bilayers allowing exocytosis of vesicle contents [39].

An alternative model to the conventional exocytosis process is "kiss and run" (Figure 1.5): in this process the release occurs through the formation of a reversible fusion pore, a 'channel-like' protein complex that opens and closes in a partial membrane distention that enables an adaptive mechanism to keep up with high-frequency [40]. During the secretion process, the vesicle maintains its integrity without collapsing. These two processes coexist in chromaffin cells [38, 40].

The last reported process is called piecemeal degranulation (PMD) (Figure 1.5). PMD differs from basic exocytosis because the granules never fuse whit each other and neither with the plasma membrane. Granules do not open to extracellular and their structures are intact [41]. This is a controlled mechanism of chatecholamine discharge by discret granule deposits and the transfer to the cell exterior of secretory material through an outward vesicle in a small quantity providing a subtle modulation of hormone release [37], *reviewed in* [42]. With PMD there is a slow and chronic release of molecules from granules. PMD was identified in both types of cells: adrenaline- and noradrenaline-producing cells. *reviewed in* [43].

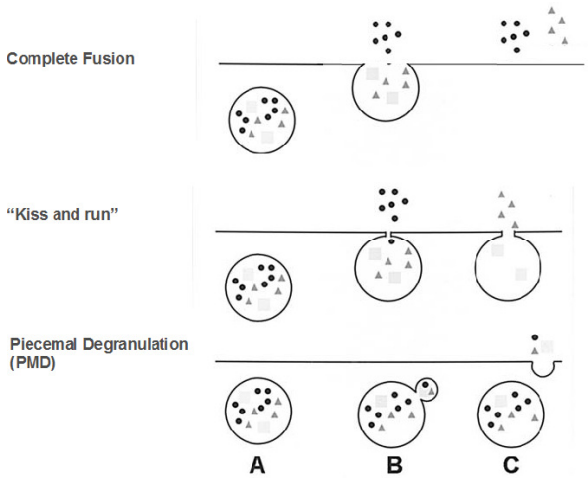


Figure 1.5 - Mechanisms of catecholamine release by exocytosis from chromaffin ganules. Schematic approach of the three processes of catecholamine release. Complete fusion, that occurs by the merging of chromaffin granule membrane with the plasma membrane and so, exocytosis of the contents of the vesicles; "Kiss and run", an alternative process where the liberation of vesicle contents occurs by formation of a reversible fusion pore that

opens and closes in a partial membrane distention. Piecemal degranulation, the last reported process, mechanism of chatecholamine discharge by discret granule deposits by small vesicles formed by chromaffin granule that keeps intact. *Adapted from [43]*

1.2 The adenosine receptors

Adenosine is an endogenous purine nucleoside continuously formed intracellular and extracellular and it is present in several tissues in mammalian organisms and has an important role in a variety of physiological processes; *reviewed* in [44]. Adenosine can act on cell surface receptors eliciting a large number of responses and so the activation of adenosine receptors (ARs) can occur in response to endogenous adenosine; *reviewed in* [45]. Adenosine produced intracellular is transported out of the cell by facilitated diffusion through a specific nucleoside transporter protein; *reviewed in* [44]. If adenosine extracellular levels are high, the adenosine is transported into cells and is degraded to inosine by adenosine deaminase. Moreover it can also be phosphorylated to AMP by adenosine kinase; *reviewed in* [44].

ARs belong to the superfamily of G protein-coupled receptors (GPCRs) and according to differences in molecular structure and pharmacological profile, four different subtypes of receptors are considered: A_1, A_{2A}, A_{2B}, and A_3 ; *reviewed in* [44]. Adenosine receptors are composed of seven transmembrane α-helices (7TM), located in a lipidic environment, with approximately 25 residues of length that are connected by intra- and extracellular loops (surrounded by an aqueous medium) ; *reviewed in* [44,46]. Adenosine receptors can also be differentiated according to their preferred mechanism of signal transduction: the subtypes A_1 and A_3 act through Gi proteins, and mediate the inhibition of adenylyl cyclase and the activity of several K^+ and Ca^{2+} channels On the other hand, the A_{2A} and A_{2B} subtypes act through the G_s proteins, causing the activation of adenylyl cyclase and thus stimulating the formation of cAMP; *reviewed in* [46].

1.2.1 Adenosine receptors subtypes

Many cell types express adenosine receptors, and the response to adenosine is individual so that several actions are associated to adenosine receptors. Different response patterns are obtained, dependent of receptor subtypes and effector molecules on the target cell. Besides some similarities between the subtypes, each of them seems to have some particularities and so different effects and functional significance in several organ tissues; *reviewed in* [47].

A_1 adenosine receptor (A_1AR) is present in several tissues and it is the most abundant adenosine receptor subtype in the brain, present in neurons and glial cells, both pre and post-synaptically. It is coupled to activation of K^+ channels and inhibition of Ca^{2+} channels both of which would inhibit neuronal activity. A_1AR is also the receptor with the highest affinity for the adenosine; *reviewed in* [48, 49]. A_1AR plays an important role in cardiovascular system and in central nervous system. The A_1AR activation has been reported to protect heart tissues from ischemia and so, selective antagonists have been used as antihypertensive. Selective antagonists of A_1AR are also been used as diuretics, in Alzheimer's disease and decrease of neuronal excitability; *reviewed in* [44, 46].

A_{2A} adenosine receptors (A_{2A}AR) are present in several tissues at high levels but only in a few regions of the brain; *reviewed in* [48]. A_{2A}AR agonists have a great potential in cardiovascular diseases treatment like in hypertension, ischemic cardiomyopathy and inflammation and also have been proposed for the treatment of neurodegenerative diseases; *reviewed in* [44, 46].

Adenosine receptors (A_{2B}AR) have been identified in almost every cell type but appear to be present at higher expression levels in various parts of the intestine and the bladder; *reviewed in* [47]. Some of the functions described linked to A2B receptors: regulation of mast cell secretion, gene expression, cell growth, intestinal functions, and vascular tone; *reviewed in* [44, 46].

A_3 adenosine (A_3AR) receptors are distributed in several peripheral organs and it is the receptor with the lowest affinity for adenosine; *reviewed in* [48]. This receptor subtype is the less known and clarified. For the A_3 receptor, it is known that the use of selective agonists may also have cardioprotective effects. A_3AR selective antagonists may be used for the treatment of asthma and inflammatory conditions; *reviewed in* [44, 46].

1.3 Adenosine receptors in adrenal gland

Adenosine acts on adenosine receptors, G-protein coupled receptors, and adenosine in basal conditions concentration, is sufficient to activate A_1, A_{2A} e A_3 receptors, if they are highly expressed. In the case of A_{2B} receptors, they need higher adenosine concentrations to be activated; *reviewed in* [50].

Besides cholinergic stimulation by splanchnic nerve, the increase of catecholamine release by stress from chromaffin cells can also be controlled by hormonal, paracrine and autocrine mechanisms, by neuropeptides and neuromodulators. The role of adenosine receptors subtypes - A_1, A_{2A}, A_{2B}, A_3 - in adrenal gland and their distribution is still not clear. Some studies tried to elucidate some of these aspects, but a lot remain unknown.

It was already described the presence of mRNA for A_1, A_{2A}, A_{2B} and A_3 receptors in rat adrenal cortical cells [51] and the presence of A_{2B} in bovine adrenal medulla [52]. Results from our laboratory (unpublished data) also showed the presence of mRNA of adenosine receptors subtypes – A_1, A_{2A} – in mouse adrenal gland.

Following the knowledge that adenosine acts on endocrine cells and immune system, modifying citokines release, interleukin-6 and TNF release was studied concerning the effect of this nucleoside. It was showed that in rat adrenal gland the effects of adenosine on the release of these citokines were mediated by A_{2A}, since an antagonist of A_{2A} receptor blocked the effects of adenosine. This study enabled A_{2A} identification in rat adrenal zone glomerulosa cortical cells [53].

In bovine adrenal cells, that express a noninactivating K^+ current (I_{AC}), experiments using adenosine and NECA, a nonselective adenosine receptor agonist demonstrated the inhibition of I_{AC} current by using agonists and antagonists of the adenosine receptors. The A_1 selective agonist CCPA, the A_{2A} selective agonist CGS 21680 and the A_3 selective agonist IBMECA inhibited I_{AC}; the specific A_1 antagonist, DPCPX, the specific A_{2A} antagonist, ZM 241385 and the A_3 specific antagonist MRS 1191 effectively blocked the inhibition of I_{AC} caused by receptors specific agonists [54]. These results also suggest the importance of adenosine receptors on K^+ current, and thereby could be putative modulators of molecules exocytosis.

However, the role of adenosine receptors on catecholamine release from adrenal chromaffin cells and which receptor is involved is still not known. Other important facts remain to be elucidated: the specific location and function of adenosine receptors subtypes in adrenal gland

and the signaling pathways of catecholamine release and synthesis associated to these receptors activity.

1.4 Aims of the present work

- Define the precise location of adenosine receptors in mouse adrenal gland.

- Optimization of a Perfusion System using mouse adrenal glands.

- Study the role of A_{2A} adenosine receptors on catecholamine release from mouse adrenal gland.

Chapter II – Materials and Methods

2.1 Immunohistochemistry

The mice adrenal glands collected, fixed with 4 % PFA, were embedded on OCT medium, frozen at – 80 º C and 7 µm and 10 µm slices were obtained using a cryostat. These sections were kept at - 80ºC for preservation.

For immunohistochemistry, the cryosections were dried at 37ºC, fixed again with 4% PFA for 45 minutes and incubated in 0.1 M glycine solution, for 30 minutes. 1 % Triton X-100 was used for cells permeabilization during 5 min. To prevent non-specific binding, slices were incubated in the blocking solution [3% (w/v) fatty acid-free bovine serum albumin (BSA) and 0.2 % Tween 20] for 1h at room temperature (RT). The slices were then incubated at 4ºC, with primary antibody, prepared in blocking solution: mouse anti-A_{2A} (1:100, Sigma-Aldrich). After incubation, slices were washed with PBS and incubated for 1h at RT with the secondary antibody also prepared in blocking solution: goat anti-mouse Alexa Fluor 647 (1:200) . The final step was the nuclei stain with Hoechst (1µg/ml in PBS) for 5 minutes. To avoid nonspecific links of secondary antibodies, negative controls without primary antibodies were performed.

After slides dry, using fluorescent mounting medium and nail polish, slides were prepared for visualization. Cells were visualized in a Zeiss PALM, coupled to an Axiocam HRc camera.

2.2 Perfusion System

As animal model it was used the adult C57BL/6 female mice (10-14 weeks). After anesthesia by halothane inhalation, the animals were sacrificed by decapitation, which was briefly followed by adrenal glands removal. Adrenal glands were immediately immersed in a cold low-calcium bicarbonate buffered saline (BBS) – 125 mM NaCl; 5mM KCl; 2mM $CaCl_2$; 1 $MgCl_2$; 26 $NaHCO_3$; 10 $C_6H_{12}O_6$; 1,25 NaH_2PO_4 (*Based on [20, 55, 57]*) - and continuously bubbled (O_2/CO_2) in holding chambers. After the extraction of surrounding fatty tissue, each adrenal gland was embedded in agar 2.5% in gelling point. Each agar block containing a gland, was cut approximated into a cube and glued to a stone block, possible to stow and immobilize in the slicing chamber of the vibratome. Slices of 250 µM were obtained, surrounded agar was removed, and the slices were kept in holding chambers containing the already described BBS solution at room temperature (RT). The slices of each gland were transferred to different chambers of the perfusion system. The perfusion system was continuously pumped with Krebs[1] solution; in a diffusion rate of 0.7 ml/min. Slices were perfused during 45 minutes with Krebs solution at 37 ° C to stabilize the slices in the new environment. Fractions were collected each minute. Several stimuli with adenosine agonists were applied in the same rate, during different periods of times. To test the effect of the A_{2A} receptor antagonist, this drug was applied 20 minutes period of pre-incubation. The fraction corresponding to each minute was collected to a different tube containing perchloric acid ($HClO_4$ 0.4 M) in order to avoid catecholamines degradation. The tubes were maintained in ice during the experience and preserved at – 80 º C.

[1] **Krebs:** 111 mM NaCl; 2.5 mM $CaCl_2$; 4.7 mM KCl; 1.2 mM MgSO4; 1.2 mM KH_2PO_4; 24.8 mM $NaHCO_3$; 11.1 mM glucose; 15 mM HEPES; pH 7.4

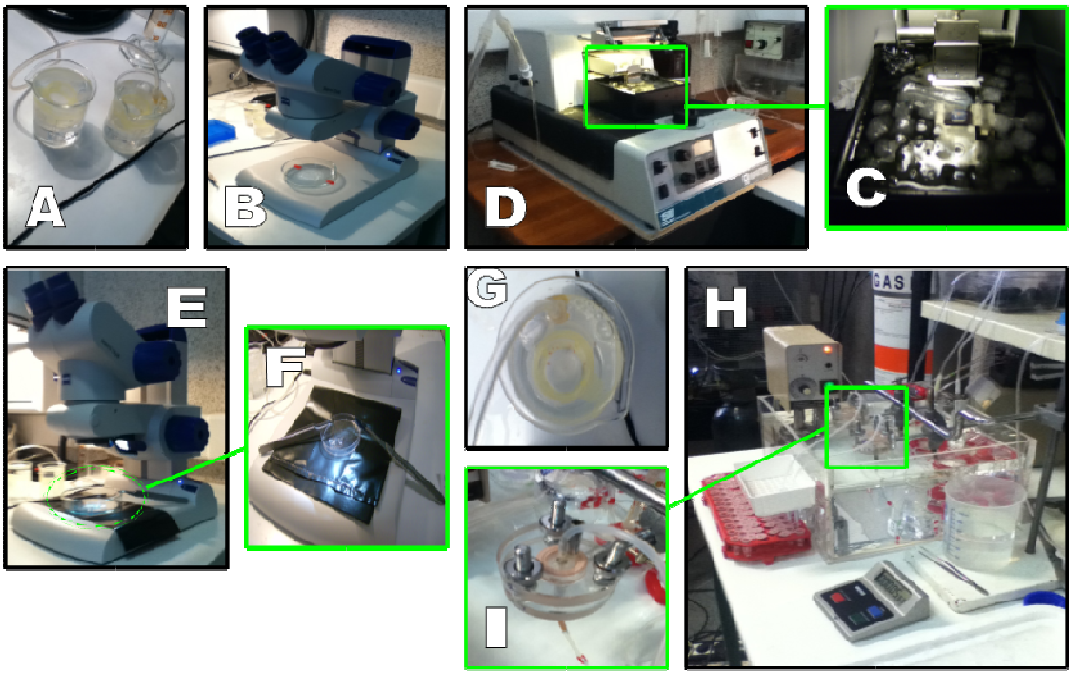


Figure 2.1 - First part of the protocol of Perfusion System and components. A- Adrenal glands after removal were kept in cold holding chambers; B- extraction of surrounding fatty tissue of adrenal glands; C- slicing chamber of the vibratome; D- vibratome; E and F- agar removal; G- holding chambers with slices at room temperature (RT), after vibratome cut; H- Perfusion System; I- Perfusion System chamber

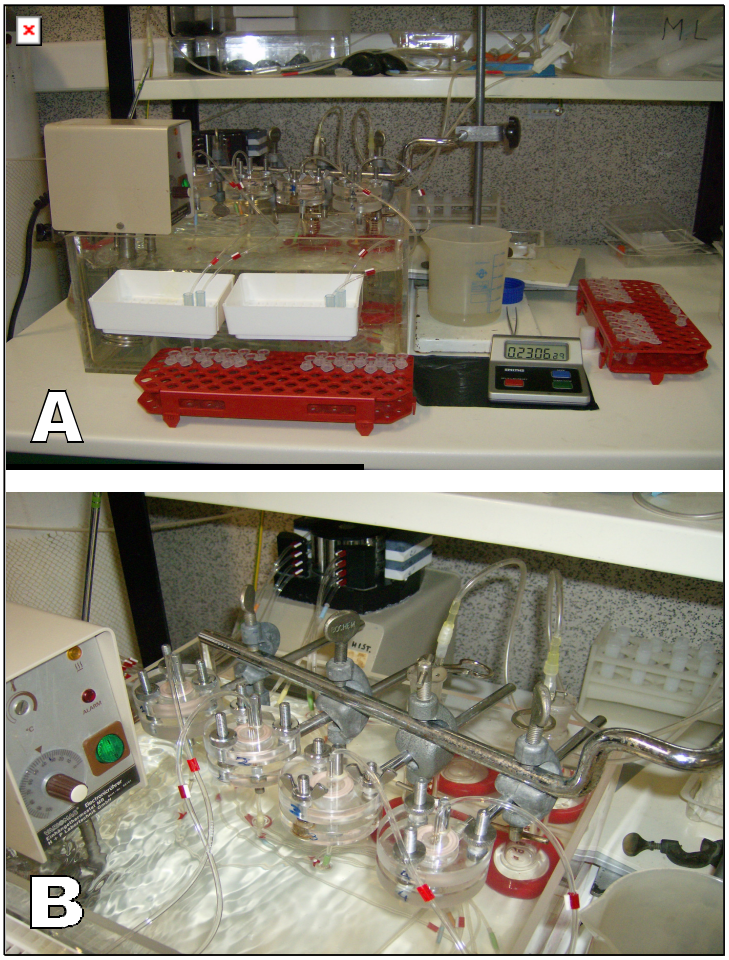


Figure 2.2 - Perfusion System during an experience. **A**- A full image of Perfusion System; **B**- A close view of perfusion chambers

2.3 Catecholamine assay by HPLC with electrochemical detection

The assay of catecholamines (NE and EP) was performed by electrochemical detection using HPLC. A portion of the total contents of each tube collected from the perfusion system, and also the intracellular (obtained by sonication) perfusion were injected directly into the HPLC.
The assay of EP and NE was carried out by HPLC "High performance liquid chromatography" with electrochemical detection. In this oxidation-reduction reaction, catecholamines are converted into quinones, by generating an electric current that is directly proportional to the amount of catecholamines present in the sample or standard. The energy required to initiate or increase this electrochemical reaction is given by the potential difference between the reference electrode and working electrode of the electrochemical detector. Around 120 µL of all the samples were injected directly into the HPLC.

Catecholamines eluted in the mobile phase[2] were separated by chromatography using an octadecylsilane column of 4 mM, 75 mm/ 4 mm (Merck Supersphere 100 RP18) and HPLC Gilson 234 connected to an electrochemical detector (ESA Coulochem II model 5200). This technique is based on a redox reaction that occurs on working electrode of electrochemical detector. In this system, the sample was initially subject to a potential of +300 mV in conditioning cell, after in the first electrode of the analytical cell to a potential +600 mV and finally, a second electrode of analytical cell at a potential of -600 mV. These potential differences have reduced catecholamines. The signal resulting from this reduction, after amplification, was broadcast to a computer with chromatography software and the analysis was performed (UniPoint Version 5.11). The identification and calibration of the peaks obtained in the chromatograms was made with known amounts of EP and NP with the limit of detection was 0.125 pmol.

[2] **Mobile Phase:** 50 mM sodium phosphate, 50 mM sodium acetate, 0.5 mM SDS, 0.4 mM EDTA, 12% acetonitrile, pH=3.2

Chapter III – Results

3.1 Optimization of a Perfusion System of mouse adrenal gland slices

The perfusion system of mouse adrenal glands is a protocol for catecholamine analysis that was established for the first time in our laboratory, which required experimental optimizations that are described in this section with some detail.

3.1.1 Optimization of adrenal slices

The development of perfusion system experience requires the optimization of two important details: the complete removal of surrounding adipose tissue and the determination of correct agar concentration, 2.5% (values between 2-3%). The removal of surrounding adipose tissue was an important step to take in account before the immersion in agar because when the adipose tissue was not completely removed, the remaining fatty tissue around the gland induced the detachment of adrenal gland from the agar (data not shown) and was not possible to obtain the adrenal slices in the vibratome.

The optimization of the agar concentration was another important point to obtain adrenal slices. 2.5 % of agar was the optimal concentration to obtain the adrenal slices.

The combination of these two important key points, the adipose tissue removal and the agar concentration, made possible to obtain adrenal gland in a conserved way so that experimental protocol could be correctly continued.

Another relevant aspect in the optimization of the Perfusion System was the thickness of mouse adrenal gland slices. Several experiments were performed with slices of different thicknesses: 100, 250 and 500 µm (Table 1). Adrenal gland slices of 100 µm were fragile and some of them shown an apparent loss of adrenal gland integrity. It seemed that especially adrenal medulla had lost consistency and appeared to get fragmented. And the experiments of perfusion with these slices we were able to detect a basal catecholamine release by HPLC with electrochemical detection. However, when slices were perfused with the addition of a solution containing a stimulating compound, it was not possible to see any catecholamine release from adrenal slices.

The 500 µm slices kept their morphology integrity, but analysis by HPLC-ED revealed the same profile of catecholamine release of 100 µm slices: basal catecholamine release was possible to

detect, but stimulated catecholamine release, using some compound that is known to increase catecholamine release, it was not possible to observe any catecholamine release.

Mouse adrenal slices with 250 µm of thickness had a conserved appearance of cortex and medulla and so the integrity was maintained. In this case was even possible to obtain a considerable number of slices, this number depending on adrenal gland position on agar. HPLC-ED analysis revealed that only in this case and was possible to detect basal and stimulated catecholamine release. All these results are summarized on Table 1. According to these results, all subsequent experiments were performed with 250 µm adrenal slices.

Table 1 - Appearance of mouse adrenal gland slices and profile of catecholamine release: basal or stimulated (this two parameters by HPLC-ED analysis), according to different thicknesses, 100, 250 and 500 µm.

Slices thickness (µm)	Slices appearance	Basal catecholamine release	Stimulated catecholamine release
100	Lost of integrity	Detectable	No response
250	Integrity maintenance	Detectable	Detectable
500 or more	Integrity maintenance	Detectable	No response

3.1.2 Optimization of the Perfusion System protocol

For the optimization of catecholamine release stimulus of slices from mouse adrenal gland, several perfusion system experiences were performed to define the protocol model: the best stimulus (positive control), duration of stimulus and best conditions for basal catecholamine release. All experiences were then performed obeying to the same profile, illustrated in Figure 3.1.

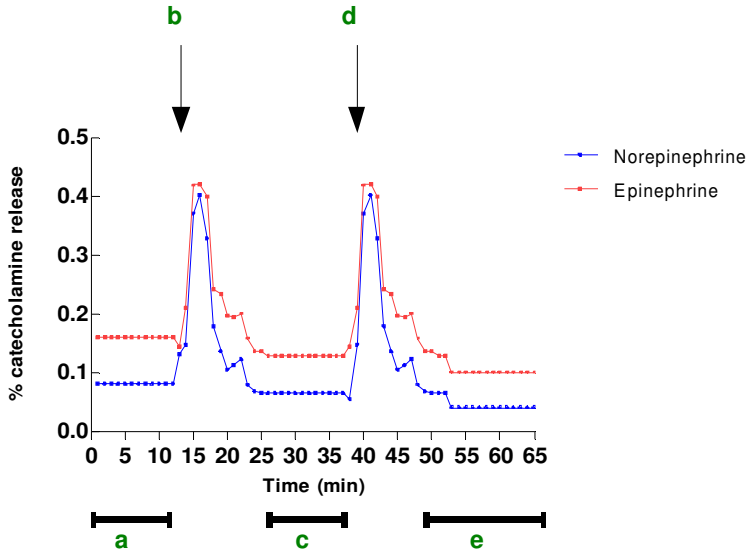


Figure 3.1 - Profile of catecholamine release after two stimuli. Percentage of catecholamine release (norepinephrine and epinephrine) induced by two different stimuli over time. a, c and e corresponds to basal fractions, obtained by perfusion of adrenal slices with a Krebs solution; b and d correspond to two different time stimuli application.

In the Perfusion System, mouse adrenal gland slices were placed in perfusion chambers and cells were maintained with the Krebs solution for 45 minutes to obtain stability recovery; all experiments were performed according to the same experimental protocol, as represented in the image (Figure 3.1). Slices were being perfused with Krebs solution during a period of time between 5 and 10 minutes (a). After this time, the first stimulus was applied to the slices, during 5, 10 or 30min, according to the experience that was being analyzed (b). Next, chambers were perfused with Krebs solution again, so that the slices recover from the first stimulus (c), and after a variable period of time, another stimulus was applied (d). This second stimulus could be the same or different stimulus compared to the first. When the application of the second stimulus finished, mouse adrenal gland slices were perfused again with Krebs solution in order to decrease catecholamine release to basal catecholamine levels (e). The values of area under curve (AUC) where then calculated, for both NE and EP curves.

3.1.2.1 Catecholamine release induced by KCl

The potassium chloride is a well know compound that induce catecholamine release from chromaffin cells [60]. So, the first experiment for the optimization of the stimulus that induce catecholamine release from mouse adrenal gland slices catecholamine release was the KCl, 50mM, during 10 minutes. KCl caused a large increase on catecholamine release (Figure 2), however, after this huge catecholamine release, catecholamine levels did not return to basal values (Figure 3.2).

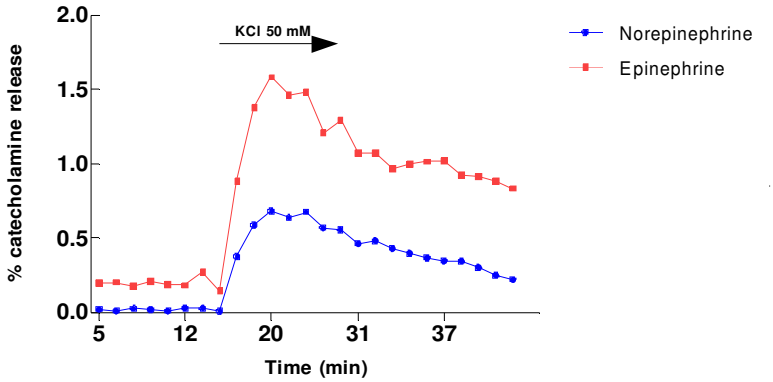


Figure 3.2 - KCl (50 mM) increases catecholamine release (norepinephrine and epinephrine). Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to total catecholamine slice content. Catecholamine release was quantified using HPLC-ED, as described in section 2.

3.1.2.2 Catecholamine release induced by Interleukin-1ß

Interleukin-1 ß (10ng/mL)is known to increase catecholamines release from human and mouse chromaffin cells in culture [61, 62].The perfusion with IL-1 ß for 5 or 10 minutes did not change catecholamine release from mouse adrenal slices (Figure 3.3).

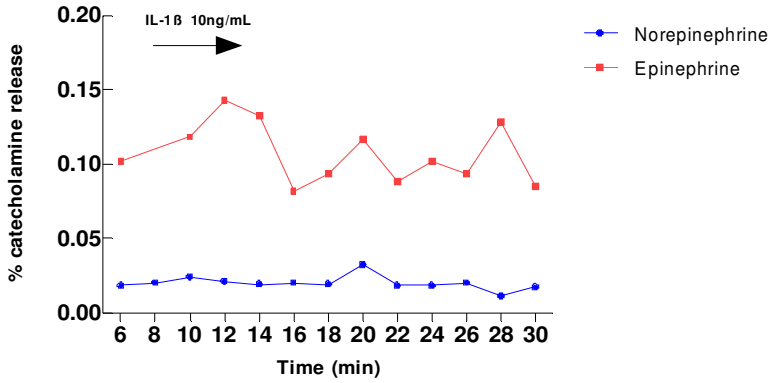


Figure 3.3 - Catecholamine release (norepinephrine and epinephrine) induced by IL-1 ß . Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to total catecholamine slice content. Catecholamine release was quantified using HPLC-ED, as described in section 2.

3.1.2.3 Catecholamine release induced by Nicotine

Using the same protocol, basal catecholamine levels were collected, and nicotine stimulus was applied in the concentration of 1 mM during 10 minutes. Nicotine (1mM) increased catecholamine release, compared to basal release. After 12 minutes (approximately), catecholamine release returned to the basal values (Figure 3.4) [59].

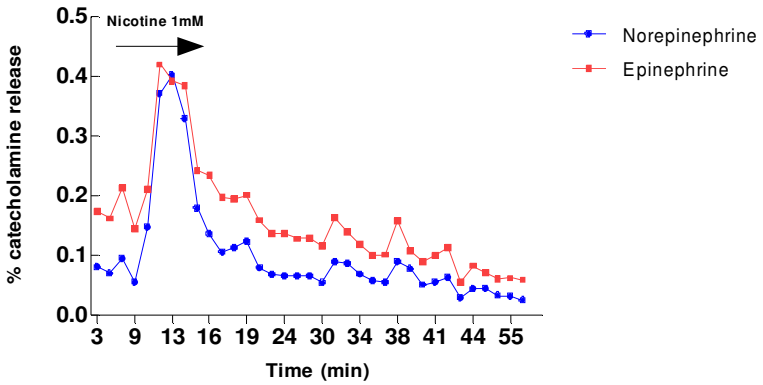


Figure 3.4 - Nicotine 1 mM increases catecholamine release (norepinephrine and epinephrine). Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2. (n=3-6)

Lower concentrations of nicotine were also tested: 100 µM, 250 µM and 500 µM.

Nicotine 100 µM did not induced an increase on catecholamine ne release (Figure 3.5).

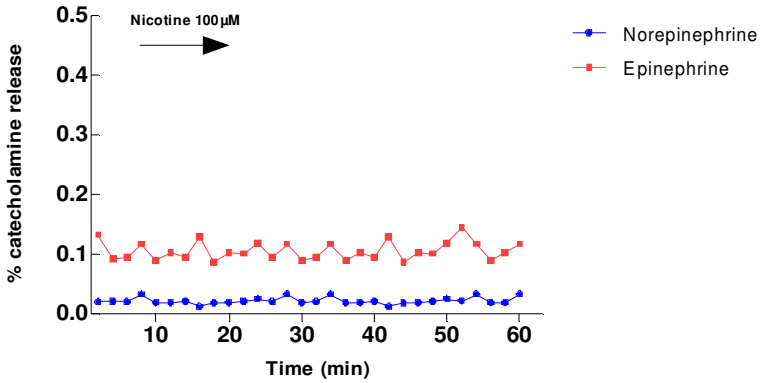
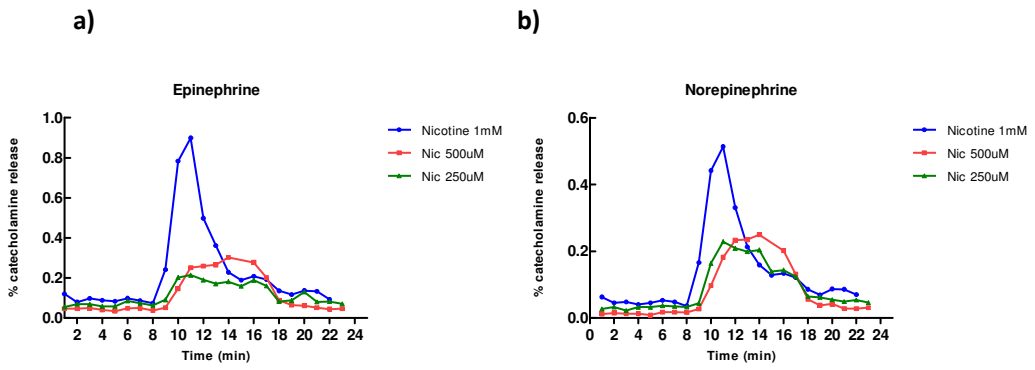


Figure 3.5 - Catecholamine release (norepinephrine and epinephrine) induced by Nicotine 100 µM. Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2.

After these experiments, was possible to define the interval of nicotine concentration where that is a visible response and an effective increase in catecholamine release: 250 µM – 1mM (Figure 3.6). Several experiments were performed with different concentrations of nicotine, 250 µM, 500 µM and 1mM, and it was possible to compare the different profiles of catecholamine release using higher or smaller concentrations. Both in EP and NE, nicotine 1mM caused a higher response but with a faster decrease when compared with nicotine 500µM and 250µM, that have a larger response, but not so high (Figure 3.6 a) and b)). AUC of all the experiences with different concentrations of nicotine where determined, and made possible to observe that nicotine 1mM causes an higher catecholamine release, while nicotine 500 µM and 250 µM have smaller and similar values.



c)

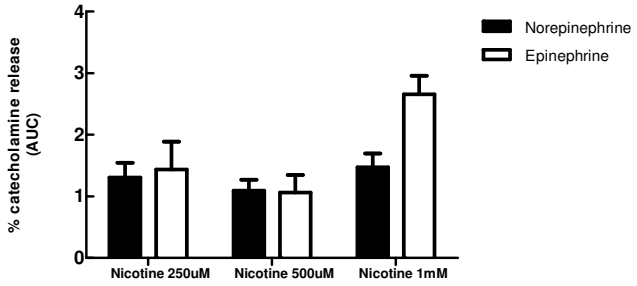


Figure 3.6 - Catecholamine release, epinephrine (a) and norepinephrine (b), induced by Nicotine 250 µM, 500 µM and 1mM. Catecholamine release (EP an NE) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2. (c)Catecholamine release (EP and NE) induced by Nicotine 250 µM, 500 µM and 1mM calculated by AUC, as described above in this section in figure 3.1. (n=3-6)

3.1.2.4 The effect of two stimuli in catecholamine release

Another important aspect in the optimization of catecholamine release stimulus, was to observe if a response occurs when the application of two stimuli, in different times. In previous experiments, using Nicotine 1mM, it was possible to conclude that after the first response of chromaffin cells, enhancing cathecolamine release after the first stimulus, it was impossible to obtain a second response using an equal second stimulus. Some experiments using A_{2A} receptor agonist CGS 21680 (300nM) did not increase the catecholamine release. However, when the firs stimulus applied was CGS 21680, known to cause no effect that was response to the second stimulus, in this case Nicotine 1mM.

According to these results it was only possible to obtain only one response to one effective stimulus, and that response (catecholamine release) is independent of the time tested (maximum 90 minutes).

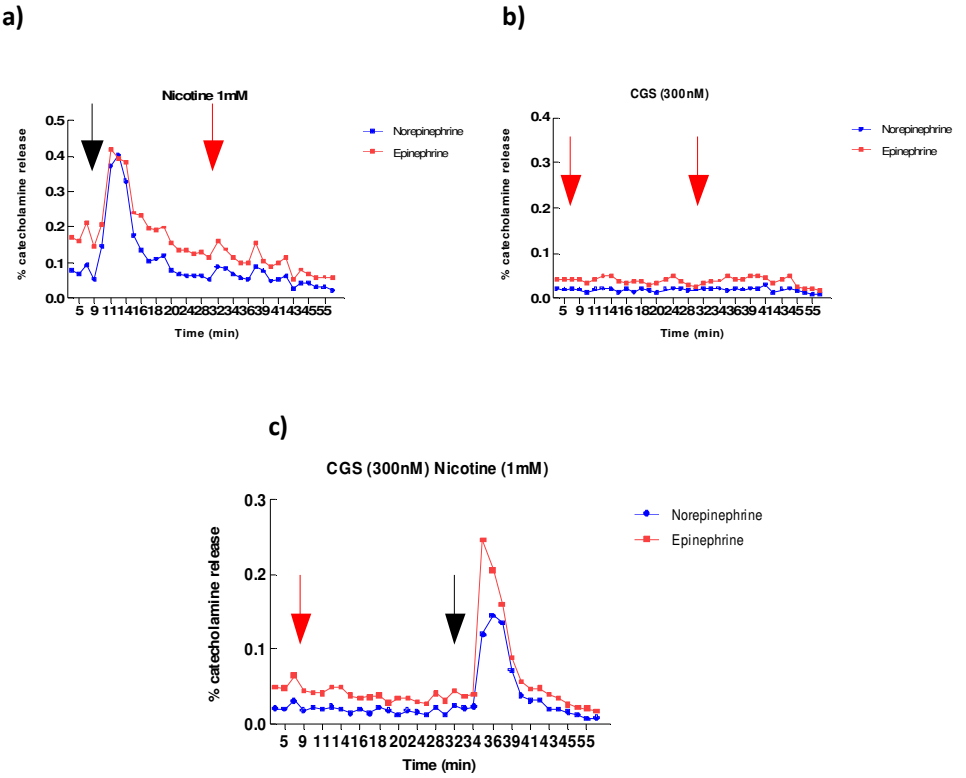


Figure 3.7 - Percentage of catecholamine release (norepinephrine and epinephrine) over time induced by two Nicotine 1mM stimuli (a), two CGS 300 nM stimuli (b) and a stimulus of CGS followed by one of Nicotine 1mM (c). Catecholamine release (epinephrine and norepinephrine) induced by two Nicotine 1mM stimuli (a), two CGS 300 nM stimuli (b) and a stimulus of CGS followed by one of Nicotine 1mM (c). Catecholamine release (NA and AD) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2.

3.2 Immunohistochemistry of mouse adrenal glands

By immunohistochemistry it was possible to identify the precise location of adenosine receptors in the medulla of mouse adrenal gland.

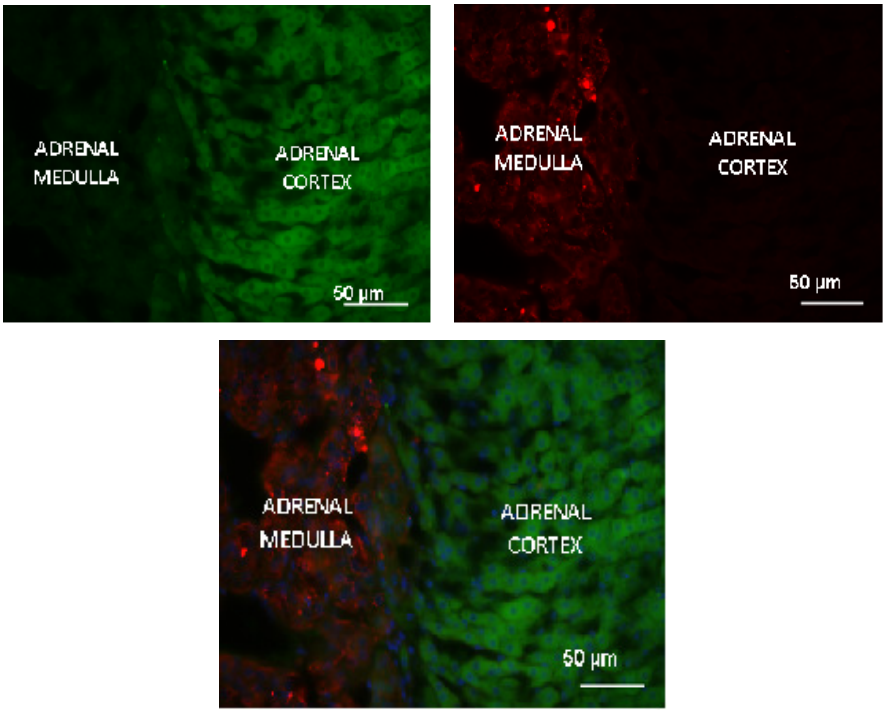


Figure 3.8 - Adenosine receptors A_{2A} are located in adrenal gland medulla. Representative images of A_{2A} adenosine receptors (red) in mouse adrenal gland. Cortex cells have autofluoresence (green), which leads to an easy vizualization of adrenal cortex and medulla. Nuclei were labeled with Hoechst.

3.3 Role of adenosine receptors on catecholamine release from mouse adrenal gland

3.3.1 Effect of A_{2A} receptor agonist and antagonist on catecholamine release induced by Nicotine 500 µM

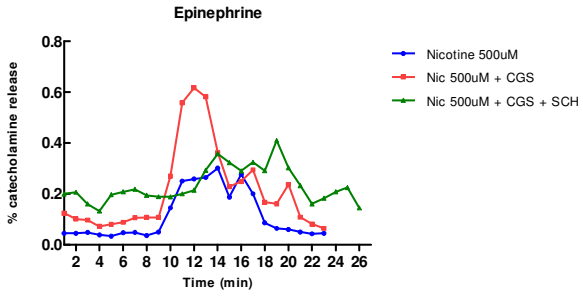
To test the effect of A_{2A} adenosine receptor in catecholamine release from mouse adrenal gland, the perfusion protocol was developed using A_{2A} receptor agonist, CGS 21680 (300nM) and A_{2A} antagonist, SCH 58261 (500 nM). Their effects were studied on basal catecholamine release and on catecholamine release induced by nicotine.

. CGS and SCH alone did not change basal catecholamine release compared to basal values (data not shown).

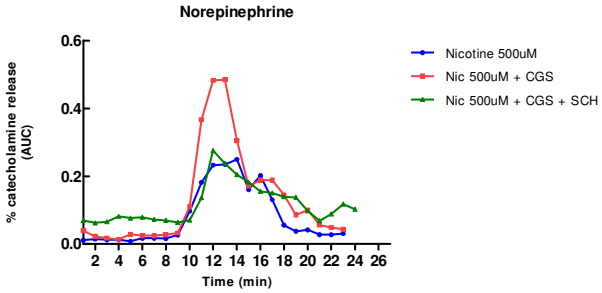
It was possible to observe that CGS 21680 (300 nM) increases catecholamine release induced by Nicotine 500 µM and with SCH 58261 (500nM) it was possible to observe a decrease of catecholamine release, when compared with nicotine 500 µM (Figure 3.9).

These effects, analyzed by several experiments, are also studied/compared in total percentage of catecholamine release and in the amplitude of the stimulus, calculated by the difference between the peak and the baseline (Figure 3.9 c and d).

a)



b)



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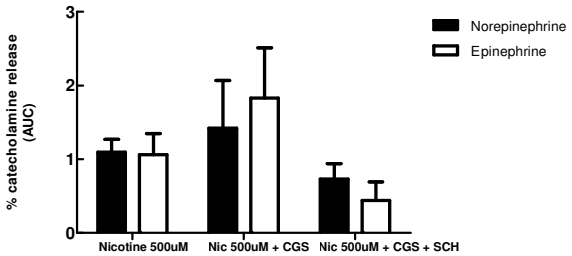


d)

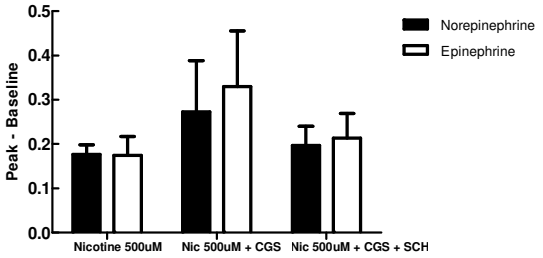


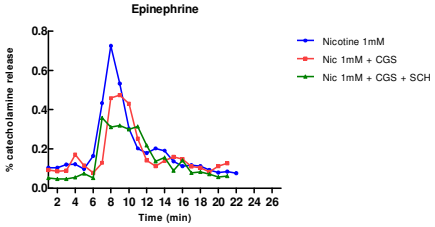
Figure 3.9 - Catecholamine release induced by A_{2A} activation in the presence of Nicotine. Catecholamine release induced by Nicotine 500 µM, Nicotine 500 µM + CGS 300nM, Nicotine 500µM + CGS 300nM + SCH 500nM of epinephrine and norepinephrine (a ,b). Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2; Calculated by area under curve (AUC), as described above in this section (c); Difference between peak of catecholamine release and baseline (d) (n = 3 – 6).

3.3.2 Effect of CGS and SCH on catecholamine release induced by Nicotine 1 mM

The perfusion protocol was also developed to study the effects of A_{2A} agonist, CGS 21680 (300nM) and A_{2A} antagonist, SCH 58261 (500 nM) on catecholamine release from mouse adrenal gland, mediated by nicotine 1 mM.

Using nicotine 1mM, the effect of CGS 21680, enhancing catecholamine release was not visible in EP and NE levels, however SCH 58261 (500 nM) seems to decrease catecholamine release values, compared with nicotine 1mM and nicotine 1mM and CGS.

a)



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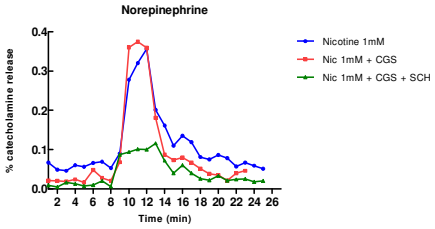

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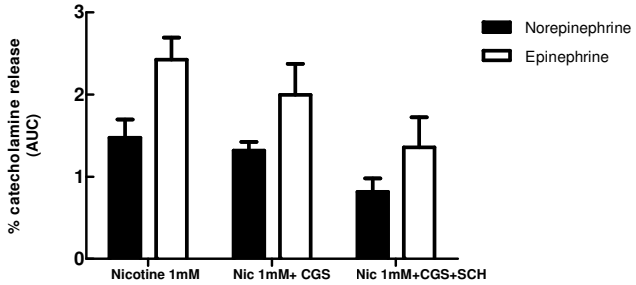

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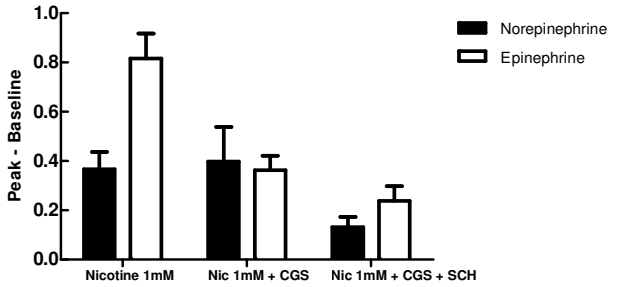

Figure 3.10 - Catecholamine release induced by A_{2A} activation in the presence of Nicotine. Catecholamine release induced by Nicotine 1mM, Nicotine 1mM + CGS 300nM, Nicotine 1mM + CGS 300nM + SCH 500nM of epinephrine and norepinephrine (a ,b). Catecholamine release (EP and NE) is expressed as the percentage of catecholamine release compared to intracellular content. Catecholamine release was quantified using HPLC-ED, as described in section 2; Calculated by area under curve (AUC), as described above in this section (c); Difference between peak of catecholamine release and baseline (d) (n = 3 – 6).

Chapter IV – Discussion

Optimization of a Perfusion System using mouse adrenal glands

The Perfusion System used in the present work was optimized in order to perform catecholamine release experiments from mouse adrenal gland. The two first general conditions optimized were the removal of adipose tissue and agar concentration. These are two significant details of the protocol that were determinant to obtain adrenal slices with functional chromaffin cells.

Mouse adrenal glands when collected are surrounded by adipose tissue that needs to be carefully removed; otherwise when adrenal glands are placed in the cut chamber of vibratome, loss of gland slices integrity occurs.

To use the vibratome, adrenal gland needs to be immobilized in order that the blade cuts slices with the desired thickness. For that purpose, agar in gelling point was used to immerse the gland that after solidification was able to sustain the gland and obtain intact slices. Some concentrations were tested based in what was described by others [20, 55, 57] and the agar concentration defined and used in this protocol was 2.5 %. In fact, after agar solidification a fairly dense cube was obtained and when placed in cut chamber was able to sustain the gland to obtain slices. With other concentrations tested (below 2.5 %), the agar was not able to sustain correctly the gland.

The other condition optimized was the adrenal slices thickness. The ideal adrenal slices thickness should guaranty that most cells of the tissue are functional and enable the drugs solutions to reach almost all cells. As can be seen in Table 1 of Chapter III, only the slices with 250 µm of thickness may have enough functional chromaffin cells that were able to release detectable basal catecholamines and respond to stimuli. Slices of 100 µm of thickness may have too many deteriorated cells, induced by blade cut, that do not allow that they respond to stimuli. The slices with 500 µm, although maintained apparent slice integrity did not respond to stimuli, maybe because sometimes these thickness slices correspond to cutting the gland into two parts which may difficult the access of stimuli to inner chromaffin cells. All these protocol adaptations were based in what was described [20, 55, 56, 58] in studies with adrenal glands slices for different purposes.

To design the appropriate protocol of Perfusion System, a template for the optimization of catecholamine release stimuli was tested. The experiences were performed using several durations and different stimuli in order to define the protocol parameters. As can be seen in Figure 3.1 of Chapter III a template for the analysis of catecholamine release during time was

optimized, according to stimulus application and slices recovery, after several optimization experiences.

KCl induces depolarization that will induce high levels of catecholamine release from chromaffin cells [60]. Using mouse adrenal slices in perfusion system, KCl (50 mM) induced a high increase on catecholamine release that did not return to basal levels, at least during the time analyzed, as it was expected.

In our laboratory, we showed that IL-1 β (10 ng/ml, 10 min) increased catecholamine release from isolated human and mice chromaffin cells in culture [61]. These results indicated that IL-1 β was also a suitable to be used in the Perfusion System to evaluate catecholamine release from mouse adrenal slices. However, in the present work the IL-1 β (10ng/mL) did not stimulate catecholamine release from mouse adrenal slices. More studies are needed to understand this lack of stimulatory effect of IL-1β.

Several concentrations of nicotine were used to stimulate catecholamine release from mouse adrenal slices, since nicotine is known for stimulate this process: 1 mM, 500 µM, 250 µM and 100 µM. Nicotine 100 µM increases catecholamine release from isolated human and mice chromaffin cells [62, 63]. However, nicotine 100 µM did not stimulate catecholamine release from adrenal slices in the perfusion system. A possible explanation for the lack of effect of this concentration could be the access of nicotine to chromaffin cells. Using higher concentration of nicotine (1 mM, 500 µM, 250 µM) it was possible to observe a significant increase on catecholamine release from mouse adrenal gland slices, when compared with basal levels. Nicotine 1 mM caused a higher stimulation of catecholamine release, as expected. A possible explanation for these results is the access of nicotine to all cells of the adrenal slices. Probably only higher concentrations of this drug are able to stimulate all the nicotinic receptors.

Another important aspect that was analyzed, was the possibility of perform the application of two stimuli (different or not) to the adrenal slices placed in the same perfusion chamber. That would be useful to use one the first stimulus as control in the same group of slices. However, as showed in the results section, after a first effective stimulus (nicotine 1 mM) on chromaffin cells a second stimulus did not increase catecholamine release. When the first drug application did not increase catecholamine release (CGS 21680 in this case), a second effective stimulus (nicotine 1mM) increased catecholamine release. In the perfusion system used, it was possible to use only one stimulus, independently of the time tested between the two drugs application (90 minutes). After an effective stimulus, it is possible that chromaffin cells had release a significant amount of catecholamine, and so, chromaffin cells will not be able to respond to a

second effective stimulus. Another hypothesis, is that may occur a desensitization of the receptors, in this case, nicotinic receptors and that explain the detection of only the basal levels of catecholamine release [64].

Based in the results achieved, it is possible to enunciate some advantages and disadvantages of this Perfusion System to study the release of catecholamine from adrenal gland slices.

Using the protocol of Perfusion System with adrenal slices, contrary to cell cultures, a considerable less quantity of animals are used. In this case a single animal can be used at least for two experimental different conditions. Another very important aspect is that, with slices, the adrenal gland structure is maintained, the interactions between cortex and medulla still almost intact and so, this protocol came much closer to the physiological conditions.

However, like was already said, the protocol still needs several optimizations. There still are some disadvantages of the Perfusion System: the concentrations of the several compounds used were very high, which lead to specificity questions, namely of nicotine and A_{2A} agonist and antagonist. Because it is impossible to apply two stimuli in the same perfusion chamber with the slices, that makes impossible to have a correct positive control and that leads also to a great variability between experiences.

Role of adenosine receptor A_{2A} in catecholamine release from mouse adrenal glands

It was already known from results of our laboratory (unpublished data) and by others that mRNA for adenosine receptors subtypes – A_1, A_{2A} – was present in mouse adrenal gland, besides some studies of other authors. (See Chapter I- Section 1.3)

By immunohistochemistry of adrenal gland slices obtained by cryostat, it was possible to identify the exact location of A_{2A} adenosine receptors. Since cortical cells have intrinsic autofluorescence (green), it was possible to define the location of mouse adrenal cortex and medulla. It was shown that A_{2A}ARs are located only in the adrenal medulla and not in the adrenal cortex. Next experiments were performed analyzing A_{2A}ARs, since they are located in adrenal medulla, where chromaffin cells are.

The role of adenosine receptors in catecholamine release is still not known. In this work, using the established Perfusion System, we studied the effect of agonist and antagonist of the A_{2A} receptor – CGS 21680 and SCH 58261, respectively – on catecholamine release from mouse adrenal slices.
It was possible to observe that the A_{2A} receptor agonist (CGS 21680) had no effect on catecholamine release from slice adrenal chromaffin cell. These results could indicate that A_{2A} activation had no role on catecholamine release. However, when adrenal glands slices were incubated with nicotine 500 µM in the presence of CGS 21680 300 nM, it was possible to observe an increase of catecholamine compared to nicotine alone. Moreover, the A_{2A} receptor antagonist (SCH 58261) decreases catecholamine release induced by nicotine. These results indicate that A_{2A} receptors may modulate catecholamine release induced by nicotine.

When adrenal slices were incubated with nicotine 1mM, the A_{2A} receptor agonist did not increase catecholamine release, unlike the effect mediated by nicotine 500 µM. When is applied additionally the A_{2A} receptor antagonist, a lower catecholamine release was observed, compared to nicotine was observed. The analysis of the difference between the peak of catecholamine release and the baseline values showed a decrease of catecholamine release with the agonist and the antagonist of A_{2A}.

The absence of an increase of catecholamine release in the presence of nicotine 1 mM might indicate that cells have released a high amount of catecholamine that, when an extra stimulus is added it is not possible to observe any change on catecholamine release. However, the fact that the selective antagonist inhibits this stimulatory effect indicates the specificity of A_{2A} activation on modulation of catecholamine release.

Another hypothesis is the receptor type and morphology: nicotinic receptors are ionotropic receptors, directly linked with ion channels, and so, their activation in adrenal chromaffin cells may be quick and causing a fast membrane depolarization and an observable response.

It is possible to achieve a main conclusion of this part of the study: A_{2A}AR appears to have a stimulatory effect on catecholamine release induced by nicotine from mouse adrenal gland, in concordance with other results from our lab (unpublished data) with chromaffin cells primary cultures.

Chapter V – Main Conclusions

The elaboration of this project allows defining some main conclusions:

- Perfusion system protocol and several conditions were optimized leading to a new and more physiological approach in studies with mouse adrenal gland.
- Based on the previous knowledge of A_{2A}AR presence in mouse adrenal gland, this receptor was identified in mouse adrenal gland medulla (chromaffin cells) by immunohistochemistry.

  A_{2A}AR appears to have a modulator role on catecholamine release induced by nicotine.

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