

**ENDOGENOUS PURINES AS POTENTIAL
PHARMACOLOGICAL TARGETS TO CONTROL
MYENTERIC NEUROTRANSMISSION**

MARGARIDA DUARTE CERQUEIRA MARTINS DE ARAÚJO

Tese de doutoramento em Ciências Biomédicas

2011

MARGARIDA DUARTE CERQUEIRA MARTINS DE ARAÚJO

**ENDOGENOUS PURINES AS POTENTIAL PHARMACOLOGICAL
TARGETS TO CONTROL MYENTERIC NEUROTRANSMISSION**

Tese de Candidatura ao grau de Doutor em
Ciências Biomédicas submetida ao Instituto
de Ciências Biomédicas de Abel Salazar da
Universidade do Porto

Orientador – Professor Doutor Paulo Jorge
da Silva Correia de Sá

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas
de Abel Salazar da Universidade do Porto

MARGARIDA DUARTE CERQUEIRA MARTINS DE ARAÚJO

**ENDOGENOUS PURINES AS POTENTIAL PHARMACOLOGICAL
TARGETS TO CONTROL MYENTERIC NEUROTRANSMISSION**

Dissertation for applying to a Doctor degree
in Biomedical Sciences submitted to the
Instituto de Ciências Biomédicas de Abel
Salazar da Universidade do Porto

Supervisor – Professor Doutor Paulo Jorge
da Silva Correia de Sá

Category – Full Professor

Affiliation – Instituto de Ciências Biomédicas
de Abel Salazar da Universidade do Porto



This work was supported by a FCT Doctoral degree grant, SFRH/BD/29044/2006, POPH - QREN/FSE funding.



Trabalho realizado no Laboratório de Farmacologia e Neurobiologia – Unidade Multidisciplinar de Investigação Biomédica (UMIB, Instituto de Ciências Biomédicas de Abel Salazar - Universidade do Porto (ICBAS-UP), sob a orientação científica do Professor Doutor Paulo Jorge da Silva Correia de Sá

Experimental work conducted at the Farmacologia e Neurobiologia Laboratory – Unit for Multidisciplinary Investigation in Biomedicine (UMIB), Instituto de Ciências Biomédicas de Abel Salazar - Universidade do Porto (ICBAS-UP), under the scientific supervision of Professor Doutor Paulo Jorge da Silva Correia de Sá

As opiniões expressas nesta publicação são da exclusiva responsabilidade do seu autor

The opinions expressed in this publication are of the exclusive responsibility of its author

À família onde nasci, à família que criei...

TABLE OF CONTENTS

Abbreviation list	1
Abstract	3
Resumo	5
1) Introduction	9
1.1 The Enteric nervous system	9
1.2 Cholinergic transmission in the ENS	17
1.2.1 Cholinergic neurotransmission	17
1.2.2 Nicotinic receptors in the ENS	19
1.2.3 Muscarinic receptors in the ENS	21
1.3 Purinergic signalling in the ENS: role of ATP	26
1.3.1 Purinergic neurotransmission	26
1.3.2 ATP releasing pathways	28
1.3.3 P2 Purinergic Receptors	30
1.3.4 ATP degrading enzymes	34
1.3.5 Purinergic neurotransmission in the gut	37
1.4 Purinergic signalling in the ENS: role of Adenosine	41
1.4.1 Adenosine as a neuromodulator	41
1.4.2 Adenosine generation and transport	43
1.4.3 P1 Adenosine receptors	44
1.4.4 Mechanisms to decrease extracellular adenosine levels	47
1.4.5 Adenosine neuromodulation in the gut	48
2) Aims	51

3)	Materials and Methods	53
3.1	Biological sample preparation	53
3.2	Experimental Procedures	55
3.2.1	[³ H]-Acetylcholine release experiments	55
3.2.2	Enzymatic kinetic experiments and high-performance liquid chromatography (HPLC) analysis	57
3.2.3	Release of adenine nucleosides and adenosine deaminase	59
3.2.4	Adenosine deaminase assay	61
3.2.5	Contraction recordings	61
3.3	Statistics	62
3.4	Drugs	63
4)	Results	65
4.1	Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on ACh release from myenteric motoneurons;	67
4.1.1	Rationale	67
4.1.2	Pattern of extracellular catabolism of adenine nucleotides and adenosine formation in the LM-MP of the rat ileum	68
4.1.3	Relative contribution of ecto-ATPase (forming ADP) and ecto-ATPDase (bypassing ADP formation) pathways for ATP catabolism in the LM-MP	70
4.1.4	ATP transiently facilitates [³ H]ACh release due to the activation of P2X receptors on myenteric nerve terminals	73
4.1.5	Adenine nucleotides inhibit [³ H]ACh release directly, through activation of P2Y ₁ purinoceptor, and indirectly, by formation of adenosine leading to A ₁ receptor activation	76
4.1.6	Stimulation of inhibitory ADP-sensitive P2Y ₁ purinoceptors may be cut short by sequential activation of adenosine A ₁ inhibitory receptors on myenteric motoneurons	79

4.1.7 – Discussion	81
4.2 Dual effects of endogenous ADO on ACh release from myenteric motoneurons are mediated by inhibitory A ₁ and facilitatory A _{2A} receptors: on the role of secreted adenosine deaminase	88
4.2.1 – Rationale	88
4.2.2 – Myenteric motoneurons possess both A ₁ inhibitory and A _{2A} facilitatory adenosine receptors modulating evoked [³ H]ACh release	90
4.2.3 – Endogenous adenosine preferentially activates facilitatory A _{2A} receptors on myenteric motoneurons	92
4.2.4 – Myenteric neurons are the main source of endogenous ADO	94
4.2.5 – Involvement of ADA and ADO uptake in the regulation of the extracellular concentration of adenosine in the rat myenteric plexus	96
4.2.6 – Synaptic ADO accumulation facilitates the evoked [³ H]ACh release from stimulated myenteric motoneurons via A _{2A} receptors activation	98
4.2.7 – Adenosine deaminase is secreted to the bathing fluid in parallel with adenosine upon stimulating the LM-MP [³ H]ACh release from myenteric motoneurons	101
4.2.8 – Contribution of the ecto-5'-nucleotidase pathway for adenosine modulation of [³ H]ACh release from myenteric motoneurons	102
4.2.9 – Discussion	106
4.3 Muscarinic M ₃ facilitation of ACh release from myenteric neurons depends on ADO outflow leading to activation of A _{2A} receptors	113
4.3.1 – Rationale	113
4.3.2 – Influence of the train length on the origin of ADO facilitating [³ H]ACh release from myenteric neurons	115
4.3.3 – Influence of the train length on muscarinic autoreceptors activity regulating [³ H]ACh release from myenteric motoneurons	116
4.3.4 – Muscarinic M ₃ autoreceptors facilitate de release of adenine nucleosides from stimulated myenteric neurons	118
4.3.5 – Muscarinic M ₃ facilitation of [³ H]ACh release depends on endogenous ADO accumulation leading to excitatory A _{2A} receptor activation	119

4.3.6 - Agonist-induced muscarinic M ₃ facilitation of [³ H]ACh release is mediated by endogenous adenosine via A _{2A} receptors	121
4.3.7 – Discussion	124
5) Summary and Main Conclusions	129
6) Future perspectives	131
7) Acknowledgements	133
8) Bibliography	135

ABBREVIATION LIST

AC – adenylyl cyclase
ACh – acetylcholine
AChE – acetylcholinesterase
AK – adenosine kinase
ADA – adenosine deaminase
ADK – adenylylate kinase
ADO – adenosine
ADP – adenosine diphosphate
AMP – adenosine monophosphate
ANS – autonomic nervous system
AP – alkaline phosphatase
ATP – adenosine triphosphate
cAMP – cyclic AMP
CGRP – calcitonin gene-related peptide
ChAT – choline acetyltransferase
CHT – choline transporter
CM – circular muscle
CNS – central nervous system
CNTs – concentrative nucleoside transporters
DAG – diacylglycerol
E-5'-N – ecto-5'-nucleotidase
ENS – enteric nervous system
ENTs – equilibrative nucleoside transporters
ERK – extracellular signal regulated kinase
fEPSPs – fast excitatory post-synaptic potentials
GABA – γ -aminobutyric acid
GI – gastrointestinal
Glu – glutamate
5-HT – 5-hydroxy-tryptamine (or serotonin)
ICC – interstitial cells of Cajal
INO – inosine
IPANS – intrinsic primary afferent neurons
IP₃ – inositol 1,4,5-trisphosphate
LDH – lactate dehydrogenase
LM – longitudinal muscle

mAChR – muscarinic acetylcholine receptors
MAPK – mitogen activated protein (MAP) kinase
MM – muscularis mucosae
MP – myenteric plexus
Muc – mucosa
NA – noradrenaline
nAChR – nicotinic acetylcholine receptors
NK – neurokinin
NO – nitric oxide
NOS – nitric oxide synthase
NPP – nucleotide pyrophosphatase / phosphodiesterases
NPY – neuropeptide Y
NTPDase – nucleoside triphosphate diphosphohydrolases
PACAP – pituitary adenylyl cyclase-activating peptide
PAK1 – *p21*-activated protein kinase 1
PKC – protein kinase C
PI 3-kinase – phosphatidylinositol 3-kinase
PLA₂ – phospholipase A₂
PLC – phospholipase C
PKA – protein kinase A
PKC – protein kinase C
S – synaptic potential
SAH – S-adenosyl-homocysteine
SAH H – S-adenosyl-homocysteine hydrolase
sEPSPs – slow excitatory post-synaptic potentials
SOM – somatostatin
SMP – submucous plexus
SP – substance P
TK – tachykinin
TM – transmembrane domain
TTX – tetrodotoxin
UDP – uridine diphosphate
UMP – uridine monophosphate
UTP – uridine triphosphate
VAChT – vesicular acetylcholine transporter
VIP – vasoactive intestinal peptide

ABSTRACT

The Enteric Nervous System (ENS) is considered as our *second brain* because it contains a comparable number of neurons, complexity and networking as the real brain. The ENS contains about 500 million neurons of 20 different functional classes, distributed in two major plexi (myenteric and submucous) and is the only substantial grouping of neurons outside the CNS that forms circuits capable of autonomous reflex activity. The myenteric (Auerbach's) plexus lies between the circular and the longitudinal smooth muscles along the entire gastrointestinal (GI) tract, and is predominantly involved in the regulation of gut motility. Acetylcholine (ACh) is widely accepted as the principal excitatory neurotransmitter in the GI tract, but there was considerable debate associated with the identification of inhibitory neurotransmitters. It is now recognized that these neurons release cotransmitters, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP). In spite of its difficult beginning, purinergic neurotransmission grew strong and, in essence, stands like Geoffrey Burnstock described it in 1972. ATP activates P2 purinoceptors, acting both as a postjunctional modulator (enhancing the responses of their cotransmitter) and as a prejunctional modulator of transmitter release. The sequential degradation of adenine nucleotides (via ecto-nucleotidase pathway) not only terminates ATP signalling but also generates intermediates with distinct signalling properties, such as adenosine diphosphate (ADP) and adenosine. Adenosine acts as an extracellular signalling molecule that influences synaptic transmission but, contrasting with ATP, it is neither stored nor released as a classical neurotransmitter, being generally considered as a neuromodulator in central and peripheral nervous systems. Knowing that ACh, ATP and adenosine extracellular levels are increased during GI pathological conditions, we decided to investigate the purinergic modulation of ACh release in the myenteric plexus of the rat ileum. We hope to contribute to a better understanding of these neurotransmitters/neuromodulators actions not only individually, but also considering the reciprocal influence on each other, in order to find a new integrative approach for the management of GI motility dysfunctions.

To accomplish this purpose, quantification of spontaneous and electrically-evoked ACh and adenine nucleosides release was determined in the absence and in the presence of distinct pharmacological tools (*e.g.* P1, P2 and muscarinic receptors agonists/antagonists). This integrative approach also took into account the mechanisms interfering with the extracellular levels of adenine nucleotides and nucleosides which might influence the purinergic tonus; in view of this, enzymatic inhibitors, substances that influence the release probability (*e.g.* voltage gated channel inhibitors) and distinct nerve stimulation conditions, were also tested.

Data presented in this thesis made it possible to conclude that ATP transiently activates facilitatory P2X₂ receptors, mediating spontaneous ACh release from myenteric motoneurons. The rat myenteric plexus exhibits significant NTPDase1 activity rapidly converting ATP into AMP, which is then dephosphorylated into adenosine by ecto-5'-nucleotidase. Activation of inhibitory P2Y₁ purinoceptors by ADP generated alternatively via ATPase has also been detected, whenever NTPDase1 becomes saturated by high ATP amounts. Interestingly, the P2Y₁-receptor-mediated inhibition of evoked ACh release may be cut short by sequential activation of presynaptic inhibitory adenosine A₁ receptors. Although there is the possibility of adenosine being originated from the catabolism of released ATP, our findings indicate that the nucleoside is predominantly released as such from stimulated myenteric neurons. In this context, adenosine plays a predominant tonic facilitatory action on ACh release through the activation of presynaptic A_{2A}-receptors. Data also indicate that extracellular deamination represents the most efficient mechanism regulating synaptic adenosine levels. Significant adenosine deaminase secretion from stimulated myenteric neurons along with a less efficient equilibrative nucleoside transport system may restrict endogenous adenosine actions to the release/production region at the myenteric cholinergic synapse, leading to the preferential activation of prejunctional facilitatory A_{2A} receptors. This "enzymatic barrier", which has been hypothesized for the first time in this study, may block the diffusion of exogenously added adenosine towards the active zones. Consequently, exogenously added adenosine can only activate extrajunctional inhibitory A₁ receptors if adenosine deaminase at the synaptic cleft is pharmacologically inhibited with erythro-9(2-hydroxy-3-nonyl) adenine (EHNA). Another major strength of this work is the demonstration that, besides purinergic neuromodulation, ACh is also capable of modulate its own release from rat myenteric neurons, through the activation of muscarinic inhibitory M₂ (predominant during brief stimulation periods) and facilitatory M₃ autoreceptors. Muscarinic M₃ positive feedback mechanism only becomes evident during sustained nerve activity, as it depends on extracellular adenosine accumulation leading to activation of facilitatory A_{2A} receptors.

In summary, this study contributed to reveal the existence of a well-coordinated "purinergic cascade" integrating ecto-NTPDase enzymes, P1 and P2 purinoceptors, and nucleoside inactivation systems (both deamination and cellular uptake) in the myenteric plexus of the rat ileum. These complex interactions, which are highly dependent on extracellular levels of purines, may provide fine tuning regulation of GI motility during physiological conditions. One may also hypothesize that these complexities might be exaggerated under pathological conditions, whenever the level of extracellular purines rises. Therefore, attempts to manipulate the mechanisms described in this study may uncover putative therapeutic targets to restore intestinal motility.

RESUMO

O Sistema Nervoso Entérico (SNE) é considerado o nosso *segundo cérebro* devido ao seu tamanho, complexidade e semelhança organizativa. O SNE contém cerca de 500 milhões de neurónios de 20 classes funcionais diferentes organizados em dois plexos nervosos (mioentérico e submucoso), sendo o único grupo substancial de neurónios fora do SNC capaz de formar circuitos que desenvolvem actividade reflexa autónoma. O plexo mioentérico está localizado entre o músculo liso circular e longitudinal ao longo de todo o trato gastrointestinal (GI), sendo o principal responsável pela regulação da actividade contráctil intestinal. A acetilcolina (ACh) é amplamente aceite como o principal neurotransmissor excitatório do aparelho GI, mas a identificação dos transmissores dos neurónios inibitórios tem sido alvo de debate. Reconheceu-se recentemente que estes neurónios libertam co-transmissores como o monóxido de azoto (NO), o péptido intestinal vasoactivo (VIP) e a adenosina trifosfato (ATP). Apesar de um início atribulado, a neurotransmissão purinérgica foi aceite e mantém-se essencialmente como Geoffrey Burnstock a descreveu em 1972. O ATP activa receptores purinérgicos P2, actuando quer como modulador pós-juncional (exacerbando a resposta do co-transmissor), quer como modulador pré-juncional da libertação de neurotransmissores. A metabolização sequencial dos nucleótidos de adenina (através da cascata das ectonucleotidases) não termina apenas com a sinalização promovida pelo ATP, como ainda origina intermediários com propriedades sinalizadoras diferentes, como o difosfato de adenosina (ADP) e a adenosina. A adenosina actua como uma molécula sinalizadora extracelular que influencia a transmissão sináptica mas, contrastando com o ATP, não é armazenada nem libertada como um neurotransmissor clássico, sendo geralmente considerada como um neuromodulador da libertação de neurotransmissores no sistema nervoso central e periférico. Sabendo que os níveis extracelulares de ACh, ATP e adenosina aumentam quando existem alterações fisiopatológicas no aparelho digestivo, decidimos investigar a neuromodulação purinérgica da libertação de ACh no plexo mioentérico de íleo de ratazana. Esperamos assim contribuir não só para a compreensão das acções individuais destes neurotransmissores/neuromoduladores, mas considerando também a sua influência recíproca, no sentido de encontrar uma nova abordagem integrativa para o tratamento das disfunções GI.

Para alcançar este objectivo, neste trabalho quantificaram-se os níveis extracelulares basais e após-estimulação eléctrica de campo de ACh e nucleótidos de adenina na ausência e na presença de diversos fármacos (*e.g.* agonistas/antagonistas dos receptores P1, P2 e muscarínicos). Esta abordagem integrativa considerou ainda os mecanismos que interferem com os níveis extracelulares dos nucleótidos e nucleósidos

de adenina, tendo sido testados inibidores enzimáticos, inibidores dos canais dependentes da voltagem e estimulação eléctrica com diferentes tempos de duração.

Os resultados apresentados nesta dissertação permitem concluir que o ATP activa de forma transitória receptores facilitatórios P2X₂ causando, por si só, a libertação de ACh a partir de neurónios mioentéricos. No plexo mioentérico do íleo de rato, a NTPDase1 (CD39 ou apirase) possui uma actividade significativa convertendo rapidamente ATP em AMP, que é por sua vez desfosforilado em adenosina pela ecto-5'-nucleotidase. Paralelamente, o ATP pode ser metabolizado em ADP pela ATPDase, sempre que os níveis extracelulares de ATP sejam suficientes para saturar a enzima NTPDase1. A formação de ADP favorece a activação de receptores inibitórios P2Y₁, cuja actividade sobre a libertação de ACh induzida por estimulação eléctrica é controlada pela activação sequencial dos receptores inibitórios A₁ da adenosina.

Apesar da adenosina poder ser gerada a partir do catabolismo dos nucleótidos de adenina libertados, os resultados apresentados neste trabalho mostram que os neurónios entéricos parecem ser a principal fonte de adenosina no plexo mioentérico. Neste contexto, a adenosina libertada *per se* a partir dos neurónios mioentéricos parece exercer um efeito facilitatório tónico preferencial sobre a libertação de ACh através da activação de receptores A_{2A}. No que respeita aos mecanismos de inactivação da adenosina no plexo mioentérico, a desaminação extracelular representa o mecanismo mais eficiente para controlar os níveis de adenosina. Este sistema de inactivação pode, no entanto, ser complementado por outro (por ventura, menos eficiente) que depende do sistema de recaptação equilibrativo de nucleósidos. Neste trabalho mostrou-se, pela primeira vez, que a desaminase da adenosina pode ser secretada paralelamente com a libertação de adenosina a partir de neurónios mioentéricos estimulados electricamente. Esta “barreira enzimática” promove a activação tónica preferencial dos receptores A_{2A} da adenosina localizados na região pré-juncional da sinapse mioentérica, mas impossibilita que a adenosina aplicada exogenamente se difunda no sentido das zonas activas. Assim, verificou-se que a aplicação exógena do nucleósido activa predominantemente receptores extra-juncionais inibitórios do subtipo A₁, a menos que seja inibida a desaminase da adenosina com eritro-9(2-hidroxi-3-nonil)adenina (EHNA).

Outro ponto forte deste trabalho reside na demonstração de que, para além da neuromodulação purinérgica, a ACh é também capaz de modular a sua própria libertação a partir de neurónios mioentéricos de ratazana através da activação de auto-receptores muscarínicos inibitórios M₂ (que predominam durante estímulos breves) e facilitatórios M₃. O mecanismo de regulação positiva mediada pelos receptores muscarínicos M₃ só foi evidenciado durante a actividade nervosa sustentada, pois depende da acumulação extracelular de adenosina que favorece a activação de receptores A_{2A} facilitatórios.

No seu conjunto, os resultados experimentais obtidos parecem indicar a existência de uma “cascata purinérgica” bem coordenada envolvendo ectoNTPDases, receptores P1 e P2 e sistemas de inativação da adenosina no plexo mioentérico de íleo de ratazana. A actividade resultante desta “cascata purinérgica” depende dos níveis extracelulares das purinas, que podem sofrer alterações subtis durante patologias intestinais, abrindo perspectivas muito interessantes para a sua manipulação nas doenças gastrointestinais. Assim, é possível que a utilização de inibidores enzimáticos e agonistas/antagonistas dos receptores purinérgicos possam ter utilidade terapêutica futura nas alterações da motilidade gastrointestinal.

1 - INTRODUCTION

1.1 - The Enteric Nervous System (ENS)

Neural networks for the control of digestive functions are positioned at many levels: the central nervous system (CNS), spinal cord, prevertebral sympathetic ganglia and in the wall of the specialized organs that make up the digestive system. The enteric nervous system (ENS) is found in the walls of the entire gastrointestinal (GI) tract from the oesophagus to the anus, associated glands (salivary glands, the pancreas) and the gallbladder (Buckley *et al.* 1986; Delacretaz 2006; Furness 2006; 2009; Furness *et al.* 2009; Hansen 2003a; 2003b; Timmermans *et al.* 2001). The ENS is a part of the autonomic nervous system (ANS) which integrates motility, secretions, blood flow, and immune responses into organised patterns of organic function through neural reflexes (Furness *et al.* 2009; Hansen 2003a). The ENS is of special interest because it is the only substantial grouping of neurons outside the CNS that forms circuits capable of autonomous reflex activity. It contains about 500 million neurons of 20 different functional classes. Because of its size, complexity and structural similarities, the ENS has been compared to the CNS and is considered, by many, as our *second brain*. This was discovered almost 150 years ago, and several remarkably insightful hypotheses about its functions were made back then. In the following decades the ENS was neglected, and only in the last 20-30 years new techniques have provided valuable information on the structural complexity, connectivity, neuron types, transmitters and cell physiology of enteric neurons, encouraging exciting new ideas (Brookes *et al.* 2006; Furness 2006; Gershon 1999; Hansen 2003a).

The ENS is formed by a number of interconnected networks of neurons, their axons and enteric glial cells. It was in the second half of the nineteenth century that two German physicians, Georg Meissner (1829-1905) and Leopold Auerbach (1828-1897), working in different laboratories, clearly described the existence of these ganglionated plexi within the walls of the digestive tract. Following their discovery, enteric ganglia attracted considerable attention and detailed information was obtained regarding enteric neurons type (Ramon Y Cajal), microarchitecture (A.S. Dogiel) and neurochemistry (J.N. Langley) (Furness 2006; Hansen 2003a; 2003b; Ruhl 2005). Although their descriptions were based on quite primitive nerve tissue revealing techniques, they were not superseded in the subsequent one hundred years, so enteric plexus arrangements remain basically as described back then.

The ENS has three major ganglionic plexi (the mucosal, the submucous and the myenteric) (Figure 1.1), with several aganglionic plexi subtypes (Table 1.1), but there are marked differences between species and gastrointestinal regions (Brookes & Costa 2006; Furness 2006; Furness *et al.* 2009; Hansen 2003a).

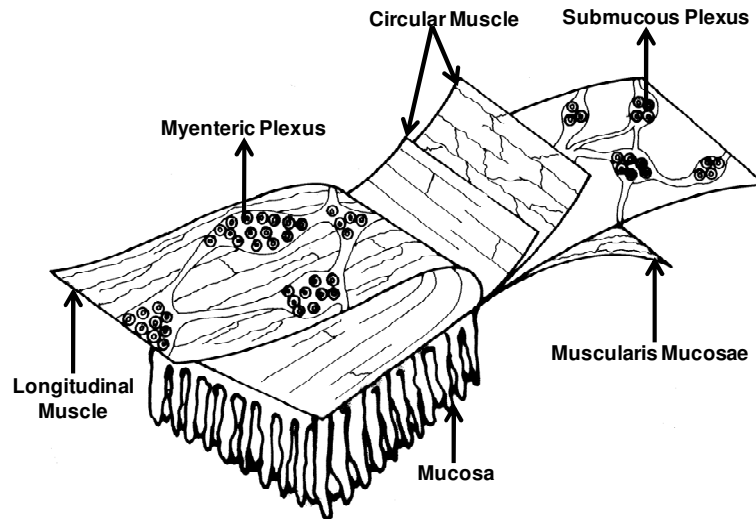


Figure 1.1 – The enteric plexi seen in a whole mount view. There are two major ganglionic plexi, the myenteric and the submucous plexus, in addition to plexi of nerve fibres in the muscle, in the mucosa and around the arterioles. Original drawing by *Duarte Monteiro*, published with the author's permission.

Table 1.1 – Major enteric plexus types and related subtypes.

<i>Major types (ganglionated)</i>	<i>Subtypes (aganglionated)</i>
	Subserous plexus
Myenteric (Aurbach's) Plexus (bigger enteric ganglia)	Longitudinal muscle plexus Circular muscle plexus Deep muscular plexus
Submucous (Meissner's) Plexus (smaller enteric ganglia)	Plexus submucous extremus Outer submucous plexus Intermediate submucous plexus Inner submucous plexus
Mucosal Plexus (small groups of nerve cell bodies)	Subglandular plexus Periglandular plexus Vascular plexus Villous subepithelial subplexus

Some authors consider the mucosal plexus the association of small groups of nerve cell bodies that occur in the lamina propria of the intestinal mucosa (close to the

inner submucous plexus). Most enteric nerve cells are found in two sets of ganglia that constitute the submucous and the myenteric plexi (Furness 2006; Hansen 2003a).

Amongst the connective tissue between the mucosa and the circular muscle layer is positioned the submucous plexus (SMP). This plexus regulates the secretomotor and vasomotor activities of the mucosa of small and large intestines. Their interconnecting strands are thinner and enteric ganglia smaller than those of the myenteric plexus (MP). The outer MP lays between the circular and the longitudinal smooth muscle layers along the entire gastrointestinal (GI) tract, and is predominantly involved in the regulation of contractile activities of the external musculature (Furness 2000; Furness 2006; Hansen 2003a; Lomax *et al.* 2000). Cell clusters are interconnected by nerve fibre bundles: primary interganglionic fibre tracts, secondary and tertiary fibres projecting to the effector systems (muscle cells, glands, blood vessels, and immune cells). Together, ganglia and intermodal strands make up the primary meshwork of the MP. Thinner nerve fibre bundles constitute the secondary component of this plexus, which runs parallel to the circular muscle. Nerve processes from the secondary strands to the circular muscle are called the tertiary plexus that meanders the space between other MP meshwork, found only where the longitudinal muscle is thin; in such regions, few nerve fibres are found within the longitudinal layer (Figure 1.2) (Brookes & Costa 2006; Furness 2000; Furness 2006; Hansen 2003a; Lomax & Furness 2000).

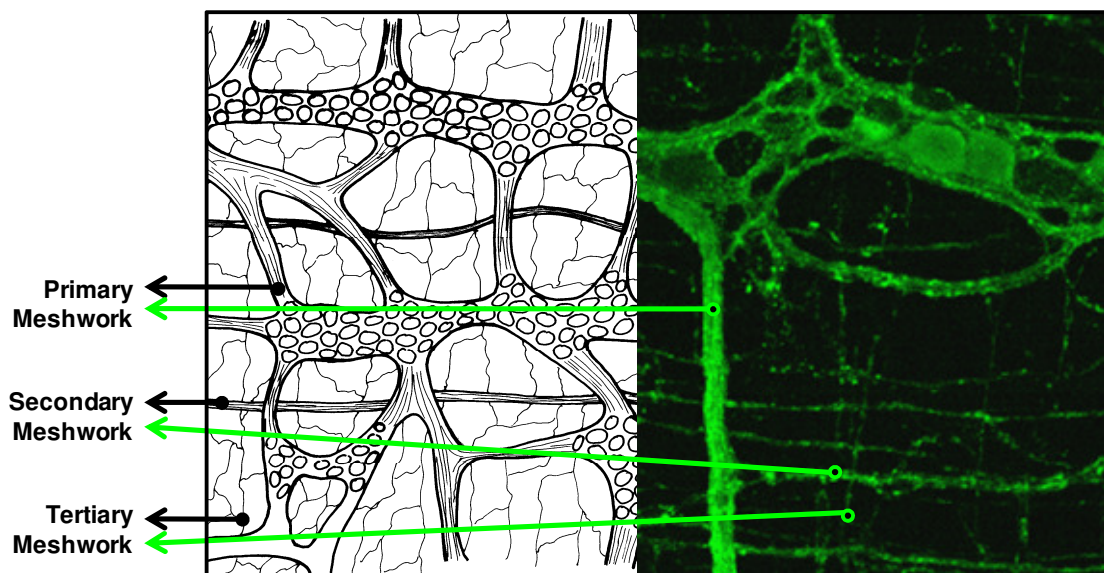


Figure 1.2 – Schematic representation and immunolabeling of a whole mount preparation of rat ileum myenteric plexus. Identification of the primary, secondary and tertiary components of the myenteric plexus. Original drawing by *Duarte Monteiro* and original immunofluorescence confocal microscopy image obtained by *Fátima Ferreirinha*, using mouse antisera against protein gene product 9.5. (PGP, 1/1600, 40x). Published with the author's permission.

The ENS has far more neurons than any other peripheral organ, being the number of neurons equal to those found in the spinal cord. Neuronal plexi occur around the gut tube from the simplest animals, such as hydra, to highly developed mammals. It is originated from neural crest cells that colonize the gut during intra-uterine life. It becomes functional in the last third of human gestation but continues to develop following birth. Enteric neurons number decreases in mammals of advanced age (Brookes & Costa 2006; Furness 2006; Wood 2006b).

Essentially, all functional types of enteric neurons have been characterized by their morphology, neurochemical properties, cell physiology and projections to target cells (Table 1.2). For example, the small intestine of the guinea-pig has 15 different types of enteric neurons, slightly more in the colon and slightly less in the stomach and oesophagus (Table 1.3 and Figure 1.3). Their orthologs in human and several other species have also been identified (Brookes 2001; Costa *et al.* 1996; Costa *et al.* 1986; Furness 2000; Hansen 2003a).

Table 1.2 – Enteric neurons basic classification.

Criteria	Classes
Morphology	Dogiel types I-VII
Electrical	Types S (synaptic) and AH (after hyperpolarisation)
Chemical	Transmitters and other markers
Functional	Sensory, interneuron, motor and intestinofugal

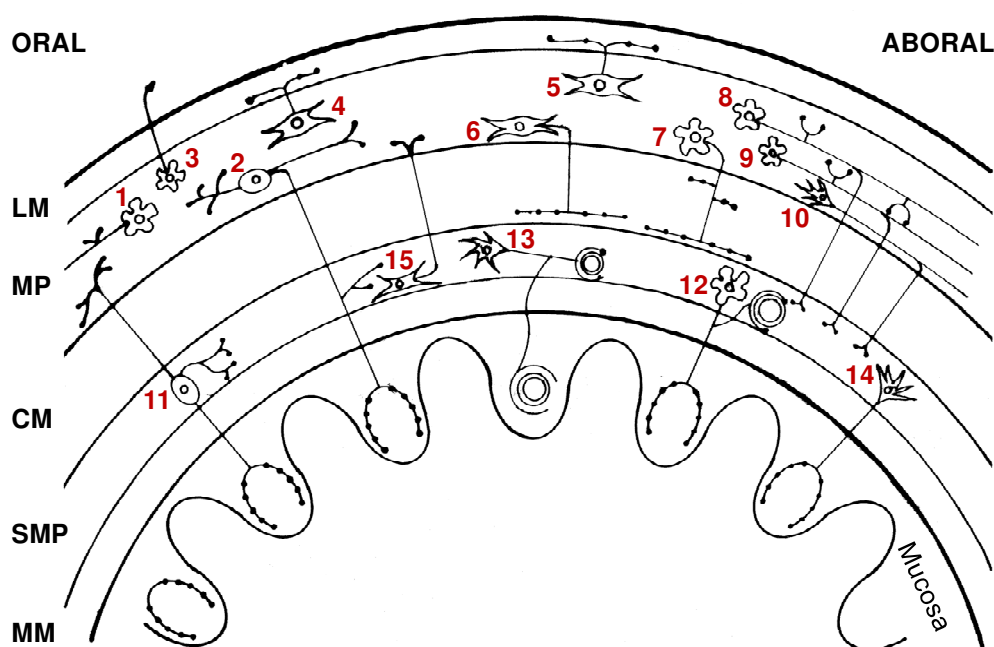


Figure 1.3 - Types of neurons of guinea-pig small intestine. See corresponding legend in Table 1.3. Original drawing by *Duarte Monteiro*, published with the author's permission. LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SMP, submucous plexus; MM, muscularis mucosae.

Table 1.3 – Type, function, proportion, shape and chemical coding of guinea-pig small intestine enteric neurons (the numbers in parentheses are the identifying numbers of neurons in Figure 1.3).

Functional definition	Proportion	Shape	Chemical coding	Transmitter/comments
Myenteric neurons				
Excitatory circular muscle motor neurons (6)	12%	Dogiel type I	Short: ChAT/TK/ENK/GABA Long: ChAT/TK/ENK/NFP	To all regions, primary transmitter ACh, co-transmitter TK
Inhibitory circular muscle motor neurons (7)	16%	Dogiel type I	Short: NOS/VIP/PACAP/ ENK/NYP/GABA Long: NOS/VIP/PACAP/ dynorphin/BN/NFP	Several co-transmitters with varying prominence: NO, ATP, VIP, PACAP
Excitatory longitudinal muscle motor neurons (4)	25%	Small Dogiel type I	ChAT/calretinin/TK	Primary transmitter ACh, co-transmitter TK
Inhibitory longitudinal muscle motor neurons (5)	~2%	Dogiel type I	NOS/VIP/GABA	Several co-transmitters with varying prominence: NO, ATP, VIP, PACAP
Ascending interneurons (local reflex) (1)	5%	Large Dogiel type I	ChAT/calretinin/TK/ENK	Primary transmitter ACh, co-transmitter TK
Descending interneurons (local reflex) (8)	5%	Dogiel type I	ChAT/NOS/VIP/BN/NPY	Primary transmitter ACh, ATP may be a co-transmitter
Descending interneurons (secretomotor and motility reflex) (9)	2%	Dogiel type I	ChAT/5-HT	Primary transmitters ACh, 5-HT (as 5-HT ₃ receptors)
Descending interneurons (migrating myoelectric complex) (10)	4%	Dogiel type III (filamentous)	ChAT/SOM	Primary transmitters ACh
Myenteric intrinsic primary afferent neurons (IPANS) (2)	26%	Dogiel type II	ChAT/TK/Orexin/IB4/ NeuNcyt/NK _{3r} /calbindin	Primary transmitters ACh and TK (or CGRP)
Intestinofugal neurons (3)	<1%	Dogiel type II	ChAT/BN/VIP/NOS/ CCK/ENK	Primary transmitter ACh, co-transmitter VIP
Submucous neurons				
Non-cholinergic secretomotor / vasodilator neurons (12)	45%	Dogiel type I	VIP/CART/CRF/GAL/PACA P/NMU/ (ChAT in humans)	Primary transmitter VIP. Some may have cell bodies in myenteric ganglia
Cholinergic secretomotor / vasodilator neurons (13)	15%	Stellate	ChAT/calretinin/dynorphin	Primary transmitter ACh
Cholinergic secretomotor (non-vasodilator) neurons (14)	29%	Type IV	ChAT/NPY/CCK/SOM/ CGRP/dynorphin	Primary transmitter ACh. Some may have cell bodies in myenteric ganglia
Uni-axonal neurons projecting to the myenteric plexus (15)	1%	Uni-axonal with thin dendrites	VIO (and maybe NOS)	Possibly displaced myenteric interneurons
Submucous intrinsic primary afferent neurons (IPANS) (11)	11%	Dogiel type II	ChAT/TK/Orexin/IB4/ NeuNcyt/(calbindin)	Primary transmitters ACh (and maybe TK or CGRP)

ACh, acetylcholine; *BN*, bombesin; *CART*, cocaine and amphetamine-regulated transcript peptide; *CCK*, cholecystokinin; *CGRP*, calcitonin gene-related peptide; *ChAT*, choline acetyltransferase; *CRF*, corticotrophin-releasing factor; *ENK*, enkephalin; *GABA*, gamma amino butyric acid; *GAL*, galanin; *GRP*, gastrin releasing peptide; *5-HT*, 5-hydroxy-tryptamine; *IB4*, isolectin B4; *NeuNcyt*, cytoplasmatic immunoreactivity for the neuronal nuclear protein NeuN; *NFP*, neurofilament protein; *NK*, neurokinin; *NOS*, nitric oxide synthase; *NPY*, neuropeptide Y; *PACAP*, pituitary adenylyl cyclase activating peptide; *SOM*, somatostatin; *TK*, tachykinin; *VIP*, vasoactive intestinal polypeptide.

Regarding the pharmacology of transmission in the ENS, extensive immunohistochemical studies demonstrate that each enteric neuron contains several substances whose chemistry and receptor pharmacology are consistent with the

neurotransmitters released and associated function. Essentially, each enteric neuron has a primary transmitter and may also have secondary transmitters (acting on the same post-synaptic cells) and/or modulatory mediators (acting presynaptically) (Figure 1.4):

- *IPANs* (intrinsic primary afferent neurons) - occur in the small and large intestines; their nerve endings are in the mucosa and respond to physiological stimuli. These neurons exhibit other processes that form synapses with interneurons and motor neurons, as well as with other IPANs. They have distinctive electrophysiological characteristics (focal stimulation induces fast excitatory synaptic potentials followed by long-lasting membrane after hyperpolarisation), hence having been termed type AH neurons. The primary transmitter, released from entero-endocrine cells, is 5-hydroxytryptamine (5-HT or serotonin). The main receptors involved are 5-HT₃, 5-HT₄ and 5-HT_{1P} (Costa *et al.* ; Furness 2006; Hansen 2003b).

- *Interneurons* - are mono-axonal neurons that generally have type S electrophysiological properties (focal stimulation induces fast excitatory synaptic potentials). These neurons project through small distances along the gut wall and form “chains of neurons” connecting with each other and with motor neurons. ACh is the major transmitter for fast excitatory transmission, activating postsynaptic nicotinic receptors. Other substances released from these neurons include ATP (acting at P2X receptors) and 5-HT (acting at 5-HT₃ receptors). Concerning slow excitatory transmission, the mediators involved are tachykinins (acting via NK₁ and NK₃ receptors), CGRP, ATP (activating at P2Y receptors), 5-HT and others. Transmitters of slow inhibitory transmission to enteric nerves include noradrenaline, somatostatin and possibly 5-HT. Modulation of presynaptic release of neurotransmitters occurs by the activation of inhibitory (muscarinic and/or 5-HT_{1P}) and facilitatory (5-HT₄) receptors (Costa *et al.* ; Furness 2006; Hansen 2003b; Wood 2006b). Recently, other pre-synaptic modulators have been found (see below).

- *Motor neurons* - are mono-axonal neurons, almost all of which have electrophysiological type S properties. They include excitatory and inhibitory muscle motor neurons, secretomotor neurons, secretomotor/vasodilator neurons and neurons that innervate entero-endocrine cells. The primary transmitters of secretomotor neurons that control fluid secretion in the small and large intestines are VIP (with PACAP) and ACh. ACh is the primary transmitter on roughly 30% of excitatory motor neurons to the GI smooth muscle. ACh acts through muscarinic receptors on smooth muscle fibres and “pacemaker”-like interstitial cells of Cajal (ICC), which are situated in close apposition between nerve endings and smooth muscle fibres. Tachykinins are secondary transmitters. They activate NK₁ and NK₂ receptors on both muscle and ICC. ACh, GABA and opioid peptides are modulators of these excitatory motor neurons, inhibiting transmitter release. The primary transmitter of inhibitory motor neurons to GI smooth

muscle is nitric oxide (Galligan *et al.* 1991). These neurons release secondary transmitters, such as VIP, PACAP and ATP, which activate smooth muscle fibres indirectly through gap junctions connecting ICC to the muscle (Costa *et al.* 1996; Furness 2006; Hansen 2003a; 2003b; Lomax & Furness 2000; Wood 2006b).

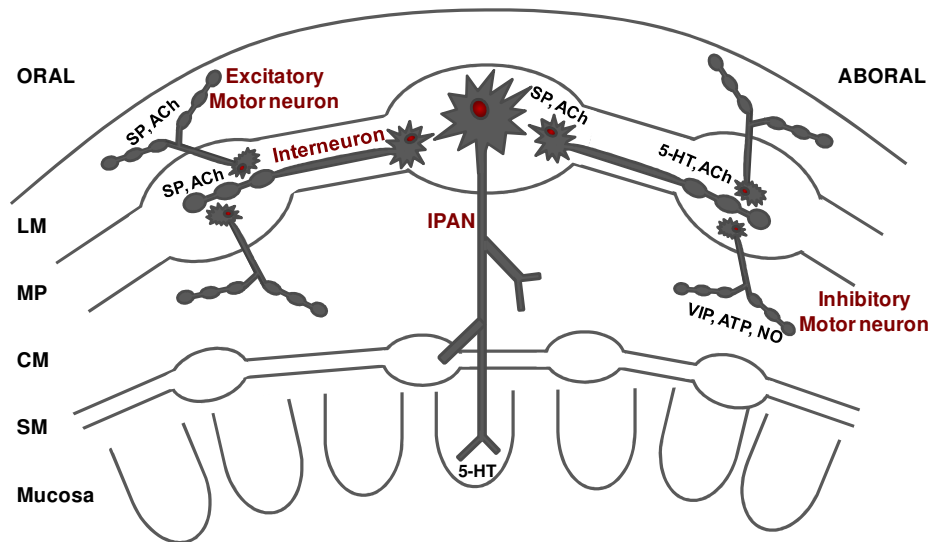


Figure 1.4 – Enteric neurons and neurotransmitters involved in identified functional enteric synapses. LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SM, submucous plexus; IPAN, intrinsic primary afferent neurons.

It is now clearer the functional description of W.M. Bayliss and E.H. Starling of the ENS that originated Neurogastroenterology. A century ago, they discovered “the law of the intestine” that was maintained even when all nerve communication between the gut and the CNS was cut, a “local nervous mechanism”. Most of the motility-controlling circuits have been gradually unravelled in the last twenty five years, based on the identification of individual neurons, neurotransmitters involved and circuits to which they belong. For example, what we now know as the “peristaltic reflex” is the result of mucosal stimulation with subsequent 5-HT release from enterochromaffin cells to IPANs (activating 5-HT_{1P} and 5-HT₄ receptors) and extrinsic primary vagal and spinal afferents (activating 5-HT₃ receptors) control. These sensory neurons release substance P (SP), acetylcholine (ACh), glutamate and calcitonin gene-related peptide (CGRP) to interneurons. Excitatory interneurons release SP and ACh orally to excitatory motor neurons, whereas 5-HT and ACh are released aborally to inhibitory motor neurons. Excitatory motor neurons release SP and ACh to muscles, whereas inhibitory motor neurons release nitric oxide (NO) (Galligan & North 1991), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP) to muscles. Efferent sympathetic neurons release noradrenaline (NA), somatostatin (SOM), and neuropeptide Y, while efferent parasympathetic neurons release ACh

(Bertrand *et al.* 2000b; Bornstein *et al.* 2004; Furness 2006; Galligan 1999; 2002a; Hansen 2003a; 2003b; Wood 2006b).

The gathering of all of this immunohistochemical information in ENS led to the creation of a “chemical coding hypothesis”, which states that each class of functionally differentiated neurons contains a unique combination of chemical markers. Amongst them, primary transmitters appear to be highly conserved across mammalian species. Thus, when a substance is a primary neurotransmitter (McConalogue *et al.* 1994) (Table 1.4), it appears to be present in all neurons that have equivalent roles in different species and regions of the gastrointestinal tract. In contrast, subsidiary transmitters and neuromodulators of equivalent neurons in different regions are not necessarily the same (Furness 2006; Furness *et al.* 1987; Goyal *et al.* 1996; McConalogue & Furness 1994).

Table 1.4 – Transmitters found in the Enteric Nervous System.

Amines

Acetylcholine (**ACh**)
Noradrenaline (**NA**)
Serotonin (5-hydroxytryptamine, **5-HT**)

Amino acids

γ -Aminobutyric acid (**GABA**)
Glutamate (**Glu**)

Purines

Adenosine triphosphate (**ATP**)
Adenosine (**ADO**)

Gases

Nitric oxide (**NO**)
Carbon monoxide (**CO**)

Peptides

Calcitonin gene-related peptide (**CGRP**)
Cholecystokinin (**CCK**)
Galanin (**GAL**)
Gastrin-releasing peptide (**GRP**)
Neuromedin U (**NMU**)
Neuropeptide Y (**NPY**)
Neurotensin (**NTS**)
Opioids (Dynorphin; Enkephalins and Endorphins)
Peptide YY (**PYY**)
Pituitary adenylyl cyclase-activating peptide (**PACAP**)
Somatostatin (**Som**)
Substance P (**SP**)
Thyrotropin-releasing hormone (**TRH**)
Vasoactive intestinal contractor (and endothelin) (**VIC**)
Vasoactive intestinal polypeptide (**VIP**)

In spite of this, the functional role of many of neurotransmitters and neuromodulators found in the enteric nervous system is unknown. Further investigation is required to provide more information regarding the function of these mediators, as well as their involvement in the pathophysiology of the GI tract.

1.2 Cholinergic Transmission in the ENS

1.2.1 Cholinergic neurotransmission

Acetylcholine (ACh), in addition to being the transmitter of preganglionic autonomic and postganglionic parasympathetic nerves, is the principal excitatory transmitter in the gastrointestinal (GI) tract. ACh is involved in the control of almost any function within this system, and is the primary transmitter of vagal and pelvic preganglionic neurons, of enteric interneurons, of some secretomotor neuron in the intestine and of motor neurons controlling gastric acid secretion. ACh and tachykinins are co-primary transmitters of muscle motor neurons, but ACh induces the larger response in the normal GI tract (Harrington *et al.* 2010; McConalogue & Furness 1994; Tobin *et al.* 2009).

The release of ACh from neurons innervating the GI tract was described in the beginning of the discovery of chemical transmission - a rewarded idea that Sir Henry Dale shared with Otto Loewi when they received the Nobel Prize of Physiology and Medicine in 1936. ACh neural origin was then confirmed by the neurotransmitter release reduction induced by the application of tetrodotoxin, botulinum toxin or Ca^{2+} ions removal (Furness 2006; Furness *et al.* 1987; Molenaar *et al.* 1970). Initially, cholinergic neurons were identified using an assay to detect acetylcholinesterase (AChE, EC 3.1.1.7) enzymatic activity at the site of active ACh synthesis (Johnson *et al.* 1996). AChE histochemistry was used to demonstrate enlarged nerve trunks for the diagnosis of Hirschsprung's disease (Dale *et al.* 1979). Then, all enzymes and transport proteins mediating ACh biosynthesis and synaptic release were used as markers for different components of the cholinergic circuitry, including nerve cell bodies and nerve fibres. Briefly, upon stimulation of cholinergic presynaptic terminals, ACh is released from vesicles into the synaptic cleft. ACh binds to postsynaptic or presynaptic ACh receptors, either nicotinic or muscarinic receptors. Within the synaptic cleft, ACh is inactivated and broken down by acetylcholinesterase. The free choline is then transported into the nerve fibre cytoplasm, by the high affinity choline transporter, where it is acetylated by choline acetyltransferase (ChAT, EC 2.3.1.6) to generate *de novo* ACh. The neurotransmitter is then packaged into synaptic vesicles through the vesicular acetylcholine transporter (VACHT), waiting for a new excitation period to release ACh by exocytosis (Figure 1.5) (Augustine 2004; Hebb *et al.* 1964; Potter 1970; Westfall *et al.* 2005). ChAT was used in the ENS to label cholinergic neurons in many species (Furness *et al.* 1984; Schemann *et al.* 1993). ChAT immunoreactivity is also present in nerve fibres, since it is synthesised in neuronal cell bodies and transported to nerve terminals where it catalyses ACh synthesis. More

recently, VACHT (Figure 1.6) was used as a selective marker to identify cholinergic nerve fibres and cholinergic nerve terminals (Arvidsson *et al.* 1997). The latest marker for cholinergic elements is the high affinity choline transporter (CHT), since presynaptic synthesis of ACh requires a steady supply of choline, which is acquired by the CHT (Lecomte *et al.* 2005; Lips *et al.* 2002; Okuda *et al.* 2000).

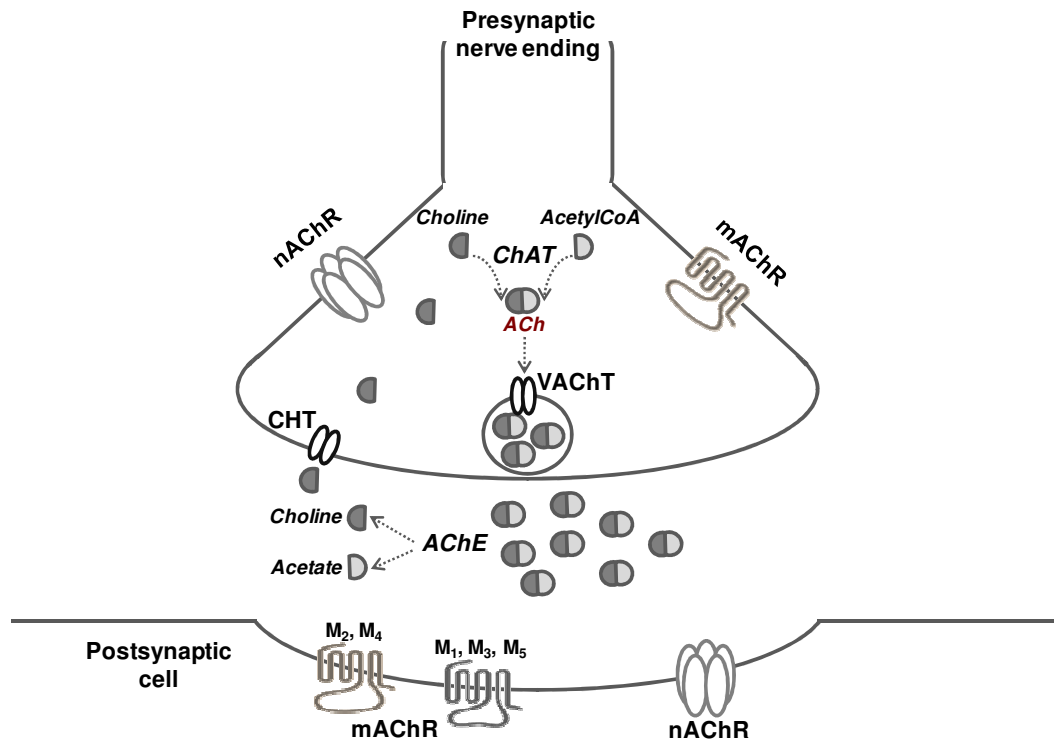


Figure 1.5 – Schematic representation of ACh biosynthesis, synaptic release, presynaptic and postsynaptic ACh receptors, either nicotinic (nAChR) or muscarinic (mAChR). *ChAT*, acetyltransferase; *VACHT*, vesicular acetylcholine transporter; *AChE*, acetylcholinesterase; *CHT*, high affinity choline transporter.

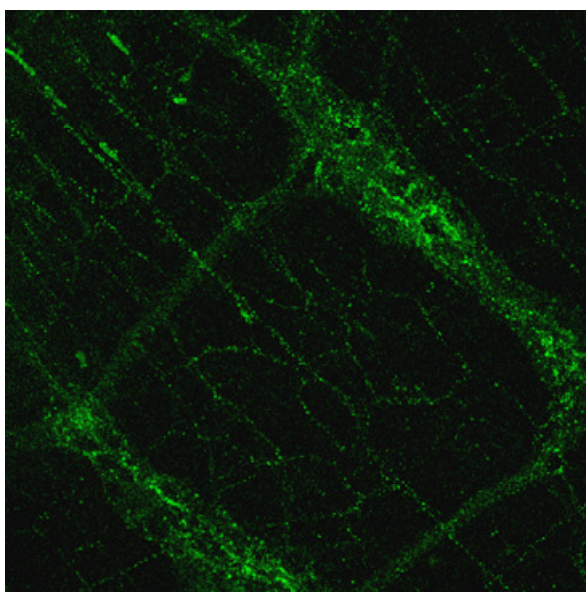


Figure 1.6 – Immunolabeling of a whole mount preparation of rat ileum myenteric plexus. Original immunofluorescence confocal microscopy image by *Fátima Ferreirinha*, using goat antisera against vesicular acetylcholine transporter (VACHT, 1/1500, 40x). Published with the author's permission.

As described previously, cholinergic transmission within the ENS is mediated by two classes of receptors: nicotinic (nAChR) and muscarinic (mAChR). nAChR are ligand-gated ion channels (Lukas *et al.* 1999), whereas mAChR receptors are G-protein-coupled receptors (Caulfield *et al.* 1998). At the cholinergic synapse, both classes of receptors are present on effector cells (postsynaptic receptors) or on nerve terminals (presynaptic receptors), where they act as autoreceptors regulating ACh release from nerve terminals (Buckley & Burnstock 1986; Galligan 1999). According to their nature, ACh receptors generate variable postsynaptic potentials: nAChR mediate fast excitatory post-synaptic potentials (fEPSPs) whereas mAChR mediate slow excitatory post-synaptic potentials (sEPSPs) (Furness 2009).

1.2.1 Nicotinic Receptors in the ENS

Within the ENS, nicotinic receptors are required for rapid neurotransmission, in order to propagate reflexes quickly and produce fast responses to stimuli (Galligan 2002b). As this receptor/ion channel complexes do not require generation of intracellular second messenger molecules to convey the primary signal, response latencies (< 1 ms) and durations (< 100 ms) are short. Neuronal nAChRs are composed of different combinations of α and β subunits and five subunits assemble to form a functional receptor. There are eight α (α_2 - α_9) and three β (β_2 - β_4) subunits and the properties of different nAChR subtypes are determined by the specific subunit composition of the receptor pentamer (Augustine 2004; Galligan *et al.* 2004; Lukas *et al.* 1999; Westfall & Westfall 2005). The subunit compositions of nAChR of myenteric neurons have been investigated by agonist rank-order potency and immunohistochemistry in the guinea-pig. The experiments indicate that the predominant expression is of nAChR composed of α_3 , α_5 , β_2 , and β_4 subunits. These subunits may combine in a homogeneous population of receptors with unique pharmacological properties, or multiple receptors of different subunit composition may be expressed by individual neurons (Furness 2006; Galligan 2002a; Galligan & North 2004; Zhou *et al.* 2002). Nicotinic receptor activation is the predominant mechanism for cholinergic neurotransmission in enteric ascending reflex pathways, with nAChR on ascending and circumferential motor pathways. Nicotinic receptors have a minor role in mediating cholinergic transmission within the descending inhibitory reflex (Bian *et al.* 2004; Galligan 2002a; Galligan & North 2004).

It is well established that nAChR are localized on the somatodendritic region of interneurons and motor neurons where they mediate excitation. However, nAChR may be localized on nerve endings mediating neurotransmitters release (Duarte-Araújo *et al.* 2004b; Galligan 2002b; Mandl *et al.* 2007; Mandl *et al.* 2003). The presynaptic localization of nAChR was firstly suggested to explain why non-cholinergic contractions of the longitudinal muscle of guinea pig, caused by nicotine and dimethy-phenyl-piperazinium (DMPP), were only partly inhibited by tetrodotoxin that blocks axonal action potentials. Galligan concluded that nerve terminal nAChR could mediate release of tachykinin peptides at the neuroeffector junction, between motor neurons and the circular and longitudinal muscle layers (Galligan 1999). Furthermore, it was suggested that nerve terminal nAChR could act as facilitatory autoreceptors, responding to ACh co-released from the same nerves that were releasing SP/NKA. Functional neurochemical evidence suggests that these receptors can be activated only by a higher concentration of agonists (Duarte-Araújo *et al.* 2004b; Mandl & Kiss 2007; Mandl *et al.* 2003).

Furthermore, data published in 2004 by our laboratory demonstrated that DMPP concentration-dependently increased ACh release in longitudinal muscle – myenteric plexus preparations of the rat ileum. DMPP-induced ACh outflow was attenuated by nAChR antagonists (hexamethonium and tubocurarine) application or by removing external Ca^{2+} (plus EGTA). In contrast to veratridine (Na^+ channel activator)-induced ACh release, DMPP-induced outflow was resistant to tetrodotoxin (Na^+ channel blocker) and cadmium (non-selective voltage-sensitive Ca^{2+} channel blocker). These results indicated that nAChR-induced ACh release is triggered by the influx of Ca^{2+} , independent of voltage-sensitive calcium channels, presumably directly through the nAChR located on myenteric axon terminals (Figure 1.7) (Duarte-Araújo *et al.* 2004b).

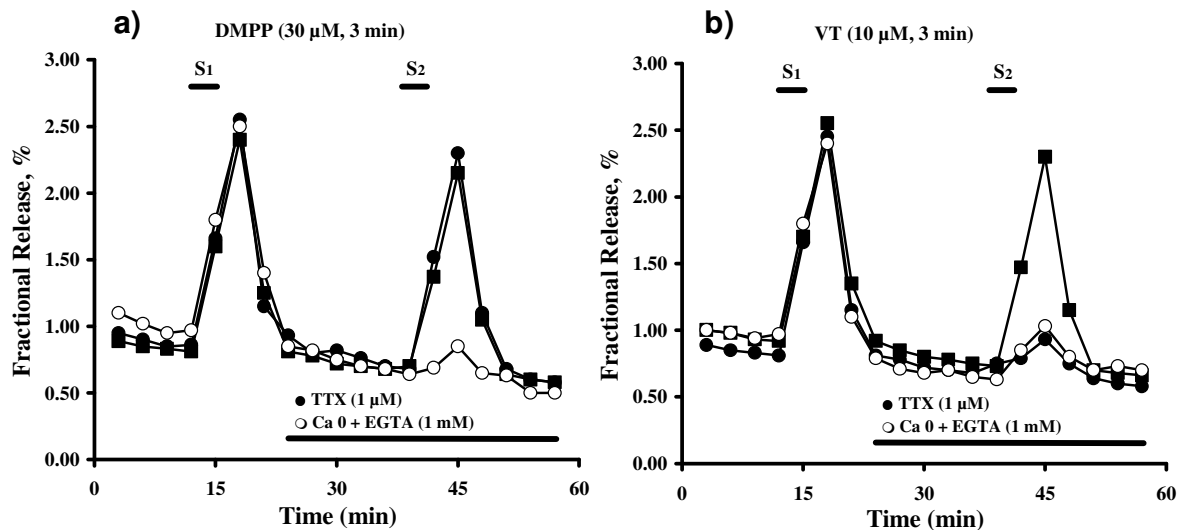


Figure 1.7 – Time course of tritium outflow from the longitudinal muscle-myenteric plexus preparation of the rat ileum taken from typical experiments: role of external Ca^{2+} and action potentials generation. Briefly, $[^3\text{H}]$ -ACh release was stimulated with (a) the nicotinic acetylcholine receptor (nAChR) agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP, 30 μM); (b) the Na^+ channel activator, veratridine (VT, 10 μM), applied twice (S₁ and S₂). DMPP-induced tritium outflow was resistant to tetrodotoxin (Na^+ channel blocker) but not to a Ca^{2+} -free solution (Ca0 + EGTA, 1 mM), in contrast to ACh release induced by veratridine, that decreased in the presence of VT and in the absence of calcium. Adapted from (Duarte-Araújo *et al.* 2004b).

Accumulated evidences indicate that nerve terminal nAChRs mediate ACh release positive feedback from myenteric motoneurons, which might play an important role in the regulation of gastrointestinal motility. Since these presynaptic nAChRs seem to be more sensitive to nicotinic ligands than somatodendritic nAChRs, they can become primary targets of exogenous compounds such as nicotine (Duarte-Araújo *et al.* 2004b; Mandl & Kiss 2007).

1.2.2 Muscarinic Receptors in the ENS

ENS cholinergic transmission is not only about ligand-gated ion channels activation; ACh also activates G-protein-coupled (GPCRs) muscarinic receptors (mAChR) (Caulfield & Birdsall 1998). These metabotropic receptors mediate slow developing (onset > 50 ms) and long-lasting (seconds to min) changes based on a multistep process that includes agonist binding and subsequent activation of one or more intracellular signalling pathways which target and alter ion channel function (Bertrand *et al.* 2000a; Brookes 2001; Galligan & North 2004; Sakamoto *et al.* 2007; Westfall & Westfall 2005). The

mAChR mediate slow excitatory and inhibitory responses, both post and presynaptically, contributing to the multiple roles that ACh has in GI physiology. Their diverse distribution and subtype coupling to various G-proteins elicits variable intracellular responses upon activation. There are five mAChR subtypes: M_1 , M_3 and M_5 receptors stimulate the phosphoinositol cascade (via G_q/G_{11}), whereas M_2 and M_4 mediate inhibition of adenylate cyclase (via G_i/G_o). So, mAChR activation either depolarises a cell, resulting in the initiation of another action potential, or hyperpolarises a cell, inhibiting additional action potentials (Brown *et al.* 2005; Challiss *et al.* 2009; Tobin *et al.* 2009).

Although it has been established that mAChR subtypes are variably distributed over muscle and ganglia within the intestine, the majority of the data on mAChR location within the ENS has been implied from neurophysiological, pharmacological and expression studies rather than histochemical studies. Surprisingly, Geoffrey Burnstock (the researcher who first suggested co-transmission and set off purinergic signalling) described, in the early 80s, using non-selective radio-ligand binding, that the majority of mAChR were associated with musculature, even though they could also be found in myenteric neurons (Buckley *et al.* 1984; Buckley & Burnstock 1986). Despite this promising start, the localisation of mAChR has been plagued by a lack of receptor-specific antibodies. Furthermore, the development of subtype specific agonists and antagonists has proven to be very difficult to achieve, since the orthosteric binding pocket of the mAChR family is highly conserved. Moreover, the interpretation of pharmacological data is not always simple, as several studies show that mAChR form exists in dimeric or oligomeric arrays, and this conformation can be affected and induced by experimental treatments (Challiss & Tobin 2009; Pradidarcheep *et al.* 2009; Zeng *et al.* 2000).

Taken together, physiological studies clearly demonstrate the importance of muscle mAChR in mediating contraction and thus have guided subsequent studies on mAChR in the ENS. As a consequence, mAChR present on muscle layers and their role in intestinal physiology is well characterised. This contrasts with the relative lack of information regarding neuronal mAChR's location and function within the ENS.

Originally, mAChR mediating the metabotropic effects of acetylcholine at nonneuronal effector cells were thought to be of the muscarinic M_3 receptor subtype (Brown & Taylor 2005; Challiss & Tobin 2009; Goyal 1988). Presently, immunohistochemistry studies in the GI tract of several species show mAChR (M_1 , M_2 and M_3) distribution on: muscle layers and ICC; myenteric and submucous ganglia; nerve fibres in the circular muscle and within ganglia; on mucosal epithelial cells and on submucous blood vessels (Harrington *et al.* 2007; 2008; Lecci *et al.* 2002; Takeuchi *et al.*

2007; Takeuchi *et al.* 2006). There are no functional or histochemical studies determining the location of M₅ within the ENS, but M₄ have been located on myenteric neurons where they seem to regulate ACh release (Takeuchi *et al.* 2005). Even so, muscarinic M₄ and M₅ receptor subtypes expression and location within the ENS are still considered ambiguous (Mansfield *et al.* 2003; Pradidarcheep *et al.* 2009).

Regarding the GI smooth muscle, a heterogeneous population of mAChR is expressed in order to mediate transmission from final motor neurons onto muscle. Many studies, both functional and anatomical, show that the majority of mAChR present on cell membranes of muscle cells are M₂ (70–80%) and M₃ (20–30%) receptors subtype, with no measurable quantities of M₁ or M₄ receptor subtypes (Tobin *et al.* 2009; Uchiyama *et al.* 2004). These mAChR mediate cholinergic-evoked muscle contraction and, even though M₃ are less abundant than M₂ on smooth muscle cells, functionally they are the dominant receptor mediating muscle contraction (Uchiyama & Chess-Williams 2004; Unno *et al.* 2005). The signalling mechanisms of the M₂ and M₃ receptors subtype within muscle differ. M₂ activation leads to adenylate cyclase inhibition, whereas the M₃ receptor subtype mediates phosphoinositide hydrolysis, resulting in calcium mobilization and muscle contraction (Sakamoto *et al.* 2007; Tobin *et al.* 2009). The role of the M₃ receptor in mediating contractile reflexes is clear-cut, while muscle contraction-induced by the M₂ receptor subtype is more complex. In the rat ileal smooth muscle, M₂ activation has specifically opposed increased levels of cAMP induced by adrenergic stimulation of adenylate cyclase. Therefore, the dual effect of mAChR on the contraction of smooth muscle is via a direct M₃-mediated contraction and an indirect M₂-mediated inhibition of the relaxation (Eglen *et al.* 1994; Ehlert *et al.* 1997; Murthy 2006; Murthy *et al.* 1997; Uchiyama & Chess-Williams 2004). However, stimulation of M₃ and M₂ receptors is also linked to additional transduction mechanisms that includes the activation of cytoplasmic phospholipase A₂ (cPLA₂) via different mechanisms: Sustained stimulation of PLA₂ activity, via M₃ receptors, involves sequential activation of G_{q/11} → PLC-β1 → PKC → ERK1/2 → cPLA₂, whereas sustained stimulation, via M₂ receptors, involves sequential activation of G_{i/o} → PI 3-kinase → Rho GTPases Cdc42/Rac1 → PAK1 → p38 MAPK → cPLA₂. Both pathways result in phosphorylation of cPLA₂, with additive effects (Gerthoffer 2005; Murthy *et al.* 2003; Zhou *et al.* 2003). Although the segregation of M₃ and M₂ signals is probably not absolute, M₂ signalling appears to modulate Ca²⁺ and non-selective cation channels, cross-bridge function and actin cytoskeleton remodelling (Gerthoffer 2005; Murthy 2006).

The effects of ACh on muscle contraction are not only due to mAChR on the smooth muscle but also by a modulatory role of these receptors on transmitter release from stimulated nerve fibres projecting onto muscle cells (Kilbinger *et al.* 1980a; Takeuchi *et al.* 2007). The functional relevance of mAChR on nerve fibres innervating the muscle is to regulate the amount of ACh release, thus modulating cholinergic-mediated muscle contraction. It has been described that presynaptic M₁ facilitate whereas M₂ receptors subtype inhibit ACh release from nerves fibres targeting muscle fibres (Kilbinger *et al.* 1980b; Soejima *et al.* 1993). Recently, both M₁ and M₂ receptors have been localised to nerve fibres in guinea-pig and human intestine: M₁ immunoreactivity co-localised with vesicular acetylcholine transporter (VACHT) and nitrous oxide synthase (NOS, EC 1.14.13.39) labelled nerve fibres, while M₂ immunoreactivity co-localised with synaptophysin (in varicosities) and nerve fibres containing substance P (SP), VACHT and NOS (Harrington *et al.* 2007; 2008; Takeuchi *et al.* 2007). Regarding M₃ receptors subtype presence on nerve fibres, given that specific antibody, agonist and antagonist drugs have been difficult to obtain, their localization and function is conflicting and requires further investigation (Pradidarcheep *et al.* 2009; Soejima *et al.* 1993).

But GI tract neuroeffector junctions are much more complicated than enteric nerve terminals lying closely apposed to smooth muscle cells. Ultrastructural studies showed that, rather than enteric motor nerves innervating smooth muscle cells directly, junctional specializations exist between enteric nerve terminals and interstitial cells of Cajal (ICC) (Sanders & Ward 2006; Ward *et al.* 2000). Cholinergic innervation depends on the activation of specific receptors on ICC, since functional neurotransmission cannot occur in the absence of these cells (Sanders *et al.* 2006; Ward *et al.* 2000; Ward *et al.* 2001a; 2001b).

Even though ICC were identified over 100 years ago (Cajal, 1909), the characteristics and functional importance of ICC to intestinal motility were only elucidated recently, due to advances in intracellular electrophysiology, the production of ICC knock-out mice and mainly to the development of immunohistochemical markers (Burns *et al.* 1997; Chen *et al.* 2007b; Sanders & Ward 2007). The immunohistochemical labelling of ICC against the antigen c-kit (receptor tyrosine kinase) has identified at least 6 types of ICC within the wall of the intestine, with distributions of ICC varying between regions of GI tract. In 2009, a new selective marker for ICC appeared: Ano1 labels all classes of ICC but, contrary to c-Kit, it does not label mast cells (Chen *et al.* 2007b; Gomez-Pinilla *et al.* 2009). Even though both M₂ and M₃ receptors subtype expression (mRNA) has been detected in ICC isolated from murine ileum, only M₃ receptors have been shown to mediate transmission from myenteric motor nerves, but there are several other conflicting

results (Chen *et al.* 2007a; Lecci *et al.* 2002; So *et al.* 2009). The mixed results in localising mAChR on ICC can either be a true reflection of regional and species differences or artifacts that occur as a consequence of using different techniques and antibodies (Harrington *et al.* 2008; So *et al.* 2009).

In addition to gastrointestinal tract neuroeffector junction complexity, the various patterns of intestinal motility are mediated by intricate enteric circuits. The characteristic peristaltic motility movement is facilitated by local enteric circuits within the myenteric plexus, with ascending excitation and descending inhibition of smooth muscle (Bornstein *et al.* 2004; Furness 2006; Wood 2006b). The cholinergic neural components of myenteric circuits controlling muscle reflexes and the location of muscarinic receptors within these circuits have been described in some detail (Harrington *et al.* 2010).

Regarding postsynaptic mAChR within the myenteric ganglia, M₁, M₃ and M₄ receptors subtype have recently been described. Within the guinea-pig intestine, M₁ receptors were localised not only on cholinergic neurons, but also on tachykinergic and nitrergic neurons (Harrington *et al.* 2007). Based on neurochemical profiling combined with functional studies, the types of neurons bearing M₁ are most likely intrinsic sensory neurons, ascending interneurons and a population of descending interneurons receiving input from sensory neurons (Harrington *et al.* 2010; Johnson *et al.* 1996). The myenteric neurons bearing M₃ and M₄ subtypes require further classification.

On the other hand, the myenteric ganglia synaptic release of ACh is regulated by inhibitory muscarinic presynaptic autoreceptors (Fosbraey and Johnson, 1980). Although M₂ receptors are known to be present on cholinergic nerves and all peptide-containing nerve fibres within the myenteric ganglia, it was only recently that they were co-localised with VACHT and SP containing nerves (Harrington *et al.* 2008; Kilbinger & Wessler 1980a).

Considering all the above, the expression “ACh is the principal transmitter in GI tract” seems to be an oversimplification of the complexity that surrounds cholinergic transmission in the ENS, antagonized by nicotinic and muscarinic receptors, located pre and/or postsynaptically in the somatodendritic region and neuroeffector junctions. In the myenteric plexus, nAChR mainly mediate fast enteric ascending reflexes, whereas postsynaptic M₁ mAChR subtype has a broader neuronal distribution. Ganglionic release of ACh seems to be regulated by presynaptic inhibitory M₂ mAChR subtype, while neurotransmitter release from nerve terminals onto the muscle is controlled by presynaptic nicotinic autoreceptors. The neuroeffector region is also very rich in mAChR, since apparently M₁ and M₂ receptor subtypes are expressed in nerve endings, whereas M₂ and M₃ receptors predominate in smooth muscle cells and ICC.

1.3 - Purinergic signalling in the ENS: role of ATP

1.3.1 Purinergic neurotransmission

In contrast to the identification of the primary neurotransmitter of the enteric excitatory neurons (acetylcholine, ACh) in the first half of the 20th century, there has been considerable difficulty and substantial debate associated with the identification of the transmitter of inhibitory neurons. It is now recognized that these neurons release cotransmitters, including adenosine triphosphate (ATP), nitric oxide (NO) and peptides, like the vasoactive intestinal peptide (VIP) (Bornstein *et al.* 2004; Brookes & Costa 2006; Furness 2009; Furness *et al.* 1973). During the 1950s and early 1960s, it was generally believed that there were two transmitters of peripheral autonomic neurons, ACh and noradrenaline (NA), with opposite functions. So, if the excitatory transmitter was ACh the inhibitory transmitter should be NA. It was also thought that each neuron could only synthesize, store and release a single substance, which became known as 'Dale's principle' (even though Dale himself has never defined it as such) (Burnstock 2009a; 2009b). However, when compounds that block noradrenergic transmission became available, a young research leader named Geoffrey Burnstock and colleagues decided to stimulate the nerves supplying the smooth muscle of the guinea-pig taenia coli in the presence of atropine and guanethidine. The idea was to block both cholinergic and adrenergic neurotransmission to see depolarisation and contraction in response to direct stimulation of the muscle. However, to their surprise, the responses to single stimuli were rapid hyperpolarisations and relaxation, the latter blocked by a newly discovered puffer fish toxin, a nerve conduction blocker – tetrodotoxin (TTX). Since that response could not be accounted for neither of the known peripheral transmitters, it was called non-adrenergic, non-cholinergic (NANC) transmission (Burnstock 2007a; 2009c; Burnstock *et al.* 1964; Burnstock *et al.* 1966). After a short period of time, evidence gathered showed that the transmitter released from these neurons was ATP. At that occasion, the major arguments in favour of ATP was that it mimicked the effects of nerve stimulation and that metabolites of ATP were released from the tissue when intramural nerves were stimulated, but it was difficult to persuade the scientific community that ATP satisfied all the criteria needed to be established as a neurotransmitter (Burnstock 1972; 2009c; Burnstock *et al.* 1970; Burnstock *et al.* 1978; Burnstock *et al.* 1972b).

Regrettably, the lack of effective and specific antagonists for ATP muscle receptors made it difficult to prove so, the “purinergic neurotransmission hypothesis” was often ridiculed at meetings and workshops over the next 25 years (Burnstock 2009b; 2009c). However, while the scientific community was struggling to accept this high-energy phosphate-containing intracellular molecule as neurotransmitter, Geoffrey Burnstock was pursuing another dogma: the ‘Dale’s principle’. The fact was that there were a number of hints in the literature (from both vertebrate and invertebrate experiments) that this might not be universally true, which led Burnstock to introduce the cotransmitter hypothesis in 1976 (Burnstock 1976a; 1978; 2009c). There are now substantial evidences showing that ATP is a cotransmitter with classical transmitters in most nerves at the peripheral and central nervous systems (CNS) (Figure 1.8), although the proportions vary between tissues and species as well as in different developmental and pathophysiological circumstances. Moreover, ATP released as a cotransmitter can act both as a postjunctional modulator, enhancing the responses of their cotransmitter, and as a prejunctional modulator of transmitter release (Figure 1.8) (Burnstock 1981; 2004).

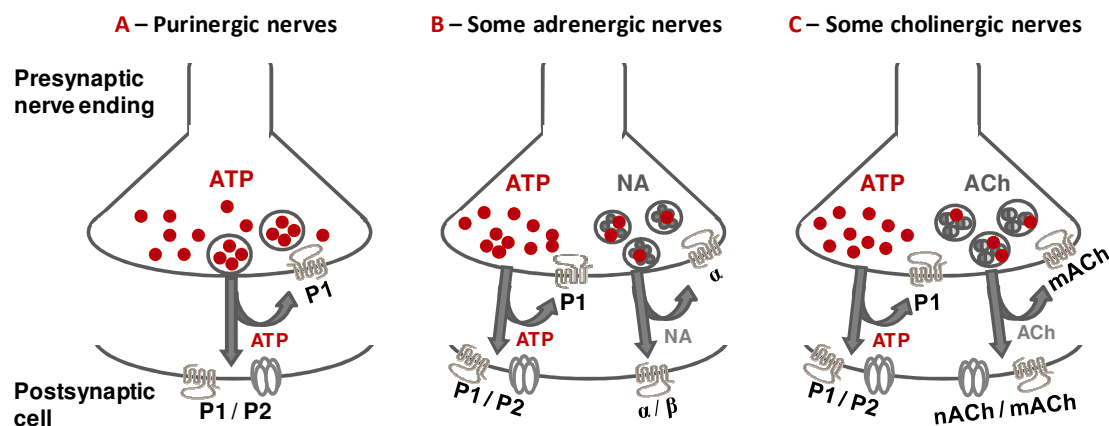


Figure 1.8 - Schematic representation of presynaptic neuromodulation of transmitter release. A) Reduction of ATP release by activation of presynaptic P1 purinoceptors. B) Reduction of noradrenaline release by activation of presynaptic α -adrenoceptors and P1 purinoceptors. C) Reduction of ACh release by activation of presynaptic muscarinic and P1 purinoceptors.

In spite of its difficult beginning, “purinergic neurotransmission” grew strong and there is now substantial evidence to support widespread purinergic signalling in both neuronal and non-neuronal systems. In essence, the purinergic neurotransmission hypothesis stands like Geoffrey Burnstock described in 1972: “ATP, stored inside vesicles in nerve varicosities, is released by exocytosis to act on postjunctional receptors for ATP on smooth muscle. ATP is broken down extracellularly by ATPases and 5-nucleotidase to generate adenosine, which is taken up by varicosities to be resynthesized and reincorporated into synaptic vesicles. Adenosine is broken down further on by adenosine deaminase to inosine and removed by the circulation” (Burnstock 1972; 1981; 2007a; Sperlagh *et al.* 2007a; Williams *et al.* 2000).

1.3.2 ATP releasing pathways

A significant progress on the purinergic machinery description occurred in the last few years, and since ATP is a highly polarized molecule which cannot pass freely the cell membrane, the nucleotides releasing pathways are still matter of debate. Besides nonspecific massive leakage of nucleotides that occurs upon cell lysis, three non-lytic mechanisms of nucleotide efflux represent a distinct and important route of nucleotide appearance in the extracellular milieu (Bours *et al.* 2006; Burnstock 2007a; Sperlagh *et al.* 2007a; Yegutkin 2008). The proposed cellular mechanisms include (1) electrodiffusional movement through membrane ion channels, including connexin hemichannels, stretch- and voltage-activated channels, (2) facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters and (3) classical cargo-vesicle trafficking and exocytotic granule secretion (Figure 1.9) (Burnstock 1981; Fitz 2007; Lazarowski *et al.* 2003; Schwiebert *et al.* 2003; Sperlagh *et al.* 2007a).

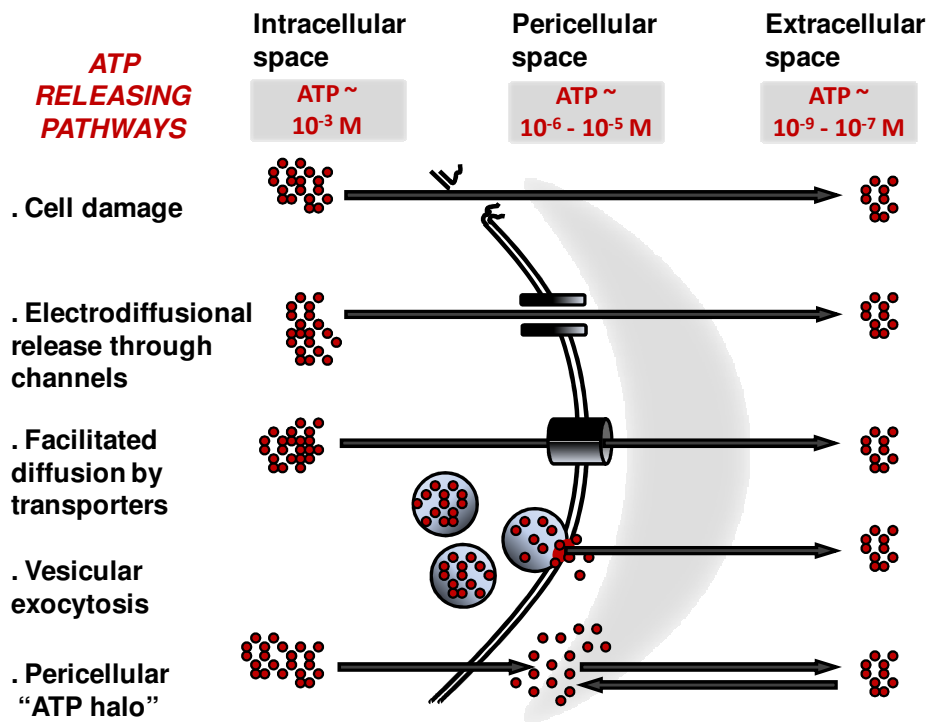


Figure 1.9 - Schematic representation of nucleotide-releasing pathways: massive nucleotide leakage upon cell damage; electrodiffusional movement through ATP release channels; facilitated diffusion by nucleotide-specific transporters; vesicular exocytosis and the pericellular "ATP halo".

However, considering that most P2 receptors activation occurs at nucleotide concentrations within the range of 10^{-6} to 10^{-4} M, it was difficult to explain the nanomolar ATP levels reported in extracellular fluids. Recent innovative nucleotide sensing assays (other than direct addition of luciferin/luciferase to bathing medium of cell suspensions) provided independent evidence that most of the released ATP is retained in the pericellular space without significant convection into the extracellular milieu. These cell surrounding ATP concentrations are sufficient for triggering purinergic responses - a phenomenon recently described as "ATP halo" (Figure 1.9) (Corriden *et al.* 2007; Llaudet *et al.* 2005; Nakamura *et al.* 2006; Sperlagh *et al.* 2007a; Yegutkin 2008).

1.3.3 P2 Purinergic Receptors

After being released to extracellular space, and in spite of its short half-life, effective concentrations of nucleotides can be reached in the synapse and P2 purinergic receptors activation occurs with subsequent cellular effect. Purinergic receptors were first defined in 1976. Two years later, two types of purinoceptors were distinguished: P1 (more sensitive to adenosine, competitively blocked by methylxanthines) and P2 (more sensitive to ATP/ADP, not competitively blocked by quinidine, apamin and some other non-specific drugs) (Burnstock 1976b; 1981). It was not until 1985 that a proposal suggesting a pharmacological basis for distinguishing two types of P2 receptors (P2X and P2Y) was made, and during the 90's, several G protein-coupled and ion-gated P2 receptors were cloned (Burnstock *et al.* 1985; Valera *et al.* 1994; Webb *et al.* 1993). That led Abbracchio and Burnstock to propose in 1994, on the basis of molecular structure and transduction mechanisms, that purinoceptors should belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. This nomenclature has been widely adopted and, since then, seven P2X and eight P2Y receptor subtypes were recognized, including receptors that are sensitive to purines as well as pyrimidines – UTP and UDP (uridine tri- and diphosphate, respectively) (Abbracchio *et al.* 1994; Burnstock 2007a; 2007b; Ralevic *et al.* 1998).

Ionotropic P2X receptors are nonselective cation channels, consisting of at least three subunits, forming a stretched trimer or a hexamer of conjoined trimers. P2X receptor subunits are 379-595 amino acid long polypeptide chains, having two transmembrane domains (TMD) and a large extracellular loop. TMD1 is involved with channel gating whereas TMD2 forms the lining of the ion pore (Burnstock 2007b; Ralevic & Burnstock 1998; Sperlagh *et al.* 2007a; Valera *et al.* 1994). Until now, seven members of this receptor family have been identified molecularly, numbered from P2X1 to P2X7. They show 30–50% sequence identity at the amino acid level and have individual kinetics and pharmacological phenotype. Basically, P2X receptors are sensitive to ATP and to its various synthetic analogues but not to AMP and adenosine (more detailed in Table 1.5) (Burnstock 2007b; Gever *et al.* 2006; Jarvis *et al.* 2009; Sperlagh *et al.* 2007a). P2X ligand-gated ion channel receptors are permeable to both monovalent (Na^+ , K^+) and divalent (Ca^{2+}) cations and the activation of the receptor generates an inward current leading to the local depolarization of the cell membrane. In addition, the Ca^{2+} influx through the receptor-ion channel complex can directly trigger transmitter release (Burnstock 2007a; 2007b; 2008a; Sperlagh *et al.* 2007a). Current genetic, biochemical, pharmacological and/or physiological evidence indicates that the extended family of

functional P2X receptors includes six homomeric channels composed of P2X1, P2X2, P2X3, P2X4, P2X5 or P2X7 subunits (P2X6 receptors do not seem to form a functional homomeric receptor without extensive glycosylation) and seven heteromeric channels that involve subunit pairings of P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 or P2X4/7 (Figure 1.10) (Burnstock 2007b; Nicke *et al.* 2005; North 2002; Sperlagh *et al.* 2007a).

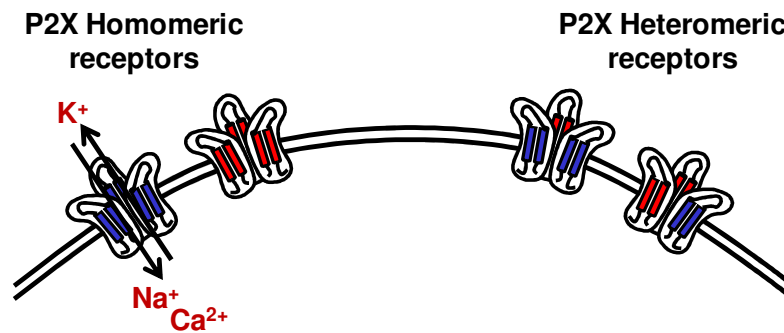


Figure 1.10 - Schematic representation of ionotropic P2X homomeric (*e.g.* P2X1, P2X2, P2X3, P2X4, P2X5 or P2X7) and heteromeric receptors (*e.g.* P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 or P2X4/7).

Since P2X receptors subunits can combine to form either homomultimers or heteromultimers, their pharmacology is complicated (see *e.g.* Jarvis and Khakh 2009). On Table 1.5, the main characteristics of P2X homomultimers receptors are described (distribution, agonists, antagonists and transduction mechanisms). Nevertheless, transgenic mice genetically deficient in individual P2X receptor subtypes are also being used for further P2X receptor identification (Burnstock 2007b; Gever *et al.* 2006; Jarvis & Khakh 2009; Sperlagh *et al.* 2007a).

Whereas P2X ionotropic receptors convey rapid changes in the neuronal excitability on the millisecond timescale, P2Y metabotropic receptors act on a longer second timescale, appropriate for the fine-tuning of synaptic transmission (Abbracchio *et al.* 2006; Sperlagh *et al.* 2007a).

Table 1.5 – Characteristics of P2 purinoceptors. .

Receptor	Main distribution	Agonists	Antagonists	Transduction mechanisms
P2X				
P2X1	smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	α, β -meATP = ATP = 2-MeSATP, L β, γ -meATP (rapid desensitisation)	TNP-ATP, IP ₅ I, NF023, NF449	intrinsic cation channel (Ca ²⁺ and Na ⁺)
P2X2	smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP $\geq$ ATP γ S $\geq$ 2-MeSATP $\gg$ α, β -meATP (pH + zinc-sensitive)	suramin, isoPPADS, RB2, NF770, NF279	intrinsic ion channel (particularly Ca ²⁺)
P2X3	sensory neurons, NTS, some sympathetic neurons	2-MeSATP $\geq$ ATP $\geq$ α, β -meATP $\geq$ AP ₄ A (rapid desensitisation)	TNP-ATP, PPADS, A317491, NF110, Ip ₅ I, phenol red	intrinsic cation channel
P2X4	CNS, testis, colon	ATP $\gg$ α, β -meATP, CTP, Ivermectin potentiation	TNP-ATP (weak), BBG (weak), phenolphthalein	intrinsic ion channel (especially Ca ²⁺)
P2X5	proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP $\gg$ α, β -meATP, ATP γ S	suramin, PPADS, BBG	intrinsic ion channel
P2X6	CNS, motor neurons in spinal cord	(does not function as homomultimer)	-	intrinsic ion channel
P2X7	apoptotic cells (e.g. immune cells), pancreas, skin	BzATP $>$ ATP $\geq$ 2-MeSATP $\gg$ α, β -meATP	BBG, KN62, KN04, O-ATP, A438079, MRS2427, Coomassie blue	intrinsic cation channel and a large pore with prolonged activation
P2Y				
P2Y₁	epithelial and endothelial cells, platelets, immune cells, osteoclasts	2-MeSADP = ADP β S $>$ 2-MeSATP = ATP, MRS2365	MRS2179, MRS2500, MRS2279, PIT	G _q /G ₁₁ ; PLC- β activation
P2Y₂	immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP = ATP, UTP γ S, INS 37217, INS 365	suramin $\gg$ RB2, ARC126313	G _q /G ₁₁ and possibly G _i ; PLC- β activation
P2Y₄	endothelial cells	UTP $\geq$ ATP, UTP γ S, INS 37217	RB2 $>$ suramin	G _q /G ₁₁ and possibly G _i ; PLC- β activation
P2Y₆	some epithelial cells, placenta, T cells, thymus	UDP $\gg$ UTP $>$ ATP, UTP γ S, IDP	MRS2578	G _q /G ₁₁ ; PLC- β activation
P2Y₁₁	spleen, intestine, granulocytes	AR-C67085MX $>$ BzATP $\geq$ ATP γ S $>$ ATP	suramin $>$ RB2, NF157, 5'-AMPS	G _q /G ₁₁ and G _s ; PLC- β activation
P2Y₁₂	platelets, glial cells	2-MeSATP $\geq$ ADP $\gg$ ATP	CT50547, AR-C69931MX, INS49266, AZD6140, PSB0413, ARL66096, 2-MeSAMP	G _{i/o} ; inhibition of adenylate cyclase
P2Y₁₃	spleen, brain, lymph nodes, bone marrow	ADP = 2-MeSADP $\gg$ ATP = 2-MeSATP	MRS2211, 2-MeSAMP	G _{i/o}
P2Y₁₄	placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP glucose = UDP-galactose	-	G _{i/o}

The first P2Y receptors were cloned in 1993 and since then several other subtypes have been isolated by homology cloning and a subscript was assigned based on cloning chronology. At present, there are eight accepted mammalian P2Y receptors: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ (Table 1.5). The missing numbers represent either non-mammalian orthologs or receptors having some sequence homology to P2Y receptors, but for which there is no functional evidence of responsiveness to nucleotides (Abbracchio *et al.* 2006; Burnstock 2007a; Webb *et al.* 1993). All P2Y receptors belong to G protein-coupled receptors, having seven hydrophobic transmembrane domains. ATP binding sites were identified on the external side of TMD3 and TMD7.

Some P2Y receptor subtypes are activated mostly by nucleoside diphosphates (P2Y_{1,6,12,13}), while others are activated mainly by nucleoside triphosphates (P2Y_{2,4,11}). Three P2Y receptor subtypes are activated by both purine and pyrimidine nucleotides (P2Y_{2,4,6}), and others by purine nucleotides alone (P2Y_{1,11,12,13}) (more detailed in Table 1.5) (Abbracchio *et al.* 2006; Burnstock 2007a; Sperlagh *et al.* 2007b). As for the signal transduction pathways activated by various subtypes of P2Y receptors, P2Y_{1,2,4,6,11} receptors are coupled to G_{q/11} proteins to stimulate phospholipase C, followed by increases in inositol phosphates and mobilization of Ca²⁺ from intracellular stores; furthermore, the P2Y₁₁ receptor mediates an increase in adenylyl cyclase activity. Additionally, P2Y_{12,13,14} receptors are coupled to G_{i/o} proteins to inhibit adenylyl cyclase activity followed by a decrease in intracellular cAMP levels (Figure 1.11). The activation of G_i protein-coupled P2Y receptors leads to the voltage-dependent inhibition of N-type voltage-sensitive Ca²⁺ channels, directly or indirectly, with subsequent inhibition of neurotransmitter release (Abbracchio *et al.* 2006; Burnstock 2007b; Hussl *et al.* 2006; Sperlagh & Vizi 2007b; von Kugelgen 2006).

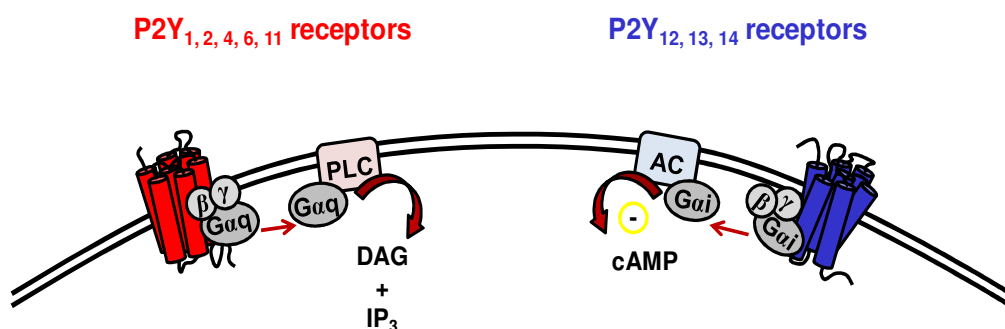


Figure 1.11 - Schematic representation of major P2Y receptors signalling pathways. P2Y_{1,2,4,6,11} receptors-induced activation of phospholipase C (PLC) occurs through G_q proteins, leading to intracellular increase of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Activation of P2Y_{12,13,14} receptors commonly inhibits adenylyl cyclase (AC) activity through activation of G_i proteins, decreasing intracellular cyclic AMP (cAMP) levels.

Although available P2Y receptor ligands display some subtype selectivity, identification of individual P2Y receptors requires careful pharmacological analysis and, if possible, the combined use of receptor knockout animals. Moreover, an exclusion of the involvement of adenosine receptors in the effect of nucleotides used as P2Y agonists should be performed. Potential heteromerization of P2Y receptor subtypes with each other and with A₁ adenosine receptors should also be considered (Abbracchio *et al.* 2006; Hussl & Boehm 2006; Nakata *et al.* 2005; Suzuki *et al.* 2006; von Kugelgen 2006).

1.3.4 ATP degrading enzymes

After ATP release and P2X and/or P2Y receptors activation, ATP is rapidly broken down to ADP, AMP and adenosine (ADO), either sequentially or bypassing ADP formation. Extracellular concentrations of nucleotides are accurately regulated by several groups of membrane-associated ecto-enzymes. These ecto-enzymes belong to four different groups: alkaline phosphatases (AP, EC 3.1.3.1), ecto-nucleotide pyrophosphatase / phosphodiesterases (E-NPP, EC 3.1.4.1), ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase, EC 3.6.1.5) and ecto-5'-nucleotidase (E-5'-NT, 3.1.3.5) (Burnstock 2007a; Lazarowski *et al.* 2003; Matsuoka *et al.* 2004; Zimmermann 2000). Amongst them, alkaline phosphatases are ubiquitous enzymes that display broad substrate specificity towards different phosphomonoesters and other phosphorylated compounds with little selectivity (Lazarowski *et al.* 2003; Yegutkin 2008). NPPs also have broad substrate specificity, since they can hydrolyze phosphodiester bonds of nucleotides and nucleic acids and pyrophosphatase bonds of nucleotides and nucleotide sugars so they can, for example, do the cleavage of ATP to AMP and inorganic pyrophosphate (Pedata *et al.* 2007) and the conversion of cAMP to AMP. The E-NPP family consists of seven structurally related ecto-enzymes (NPP1 through NPP7) that are numbered according to their order of cloning. Only the first three members of this family, NPP1, NPP2 and NPP3 (see previous/alternative names in Table 1.6) are capable of hydrolysing various nucleotides and are therefore relevant in the context of the purinergic signalling cascade (Burnstock 2007a; Stefan *et al.* 2006; Yegutkin 2008; Zimmermann 2000).

Table 1.6 – Ecto-enzymes involved in extracellular adenine nucleotides / nucleosides catabolism.

Ecto-enzyme Family	Ecto-enzyme (common name)	Ecto-enzyme (alternative name)	Catalyzing reactions
AP (EC 3.1.3.1)	Alkaline phosphatases		ATP→ADP→AMP→ADO
E-NPP (EC 3.1.4.1)	NPP1 NPP2 NPP3	PC-1 Autotaxin, PD-I α gp130 ^{RB13-6} , B10, PD-I β	ATP→AMP ATP→AMP (LPC→LPA) ATP→AMP
E-NTPDase (EC 3.6.1.5)	NTPDase1 NTPDase2 NTPDase3 NTPDase8	Ecto-apyrase, CD39 Ecto-ATPase, CD39L1 HB6, CD39L3 liver canalicular ecto-ATPase, hATPDase	ATP→AMP / ADP→AMP ATP→ADP ATP→ADP→AMP ATP→ADP→AMP
E-5'-N (EC 3.1.3.5)	Ecto-5'-NTDase	CD73	AMP→ADO

ADO, adenosine; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid.

E-NTPDase family is capable of hydrolysing nucleoside tri and/or diphosphates but not monophosphates and requires millimolar concentrations of Ca^{2+} and Mg^{2+} for maximal activity. NTPDases have diverse membrane topography, catalytic properties and substrate affinity. NTPDase4-7 are intracellular enzymes that preferentially hydrolyze nucleoside diphosphates and triphosphates apart from ATP. The principal substrates of NTPDases4 and 7 are uridine di and triphosphate (UDP and UTP), while NTPDase5 and NTPDase6 hydrolyze preferentially UDP and GDP, respectively (Figure 1.12). The last two enzymes can be cleaved from the endoplasmic reticulum (NTPDase5) and Golgi apparatus (NTPDase6) where they are localised, and released from cells in a soluble form (Burnstock 2007a; Matsuoka & Ohkubo 2004; O'Keeffe *et al.* 2010; Robson *et al.* 2006; Yegutkin 2008). Four NTPDases are typical cell surface-located enzymes with the catalytic site facing extracellularly (NTPDase 1, 2, 3, 8). NTPDase1 hydrolyzes ATP and ADP with similar rates, whereas NTPDase2 hydrolyzes ATP with high selectivity over ADP. NTPDase3 and 8 hydrolyze ATP with some selectivity over ADP (Figure 1.12). Since NTPDases1, 2, 3 and 8 efficiently hydrolyzed ATP with K_m values in the micromolar range (K_m values for ATP of murine NTPDase1, 2, 3 and 8 are approximately 12 μM , 37 μM , 11 μM and 13 μM , respectively), they should terminate the effects exerted by these nucleotide agonists at P2X1-7 and P2Y_{2,4,11} receptors. Since NTPDase1 does not allow accumulation of ADP, it should terminate the activation of P2Y_{1,12,13} receptors far more efficiently than the other NTPDases. In contrast, NTPDases2, 3 and 8 are expected to promote the activation of ADP specific receptors, since they produce ADP accumulation (Burnstock 2007a; Kukulski *et al.* 2005; Lazarowski *et al.* 2003; Matsuoka & Ohkubo

2004; Zimmermann 2000). Furthermore, NTPDase1 catalyzes the key step for extracellular breakdown of ATP and ADP (both important signalling molecules) and is responsible for extracellular adenosine production by generation of AMP. As such, pharmacological inhibition or genetic deletion of NTPDase1 is associated with attenuated extracellular adenosine levels and defects in extracellular adenosine signalling, associated with elevated ATP or ADP signalling effects (Atkinson *et al.* 2006; Reutershan *et al.* 2009).

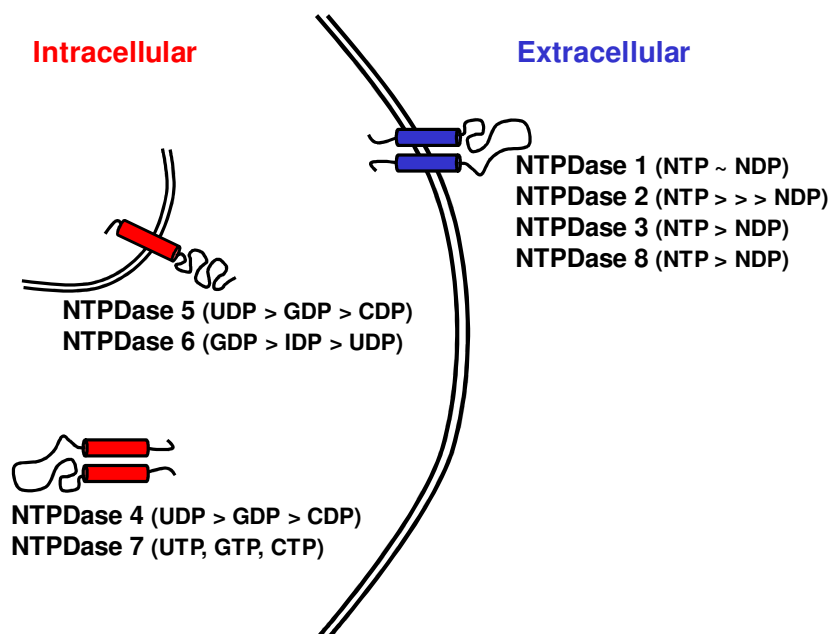


Figure 1.12 – Cellular localization of NTPDases (extracellular: blue TMD and intracellular: red TMD), major substrate preferences and predicted topography. NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate.

The final step of ATP extracellular inactivation is the hydrolysis of AMP by the ecto-5'-NTDase (E-5'-N), a zinc-binding metalloenzyme (see previous/alternative names in Table 1.6) that originates adenosine (ADO), the endogenous ligand for P1 receptors. Seven human 5'-nucleotidases have been isolated and characterised. Five of the enzymes are located in the cytosol, one in the mitochondrial matrix and the other one, attached to the outer plasma membrane, is E-5'-N (Sperlach *et al.* 2007a; Strater 2006; Yegutkin 2008). Contrary to related intracellular 5'-nucleotidases, this ecto-enzyme shows no activity towards nucleoside 2'- and 3'-monophosphates. E-5'-N has a high affinity activity for AMP, with a K_m value within the range of 5 to 20 μ M (Le Hir *et al.* 1989; Strater 2006; Turnay *et al.* 1992; Yegutkin 2008; Zimmermann 2000). Genetic deletion or pharmacological inhibition of E-5'-NT is associated with elevated AMP and attenuated

adenosine levels within the extracellular fluids. Comparative measurements of E-5'-NT activity in wild-type or E-5'-NT $-/-$ mice revealed that E-5'-NT levels are particularly high in the intestine, brain, kidneys and lungs (Hart *et al.* 2008; Thompson *et al.* 2004). The molecular identification of individual ecto-nucleotidases subtypes, genetic engineering, mutational analyses, and the generation of subtype-specific antibodies have not only led to considerable insight into enzyme structure and function, but have also defined physiological and pathophysiological functions of ecto-nucleotidases in a substantial variety of tissues (Bianco *et al.* 2005; Bonan *et al.* 2000; Zimmermann 2000).

Nevertheless, it is worth noting that, at least in principle, the duration and magnitude of purinergic signalling can be coordinated via two opposite, ATP-consuming and ATP-regenerating pathways. Sequential nucleotide breakdown to adenosine and further to inosine is counterbalanced by re-synthesis of high-energy phosphoryls through backward phosphotransfer reactions, catalyzed by adenylate kinase (ADK, EC 2.7.4.10), for example (Donaldson *et al.* 2002; Lazarowski *et al.* 1997; Yegutkin *et al.* 2001). Although under normal physiological conditions ecto-nucleotide kinases do not seem to compete with ecto-nucleotidases activities, under pathological conditions, the extracellular levels of nucleotides increase and ecto-nucleotide kinases may exert a limited effect (see Figure 1.13 in Section 1.4.1). Acute changes in the specific ratios of nucleoside mono-, di- and triphosphates, in conjunction with feed-forward inhibition of ecto-5'-nucleotidase activity by its precursors (*e.g.* adenosine formation is delayed with increasing concentrations of ATP and/or ADP), could determine the shift towards nucleotide synthesis or degradation (Cunha 2001; Magalhães-Cardoso *et al.* 2003; Redegeld *et al.* 1999; Yan *et al.* 1999; Yegutkin *et al.* 2002).

1.3.5 Purinergic neurotransmission in the gut

Considering what is now known about ATP release, P2 receptors activation and the catabolism of extracellular adenine nucleotides, it is interesting to remember that the concept of purinergic signalling arose from experiments designed to find the identity of the NANC inhibitory neurotransmitter in the gut (Burnstock 1972; Burnstock *et al.* 1970; Burnstock *et al.* 1972a). It has taken many years and a considerable effort for the importance of the various roles of ATP as a physiological messenger in the gut to be recognized (Bornstein 2008; Burnstock 2008c; Ren *et al.* 2008). P2X and P2Y receptor subtypes have been identified on myenteric, submucous, motor, sensory and interneurons involved in synaptic neurotransmission, neuromodulation and reflex activities, including ascending excitatory and descending inhibitory pathways.

Although functional validation should be added to some initial data, it is now possible to know the geographic distribution of P2 purinoceptors on neuronal and non-neuronal cells in the gut (Burnstock 2008b). Extrinsic vagal and sacral parasympathetic nerves connect with NANC inhibitory neurons in the myenteric plexus expressing P2X₂, P2X₃, P2Y₁, P2Y₆ receptors, as well as with cholinergic motor neurons; these neurons are also activated by descending interneurons (Castelucci *et al.* 2003; Wood 2006b; Xiang *et al.* 2004; 2006). Extrinsic sympathetic nerves modulate motility via excitatory motor neurons and constrict blood vessels in the gut via P2X₁ receptors. Extrinsic sensory nerves, arising from cell bodies in dorsal root ganglia and with subepithelial terminals, mediate nociception (Bertrand *et al.* 2002; Bornstein 2008; Evans *et al.* 1992). Intrinsic sensory neurons, in both myenteric and submucous plexi, express P2X₂, P2X₃ receptors and P2Y₁₂ receptors; they connect with motor pathways involved in peristalsis (Bertrand & Bornstein 2002; Bian *et al.* 2004; Xiang & Burnstock 2006). Excitatory motor neurons express P2X₂, P2X₃, P2X_{2/3}, P2X₅ and P2Y₂ receptors and connect with both interneurons and secretomotor neurons. Interneurons express P2X₂ and P2X₃ receptors (Bertrand & Bornstein 2002; Bian *et al.* 2003; Xiang *et al.* 2005). P2X₇ receptors appear to act as prejunctional modulators of both motor and interneurons (Hu *et al.* 2001). Enteric glial cells express P2Y₄ and P2X₇ receptors, while interstitial cells of Cajal express P2X₂, P2X₅ and P2Y₄ receptor subtypes (Hu *et al.* 2001; Ruan *et al.* 2005; Van Nassauw *et al.* 2006).

Although P2 receptors distribution in ENS is rather complex, it has proven to be helpful to unravel several neurogastroenterology dilemmas. One of them relates to synaptic transmission and neuromodulation in enteric ganglionic neurons. Intracellular electrophysiological recordings indicate that ATP regulates synaptic transmission by pre- as well as postsynaptic modulation mechanisms. Neuro-neuronal fast synaptic potentials, in both ascending and descending reflex pathways to the longitudinal and circular muscles, seem to be mediated by P2X receptors composed of P2X₂ and P2X₃ subunits, whereas slow synaptic potentials involve P2Y₁ receptors (Bertrand & Bornstein 2002; Bian *et al.* 2003; Kimball *et al.* 1995; Wood 2006b; Xiang & Burnstock 2004).

Regarding intramural enteric sensory neurons, ATP appears to activate intestinal submucous terminals of intrinsic sensory neurons, supporting the hypothesis that ATP released from mucosal epithelial cells has a dual action on P2X₃ and/or P2X_{2/3} receptors in the subepithelial sensory nerve plexus. These receptors may contribute to the detection of intraluminal pressure increases and initiation of reflex contractions (Bian *et al.* 2004; Burnstock 2001; Wynn *et al.* 2003). Purinergic mechanosensory transduction has also been implicated in reflex control of intestinal secretion, whereby ATP released from

mucosal epithelial cells acts on P2Y₁ receptors on enterochromaffin cells to release 5-HT, which leads to regulation of secretion either directly or via intrinsic reflex activity (Cooke *et al.* 2003; Wood 2006a).

Additionally, ATP receptors are also expressed in enteric non-neuronal cells. Interstitial cells of Cajal (ICC) are innervated by enteric nerves and express P2X₂, P2X₅ and P2Y₄ receptors. They seem to induce intracellular calcium oscillations, modulating ICCs pacemaker activity (Burnstock *et al.* 2002; Furuzono *et al.* 2005; Van Nassauw *et al.* 2006). Regarding enteric glia, which out-numbers enteric neurons by at least 2:1, it responds to ATP and UTP (increasing intracellular calcium) probably via P2Y₂ or P2Y₄ receptors activation. Immunohistochemical studies also showed expression of P2X₇ receptors on S100 immunolabelled enteric glial cells. Nevertheless, P2 receptors role on enteric glial cells and its eventual analogy with neuron–glial cell interactions in the CNS remains to be elucidated (Van Nassauw *et al.* 2006; Vanderwinden *et al.* 2003).

Although the knowledge gathered on ATP receptors distribution and their role in a variety of physiological activities in the gastrointestinal tract of mammals (including man) have increased considerably over the past years, the pathophysiological conditions under which they act remain unclear. Following gut injury, ischemia and during inflammation, the excitability of visceral afferent nerves is enhanced and several substances, like ATP, are released from various sources causing sensitization of afferent nerves to mechanical and/or chemical stimuli. ATP receptors represent potential targets for drug treatment aimed at attenuating the inappropriate visceral sensation and subsequent reflex activities that underlie abnormal bowel function and visceral pain (Bornstein 2008; Burnstock 2006; Ren & Bertrand 2008). Until now, P2 receptors expression and/or function were found altered in aganglionic intestine in Hirschsprung's disease (decrease in P2X₃, P2Y₁ and P2Y₂ receptors, which may explain the contracted state of the disease) and in Chaga's disease (enhancement of P2X₇ expression receptor during acute phase) (Coutinho 1998; Facer *et al.* 2001; O' Donnell *et al.* 2008). Regarding motility disorders, like postoperative ileum, it has been generally suggested that P2X agonists may enhance gastrointestinal propulsion and secretion, which could be useful for treating constipation-predominant irritable bowel syndrome (IBS), while P2X antagonists might be useful for treating diarrhoea-predominant inflammatory bowel diseases (IBD) (Szentpali *et al.* 2001; Xiang & Burnstock 2004). In IBS, P2X₃ receptor expression seems to be increased in the enteric plexi and P2Y₆ and P2X₇ also seem to be implicated, suggesting a complex potential role in dysmotility and pain (Burnstock 2008c; Vanderwinden *et al.* 2003).

Although P2 receptors interest as potential therapeutic targets has just begun, it has not been accompanied by equal curiosity in pharmacological manipulation of ATP release and degradation mechanisms under pathological conditions. Extracellular ATP catabolism leads to adenosine formation and subsequent P1 receptors activation so, in our opinion, a more integrative view of purinergic cascade alteration during pathophysiological conditions should be taken into consideration.

1.4 – Purinergic signalling in the ENS: role of adenosine

1.4.1 Adenosine as a neuromodulator

In the early 70's, Geoffrey Burnstock was struggling for the acceptance of the purinergic neurotransmission hypothesis by the scientific community. Back then, adenosine was considered as a product of ATP catabolism that could be further broken down by adenosine deaminase (ADA, EC 3.5.4.4) to inosine and removed by the circulation (Burnstock 1981; Burnstock *et al.* 1972b). However, Drury and Szent-Gyorgyi and his group, back in 1927, demonstrated something very different. They described their surprise when, after an intravenous injection of cardiac tissue extracts, a transient disturbance of the cardiac rhythm and slowing of the heart rate was observed. After several purification steps, they were able to identify an “adenine compound” as the biologically active compound (Burnstock 2007b; 2009b; Drury *et al.* 1929; Eltzschig 2009).

More than 50 years have passed since the heart rate-slowng effect of “adenine compounds” was discovered and the clinical use of adenosine in supraventricular tachycardia has emerged (Belhassen *et al.* 1984; Burnstock 2007b; Eltzschig 2009). In between, Ginsborg and Hirst (1972) studied the effects of adenosine in the rat phrenic nerve diaphragm preparation, demonstrating the role of adenosine as an inhibitory modulator of transmitter release. The hypothesis that all adenine-based purines were acting by a common mechanism emerged when, in the same preparation, both ATP and ADP depressed ACh release (Ginsborg *et al.* 1972; Gonçalves *et al.* 2008; Ribeiro *et al.* 1975). These findings originated confusion in the scientific community, since rapid extracellular breakdown of ATP to adenosine could be misleading. However, in 1978 Geoffrey Burnstock clarified this problem by proposing a basis for distinguishing two types of purinergic receptors: P1 adenosine-sensitive receptors (which were antagonized by methylxanthines) and P2 receptors, activated by the adenine nucleotides ATP and ADP. Furthermore, he extended the purinergic neurotransmission concept, by identifying the post-junctional receptors as P2, while the pre-junctional P1 receptors mediated negative feedback responses (Burnstock 1981; 2007b). At about the same time, two subtypes of the P1 (adenosine) receptor were recognised, since adenosine was either inhibiting or stimulating adenylate cyclase activity, indicating the existence of two P1 receptors that differ functional and pharmacologically (Londos *et al.* 1980; van Calker *et al.* 1979). In 1989, complementary DNAs encoding two different P1 receptor subtypes (named A₁ and A₂) were isolated, and A₂ receptor was identified in cholinergic synapses (increasing ACh release) almost simultaneously in the CNS and at the cholinergic nerve terminals of the neuromuscular junction (Brown *et al.* 1990; Correia-de-Sá *et al.* 1991; Libert *et al.* 1989).

So, adenosine acts as an extracellular signalling molecule that influences synaptic transmission but, contrasting with ATP, it is neither stored nor released as a classical neurotransmitter, being generally considered as a neuromodulator of neurotransmitters release in central and peripheral nervous systems (Gonçalves & Queiróz 2008; Sebastião *et al.* 2000; Sebastião *et al.* 2009). Adenosine is a ubiquitous molecule that, under normal conditions, is continuously formed both intra and extracellularly. The extracellular basal levels of this adenine nucleoside have been roughly estimated between 20nM to 100nM, but they can rapidly rise to the micromolar range if ischemic stress, inflammation or other insults (that can eventually determine cell death) occur (Figure 1.13) (Antonioli *et al.* 2008a; Fredholm *et al.* 2001; Jacobson 2009; Pedata *et al.* 2007).

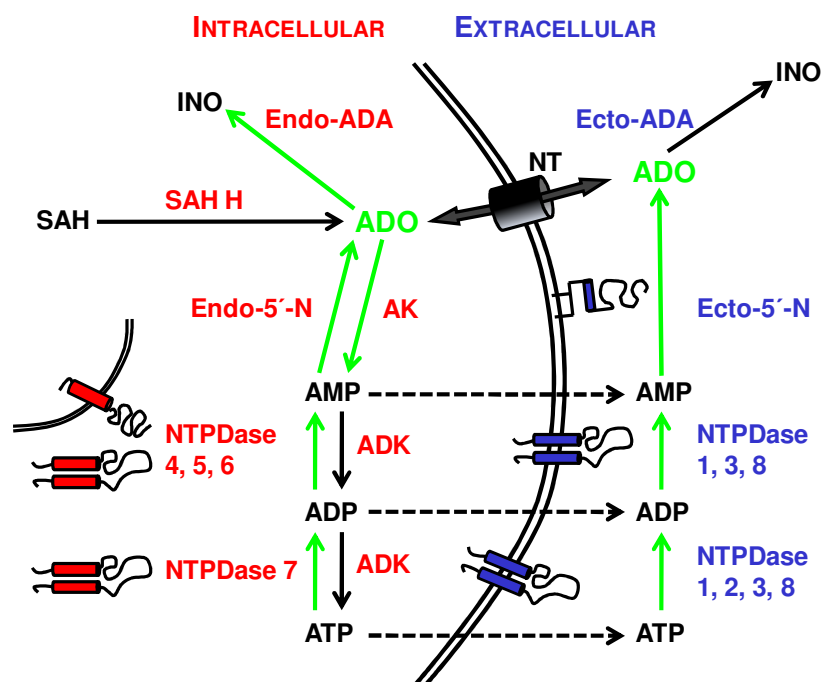


Figure 1.13 - Schematic diagram illustrating the biosynthesis and catabolism of adenosine. Under physiological conditions, adenosine is formed mainly at intracellular level from S-adenosylhomocysteine (SAH) by SAH hydrolase (SAH H) and transported across cell membranes by nucleoside transporters (NT). Intracellularly adenosine (ADO) undergoes rapid phosphorylation to adenosine monophosphate (AMP) by adenosine kinase (AK) and then to adenosine di (ADP) and triphosphate (ATP) by adenylate kinase (Govindarajan *et al.* 2007). ADO intracellular levels are maintained due to ADO bidirectional transport through NT and deamination to inosine (INO) by endo-adenosine deaminase (endo-ADA). Pathological conditions (green arrows) are associated with increased intracellular and extracellular dephosphorylation of adenine nucleotides by nucleoside triphosphate diphosphohydrolases (NTPDase) and subsequent ADO formation by 5'-nucleotidase (5'-N). In the extracellular environment, ADO concentrations are controlled by ecto-adenosine deaminase (ecto-ADA).

1.4.2 Adenosine generation and transport

The intracellular production of adenosine is mediated either by an intracellular 5'-nucleotidase or by S-adenosyl-homocysteine (SAH) hydrolase (SAH H, EC 3.3.1.1). Hydrolysis of SAH is not closely linked to the energy state of the cell and provides one-third of adenosine production under normoxic conditions in the heart, but does not seem to significantly influence adenosine levels either under normoxic or ischemic conditions in the brain (Latini *et al.* 2001; Pedata *et al.* 2007; Schepp *et al.* 2008). Regarding intracellular AMP catabolism, two soluble 5'-nucleotidases are found in cytosolic fractions, but one of them is 15-20 times more active on AMP than IMP. Because their K_m values for AMP are much higher (1 ± 14 mM) than the intracellular concentration of AMP under physiological conditions (0.1 ± 0.5 mM), some authors consider that both enzymes respond only to a large increase in AMP concentration during increased metabolic activity (Figure 1.13) (Antonioli *et al.* 2008a; Latini & Pedata 2001; Pedata *et al.* 2007).

In normal conditions, adenosine is present in a higher concentration inside than outside the cell, and does not freely diffuse across the cell membrane. Intracellularly generated adenosine is transported into the extracellular space mainly via specific bi-directional transporters, through facilitated diffusion (Figure 1.13). Two major classes of nucleoside transport systems have been described in mammalian cells, the equilibrative and the concentrative transport systems (Antonioli *et al.* 2008b; Fredholm *et al.* 2001; Latini & Pedata 2001). The equilibrative transport system is subdivided in two types (*es* and *ei*) based on their sensitivity to nitrobenzylthioinosine (NBTI). The *es* transport system is highly sensitive to inhibition by NBTI ($K_i = 0.1$ to 10 nM), whereas *ei* is NBTI-insensitive but sensitive to dipyridamole. Four transporters belonging to the equilibrative transport system have been identified and cloned (ENT1, ENT2, ENT3 and ENT4). All transporters are able to transport adenosine, but they have different ability to transport other nucleosides and nucleobases (Table 1.7) (King *et al.* 2006; Podgorska *et al.* 2005). The concentrative transport system is sodium-dependent and the movement of the nucleoside occurs regardless of its concentration gradient. Three electrogenic mammalian active transporter isoforms have been described (CNT1, CNT2 and CNT3). They differ in the nature of cation coupling and substrate specificity (further detailed in Table 1.7) (King *et al.* 2006; Latini & Pedata 2001; Podgorska *et al.* 2005). Although nucleoside salvage is a primary function of these specialized carrier proteins, they also have the potential to regulate synaptic levels of neuroactive purines such as adenosine, indirectly modulating physiological processes through P1 purinoceptors activation.

Table 1.7 – Nucleoside transport systems and their specificity.

Nucleoside transport system	Protein	Substrate selectivity (K_m for adenosine)	Co-transported ion
ENT Family			
es (equilibrative, sensitive to NBTI)	ENT1	Purine and pyrimidine nucleosides (~ 40 μ M)	None
ei (equilibrative, insensitive to NBTI)	ENT2	Purine and pyrimidine nucleosides and some nucleobases (~ 140 μ M)	None
ei (equilibrative, insensitive to NBTI)	ENT3	Purine and pyrimidine nucleosides and some nucleobases (~ 1.9 mM)	Unclear, but optimal transport at pH 5.5
es (equilibrative, sensitive to NBTI)	ENT4	Organic cations, including monoamines	None. Electrogenic transport of cationic substrates
CNT Family			
concentrative, insensitive to NBTI	CNT1	Pyrimidine nucleosides, adenosine	Na^+ ; stoichiometry (ion:nucleoside) 1:1
concentrative, insensitive to NBTI	CNT2	Purine nucleosides, uridine, formycin B (8 μ M)	Na^+ ; stoichiometry (ion:nucleoside) 1:1
concentrative, insensitive to NBTI	CNT3	Purine and pyrimidine nucleosides (15 μ M)	Na^+ , Li^+ or H^+ ; stoichiometry (ion:nucleoside) 2:1 for Na^+ or $\text{Na}^{2+} \text{H}^+$, or 1:1 for H^+ alone

1.4.3 P1 Adenosine receptors

Adenosine acts on cell surface receptors that are coupled to intracellular signalling cascades. Four different P1 receptor subtypes called (according to IUPHAR): A_1 , A_{2A} , A_{2B} and A_3 have been characterized and cloned from several mammalian species, including man. All are members of the rhodopsin like family of G protein-coupled receptors (GPCRs) and share an extensive sequence similarity between species for the A_1 , A_{2A} , A_{2B} receptors, whereas A_3 receptors are more variable (Fredholm *et al.* 2001; Frenguelli *et al.* 2009; Jacobson 2009). As GPCRs, they are composed by a single polypeptide chain forming seven transmembrane helices (TMs), with an extracellular short N-terminus and a cytosolic C-terminus. The A_{2A} receptor has a long C-terminal segment of more than 120 amino acid residues, which is not required for coupling to G_s , but can serve as a binding site for “accessory” proteins (like α -actinin, ARNO, USP4 and translin-associated protein-

X) or to form heteromeric complex with the D₂ (dopamine receptor) or mGlu₅ (metabotropic glutamate receptor), for example (Ciruela *et al.* 2010; Jacobson 2009; Zezula *et al.* 2008). Adenosine receptors are historically coupled to adenylylate cyclase. The A₁ and A₃ receptors inhibit the production of cyclic AMP through coupling to G_i, whereas A_{2A} and A_{2B} subtypes are coupled to G_s or G_o to stimulate adenylylate cyclase (Figure 1.14).

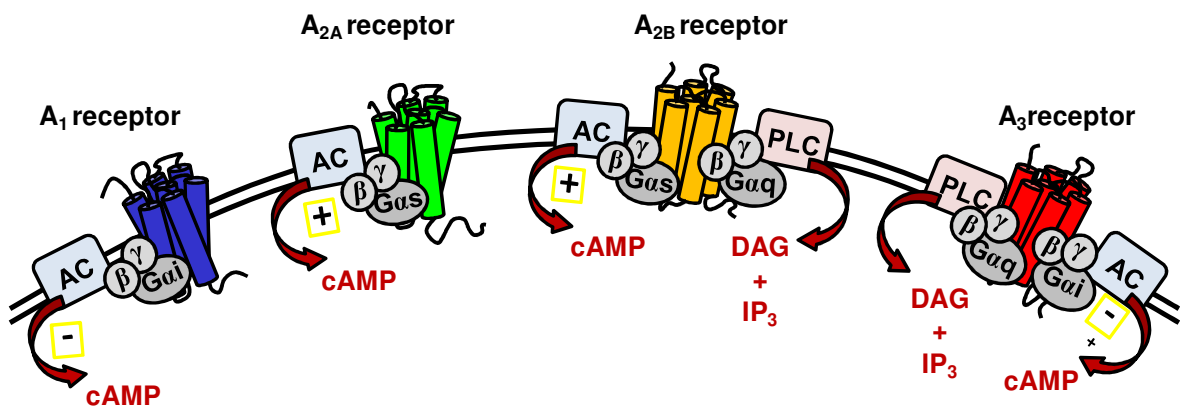


Figure 1.14 - Schematic diagram illustrating major adenosine receptors signalling pathways. Activation of the A₁ and A₃ adenosine receptors commonly inhibits adenylylate cyclase (AC) activity through activation of pertussis toxin-sensitive G_i proteins, decreasing intracellular cyclic AMP (cAMP) levels. Activation of the A_{2A} and A_{2B} adenosine receptors usually increases AC activity through activation of G_s proteins, with subsequent increase in intracellular cAMP concentration. A_{2B} adenosine receptors-induced activation of phospholipase C (PLC) occurs through G_q proteins, leading to intracellular increase of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃).

The adenosine receptor with the lowest affinity for adenosine ($K_i > 1 \mu\text{M}$), the A_{2B} subtype, is also coupled to G_{q/11} activating phospholipase C. A₁ and A_{2A} receptor subtypes exhibit the highest affinity for adenosine (K_i 10 – 30 nM, respectively), whereas the affinity of the A₃ receptor for adenosine is intermediate ($K_i \sim 1 \mu\text{M}$) in rodents (Ciruela *et al.* 2010; Fredholm *et al.* 2001; Frenguelli *et al.* 2009; Jacobson 2009). Some authors consider that the A₃ receptor, besides being negatively coupled to adenylylate cyclase, may also interact directly with G_s proteins (Figure 1.14). It is also worth noting that the A₃ receptor is insensitive to methylxanthines, which has been the main drawback in its characterization and delayed its inclusion in P1 adenosine receptors subfamily. Furthermore, effector mechanisms other than adenylylate cyclase and phospholipase C, may be initiated through the β, γ subunits of G proteins, which can also lead to the coupling of adenosine receptors to ion channels. For instance, activation of the A₁ receptor subtype induces the influx of calcium ions or the efflux of potassium ions. The activation of phosphoinositide 3-kinase

(PI3K), mitogen-activated protein kinases (MAPKs), and extracellular receptor signal-induced kinase (ERK) can also result from adenosine receptors activation, influencing cells differentiation, proliferation and apoptosis (Table 1.8) (Burnstock 2007b; Ciruela *et al.* 2010; Jacobson 2009; Jacobson *et al.* 2006).

Over the past decades, selective agonists and antagonists for adenosine receptors have been developed, which have been instrumental for subtype characterization. The main approach for discovering adenosine receptor agonists has been the modification of adenosine itself. Modification of the xanthine nucleus (caffeine and theophylline derivatives) led to the development of adenosine receptors antagonists (Table 1.8). Nonetheless, a number of new chemical structures are being developed as adenosine receptor antagonists. Like in other GPCR, amino acids in the third and seventh transmembrane domains are considered critical for agonist binding. Agonist potency is strongly dependent on receptor number and on the assay used. Keeping this in mind, data from former studies on the absolute and relative potencies for available adenosine agonists must be used cautiously whenever one wants to classify adenosine receptors (Burnstock 2007b; Fredholm *et al.* 2001; Frenguelli *et al.* 2009; Jacobson & Gao 2006).

Table 1.8 – Characteristics of P1 adenosine receptors.

Receptor	Main distribution	Agonists	Antagonists	Transduction mechanisms
P1				
A₁	brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA, CPA, S-ENBA, CVT-510	DPCPX, N-0840, MRS1754, WRC-0571	G _{i/o} Inhibits AC (↓ cAMP) Activates PLC (↑ IP3/DAG) Inhibits Ca ²⁺ channels
A_{2A}	brain, heart, lungs, spleen	CGS 21680, HE-NECA, CVT-3146	KF17837, SCH58261, ZM241385, KW 6002	G _{s/o} Activates AC (↑ cAMP) Inhibits Ca ²⁺ channels
A_{2B}	large intestine, bladder	NECA (non-selective)	MRE2029-F20, MRS1706, MRS17541, Enprofylline,	G _s Activates AC (↑ cAMP) Activates Ca ²⁺ channels G _{q/11} Activates PLC (↑ IP3/DAG)
A₃	lung, liver, brain, testis, heart	2-Cl-IB-MECA, IB-MECA, DBXRM, VT160	MRS1220, L-268605, MRS1191, MRS1523, VUF8504	G _i Inhibits AC (↓ cAMP) G _{q/11} Activates PLC (↑ IP3/DAG)

1.4.4 Mechanisms to decrease extracellular adenosine levels

In order to terminate P1 receptor activation, a decrease in extracellular adenosine levels must occur. When adenosine levels in the extracellular space are high, adenosine is transported into neighbouring cells by uptake through the bi-directional nucleoside transport system (see Table 1.7, Section 1.4.2). Once in the cytosol, adenosine undergoes rapid phosphorylation to AMP by adenosine kinase (AK, EC 2.7.1.20) (Govindarajan *et al.* 2007); deamination to inosine by adenosine deaminase (ADA, EC 3.5.4.4) plays a role when the capacity of AK is exceeded (see Figure 1.13, Section 1.4.1). Inosine can then be phosphorylated by a purine nucleoside phosphorylase (PNP) into hypoxanthine, which is finally metabolised via xanthine oxidase to the stable end-reaction product, uric acid (Antonioli *et al.* 2008b; Fredholm *et al.* 2001; Latini & Pedata 2001; Yegutkin 2008). AK is a low capacity and low K_m enzyme (K_m for adenosine ~ 100 nM), whereas ADA is a high capacity and high K_m enzyme (K_m for adenosine 20-100 μ M). When ADA is blocked or genetically deleted, the capacity of AK is exceeded and adenosine levels rise dramatically. Conversely, AK is important to maintain low adenosine levels (within the physiological range), as well as adenine nucleotides depots. Targeted deletions of these enzymes, as well as drug enzymatic inhibitors, are important tools to investigate adenosine pathophysiological roles (Boison 2006; Fredholm 2007; Fredholm *et al.* 2001). Along with cytosolic localization, ADA can be expressed as an ectoenzyme on the surfaces of several cells. Besides the main role of ecto-ADA in degrading extracellular adenosine, recent evidence points to an extraenzymatic functional role through direct protein-protein interactions. It has been shown that ecto-ADA, associated to adenosine A₁ receptors, can act as a positive modulator for adenosine binding and efficient coupling to the signal transduction machinery. This heterotypic interaction was also recently described for adenosine A_{2B} receptor subtype (Ciruela *et al.* 2010; Latini & Pedata 2001; Yegutkin 2008).

Considering adenosine biosynthesis and catabolism, one can understand that the extracellular levels of the nucleoside depend on the balance between adenosine outflow plus extracellular ATP breakdown *versus* extracellular adenosine uptake, phosphorylation into AMP and deamination into inosine. Extracellular levels determine adenosine receptors activation and subsequently the cellular effects of the nucleoside. Adenosine is considered as a local modulator with a general cytoprotective function in the body, since it has been proven that it increases the ratio of oxygen supply to demand, protects against ischaemic damage by cell conditioning, triggers anti-inflammatory responses and promotes angiogenesis (Fredholm 2007; Jacobson & Gao 2006; Linden 2005).

Adenosine short-life in the circulation allows its clinical use in the treatment of paroxysmal supraventricular tachycardia and in radionuclide myocardial perfusion imaging (Belhassen & Pelleg 1984; Delacretaz 2006). Many selective and potent synthetic adenosine receptor agonists with much longer effects in the body than native adenosine have promising therapeutic applications (Cerqueira 2006; Eltzschig 2009; Jacobson & Gao 2006). In fact, the development of potent and selective synthetic agonists and antagonists of adenosine receptors has been the subject of medicinal chemistry research for more than three decades. Agonists potential therapeutic applications are based on adenosine anti-inflammatory (A_{2A} and A_3), cardioprotective (A_1 , A_{2B} and A_3), cerebroprotective (A_1 and A_3) and antinociceptive (A_1) properties, but many others are in line to be investigated. Potent and selective adenosine antagonists display therapeutic potential as kidney protective (A_1), antifibrotic (A_{2A}), neuroprotective (A_{2A}), antiasthmatic (A_{2B}) and antiglaucoma (A_3) drugs. Although promising, only the recent CF101 (A_3 adenosine receptor agonist) was approved for the treatment of autoimmune inflammatory diseases and, at this moment, CF101 is in the final clinical trials phase for patients with rheumatoid arthritis, psoriasis and dry eye syndrome (Eltzschig 2009; Fishman 2010; Jacobson & Gao 2006).

1.4.5 Adenosine neuromodulation in the gut

An integrated regulatory interplay between the enteric nervous system (ENS), smooth muscle and the mucosal/immune system is necessary to ensure intestinal functions, maintain a homeostatic status and guarantee adaptative responses in pathological conditions. This complex network is regulated by several mediators and there is convincing evidence indicating that adenosine is one of the most important modulating agents (Bornstein 2008; Christofi 2001; Ren & Bertrand 2008; Wood 2006a). The wide distribution of adenosine receptor subtypes as well as enzymes and transporters that regulate adenosine extracellular levels in different gut regions, of both rodents and humans, suggests a complex role for this mediator in the regulation of gastrointestinal functions (Table 1.9) (Antonioli *et al.* 2008b; Christofi 2008). Studies performed in rodents and humans have shown a significant involvement of adenosine in the control of intestinal secretion, motility and sensation, via activation of P1 purinoceptors. These findings prompted the study of adenosine involvement in the pathophysiology of diverse intestinal disorders (*e.g.* inflammatory bowel diseases (IBDs), intestinal ischemia and post-operative ileus) and related dysfunctions, such as gut dysmotility, diarrhoea and abdominal discomfort/pain (Antonioli *et al.* 2008a; Christofi 2008; Cooke *et al.* 2003).

Several studies suggest that adenosine contributes to the modulation of enteric immune and inflammatory responses, leading to anti-inflammatory actions. Therefore, research efforts are attempting to unravel adenosine role in the pathophysiology of bowel inflammation and related gut dysfunctions, as well as to evaluate possible therapeutic implications of its pharmacological modulation.

Table 1.9 – Small Intestinal localization of adenosine receptor subtypes, enzymes and transporters in humans and rats.

Small Intestine Region	A ₁		A _{2A}		A _{2B}		A ₃		ADA		E-5'-N		ENTs		CNTs	
	H	R	H	R	H	R	H	R	H	R	H	R	H	R	H	R
Mucosa	-	+	+	n.a.	+	n.a.	+	+	+	+	+	n.a.	+	+	+	+
Submucous plexus	-	+	+	n.a.	+	n.a.	+	+	n.a.	+	n.a.	n.a.	n.a.	+	n.a.	+
Circular muscle	+	+	-	n.a.	-	n.a.	n.a.	+	+	+	n.a.	+	n.a.	+	n.a.	+
Myenteric plexus	+	+	+	n.a.	+	n.a.	+	+	+	+	n.a.	+	n.a.	+	n.a.	+
Longitudinal muscle	+	+	+	n.a.	-	n.a.	+	+	+	+	n.a.	+	n.a.	+	n.a.	+

Present (+); absent (-); data not available (**n.a.**); human (H) and rat (R).

Adenosine deaminase (ADA); ecto-5'-nucleotidase (E-5'-N); equilibrative nucleoside transporters (ENTs); concentrative nucleoside transporters (CNTs).

Human data was assembled mainly from immunohistochemistry (Christofi *et al.* 2001; Govindarajan *et al.* 2007; Puffinbarger *et al.* 1995; Sakamoto *et al.* 1993; Strohmeier *et al.* 1997) and mRNA (Christofi *et al.* 2001; Podgorska *et al.* 2005) studies. Pharmacological studies in human intestine are scarce (Namiot *et al.* 1992). Rat data was assembled from pharmacological (Coupar 1999; Fozard *et al.* 2003; Obata *et al.* 1998); immunohistochemistry (Dinjens *et al.* 1989; Giron *et al.* 2008; Puffinbarger *et al.* 1995) and mRNA (Antonioli *et al.* 2006; Dixon *et al.* 1996; Kadowaki *et al.* 2000; Lu *et al.* 2004) studies.

Under inflammatory conditions, purinergic signalling pathways are subjected to dynamic changes in the expression and/or function of ectoenzymes and adenosine receptors, with variations of extracellular adenosine levels and receptor activation, in order to fine-tuning inflammatory/immune responses, with consequent limitation of inflammatory tissue injury (Antonioli *et al.* 2008a; Bours *et al.* 2006; Rybaczyk *et al.* 2009b). The knowledge gained from both *in vitro* and *in vivo* studies in animal models of bowel inflammation encourage the development of novel therapeutic strategies. In recent years, most of the drugs tested in experimental models of bowel inflammation were A_{2A} (ATL-146e or CGS 21680), A_{2B} (ATL-801) and A₃ (IB-MECA) adenosine receptors agonists or

ADA (Pentostatin or APP), AK (GP515) or ENT (dipyridamole) inhibitors (Antonioli *et al.* 2008b; Kolachala *et al.* 2008). Their effects on adenosine pathways were evaluated, but despite encouraging results, several aspects remain unclear and deserve extensive investigation, so none of these drugs is at the moment being developed for treatment of human gastrointestinal disorders.

As explained in the last two chapters, the role of purinergic signalling in the gastrointestinal tract is extremely complex, since tissues and cells often co-express multiple purinergic receptors, sometimes with opposing effects. Several tiers of regulation were identified, allowing modulation of cellular function (receptor agonist or antagonist drugs) or extracellular levels of adenine nucleosides and nucleotides (enzymatic or transporter blockers). Almost forty years after it was first described, the interdependence between ATP and adenosine extracellular levels and consequent P2 and/or P1 receptor activation is now clearer (Figure 1.15), but consequences of altering purinergic signalling cascade are rarely taken into consideration.

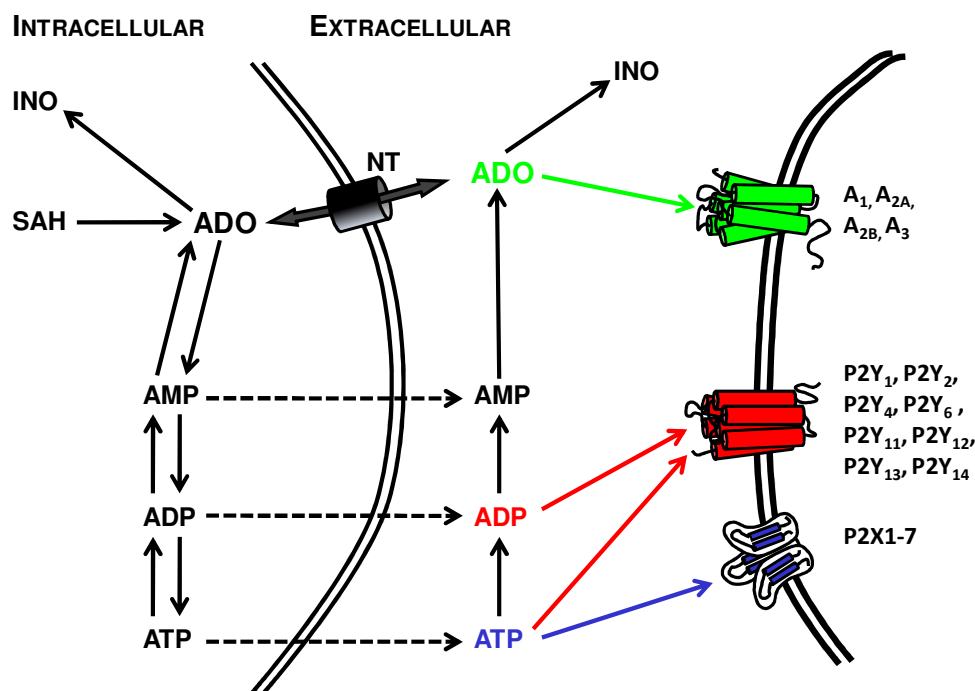


Figure 1.15 – Simplified schematic representation of extracellular ATP and adenosine biosynthesis and catabolism. In the extracellular space ATP and its breakdown products, ADP and adenosine, activate purinergic P2 (P2X1-7 and P2Y₁, 2, 4, 6, 11, 12, 13, 14) and P1 (A₁, A_{2A}, A_{2B} and A₃) receptor subtypes. S-adenosylhomocysteine (SAH); bidirectional nucleoside transporter (NT).

2 - AIMS

As briefly reviewed in the introduction, the concept of purinergic signalling arose from experiments designed to find the identity of the NANC inhibitory neurotransmitter in the gut (Burnstock 1972; Burnstock *et al.* 1970; Burnstock *et al.* 1972a). Nowadays, P2X and P2Y receptors have been identified on interneurons, myenteric, submucous, motor and sensory neurons that are involved in synaptic neurotransmission, neuromodulation and reflex activities including ascending excitatory and descending inhibitory pathways in the GI tract. Additionally, ATP receptors are also expressed in enteric non-neuronal cells, like interstitial cells of Cajal (ICC) and enteric glia, but their role remains to be elucidated.

Regarding adenosine, back in the early 70's, it was just considered as a byproduct of ATP catabolism. At the present time, the wide distribution of adenosine receptor subtypes, as well as enzymes and transporters that regulate adenosine extracellular levels in different gut regions, suggests a complex role for this mediator in the regulation of GI functions (Antonioli *et al.* 2008b; Christofi 2008) and deserves further investigations. Studies performed in rodents and humans have shown a significant involvement of adenosine in the control of intestinal secretion, motility and sensation, via activation of P1 purinergic receptors.

Curiously, almost forty years after it was first described, the general interdependence between ATP and adenosine extracellular levels and subsequent P2 and/or P1 receptor activation are still undervalued, so the full consequences of altering purinergic signalling are rarely overviewed.

Considering all the above premises, the main objective of the present thesis is to contribute to the description and interpretation of the “*purinergic cascade*” in the myenteric plexus of the rat ileum. To accomplish this purpose, the neuromodulatory role of ATP and its active metabolites (like ADP and adenosine) on ACh release will be addressed in an integrative way, taking into account not only the activation of purinergic receptors (and others that interact with them) but also the factors that influence adenine nucleotides and nucleosides extracellular levels. Therefore, quantification of spontaneous and electrically-evoked ACh and adenine nucleoside release will be determined in the absence and in the presence of distinct pharmacological tools (*e.g.* P1, P2 and muscarinic receptors agonists/antagonists) in the longitudinal muscle-myenteric plexus (LM-MP) of the rat ileum. Results to be obtained will also take into account the mechanisms interfering with the extracellular levels of adenine nucleotides and nucleosides which might influence the purinergic tonus; to this end, enzymatic inhibitors, substances that influence the release

probability (*e.g.* voltage gated channel inhibitors) and distinct nerve stimulation conditions, will be used.

We believe that knowing purinergic signalling dynamics in the LM-MP preparation is essential to unravel new therapeutic targets besides P1/P2 receptors agonist or antagonist drugs, since most gastrointestinal pathophysiological conditions are accompanied by an alteration in extracellular levels of adenine nucleotides or nucleosides.

3 – MATERIALS AND METHODS

3.1 - Biological sample preparation

Wistar rats of either sex, weighing approximately 200 g (Charles River, Barcelona, Spain), were kept in holding rooms with proper ventilation, stable temperature (20-24 °C) and relative humidity (45-65%) intervals, in accordance with the specie (*Rattus norvegicus*) requirements. A regular light (06.30–19.30 h)/dark (19.30–06.30 h) cycle with food and water *ad libitum* were also assured. The animals were killed by decapitation, using a small rodent guillotine (DCPA from WPI). All animal procedures were performed according to the Portuguese and European Guidelines for animal Care and approved by Direcção Geral de Veterinária.

Sections of approximately 10 cm long of the rat ileum (excluding the terminal 2 cm that are closest to the ileo–caecal junction) were excised from the small intestine of each animal (Figure 3.1) by cautious trimming of the adjacent mesentery. Ileum fragments were then immersed and subsequently flushed of its content with gassed (95% O₂ and 5% CO₂) Tyrode's solution containing (mM): NaCl, 137; KCl, 2.7; CaCl₂, 1.8; MgCl₂, 1; NaH₂PO₄, 0.4; NaHCO₃, 11.9; glucose, 11.2; and choline, 0.001 (Figure 3.2a).

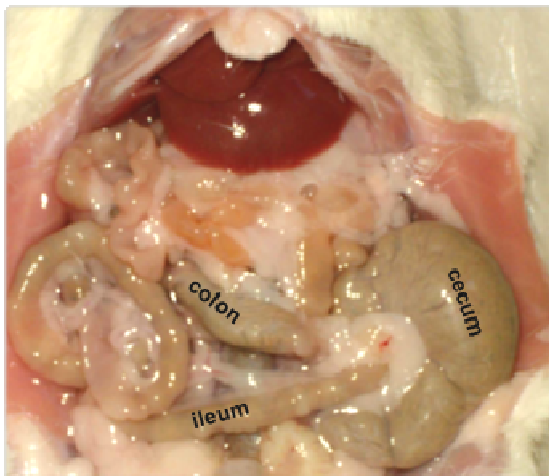


Figure 3.1 - Photography of the rat gastrointestinal tract after opening the abdominal cavity. The ileum, cecum and colon portions of the rat intestine are identified.

Longitudinal muscle strips with the myenteric plexus attached were prepared by the method first described by Ambache during the fifties in the rabbit intestine and, later on, modified to use in the guinea pig ileum (Ambache 1954; Paton *et al.* 1969; Paton *et al.* 1968; Rang 1964). Briefly, fragments of the ileum were pulled over a glass pipette. The serosa and the underlying longitudinal muscle were carefully sectioned along their longitudinal axis in the opposite side of the mesenteric border with a scalpel (Figure 3.2b).

The longitudinal muscle with the adherent myenteric plexus was detached from the underlying circular muscle layer by gently rubbing the incision with gauze pads (soaked in Tyrode's solution), working along and around the circumference of the whole ileum (Figure 3.2c). The average dry weight of longitudinal muscle-myenteric plexus (LM-MP) preparations of the rat ileum was 24.01 ± 0.55 mg ($n= 347$).

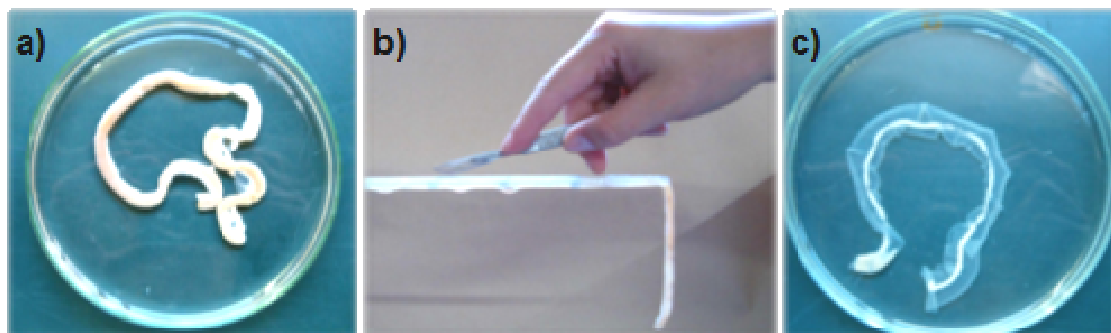


Figure 3.2 - Illustration of the method used to obtain longitudinal muscle – myenteric plexus preparations of the rat ileum. (a) Entire ileum immersed in Tyrode's solution. (b) Ileum fragment pulled over a glass pipette and the serosa and the underlying longitudinal muscle sectioned with a scalpel. (c) Final view of a longitudinal muscle-myenteric plexus strip immersed in Tyrode's solution.

3.2– Experimental Procedures

3.2.1 - [^3H] Acetylcholine release experiments

The procedures used for labelling the preparations and measuring evoked [^3H]ACh release were described previously and were applied with minor modifications (Correia-de-Sá *et al.* 1991). Briefly, LM-MP strips were mounted in vertical perfusion chambers of 3 mL capacity (Figure 3.3a), heated at 37°C. After a 30 min equilibration period, essential to remove tissue debris that resulted from LM-MP preparation dissection, the preparations were incubated in Tyrode's solution containing 1 μM [^3H]choline (specific activity 2.5 $\mu\text{Ci/nmol}$) for 40 min (Figure 3.4). During the loading period, the preparations were continuously stimulated with supramaximal square wave pulses of 1 ms duration delivered at 1 Hz frequency through an SD9 stimulator (Grass Instrument, Quincy, USA) (Figure 3.3c), and two platinum electrodes placed above and below the suspended muscle strip (electrical field stimulation; EFS). Following loading, muscle strips were washed by superfusion (15 mL/min) with Tyrode's solution containing hemicholinium-3 (10 μM), which remained in the bathing solution until the end of the experiment to prevent choline uptake (Takagi *et al.* 1970) (Figure 3.4). After a 60-min period of washout, bath samples (2.8 mL) were automatically collected every 3 min by emptying and refilling the organ bath with the solution in use (Figure 3.4), by using a fraction collector (Gilson, FC 203B, France) (Figure 3.3d) coupled to a peristaltic pump (Gilson, Minipuls3, France) programmed device (Figure 3.3b).

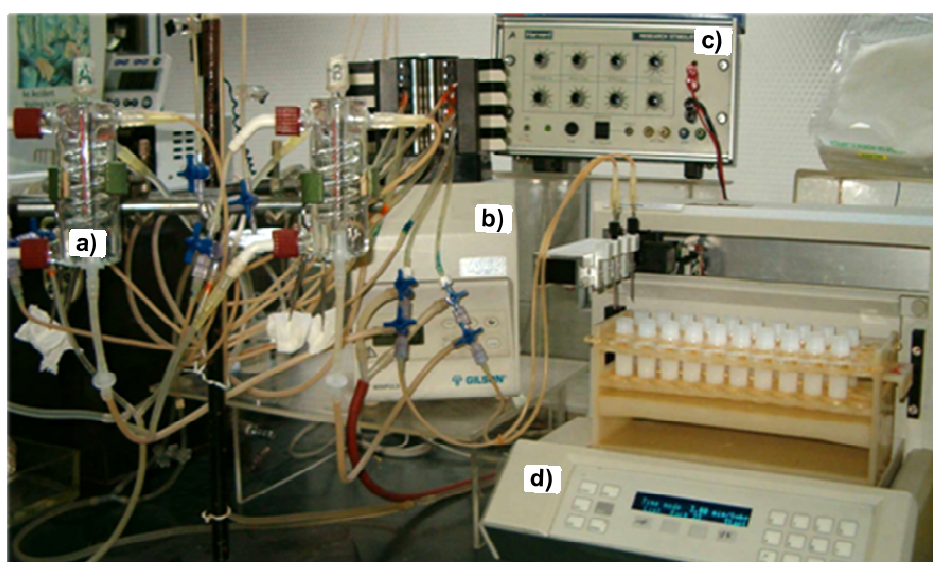


Figure 3.3 - Set-up for [^3H]ACh release experiments from LM-MP of the rat ileum. (a) Vertical perfusion chambers with LM-MP strip placed between platinum electrodes; (b) peristaltic pump; (c) electrical stimulator (to perform electrical field stimulation) and (d) fraction collector.

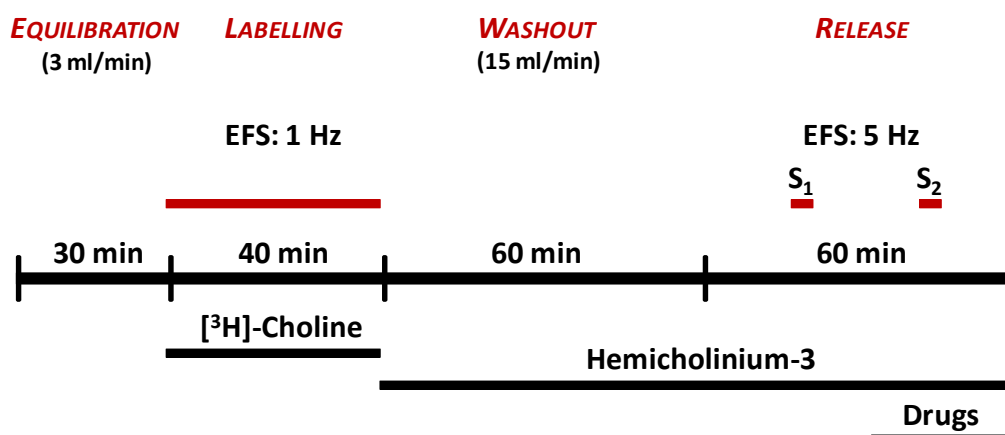


Figure 3.4 - $[^3\text{H}]$ ACh release protocol. The protocol involves four different periods: equilibration, labelling, washout and release. Electrical field stimulation (EFS, dark red lines) was applied during the labelling period (EFS: 1 ms, 1 Hz) and during S_1 (initiated 12 min after the beginning of the release period) and S_2 (initiated 39 min after the beginning of the release period) stimulation periods (EFS: 1 ms, 5 Hz, 200 – 1350 pulses), inducing $[^3\text{H}]$ ACh release. Test drugs were added 15 min before S_2 and were present up to the end of the experiments.

Aliquots (0.4 mL) of the incubation medium were added to 3.5 mL of Packard Insta Gel II (USA) scintillation cocktail. The tritium content of the samples was measured by liquid scintillation spectrometry (% counting efficiency: $40 \pm 2\%$) after appropriate background subtraction, which did not exceed 5% of the tritium content of the samples. The radioactivity was expressed as disintegrations per minute (dpm) per gram of dry weight of the tissue, determined at the end of the experiment. After the loading and washout periods, the preparation contained $(10648 \pm 324) \times 10^3$ dpm/g, and the resting release was $(115 \pm 18) \times 10^3$ dpm/g in 3 min ($n = 8$). When the fractional release was calculated, this value was $1.08 \pm 0.14\%$ of the radioactivity present in the tissue in the first collected sample.

$[^3\text{H}]$ -acetylcholine release was evoked by EFS, starting in the twelfth (S_1) and thirty-ninth (S_2) min after the beginning of the release period (Figure 3.4), each consisting of 200, 500, 750 or 1350 square wave pulses of 1 ms duration delivered at a 5 Hz frequency (see Table 4.1, Section 4.2.8). The method of stimulation was the same as during the labelling period, except for the stimulation rate. Others have used this methodology to study the contractile responses induced by electrical stimulation of small intestine muscle strips (De Man *et al.* 2003).

In some experiments, $[^3\text{H}]$ ACh release was induced by 3 min application of ATP, only in S_2 [EFS in S_1 and ATP (1–300 μM) in S_2] or in either period [without EFS, ATP (100 or 300 μM) in S_1 and S_2]. As 97% of the radioactivity release in response to EFS is $[^3\text{H}]$ ACh (Kilbinger *et al.* 1985), no attempt was made to separate labelled choline from

ACh. Prevention of tritium outflow in the absence of external calcium (Ca^{2+} 0 + EGTA 1 mM) and in the presence of 1 μM of tetrodotoxin (TTX) (Duarte-Araújo *et al.* 2004b) indicates that evoked [^3H]ACh release results from vesicle exocytosis of depolarized nerve terminals. In both cases, evoked [^3H] ACh release was calculated by subtracting the basal from the total tritium outflow during the stimulation period.

Test drugs were added 15 min before S_2 and were present up to the end of the experiments. The change in the ratio between the evoked [^3H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. When we evaluated the modifications of the effect of tested drugs by a modifier, this modifier was applied 15 min before starting sample collection and hence was present during S_1 and S_2 . When the same drug was present in S_1 and S_2 , the S_2/S_1 ratios were not significantly ($P > 0.05$) different from those obtained in control conditions, that is, without addition of drugs. None of the drugs, with the exception of ATP, changed significantly ($P > 0.05$) basal tritium outflow (see Figure 4.4, Section 4.1.4).

3.2.2 – Enzymatic kinetic experiments and high-performance liquid chromatography (HPLC) analysis

For kinetic experiments of nucleotide or nucleoside catabolism, LM-MP strips were mounted in a 3 mL organ bath and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution kept at 37°C. Because enzymatic activity might not be linearly related to the weight of the preparations, care was taken to use preparations of similar weight (23.31 ± 1.07 mg, $n= 82$). After a 5-min washout and a 30-min equilibration period, the organ bath was emptied and a 30 μM solution of adenine nucleotides or nucleosides in Tyrode's solution was added to the preparations at zero time. Samples of 75 μL were collected from the organ bath at different times up to 45 min for HPLC analysis of the variation of substrate disappearance and product formation (Figure 3.5). Chromatographic separation of ATP and its products of metabolism was carried out by reverse-phase HPLC analysis (using a Merck LaChrom Elite HPLC with UV detection) and followed the method described earlier by Cunha & Sebastião (1991), with minor modifications. Two eluent solutions were used: 100 mM KH_2PO_4 at pH = 7 (solution A) and 100 mM KH_2PO_4 with 30% (v/v) methanol (solution B) at pH = 7. The elution program was as follows: 12 min with a mixture of 96% of solution A plus 4% solution B, followed by a 5-min linear gradient, until solution B reaches 25% v/v. The flow was maintained at 1.25 mL/min. Chromatographic identification of ATP metabolites was performed by comparing the

retention time of ATP, ADP, AMP, adenosine, inosine and hypoxanthine with high purity standards under similar chromatographic conditions. Quantification of adenine nucleotides and nucleosides in the samples was performed by the external standard method (Cunha *et al.* 1991).

In some of the experiments, separation of adenine nucleosides was accomplished using an isocratic elution program, which is faster than the method described above. To this end, the elution program involved a mixture of 65% of solution A plus 35% of solution B during 10 min at a flow rate of 1.00 mL/min. The injection volume (20 μ L) of both standard and test samples was kept constant throughout the assay.

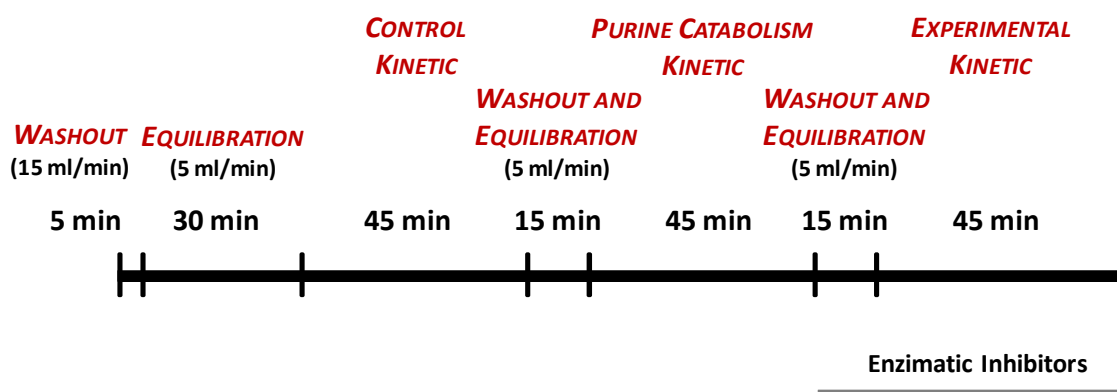


Figure 3.5 - Protocol used in the kinetic experiments of ATP metabolism and adenosine formation at the LM-MP of the rat ileum. After a 35-min washout and equilibration periods, bath samples were collected during 45 min in control conditions, i.e. with only Tyrode's solution added to the preparation. The samples were retained for HPLC analysis of the spontaneous release of adenine nucleotides and nucleosides from the preparation. After a second 15-min washout and equilibration period, preparations were incubated with 30- μ M adenine nucleotides or nucleosides made up in Tyrode's solution (zero time). Samples were collected at different times up to 45 min for HPLC analysis as described previously. Whenever using enzymatic inhibitors, these drugs were superfused during 15 min prior to addition of the 30- μ M adenine nucleotides or nucleosides. In these circumstances, a third period of sample collection was performed in a similar manner as the second collection period, still in the presence of the inhibitor.

The actual concentrations of ATP, ADP, AMP, adenosine, inosine and hypoxanthine were expressed in μ M. Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analysed for each progress curve: half-degradation time of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment. At the end of experiments, the remaining

incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity, by the method of Stolzenbach (Pesce *et al.* 1964; Stolzenbach *et al.* 1976). The negligible (0.12 ± 0.01 U/mL, $n=20$) activity of lactate dehydrogenase in bath samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period (Keiding *et al.* 1974). Incubation of the preparations for 45 min with the bathing solution (*i.e.* with no added substrate) only produced spontaneous release of a compound with the same time of retention of inosine monophosphate (IMP), whose concentration was never higher than 2 μ M. The spontaneous degradation of adenine nucleotides and adenosine at 37°C in the absence of the preparation was negligible (0–5%) over 45 min.

3.2.3 - Release of adenine nucleosides and adenosine deaminase

To follow the stimulation-evoked release of adenine nucleosides and adenosine deaminase (ADA, EC 3.5.4.4), the preparations were incubated as for the release of [^{3} H]ACh, except that no [^{3} H]choline was added to the Tyrode's solution. After a 30-min equilibration period, the preparations were incubated with 2.8 ml of gassed (95% O₂ and 5% CO₂) Tyrode's solution, which was changed every 15 min by emptying and refilling the organ bath with the solution in use. As for the release of [^{3} H]ACh, the preparations were electrically stimulated once, 30 min after starting sample collection (zero time), using 3000 square wave pulses of 1 ms duration delivered at a 5 Hz frequency (Figure 3.6); for comparison purposes, we used two different stimulation patterns changing either the frequency (1 Hz) and/or the number of pulses (600 or 1350) delivered to the preparations (see Table 4.3, Section 4.3.3).

In these experiments, two samples were collected before stimulus application and two other samples were collected after electrical stimulation and retained for analysis. The first sample (containing only Tyrode's solution) was collected 15 min after starting the release period; the second sample was collected 15 min after drug application, *i.e.* 30 min after starting sample collection and immediately before stimulus application; the third and fourth samples, corresponding to post-stimulation periods, were collected respectively 45 and 60 min after starting sample collection (Figure 3.6).

Bath aliquots (250 μ l) were frozen in liquid nitrogen immediately after samples collection, stored at -20 °C (the enzymes are stable for at least 4 weeks) and analysed by HPLC with UV detection, within 1 week of collection, to measure adenine nucleosides. Some aliquots were used for determination of ADA activity (see Section 3.2.4).

Stimulation-induced modifications of ADA activity were compared with changes in LDH activity in the same collected samples. LDH activity was used as indicator of cell integrity during the stimulation period.

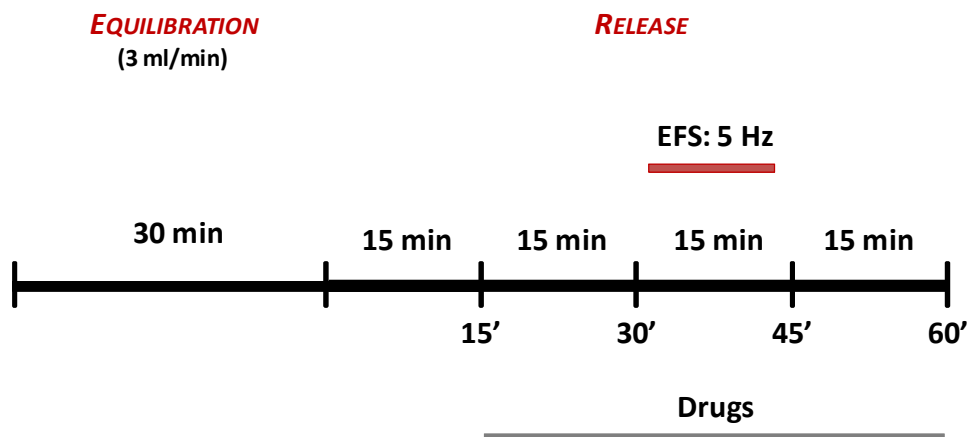


Figure 3.6 – Protocol used to measure EFS-induced endogenous adenine nucleosides release from LM-MP of the rat ileum. After a 30-min equilibration, samples were collected once every 15-min by emptying and refilling the organ bath with Tyrode's solution. Test drugs were added from the second collection period onwards. Preparations were stimulated (EFS, 1 ms pulse duration delivered at 5 Hz frequency) during 10 min, i.e. from the 35th to the 45th min after starting sample collection. Thus, the third and fourth collected samples corresponded to post-stimulation periods.

Nerve evoked release of adenine nucleosides was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenine nucleosides determined after stimulus application. When testing the ability of a modifier to affect adenine nucleosides and ADA release, the modifier was added to the bath 15 min before starting sample collection and was present throughout the assay.

3.2.4 - Adenosine deaminase assay

Total adenosine deaminase (ADA, EC 3.5.4.4) activity was determined by an enzymatic spectrophotometric method, using an ADA activity kit (Roche Diagnostics, Switzerland) on a Cobas Mira S autoanalyser, according to Giusti's method (Giusti 1974). Briefly, this assay is based on the enzymatic deamination of adenosine to inosine, which is converted to hypoxanthine by purine nucleoside phosphorylase. Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase. H_2O_2 is further reacted with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine that generates, in the presence of peroxidase, a quinone dye. After adding the kit reagents, bath samples (15 μ l) were incubated at 37°C during 3 min. The catalytic activity of ADA in the sample is directly proportional to absorbance measured at 540 nm. Control samples (ADA, 0.05–0.5 U/ml) were used to determine the precision of this assay. Some samples were also tested for adenosine (10 μ M) disappearance and inosine formation by HPLC analysis.

3.2.5 - Contraction recordings

LM-MP strips were mounted along the longitudinal axis in 10-mL capacity perfusion chambers heated at 37°C (Figure 3.7a). One end of the segment was attached to the base of the holder using a 4-0 silk suture whereas the other end was attached to an isometric force transducer (Figure 3.7b). The changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments, Colorado Springs, CO, USA) (Figure 3.7c and 3.7d). Tissues were preloaded with 0.5 g of tension and allowed to equilibrate for 90 min in Tyrode's solution kept at 37°C. Preparations in which the repeated administration of 1 μ M ACh induced equal-in-amplitude contractile responses were used in the experiments. Contractile responses were elicited by cumulative applications of oxotremorine (0.003–300 μ M) or by electrical field stimulation (EFS, 1 ms, 60 V, 5 Hz), in the absence and in the presence of test drugs. EFS of the intrinsic nerves was applied for 40 seconds by a pair of silver electrodes (0.45 mm thick) diametrically opposed on the organ bath walls, from a Harvard stimulator. The response to EFS is a complex of changes in the contractility of the longitudinal muscle and consists of more than one component. Axon potential blockade with 1 μ M tetrodotoxin was used to demonstrate that EFS-induced contractions resulted mainly from stimulation of intrinsic myenteric neurons. Drugs were applied directly to the organ bath in volumes of 20 to 60 μ L.

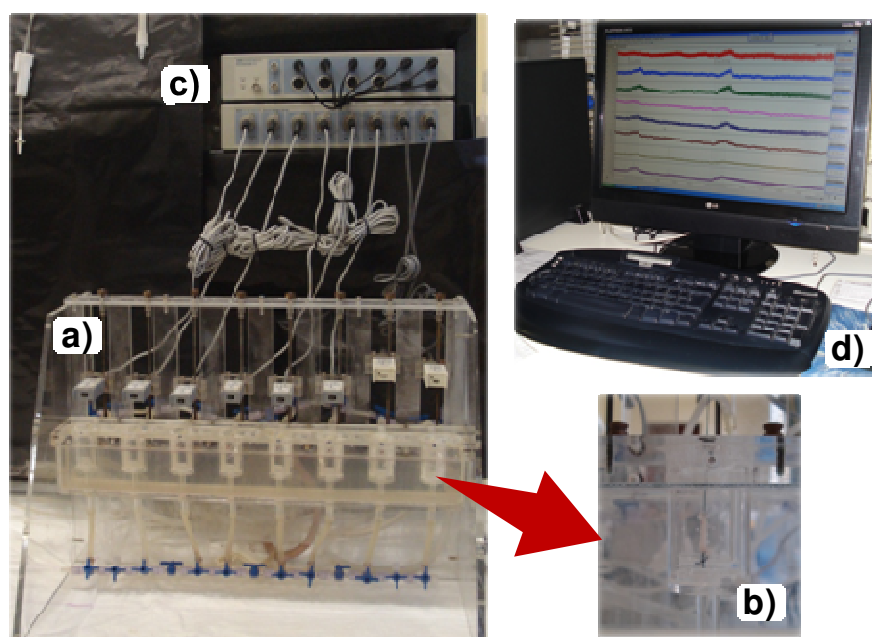


Figure 3.7 - Set-up used for contraction recordings from LM-MP strips of the rat ileum. (a) System of eight perfusion chambers; (b) enlarged view of a LM-MP strip tied up between the holder and the force transducer in a perfusion chamber; (c) PowerLab data acquisition system and (d) view of the Power lab program in the computer screen.

3.3 – Statistics

Values are presented as mean $\pm$ SEM (standard error of the mean), with n indicating the number of animals used for a particular group of experiments. Statistical analysis of data was carried out by using paired or unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's modified t-test (Hox 2010). A value of $P < 0.05$ was considered to represent a significant difference.

3.4 - Drugs

Table 3.1 – Drugs and reagents used in the experimental work.

Abbreviation	Designation	Function	Supplier	Stock solution
ADA	Adenosine deaminase (type IV)	Deamination of adenosine into inosine	Sigma (St Louis, MO, USA)	
ADA activity kit	Spectrophotometric ADA activity kit	Quantification of ADA activity	Roche Diagnostics, Switzerland	
ADO	Adenosine	Adenine nucleoside		
ADP	Adenosine 5'-diphosphate	Adenine nucleotide	Sigma (St Louis, MO, USA)	
ADPβS	Adenosine 5'[[β -thio] diphosphate	ADP stable analogue, P2Y ₁ agonist	Sigma (St Louis, MO, USA)	3 mM stock solution in distilled water
AF-DX 116	11-[[2-1[(diethylamino) methyl-1-Piperidiny]-acetyl]- 5,11-dihydro-6H-pyrido [2,3-b][1,4] benzodiazepine-6-one	Muscarinic M ₂ acetylcholine receptor antagonist	Tocris Cookson Inc. (UK)	50 mM stock solutions in DMSO
AMP	Adenosine 5'-monophosphate	Adenine nucleotide	Sigma (St Louis, MO, USA)	
AOPCP	α,β -methylene ADP	Nucleotide ecto-5'-NTDase inhibitor	Sigma (St Louis, MO, USA)	
Atropine	Atropine sulphate	Antagonist of muscarinic acetylcholine receptors	Sigma (St Louis, MO, USA)	
ATP	Adenosine 5'-triphosphate	Adenine nucleotide	Sigma (St Louis, MO, USA)	
ARL 67156	6-N,N-diethyl-D- β,γ dibromomethylene-D-adenosine-5-triphosphate	Inhibition of ecto-ATPase	Tocris Cookson Inc. (UK)	
ATPγS	adenosine 5'-[γ -thio] triphosphate	Stable ATP analogue, P2X and P2Y receptor agonist	Sigma (St Louis, MO, USA)	
[³H]-choline	[methyl- ³ H]choline chloride	Tritiated acetylcholine precursor (radioactive)	Amersham (UK)	ethanol solution, 80 Ci mM
CGS 21680C	2-[4-(2- <i>p</i> -carboxyethyl) phenylamino]-5'-N-ethylcarboxamido adenosine	Selective adenosine A _{2A} receptor agonist	Research Biochemicals, Natick, MA, USA	
Choline chloride	Choline chloride	Acetylcholine precursor	Sigma (St Louis, MO, USA)	
Con A	Concanavalin A	Non-nucleotide ecto-5'-nucleotidase inhibitor	Sigma (St Louis, MO, USA)	
4-DAMP	4-diphenylacetoxo- <i>N</i> -methylpiperidine methiodide	Muscarinic M ₃ acetylcholine receptor antagonist	Sigma (St Louis, MO, USA)	
DMSO	dimethylsulphoxide	Polar aprotic solvent		
DPCPX	1,3-dipropyl-8-cyclopentyl xanthine	Selective adenosine A ₁ receptor antagonist	Research Biochemicals, Natick, MA, USA	5 mM stock solution in 99% DMSO/1% NaOH 1M (v.v ⁻¹)
Dipy	Dipyridamole	Equilibrative nucleoside transporter inhibitor	Boehringer Ingelheim, Germany	
EHNA	erythro-9(2-hydroxy-3-nonyl) adenine	ADA inhibitor	Research Biochemicals, Natick, MA, USA	5 mM stock solution in ethanol
EGTA	ethylene glycol- <i>bis</i> (β -aminoethylether)-N,N,N',N'-tetra acetic acid	Calcium chelating agent	Sigma (St Louis, MO, USA)	
H-89	<i>N</i> -[2-(<i>p</i> -bromocinnamyl amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride	Protein kinase A inhibitor	Sigma (St Louis, MO, USA)	
Hemicholinium-3	hemicholinium-3	Blockade of choline uptake	Sigma (St Louis, MO, USA)	
ITU*	5-iodotubericidin	AK inhibitor	Research Biochemicals, Natick, MA, USA	5 mM solution in DMSO

Material and Methods

J104129	(α R)- α -Cyclopentyl- α -hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate	Muscarinic M ₃ acetylcholine receptor selective antagonist	Tocris Cookson Inc. (UK)	10 mM stock solutions in DMSO
2-MeSADP	2-methylthio-adenosine diphosphate	Stable ADP analogue, P2Y _{1,12,13} agonist	Sigma (St Louis, MO, USA)	10 mM stock solution in distilled water
α,β-MeATP	α,β -methylene adenosine 5'-triphosphate	Stable ATP analogue, activates preferentially P2X _{1,3} receptor subunits	Sigma (St Louis, MO, USA)	
McN-A-343	4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butyryl trimethylammonium chloride	Muscarinic M ₁ acetylcholine receptor selective agonist	Sigma (St Louis, MO, USA)	
MRS 2179	2'-deoxy-N6-methyl adenosine 3',5'-diphosphate diammonium salt	Selective P2Y ₁ receptor antagonist	Tocris Cookson Inc. (UK)	3 mM stock solution in distilled water
MT-3	muscarinic toxin 3	Muscarinic M ₄ acetylcholine receptor selective antagonist	Alomone Laboratories, Jerusalem, Israel	
MT-7	muscarinic toxin 7	Muscarinic M ₁ acetylcholine receptor selective antagonist	Peptide Institute Inc., Osaka, Japan	
NBTI	S-(p-nitrobenzyl)-6-thioinosine	Equilibrative nucleoside transporter inhibitor	Sigma (St Louis, MO, USA)	10 mM stock solutions in DMSO
NIF*	nifedipine	L-type voltage sensitive Ca ²⁺ channel blocker	Sigma (St Louis, MO, USA)	10 mM stock solution in ethanol
Oxo	oxotremorine sesquifumarate	Agonist of muscarinic acetylcholine receptors	Sigma (St Louis, MO, USA)	
Physio	physostigmine	Acetylcholinesterase inhibitor	Sigma (St Louis, MO, USA)	
PPADS*	pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) tetrasodium salt	Non-selective P2X receptor antagonist	Sigma (St Louis, MO, USA)	3 mM stock solution in distilled water
RB-2*	Reactive blue-2	P2 receptor antagonist	Sigma (St Louis, MO, USA)	
R-PIA	R-N ⁶ -phenylisopropyl adenosine	Selective adenosine A ₁ receptor agonist	Sigma (St Louis, MO, USA)	50 mM stock solution in DMSO*
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl) adenosine -5'-triphosphate	Preferential P2X _{1,3} receptor antagonist	Tocris Cookson Inc. (UK)	10 mM stock solution in distilled water
TTX	tetrodotoxin	Inhibition of voltage sensitive sodium channels, blocking axonal conduction	Tocris Cookson Inc. (UK)	
ZM 241385	(4-(2-[7-amino-2-(2-furyl[1,2,4]-triazolo {2,3-a}{1,3,5}triazin-5-yl-aminoethyl)-phenol	Selective adenosine A _{2A} receptor antagonist	Tocris Cookson Inc. (UK)	5 mM stock solutions in DMSO

* These drugs were kept protected from the light to prevent photodecomposition.

All stock solutions were stored as frozen aliquots at -20 °C. Dilutions of these stock solutions were made daily and appropriate solvent controls were used. All the other compounds were dissolved in Tyrode's solution. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v·v⁻¹), were observed. The pH of the superfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

4 - RESULTS

The author's contribution to the results presented in this thesis includes the majority of the experimental data, excluding HPLC analysis that was kindly performed and/or supervised by Dra. Maria Teresa Magalhães-Cardoso (Laboratório de Farmacologia e Neurobiologia, ICBAS-UP). Determination of ADA and LDH activities were performed by Ms. Maria Júlia Carvalho and Ms. Maria Isabel Garrido (Laboratório de Química Clínica – Hospital Geral de Santo António, SA, Porto). The author participated in all scientific discussions and conclusions of the present work. The scientific content of the present thesis has been included in the publication of the following original articles:

Duarte-Araújo M, Nascimento C, Timóteo MA, Magalhães-Cardoso T & Correia-de-Sá P (2004). Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A_{2A} and extrajunctional inhibitory A_1 receptors. *Br J Pharmacol.* 141(6), 925-934. (Duarte-Araújo *et al.* 2004a).

Correia-de-Sá P, Adães S, Timóteo MA, Vieira C, Magalhães-Cardoso T, Nascimento C & **Duarte-Araújo M** (2006). Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: on the role of secreted adenosine deaminase. *Auton Neurosci.* 126-127, 211-224. (Correia-de-Sá *et al.* 2006).

Duarte-Araújo M, Nascimento C, Timóteo MA, Magalhães-Cardoso MT & Correia-de-Sá P (2009). Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on acetylcholine release from myenteric motoneurons. *Br J Pharmacol.* 156(3), 519-533. (Duarte-Araújo *et al.* 2009).

Vieira C, **Duarte-Araújo M**, Adães S, Magalhães-Cardoso T & Correia-de-Sá P (2009). Muscarinic M_3 facilitation of acetylcholine release from rat myenteric neurons depends on adenosine outflow leading to activation of excitatory A_{2A} receptors. *Neurogastroenterol Motil.* 21(10), 1118-1128. (Vieira *et al.* 2009).

Other papers or manuscripts where the author's conjoint work was published during her doctoral studies:

Duarte-Araújo M, Timóteo MA & Correia-de-Sá P (2004). Adenosine activating A_{2A} -receptors coupled to adenylate cyclase/cyclic AMP pathway downregulates nicotinic autoreceptor function at the rat myenteric nerve terminals. *Neurochem Int.* 45(5), 641-651. (Duarte-Araújo *et al.* 2004b).

Vieira C, Ferreirinha MF, Silva I, **Duarte-Araújo M** & Correia-de-Sá P (2011). Localization and function of adenosine receptor subtypes at the longitudinal muscle – myenteric plexus of the rat ileum. *Neurochem Int.* 59(7), 1043-1055. (Vieira *et al.* 2011).

4.1 – Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on ACh release from myenteric motoneurons;

4.1.1 Rationale

ATP generally produces hyperpolarisation and relaxation of the gut; however, conflicting results showing ATP contribution to excitatory neurotransmission in some regions of the gut from various animal species, have been reported (*e.g.* Giaroni *et al.*, 2002; Barthó *et al.*, 2006; Zizzo *et al.*, 2007). P2Y₁ receptors were identified in enteric neurons and smooth muscle cells by immunohistochemistry; reactivity to P2Y₂ receptors sensitive to uridine nucleosides was also found in a smaller number of neurons (Giaroni *et al.*, 2002). Electrophysiological studies indicate that ATP mediates slow excitatory post-synaptic potentials (sEPSP) in NOS-containing descending interneurons in the myenteric plexus through a yet unidentified P2Y receptor, while the ADP-sensitive P2Y₁ receptor seems to be involved in sEPSP in submucous S neurons (Galligan, 2002). Additionally, ATP is the predominant non-cholinergic fast synaptic transmitter in murine enteric neurons through the activation of P2X receptors. Neuro-neuronal fast synaptic potentials, in both ascending and descending reflex pathways to the longitudinal and circular muscles, are mediated by P2X₂ and P2X₃ subunit-containing receptors (Bertrand & Bornstein 2002; Bian *et al.* 2003; Galligan 2002a; Wood 2006a; Xiang & Burnstock 2004).

The study of nucleotide receptors and their functions is complicated by the presence on the cell surface of enzymes that rapidly break down extracellular nucleotides into nucleosides, the ecto-nucleotidases (Yegutkin 2008; Zimmermann 2000). One nucleoside in particular, adenosine, directly activates P1 receptors located on smooth muscle fibres and enteric neurons, where it modulates the release of excitatory neurotransmitters, like substance P and acetylcholine (Duarte-Araújo *et al.* 2004a; Moneta *et al.* 1997; Nicholls *et al.* 1996). The myenteric plexus contains the enzymes responsible for the formation of adenosine from ATP released from the activated smooth muscle cells (Nitahara, Kittel *et al.* 1995) as well as from stimulated myenteric neurons (Nitahara *et al.* 1995; White *et al.* 1982). Although the extracellular catabolism of ATP via the ecto-nucleotidase pathway contributes only partially to the total interstitial nucleoside concentration in the myenteric plexus (this thesis; see also Correia-de-Sá *et al.* 2006), it might be relevant under pathological conditions (*e.g.* intestinal ischemia, chronic inflammation) when extracellular ATP levels increase (Bogers *et al.* 2000; Bornstein 2008; Burnstock 2008b; Correia-de-Sá *et al.* 2006)

Among the nucleotidases, four members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, namely NTPDase1, ATPDase, NTPDase3 and NTPDase8, and two members of the ecto-nucleotide pyrophosphatases / phosphodiesterases (E-NPP) family, NPP1 and NPP3, are located at the plasma membrane and hydrolyse extracellular nucleotides (Kukulski *et al.* 2005; Stefan *et al.* 2006; Zimmermann 2000) (for further details, see Introduction). The final nucleotide, AMP, is hydrolysed to adenosine and Pi by ecto-5'-nucleotidase (E-5'-N), a glycosylphosphatidylinositol-anchored enzyme located at the cell surface. It is interesting to note that E-5'-N activity is concentrated in the myenteric smooth muscle cell layer (Nitahara *et al.* 1995). The sequential degradation of adenine nucleotides not only terminates ATP signalling but also generates intermediates with distinct signalling properties. The relative contribution of distinct ecto-nucleotidase species to the modulation of purinergic signalling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific membrane domains, but also on substrate availability and substrate preference. In the present work, we studied the pattern of extracellular ATP catabolism with particular emphasis on the relative contribution of ecto-ATPDase (an ADP forming ecto-NTPDase that hydrolyzes ATP with higher selectivity over ADP - probably NTPDase2) and of NTPDase1 (bypassing ADP formation) pathways, in order to assess their role in controlling [³H]acetylcholine ([³H]ACh) release from the myenteric plexus of the rat ileum.

4.1.2 Pattern of extracellular catabolism of adenine nucleotides and adenosine formation in the LM-MP of the rat ileum

Figure 4.1 illustrates the time course of the extracellular catabolism of adenine nucleotides (ATP, ADP and AMP) in the LM-MP of the rat ileum. ATP (30 μ M) was catabolised with a half-degradation time of 6.9 ± 0.7 min ($n = 6$). The ATP metabolites detected in the bath were ADP, AMP, adenosine, inosine and hypoxanthine, whose concentrations increased with time. AMP was the first metabolite to appear in the bath, reaching a peak concentration of 6.86 ± 0.78 μ M at 15 min (Figure 4.1a). The concentration of ADP attained a maximum of 3.98 ± 0.51 μ M also at 15 min, whereas adenosine reached a maximum concentration of 7.85 ± 1.77 μ M only 30 min after ATP (30 μ M) application. The concentration of inosine increased continuously up to 45 min, reaching 13.71 ± 2.30 μ M. The formation of hypoxanthine was almost negligible (1.99 ± 0.20 μ M at 45 min).

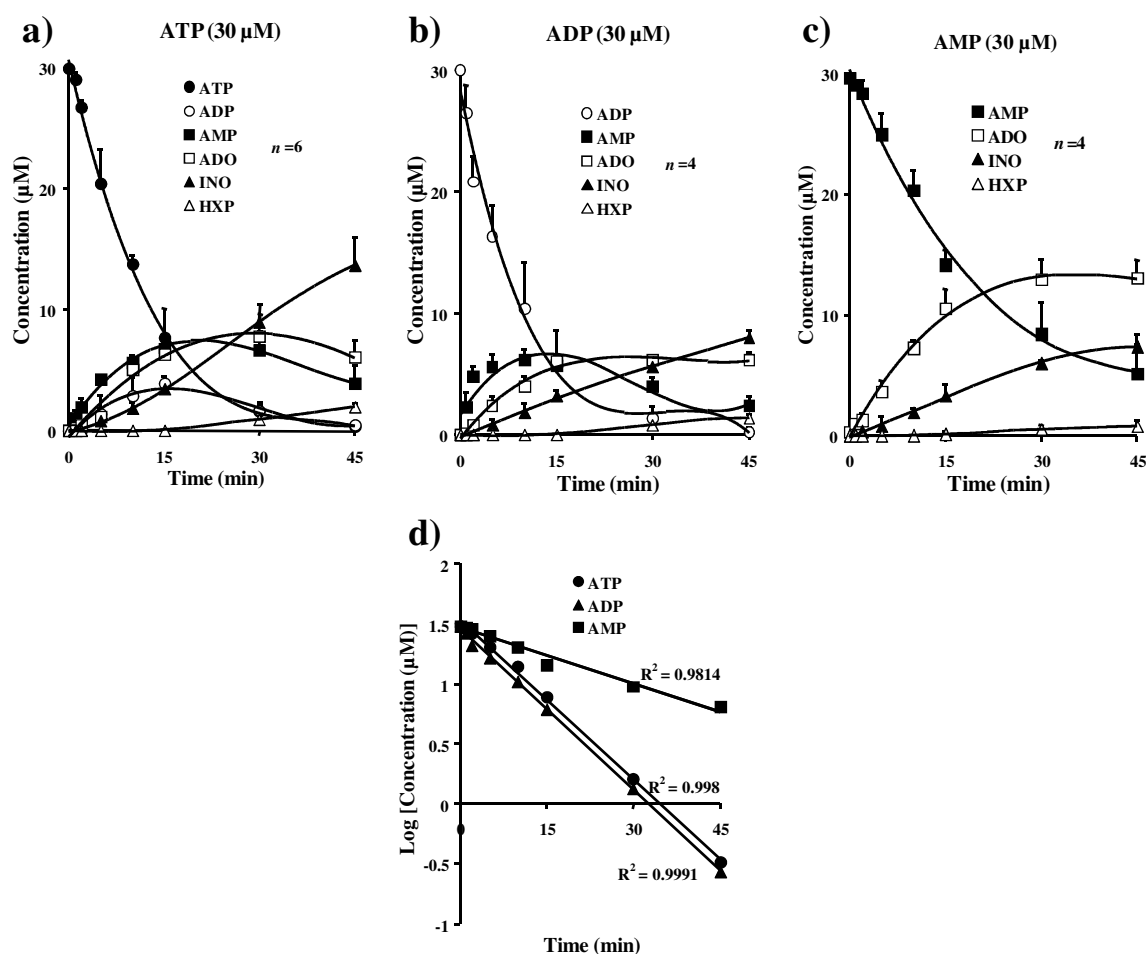


Figure 4.1 Time course of extracellular ATP (a), ADP (b) and AMP (c) metabolism in the myenteric plexus of the rat ileum. Adenine nucleotides (30 μM) were added at time zero to the preparations, and samples (75 μL) were collected from the bath at indicated times on the abscissa. Each collected sample was analysed by high-performance liquid chromatography to separate and quantify ATP, ADP, AMP, adenosine (ADO), inosine (INO) and hypoxanthine (HXP). Averaged results obtained in (a) six or (b and c) four experiments; the vertical bars represent SEM and are shown when they exceed the symbols in size. In control conditions, without the addition of ATP, ADP or AMP, only inosine monophosphate could be detected in the bath, reaching a maximum concentration (1.65 μM) at the end of 45 min. In (d), are represented the semi-logarithmic progress curves obtained by polynomial fitting of the catabolism of ATP (30 μM), ADP (30 μM) and AMP (30 μM). Note that ATP, ADP and AMP linearly disappeared from the bath; the calculated half-degradation times appear in the text.

As shown in Figure 4.1b, extracellular ADP (30 μM) was catabolised with a half-degradation time (6.6 ± 1.6 min, $n = 4$) comparable to that of ATP (30 μM) metabolism (see also, Figure 4.1d). ADP catabolism led rapidly to AMP formation, which was then sequentially metabolized into adenosine, inosine and hypoxanthine (Figure 4.1b). The maximum concentration of AMP (6.16 ± 0.88 μM) was obtained at 10 min. Adenosine reached a maximum concentration of 6.16 ± 0.39 μM at 15 min. The concentration of

inosine increased continuously up to 45 min, reaching $8.02 \pm 0.62 \mu\text{M}$, while formation of hypoxanthine was still negligible ($1.43 \pm 0.23 \mu\text{M}$ at 45 min).

Extracellular AMP (30 μM) was catabolised with a half-degradation time of 15 ± 2.4 min. The AMP metabolites detected in the bath were adenosine, inosine and hypoxanthine, whose concentrations in the bathing fluid increased progressively reaching maximum values of $12.95 \pm 2.21 \mu\text{M}$ at 30 min, $7.37 \pm 0.96 \mu\text{M}$ at 45 min and $1.67 \pm 0.92 \mu\text{M}$ at 45 min, respectively (Figure 4.1c). Given the linearity of the kinetics of ATP, ADP and AMP degradation shown in Figure 4.1d, the analysis of the corresponding half-degradation time values clearly indicates that extracellular catabolism of AMP, through the ecto-5'-nucleotidase (E-5'-N), is the rate-limiting step in the generation of adenosine from exogenously added adenine nucleotides in the myenteric plexus.

The presence of *p*-nitrophenylphosphate (*p*-NPP) in a saturating concentration (1 mM) did not alter the degradation kinetics of ATP, ADP or AMP, suggesting that the contribution of non-specific phosphatases, such as alkaline phosphatase, to the extracellular catabolism of adenine nucleotides is negligible (data not shown).

4.1.3 Relative contribution of ecto-ATPase (forming ADP) and ecto-ATPDase (bypassing ADP formation) pathways for ATP catabolism in the LM-MP

During the first 2 min of ATP degradation, there was a stoichiometric conversion of ATP into AMP without detectable formation of ADP (Figure 4.1a). These findings demonstrate the presence of NTPDase1 (ATP diphosphohydrolase or apyrase) activity with a strong preference for nucleotide 5'-triphosphates in the rat myenteric plexus, through which ATP is hydrolysed directly into AMP with minimal sequential breakdown to form ADP (see Figure 4.10, Section 4.1.7).

To evaluate the relative contributions of these two pathways, we investigated the influence of the structural analogue of ATP that selectively inhibits ecto-ATPDase activity, ARL 67156 (6-N,N-diethyl-D- β,γ -dibromomethylene ATP, formerly known as FPL 67156) (Crack *et al.* 1995), on the kinetics of ATP (30 μM) catabolism in the rat myenteric plexus. When applied in a concentration (100 μM) near the IC_{50} value to inhibit ecto-ATPDase activity, ARL 67156 moderately decreased ATP hydrolysis (Figure 4.2a), without significantly ($P > 0.05$) affecting the generation profile of AMP (Figure 4.2c) and adenosine (Figure 4.2d). The way ARL 67156 affected ATP catabolism was to delay ADP formation, that is, the concentrations of ADP reach maximum values ($\sim 4 \mu\text{M}$) at 15 min and 30 min of incubation in the absence and in the presence of ARL 67156 (100 μM), respectively (Figure 4.2b).

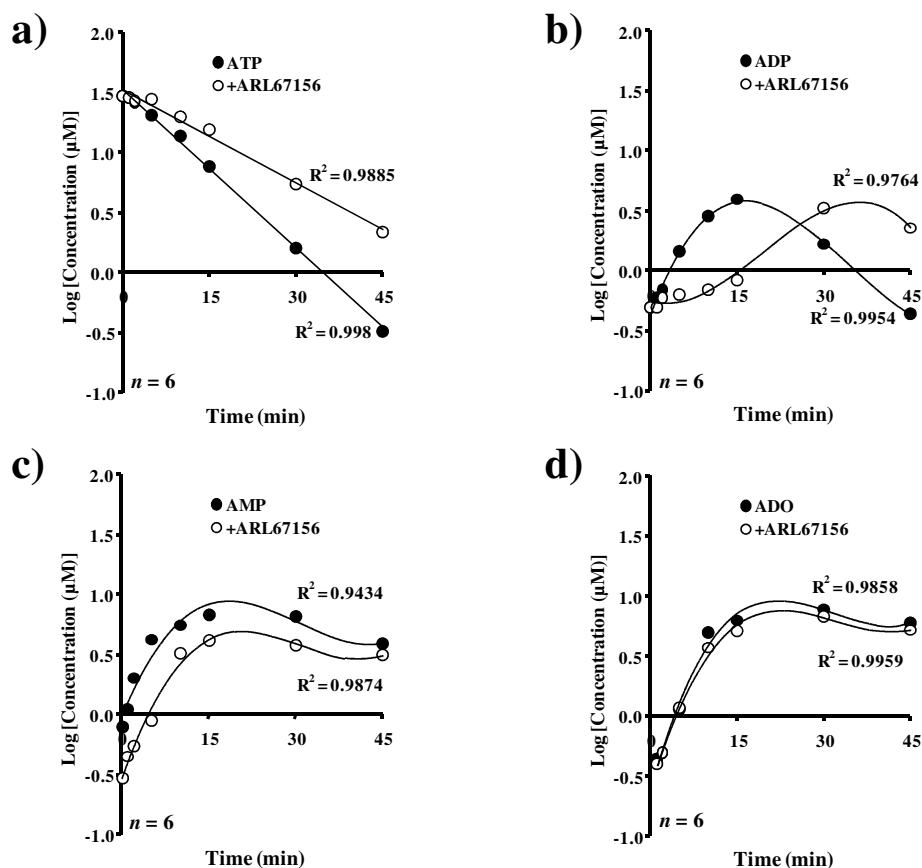


Figure 4.2 Semi-logarithmic progress curves obtained for the extracellular catabolism of ATP in the absence and in the presence of the ecto-ATPase inhibitor, ARL 67156 (100 μM). ATP (30 μM) was added at time zero to the preparations in the absence and in the presence of ARL 67156. Samples (75 μL) were collected at the times indicated on the abscissa and retained for high-performance liquid chromatography to separate and quantify (a) ATP, (b) ADP, (c) AMP and (d) adenosine (ADO). Both progress curves were obtained from the same preparations; in the absence of ARL 67156, time-matched results did not significantly ($P > 0.05$) differ from the control situation (Figure 4.1a). Semi-logarithmic curves were obtained by polynomial fitting from an average of six experiments; for the sake of clarity, bars representing SEM are not shown. Note that ARL 67156 reduced (a) ATP catabolism and delayed (b) the formation of ADP, without affecting the profile of (c) AMP and (d) ADO generation. In control conditions, without the addition of ATP, only inosine monophosphate could be detected in the bath, reaching a maximum concentration (1.15 μM) at the end of 45 min.

Figure 4.3 illustrates the kinetics of ATP breakdown and metabolite formation, with increasing initial concentrations of the substrate (10-100 μM). The rate of ATP hydrolysis leading to ADP, AMP and adenosine formation was the same, irrespective of the initial concentration of ATP applied to the incubation fluid (Figure 4.3a). Interestingly, dephosphorylation of ATP leading to ADP formation increased progressively, compared with the direct conversion of ATP into AMP when the concentration of ATP applied to the bath increased from 10 to 100 μM (Figure 4.3b). The absolute amount of AMP detected

following 15 min incubation with ATP (10–100 μM) remained essentially unchanged ($\sim 7 \mu\text{M}$). Taken together, these results support the idea that the ADP-generating ecto-ATPase pathway may function as an alternative for ATP breakdown whenever the substrate concentration rises to levels high enough to saturate the preferential NTPDase1 pathway.

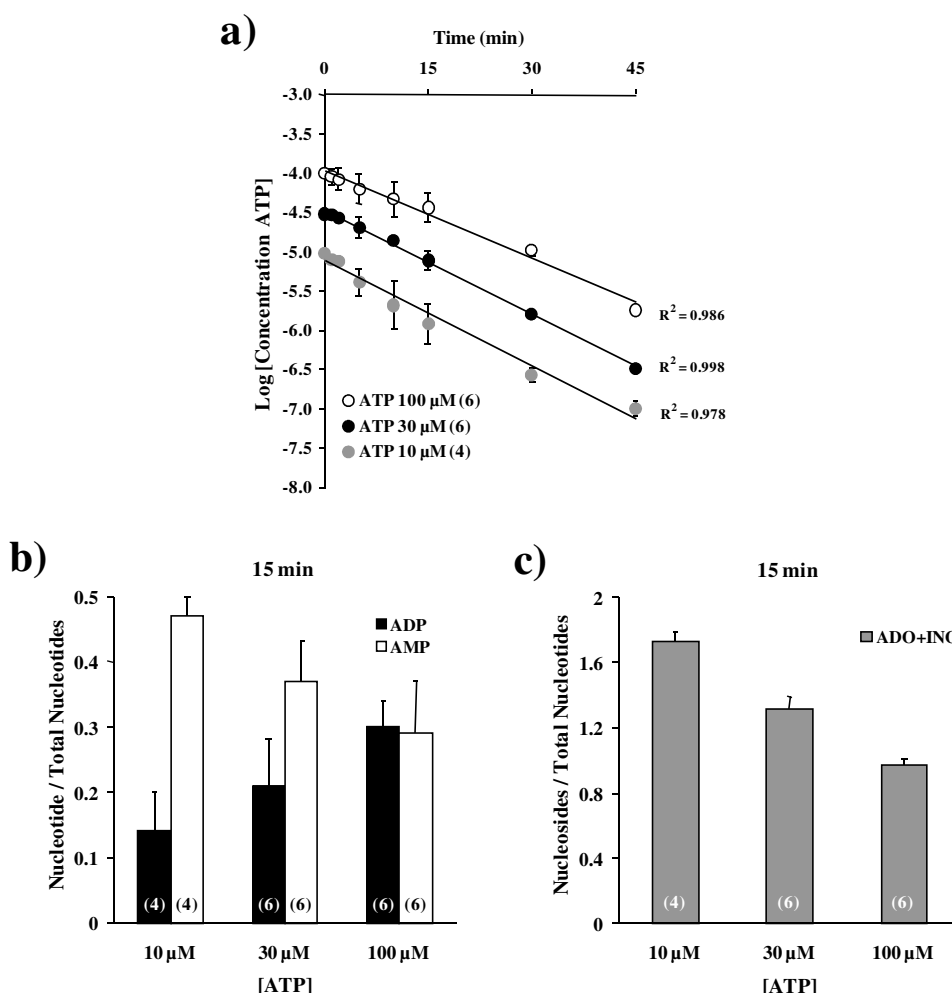


Figure 4.3 - Kinetics of extracellular ATP breakdown and metabolite formation upon increasing the initial concentration of the substrate. ATP (10–100 μM) was added at time zero to the preparations. Samples (75 μL) collected at the times indicated on the abscissa were retained for high-performance liquid chromatography to separate and quantify (a) ATP, and its metabolites (b) ADP or AMP and (c) adenosine (ADO) plus inosine (INO). In (a), semi-logarithmic progress curves were obtained by polynomial fitting of the catabolism of ATP (10–100 μM). Note that the kinetics of ATP breakdown was not affected by the concentration of the initial substrate. In (b), it is shown the relative amount of ADP and AMP as compared with the total amount of nucleotides [(ATP + ADP + AMP)] in the bath, following 15 min incubation with ATP (10–100 μM). In (c), the ratio [nucleosides]:[total nucleotides], following 15 min incubation with ATP (10–100 μM) as a direct measure of the activity of ecto-5'-nucleotidase, is shown. Data shown are pooled from the number of experiments shown in parentheses. The vertical bars represent SEM and are shown when they exceed the symbols in size.

Formation of extracellular adenosine from the catabolism of adenine nucleotides is carried out by ecto-5'-nucleotidase (E-5'-N), which may be subjected to "feed-forward" inhibition by ATP and/or ADP (Cunha 2001; Naito *et al.* 1985). This inhibition can be evaluated by quantifying the ratio [Nucleosides] : [Total nucleotides], which is a direct measure of the activity of ecto-5'-nucleotidase. Figure 4.3c shows that this ratio decreased when the concentration of ATP increased from 10 to 100 μ M. In such circumstances, adenosine formation was delayed indicating that ATP did inhibit ecto-5'-nucleotidase (E-5'-N) in this preparation. Exposure of the myenteric plexus to a higher concentration of AMP (100 μ M) did not alter its rate of hydrolysis or the pattern of appearance of its metabolites (data not shown).

4.1.4 ATP transiently facilitates [^{3} H]ACh release due to the activation of P2X receptors on myenteric nerve terminals

Exogenously applied ATP caused a dual effect on [^{3} H]ACh release from myenteric motoneurons of the rat ileum (Figure 4.4). ATP (1-300 μ M) transiently increased spontaneous [^{3} H]ACh release in a concentration-dependent manner (see also, Figure 4.5a); tritium outflow returned to the pre-stimulation levels by 9–12 min after ATP application. Following 15 min of application, ATP (30 μ M) decreased [^{3} H]ACh release from stimulated (EFS, 200 pulses of 1 ms duration applied at a 5 Hz frequency) myenteric motoneurons (Figure 4.4).

It appears that the facilitatory effect of ATP results from activation of ionotropic P2X receptors, because ATP-induced tritium outflow was nearly abolished in the presence of TNP-ATP (10 μ M), which blocks preferentially P2X receptors in the micromolar concentration range without affecting P2Y receptor activity (Virginio *et al.* 1998) (Figure 4.5b). The non-selective P2 receptor antagonist, PPADS (10 μ M), also reduced ATP-induced [^{3} H]ACh release by $73 \pm 8\%$ ($n = 6$) (Figure 4.5b). α,β -methyleneATP (30 μ M), a potent agonist of P2X1 and P2X3 receptors (North 2002; Ralevic & Burnstock 1998), was devoid of effect on the spontaneous [^{3} H]ACh release.

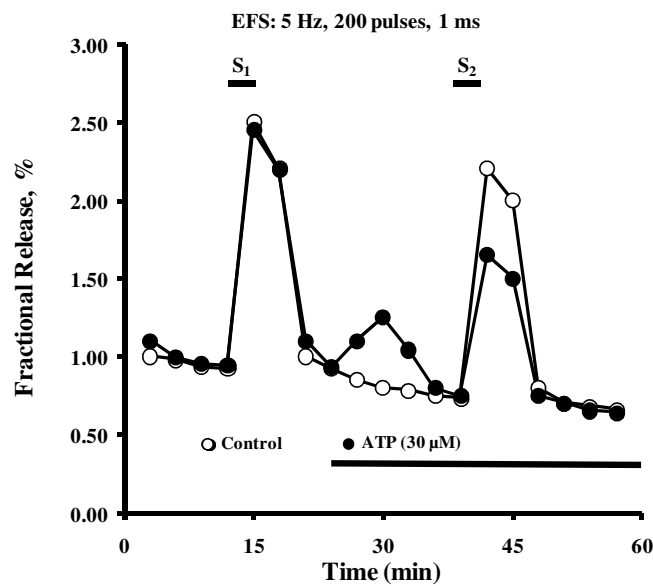


Figure 4.4 - Effect of ATP (30 μM) on tritium outflow from the longitudinal muscle-myenteric plexus of the rat ileum. The time course of tritium outflow, taken from a typical experiment, is shown. Tritium outflow (ordinate) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional Release, %). The release of [^3H]acetylcholine ([^3H]ACh), in response to electrical field stimulation (EFS; 200 pulses of 1 ms duration delivered at a 5 Hz frequency), was elicited twice during the periods indicated (S_1 and S_2). ATP (30 μM) was applied 15 min before S_2 (as represented by the horizontal bar). The time course of tritium outflow in a control situation, that is, in the absence of ATP, is also shown for comparison. Note that ATP (30 μM) transiently increased the resting tritium outflow, but decreased the release of [^3H]ACh from the stimulated myenteric plexus.

The facilitatory effect of ATP (100 μM) on [^3H]ACh outflow was dependent on Ca^{2+} influx from the extracellular media, but was insensitive to blockade by TTX (reduction by $3 \pm 8\%$, $n = 6$) (Figure 4.5c), when this toxin was applied in a concentration (1 μM) that fully blocked tritium outflow caused by EFS (Duarte-Araújo *et al.* 2004b). Resistance to TTX (1 μM), which blocks Na^+ influx thereby blocking axonal conduction, indicates that the ionotropic P2X receptors are most probably located on cholinergic nerve terminals.

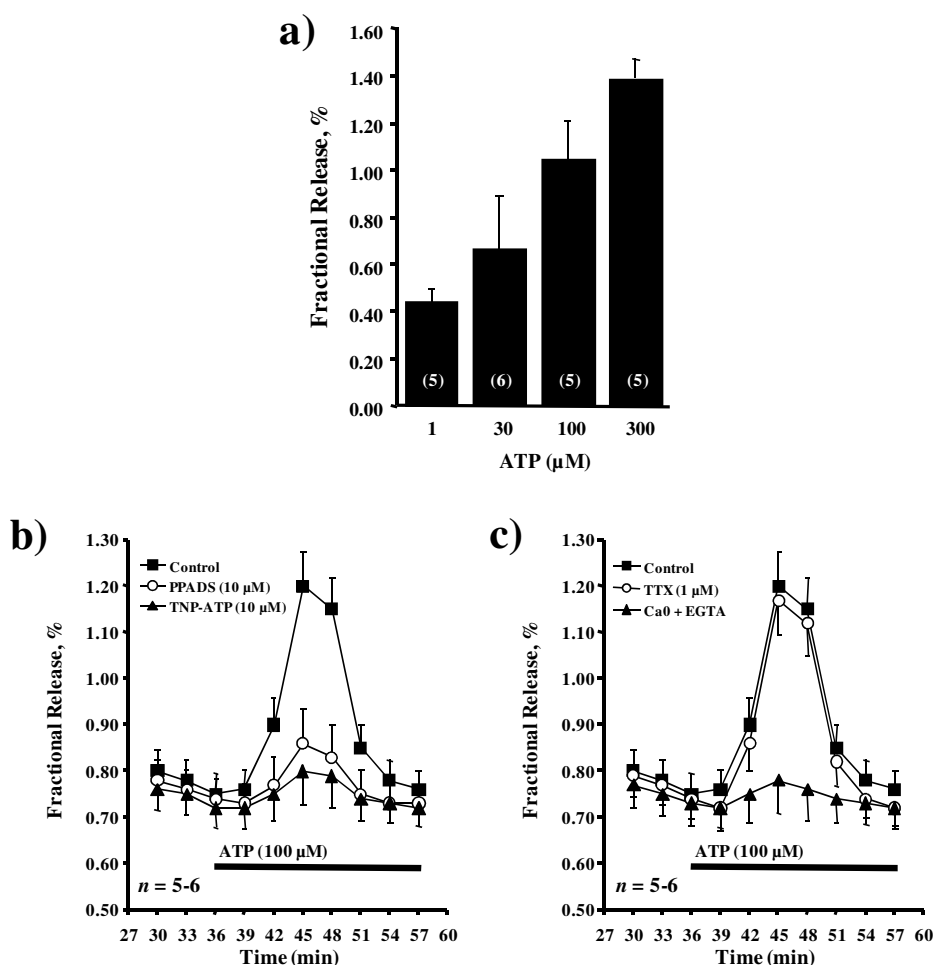


Figure 4.5 - Extracellular ATP transiently activates P2X receptors mediating spontaneous [^3H]ACh release from myenteric nerve terminals. In (a), the ordinates represent spontaneous tritium outflow (as Fractional Release %; see Methods) induced by increasing extracellular ATP concentrations (1–300 μM). ATP was applied as indicated in Figure 4.4. Each column represents pooled data from five to six experiments. The vertical bars represent SEM. In (b) and (c) it is shown the time course of [^3H]ACh release induced by ATP (100 μM , arrow) in the absence and in the presence of (b) two non-selective P2X antagonists, PPADS (10 μM) and TNP-ATP (10 μM), (c) TTX (tetrodotoxin, 1 μM , an action potential generation blocker) and Tyrode's solution without Ca^{2+} plus EGTA (1 mM). Tritium outflow (ordinates) is expressed as Fractional Release, %. The abscissa indicates the times at which the samples were collected. Each point is pooled data ($\pm\text{SEM}$) from five to six experiments. None of the drugs changed spontaneous tritium outflow.

4.1.5 Adenine nucleotides inhibit [³H]ACh release directly, through activation of P2Y₁ purinoceptor and indirectly, by formation of adenosine leading to A₁ receptor activation

The inhibitory effect of ATP (30 μ M, $-25 \pm 3\%$, $n = 6$) on stimulation-induced [³H]ACh release was mimicked by the non-selective P2 agonist, ATP γ S (30 μ M, $-22 \pm 3\%$, $n = 6$), but not by the preferential P2X agonist, α,β -methyleneATP (30 μ M) (Figure 4.6a). These findings suggest that ATP inhibition of evoked [³H]ACh release from myenteric motoneurons is not due to fast desensitization of facilitatory prejunctional P2X purinoceptors operated by α,β -methyleneATP (30 μ M). Exogenous ADP also decreased [³H]ACh release from stimulated myenteric motoneurons, respectively by $22 \pm 4\%$ ($n = 6$) and $37 \pm 1\%$ ($n = 6$), when the nucleotide was applied in 30 and 100 μ M concentrations (see Figure 4.6b); ADP (30 and 100 μ M) failed to increase spontaneous [³H]ACh release. The inhibitory effect of ADP on evoked [³H]ACh release was reproduced by its stable analogue, ADP β S (30 μ M, $-27 \pm 3\%$, $n = 6$) (Figure 4.6b), but not by 2-MeSADP (30 μ M). This might be due to the chemical instability of 2-MeSADP (30 μ M) (von Kugelgen *et al.* 2000), as this drug is extensively hydrolysed (more than 80% following a 15 min period of incubation) in the longitudinal muscle-myenteric plexus (data not shown). It is worth noting that none of the stable ATP analogues used in this study affected the activity of NTPDases (Yegutkin *et al.* 2000).

The inhibitory effect of ADP (100 μ M, $-37 \pm 1\%$, $n = 6$) was partially reduced by MRS 2179 (0.3 μ M, $-24 \pm 4\%$, $n = 5$, a selective P2Y₁ antagonist), by ADA, the enzyme that inactivates endogenous adenosine (0.5 U/mL, $-26 \pm 5\%$, $n = 6$), and by DPCPX, a selective adenosine A₁ antagonist (10 nM, $-28 \pm 7\%$, $n = 5$). The non-selective P2 antagonists, PPADS (10 μ M) and RB-2 (30 μ M), failed to modify ADP (100 μ M) inhibition of evoked [³H]ACh release. Co-application of MRS 2179 (0.3 μ M) plus ADA (0.5 U/mL) completely abolished ADP inhibition of [³H]ACh release from stimulated myenteric motoneurons (Figure 4.7). These findings suggest that ADP acts directly, via ADP-sensitive P2Y₁ purinoceptors, and indirectly, via adenosine formation leading to adenosine A₁ receptor activation, to reduce the evoked release of [³H]ACh. A similar pattern of the ability of ATP to inhibit evoked [³H]ACh release from myenteric motoneurons was shown by using MRS 2179 (0.3 μ M) and ADA (0.5 U/mL). In these conditions, the inhibitory P2Y₁ receptors activated by ADP, generated via ecto-ATPDase, play a major role compared with the adenosine A₁ receptors, when the concentration of ATP was increased from 30 to 100 μ M. That is, the attenuation of the inhibitory effect of ATP (100 μ M, $-30 \pm 3\%$, $n = 5$) was greater in the presence of MRS 2179 (0.3 μ M, $-19 \pm 2\%$, $n = 6$) than in the presence of ADA (0.5 U/mL, $-24 \pm 5\%$, $n = 4$), while both drugs had a similar effect (reduction by $\sim 19\%$) with 30 μ M ATP.

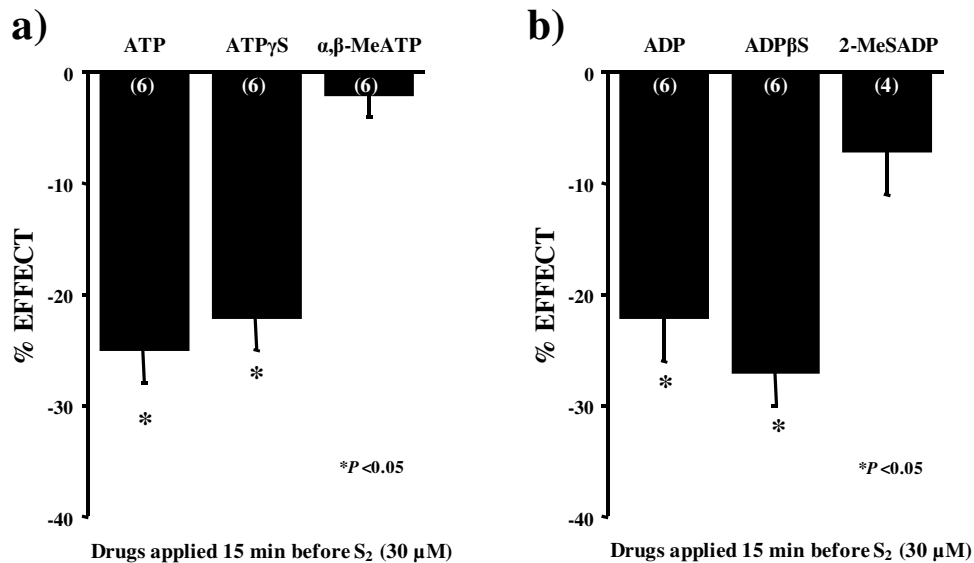


Figure 4.6 - Effects of exogenously added adenine nucleotides on $[^3\text{H}]\text{ACh}$ release from myenteric neurons stimulated with electrical field stimulation (EFS). The release of $[^3\text{H}]\text{ACh}$ in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S_1 and S_2). (a) Nucleotide triphosphates (ATP, $\text{ATP}\gamma\text{S}$ and $\alpha,\beta\text{-MeATP}$) and (b) diphosphates (ADP, $\text{ADP}\beta\text{S}$ and 2-MeSADP) were added 15 min before S_2 in a 30 μM concentration. The ordinates are percentage changes in S_2/S_1 ratios compared with controls. Each column represents pooled data ($\pm\text{SEM}$) from an n number of experiments shown in parentheses. $*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) when compared with zero percentage of change.

On their own, the selective P2Y_1 receptor antagonist, MRS 2179 (0.3 μM), facilitated $[^3\text{H}]\text{ACh}$ release from stimulated myenteric motoneurons by $28 \pm 2\%$ ($n = 6$) (Figure 4.8), whereas the non-selective P2 antagonists, PPADS (10 μM) and RB-2 (30 μM), increased $[^3\text{H}]\text{ACh}$ release only by $7 \pm 3\%$ ($n = 5$) and $2 \pm 7\%$ ($n = 4$), respectively (data not shown). A higher concentration of MRS 2179 (1 μM) did not increase further the facilitatory action ($28 \pm 6\%$, $n = 4$) of the P2Y_1 receptor antagonist (data not shown). Inhibition of ecto-ATPDase with ARL 67156 (100 μM) prevented MRS 2179 (0.3 μM)-induced facilitation ($-9 \pm 7\%$, $n = 6$) of evoked $[^3\text{H}]\text{ACh}$ release (Figure 4.8), demonstrating that endogenous ADP tonically activates inhibitory P2Y_1 receptors on myenteric nerve terminals. MRS 2179 does not affect NTPDase activities in the concentration range commonly used to inhibit P2Y_1 receptors (Munkonda *et al.* 2007). The magnitude of the P2Y_1 receptor-mediated inhibitory tonus might be positively correlated with the stimulation train length, that is, with the amount of ADP generated at the myenteric synapse. To confirm this hypothesis, we performed experiments increasing the number of pulses delivered to the preparation from 200 to 500, keeping constant the stimulation frequency (5 Hz) and the pulse width (1 ms). In these circumstances,

facilitation of evoked [^3H]ACh release by MRS 2179 (0.3 μM) was increased to $36 \pm 3\%$ ($n = 4$) (data not shown).

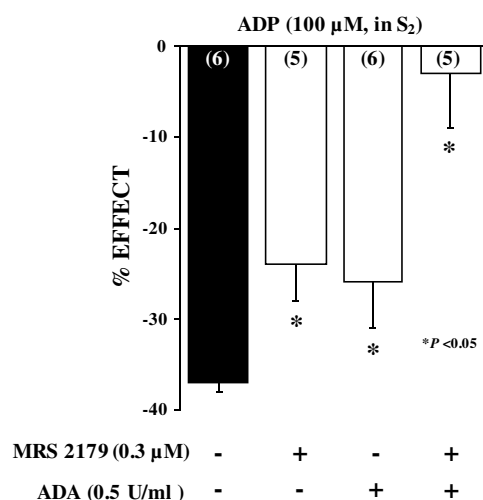


Figure 4.7 - Effect of exogenously added ADP on [^3H]ACh release from myenteric neurons stimulated by EFS, in the absence and in the presence of the selective P2Y₁ receptor antagonist, MRS 2179 (0.3 μM) and of ADA (0.5 U/mL). The release of [^3H]ACh in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S_1 and S_2). ADP (100 μM) was added 15 min before S_2 ; MRS 2179 and/or ADA were added to the incubation media at the beginning of the release period (time zero) and were present throughout the assay, including S_1 and S_2 . The ordinates are percentage changes in S_2/S_1 ratios compared with controls. The average S_2/S_1 ratios, in the presence of MRS 2179 (0.87 ± 0.04 , $n = 6$) and of ADA (0.85 ± 0.06 , $n = 4$) (without ADP), were not significantly ($P > 0.05$) different from the control value (0.83 ± 0.11 , $n = 4$; data not shown). Each column represents pooled data ($\pm\text{SEM}$) from an n number of experiments shown in parentheses. $*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test), significant differences compared with the effect of ADP in the absence of MRS 2179 and/or ADA.

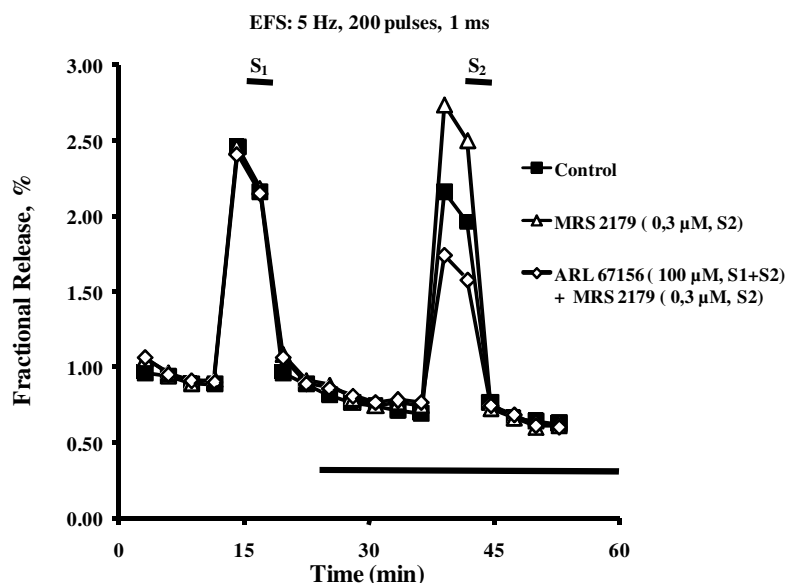


Figure 4.8 - Tonic P2Y₁-mediated inhibition of [³H]-ACh release from stimulated myenteric neurons depends on ecto-ATPDase activity. The time course of tritium outflow taken from a typical experiment is shown. Tritium outflow (ordinate) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional Release, %). The release of [³H]ACh in response to electrical field stimulation (EFS; 200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice during the periods indicated (S₁ and S₂). The selective P2Y₁ antagonist, MRS 2179 (0.3 μM), was applied 15 min before S₂ (as represented by the horizontal bar) in the absence (open triangles) and in the presence (open lozenges) of the NTPDase2 inhibitor, ARL 6715639 (100 μM). The time course of tritium outflow in control conditions (in the absence of test drugs) is also shown for comparison (filled squares). None of the drugs modified basal tritium outflow.

4.1.6 Stimulation of inhibitory ADP-sensitive P2Y₁ purinoceptors may be cut short by sequential activation of adenosine A₁ inhibitory receptors on myenteric motoneurons

Figure 4.9 shows that two stable analogues of ADP and adenosine, respectively ADPβS (0.3–30 μM) and R-PIA (30–300 nM), both inhibited the release of [³H]ACh in a concentration dependent manner. The selective P2Y₁ receptor antagonist, MRS 2179 (0.3 μM), prevented the inhibitory effect of ADPβS (30 μM, *n* = 6) on evoked [³H]ACh release (Figure 4.9a). Blockade of the adenosine A₁ receptor with DPCPX (10 nM) significantly (*P* < 0.05) enhanced the inhibitory action of ADPβS (30 μM; *n* = 6), compared with control. Likewise, inactivation of endogenous adenosine with ADA (0.5 U/mL) augmented ADPβS (30 μM; *n* = 4) inhibitory action to 51 ± 4% (*n* = 4) (data not shown). On the contrary, the selective A₁ receptor antagonist, DPCPX (10 nM), fully blocked inhibition of [³H]ACh release caused by R-PIA (300 nM), whereas MRS 2179 (0.3 μM) was devoid of effect (Figure 4.9b).

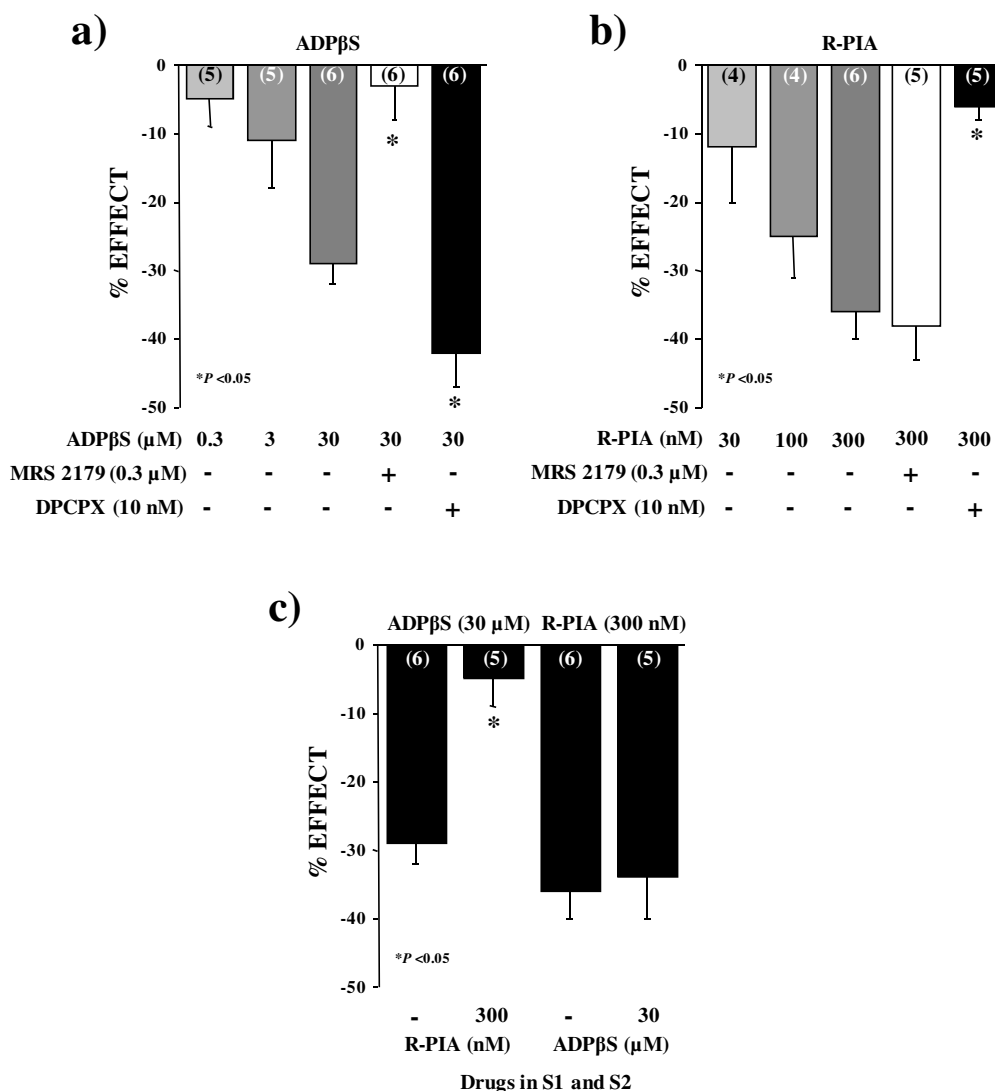


Figure 4.9 - Crosstalk between inhibitory P2Y₁ and A₁ receptors regulating [³H]ACh release from myenteric neurons stimulated by EFS. The release of [³H]ACh in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S₁ and S₂). The ordinates are percentage changes in S₂/S₁ ratios compared with controls. (a) ADPβS (0.3–30 μM) and (b) R-PIA (30–300 nM) were added 15 min before S₂; MRS 2179 (0.3 μM) and DPCPX (10 nM) were added to the incubation media at the beginning of the release period (time zero) and were present throughout the assay, including S₁ and S₂. The average S₂/S₁ ratios in the presence of MRS 2179 (0.3 μM, 0.87 ± 0.04, *n* = 6) and of DPCPX (10 nM, 0.89 ± 0.07, *n* = 6) alone were not significantly (*P* > 0.05) different from the control value (0.83 ± 0.11, *n* = 4; data not shown). Each column represents pooled data (±SEM) from an *n* number of experiments shown in parentheses. **P* < 0.05 (one-way ANOVA followed by Dunnett's modified *t* test), significant differences compared with the inhibitory effects of (a) ADPβS (30 μM) and (b) R-PIA (300 nM) in control conditions, respectively. In (c), ADPβS (30 μM) was applied 15 min before S₂ in the absence and in the presence of R-PIA (300 nM, applied in S₁ and S₂), and vice versa. **P* < 0.05, significant differences compared with the inhibitory effects of ADPβS (30 μM) or R-PIA (300 nM) in the absence of the other modulator, respectively.

Application of the adenosine A₁ receptor agonist R-PIA (300 nM) during the whole assay, including S₁ and S₂, attenuated the inhibitory action of ADPβS (30 μM) on [³H]ACh release from stimulated myenteric motoneurons (Figure 4.9c). In contrast, pretreatment of the preparations with ADPβS (30 μM, in S₁ and S₂) failed to affect the inhibitory effect of R-PIA (300 nM) (Figure 4.9c). These results suggest that activation of inhibitory P2Y₁ purinoceptors may be cut short by subsequent activation of inhibitory A₁ receptors by adenosine.

4.1.7 Discussion

Data suggest that ATP transiently activates facilitatory P2X receptors mediating spontaneous [³H]ACh release from myenteric motoneurons. P2X facilitation may be rapidly terminated by extracellular ATP catabolism into active adenine nucleotides, such as ADP and adenosine. As a consequence of the rapid conversion of ATP directly into AMP, catalysed by NTPDase1, and subsequent AMP dephosphorylation to adenosine by the ecto-5'-nucleotidase (E-5'-N), the magnitude of ATP excitation may be ultimately regulated by the nucleoside adenosine activating presynaptic inhibitory adenosine A₁ receptors. In addition, activation of P2Y₁ purinoceptors by ADP, generated alternatively via ecto-ATPDase (probably NTPDase2), might be functionally relevant to restrain stimulation-induced ACh exocytosis, particularly when ATP accumulation reaches the V_{max} for NTPDase1. Stimulation of ADP-sensitive inhibitory P2Y₁ purinoceptors regulating [³H]ACh release may be cut short by sequential activation of inhibitory A₁ receptors by adenosine, generated from the catabolism of released adenine nucleotides (Figure 4.10).

The kinetics of the extracellular conversion of ATP into other metabolically active derivatives, namely ADP and adenosine, may be functionally relevant to their role in controlling synaptic efficiency. Histochemical studies have shown that the myenteric plexus contains the enzymes responsible for the catabolism of ATP into adenosine (Nitahara *et al.* 1995; Seigny *et al.* 1998). Co-expression of NTPDase1, causing dephosphorylation of ATP directly into AMP with only a modest formation of ADP and generating sequentially ADP and AMP, has been shown in some nerve structures, demonstrating that complex pathways regulate the extracellular metabolism of adenine nucleotides (Kegel *et al.* 1997; Vlajkovic *et al.* 2002). Experimental data strongly indicates that NTPDase1 participates in the termination of P2 receptor-mediated signal transmission, whereas the function of ecto-ATPDase remains a matter of speculation. Our results indicate that ATP (30 μM) is metabolized ($t_{1/2} = 6.9 \pm 0.7$ min) predominantly into AMP, which is subsequently dephosphorylated into adenosine ($t_{1/2} = 15.04 \pm 2.42$ min) by

E-5'-N in the myenteric plexus of the rat ileum; appearance of ADP in the incubation fluid was absent during the first 2 min (see Figure 4.1a, Section 4.1.2). The alternative pathway, that converts ATP into ADP, becomes more relevant as the extracellular ATP concentration increased to 100 μ M (see Figure 4.3, Section 4.1.3). In these circumstances, besides ADP accumulation in the extracellular milieu, we observed a delay of adenosine formation probably due to feed-forward inhibition by increased levels of extracellular ATP (Cunha 2001; Naito & Lowenstein 1985).

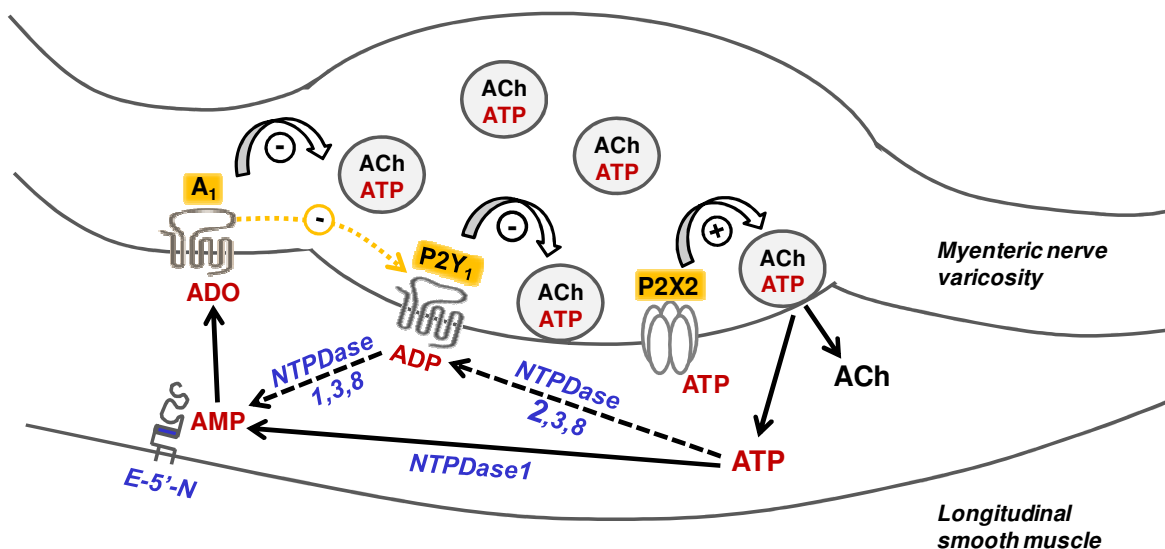


Figure 4.10 - Schematic representation of the modulatory role of ATP and its metabolites on [³H]ACh release from myenteric neurons of the rat ileum. The myenteric plexus contains the enzymes responsible for the catabolism of ATP released from activated smooth muscle cells as well as from stimulated myenteric neurons. ATP is converted preferentially into AMP via NTPDase1, which is then sequentially dephosphorylated into adenosine (ADO) by ecto-5'-nucleotidase (E-5'-N); the alternative pathway, conversion of ATP into ADP via ecto-ATPDase, only becomes relevant upon increasing the extracellular concentration of ATP. Understanding the effective contribution of the ATP breakdown, via ecto-ATPDase (generating ADP) and via NTPDase1 (bypassing ADP formation), is of central importance to predict the fine tuning of purinergic control of gut motility. Data suggest that ATP transiently activates facilitatory P₂X₂ receptors mediating spontaneous [³H]ACh release, while ATP metabolites, like ADP and ADO, interplay to control evoked transmitter release by activating inhibitory P₂Y₁ and A₁ receptors, respectively.

These findings are in good agreement with the observations in porcine cortical synaptosomes and in C6 glioma cells, where the calculated K_m value for NTPDase1, in respect to ATP as a substrate, was several (three) times lower in comparison with the corresponding values for ecto-ATPDase (Grobben *et al.* 1999; Kukulski *et al.* 2003). Because both enzymes have a similar molecular activity, these findings suggest that when

ATP concentration in the extracellular space is low (close to the V_{max} for NTPDase1), ATP will be hydrolysed preferentially by NTPDase1 than by ecto-ATPase. NTPDase1 seems to have similar affinities as far as ATP and ADP are concerned (see Figure 4.1, Section 4.1.2). The delay in ADP formation upon inhibiting ecto-ATPDase with ARL 67156 (Crack *et al.* 1995), without compensatory changes in AMP formation (see Figure 4.2, Section 4.1.3), suggests that the ecto-ATPDase pathway works probably as an alternative pathway for inactivating ATP when its concentration reaches high levels in the myenteric synapse. Therefore, Ecto-ATPDase may be responsible for the removal of high, toxic, concentrations of ATP on one hand and, on the other hand, for the production of a compensatory signal molecule, ADP, which can control neuronal activity during sustained firing, hypoxia or ischemia and inflammatory insults of the enteric nervous system.

In the myenteric plexus, all enteric neurons, except NO synthase-immunoreactive inhibitory muscle motoneurons, contain choline acetyltransferase (ChAT) (Furness 2000), suggesting that myenteric neurons are mostly cholinergic and that ATP and ACh might be co-released. Here, we provided evidence indicating that cholinergic nerve terminals of the myenteric plexus of the rat ileum possess TNP-ATP-sensitive P2X receptors triggering the release of [^{3} H]ACh in the absence of action potential generation (Barthó *et al.* 2006). A common denominator to all P2X receptor subtypes is a Ca^{2+} influx through non-selective cation channels (also permeant to Na^+ and/or K^+) promoted by purines acting through the receptor channel itself (see Figure 4.5c, Section 4.1.4). ATP can also inhibit the membrane potassium conductance in enteric neurons (Barajas-Lopez *et al.* 1994). Via these mechanisms, ATP can cause membrane depolarization leading to a secondary opening of voltage-gated Ca^{2+} channels and to transmitter release. ATP-induced increase in basal [^{3} H]ACh release was dependent on extracellular Ca^{2+} but was resistant to blockade of axonal conduction by TTX, indicating that its action is likely to be independent on generation of action potentials and has to be mediated by P2X receptors present on myenteric nerve endings. Likewise, ATP can increase ACh release from isolated nerve terminals (synaptosomes) from the guinea-pig myenteric plexus (Barthó *et al.* 2006; Reese *et al.* 1982). Murine enteric neurons express mainly P2X2 and P2X3 subunit-containing receptors, although unusual purinoceptor subtypes, sharing some properties with P2X4 and P2X6, may also be involved in ATP-induced currents in myenteric neurons (Barajas-Lopez *et al.* 1996; Galligan 2002a). The involvement of the P2X3 receptor is unlikely because there was almost no response to α,β -MeATP, which potently activates homomeric P2X receptors composed by P2X1 or P2X3 subunits and P2X2/3 heteromeric receptors, but does not activate P2X2 homomeric receptors (North 2002; Ralevic & Burnstock 1998; Zhou *et al.* 1996); fast desensitizing ($\tau < 1$ s) P2X3 receptors are only present in approximately 10% of myenteric neurons (Zhou & Galligan 1996), which are

mainly AH (intrinsic intestinal sensory) neurons but not S motoneurons and interneurons (Burnstock 2007b; Furness 2000). Therefore, ATP-induced [^3H]ACh release from non-stimulated myenteric neurons of the rat ileum is most likely due to the activation of prejunctional receptors containing P2X2 subunits, which are sensitive to micromolar concentrations of trinitrophenyl-substituted nucleotides, especially TNP-ATP, as well as to non-nucleotide compounds, like PPADS (see Figure 4.5b, Section 4.1.4) (North 2002; Virginio *et al.* 1998). Using electrophysiological, single-cell Ca^{2+} imaging and molecular biology techniques, Ohta and collaborators (2005) demonstrated that ATP mainly activates excitatory P2X2 receptors resulting in Ca^{2+} influx in primary cultures of myenteric neurons isolated from the neonatal rat intestine (Ohta *et al.* 2005). Our suggestion that homomeric P2X2 receptors are expressed in myenteric motoneurons is supported by immunohistochemical studies in the guinea-pig intestine and by data derived from P2X2 receptor knockout mice (Castelucci *et al.* 2002; Ren *et al.* 2003).

Ionotropic P2X and metabotropic P2Y receptors are co-expressed in many cell types. The relative contribution of Ecto-ATPDase (forming ADP) versus NTPDase1 (bypassing ADP formation) pathway seems critical to define the extracellular inactivation of ATP (the natural ligand for both P2X and P2Y receptors) and formation of biologically active metabolites (*e.g.* ADP and adenosine) capable of regulating neurotransmitter release via P2Y and P1 receptor activation. Here, we showed that ATP and its stable analogue, $\text{ATP}\gamma\text{S}$, but not $\alpha,\beta\text{-MeATP}$, consistently inhibited electrically evoked [^3H]ACh release from myenteric neurons (see Figure 4.6a, Section 4.1.5). Applied in similar experimental conditions, ADP and the enzymatically stable ADP analogue, $\text{ADP}\beta\text{S}$, mimicked the inhibitory action of ATP (see Figure 4.6b, Section 4.1.5). We suspect that inhibition of electrically evoked transmitter release by adenine nucleotides may be mediated by P2Y_1 receptors, because the selective P2Y_1 antagonist, MRS 2179, counteracted the inhibitory action of $\text{ADP}\beta\text{S}$ (see Figure 4.9a, Section 4.1.6) (von Kugelgen & Wetter 2000). The non-selective P2 receptor antagonists, PPADS and RB-2, produced puzzling results versus ADP-induced inhibitory response. These antagonists are reported to block P2Y_1 receptors, but contrasting data have been published (von Kugelgen & Wetter 2000). For example, PPADS was ineffective as an antagonist at rabbit aortic endothelial P2Y_1 receptors (Ralevic & Burnstock 1998). Sensitivity to MRS 2179 discounts an effect mediated by distinct $\text{ADP}\beta\text{S}$ -sensitive $\text{P2Y}_{6,12,13}$ receptors. The involvement of $\alpha,\beta\text{-MeATP}$ -sensitive $\text{P2Y}_{2,4,11}$ receptors can also be dismissed, as this compound was devoid of effect on evoked [^3H]ACh release (see Figure 4.6a, Section 4.1.5) (Abbracchio *et al.* 2006). Electrophysiological, immunohistochemical and molecular studies have demonstrated inhibitory P2Y_1 receptors on myenteric and submucous neurons of rodents (Galligan 2002a; Giaroni *et al.* 2002; Hu *et al.* 2003), as well as of men

(Gallego *et al.* 2006; Wunderlich *et al.* 2008). Purinergic neurotransmission is known to occur between neurons in the enteric nervous system. Whether the P2Y₁ receptor inhibition of [³H]ACh release is mediated via the production of NO from inhibitory nerves is controversial (Giaroni *et al.* 2002; Zizzo *et al.* 2007). However, unpublished experiments from our laboratory (performed by Isabel Silva) indicate that in LM-MP preparations neither increasing (L-arginine, 470 μM and SIN-1, 10 μM) nor decreasing (L-NAME, 100 and 300 μM) NO production results in significant modification of ACh release, thus suggesting that P2Y₁ receptor inhibition is not mediated via the NO signalling pathway. Tonic activation of inhibitory P2Y₁ receptors might also be physiologically relevant during high levels of enteric nerve activity, as predicted from augmentation of [³H]ACh release in the presence of MRS 2179, particularly when stimulation train duration was prolonged (from 200 to 500 pulses). In these circumstances, ATP released from stimulated myenteric neurons might reach such high levels that AMP generation via NTPDase1 is saturated and ATP catabolism is partially diverted to the formation of ADP via the Ecto-ATPDase pathway, with ADP being the most potent natural agonist of inhibitory P2Y₁ receptors (Ralevic & Burnstock 1998). This hypothesis was further supported by the sensitivity of MRS 2179 facilitation of [³H]ACh release from stimulated myenteric neurons to the inhibition of Ecto-ATPDase by ARL 67156 (see Figure 4.8, Section 4.1.5). Up-regulation of Ecto-ATPDase at the transcriptional level has been observed in response to prolonged stimulation of P2Y₁ receptors (Lu *et al.* 2007). This up-regulation might reflect a feedback circuit to regulate excess of extracellular ATP under certain pathological conditions, such as ischaemic insults and chronic inflammation.

The inhibitory effect of ADP on evoked [³H]ACh release was not fully blocked by MRS 2179 and complete blockade required co-application of the P2Y₁ receptor antagonist together with ADA (see Figure 4.7, Section 4.1.5). This contrasts with ADPβS, which does not generate adenosine. ADA rapidly removes adenosine in the bathing solution, and it may prevent the activation of inhibitory adenosine A₁ receptors present on myenteric neurons (Duarte-Araújo *et al.* 2004a). The selective adenosine A₁ receptor antagonist, DPCPX (10 nM), also partially attenuated the inhibitory action of ADP (100 μM). These findings suggest that ADP acts directly, via ADP-sensitive P2Y₁ purinoceptors, and indirectly, via adenosine formation leading to adenosine A₁ receptor activation, to reduce the release of [³H]ACh from stimulated myenteric motoneurons. The adenosine A₁ receptors have a high affinity for adenosine and are widely distributed in mammals (including man) regulating transmitters release. Adenosine exerts an inhibitory action in the enteric nervous system mediated by neuronal A₁ receptors, sensitive to DPCPX (10 nM) (Barajas-Lopez *et al.* 1996; Correia-de-Sá *et al.* 2006; Duarte-Araújo *et al.* 2004a; Nitahara *et al.* 1995).

While the E-5'-N pathway modulates the rate of adenosine formation from the catabolism of released ATP, via AMP-forming NTPDase1, alternative ATP hydrolysis, via ecto-ATPDase, controls the amount of ADP generated at the myenteric synapse. The combined effect of both pathways, in parallel with feed-forward inhibition of E-5'-N by high ATP amounts, might serve to regulate the local concentration of ADP and adenosine available to interact with inhibitory P2Y₁ and adenosine A₁ receptors, respectively. It is also interesting to note that E-5'-N activity is concentrated in the smooth muscle plasma membrane, which may lead to a delay in the accumulation of adenosine that is produced some distance away from the receptor sites (Nitahara *et al.* 1995; Seigny *et al.* 1998). Heinemann and collaborators (1999) showed that the P1 receptor antagonist, 8-phenyltheophylline, attenuated the peristalsis-inhibiting effect of low (10 μ M) ATP concentrations, but this antagonism was less robust upon increasing the concentration of the nucleotide (Heinemann *et al.* 1999). We showed a similar pattern on the ability of ATP to inhibit evoked [³H]ACh release from myenteric motoneurons using ADA (0.5 U/mL), suggesting that P2Y₁ receptors activated by ADP, generated via ecto-ATPDase, play a greater role as ATP concentrations are raised.

Thus, our results are in line with previous observations suggesting that ATP and its metabolites, namely ADP and adenosine, form a 'purinergic cascade' leading to complex interactions between P1 and P2 receptors needed to control neurotransmission in several synapses (Ralevic & Burnstock 1998). Here, we demonstrated that activation of ADP-sensitive P2Y₁ purinoceptors may be cut short by sequential activation of inhibitory A₁ receptors by adenosine. Our results, showing that ADP β S and MRS 2179 were unable to change the inhibitory effect of R-PIA (see Figure 4.9b and 4.9c, Section 4.1.6), which potently activates adenosine A₁ receptors, are in disagreement with the previously reported reduction in the adenosine A₁ ligand affinity observed in cells possessing A₁/P2Y₁ heterodimers (Yoshioka *et al.* 2001). Although heteromerization between adenosine A₁ and P2Y₁ receptors may provide the molecular basis to explain hybrid pharmacology of these receptors, that is, atypical P2Y₁ receptors that are sensitive to adenosine A₁ receptor antagonists, this might not occur on cholinergic neurons of the rat myenteric plexus as DPCPX favoured, instead of decreasing, the ability of ADP β S to inhibit evoked [³H]ACh release (see Figure 4.9a, Section 4.1.6).

Through these complex interactions, gradients of ATP breakdown products (ADP and adenosine) may provide fine tuning of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischemia and chronic inflammation, when extracellular ATP levels become increased (De Man *et al.* 2003; Milusheva *et al.* 1990). Up to 1% of intracellular ATP may be released in response to stimuli. Given that intracellular ATP is around 3–6 mM, such an increase has marked

consequences for the kinetics of P2 and P1 receptors activation, the latter being activated by secondary formation of adenosine through the ecto-nucleotidase pathway. Since the original purinergic receptor hypothesis and classification by Burnstock (1981), extensive investigation has established their indispensable role in cellular homeostasis and raised excitement over the potential of developing therapeutic targets in many pathological conditions. As our understanding of the underlying signalling and pathophysiological implications of the ecto-nucleotidase pathway evolves, the potential for new therapies will expand (Gendron *et al.* 2002).

4.2 – Dual effects of endogenous ADO on ACh release from myenteric motoneurons are mediated by inhibitory A₁ and facilitatory A_{2A} receptors: on the role of secreted adenosine deaminase

4.2.1 Rationale

Adenosine, which is neither stored nor released as a classical neurotransmitter, is a recognized neuromodulator in the peripheral and central nervous systems. This nucleoside controls the release of neurotransmitters by activating inhibitory A₁ and facilitatory A_{2A} adenosine receptors, which may coexist in the same nerve terminal (Correia-de-Sá *et al.* 1991; Sebastião & Ribeiro 2000). Besides the well-characterized inhibitory effect of adenosine in the gastrointestinal tract operated by neuronal A₁ receptors (Barajas-Lopez *et al.* 1996; Broad *et al.* 1992; De Man *et al.* 2003; Gustafsson *et al.* 1985; Nitahara *et al.* 1995), the involvement of A₂ receptors mediating excitation of myenteric neurons was reported with conflicting results (Christofi *et al.* 1994; Tomaru *et al.* 1995). The coexistence of both subtypes of high-affinity adenosine receptors in cholinergic neurons prompted the question of how adenosine chose to activate A₁ or A₂ receptors, and under which conditions does it discriminate between these receptors.

The way adenosine builds its influence to control neuronal communication depends on the extracellular concentration of the nucleoside, which is achieved by balancing extracellular adenosine generation and inactivation mechanisms (cellular uptake and/or extracellular deamination) (Correia-de-Sá *et al.* 1996; Gonçalves *et al.* 1993). Extracellular inactivation may restrict adenosine actions to the release/production sites and may limit diffusion of the exogenously added nucleoside towards the active zones (Daly 1982; Dowdall 1978). In the myenteric plexus of the guinea-pig ileum, adenosine may be released as such in response to electrical stimulation (Begg *et al.* 2002). This release is thought to be mediated via facilitated diffusional nucleoside transporters and can be regulated by endogenous neuromediators co-released from myenteric neurons, such as ACh and ATP (Correia-de-Sá & Ribeiro 1996). In view of the role of endogenous ACh in the control of myenteric synaptic transmission, we thought it was important to evaluate how this transmitter affects adenosine release. Parallel formation of adenosine from the hydrolysis of ATP released from activated smooth muscle cells, as well as from stimulated myenteric neurons, is also possible (Nitahara *et al.* 1995; White & Leslie 1982). Histochemical studies showed that the myenteric plexus is equipped with enzymes responsible for the hydrolysis of ATP into adenosine, through the ecto-nucleotidase pathway (Nitahara *et al.* 1995; Zimmermann 2000).

Therefore, it is conceivable that adenosine contributes to an overall homeostatic role on enteric excitability during pathological conditions (*e.g.* hypoxia, inflammation,

postoperative ileus) where endogenous adenosine becomes elevated (De Man *et al.* 2003; Kadowaki *et al.* 2003; Milusheva *et al.* 1990). The existence of topographically distinct sources of extracellular adenosine, two nucleoside inactivation mechanisms and at least two types of adenosine receptors with opposite physiological functions, makes it critical to understand how these systems interplay to control adenosine actions on [³H]ACh release from depolarized myenteric motoneurons of the rat ileum.

Knowing that ACh is regarded as the major excitatory neurotransmitter in the myenteric plexus and the prime regulator of gastrointestinal motility (Costa *et al.* 1996; Furness 2006), the present study was undertaken to investigate the kinetics and pharmacology of adenosine generation, in order to probe its role to control the electrically evoked [³H]ACh release from isolated myenteric plexus-longitudinal muscle of the rat ileum. To characterize the enzymatic pathways responsible for the extracellular formation and removal of adenosine leading to a differential activation of inhibitory A₁ and excitatory A_{2A} receptors in the rat ileum, we used several pharmacological inhibitors designed to either limit or increase the availability of endogenous adenosine. The results obtained by manipulating the endogenous levels of adenosine were compared with the effects of the nucleoside itself, of the adenosine precursor, AMP, and of two stable adenosine analogues displaying a high degree of selectivity for A₁ or A_{2A} receptors, all applied exogenously to the bathing fluid.

4.2.2 Myenteric motoneurons possess both A₁ inhibitory and A_{2A} facilitatory adenosine receptors modulating evoked [³H]ACh release

Exogenously applied adenosine (0.3-300 μ M) decreased electrically evoked [³H]ACh release from myenteric motoneurons of the rat ileum in a concentration-dependent manner (Figure 4.11). In the presence of the A₁ receptor antagonist DPCPX (10 nM), adenosine (0.3 and 30 μ M) significantly ($P<0.05$) increased, rather than decreased, tritium outflow. When adenosine was applied at a higher concentration (300 μ M), inhibition of [³H]ACh release was completely prevented by DPCPX (10 nM), but the facilitatory effect was no longer observed.

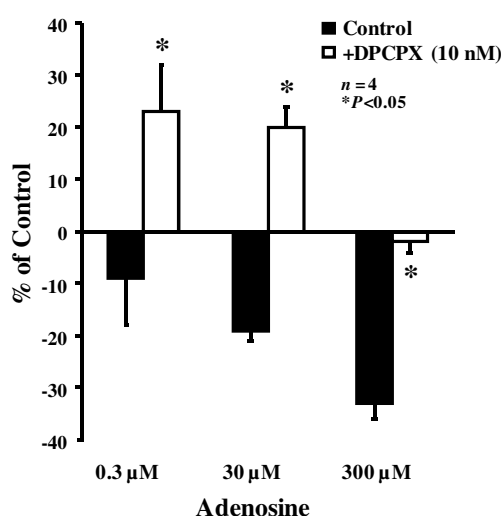


Figure 4.11 - Effect of exogenously added adenosine on [³H]ACh release from myenteric motoneurons stimulated electrically in the absence (filled bars) and in the presence (open bars) of the adenosine A₁ receptor antagonist, DPCPX (10 nM). Adenosine was added 15 min before S₂ and remained in the bath until the end of the experiments. DPCPX was added to the incubation media since the beginning of the release period (time zero), *i.e.* it was present during the whole assay including S₁ and S₂. The ordinates are percentage changes in S₂/S₁ ratios as compared to controls. Zero percent represents identity between ratios; Positive and negative values represent facilitation and inhibition of evoked [³H]ACh release, respectively. The average S₂/S₁ ratio in the presence of DPCPX (0.89±0.07, $n=6$) was not significantly ($P>0.05$) different from the control value (0.83±0.11, $n=4$) (see Table 4.1). Each column represents pooled data (±SEM) from four experiments. * $P<0.05$ (one-way ANOVA followed by Dunnett's modified t test) represent significant differences as compared with the effect of the same concentration of adenosine in the absence of DPCPX.

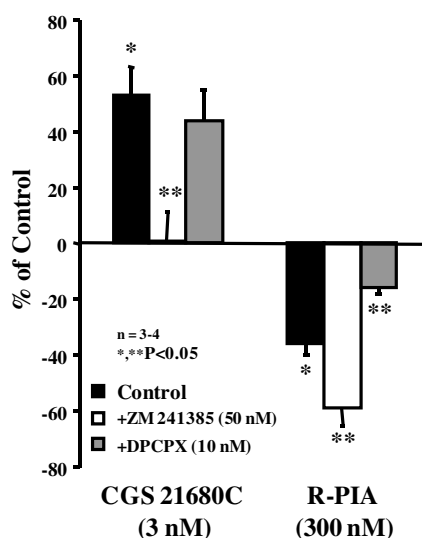


Figure 4.12 - Actions of selective adenosine A_1 and A_{2A} receptor antagonists on the effects of two stable adenosine analogues, CGS 21680C (an A_{2A} receptor agonist) and R-PIA (an A_1 receptor agonist), on [^{3} H]ACh release from myenteric motoneurons stimulated electrically. CGS 21680C (3 nM) and R-PIA (300 nM) were applied 15 min before S_2 . The adenosine receptor antagonists, ZM 241385 (50 nM) and DPCPX (10 nM), were added to the incubation media since the beginning of the release period (time zero), *i.e.* they were present during the whole assay including S_1 and S_2 . The ordinates are percentage changes in S_2/S_1 ratios as compared to controls. Zero percent represents identity between ratios; Positive and negative values represent facilitation and inhibition of evoked [^{3} H]ACh release, respectively. The average S_2/S_1 ratio in the presence of DPCPX (10 nM, 0.89 ± 0.07 , $n=6$) and ZM 241385 (50 nM, 0.89 ± 0.03 , $n=5$) were not significantly ($P > 0.05$) different from the control value (0.83 ± 0.11 , $n=4$) (see Table 4.1). The data are means ($\pm$ SEM) of three to four individual experiments. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the control; ** $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences as compared with the effect of CGS 21680C or R-PIA, in the absence of the adenosine receptor antagonists.

The selective adenosine A_1 receptor agonist, R-PIA (100-300 nM), significantly ($P < 0.05$) decreased the evoked tritium outflow in a concentration-dependent manner. Inhibition of transmitter release by R-PIA (300 nM) was attenuated in the presence of the A_1 receptor antagonist DPCPX (10 nM), but it was enhanced upon blocking A_{2A} receptors with ZM 241385 (50 nM) (Figure 4.12). In contrast, activation of adenosine A_{2A} receptors with CGS 21680C (1-3 nM) facilitated the evoked release of [^{3} H]ACh in a concentration-dependent manner. A higher concentration (10 nM) of CGS 21680C also increased evoked tritium outflow ($P < 0.05$), but the increase was smaller ($+ 22 \pm 4\%$, $n=3$) than that observed with 3 nM of CGS 21680C. The facilitatory effect of CGS 21680C (3 nM) was abolished in the presence of ZM 241385, but was virtually unaffected by DPCPX (Figure 4.12).

4.2.3 Endogenous adenosine preferentially activates facilitatory A_{2A} receptors on myenteric motoneurons

To study the net tonic action of endogenous adenosine on electrically (200 pulses of 1 ms duration delivered at a 5 Hz frequency) evoked [³H]ACh release from myenteric motoneurons, we compared the effect of adenosine deaminase (ADA), the enzyme that inactivates adenosine into inosine, with the effects resulting from the blockade of adenosine A₁ and A_{2A} receptors with DPCPX and ZM 241385, respectively (Figure 4.13) (Arch *et al.* 1978).

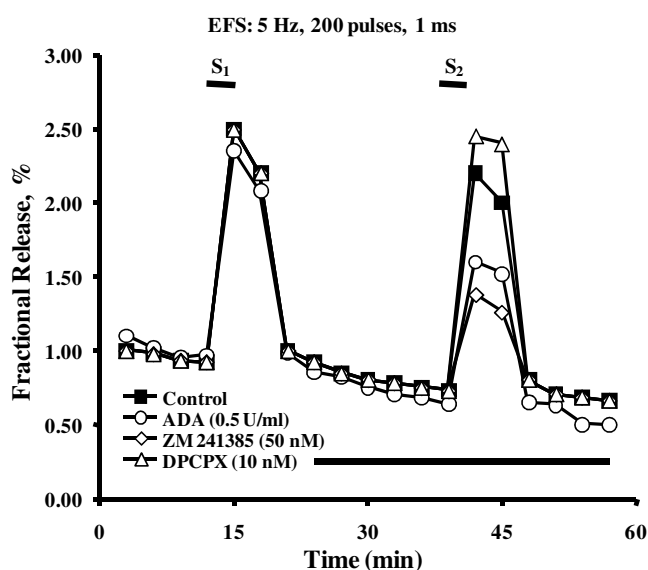


Figure 4.13 - Effects of ADA (circles), ZM 241385 (lozenges) and DPCPX (triangles) on [³H]-ACh release from myenteric neurons. Shown is the time course of tritium outflow from the longitudinal muscle-myenteric plexus of the rat ileum taken from typical experiments. Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period. The abscissa indicates the times at which the samples were collected. The release of [³H]ACh, in response to electrical field stimulation (200 pulses of 1 ms duration delivered at a 5 Hz frequency), was elicited twice during the periods indicated (S₁ and S₂). ADA (0.5 U/mL), ZM 241385 (50 nM) and DPCPX (10 nM) were applied 15 min before S₂, as represented by the horizontal bar. The time course of tritium outflow in a control situation (in the absence of test drugs) is also shown for comparison (filled squares). None of the drugs used modified basal tritium outflow.

ADA (0.5 U/ml) inhibited the evoked [³H]ACh release by $-31 \pm 6\%$ ($n=7$), indicating that endogenous adenosine is producing a predominant tonic facilitatory action on neurotransmitter release in the rat myenteric plexus (Figure 4.13). DPCPX (10 nM) slightly increased the release of [³H]ACh by $17 \pm 4\%$ ($n=4$) (Figure 4.13). In contrast, the adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM), maximally decreased the evoked

tritium outflow by $37 \pm 10\%$ ($n=6$) (Figure 4.13), an effect that was not statistically ($P>0.05$) different from the inhibition detected upon removal of all endogenous adenosine with ADA (0.5 U/mL). The concentrations of DPCPX (10 nM) and ZM 241385 (50 nM) used in the present study are within the range usually required to selectively block adenosine A_1 and A_{2A} receptors in functional studies, respectively (Cunha 2001; Oliveira *et al.* 2002). Higher concentrations of these antagonists did not further increase their action on the evoked [^{3} H]ACh release, *i.e.* DPCPX (50 nM) facilitated the release of [^{3} H]ACh by $21 \pm 8\%$ ($n=4$), while ZM 241385 (100 nM) produced an inhibitory effect of $37 \pm 6\%$ ($n=4$). These results indicate that endogenous adenosine exerts a predominant facilitatory action by activating A_{2A} receptors on myenteric neurons.

4.2.4 Myenteric neurons are the main source of endogenous ADO

Electrical stimulation of the LM-MP, at a frequency of 5 Hz for 10 min, led to an increased accumulation of adenine nucleosides, consisting mostly of adenosine, inosine and hypoxanthine (Figure 4.14). Adenine nucleosides rose from an average basal value of 15.1 ± 1.3 pmol/mg tissue to a total value of 26.4 ± 0.9 pmol/mg tissue ($n=6$) in the incubation media following LM-MP stimulation. The evoked adenosine outflow was time and frequency dependent, as it was significantly ($P<0.05$) reduced by decreasing the train duration to 4.5 min or the frequency of stimulation to 1 Hz (data not shown).

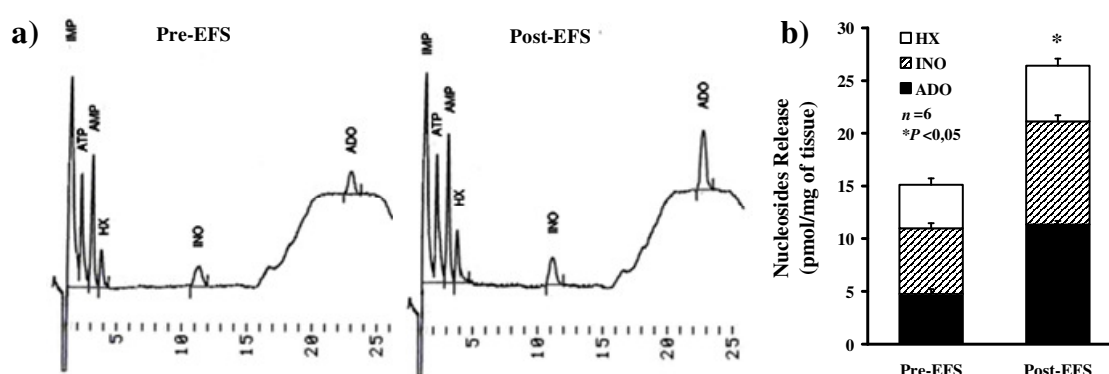


Figure 4.14 - Effect of electrical field stimulation (EFS) on extracellular accumulation of purines in the LM-MP of the rat ileum. The preparations were incubated with Tyrode's solution for 15 min and then subjected to EFS (5 Hz frequency, 3000 pulses and 1 ms duration). Samples collected from the incubation media were analysed by HPLC to separate and quantify purine nucleotides and nucleosides. Panel (a) depicts HPLC chromatograms obtained from samples collected immediately before (Pre-EFS) and after (Post-EFS) stimulation of the LM-MP. EFS elicited an increment in the purines content of the bathing fluid. Panel (b) illustrates the composition of purine nucleosides in both samples collected under basal conditions (Pre-EFS) and after (Post-EFS) stimulation of the LM-MP expressed in pmol/mg of tissue, as determined by HPLC analysis. The following purine nucleosides were found in the incubation media: adenosine, inosine and hypoxanthine. Note that the amount of adenosine and inosine were significantly increased in response to electrical stimulation of the LM-MP. Data represent the means ($\pm$ SEM) of six individual experiments. * $P<0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the total amount of purine nucleosides detected before stimulus application.

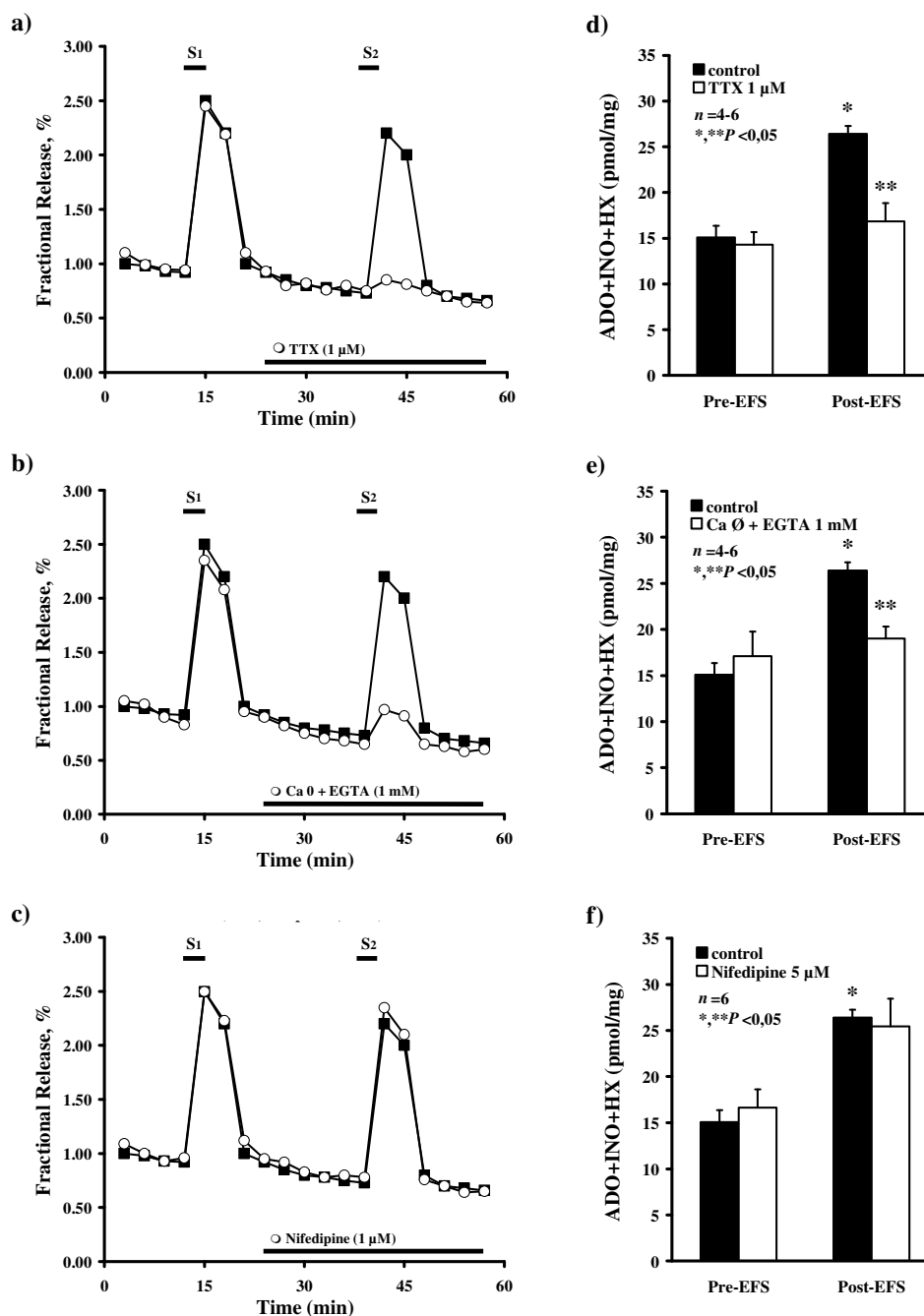


Figure 4.15 - Influence of tetrodotoxin (TTX, 1 μM), extracellular Ca²⁺ deprivation (Ca⁰ + EGTA, 1 mM) and nifedipine (1 μM) on stimulation-evoked release of [³H]ACh and purine nucleosides from the LM-MP of the rat ileum. TTX, Ca²⁺-free solution and nifedipine were in contact with the preparations for at least 15 min before stimulus application. Panels (a-c) show the time course of tritium outflow from the LM-MP taken from typical experiments. Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period. The release of [³H]ACh was elicited twice, in response to electrical field stimulation (5 Hz frequency, 200 pulses of 1 ms duration), which was applied at the 12th (S₁) and 39th (S₂) min after the end of the washout period. Panels (d-f), illustrate the purine nucleosides content of bath samples collected immediately before (Pre-EFS) and after (Post-EFS) LM-MP stimulation expressed in pmol/mg of tissue, as determined by HPLC analysis. Data

represent the means ($\pm$ SEM) of an n number of individual experiments. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the total amount of purine nucleosides detected before stimulus application; ** $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences as compared with the evoked outflow of purine nucleosides in the absence of TTX, Ca^{2+} -free solution and nifedipine.

As illustrated in Figure 4.15, stimulus-evoked release of adenosine was dependent on extracellular Ca^{2+} and on neuronal activity, since omission of Ca^{2+} in the Tyrode's solution or application of 1 μM of tetrodotoxin essentially abolished the nucleoside outflow. Likewise, the evoked [^3H]ACh release from myenteric motoneurons was prevented in the absence of external Ca^{2+} ($\text{Ca}^{2+}_0 + \text{EGTA}$, 1 mM) or in the presence of 1 μM tetrodotoxin (Figure 4.15; see also (Duarte-Araújo *et al.* 2004b), indicating that [^3H]ACh release results from vesicle exocytosis of depolarised nerve terminals. In contrast to tetrodotoxin, which blocks nerve evoked smooth muscle contractions, the L-type voltage-sensitive Ca^{2+} channel (Ca_v1) blocker, nifedipine (1 μM), depressed ACh-induced contraction of the longitudinal muscle (data not shown) without affecting the release of [^3H]ACh from stimulated LM-MP (Figure 4.15c). Nifedipine was also unable to affect the accumulation of adenine nucleosides in bath samples collected immediately after electrical field stimulation of the LM-MP (Figure 4.15f). The results indicate that myenteric neurons are the main source of adenosine released from stimulated LM-MP of the rat ileum.

4.2.5 Involvement of ADA and adenosine uptake in the regulation of the extracellular concentration of adenosine in the rat myenteric plexus

As illustrated in the progress curve of extracellular adenosine disappearance in the rat myenteric plexus (Figure 4.16), the bath concentration of adenosine (30 μM) decreased with time following its addition to the preparation. The only metabolites detected in the bath were inosine and hypoxanthine (Figure 4.16). The inosine concentration increased continuously up to 45 min, reaching a value of $14.51 \pm 1.98 \mu\text{M}$ ($n=4$). The absence of AMP formation from adenosine suggests that no extracellular adenosine kinase (AK, EC 2.7.1.20) activity is present in this preparation. When inosine (30 μM) was added to the preparation, it disappeared slowly and only small amounts of hypoxanthine ($1.94 \pm 0.29 \mu\text{M}$) appeared after 45 min ($n=2$) (data not shown).

In the presence of the nucleoside transport inhibitor, dipyridamole (0.5 μM) (Griffith *et al.* 1996), disappearance of adenosine (30 μM) from the bath was slower while the formation of inosine remained virtually unchanged (Figure 4.16). The average half

disappearance time of adenosine was increased from 34.10 ± 1.30 min, in control conditions, to 75.6 ± 9.4 min in the presence of dipyridamole ($n=3$); these values were estimated from polynomial fitting of semi-logarithmic progress curves of adenosine catabolism, in the absence and in the presence of dipyridamole. The amounts of adenosine present in the bath 45 min after addition to the preparations were 11.89 ± 0.36 μM ($n=4$) in the absence and 20.10 ± 0.62 μM ($n=3$) in the presence of dipyridamole (0.5 μM), in the same experiments. Increasing the concentration of dipyridamole to 2 μM did not further increase the amount of adenosine remaining in the bath nor prolonged its time course. No inhibition of the enzymatic activity of adenosine deaminase (0.0005-0.5 U/ml) was demonstrated in the presence of dipyridamole (2 μM) (data not shown), ruling out the possibility of an inhibitory influence of dipyridamole on adenosine deamination (Hopkins *et al.* 1971).

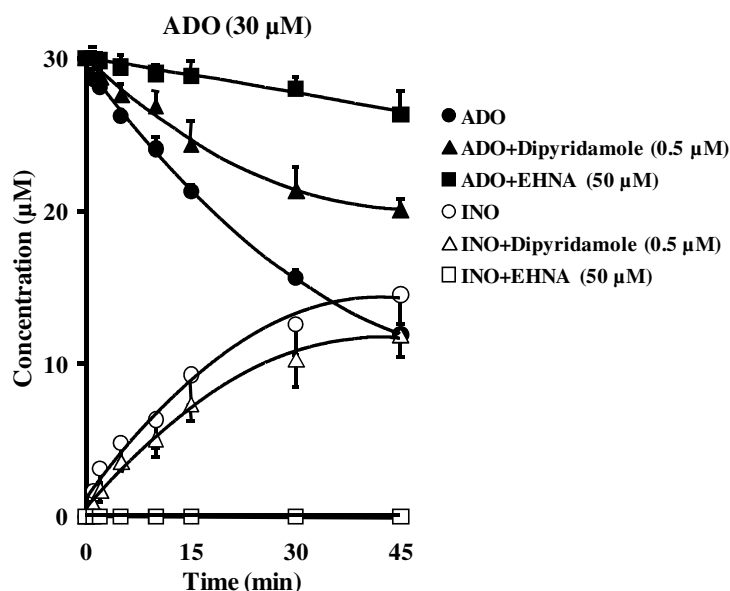


Figure 4.16 - Effects of dipyridamole and EHNA on adenosine (ADO, filled symbols) disappearance and inosine (INO, open symbols) formation in the myenteric plexus of the rat ileum. Adenosine (30 μM) was incubated at zero time with LM-MP preparations, in the absence (circles) and in the presence of either dipyridamole (0.5 μM , triangles) or EHNA (50 μM , squares). Each collected sample was analysed by HPLC to separate and quantify adenosine and its metabolites. Average results obtained in 3-4 experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. The progress curves, using each adenosine inactivation modifier, were obtained from the same preparation. Hypoxanthine was also detected in the bath after 15 min incubation, in concentrations ranging from 0.48-1.99 μM , but was not represented for the sake of clarity; the concentration of hypoxanthine was unaltered in the presence of dipyridamole (0.5 μM), but it was virtually undetectable during incubation with EHNA (50 μM). Note that EHNA (50 μM) completely blocked inosine (open squares) formation from adenosine.

S-(p-nitrobenzyl)-6-thioinosine (NBTI, 30 μ M, $n=4$), another compound designed at inhibiting equilibrative nucleoside transporters (Griffith & Jarvis 1996), did not alter either the rate of adenosine disappearance (33.70 ± 0.40 min) nor the rate of inosine generation, thus indicating that the adenosine transporter present at the rat myenteric plexus is insensitive to NBTI (e). Although concentrative nucleoside transport is found in the gastrointestinal system of a wide variety of species, since ouabain (2 mM, $n=2$) did not affect the kinetics of adenosine disappearance and inosine appearance in the bath (data not shown), it is highly improbable that a sodium-dependent concentrative nucleoside transport system is responsible for the adenosine uptake in the rat myenteric plexus.

The adenosine deaminase inhibitor, erythro-9(2-hydroxy-3-nonyl) adenine (EHNA, 50 μ M, $n=4$) (Agarwal *et al.* 1977), prevented adenosine disappearance from the bath by more than 90% at 45 min and virtually abolished extracellular formation of inosine (Figure 4.16). These results suggest that, in addition to the equilibrative nucleoside uptake system sensitive to dipyrindamole, the LM-MP of the rat ileum displays a very intense ecto-adenosine deaminase activity, which may act cooperatively to control extracellular adenosine accumulation.

4.2.6 Synaptic ADO accumulation facilitates the evoked [^{3} H]ACh release from stimulated myenteric motoneurons via A_{2A} receptors activation

Taken the results obtained from the analysis of adenosine kinetics in the rat LM-MP, we used dipyrindamole (0.5 μ M) and EHNA (50 μ M) to promote extracellular adenosine accumulation by inhibiting the nucleoside inactivation. Application of dipyrindamole (0.5 μ M) and EHNA (50 μ M) significantly ($P < 0.05$) increased the evoked [^{3} H]ACh release by $27 \pm 7\%$ ($n=5$) and by $81 \pm 12\%$ ($n=4$), respectively (Figure 4.17). When applied in a higher concentration, dipyrindamole (2 μ M) did not facilitate further the release of transmitter (data not shown), agreeing with the findings obtained in the kinetic studies, suggesting that adenosine uptake was maximally inhibited with 0.5 μ M dipyrindamole. Since the maximal facilitatory effect of EHNA had a higher magnitude as compared to dipyrindamole, it seems likely that deamination is relatively more efficient than uptake in regulating extracellular adenosine levels at the rat myenteric plexus.

It has been reported that intracellular metabolism of adenosine may directly affect the extracellular concentration of adenosine through the nucleoside facilitated transport system (Thorn *et al.* 1996). Of the enzymes that metabolise adenosine, adenosine kinase has the highest affinity (Arch & Newsholme 1978) so, under baseline conditions, it is most likely to affect the rate of intracellular adenosine catabolism (Arch & Newsholme 1978; Lloyd *et al.* 1995). The inhibitor of adenosine kinase, 5'-iodotubercidin (ITU), has been

instrumental to increase extracellular adenosine levels in the nervous tissue (Golembiowska *et al.* 1996; Lloyd & Fredholm 1995; Pak *et al.* 1994). Application of ITU (10 μ M) increased the release of [^{3} H]ACh induced by electrical field stimulation of the myenteric plexus. The facilitatory effect of ITU (10 μ M, $+57\pm14\%$, $n=4$) was twice the effect of dipyridamole (0.5 μ M, $+27\pm7\%$, $n=5$) (Figure 4.17), ruling out the possibility that ITU might be facilitating transmitter release mainly because of its ability to inhibit adenosine uptake (Lloyd & Fredholm 1995). Furthermore, pre-treatment with dipyridamole, applied during the whole assay including S_1 and S_2 , prevented facilitation caused by ITU (10 μ M, $-4\pm10\%$, $n=3$) (data not shown). Thus, facilitation of transmitter release caused by inhibiting intracellular adenosine kinase is probably due to an outward membrane transport of adenosine, sensitive to dipyridamole.

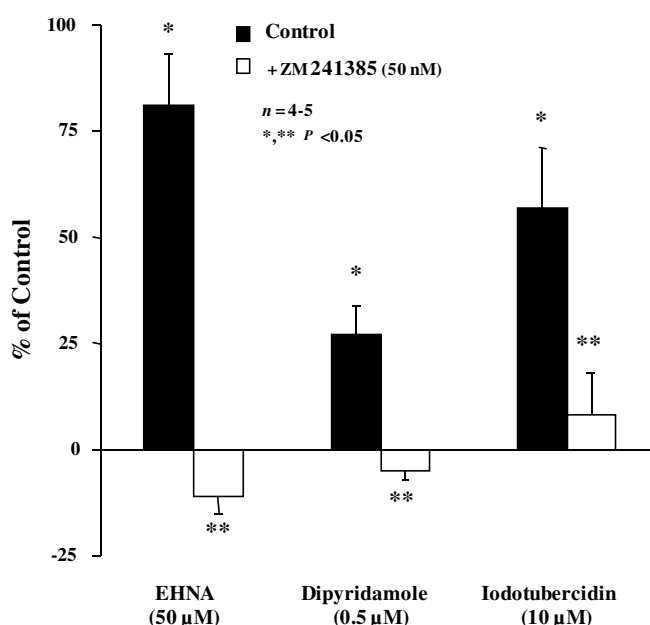


Figure 4.17 - Action of the selective adenosine A_{2A} receptor antagonist, ZM 241385, on the facilitatory effects induced by EHNA, dipyridamole and ITU on [^{3} H]ACh release from myenteric motoneurons, stimulated electrically. EHNA (50 μ M), dipyridamole (0.5 μ M) and ITU (10 μ M) were applied 15 min before S_2 . The adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM), was added to the incubation media since the beginning of the release period (time zero), *i.e.* it was present during the whole assay, including S_1 and S_2 . The ordinates are percentage changes in S_2/S_1 ratios, as compared to controls. Zero percent represents identity between ratios; Positive and negative values represent facilitation and inhibition of evoked [^{3} H]ACh release, respectively. The average S_2/S_1 ratio, in the presence of ZM 241385 (50 nM, 0.89 ± 0.03 , $n=5$), was not significantly ($P>0.05$) different from the control value (0.83 ± 0.11 , $n=4$) (see Table 4.1, Section 4.2.8). The data are means ($\pm$ SEM) of four to five individual experiments. * $P<0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences from the control; ** $P<0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences as compared with the effect of EHNA, dipyridamole or ITU, in the absence of ZM 241385.

To investigate whether facilitatory responses to dipyridamole (0.5 μ M), EHNA (50 μ M) and ITU (10 μ M) were mediated by extracellular adenosine accumulation activating presynaptic A_{2A} receptors, we compared the effects of these agents in the absence and in the presence of ZM 241385 (50 nM). Like the facilitation caused by CGS 21680C (3 nM) (see Figure 4.12, Section 4.2.2), the facilitatory effects of dipyridamole, EHNA and ITU on evoked [^{3} H]ACh release were all prevented by ZM 241385 (50 nM) (Figure 4.17).

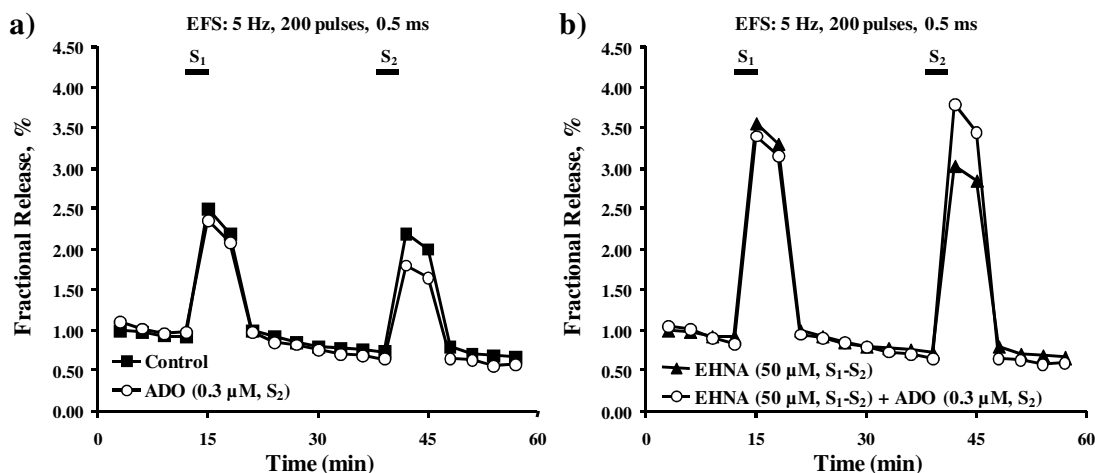


Figure 4.18 - Effect of exogenously added adenosine (ADO, 0.3 μ M) on [^{3} H]-ACh release from myenteric motoneurons stimulated electrically, (a) in the absence and (b) in the presence of the ADA inhibitor, EHNA (50 μ M). Shown is the time course of tritium outflow from the LM-MP of the rat ileum taken from typical experiments. Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue, at the beginning of the collection period. The abscissa indicates the times at which the samples were collected. The release of [^{3} H]ACh, in response to electrical field stimulation (200 pulses of 1 ms duration delivered at a 5 Hz frequency), was elicited twice during the periods indicated (S_1 and S_2). ADO (0.3 μ M, open circles) was applied 15 min before S_2 . EHNA (50 μ M, filled triangles) was added to the incubation media since the beginning of the release period (time zero), *i.e.* it was present during the whole assay including S_1 and S_2 . The time course of tritium outflow in control conditions (in the absence of test drugs) is also shown for comparison (filled squares). The average S_2/S_1 ratio in the presence of EHNA (50 μ M, 0.85 ± 0.06 , $n=4$) was not significantly ($P>0.05$) different from the control value (0.83 ± 0.11 , $n=4$). None of the drugs used modified basal tritium outflow.

Extracellular inactivation may also account for failure of exogenous adenosine to reach receptors located at highly protected regions, like most neuro-effector synapses (Daly 1982; Dowdall 1978). Inhibition of ecto-ADA activity with EHNA (50 μ M), present during the whole assay including S_1 and S_2 , transformed the A_1 -receptor mediated inhibition of evoked [^{3} H]ACh release by exogenous adenosine (0.3 μ M, $19 \pm 5\%$, $n=4$)

(Figure 4.18a) into a consistent facilitatory effect ($+34\pm 8\%$, $n=4$) (Figure 4.18b). Data indicate that extracellular ADA might control deliverance of exogenously added adenosine to facilitatory A_{2A} receptors located at the synaptic region.

4.2.7 Adenosine deaminase is secreted to the bathing fluid in parallel with adenosine upon stimulating the LM-MP

Figure 4.19 illustrates the progress curves of extracellular adenosine (30 μM) disappearance and inosine formation in the incubation media, in the presence and after removing the LM-MP from the organ bath. The concentration of adenosine (30 μM) reached a value of 11.89 ± 0.36 μM ($n=4$) after a 45-min incubation with the LM-MP. Surprisingly, the adenosine concentration also decreased significantly ($P<0.05$) to 24.99 ± 1.70 μM ($n=4$) over the 45-min incubation period, after removing the preparation from the organ bath. The inosine concentration increased continuously over 45 min, reaching values of 14.51 ± 1.98 μM ($n=4$) and 7.27 ± 0.84 μM ($n=4$) in the presence and in the absence of the LM-MP, respectively. The results suggest that a residual ($\sim 40\%$) ADA activity is left in the incubation media after removing the preparation.

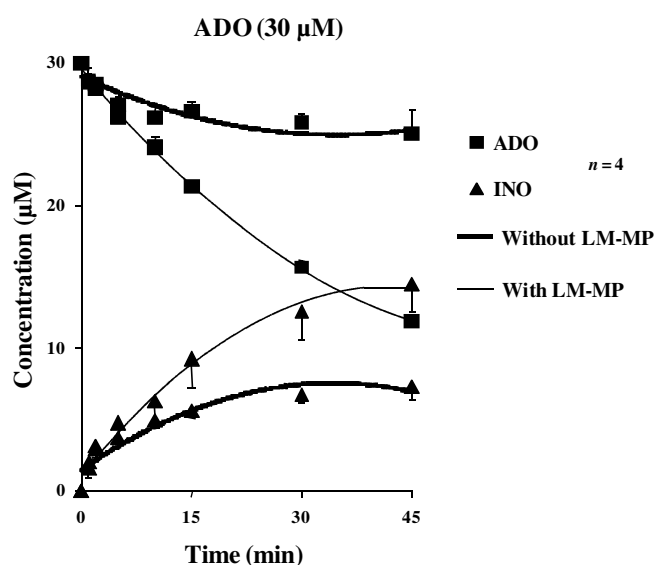


Figure 4.19 - Kinetics of adenosine (ADO, 30 μM , squares) disappearance and inosine (INO, triangles) formation in the LM-MP of the rat ileum: Residual deaminase activity in the incubation fluid, after removing the preparation from the organ bath. ADO (30 μM) was incubated at zero time, either in the presence of the LM-MP or after its removal from the organ bath (see Materials and Methods). Collected samples were analysed by HPLC to separate and quantify adenosine and inosine. Average results obtained in four experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size.

Then, we tested whether soluble ADA activity increased in the bath following depolarisation of the LM-MP. Electrical stimulation (5 Hz frequency, 3000 pulses of 1 ms duration) of the preparation led to an increased activity ($P>0.05$) of ADA in samples collected from the organ bath (Figure 4.20), without any modification of lactate dehydrogenase (LDH) activity, thus indicating that the increased ADA activity was not due to cell damage. Surprisingly, adenosine and its deamination products (inosine and hypoxanthine) also accumulated in the incubation buffer (see Figure 4.14, Section 4.2.4) in parallel with the stimulation-induced enhancement of ADA activity. This implies that the amount of endogenous adenosine appearing in the bath effluent may be largely underestimated, as compared with the levels of the nucleoside in the biophase, due to the activity of ADA secreted by the LM-MP.

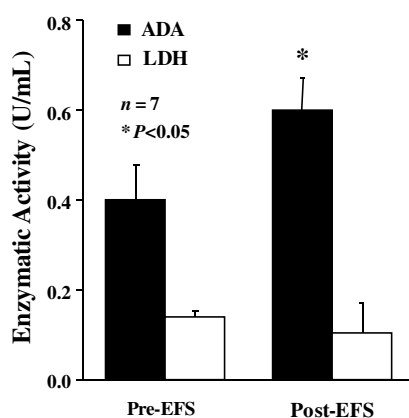


Figure 4.20 - ADA activity increases in the incubation fluid, following electrical stimulation (EFS) of the LM-MP of the rat ileum. The preparations were incubated with Tyrode's solution for 15 min and then subjected to EFS (5 Hz frequency, 3000 pulses, 1 ms of duration). Total ADA activity of samples collected from the incubation media was determined at 37°C by enzymatic spectrophotometric analysis (Giusti 1974). The activity of intracellular LDH remained essentially unchanged in sample duplicates collected following LM-MP stimulation, indicating that ADA secretion is unlikely to result from membrane cell damage. The data are means ($\pm$ SEM) of seven individual experiments. * $P<0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences as compared with enzymatic activity before stimulus application.

4.2.8 Contribution of the ecto-5'-nucleotidase pathway for adenosine modulation of [^{3} H]ACh release from myenteric motoneurons

As previously mentioned (see Figure 4.1c, Section 4.1.2), the bath concentration of the adenosine precursor, AMP (30 μ M), decreased continuously with time reaching a concentration of 5.14 ± 2.41 μ M ($n=3$) after a 45 min incubation with the LM-MP of the rat ileum. The average half degradation time of exogenously added AMP was 15.04 ± 2.41

min. AMP was dephosphorylated into adenosine, whose concentration in the bathing fluid increased progressively to a maximum of $12.95 \pm 2.21 \mu\text{M}$ at 30 min (Figure 4.21). Exposure of the myenteric plexus to a higher concentration of AMP (100 μM) did not alter its rate of hydrolysis or the pattern of appearance of its metabolites (data not shown). Figure 4.21 also shows the time course of AMP degradation in the presence of two chemically distinct ecto-5'-nucleotidase inhibitors, the ADP analogue α,β -methylene ADP (AOPCP) (Naito & Lowenstein 1985) and the non-nucleoside lectin, concanavalin A (Con A) (Naito & Lowenstein 1985; Riordan *et al.* 1974; Stefanovic *et al.* 1975). These two membrane-impermeable compounds, AOPCP (200 μM) and Con A (0.1 mg/mL), proportionally inhibited AMP degradation and adenosine formation by about 60%. In the presence of AOPCP and Con A, the average half degradation time of exogenously added AMP was increased to 45.82 ± 2.79 min ($n=5$) and 34.15 ± 1.23 min ($n=3$), respectively. Increasing the concentration of Con A to 0.2 mg/mL did not significantly ($P>0.05$) increased the amount of AMP remaining in the bath nor prolonged its time course.

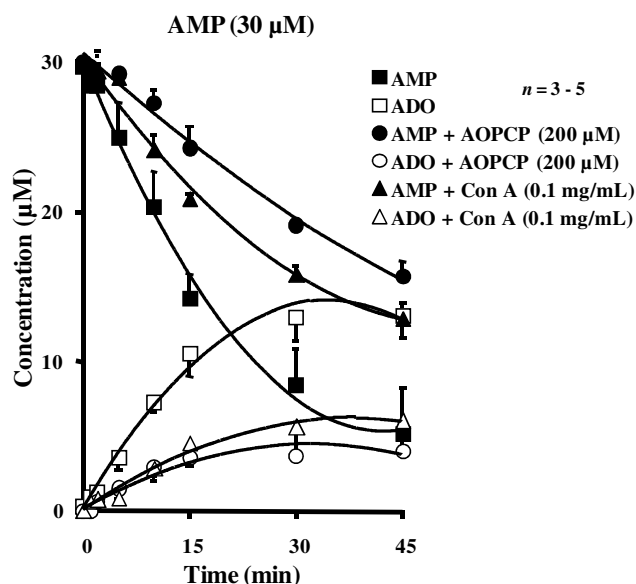


Figure 4.21 - Inhibitory effects of α,β -methylene ADP (AOPCP) and concanavalin A (Con A) on the catabolism of AMP (filled symbols) and formation of adenosine (ADO, open symbols) in the myenteric plexus of the rat ileum. AMP (30 μM) was incubated at zero time with LM-MP preparations in a 3-ml organ bath at 37°C, in the absence (squares) and in the presence of AOPCP (200 μM , circles) or Con A (0.1 mg/mL, triangles). Both progress curves were obtained from the same preparation. Samples (75 μl) were collected at the times indicated on the abscissa and analysed by HPLC. Average results obtained in three to five experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. Inosine formation was detected in the bath from the second min, in concentrations ranging from 0.39-7.37 μM . Hypoxanthine was formed linearly from the fifteenth minute (0.23-1.67 μM) of incubation. None of these compounds were represented for the sake of clarity. In the presence of AOPCP (200 μM) and Con A (0.1 mg/mL), inosine formation was only detected in the bath from the tenth minute in concentrations ranging from 0.07-2.23 μM , while hypoxanthine was virtually undetectable.

AMP (30 and 100 μ M) facilitated the electrically evoked release of [^{3} H]ACh from myenteric motoneurons by $21\pm 8\%$ ($n=3$) and $39\pm 9\%$ ($n=5$), respectively. As shown in Figure 4.22, the facilitatory effect of AMP (100 μ M) was prevented both by ADA (0.5 U/mL) and Con A (0.1 mg/mL). This ability of Con A to block the facilitatory effect of AMP might not be due to an inhibitory effect of Con A itself, since Con A (0.1 mg/mL) did not modify the evoked [^{3} H]ACh release under the present stimulation conditions (200 pulses of 1 ms duration delivered at a 5 Hz frequency) (see Table 4.1, this Section). Taken together, these observations suggest that AMP has to be catabolised extracellularly into adenosine to facilitate transmitter release. The activation of the adenosine A_{2A} receptors is probably responsible for the facilitatory effect of exogenously applied AMP (100 μ M), since the selective A_{2A} receptor antagonist, ZM 241385 (50 nM), prevented AMP-induced facilitation, while this was virtually unaffected by the adenosine A_1 receptor antagonist, DPCPX (10 nM) (Figure 4.22).

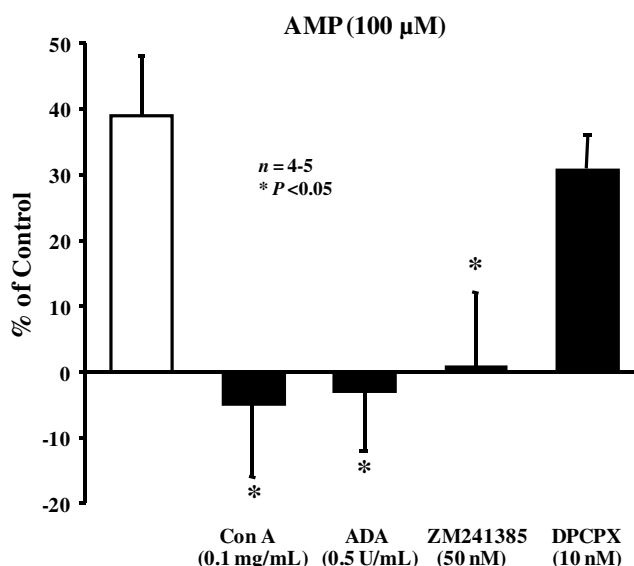


Figure 4.22 - The facilitatory effect of AMP on the evoked [^{3} H]ACh release from myenteric motoneurons depends on the activation of presynaptic A_{2A} receptors by adenosine generated extracellularly. AMP (100 μ M) was applied 15 min before S_2 . Con A (0.1 mg/mL), ADA (0.5 U/mL), ZM 241385 (50 nM) and DPCPX (10 nM) were present during the whole assay, including S_1 and S_2 . The ordinates are percentage changes in S_2/S_1 ratios, as compared to controls. Zero percent represents identity between ratios; Positive and negative values represent facilitation and inhibition of evoked [^{3} H]ACh release, respectively. The average S_2/S_1 ratios, in the presence of Con A, ADA, ZM 241385 or DPCPX, were not statistically ($P>0.05$) different from the control value (see Table 4.1). The data are means ($\pm$ SEM) of four to five individual experiments. * $P<0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences as compared with the facilitatory effect of AMP alone.

In view of the regulatory potential of the ecto-nucleotidase pathway for extracellular adenosine accumulation, we decided to investigate the role of endogenous adenosine, formed from released adenine nucleotides, in relation to the activation of nearby facilitatory adenosine A_{2A} receptors modulating [^3H]ACh release from myenteric motoneurons. For this purpose, we compared the influence of the train length on the inhibitory effect of ADA, which removes all the endogenous extracellular adenosine (see Figure 4.13, Section 4.2.3), with the effects of AOPCP and Con A, which prevent only the formation of adenosine from the extracellular catabolism of released adenine nucleotides (Table 4.1). In contrast to ADA (0.5 U/mL), which consistently inhibited the evoked [^3H]ACh release by 30-40%, depending on the stimulation train length, AOPCP (200 μ M) and Con A (0.1 mg/mL) failed to modify [^3H]ACh release when the train length delivered to the myenteric plexus was shorter than 2.5 min (Table 4.1). However, upon increasing the train length to 4.5 min (keeping the stimulation frequency at 5 Hz), inhibition of adenosine formation from the catabolism of released nucleotides, with either Con A (0.1 mg/mL) or AOPCP (200 μ M), significantly reduced the release of [^3H]ACh (Table 4.1), an effect prevented by the A_{2A} receptor antagonist, ZM 241385 (50 nM).

Table 4.1 - Influence of the number of pulses (train length) on the tonic effect of adenosine on electrically evoked [^3H]ACh release from LM-MP of the rat ileum.

Number of Pulses	Control		Drugs in S_2 (% of Control)		
	S_1 (10^3 dpm/g)	S_2/S_1	ADA (0.5 U/mL)	Con A (0.1 mg/ml)	AOPCP (200 μ M)
200 pulses	44 $\pm$ 5 (15)	0.83 $\pm$ 0.11 (4)	-31 $\pm$ 6% (7)*	+4 $\pm$ 8% (4)	-1 $\pm$ 9% (4)
750 pulses	94 $\pm$ 11 (18)	0.84 $\pm$ 0.07 (4)	-32 $\pm$ 9% (4)*	-13 $\pm$ 4% (4)	+2 $\pm$ 9% (5)
1350 pulses	220 $\pm$ 14 (8)	0.85 $\pm$ 0.05 (4)	-38 $\pm$ 3% (4)*	-41 $\pm$ 4% (4)*	-36 $\pm$ 5% (3)*

Evoked [^3H]ACh release was elicited by two trains (S_1 and S_2) of electrical field stimulation consisting of 200, 750 or 1350 pulses delivered at a 5 Hz frequency (1 ms pulse duration). Values for S_1 and S_2/S_1 are means ($\pm$ SEM). ADA (0.5 U/mL), Con A (0.1 mg/mL) and AOPCP (200 μ M) were applied 15 min before S_2 . Drug effects were measured on the stimulation (S_2)-evoked release of ACh and are expressed as percentage changes ($\pm$ SEM) in S_2/S_1 ratios, as compared to controls. Zero percent represents identity between ratios; Positive and negative values represent facilitation and inhibition of evoked [^3H]ACh release, respectively. The number of experiments is between parentheses. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the control.

4.2.9 Discussion

In this study, we provide evidence indicating that synaptic accumulation of endogenous adenosine has a predominant tonic facilitatory action on ACh release from electrically stimulated myenteric motoneurons, via the activation of adenosine A_{2A} receptors. Although adenosine may be formed from the extracellular catabolism of released adenine nucleotides through the ecto-nucleotidase pathway, this might not represent the major source of endogenous adenosine modulating short-term cholinergic neurotransmission in the rat myenteric plexus. Data indicate that myenteric neurons are the main source of adenosine released from the stimulated LM-MP of the rat ileum. Another major strength of the present work is the demonstration that extracellular deamination represents the most efficient mechanism regulating synaptic adenosine levels and, consequently, tonic activation of facilitatory A_{2A} receptors on myenteric nerve terminals. Besides the very intense ecto-adenosine deaminase activity in this tissue, a less efficient (NBTI-insensitive, *er*) nucleoside transport system may also contribute to inactivate extracellular adenosine. Due to the competence of both inactivation mechanisms, endogenous adenosine actions may be restricted to the release/production region at the myenteric cholinergic synapse, while exogenously added adenosine seems to activate preferentially extrajunctional inhibitory A_1 receptors (Figure 4.23, this Section).

In addition to inhibitory adenosine A_1 receptors expressed on both cholinergic and tachykinergic myenteric neurons, activation of A_{2A} receptors may facilitate depolarization of enteric neurons and increase ileal contractions elicited by electrical field stimulation (Broad *et al.* 1992; Christofi & Wood 1994; Gustafsson *et al.* 1985; Tomaru *et al.* 1995). In the present work, we showed that exogenously applied adenosine consistently inhibited the electrically evoked [^{3} H]ACh release. The finding that DPCPX (10 nM) not only counteracted the inhibitory action of adenosine but also converted it into a facilitatory effect on [^{3} H]ACh release from myenteric motoneurons, indicates that exogenous adenosine may activate facilitatory receptors providing that co-existent inhibitory A_1 receptors are blocked (see Figure 4.11, Section 4.2.2) (Correia-de-Sá *et al.* 1991; Tomaru *et al.* 1995). Using two stable adenosine analogues displaying high subtype selectivity for adenosine receptors, R-PIA (100-300 nM) and CGS 21680C (1-10 nM) (Fredholm *et al.* 2010a; 2010b), we clearly demonstrated the co-existence of inhibitory A_1 and facilitatory A_{2A} receptors modulating [^{3} H]ACh release from the rat myenteric motoneurons (see Figure 4.12, Section 4.2.2). The presence of two adenosine receptors, A_1 and A_{2A} , raised the question of how endogenous adenosine would interact with each receptor subtype, and which would be the prevailing response. Tonic adenosinergic regulation in the ileum has

been addressed before with conflicting results. Most of the studies, using the guinea-pig ileum as a model, provided indications that adenosine inhibitory tonus is mediated by A_1 receptors located at myenteric motoneurons (Christofi *et al.* 1993; Lee *et al.* 2001; Nitahara *et al.* 1995). This was observed in spite of the fact that in some studies the A_1 antagonist, DPCPX, failed to affect the contractile responses to electrical stimulation (Nitahara *et al.* 1995; Tomaru *et al.* 1995). Differences were also found in the frequency of stimulation, as those authors used lower frequencies (0.1-2 Hz) than that used here (5 Hz), which may, therefore, have been insufficient to cause a significant release/accumulation of extracellular adenosine (De Man *et al.* 2003). Overall, these discrepancies indicate that the mechanism of adenosine modulation of cholinergic transmission in the myenteric plexus may differ between species and between the conditions under which signals were recorded.

The present results showed that selective blockade of A_{2A} -receptors (with ZM 241385, 50 nM) inhibited the evoked [^{3} H]ACh release, while antagonism of A_1 -receptors (with 10 nM of DPCPX) produced a small but consistent increase in transmitter release (see Figure 4.13, Section 4.2.3). It was previously demonstrated that pre-treatment with DPCPX shifts the balance between A_1 and A_{2A} receptor activation towards the activation of A_{2A} receptors (Correia-de-Sá & Ribeiro 1996). The fact that ZM 241385 action mimicked the inhibition caused by removing extracellular adenosine with ADA (0.5 U/mL) is consistent with the idea that endogenous adenosine exerts a predominant tonic facilitatory effect (of about 30%) on cholinergic neurotransmission. Furthermore, the increase of adenosine extracellular levels (using inhibitors of adenosine deaminase, nucleoside transporters or adenosine kinase) facilitates ACh release in a manner sensitive to A_{2A} receptor blockade (see Figure 4.17, Section 4.2.6). To our knowledge, this is the first report showing that endogenous adenosine tonically facilitates the release of ACh from enteric motoneurons through the activation of adenosine A_{2A} receptors.

In this study, we also provided evidence suggesting that myenteric neurons are the main source of adenosine released from electrically-stimulated LM-MP of the rat ileum. Multiple forms of depolarization can release adenosine from neuronal sources (Sawynok *et al.* 2003). Our findings clearly indicate that the nucleoside outflow is Ca^{2+} - and action potential-dependent, but it remains unaltered by blocking smooth muscle contractions with nifedipine (see Figure 4.15, Section 4.2.4). Normally, Ca^{2+} -dependent neurotransmitter release indicates a vesicular storage and exocytotic release, but there is no evidence for vesicular storage and release of adenosine. Neuronal adenosine release occurs in response to an increase in energy demand (depolarization) or a mismatch in the demand of energy and ATP resynthesis (ischemia, hypoxia) or through receptor-operated mechanisms, and such release originates from metabolic pools (Latini & Pedata 2001).

The intracellular Ca^{2+} requirement for adenosine release from myenteric neurons may reflect actions on intracellular processes that modify metabolic production of adenosine or promote the efflux of nucleoside through equilibrative transporters. For instance, intracellular Ca^{2+} increase can lead to protein kinase C activation that subsequently stimulates 5'-nucleotidase and/or inhibits adenosine kinase, leading to intracellular adenosine accumulation and efflux from the cell (Obata *et al.* 2001; Sinclair *et al.* 2000). In line with this concept, we showed that ITU-induced adenosine kinase inhibition increased extracellular adenosine accumulation, whereas blockade of the nucleoside membrane transport with dipyridamole produced an opposite effect.

Another major strength of the present work is the demonstration that extracellular deamination represents the most efficient mechanism regulating synaptic adenosine levels (Franco *et al.* 1988) and, consequently, tonic activation of facilitatory $\text{A}_{2\text{A}}$ receptors on myenteric nerve terminals. ADA is a ubiquitous purine metabolic enzyme that was considered cytosolic for many years, but it has been found on the cell surface of a number of cell types (ecto-ADA) as well as in the interstitial fluid (exo-ADA) (Latini & Pedata 2001; Yegutkin 2008). The presence of ADA in nerve terminals has been clearly demonstrated in many brain regions and it was hypothesized that ADA-containing terminals may release adenosine or the enzyme itself, which could serve to regulate purinergic neurotransmission (Nagy *et al.* 1996). To our knowledge, this is the first study demonstrating that ADA activity increases in bath samples collected after LM-MP stimulation, in parallel with the interstitial accumulation of adenosine and its metabolites (see Figures 4.14 and 4.20, Sections 4.2.4 and 4.2.7, respectively). This implies that the amount of adenosine detected, following a period of electrical field stimulation, may be largely underestimated as compared to the nucleoside levels in the biophase. ADA secretion and adenosine release from stimulated LM-MP are not related to membrane cell damage, since the activity of intracellular LDH remained essentially unchanged in duplicates (see Figure 4.20 Section 4.2.7). Although ADA secretion increases in various diseases, especially those of the hematopoietic system, the origin and the mechanism by which interstitial activity is increased have not been fully elucidated (Ungerer *et al.* 1992). In most cases, ADA secretion differs from that of LDH and seems to be originated from monocyte/macrophage lineages, thus reflecting the involvement of the cellular immune system. It was recently reported that chronic intestinal inflammation enhanced the enteric contractile activity in part due to an impairment of purinergic modulation of cholinergic nerve activity (De Man *et al.* 2003). Because adenine nucleotides and nucleosides (and ADA itself) may be released from activated inflammatory cells (Marquardt *et al.* 1984), as well as from neighbouring myenteric neurons (Bogers *et al.* 2000; Stead *et al.* 1989), there is an increasing interest in the neuromodulatory effects exerted by adenosine during

inflammatory insults when the nucleoside levels become elevated. Moreover, adenosine and its breakdown product, inosine, have powerful anti-inflammatory effects by inhibiting the release of proinflammatory cytokines and chemokines (Mabley *et al.* 2003), reinforcing the pathophysiological interest of ADA secretion in parallel with interstitial adenosine accumulation in the myenteric plexus.

Besides the very intense ADA activity in this tissue, a less efficient dipyrnidamole-sensitive nucleoside transport system may also contribute to inactivate extracellular adenosine, as dipyrnidamole partially reduced the nucleoside inactivation leading to a smaller increase in electrically-evoked [^3H]ACh release from myenteric neurons as compared with the ADA inhibitor, EHNA (50 μM) (see Figures 4.16 and 4.17, Sections 4.2.5 and 4.2.6, respectively). Interestingly, clearing up extracellular adenosine by cellular uptake, via equilibrative nucleoside carriers, is driven by the concentration gradient of adenosine, but the same transporter may be implicated in the release process of adenosine under different conditions, especially during periods of neuronal activity when the metabolic demand increases (Latini & Pedata 2001; Sperlagh *et al.* 2003).

The competence of nucleoside inactivation mechanisms may restrict endogenous adenosine actions to the release/production region at the myenteric synapse. ADA secretion may also limit diffusion of exogenously added adenosine towards the active zones, and we showed that this constrain may be overcome by inhibiting ADA activity (see Figure 4.18, Section 4.2.6). The kinetics of extracellular adenosine inactivation, the potential nucleoside sources and the regional distribution of adenosine receptors, provide unique spatiotemporal conditions for adenosine to control excitability of the enteric nervous system. Excitatory adenosine A_{2A} receptors are co-localized with inhibitory A_1 receptors on post-ganglionic myenteric neurons, and these receptors exhibit a heterogeneous distribution in both rodent (Christofi & Wood 1994; Christofi *et al.* 2001) and human species (Christofi *et al.* 2001). Therefore, it is tempting to speculate that adenosine that is generated away from the synaptic region, being more prone to inactivation by uptake and deamination during diffusion, favours activation of inhibitory adenosine A_1 receptors located in the soma or in the axons of myenteric neurons (Barajas-Lopez *et al.* 1996). Conversely, adenosine formed at the myenteric neuro-effector junction may be a major contributor to maintain cholinergic neurotransmission through activation of the newly characterized facilitatory A_{2A} receptors. The lack of morphological studies precludes further interpretations concerning the distribution of the different elements involved in adenosine signalling in the rat myenteric plexus. It is, however, worth noting that our group recently confirmed that immunoreactivity against A_1 and A_{2A} receptors is located in different sub-regions of myenteric neurons of the rat ileum using confocal microscopy. While A_1 receptors are positioned predominantly in neuronal

cell bodies of myenteric ganglia, immunoreactivity against A_{2A} receptors decorates varicose nerve fibers, namely VACHT-positive cholinergic nerve terminals (Vieira *et al.* 2011).

Thus, regardless of the fact that adenosine can be derived from ATP released either from activated smooth muscle cells (Katsuragi *et al.* 1993; Vizi *et al.* 1992) and/or from autonomic nerve terminals (White & Leslie 1982), it is highly probable that adenosine released *per se* upon electrical stimulation (Begg *et al.* 2002) constitutes the major contributor to regulate myenteric excitability. This was inferred because inhibition of endogenous AMP breakdown into adenosine failed to modify the release of [^{3} H]ACh from stimulated (200 pulses delivered at 5 Hz frequency) myenteric motoneurons, whereas removal of all extracellular adenosine by bath application of ADA (0.5 U/mL) caused a consistent inhibitory effect. Similar results were obtained in isolated strips of the guinea-pig ileum, where inhibition of ecto-5'-nucleotidase with AOPCP yielded either no effect or a weak inhibitory effect on cholinergic neurotransmission (Katsuragi *et al.* 1993; Wiklund *et al.* 1986). Because P2-purinoceptors activation could complicate data interpretation obtained with the ADP analogue, AOPCP (200 μ M) (Naito & Lowenstein 1985), we tested the non-nucleoside inhibitor of ecto-5'-nucleotidase, Con A (0.1 mg/mL) (Riordan & Slavik 1974; Stefanovic *et al.* 1975), which was also inefficient. Adenosine originated from the catabolism of released nucleotides only became functionally relevant after prolonging the stimulation period over 4.5 min (see Table 4.1, Section 4.2.8). Sensitivity of AOPCP- and Con A-induced inhibitory effects to blockade by ZM 241385 reveals the occurrence of a time-dependent activation pattern of facilitatory A_{2A} receptors by adenosine originated via the ecto-nucleotidase pathway. Failure of ecto-5'-nucleotidase inhibitors to modify [^{3} H]ACh release during brief stimulation trains, contrasts with the facilitatory effect of the exogenously added adenosine precursor, AMP. These findings indicate that the amount of adenosine generated from released adenine nucleotides is probably not enough to activate prejunctional facilitatory A_{2A} receptors, which may be due to an insufficient amount of released adenine nucleotides. Alternatively, the postsynaptic localization of ecto-5'-nucleotidase (Nitahara *et al.* 1995) may lead to a delayed production of adenosine away from the adenosine receptor sites on myenteric nerve terminals.

It is uncertain whether the extracellular levels of adenosine achieved in the current study are physiologically relevant. However, both *in vivo* and *in vitro* models suggested that the balance between adenosine A_1 -inhibitory/ A_{2A} -facilitatory receptors might be important in regulating intestinal motility. This has been ascertained because administration of DPCPX, thereby unveiling A_{2A} -receptor-mediated effects (Correia-de-Sá *et al.* 1991; Tomaru *et al.* 1995), promoted faecal expulsion (Tomaru *et al.* 1995) and may reverse postoperative ileus (Correia-de-Sá *et al.* 1991; Kadowaki *et al.* 2003; Tomaru *et*

al. 1995) in rats. In addition, Milusheva and collaborators (1990) demonstrated that hypoxia inhibited the release of acetylcholine from the myenteric plexus, through the elevation of endogenous adenosine (Milusheva *et al.* 1990). Recently, it was reported that chronic intestinal inflammation enhanced the enteric contractile activity, in part due to a loss of the cholinergic neuromodulatory role of adenosine mediated by A₁ receptors (De Man *et al.* 2003). Desensitization of purinoceptors on enteric nerves may occur during chronic inflammation because purines, such as adenosine and ATP, are released from mast cells (Marquardt *et al.* 1984) located in the vicinity of myenteric neurons in several species, including man (Bogers *et al.* 2000; Marquardt *et al.* 1984; Stead *et al.* 1989). Since human post-ganglionic myenteric neurons co-express adenosine A₁ and A_{2A} receptors exhibiting a heterogeneous regional distribution (Christofi *et al.* 2001), it is conceivable that adenosine contributes to an overall homeostatic role on enteric excitability during pathological conditions, like ischaemic and inflammatory insults, when adenosine levels become elevated.

Despite the release of adenosine *per se* may be the main source of extracellular adenosine in most stressed cells (Cunha 2001), the pathophysiological implications of adenosine originated directly from neighbouring neurogenic, myogenic, vascular and inflammatory sources, or indirectly, as an ATP breakdown product, remain to be elucidated. According to the present data, it is tempting to speculate that adenosine that is generated away from the active zones, being more prone to inactivation by uptake and deamination during diffusion towards the synaptic region, favours the activation of neuroprotective inhibitory adenosine A₁ receptors located in the soma or in the axons of myenteric neurons (Barajas-Lopez *et al.* 1996). In contrast, adenosine formed at myenteric neuro-effector junctions might be a major contributor to maintain cholinergic neurotransmission through the activation of prejunctional facilitatory A_{2A} receptors (Figure 4.23). The intense ADA activity, due to enzymatic secretion during neuronal firing, may restrict endogenous adenosine actions to the synaptic region, leading to a preferential tonic activation of prejunctional A_{2A} receptors.

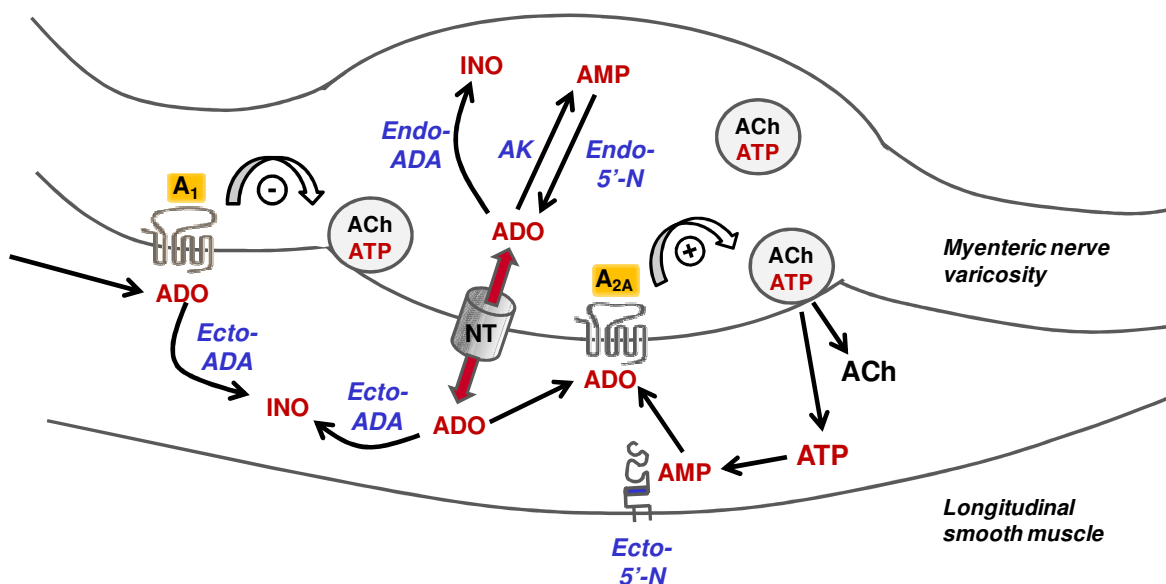


Figure 4.23 - Schematic representation of the neuromodulatory action of adenosine in the myenteric plexus of rat ileum. In response to stimulation, extracellular adenosine (ADO) can originate directly via bi-directional nucleoside transporters (NT). In addition, released ATP may be sequentially dephosphorylated by extracellular nucleotidases to form endogenous adenosine. Ecto-5'-nucleotidase (Ecto-5'-N), the limiting enzyme of the ectonucleotidase pathway, plays an important role regulating the rate of local adenosine production from adenine nucleotides. Endogenously generated adenosine can interact with facilitatory A_{2A} receptors, located on myenteric nerve varicosities, to stimulate the release of ACh release. Adenosine signalling is tightly regulated by the nucleoside inactivation mechanisms. Deamination into inosine by adenosine deaminase existing extracellularly (Ecto-ADA) represents the most efficient mechanism regulating synaptic adenosine levels. Moreover, adenosine uptake into cells, via facilitated NT, may also contribute to restrict adenosine actions to the release/production region. Note that while the facilitatory adenosine A_{2A} receptor seems to be localized at the neuro-effector region, the inhibitory A_1 receptor may be away from the sites of adenosine formation and removal hence more accessible to exogenous adenosine. For the sake of clarity, prejunctional muscarinic and P2 receptors were omitted.

4.3 – Muscarinic M₃ facilitation of ACh release from myenteric neurons depends on ADO outflow leading to activation of A_{2A} receptors

4.3.1 Rationale

Gastrointestinal motility depends on ACh release from the excitatory enteric neurons and the activation of smooth muscle muscarinic receptors (Furness 2000). A heterogeneous mixture of M₂ and M₃ receptors is expressed in enteric smooth muscle, with M₂ outnumbering the M₃ by at least fourfold, but the latter receptor is more active in mediating smooth muscle contraction (Ehlert *et al.* 1997; Matsui *et al.* 2003; Sawyer *et al.* 1998). ACh acting via muscarinic autoreceptors may also regulate its own release (North *et al.* 1985). Controversy remains on the predominant neuromodulatory role of ACh via co-expressed inhibitory and facilitatory muscarinic autoreceptors (Kilbinger & Nafziger 1985; Kurjak *et al.* 1999; Leclerc *et al.* 2002; North *et al.* 1985). ACh output from myenteric neurons declines with the increase of train length, but the muscarinic negative feedback mechanism plays only a minor role with longer trains (Kilbinger *et al.* 1983). The human myenteric plexus expresses both m2 and m3 messenger RNAs, and their expression declines in the aganglionic bowel (Oue *et al.* 2000). Conversely, other authors described that the activation of inhibitory M₂/M₄ receptors and of pirenzepine-sensitive (M₁) excitatory receptors may regulate synaptic transmission in the myenteric plexus (North *et al.* 1985; Simeone *et al.* 1996; Takeuchi *et al.* 2005). Therefore, we aimed at investigating the time course of action of facilitatory and inhibitory muscarinic receptors modulating [³H]ACh release from stimulated myenteric plexus of the rat ileum.

Consensus in data interpretation is also challenged if variations on the release of transmitters and crosstalk with endogenous neuromodulators, namely adenosine, during repetitive stimuli are not taken into account. It has already been provided functional evidence that adenosine controls synaptic transmission through a sophisticated interplay between its own receptors and receptors for other neurotransmitters/neuromodulators, namely nicotinic and muscarinic receptors (Correia-de-Sá *et al.* 1994; Duarte-Araújo *et al.* 2004b; Oliveira *et al.* 2002; Sebastião & Ribeiro 2000). The key receptor in this synaptic interplay appears to be the A_{2A} receptor which, in most neuro-effector junctions, is positively coupled to the adenylate cyclase/cyclic AMP pathway (Duarte-Araújo *et al.* 2004b; Oliveira *et al.* 2005). Previously (Section 4.2.3 in this thesis), we have demonstrated that endogenous adenosine plays a predominant facilitatory action on ACh release from myenteric neurons of the rat ileum, via the activation of facilitatory A_{2A} receptors (Duarte-Araújo *et al.* 2004a). Using immunohistochemistry and molecular biology techniques, other authors showed that human post-ganglionic myenteric neurons

express A_{2A} receptors, but presynaptic action of adenosine promoting vagal-induced gastric contractions has also been reported (Cho *et al.* 1993). These authors demonstrated that the nucleoside lacks an effect on the contractile cholinergic receptors located on enteric smooth muscle in the rat. Therefore, co-existence of facilitatory adenosine A_{2A} and muscarinic receptors on myenteric neurons prompted us to investigate how these receptors interplay to regulate [³H]ACh release.

As described before, adenosine regulation of synaptic transmission depends on its extracellular accumulation, which is achieved by balancing extracellular adenosine generation and inactivation mechanisms (cellular uptake and/or extracellular deamination) (Section 4.2.6 in this thesis). In the myenteric plexus, adenosine may be released as such, in response to electrical stimulation (Begg *et al.* 2002). This release is thought to be mediated via facilitated equilibrative nucleoside transporters, and might be regulated by endogenous neuromediators co-released from myenteric neurons, such as ACh and ATP. Parallel formation of adenosine, from the hydrolysis of ATP released from activated smooth muscle cells as well as from stimulated myenteric neurons, is also possible (Nitahara *et al.* 1995; White & Leslie 1982) although it requires prolonged periods of stimulation (see Table 4.1, Section 4.2.8). In view of the role of muscarinic autoreceptors to control myenteric synaptic activity, we thought that it was important to investigate how these receptors affect endogenous adenosine accumulation, thus contributing to the putative homeostatic effect of the nucleoside on enteric excitability (De Man *et al.* 2003; Kadowaki *et al.* 2003; Milusheva *et al.* 1990).

4.3.2 Influence of the train length on the origin of ADO facilitating [^3H]ACh release from myenteric neurons

Electrical field stimulation increased [^3H]ACh release, in a time-dependent manner, yet tritium outflow per stimulation pulse declined significantly ($P < 0.05$) with trains longer than 1350 pulses (Table 4.2).

Table 4.2 – Influence of the number of pulses (train length) on the amount of [^3H]ACh release from myenteric motoneurons of the rat ileum

Number of Pulses in S_2	S_2 (10^3 dpm/g)	S_2/S_1
200 pulses	219±13 (17)	0.83±0.11 (4)
500 pulses	484±42 (4)*	2.17±0.16 (4)*
750 pulses	683±98 (4)*	3.19±0.20 (4)*
1350 pulses	802±43 (4)*	3.57±0.31 (4)*

Evoked [^3H]ACh release was elicited by two trains (S_1 and S_2) of electrical field stimulation. While the number of pulses in S_1 was kept constant (200 pulses), S_2 consisted in 200, 500, 750 or 1350 pulses delivered at a 5 Hz frequency (1 ms pulse duration). Values for S_2 and S_2/S_1 are means ($\pm$ SEM). The number of experiments is between parentheses. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the situation where the number of pulses applied in S_1 and S_2 was identical (200 pulses).

Data from this study (see Table 4.1, Section 4.2.8) are in agreement with other authors' findings on the conclusion that the hydrolysis of released ATP contributes only partially to the total interstitial nucleoside concentration, in addition to neural release of adenosine *per se* (Katsuragi *et al.* 1993; Moneta *et al.* 1997; Wiklund & Gustafsson 1986). This was predicted since selective blockade of A_{2A} receptors with ZM 241385 (50 nM) or removal of extracellular adenosine with ADA (0.5 U/mL) caused consistent inhibitory effects in all stimulation conditions (Figure 4.24). In contrast, Con A (0.1 mg/mL), which only prevents the formation of adenosine from the extracellular catabolism of released adenine nucleotides by inhibiting ecto-5'-nucleotidase (Christofi *et al.* 2001), failed to modify [^3H]ACh release when the number of pulses within a train was <1350 (Figure 4.24).

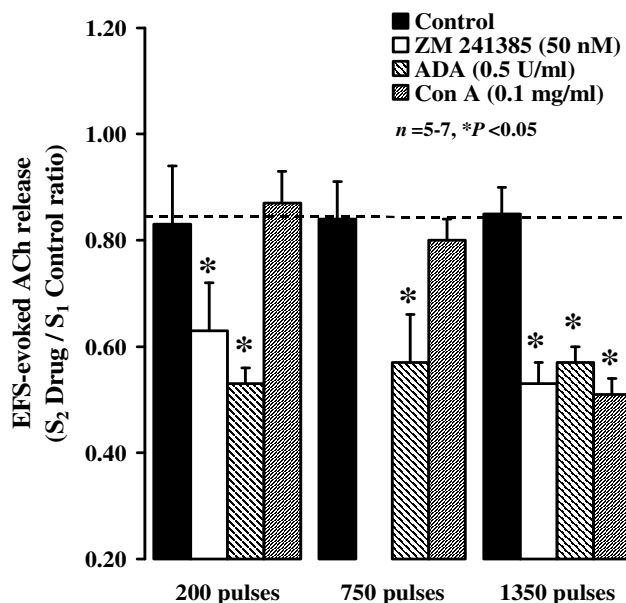


Figure 4.24 - Influence of the number of pulses (train length) on tonic activation of presynaptic adenosine receptors regulating $[^3\text{H}]\text{ACh}$ released from stimulated myenteric motoneurons of the rat ileum. After the labelling and washout periods, $[^3\text{H}]\text{ACh}$ release was induced by two trains (S_1 and S_2) of electrical field stimulation each consisting of 200, 750 or 1350 pulses delivered at a 5 Hz frequency. Adenosine modifying agents, ZM 241385 (50 nM), ADA (0.5 U/mL) and Concanavalin A (Con A, 0.1 mg/mL) were added 15 min before S_2 . The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios, *i.e.* the ratio between the evoked $[^3\text{H}]\text{ACh}$ release during the second period of stimulation (in the presence of the test drug), and the evoked $[^3\text{H}]\text{ACh}$ release during the first stimulation period (without the test drug). Each column represents pooled data from five to seven individual experiments. The vertical bars represent the SEM. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences compared to control (dark bars) obtained in the absence of test drugs and using an identical stimulation protocol.

4.3.3 Influence of the train length on muscarinic autoreceptors activity regulating $[^3\text{H}]\text{ACh}$ release from myenteric motoneurons

To study the role of cholinergic autoreceptors modulating evoked $[^3\text{H}]\text{ACh}$ release, we tested atropine (100 μM) and some selective muscarinic receptor antagonists, like 4-DAMP (100 nM), J104129 (6 nM), AF-DX 116 (10 μM), MT-3 (1 nM) and MT-7 (1 nM) (Birdsall 2010a; 2010b; 2010c; 2010d; 2010e; Challiss & Tobin 2009).

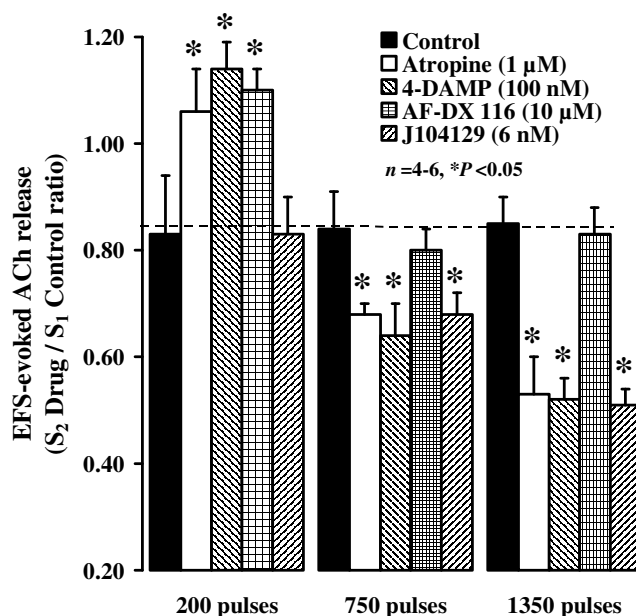


Figure 4.25 - Influence of the number of pulses (train length) on tonic activation of presynaptic muscarinic receptors regulating [³H]ACh released from stimulated myenteric motoneurons of the rat ileum. After the labelling and washout periods, [³H]ACh release was induced by two trains (S₁ and S₂) of electrical field stimulation each consisting of 200, 750 or 1350 pulses, delivered at a 5 Hz frequency. Muscarinic receptor antagonists: atropine (1 μM), 4-DAMP (100 nM), AF-DX 116 (10 μM) and J104129 (6 nM) were added 15 min before S₂. The ordinates represent evoked tritium outflow expressed by S₂/S₁ ratios, *i.e.* the ratio between the evoked [³H]ACh release, during the second period of stimulation (in the presence of the test drug), and the evoked [³H]ACh release, during the first stimulation period (without the test drug). Each column represents pooled data from four to six individual experiments, respectively. The vertical bars represent the SEM. **P* < 0.05 (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences compared to control (dark bars) obtained in the absence of test drugs and using an identical stimulation protocol.

Atropine (1 μM) and 4-DAMP (100 nM), which is a high affinity nonselective muscarinic antagonist that has been instrumental in the characterization of M₃ receptors (Griffin *et al.* 2003), had biphasic effects on evoked [³H]ACh release (Figure 4.25). Both drugs increased the release of [³H]ACh during brief stimulation trains (200 pulses), but they consistently decreased tritium outflow upon increasing the number of pulses to 750 and 1350. J104129 (6 nM), a selective M₃ receptor antagonist (*K*_i = 4.2 nM) which exhibits 120-fold selectivity over M₂ receptors (*K*_i = 490 nM) (Mitsuya *et al.* 1999), specifically reduced [³H]ACh release seen with long stimulation trains (750 and 1350 pulses), but was without effect with brief trains (200 pulses) (Figure 4.25). Higher concentrations of atropine (100 μM, *n* = 4), 4-DAMP (1 μM, *n* = 5) and J104129 (100 nM, *n* = 4) did not further increase the magnitude of their responses (data not shown). AF-DX 116 (10 μM), which

binds preferentially to M_2 and M_4 receptors (Birdsall 2010b; 2010d), increased the release of [^{3} H]ACh when the myenteric plexus was stimulated with 200 pulses, but failed to modify tritium outflow upon increasing the stimulation train length (Figure 4.25). The two most selective muscarinic toxins isolated from the venom of the green mamba (*Dendroaspis angusticeps*), MT-3 and MT-7, which exhibit high subtype selectivity respectively for M_4 ($pK_B \sim 8.7$) and M_1 receptors ($pK_B \sim 9.8$) (Adem *et al.* 1997; Birdsall 2010a; 2010d; Caulfield & Birdsall 1998), failed to affect evoked [^{3} H]ACh when they were applied in the low nanomolar (1-10 nM, $n=5$) concentration range (data not shown).

4.3.4 Muscarinic M_3 autoreceptors facilitate the release of adenine nucleosides from stimulated myenteric neurons

Table 4.3 – Myenteric neurons are the main source of adenine nucleosides released from stimulated LM-MP: On the role of muscarinic autoreceptors.

Frequency (Hz)	Number of pulses	Drugs	Evoked release of ADO + INO + HX (pmol/mg of tissue)
1 Hz	600 pulses		1.10 ± 0.60 (4)
5 Hz	1350 pulses		3.30 ± 0.60 (4)
5 Hz	3000 pulses	Control	9.40 ± 1.30 (6)
		TTX (1 μ M)	2.10 ± 0.70 (6)*
		Ca ²⁺ Ø + EGTA (1mM)	2.70 ± 1.10 (4)*
		Nifedipine (5 μ M)	8.80 ± 0.40 (6)
		AF-DX 116 (10 μ M)	8.70 ± 1.20 (4)
		4-DAMP (100 nM)	3.60 ± 0.90 (5)*
		J104129 (20 nM)	3.80 ± 0.90 (4)*

The preparations were incubated with Tyrode's solution for 15 min and then subjected to electrical field stimulation (EFS) as indicated. Samples collected from the incubation media were analysed by HPLC to separate and quantify adenine nucleosides (expressed in pmol/mg of tissue). EFS elicited an increment in adenine nucleosides content of the bathing fluid, consisting mostly of adenosine (ADO), inosine (INO) and hypoxanthine (HX) (Cho *et al.* 1993). Tetrodotoxin (1 μ M), Ca²⁺-free solution (Ca²⁺Ø + EGTA, 1 mM), nifedipine (5 μ M), AF-DX 116 (10 μ M), 4-DAMP (100 nM) and J104129 (20 nM) contacted the preparations for at least 15 min before stimulus application. Data represent the mean values ± SEM. The number of experiments is given in parentheses. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences from the release of adenine nucleosides induced by EFS (5 Hz, 3000 pulses) in the absence of test drugs.

Electrical stimulation of the LM-MP led to a time- and frequency-dependent increase in adenine nucleosides content in the bathing fluid (Table 4.3), consisting mostly of adenosine, INO and HX (Begg *et al.* 2002; Correia-de-Sá *et al.* 2006). Stimulus-evoked release of adenosine was dependent on extracellular Ca^{2+} and was sensitive to blockade of nerve action potentials by TTX (1 μM), but not to the smooth muscle relaxant nifedipine (5 μM) (Table 4.3), thus indicating that stimulated myenteric neurons are the main source of adenosine in the LM-MP preparations (see Figure 4.15, Section 4.2.4).

4-DAMP (100 nM) and J104129 (20 nM) significantly ($P < 0.05$) decreased the outflow of adenine nucleosides from stimulated myenteric neurons (Table 4.3). Release of adenosine and its metabolites was unaffected by MT-7 (1 nM, $n = 2$, data not shown) and by AF-DX 116 (10 μM) (Table 4.3). None of these antagonists modified the basal levels of the nucleoside. Inhibition of cholinesterase activity with physostigmine (10 μM) led to a substantial increase ($110 \pm 15\%$, $n = 6$, data not shown) in adenine nucleosides content in samples collected after LM-MP stimulation.

4.3.5 Muscarinic M_3 facilitation of [^3H]ACh release depends on endogenous ADO accumulation leading to excitatory A_{2A} receptor activation

Adenosine deaminase (0.5 U/mL) prevented 4-DAMP (100 nM) inhibition of [^3H]ACh release from the stimulated LM-MP (data not shown). Likewise, blockade of A_{2A} receptors activation with ZM 241385 (50 nM) abrogated the inhibitory effect of 4-DAMP (100 nM) seen with long stimulation trains (750 and 1350 pulses), but failed to affect 4-DAMP (100 nM) facilitation of [^3H]ACh release during brief trains (200 pulses). ZM 241385 (50 nM) restored the facilitatory component of 4-DAMP (100 nM) action when [^3H]ACh release was triggered by 750 pulses delivered at 5 Hz (Figure 4.26).

Because extracellular adenosine may have been originated from the hydrolysis of released adenine nucleotides, in which ecto-5'-nucleotidase is the rate limiting step of this pathway in the LM-MP (see Sections 4.2.1 and 4.2.8 in this thesis), we evaluated the effect of Con A on the positive feedback mechanism mediated by muscarinic M_3 autoreceptors. Con A (0.1 mg/mL) attenuated the inhibitory effect of 4-DAMP (100 nM) during long stimulation trains (1350 pulses), but the enzyme inhibitor failed to modify M_3 autofacilitation seen with shorter stimulation trains (750 pulses) (Figure 4.26).

Adenosine outflow, via equilibrative nucleoside transporters, may also contribute to synaptic adenosine accumulation upon depolarization of myenteric neurons (see Sections 4.2.5 and 4.2.6 in this thesis). Figure 4.26 shows that the nucleoside transport inhibitor, dipyridamole (0.5 μM), was more effective in attenuating the 4-DAMP (100 nM) inhibition

of [^3H]ACh release as compared with Con A (0.1 mg/mL), at least when 750 pulses were used.

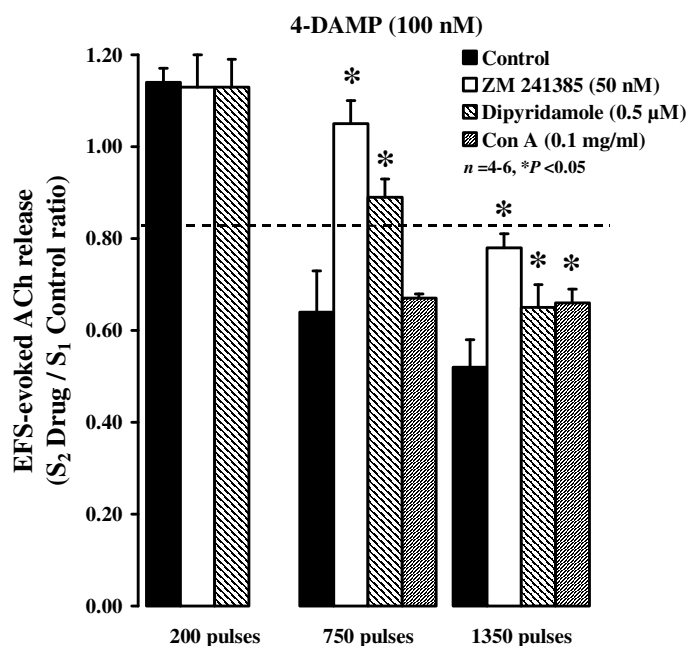


Figure 4.26 - Role of endogenous adenosine, via A_{2A} receptors, mediating muscarinic facilitation of [^3H]ACh release from stimulated myenteric neurons: influence of the stimulation train length. After the labelling and washout periods, [^3H]ACh release was induced by two trains (S_1 and S_2) of EFS each consisting of 200, 750 or 1350 pulses delivered at a 5 Hz frequency (1-ms pulse duration). The muscarinic receptor antagonist, 4-DAMP (100 nM), was applied 15 min before S_2 ; the endogenous adenosine modulators, ZM 241385 (50 nM), dipyridamole (0.5 μM) and concanavalin A (Con A, 0.1 mg/mL), were present throughout the assay, including S_1 and S_2 . The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios, *i.e.* the ratio between the evoked [^3H]ACh release during the second period of stimulation (in the presence of the test drug) and the evoked [^3H]ACh release during the first stimulation period (without the test drug). The average S_2/S_1 ratios in the presence of ZM 241385 (50 nM), dipyridamole (0.5 μM) and Con A (0.1 mg/mL) were not statistically ($P > 0.05$) different from control values obtained using identical stimulation protocols (dashed line) (see Figure 4.24 and 4.25). Each column represents pooled data from four to six individual experiments. The vertical bars represent SEM. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences as compared with the effect of 4-DAMP (100 nM) alone and using an identical stimulation protocol.

4.3.6 Agonist-induced muscarinic M₃ facilitation of [³H]ACh release is mediated by endogenous adenosine via A_{2A} receptors

To further investigate the hypothesis that endogenous adenosine acting through prejunctional A_{2A} receptors was required to induce muscarinic M₃ facilitation of transmitter release without constraints related to the amount of ACh in the biophase, we performed experiments using the enzymatically stable muscarinic receptor agonist, oxotremorine (Figure 4.27) (Birdsall 2010c).

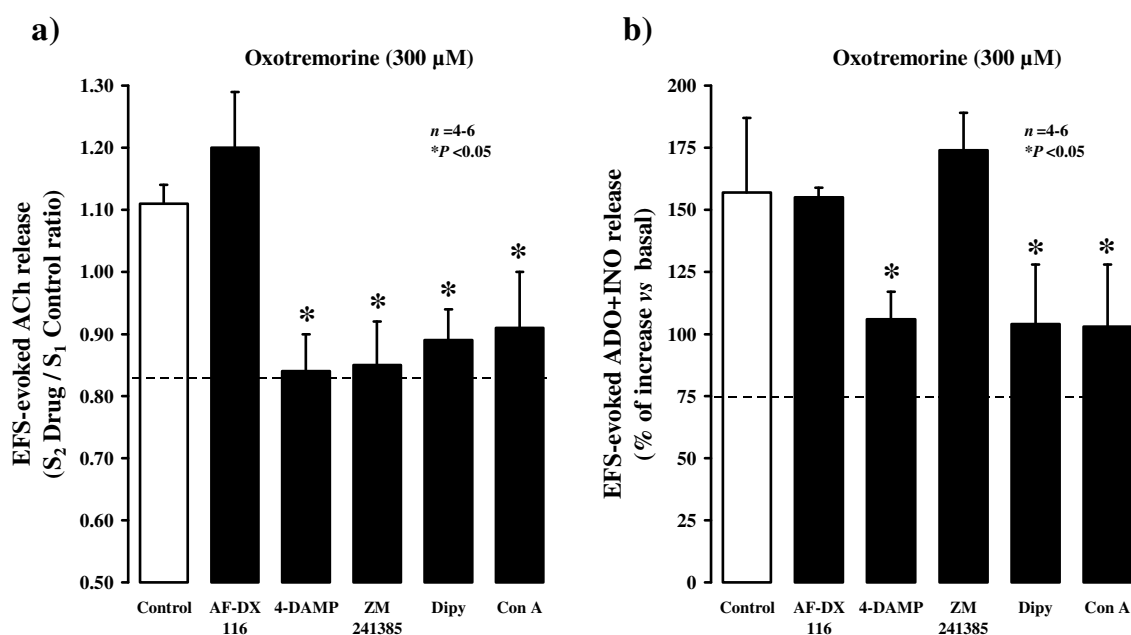


Figure 4.27 - Oxotremorine facilitates stimulation-evoked release of (a) [³H]ACh and (b) purine nucleosides of LM-MP preparations of the rat ileum. In (a) and (b), the preparations were exposed to oxotremorine (300 μM) for at least 15 min before stimulus application; AF-DX 116 (10 μM), 4-DAMP (100 nM), ZM 241385 (50 nM), dipyrindamole (Dipy, 0.5 μM) and concanavalin A (Con A, 0.1 mg/mL) were present throughout the assay. In (a), the ordinates represent evoked tritium outflow (5 Hz frequency, 200 pulses) expressed by S₂/S₁ ratios, *i.e.* the ratio between the evoked [³H]ACh release during the second period of stimulation (in the presence of the test drug) and the evoked [³H]ACh release during the first stimulation period (without the test drug). The average S₂/S₁ ratio in control conditions (*i.e.* in the absence of oxotremorine) was 0.83 ± 0.11 (*n* = 4) (dashed line). In (b), the ordinates represent percentage increases in purine nucleosides release above the baseline following stimulation (5 Hz frequency, 3000 pulses) of the LM-MP. In control conditions (*i.e.* in the absence of oxotremorine), stimulation-evoked purine nucleosides release was 75 ± 7% (*n* = 6) above the baseline (dashed line). The vertical bars represent ± SEM from an *n* number of experiments. **P* < 0.05 (one-way ANOVA followed by Dunnett's modified *t* test) represents significant differences as compared with the effect of oxotremorine alone.

Oxotremorine (300 μ M) increased both the release of [^{3} H]ACh and the outflow of adenine nucleosides from the stimulated LM-MP (Figure 4.27); the effect of oxotremorine (100–300 μ M) was concentration-dependent (data not shown). 4-DAMP (100 nM, $n = 4$) and J104129 (6 nM, $n = 4$), but not AF-DX 116 (10 nM, $n = 5$), prevented oxotremorine (300 μ M) facilitation of [^{3} H]ACh release, when those drugs were used in concentrations high enough to antagonize oxotremorine (0.003–300 μ M)-induced contractions of the longitudinal smooth muscle (Figure 4.28). The effect of oxotremorine (300 μ M) was not mimicked by the M_1 receptor agonist, McN-A-343 (10 μ M, $n = 4$) (data not shown).

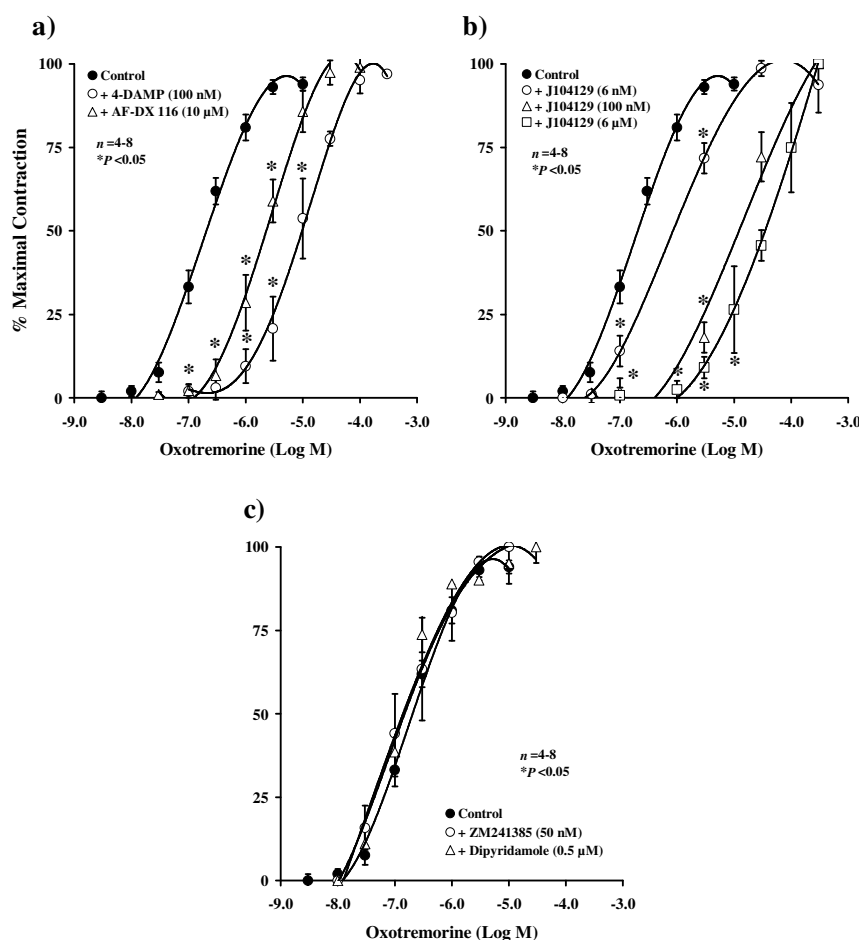


Figure 4.28 - Oxotremorine contractile responses of LM-MP preparations of the rat ileum. The concentration–response curves of oxotremorine (0.003–300 μ M) are represented in the absence and in the presence of (a) 4-DAMP (100 nM) and AF-DX 116 (10 μ M); (b) J104129 (6, 100 nM and 6 μ M); (c) ZM 241385 (50 nM) and dipyridamole (0.5 μ M). These drugs were added to the incubation fluid 15 min before application of oxotremorine. The ordinates are percentage of maximal contraction and the vertical bars represent $\pm$ SEM from an n number of experiments. $*P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represents significant differences as compared with the effect of oxotremorine alone.

Activation of muscarinic receptors and subsequent contraction of smooth muscle fibres, caused by oxotremorine (300 μ M), might not explain the extracellular accumulation of adenine nucleosides (Cho *et al.* 1993), as this effect was still present after preventing smooth muscle contraction with nifedipine, but not when nerve action potentials were blocked by TTX (Figure 4.15 and Table 4.3, Sections 4.2.4 and 4.3.3, respectively).

Interestingly, dipyridamole (0.5 μ M) and Con A (0.1 mg/mL) attenuated the facilitatory effect of oxotremorine (300 μ M), both on the release of [^{3} H]ACh and on the outflow of adenine nucleosides (Figure 4.27). In contrast, ZM 241385 (50 nM) prevented oxotremorine-induced facilitation of [^{3} H]ACh release (Figure 4.27a), but kept unchanged the outflow of adenine nucleosides (Figure 4.27b). Oxotremorine-induced contractile response was also not affected by ZM 241385 (50 nM) (Figure 4.28). Endogenous adenosine inactivation with ADA (0.5 U/mL) prevented oxotremorine (300 μ M) facilitation of [^{3} H]ACh release ($n = 2$, data not shown).

To test whether activation of the adenylate cyclase/cyclic AMP pathway by prejunctional A_{2A} receptors could modulate muscarinic M_3 facilitation of [^{3} H]ACh release, as it did with nicotinic autoreceptors at the rat myenteric nerve terminals (Duarte-Araújo *et al.* 2004b), we performed experiments with a selective inhibitor of protein kinase A, H-89 (Chijiwa *et al.* 1990). H-89 (10 μ M) prevented oxotremorine (300 μ M) facilitation of [^{3} H]ACh release ($n = 6$, data not shown) without changing the outflow of adenine nucleosides, as it was observed with the A_{2A} receptor antagonist, ZM 241385 (50 nM) (Figure 4.27).

4.3.7 Discussion

Studies in the human myenteric plexus have shown a strong expression of both m2 and m3 messenger RNAs (Oue *et al.* 2000). When co-expressed in heterologous systems, the binding and functional data suggest that both M₂ and M₃ receptors act independently of one another, without the formation of heteromeric complexes or the exhibition of a novel pharmacology (Griffin *et al.* 2003). Using selective muscarinic antagonists, we demonstrated that inhibitory M₂ and facilitatory M₃ receptor subtypes mediate a dual neuromodulatory role at the LM-MP of the rat ileum depending on the stimulation train length (number of pulses) (see Figure 4.25, Section 4.3.3). Although others (Kilbinger & Wessler 1983) have found that activation of pirenzepine-sensitive (M₁) excitatory receptors may regulate synaptic transmission in the myenteric plexus, we failed to demonstrate their participation to control [³H]ACh release using the most selective M₁ receptor antagonist, MT-7 (Adem & Karlsson 1997; Challiss & Tobin 2009; North *et al.* 1985; Simeone *et al.* 1996). One cannot, however, exclude a mixed inhibitory action mediated by M₂ and M₄ receptors, as they co-localize on myenteric neurons (Takeuchi *et al.* 2005). These authors found that atropine-induced facilitation was abolished only in the double knockout mice, suggesting that a loss of one of these subtypes can be compensated functionally by the receptor that remained. This might explain the lack of effect of the highly selective M₄ receptor antagonist, MT-3, whereas blockade of both M₂ and M₄ receptors, with AF-DX 116 or atropine, further increased stimulation-induced [³H]ACh release during brief stimulation trains (see Figure 4.25, Section 4.3.3).

Another major strength of the present study is the demonstration that adenosine released from stimulated myenteric neurons may be positively modulated by muscarinic M₃ autoreceptors. Our findings clearly indicate that the nucleoside outflow is Ca²⁺- and action potential-dependent, but it was unaltered by blocking smooth muscle contractions with nifedipine (Figure 4.15 and Table 4.3, Sections 4.2.4 and 4.3.3, respectively). We found that inhibition of the enzymatic breakdown of endogenous ACh (with physostigmine) led to an almost twofold increase in extracellular tissue levels of adenine nucleosides, following LM-MP stimulation. Purine nucleosides release was receptor-mediated as it was suppressed by blocking M₃ receptors with J104129 and 4-DAMP (see Table 4.3, Section 4.3.3) (Challiss & Tobin 2009; Mitsuya *et al.* 1999).

The intracellular Ca²⁺ requirement for adenosine release from myenteric neurons may reflect actions on intracellular processes that modify metabolic production of adenosine or promote the efflux of the nucleoside through equilibrative transporters. For instance, increases in intracellular Ca²⁺ can lead to activation of protein kinase C, which could subsequently stimulate adenosine efflux by equilibrative nucleoside transporters

(Coe *et al.* 2002). Increase in protein kinase C activity might also stimulate 5'-nucleotidase inside the cell and/or inhibit adenosine kinase, yielding to enhance intracellular accumulation and efflux of adenosine from the cell (Obata *et al.* 2001; Sinclair *et al.* 2000) (Sections 4.2.6 and 4.2.8 in this thesis). The participation of protein kinase C on the efflux of adenosine is further supported as the active phorbol ester, phorbol 12-myristate 13-acetate (PMA, 10 μ M), almost doubled the adenine nucleosides content following stimulation of the rat myenteric plexus (Vieira C, Duarte-Araújo M., Magalhães-Cardoso MT & Correia-de-Sá P, unpublished observations); interestingly, active PMA also increased ($50 \pm 4\%$, $n = 3$, $P < 0.05$) evoked [^{3} H]ACh release in the myenteric plexus of guinea-pig ileum (Saitoh *et al.* 1997). The ability of phorbol ester-induced protein kinase C activation to inhibit cyclic AMP phosphodiesterase activity has been shown (Irvine *et al.* 1986). Intracellular cyclic AMP may be a significant source of extracellular adenosine in neuronal tissues (Saitoh *et al.* 1997), in that cyclic AMP extruded from cells may be converted to AMP and then to adenosine by ecto-phosphodiesterases and ecto-5'-nucleotidase, respectively (Rosenberg *et al.* 1989). A functioning extracellular cyclic AMP-adenosine pathway is present in rat ileum and affects intestinal motility (Giron *et al.* 2008). Unlike the M_2 receptors that preferentially couple to $G_{i/o}$ to mediate inhibition of adenylate cyclase and Ca^{2+} influx via voltage-sensitive channels, muscarinic M_3 receptors typically couple via subunits of $G_{q/11}$ protein family to phospholipase C, leading to inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} mobilization and to protein kinase C activation (Challiss & Tobin 2009). Therefore, M_3 -like receptors, which mediate phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum, are strong candidates to promote the efflux/formation of adenosine from stimulated neurons (Candell *et al.* 1990).

Although it is highly probable that adenosine is released as such from enteric nerves upon depolarization via equilibrative dipyridamole-sensitive nucleoside transporters (see Section 4.2.4 and 4.2.5 in this thesis), an additional source of interstitial adenosine may result from the hydrolysis of released ATP (see Section 4.2.8 in this thesis) stored together with ACh in synaptic vesicles (Begg *et al.* 2002; Nitahara *et al.* 1995; White & Leslie 1982). This hypothesis was explored using the non-nucleotide lectin Con A, which has been shown to inhibit ecto-5'-nucleotidase activity in a number of tissues (Riordan & Slavik 1974; Stefanovic *et al.* 1975; Zimmermann 2000), including the rat myenteric plexus (see for *e.g.* Figure 4.21, Section 4.2.8), without causing direct actions on P2 purinoceptors. Investigating modulation of cholinergic and peptidergic neurotransmission in the myenteric plexus, other authors also reached to the conclusion that the hydrolysis of released adenine nucleotides contributes, only partially, to the total

interstitial nucleoside concentration, in addition to neural release of adenosine *per se* (Katsuragi *et al.* 1993; Moneta *et al.* 1997; Wiklund & Gustafsson 1986).

To the best of our knowledge, this is the first study to demonstrate that muscarinic M_3 autofacilitation is mediated indirectly by extracellular adenosine accumulation to the levels capable of activating excitatory A_{2A} receptors. The formation of heterodimers between M_3 and A_{2A} receptors, leading to unlike pharmacological profiles, cannot provide the explanation by which ADA affected M_3 facilitation of evoked [^3H]ACh release. In most neuro-effector junctions, adenosine A_{2A} receptors stimulate the adenylate cyclase/cyclic AMP system (Christofi *et al.* 1993; Duarte-Araújo *et al.* 2004b; Oliveira & Correia-de-Sá 2005). The participation of this pathway on the muscarinic M_3 positive feedback mechanism was demonstrated using the selective inhibitor of protein kinase A, H-89, which mimicked the effect of A_{2A} receptor blockade with ZM 241385. Interestingly, inhibition of protein kinase A in murine enteric neurons causes lethal intestinal pseudo-obstruction without any obvious malformations on the mature enteric nervous system (Howe *et al.* 2006). Whether this involves changes in neurochemical modulation, like failure of adenosine A_{2A} receptors activation on myenteric neurons, requires further investigations.

In conclusion, the major findings of this study show that ACh modulates its own release from rat myenteric neurons through the activation of muscarinic inhibitory M_2 and facilitatory M_3 autoreceptors. The M_2/M_3 receptor activation balance depends on the stimulation train length (number of pulses). Thus, while the M_2 inhibitory effect is predominant during brief stimulation periods (200 pulses) (Kilbinger & Wessler 1983), muscarinic M_3 autofacilitation becomes evident only during sustained nerve activity (>750 pulses), a situation that coincided with extracellular adenosine accumulation and increased tonic activation of prejunctional excitatory A_{2A} receptors. We provided evidence suggesting that the muscarinic M_3 positive feedback mechanism is indirectly mediated by the outflow of endogenous adenosine from myenteric neurons leading to the activation of facilitatory A_{2A} receptors (Figure 4.29). Although adenosine may be originated from the extracellular breakdown of released adenine nucleotides (*e.g.* ATP, cyclic AMP) via the ecto-nucleotidase pathway, we showed that adenosine outflow, through equilibrative nucleoside transporters, may be the main source of endogenous adenosine in the myenteric plexus.

These features may provide a purinergic re-enforcement to maintain cholinergic neurotransmission during high enteric nerve activity or whenever endogenous levels of ACh and adenosine become elevated (e.g. hypoxia, inflammation, postoperative ileum) (De Man *et al.* 2003; Kadowaki *et al.* 2003; Milusheva *et al.* 1990). Redundant mechanisms, through other muscarinic receptor subtypes or other mediators such as adenosine, might explain why M₃-mediated signals are dispensable in the mutant mouse digestive organs (Matsui *et al.* 2000). Nevertheless, in the ileum of diabetic rats exhibiting decreased enteric nerve function, an increased responsiveness of muscarinic M₃ receptors has been observed, which may be an adaptive change in response to upregulation of the inhibitory M₂ receptors in these animals (Coulson *et al.* 2002). Compensatory M₃ activity may partially offset gastrointestinal motility dysfunction in diabetics, as well as in other pathological conditions, which may lead to novel therapeutic candidates targeting adenosine-related mechanisms.

5 – Summary and Main Conclusions

Data presented in this thesis discloses the existence of a well-coordinated “purinergic cascade” that leads to P2 and P1 receptors activation in the myenteric plexus of the rat ileum. Sequential activation of neuronal purinergic receptors, namely excitatory P2X₂ and A_{2A} receptors and inhibitory P2Y₁ and A₁ receptors, together with the interplay between each other as well as with muscarinic autoreceptors, may influence ACh release and, thereby, control myenteric synaptic neurotransmission. Through these complex interactions, gradients of ATP breakdown products like ADP and adenosine may provide fine tuning of peristaltic motor performance in the gut during pathological conditions, when purine extracellular levels become elevated. Furthermore, the relevance of changing the extracellular concentration of adenine nucleotides and nucleosides to the neuromodulatory role of purines was also demonstrated, unravelling new therapeutic targets besides receptor agonists or antagonists drugs, like adenosine kinase, nucleoside transporters, adenosine deaminase and ecto-nucleotidase inhibitors.

In this context, the present scientific work led to the following conclusions:

1) In the LM-MP preparation of the rat ileum, ATP transiently activates facilitatory P2X receptors mediating spontaneous [³H]ACh release from myenteric motoneurons. P2X facilitation may be rapidly terminated by extracellular ATP catabolism into active adenine nucleotides, such as ADP and adenosine. As a consequence of the rapid conversion of ATP directly into AMP, catalysed by NTPDase1, and subsequent AMP dephosphorylation to adenosine by the E-5'-N, the magnitude of ATP excitation may be ultimately regulated by the nucleoside adenosine activating presynaptic inhibitory adenosine A₁ receptors. Additionally, activation of P2Y₁ purinoceptors by ADP, generated alternatively via Ecto-ATPDase, might be functionally relevant to restrain stimulation-induced ACh exocytosis, particularly when ATP accumulation overcomes NTPDase1 capacity. Stimulation of ADP-sensitive inhibitory P2Y₁ purinoceptors regulating [³H]ACh release may be cut short by sequential activation of inhibitory A₁ receptors by adenosine generated from the catabolism of released adenine nucleotides.

2) Myenteric neurons are the main source of adenosine released from electrically-stimulated LM-MP of the rat ileum. Our findings indicate that the nucleoside outflow is Ca²⁺- and action potential-dependent, but it remains unaltered by blocking smooth muscle contractions with nifedipine. Synaptic accumulation of neuronal adenosine release plays a

Summary and Main Conclusions

predominant tonic facilitatory action on ACh release from electrically stimulated myenteric motoneurons, via the activation of adenosine A_{2A} receptors. Although adenosine may be formed from the extracellular catabolism of released adenine nucleotides through the ecto-nucleotidase pathway, this might not represent the major source of endogenous adenosine modulating short-term cholinergic neurotransmission in the rat myenteric plexus. Extracellular deamination represents the most efficient mechanism regulating synaptic adenosine levels and, consequently, tonic activation of facilitatory A_{2A} receptors on myenteric nerve terminals. Moreover, this study demonstrates that ADA activity increases in bath samples collected after LM-MP stimulation, in parallel with the interstitial accumulation of adenosine and its metabolites, implying that the amount of adenosine detected may be largely underestimated when compared to the nucleoside levels in the biophase. Our findings are in keeping with the hypothesis that ADA secretion, along with a less efficient equilibrative nucleoside transport system, may restrict endogenous adenosine actions to the release/production region at the myenteric cholinergic synapse leading to facilitatory A_{2A} receptors activation. Such a local environment may also limit diffusion of exogenously added adenosine towards the active zones; therefore, these constrain leads to preferential activation of extrajunctional inhibitory A_1 receptors.

3) This study also indicates that ACh modulates its own release from rat myenteric neurons through the activation of muscarinic inhibitory M_2 and facilitatory M_3 autoreceptors. The M_2/M_3 receptor activation balance depends on the stimulation train length. M_2 inhibitory effect is predominant during brief stimulation periods (200 pulses), while muscarinic M_3 autofacilitation becomes evident only during sustained nerve activity (>750 pulses), a situation that coincided with extracellular adenosine accumulation and increased tonic activation of prejunctional excitatory A_{2A} receptors. This muscarinic M_3 positive feedback mechanism is indirectly mediated by the outflow of endogenous adenosine from myenteric neurons, which leads to the activation of facilitatory A_{2A} receptors.

6 – Future Perspectives

The existence of a well-coordinated “purinergic cascade” in the rat myenteric plexus raises the obvious question of how does this system adapt under pathological conditions. Our investigation group is already working on a rat model of inflammatory bowel disease induced by intraluminal application of TNBS, in order to evaluate whether changes in purinergic signalling constitute therapeutic targets. It is our understanding that if purines are capable of regulating ACh release due to a broad range of stimulatory patterns (and pathological conditions), maybe the rectification of synaptic levels of ATP and/or adenosine would be a more “physiological” approach in most circumstances.

In this work, we focused our attention on ACh release but the fact is that, in most excitatory myenteric neurons, ACh is co-released with other neurotransmitters, like tachykinins. To evaluate the interplay between substance P (SP) and purines in the myenteric plexus we are currently undertaking preliminary experiments using an animal model in which peptidergic nerve fibres are depleted (>95%) by neonatal injection of the chilli pepper venom, capsaicin.

Coordination of peristalsis does not rely exclusively on the myenteric neuro-effector junction. Interstitial “pacemaker” cells of Cajal (ICC) and enteric glia may also be involved. Although the importance of non-neuronal cells has been extensively addressed in the CNS, the integrative function of their peripheral homologues in the ENS is still largely unknown. Further studies are, therefore, required to investigate the contribution of non-neuronal cells, such as ICC and glia, to the overall purinergic control of the myenteric transmission. Myenteric nerve terminals, longitudinal smooth muscle fibres and adjacent ICCs arrange into a complex tridimensional structure (“tripartite myenteric synapse”), which is still unexplored concerning its functional role in releasing chemical mediators (such as ATP and adenosine) to control enteric neurotransmission.

All of these perspectives have a common denominator: to understand purinergic neuromodulation in the myenteric plexus in order to identify new pharmacological targets to treat gastrointestinal diseases.

7 – ACKNOWLEDGEMENTS

I would like to acknowledge the scientific supervision of Professor Doutor Paulo Jorge da Silva Correia de Sá, head of the Pharmacology and Neurobiology Laboratory, Immune-Physiology and Pharmacology Department - ICBAS-UP, where this work was carried out.

I would like to thank FCT for giving me a doctoral degree grant, SFRH/BD/29044/2006, supported by POPH - QREN/FSE.

I am very grateful to Sr. Duarte Monteiro for the original drawings included in this thesis and to Prof. Luís Baldaia, Prof. Paula Ferreira, Sr. Daniel, D. Madalena and D. Fernanda for their encouragement.

I wish to thank all colleagues from the Pharmacology and Neurobiology Lab: Prof. Graça, Dra. Alexandrina, Dra. Teresa, Laura, Miguel Faria, Prof. Adelina, Ana Rita and all final grade and master students, particularly to those that worked side by side with me in the myenteric plexus: Carlos, Sara, António, Marisa, but above all to Cátia, Isabel and Patrícia, the “plexi girls”, whose dedication and friendship I will always cherish. My special thanks also go to my cabinet colleagues: Patrícia, Fátima, Miguel Cordeiro and Bernardo, for making long days shorter. I also thank D. Suzete, D. Helena and D. Belmira, for their technical help and Mila, for everything.

I would also like to thank ICBAS laboratory animal facility staff: Dra. Luísa, Olímpia, Carla, Patrícia and specially Bárbara, for making the management of such a particular service a shared adventure. Thank you for making animal welfare compatible with human care.

I am very grateful to my dearest friends: Clara, Lau, Patrícia, Anita and Celso, with whom I will continue to divide sorrows and multiply happiness. To my oldest friends: Paula Alexandra, Tina, Elsa, Ivone, Sofia, Ana Paula, Miguel and Gama – that even though we may be apart from each other, you will always be near my heart. I want you all to know that I will always value our friendship and hope to be worthy of it.

To my family, that has always been able to show their love and support whenever needed and against all odds. I cannot forget the privilege of having had my grandmothers, uncles and my big sister as part of my life. You are all a part of me...

Finally, I am forever indebted to my best friend and husband, Paulinho, for his understanding and encouragement when it was most required. He makes me believe that raising Dinis and Sofia and maintaining this working rhythm is not only possible, but also a joy. And it really is.

8 – BIBLIOGRAPHY

- Abbracchio, M P & Burnstock, G (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* **64**(3): 445-475.
- Abbracchio, M P, Burnstock, G, Boeynaems, J M, Barnard, E A, Boyer, J L, Kennedy, C, Knight, G E, Fumagalli, M, Gachet, C, Jacobson, K A & Weisman, G A (2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* **58**(3): 281-341.
- Adem, A & Karlsson, E (1997). Muscarinic receptor subtype selective toxins. *Life Sci* **60**(13-14): 1069-1076.
- Agarwal, R P, Spector, T & Parks, R E, Jr. (1977). Tight-binding inhibitors-IV. Inhibition of adenosine deaminases by various inhibitors. *Biochem Pharmacol* **26**(5): 359-367.
- Ambache, N (1954). Separation of the longitudinal muscle of the rabbit's ileum as a broad sheet. *J Physiol* **125**(2): 53-55P.
- Antonioli, L, Fornai, M, Colucci, R, Ghisu, N, Blandizzi, C & Del Tacca, M (2006). A_{2A} receptors mediate inhibitory effects of adenosine on colonic motility in the presence of experimental colitis. *Inflamm Bowel Dis* **12**(2): 117-122.
- Antonioli, L, Fornai, M, Colucci, R, Ghisu, N, Tuccori, M, Del Tacca, M & Blandizzi, C (2008a). Pharmacological modulation of adenosine system: novel options for treatment of inflammatory bowel diseases. *Inflamm Bowel Dis* **14**(4): 566-574.
- Antonioli, L, Fornai, M, Colucci, R, Ghisu, N, Tuccori, M, Del Tacca, M & Blandizzi, C (2008b). Regulation of enteric functions by adenosine: pathophysiological and pharmacological implications. *Pharmacol Ther* **120**(3): 233-253.
- Arch, J R & Newsholme, E A (1978). The control of the metabolism and the hormonal role of adenosine. *Essays Biochem* **14**: 82-123.
- Arvidsson, U, Riedl, M, Elde, R & Meister, B (1997). Vesicular acetylcholine transporter (VACHT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol* **378**(4): 454-467.
- Atkinson, B, Dwyer, K, Enjyoji, K & Robson, S C (2006). Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: Potential as therapeutic targets. *Blood Cells Mol Dis* **36**(2): 217-222.
- Augustine, G J (2004). Neural Signaling Neuroscience D, P. Sunderland, MA, *Sinauer Associates, Inc.*: 129-164.

- Barajas-Lopez, C, Espinosa-Luna, R & Gerzanich, V (1994). ATP closes a potassium and opens a cationic conductance through different receptors in neurons of guinea pig submucous plexus. *J Pharmacol Exp Ther* **268**(3): 1397-1402.
- Barajas-Lopez, C, Huizinga, J D, Collins, S M, Gerzanich, V, Espinosa-Luna, R & Peres, A L (1996). P2x-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. *Br J Pharmacol* **119**(8): 1541-1548.
- Barthó, L, Undi, S, Benko, R, Wolf, M, Lazar, Z, Lenard, L, Jr. & Maggi, C A (2006). Multiple motor effects of ATP and their inhibition by P purinoceptor antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid in the small intestine of the guinea-pig. *Basic Clin Pharmacol Toxicol* **98**(5): 488-495.
- Begg, M, Dale, N, Llaudet, E, Molleman, A & Parsons, M E (2002). Modulation of the release of endogenous adenosine by cannabinoids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. *Br J Pharmacol* **137**(8): 1298-1304.
- Belhassen, B & Pelleg, A (1984). Electrophysiologic effects of adenosine triphosphate and adenosine on the mammalian heart: clinical and experimental aspects. *J Am Coll Cardiol* **4**(2): 414-424.
- Bertrand, P P & Bornstein, J C (2002). ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. *J Neurosci* **22**(12): 4767-4775.
- Bertrand, P P, Kunze, W A, Furness, J B & Bornstein, J C (2000b). The terminals of myenteric intrinsic primary afferent neurons of the guinea-pig ileum are excited by 5-hydroxytryptamine acting at 5-hydroxytryptamine-3 receptors. *Neuroscience* **101**(2): 459-469.
- Bertrand, P P, Thomas, E A, Kunze, W A & Bornstein, J C (2000a). A simple mathematical model of second-messenger mediated slow excitatory postsynaptic potentials. *J Comput Neurosci* **8**(2): 127-142.
- Bian, X, Ren, J, DeVries, M, Schnegelsberg, B, Cockayne, D A, Ford, A P & Galligan, J J (2003). Peristalsis is impaired in the small intestine of mice lacking the P2X3 subunit. *J Physiol* **551**(Pt 1): 309-322.
- Bian, X C, Heffer, L F, Gwynne, R M, Bornstein, J C & Bertrand, P P (2004). Synaptic transmission in simple motility reflex pathways excited by distension in guinea pig distal colon. *Am J Physiol Gastrointest Liver Physiol* **287**(5): G1017-1027.
- Bianco, F, Fumagalli, M, Pravettoni, E, D'Ambrosi, N, Volonte, C, Matteoli, M, Abbracchio, M P & Verderio, C (2005). Pathophysiological roles of extracellular nucleotides in glial cells: differential expression of purinergic receptors in resting and activated microglia. *Brain Res Brain Res Rev* **48**(2): 144-156.

- Birdsall, N J M B, Buckley, N J, Christopoulos, A, Eglen, R M, Ehlert, F, Hammer, R, Kilbinger, H.J, Lambrecht, G, Mitchelson, F, Mutschler, E, Nathanson, N M, Schwarz, R D, Tobin & A B, Wess, J (2010a). Acetylcholine receptors (muscarinic): M₁. IUPHAR database.
- Birdsall, N J M B, Buckley, N J, Christopoulos, A, Eglen, R M, Ehlert, F, Hammer, R, Kilbinger, H.J, Lambrecht, G, Mitchelson, F, Mutschler, E, Nathanson, N M, Schwarz, R D, Tobin, A B & Wess, J (2010b). Acetylcholine receptors (muscarinic): M₂. IUPHAR database.
- Birdsall, N J M B, Buckley, N J, Christopoulos, A, Eglen, R M, Ehlert, F, Hammer, R, Kilbinger, H.J, Lambrecht, G, Mitchelson, F, Mutschler, E, Nathanson, N M, Schwarz, R D, Tobin, A B & Wess, J (2010c). Acetylcholine receptors (muscarinic): M₃. IUPHAR database.
- Birdsall, N J M B, Buckley, N J, Christopoulos, A, Eglen, R M, Ehlert, F, Hammer, R, Kilbinger, H.J, Lambrecht, G, Mitchelson, F, Mutschler, E, Nathanson, N M, Schwarz, R D, Tobin, A B & Wess, J (2010d). Acetylcholine receptors (muscarinic): M₄. IUPHAR database.
- Birdsall, N J M B, Buckley, N J, Christopoulos, A, Eglen, R M, Ehlert, F, Hammer, R, Kilbinger, H.J, Lambrecht, G, Mitchelson, F, Mutschler, E, Nathanson, N M, Schwarz, R D, Tobin, A B & Wess, J (2010e). Acetylcholine receptors (muscarinic): M₅. IUPHAR database.
- Bogers, J, Moreels, T, De Man, J, Vrolix, G, Jacobs, W, Pelckmans, P & van Marck, E (2000). Schistosoma mansoni infection causing diffuse enteric inflammation and damage of the enteric nervous system in the mouse small intestine. *Neurogastroenterol Motil* **12**(5): 431-440.
- Boison, D (2006). Adenosine kinase, epilepsy and stroke: mechanisms and therapies. *Trends Pharmacol Sci* **27**(12): 652-658.
- Bonan, C D, Walz, R, Pereira, G S, Worm, P V, Battastini, A M, Cavalheiro, E A, Izquierdo, I & Sarkis, J J (2000). Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. *Epilepsy Res* **39**(3): 229-238.
- Bornstein, J C (2008). Purinergic mechanisms in the control of gastrointestinal motility. *Purinergic Signal* **4**(3): 197-212.
- Bornstein, J C, Costa, M & Grider, J R (2004). Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil* **16 Suppl 1**: 34-38.
- Bours, M J, Swennen, E L, Di Virgilio, F, Cronstein, B N & Dagnelie, P C (2006). Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther* **112**(2): 358-404.

- Broad, R M, McDonald, T J, Brodin, E & Cook, M A (1992). Adenosine A₁ receptors mediate inhibition of tachykinin release from perfused enteric nerve endings. *Am J Physiol* **262**(3 Pt 1): G525-531.
- Brookes, S J (2001). Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec* **262**(1): 58-70.
- Brookes, S J & Costa, M (2006). Functional Histoanatomy of the Enteric Nervous System. Physiology of the Gastrointestinal Tract. Johnson, L R B, K.E.; Ghishan, F.K.; Merchant, J.L.; Said, H.M.; Wood, J.D., *Elsevier, Academic Press*. **1**: 577 - 597.
- Brown, J H & Taylor, P (2005). Muscarinic receptor agonist and antagonist Goodman & Gilman's The Pharmacological Basis of Therapeutics Brunton, L L, J.; Parker, K., *McGraw-Hill Professional*.
- Brown, S J, James, S, Reddington, M & Richardson, P J (1990). Both A₁ and A_{2A} purine receptors regulate striatal acetylcholine release. *J Neurochem* **55**(1): 31-38.
- Buckley, N J & Burnstock, G (1984). Autoradiographic localisation of muscarinic receptors in guinea-pig intestine: distribution of high and low affinity agonist binding sites. *Brain Res* **294**(1): 15-22.
- Buckley, N J & Burnstock, G (1986). Localization of muscarinic receptors on cultured myenteric neurons: a combined autoradiographic and immunocytochemical approach. *J Neurosci* **6**(2): 531-540.
- Burns, A J, Herbert, T M, Ward, S M & Sanders, K M (1997). Interstitial cells of Cajal in the guinea-pig gastrointestinal tract as revealed by c-Kit immunohistochemistry. *Cell Tissue Res* **290**(1): 11-20.
- Burnstock, G (1972). Purinergic nerves. *Pharmacol Rev* **24**(3): 509-581.
- Burnstock, G (1976a). Do some nerve cells release more than one transmitter? *Neuroscience* **1**(4): 239-248.
- Burnstock, G (1976b). Purinergic receptors. *J Theor Biol* **62**(2): 491-503.
- Burnstock, G (1978). Do some sympathetic neurones synthesize and release both noradrenaline and acetylcholine? *Prog Neurobiol* **11**(3-4): 205-222.
- Burnstock, G (1981). Review lecture. Neurotransmitters and trophic factors in the autonomic nervous system. *J Physiol* **313**: 1-35.
- Burnstock, G (2001). Purine-mediated signalling in pain and visceral perception. *Trends Pharmacol Sci* **22**(4): 182-188.
- Burnstock, G (2004). Cotransmission. *Curr Opin Pharmacol* **4**(1): 47-52.
- Burnstock, G (2006). Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* **58**(1): 58-86.
- Burnstock, G (2007a). Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**(2): 659-797.

- Burnstock, G (2007b). Purine and pyrimidine receptors. *Cell Mol Life Sci* **64**(12): 1471-1483.
- Burnstock, G (2008a). Unresolved issues and controversies in purinergic signalling. *J Physiol* **586**(14): 3307-3312.
- Burnstock, G (2008b). Purinergic receptors as future targets for treatment of functional GI disorders. *Gut* **57**(9): 1193-1194.
- Burnstock, G (2008c). The journey to establish purinergic signalling in the gut. *Neurogastroenterol Motil* **20 Suppl 1**: 8-19.
- Burnstock, G (2009a). Purinergic cotransmission. *Exp Physiol* **94**(1): 20-24.
- Burnstock, G (2009b). Autonomic neurotransmission: 60 years since sir Henry Dale. *Annu Rev Pharmacol Toxicol* **49**: 1-30.
- Burnstock, G (2009c). Purinergic signalling: past, present and future. *Braz J Med Biol Res* **42**(1): 3-8.
- Burnstock, G, Campbell, G, Bennett, M & Holman, M E (1964). Innervation of the Guinea-Pig Taenia Coli: Are There Intrinsic Inhibitory Nerves Which Are Distinct from Sympathetic Nerves? *Int J Neuropharmacol* **3**: 163-166.
- Burnstock, G, Campbell, G & Rand, M J (1966). The inhibitory innervation of the taenia of the guinea-pig caecum. *J Physiol* **182**(3): 504-526.
- Burnstock, G, Campbell, G, Satchell, D & Smythe, A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**(4): 668-688.
- Burnstock, G, Cocks, T, Kasakov, L & Wong, H K (1978). Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. *Eur J Pharmacol* **49**(2): 145-149.
- Burnstock, G, Dumsday, B & Smythe, A (1972a). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br J Pharmacol* **44**(3): 451-461.
- Burnstock, G & Kennedy, C (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* **16**(5): 433-440.
- Burnstock, G & Lavin, S (2002). Interstitial cells of Cajal and purinergic signalling. *Auton Neurosci* **97**(1): 68-72.
- Burnstock, G, Satchell, D G & Smythe, A (1972b). A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *Br J Pharmacol* **46**(2): 234-242.

- Candell, L M, Yun, S H, Tran, L L & Ehlert, F J (1990). Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol Pharmacol* **38**(5): 689-697.
- Castelucci, P, Robbins, H L & Furness, J B (2003). P2X2 purine receptor immunoreactivity of intraganglionic laminar endings in the mouse gastrointestinal tract. *Cell Tissue Res* **312**(2): 167-174.
- Castelucci, P, Robbins, H L, Poole, D P & Furness, J B (2002). The distribution of purine P2X2 receptors in the guinea-pig enteric nervous system. *Histochem Cell Biol* **117**(5): 415-422.
- Caulfield, M P & Birdsall, N J (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50**(2): 279-290.
- Cerqueira, M D (2006). Advances in pharmacologic agents in imaging: new A_{2A} receptor agonists. *Curr Cardiol Rep* **8**(2): 119-122.
- Challiss, R A J & Tobin, A B (2009). Acetylcholine receptors (muscarinic), introductory chapter. IUPHAR database.
- Chen, H, Ordog, T, Chen, J, Young, D L, Bardsley, M R, Redelman, D, Ward, S M & Sanders, K M (2007a). Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. *Physiol Genomics* **31**(3): 492-509.
- Chen, H, Redelman, D, Ro, S, Ward, S M, Ordog, T & Sanders, K M (2007b). Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. *Am J Physiol Cell Physiol* **292**(1): C497-507.
- Chijiwa, T, Mishima, A, Hagiwara, M, Sano, M, Hayashi, K, Inoue, T, Naito, K, Toshioka, T & Hidaka, H (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* **265**(9): 5267-5272.
- Cho, C H, Qiu, B S, Ballard, H J & Ogle, C W (1993). Adenosine and cholinergic-induced gastric contraction in rats. *Digestion* **54**(2): 98-104.
- Christofi, F L (2001). Unlocking mysteries of gut sensory transmission: is adenosine the key? *News Physiol Sci* **16**: 201-207.
- Christofi, F L (2008). Purinergic receptors and gastrointestinal secretomotor function. *Purinergic Signal* **4**(3): 213-236.

- Christofi, F L, Hanani, M, Maudlej, N & Wood, J D (1993). Enteric glial cells are major contributors to formation of cyclic AMP in myenteric plexus cultures from adult guinea-pig small intestine. *Neurosci Lett* **159**(1-2): 107-110.
- Christofi, F L & Wood, J D (1993). Presynaptic inhibition by adenosine A₁ receptors on guinea pig small intestinal myenteric neurons. *Gastroenterology* **104**(5): 1420-1429.
- Christofi, F L & Wood, J D (1994). Electrophysiological subtypes of inhibitory P1 purinoceptors on myenteric neurones of guinea-pig small bowel. *Br J Pharmacol* **113**(3): 703-710.
- Christofi, F L, Zhang, H, Yu, J G, Guzman, J, Xue, J, Kim, M, Wang, Y Z & Cooke, H J (2001). Differential gene expression of adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors in the human enteric nervous system. *J Comp Neurol* **439**(1): 46-64.
- Ciruela, F, Albergaria, C, Soriano, A, Cuffi, L, Carbonell, L, Sanchez, S, Gandia, J & Fernandez-Duenas, V (2010). Adenosine receptors interacting proteins (ARIPs): Behind the biology of adenosine signaling. *Biochim Biophys Acta* **1798**(1): 9-20.
- Coe, I, Zhang, Y, McKenzie, T & Naydenova, Z (2002). PKC regulation of the human equilibrative nucleoside transporter, hENT1. *FEBS Lett* **517**(1-3): 201-205.
- Cooke, H J, Wunderlich, J & Christofi, F L (2003). "The force be with you": ATP in gut mechanosensory transduction. *News Physiol Sci* **18**: 43-49.
- Correia-de-Sá, P, Adães, S, Timóteo, M A, Vieira, C, Magalhães-Cardoso, T, Nascimento, C & Duarte-Araújo, M (2006). Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: on the role of secreted adenosine deaminase. *Auton Neurosci* **126-127**: 211-224.
- Correia-de-Sá, P & Ribeiro, J A (1994). Tonic adenosine A_{2A} receptor activation modulates nicotinic autoreceptor function at the rat neuromuscular junction. *Eur J Pharmacol* **271**(2-3): 349-355.
- Correia-de-Sá, P & Ribeiro, J A (1996). Adenosine uptake and deamination regulate tonic A_{2A} receptor facilitation of evoked [³H]acetylcholine release from the rat motor nerve terminals. *Neuroscience* **73**(1): 85-92.
- Correia-de-Sá, P, Sebastião, A M & Ribeiro, J A (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. *Br J Pharmacol* **103**(2): 1614-1620.
- Correia-de-Sá, P, Sebastião, A M & Ribeiro, J A (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. *Br J Pharmacol* **103**(2): 1614-1620.

- Corriden, R, Insel, P A & Junger, W G (2007). A novel method using fluorescence microscopy for real-time assessment of ATP release from individual cells. *Am J Physiol Cell Physiol* **293**(4): C1420-1425.
- Costa, M, Brookes, S J, Steele, P A, Gibbins, I, Burcher, E & Kandiah, C J (1996). Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience* **75**(3): 949-967.
- Costa, M, Furness, J B & Gibbins, I L (1986). Chemical coding of enteric neurons. *Prog Brain Res* **68**: 217-239.
- Coulson, F R, Jacoby, D B & Fryer, A D (2002). Increased function of inhibitory neuronal M2 muscarinic receptors in trachea and ileum of diabetic rats. *Br J Pharmacol* **135**(6): 1355-1362.
- Coupar, I M (1999). Characterization and tissue location of the neural adenosine receptor in the rat ileum. *Br J Pharmacol* **126**(5): 1269-1275.
- Coutinho, A H, Araújo-Jorge, T C, Persechini, P M & Coutinho-Silva, R (1998). Enhancement of P2Z-associated cell permeabilization during acute phase of Chagas' disease. *Drug Dev Res* **43**(38).
- Crack, B E, Pollard, C E, Beukers, M W, Roberts, S M, Hunt, S F, Ingall, A H, McKechnie, K C, AP, I J & Leff, P (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *Br J Pharmacol* **114**(2): 475-481.
- Cunha, R A (2001). Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. *Neurochem Res* **26**(8-9): 979-991.
- Cunha, R A & Sebastião, A M (1991). Extracellular metabolism of adenine nucleotides and adenosine in the innervated skeletal muscle of the frog. *Eur J Pharmacol* **197**(1): 83-92.
- Dale, G, Bonham, J R, Lowdon, P, Wagget, J, Rangecroft, L & Scott, D J (1979). Diagnostic value of rectal mucosal acetylcholinesterase levels in Hirschsprung's disease. *Lancet* **1**(8112): 347-349.
- Daly, J W (1982). Adenosine receptors: targets for future drugs. *J Med Chem* **25**(3): 197-207.
- De Man, J G, Seerden, T C, De Winter, B Y, Van Marck, E A, Herman, A G & Pelckmans, P A (2003). Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation. *Br J Pharmacol* **139**(1): 172-184.
- Delacretaz, E (2006). Clinical practice. Supraventricular tachycardia. *N Engl J Med* **354**(10): 1039-1051.

- Dinjens, W N, ten Kate, J, Wijnen, J T, van der Linden, E P, Beek, C J, Lenders, M H, Khan, P M & Bosman, F T (1989). Distribution of adenosine deaminase-complexing protein in murine tissues. *J Biol Chem* **264**(32): 19215-19220.
- Dixon, A K, Gubitz, A K, Sirinathsinghi, D J, Richardson, P J & Freeman, T C (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol* **118**(6): 1461-1468.
- Donaldson, S H, Picher, M & Boucher, R C (2002). Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. *Am J Respir Cell Mol Biol* **26**(2): 209-215.
- Dowdall, M J (1978). Adenine nucleotides in cholinergic transmission: presynaptic aspects. *J Physiol (Paris)* **74**(5): 497-501.
- Drury, A N & Szent-Gyorgyi, A (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol* **68**(3): 213-237.
- Duarte-Araújo, M, Nascimento, C, Alexandrina Timóteo, M, Magalhães-Cardoso, T & Correia-de-Sá, P (2004a). Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A_{2A} and extrajunctional inhibitory A₁ receptors. *Br J Pharmacol* **141**(6): 925-934.
- Duarte-Araújo, M, Nascimento, C, Timóteo, M A, Magalhães-Cardoso, M T & Correia-de-Sá, P (2009). Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on acetylcholine release from myenteric motoneurons. *Br J Pharmacol* **156**(3): 519-533.
- Duarte-Araújo, M, Timóteo, M A & Correia-de-Sá, P (2004b). Adenosine activating A_{2A}-receptors coupled to adenylate cyclase/cyclic AMP pathway downregulates nicotinic autoreceptor function at the rat myenteric nerve terminals. *Neurochem Int* **45**(5): 641-651.
- Eglen, R M, Reddy, H, Watson, N & Challiss, R A (1994). Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol Sci* **15**(4): 114-119.
- Ehlert, F J, Ostrom, R S & Sawyer, G W (1997). Subtypes of the muscarinic receptor in smooth muscle. *Life Sci* **61**(18): 1729-1740.
- Eltzschig, H K (2009). Adenosine: an old drug newly discovered. *Anesthesiology* **111**(4): 904-915.
- Evans, R J & Surprenant, A (1992). Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP. *Br J Pharmacol* **106**(2): 242-249.

- Facer, P, Knowles, C H, Tam, P K, Ford, A P, Dyer, N, Baecker, P A & Anand, P (2001). Novel capsaicin (VR1) and purinergic (P2X3) receptors in Hirschsprung's intestine. *J Pediatr Surg* **36**(11): 1679-1684.
- Fishman, P Y (2010). CF101 for the treatment of autoimmune inflammatory diseases: data from phase 2 clinical trials in patients with rheumatoid arthritis, psoriasis and dry eye syndrome. *Purinergic Signalling* **6, Supplement 1**: 1-162.
- Fitz, J G (2007). Regulation of cellular ATP release. *Trans Am Clin Climatol Assoc* **118**: 199-208.
- Fozard, J R, Baur, F & Wolber, C (2003). Antagonist pharmacology of adenosine A_{2B} receptors from rat, guinea pig and dog. *Eur J Pharmacol* **475**(1-3): 79-84.
- Franco, R, Hoyle, C H, Centelles, J J & Burnstock, G (1988). Degradation of adenosine by extracellular adenosine deaminase in the rat duodenum. *Gen Pharmacol* **19**(5): 679-681.
- Fredholm, B B (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* **14**(7): 1315-1323.
- Fredholm, B B, AP, I J, Jacobson, K A, Klotz, K N & Linden, J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**(4): 527-552.
- Fredholm, B B, Frenguelli, B G, Hills, R, Ijzerman, A P, Jacobson, K A, Linden, J, Schwabe, U & Stiles, G L (2010a). Adenosine receptors: A₁. IUPHAR database.
- Fredholm, B B, Frenguelli, B G, Hills, R, Ijzerman, A P, Jacobson, K A, Linden, J, Schwabe, U & Stiles, G L (2010b). Adenosine receptors: A_{2A}. IUPHAR database.
- Frenguelli, B G, Fredholm, B B, Hills, R, Ijzerman, A P, Jacobson, K A, Linden, J, Schwabe, U & Stiles, G L (2009). Adenosine receptors, introductory chapter. IUPHAR database.
- Furness, J B (2000). Types of neurons in the enteric nervous system. *J Auton Nerv Syst* **81**(1-3): 87-96.
- Furness, J B (2006). The enteric nervous system, *Blackwell Publishing*.
- Furness, J B (2009). Enteric Nervous System: Neural Circuits and Chemical Coding. Encyclopedia of Neuroscience. Binder, M D H, N.; Windhorst, U.; Hirsch, M.C. Heidelberg, Germany *Springer*: 1089-1095.
- Furness, J B & Costa, M (1973). The nervous release and the action of substances which affect intestinal muscle through neither adrenoreceptors nor cholinoreceptors. *Philos Trans R Soc Lond B Biol Sci* **265**(867): 123-133.
- Furness, J B, Costa, M & Keast, J R (1984). Choline acetyltransferase- and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. *Cell Tissue Res* **237**(2): 329-336.

- Furness, J B, Costa, M, Rokaeus, A, McDonald, T J & Brooks, B (1987). Galanin-immunoreactive neurons in the guinea-pig small intestine: their projections and relationships to other enteric neurons. *Cell Tissue Res* **250**(3): 607-615.
- Furness, J B, Nguyen, T V, Nurgali, K & Shimizu, Y (2009). The enteric nervous system and its extrinsic connections. Textbook of Gastroenterology. Yamada, T. Oxford, U.K., *Blackwell Publishing*: 15-39.
- Furuzono, S, Nakayama, S & Imaizumi, Y (2005). Purinergic modulation of pacemaker Ca^{2+} activity in interstitial cells of Cajal. *Neuropharmacology* **48**(2): 264-273.
- Gallego, D, Hernandez, P, Clave, P & Jimenez, M (2006). P2Y₁ receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. *Am J Physiol Gastrointest Liver Physiol* **291**(4): G584-594.
- Galligan, J J (1999). Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* **291**(1): 92-98.
- Galligan, J J (2002a). Pharmacology of synaptic transmission in the enteric nervous system. *Curr Opin Pharmacol* **2**(6): 623-629.
- Galligan, J J (2002b). Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil* **14**(6): 611-623.
- Galligan, J J & North, R A (1991). Opioid, 5-HT_{1A} and alpha 2 receptors localized to subsets of guinea-pig myenteric neurons. *J Auton Nerv Syst* **32**(1): 1-11.
- Galligan, J J & North, R A (2004). Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**: 64-70.
- Gendron, F P, Benrezzak, O, Krugh, B W, Kong, Q, Weisman, G A & Beaudoin, A R (2002). Purine signaling and potential new therapeutic approach: possible outcomes of NTPDase inhibition. *Curr Drug Targets* **3**(3): 229-245.
- Gershon, M (1999). The Second Brain: A Groundbreaking New Understanding of Nervous Disorders of the Stomach and Intestin, *Harper Collins Publishers*.
- Gerthoffer, W T (2005). Signal-transduction pathways that regulate visceral smooth muscle function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles. *Am J Physiol Gastrointest Liver Physiol* **288**(5): G849-853.
- Gever, J R, Cockayne, D A, Dillon, M P, Burnstock, G & Ford, A P (2006). Pharmacology of P2X channels. *Pflugers Arch* **452**(5): 513-537.
- Giaroni, C, Knight, G E, Ruan, H Z, Glass, R, Bardini, M, Lecchini, S, Frigo, G & Burnstock, G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**(8): 1313-1323.

- Ginsborg, B L & Hirst, G D (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *J Physiol* **224**(3): 629-645.
- Giron, M C, Bin, A, Brun, P, Etteri, S, Bolego, C, Florio, C & Gaion, R M (2008). Cyclic AMP in rat ileum: evidence for the presence of an extracellular cyclic AMP-adenosine pathway. *Gastroenterology* **134**(4): 1116-1126.
- Giusti, G (1974). Adenosine deaminase. Methods of Enzymatic Analysis. Bergmeyer, H U. New York, *Academic Press*. **II**: 1092– 1099.
- Golembiowska, K, White, T D & Sawynok, J (1996). Adenosine kinase inhibitors augment release of adenosine from spinal cord slices. *Eur J Pharmacol* **307**(2): 157-162.
- Gomez-Pinilla, P J, Gibbons, S J, Bardsley, M R, Lorincz, A, Pozo, M J, Pasricha, P J, Van de Rijn, M, West, R B, Sarr, M G, Kendrick, M L, Cima, R R, Dozois, E J, Larson, D W, Ordog, T & Farrugia, G (2009). Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. *Am J Physiol Gastrointest Liver Physiol* **296**(6): G1370-1381.
- Gonçalves, J & Queiróz, G (1993). Facilitatory and inhibitory modulation by endogenous adenosine of noradrenaline release in the epididymal portion of rat vas deferens. *Naunyn Schmiedebergs Arch Pharmacol* **348**(4): 367-371.
- Gonçalves, J & Queiróz, G (2008). Presynaptic Adenosine and P2Y Receptors. Pharmacology of Neurotransmitter Release. Sudhof, T C S, K. Berlin *Springer-Verlag*: 340-361.
- Govindarajan, R, Bakken, A H, Hudkins, K L, Lai, Y, Casado, F J, Pastor-Anglada, M, Tse, C M, Hayashi, J & Unadkat, J D (2007). In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am J Physiol Regul Integr Comp Physiol* **293**(5): R1809-1822.
- Goyal, R K (1988). Identification, localization and classification of muscarinic receptor subtypes in the gut. *Life Sci* **43**(26): 2209-2220.
- Goyal, R K & Hirano, I (1996). The enteric nervous system. *N Engl J Med* **334**(17): 1106-1115.
- Griffin, M T, Hsu, J C, Shehnaz, D & Ehlert, F J (2003). Comparison of the pharmacological antagonism of M₂ and M₃ muscarinic receptors expressed in isolation and in combination. *Biochem Pharmacol* **65**(8): 1227-1241.
- Griffith, D A & Jarvis, S M (1996). Nucleoside and nucleobase transport systems of mammalian cells. *Biochim Biophys Acta* **1286**(3): 153-181.

- Grobben, B, Anciaux, K, Roymans, D, Stefan, C, Bollen, M, Esmans, E L & Slegers, H (1999). An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. *J Neurochem* **72**(2): 826-834.
- Gustafsson, L E, Wiklund, N P, Lundin, J & Hedqvist, P (1985). Characterization of pre- and post-junctional adenosine receptors in guinea-pig ileum. *Acta Physiol Scand* **123**(2): 195-203.
- Hansen, M B (2003a). The enteric nervous system I: organisation and classification. *Pharmacol Toxicol* **92**(3): 105-113.
- Hansen, M B (2003b). The enteric nervous system II: gastrointestinal functions. *Pharmacol Toxicol* **92**(6): 249-257.
- Harrington, A M, Hutson, J M & Southwell, B R (2007). Immunohistochemical localisation of cholinergic muscarinic receptor subtype 1 (M1r) in the guinea pig and human enteric nervous system. *J Chem Neuroanat* **33**(4): 193-201.
- Harrington, A M, Hutson, J M & Southwell, B R (2008). Immunohistochemical localisation of pre-synaptic muscarinic receptor subtype-2 (M2r) in the enteric nervous system of guinea-pig ileum. *Cell Tissue Res* **332**(1): 37-48.
- Harrington, A M, Hutson, J M & Southwell, B R (2010). Cholinergic neurotransmission and muscarinic receptors in the enteric nervous system. *Prog Histochem Cytochem* **44**(4): 173-202.
- Hart, M L, Much, C, Gorzolla, I C, Schittenhelm, J, Kloor, D, Stahl, G L & Eltzschig, H K (2008). Extracellular adenosine production by ecto-5'-nucleotidase protects during murine hepatic ischemic preconditioning. *Gastroenterology* **135**(5): 1739-1750 e1733.
- Hebb, C O, Krnjevic, K & Silver, A (1964). Acetylcholine and Choline Acetyltransferase in the Diaphragm of the Rat. *J Physiol* **171**: 504-513.
- Heinemann, A, Shahbazian, A, Bartho, L & Holzer, P (1999). Different receptors mediating the inhibitory action of exogenous ATP and endogenously released purines on guinea-pig intestinal peristalsis. *Br J Pharmacol* **128**(2): 313-320.
- Hopkins, S V & Goldie, R G (1971). A species difference in the uptake of adenosine by heart. *Biochem Pharmacol* **20**(12): 3359-3365.
- Howe, D G, Clarke, C M, Yan, H, Willis, B S, Schneider, D A, McKnight, G S & Kapur, R P (2006). Inhibition of protein kinase A in murine enteric neurons causes lethal intestinal pseudo-obstruction. *J Neurobiol* **66**(3): 256-272.
- Hox, J J (2010). Multilevel Analysis: Techniques and Applications, *Routledge Academic*.

- Hu, H Z, Gao, N, Lin, Z, Gao, C, Liu, S, Ren, J, Xia, Y & Wood, J D (2001). P2X7 receptors in the enteric nervous system of guinea-pig small intestine. *J Comp Neurol* **440**(3): 299-310.
- Hu, H Z, Gao, N, Zhu, M X, Liu, S, Ren, J, Gao, C, Xia, Y & Wood, J D (2003). Slow excitatory synaptic transmission mediated by P2Y₁ receptors in the guinea-pig enteric nervous system. *J Physiol* **550**(Pt 2): 493-504.
- Hussl, S & Boehm, S (2006). Functions of neuronal P2Y receptors. *Pflugers Arch* **452**(5): 538-551.
- Irvine, F, Pyne, N J & Houslay, M D (1986). The phorbol ester TPA inhibits cyclic AMP phosphodiesterase activity in intact hepatocytes. *FEBS Lett* **208**(2): 455-459.
- Jacobson, K A (2009). Introduction to Adenosine Receptors as Therapeutic Targets. Adenosine Receptors in Health and Disease. Wilson, C N M, S.J. Berlin *Springer-Verlag*: 4-38.
- Jacobson, K A & Gao, Z G (2006). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5**(3): 247-264.
- Jarvis, M F & Khakh, B S (2009). ATP-gated P2X cation-channels. *Neuropharmacology* **56**(1): 208-215.
- Johnson, P J, Bornstein, J C, Yuan, S Y & Furness, J B (1996). Analysis of contributions of acetylcholine and tachykinins to neuro-neuronal transmission in motility reflexes in the guinea-pig ileum. *Br J Pharmacol* **118**(4): 973-983.
- Kadowaki, M, Nagakura, Y, Tokita, K, Hanaoka, K & Tomoi, M (2003). Adenosine A₁ receptor blockade reverses experimental postoperative ileus in rat colon. *Eur J Pharmacol* **458**(1-2): 197-200.
- Kadowaki, M, Takeda, M, Tokita, K, Hanaoka, K & Tomoi, M (2000). Molecular identification and pharmacological characterization of adenosine receptors in the guinea-pig colon. *Br J Pharmacol* **129**(5): 871-876.
- Katsuragi, T, Shirakabe, K, Soejima, O, Tokunaga, T, Matsuo, K, Sato, C & Furukawa, T (1993). Possible transsynaptic cholinergic neuromodulation by ATP released from ileal longitudinal muscles of guinea pigs. *Life Sci* **53**(11): 911-918.
- Kegel, B, Braun, N, Heine, P, Maliszewski, C R & Zimmermann, H (1997). An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. *Neuropharmacology* **36**(9): 1189-1200.
- Keiding, R, Horder, M, Gerhardt, W, Pitkenen, E, Tenhunen, R, Stromme, J H, Theordersen, L, Waldenstrom, J, Tryding, N & Westlund, L (1974). Recommended methods for the determination of four enzymes in the blood. *Scand. J. Clin. Lab. Invest.* **33**(4): 291-306.

- Kilbinger, H & Nafziger, M (1985). Two types of neuronal muscarine receptors modulating acetylcholine release from guinea-pig myenteric plexus. *Naunyn Schmiedebergs Arch Pharmacol* **328**(3): 304-309.
- Kilbinger, H & Wessler, I (1980a). Pre- and postsynaptic effects of muscarinic agonists in the guinea-pig ileum. *Naunyn Schmiedebergs Arch Pharmacol* **314**(3): 259-266.
- Kilbinger, H & Wessler, I (1980b). Inhibition by acetylcholine of the stimulation-evoked release of [3H]acetylcholine from the guinea-pig myenteric plexus. *Neuroscience* **5**(7): 1331-1340.
- Kilbinger, H & Wessler, I (1983). The variation of acetylcholine release from myenteric neurones with stimulation frequency and train length. Role of presynaptic muscarine receptors. *Naunyn Schmiedebergs Arch Pharmacol* **324**(2): 130-133.
- Kimball, B C & Mulholland, M W (1995). Neuroligands evoke calcium signaling in cultured myenteric neurons. *Surgery* **118**(2): 162-169; discussion 170.
- King, A E, Ackley, M A, Cass, C E, Young, J D & Baldwin, S A (2006). Nucleoside transporters: from scavengers to novel therapeutic targets. *Trends Pharmacol Sci* **27**(8): 416-425.
- Kolachala, V L, Bajaj, R, Chalasani, M & Sitaraman, S V (2008). Purinergic receptors in gastrointestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* **294**(2): G401-410.
- Kukulski, F & Komoszynski, M (2003). Purification and characterization of NTPDase1 (ecto-apyrase) and NTPDase2 (ecto-ATPase) from porcine brain cortex synaptosomes. *Eur J Biochem* **270**(16): 3447-3454.
- Kukulski, F, Levesque, S A, Lavoie, E G, Lecka, J, Bigonnesse, F, Knowles, A F, Robson, S C, Kirley, T L & Sevigny, J (2005). Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal* **1**(2): 193-204.
- Kurjak, M, Sattler, D, Schusdziarra, V & Allescher, H D (1999). Characterization of prejunctional and postjunctional muscarinic receptors of the ascending reflex contraction in rat ileum. *J Pharmacol Exp Ther* **290**(2): 893-900.
- Latini, S & Pedata, F (2001). Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem* **79**(3): 463-484.
- Lazarowski, E R, Boucher, R C & Harden, T K (2003). Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* **64**(4): 785-795.
- Lazarowski, E R, Homolya, L, Boucher, R C & Harden, T K (1997). Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. *J Biol Chem* **272**(33): 20402-20407.

- Le Hir, M, Gandhi, R & Dubach, U C (1989). Purification and properties of a 5'-nucleotidase from rat renal membranes. *Enzyme* **41**(2): 87-93.
- Lecci, A, Santicioli, P & Maggi, C A (2002). Pharmacology of transmission to gastrointestinal muscle. *Curr Opin Pharmacol* **2**(6): 630-641.
- Leclerc, P G & Lefebvre, R A (2002). Characterization of pre- and postsynaptic muscarinic receptors in circular muscle of pig gastric fundus. *Br J Pharmacol* **135**(5): 1245-1254.
- Lecomte, M J, De Gois, S, Guerci, A, Ravassard, P, Faucon Biguet, N, Mallet, J & Berrard, S (2005). Differential expression and regulation of the high-affinity choline transporter CHT1 and choline acetyltransferase in neurons of superior cervical ganglia. *Mol Cell Neurosci* **28**(2): 303-313.
- Lee, J J, Talubmook, C & Parsons, M E (2001). Activation of presynaptic A₁-receptors by endogenous adenosine inhibits acetylcholine release in the guinea-pig ileum. *J Auton Pharmacol* **21**(1): 29-38.
- Libert, F, Parmentier, M, Lefort, A, Dinsart, C, Van Sande, J, Maenhaut, C, Simons, M J, Dumont, J E & Vassart, G (1989). Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* **244**(4904): 569-572.
- Linden, J (2005). Adenosine in tissue protection and tissue regeneration. *Mol Pharmacol* **67**(5): 1385-1387.
- Lips, K S, Pfeil, U, Haberberger, R V & Kummer, W (2002). Localisation of the high-affinity choline transporter-1 in the rat skeletal motor unit. *Cell Tissue Res* **307**(3): 275-280.
- Llaudet, E, Hatz, S, Droniou, M & Dale, N (2005). Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal Chem* **77**(10): 3267-3273.
- Lloyd, H G & Fredholm, B B (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int* **26**(4): 387-395.
- Lomax, A E & Furness, J B (2000). Neurochemical classification of enteric neurons in the guinea-pig distal colon. *Cell Tissue Res* **302**(1): 59-72.
- Londos, C, Cooper, D M & Wolff, J (1980). Subclasses of external adenosine receptors. *Proc Natl Acad Sci U S A* **77**(5): 2551-2554.
- Lu, H, Chen, C & Klaassen, C (2004). Tissue distribution of concentrative and equilibrative nucleoside transporters in male and female rats and mice. *Drug Metab Dispos* **32**(12): 1455-1461.
- Lu, W, Reigada, D, Sevigny, J & Mitchell, C H (2007). Stimulation of the P2Y₁ receptor up-regulates nucleoside-triphosphate diphosphohydrolase-1 in human retinal pigment epithelial cells. *J Pharmacol Exp Ther* **323**(1): 157-164.

- Lukas, R J, Changeux, J P, Le Novere, N, Albuquerque, E X, Balfour, D J, Berg, D K, Bertrand, D, Chiappinelli, V A, Clarke, P B, Collins, A C, Dani, J A, Grady, S R, Kellar, K J, Lindstrom, J M, Marks, M J, Quik, M, Taylor, P W & Wonnacott, S (1999). International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. *Pharmacol Rev* **51**(2): 397-401.
- Mabley, J G, Pacher, P, Liaudet, L, Soriano, F G, Hasko, G, Marton, A, Szabo, C & Salzman, A L (2003). Inosine reduces inflammation and improves survival in a murine model of colitis. *Am J Physiol Gastrointest Liver Physiol* **284**(1): G138-144.
- Magalhães-Cardoso, M T, Pereira, M F, Oliveira, L, Ribeiro, J A, Cunha, R A & Correia-de-Sá, P (2003). Ecto-AMP deaminase blunts the ATP-derived adenosine A_{2A} receptor facilitation of acetylcholine release at rat motor nerve endings. *J Physiol* **549**(Pt 2): 399-408.
- Mandl, P & Kiss, J P (2007). Role of presynaptic nicotinic acetylcholine receptors in the regulation of gastrointestinal motility. *Brain Res Bull* **72**(4-6): 194-200.
- Mandl, P, Kiss, J P & Vizi, E S (2003). Functional neurochemical evidence for the presence of presynaptic nicotinic acetylcholine receptors at the terminal region of myenteric motoneurons: a study with epibatidine. *Neurochem Res* **28**(3-4): 407-412.
- Mansfield, K J, Mitchelson, F J, Moore, K H & Burcher, E (2003). Muscarinic receptor subtypes in the human colon: lack of evidence for atypical subtypes. *Eur J Pharmacol* **482**(1-3): 101-109.
- Marquardt, D L, Gruber, H E & Wasserman, S I (1984). Adenosine release from stimulated mast cells. *Proc Natl Acad Sci U S A* **81**(19): 6192-6196.
- Matsui, M, Griffin, M T, Shehnaz, D, Taketo, M M & Ehlert, F J (2003). Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M₂ receptor knockout mice. *J Pharmacol Exp Ther* **305**(1): 106-113.
- Matsui, M, Motomura, D, Karasawa, H, Fujikawa, T, Jiang, J, Komiya, Y, Takahashi, S & Taketo, M M (2000). Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M₃ subtype. *Proc Natl Acad Sci U S A* **97**(17): 9579-9584.
- Matsuoka, I & Ohkubo, S (2004). ATP- and adenosine-mediated signaling in the central nervous system: adenosine receptor activation by ATP through rapid and localized generation of adenosine by ecto-nucleotidases. *J Pharmacol Sci* **94**(2): 95-99.
- McConalogue, K & Furness, J B (1994). Gastrointestinal neurotransmitters. *Baillieres Clin Endocrinol Metab* **8**(1): 51-76.

- Milusheva, E, Sperlagh, B, Kiss, B, Szporny, L, Pasztor, E, Papasova, M & Vizi, E S (1990). Inhibitory effect of hypoxic condition on acetylcholine release is partly due to the effect of adenosine released from the tissue. *Brain Res Bull* **24**(3): 369-373.
- Mitsuya, M, Mase, T, Tsuchiya, Y, Kawakami, K, Hattori, H, Kobayashi, K, Ogino, Y, Fujikawa, T, Satoh, A, Kimura, T, Noguchi, K, Ohtake, N & Tomimoto, K (1999). J-104129, a novel muscarinic M₃ receptor antagonist with high selectivity for M₃ over M₂ receptors. *Bioorg Med Chem* **7**(11): 2555-2567.
- Molenaar, P C & Polak, R L (1970). Stimulation by atropine of acetylcholine release and synthesis in cortical slices from rat brain. *Br J Pharmacol* **40**(3): 406-417.
- Moneta, N A, McDonald, T J & Cook, M A (1997). Endogenous adenosine inhibits evoked substance P release from perfused networks of myenteric ganglia. *Am J Physiol* **272**(1 Pt 1): G38-45.
- Munkonda, M N, Kauffenstein, G, Kukulski, F, Levesque, S A, Legendre, C, Pelletier, J, Lavoie, E G, Lecka, J & Seigny, J (2007). Inhibition of human and mouse plasma membrane bound NTPDases by P₂ receptor antagonists. *Biochem Pharmacol* **74**(10): 1524-1534.
- Murthy, K S (2006). Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol* **68**: 345-374.
- Murthy, K S & Makhlouf, G M (1997). Differential coupling of muscarinic M₂ and M₃ receptors to adenylyl cyclases V/VI in smooth muscle. Concurrent M₂-mediated inhibition via G α i3 and M₃-mediated stimulation via G β γ q. *J Biol Chem* **272**(34): 21317-21324.
- Murthy, K S, Zhou, H, Grider, J R, Brautigan, D L, Eto, M & Makhlouf, G M (2003). Differential signalling by muscarinic receptors in smooth muscle: M₂-mediated inactivation of myosin light chain kinase via Gi3, Cdc42/Rac1 and p21-activated kinase 1 pathway, and M₃-mediated MLC20 (20 kDa regulatory light chain of myosin II) phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway. *Biochem J* **374**(Pt 1): 145-155.
- Nagy, J I, Yamamoto, T, Uemura, H & Schrader, W P (1996). Adenosine deaminase in rodent median eminence: detection by antibody to the mouse enzyme and co-localization with adenosine deaminase-complexing protein (CD26). *Neuroscience* **73**(2): 459-471.
- Naito, Y & Lowenstein, J M (1985). 5'-Nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. *Biochem J* **226**(3): 645-651.

- Nakamura, M, Mie, M, Funabashi, H, Yamamoto, K, Ando, J & Kobatake, E (2006). Cell-surface-localized ATP detection with immobilized firefly luciferase. *Anal Biochem* **352**(1): 61-67.
- Nakata, H, Yoshioka, K, Kamiya, T, Tsuga, H & Oyanagi, K (2005). Functions of heteromeric association between adenosine and P2Y receptors. *J Mol Neurosci* **26**(2-3): 233-238.
- Namiot, Z, Stasiewicz, J, Marcinkiewicz, M & Gorski, J (1992). Adenosine deaminase activity in the human duodenal mucosa in relation to gastric acid secretion. *J Physiol Pharmacol* **43**(2): 149-152.
- Nicholls, J, Brownhill, V R & Hourani, S M (1996). Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *Br J Pharmacol* **117**(1): 170-174.
- Nicke, A, Kerschensteiner, D & Soto, F (2005). Biochemical and functional evidence for heteromeric assembly of P2X1 and P2X4 subunits. *J Neurochem* **92**(4): 925-933.
- Nitahara, K, Kittel, A, Liang, S D & Vizi, E S (1995). A₁-receptor-mediated effect of adenosine on the release of acetylcholine from the myenteric plexus: role and localization of ecto-ATPase and 5'-nucleotidase. *Neuroscience* **67**(1): 159-168.
- North, R A (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82**(4): 1013-1067.
- North, R A, Slack, B E & Surprenant, A (1985). Muscarinic M₁ and M₂ receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. *J Physiol* **368**: 435-452.
- O' Donnell, A M & Puri, P (2008). Deficiency of purinergic P2Y receptors in aganglionic intestine in Hirschsprung's disease. *Pediatr Surg Int* **24**(1): 77-80.
- O'Keeffe, M G, Thorne, P R, Housley, G D, Robson, S C & Vlajkovic, S M (2010). Distribution of NTPDase5 and NTPDase6 and the regulation of P2Y receptor signalling in the rat cochlea. *Purinergic Signal* **6**(2): 249-261.
- Obata, T, Kubota, S & Yamanaka, Y (2001). Histamine increases interstitial adenosine concentration via activation of ecto-5'-nucleotidase in rat hearts in vivo. *J Pharmacol Exp Ther* **298**(1): 71-76.
- Obata, T & Yamanaka, Y (1998). Adenosine deaminase activity in rat intestine: assay with a microdialysis technique. *Comp Biochem Physiol A Mol Integr Physiol* **119**(1): 309-313.
- Ohta, T, Kubota, A, Murakami, M, Otsuguro, K & Ito, S (2005). P2X2 receptors are essential for [Ca²⁺]_i increases in response to ATP in cultured rat myenteric neurons. *Am J Physiol Gastrointest Liver Physiol* **289**(5): G935-948.

- Okuda, T, Haga, T, Kanai, Y, Endou, H, Ishihara, T & Katsura, I (2000). Identification and characterization of the high-affinity choline transporter. *Nat Neurosci* **3**(2): 120-125.
- Oliveira, L & Correia-de-Sá, P (2005). Protein kinase A and Ca(v)1 (L-Type) channels are common targets to facilitatory adenosine A_{2A} and muscarinic M₁ receptors on rat motoneurons. *Neurosignals* **14**(5): 262-272.
- Oliveira, L, Timóteo, M A & Correia-de-Sá, P (2002). Modulation by adenosine of both muscarinic M₁-facilitation and M₂-inhibition of [³H]-acetylcholine release from the rat motor nerve terminals. *Eur J Neurosci* **15**(11): 1728-1736.
- Oue, T, Yoneda, A, Shima, H & Puri, P (2000). Muscarinic acetylcholine receptor expression in aganglionic bowel. *Pediatr Surg Int* **16**(4): 267-271.
- Pak, M A, Haas, H L, Decking, U K & Schrader, J (1994). Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology* **33**(9): 1049-1053.
- Paton, W D & Vizi, E S (1969). The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *Br J Pharmacol* **35**(1): 10-28.
- Paton, W D & Zar, M A (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J Physiol* **194**(1): 13-33.
- Pedata, F, Melani, A, Pugliese, A M, Coppi, E, Cipriani, S & Traini, C (2007). The role of ATP and adenosine in the brain under normoxic and ischemic conditions. *Purinergic Signal* **3**(4): 299-310.
- Pesce, A, McKay, R H, Stolzenbach, F, Cahn, R D & Kaplan, N O (1964). The Comparative Enzymology of Lactic Dehydrogenases. I. Properties of the Crystalline Beef and Chicken Enzymes. *J Biol Chem* **239**: 1753-1761.
- Podgorska, M, Kocbuch, K & Pawelczyk, T (2005). Recent advances in studies on biochemical and structural properties of equilibrative and concentrative nucleoside transporters. *Acta Biochim Pol* **52**(4): 749-758.
- Potter, L T (1970). Synthesis, storage and release of [¹⁴C]acetylcholine in isolated rat diaphragm muscles. *J Physiol* **206**(1): 145-166.
- Pradidarcheep, W, Stallen, J, Labruyere, W T, Dabhoiwala, N F, Michel, M C & Lamers, W H (2009). Lack of specificity of commercially available antisera against muscarinergic and adrenergic receptors. *Naunyn Schmiedeberg's Arch Pharmacol* **379**(4): 397-402.

- Puffinbarger, N K, Hansen, K R, Resta, R, Laurent, A B, Knudsen, T B, Madara, J L & Thompson, L F (1995). Production and characterization of multiple antigenic peptide antibodies to the adenosine A_{2B} receptor. *Mol Pharmacol* **47**(6): 1126-1132.
- Ralevic, V & Burnstock, G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**(3): 413-492.
- Rang, H P (1964). Stimulant Actions of Volatile Anaesthetics on Smooth Muscle. *Br J Pharmacol Chemother* **22**: 356-365.
- Redegeld, F A, Caldwell, C C & Sitkovsky, M V (1999). Ecto-protein kinases: ecto-domain phosphorylation as a novel target for pharmacological manipulation? *Trends Pharmacol Sci* **20**(11): 453-459.
- Reese, J H & Cooper, J R (1982). Modulation of the release of acetylcholine from ileal synaptosomes by adenosine and adenosine 5'-triphosphate. *J Pharmacol Exp Ther* **223**(3): 612-616.
- Ren, J & Bertrand, P P (2008). Purinergic receptors and synaptic transmission in enteric neurons. *Purinergic Signal* **4**(3): 255-266.
- Ren, J, Bian, X, DeVries, M, Schnegelsberg, B, Cockayne, D A, Ford, A P & Galligan, J J (2003). P2X₂ subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. *J Physiol* **552**(Pt 3): 809-821.
- Reutershan, J, Vollmer, I, Stark, S, Wagner, R, Ngamsri, K C & Eltzschig, H K (2009). Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J* **23**(2): 473-482.
- Ribeiro, J A & Walker, J (1975). The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. *Br J Pharmacol* **54**(2): 213-218.
- Riordan, J R & Slavik, M (1974). Interactions of lectins with membrane glycoproteins. Effects of concanavalin A on 5'-nucleotidase. *Biochim Biophys Acta* **373**(3): 356-360.
- Robson, S C, Seigny, J & Zimmermann, H (2006). The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal* **2**(2): 409-430.
- Rosenberg, P A & Dichter, M A (1989). Extracellular cAMP accumulation and degradation in rat cerebral cortex in dissociated cell culture. *J Neurosci* **9**(8): 2654-2663.
- Ruan, H Z & Burnstock, G (2005). The distribution of P2X₅ purinergic receptors in the enteric nervous system of mouse. *Cell Tissue Res* **319**(2): 191-200.
- Ruhl, A (2005). Glial cells in the gut. *Neurogastroenterol Motil* **17**(6): 777-790.

- Rybaczky, L, Rozmiarek, A, Circle, K, Grants, I, Needleman, B, Wunderlich, J E, Huang, K & Christofi, F L (2009b). New bioinformatics approach to analyze gene expressions and signaling pathways reveals unique purine gene dysregulation profiles that distinguish between CD and UC. *Inflamm Bowel Dis* **15**(7): 971-984.
- Saitoh, N, Fujimoto, R, Ishii, T, Nishio, H, Takeuchi, T & Hata, F (1997). Muscarinic autoinhibition and modulatory role of protein kinase C in acetylcholine release from the myenteric plexus of guinea pig ileum. *Jpn J Pharmacol* **74**(2): 155-163.
- Sakamoto, J, Watanabe, T, Teramukai, S, Akiyama, S, Morimoto, T, Takagi, H, Nakazato, H, Ueda, R & Takahashi, T (1993). Distribution of adenosine deaminase binding protein in normal and malignant tissues of the gastrointestinal tract studied by monoclonal antibodies. *J Surg Oncol* **52**(2): 124-134.
- Sakamoto, T, Unno, T, Kitazawa, T, Taneike, T, Yamada, M, Wess, J, Nishimura, M & Komori, S (2007). Three distinct muscarinic signalling pathways for cationic channel activation in mouse gut smooth muscle cells. *J Physiol* **582**(Pt 1): 41-61.
- Sanders, K M, Koh, S D & Ward, S M (2006). Interstitial cells of cajal as pacemakers in the gastrointestinal tract. *Annu Rev Physiol* **68**: 307-343.
- Sanders, K M & Ward, S M (2006). Interstitial cells of Cajal: a new perspective on smooth muscle function. *J Physiol* **576**(Pt 3): 721-726.
- Sanders, K M & Ward, S M (2007). Kit mutants and gastrointestinal physiology. *J Physiol* **578**(Pt 1): 33-42.
- Sawyer, G W & Ehlert, F J (1998). Contractile roles of the M₂ and M₃ muscarinic receptors in the guinea pig colon. *J Pharmacol Exp Ther* **284**(1): 269-277.
- Sawynok, J & Liu, X J (2003). Adenosine in the spinal cord and periphery: release and regulation of pain. *Prog Neurobiol* **69**(5): 313-340.
- Schemann, M, Sann, H, Schaaf, C & Mader, M (1993). Identification of cholinergic neurons in enteric nervous system by antibodies against choline acetyltransferase. *Am J Physiol* **265**(5 Pt 1): G1005-1009.
- Schepp, C P & Reutershan, J (2008). Bench-to-bedside review: adenosine receptors-promising targets in acute lung injury? *Crit Care* **12**(5): 226.
- Schwiebert, E M & Zsembery, A (2003). Extracellular ATP as a signaling molecule for epithelial cells. *Biochim Biophys Acta* **1615**(1-2): 7-32.
- Sebastião, A M & Ribeiro, J A (2000). Fine-tuning neuromodulation by adenosine. *Trends Pharmacol Sci* **21**(9): 341-346.
- Sebastião, A M & Ribeiro, J A (2009). Adenosine Receptors and the Central Nervous System. *Adenosine Receptors in Health and Disease*. Wilson, C N M, S.J. Berlin Springer-Verlag 471 - 534.

- Sevigny, J, Grondin, G, Gendron, F P, Roy, J & Beaudoin, A R (1998). Demonstration and immunolocalization of ATP diphosphohydrolase in the pig digestive system. *Am J Physiol* **275**(3 Pt 1): G473-482.
- Simeone, D M, Kimball, B C & Mulholland, M W (1996). Acetylcholine-induced calcium signaling associated with muscarinic receptor activation in cultured myenteric neurons. *J Am Coll Surg* **182**(6): 473-481.
- Sinclair, C J, Shepel, P N, Geiger, J D & Parkinson, F E (2000). Stimulation of nucleoside efflux and inhibition of adenosine kinase by A₁ adenosine receptor activation. *Biochem Pharmacol* **59**(5): 477-483.
- So, K Y, Kim, S H, Sohn, H M, Choi, S J, Parajuli, S P, Choi, S, Yeum, C H, Yoon, P J & Jun, J Y (2009). Carbachol regulates pacemaker activities in cultured interstitial cells of Cajal from the mouse small intestine. *Mol Cells* **27**(5): 525-531.
- Soejima, O, Katsuragi, T & Furukawa, T (1993). Opposite modulation by muscarinic M1 and M3 receptors of acetylcholine release from guinea pig ileum as measured directly. *Eur J Pharmacol* **249**(1): 1-6.
- Sperlagh, B, Heinrich, A & Csölle, C (2007a). P2 receptor-mediated modulation of neurotransmitter release-an update. *Purinergic Signal* **3**(4): 269-284.
- Sperlagh, B, Szabo, G, Erdelyi, F, Baranyi, M & Vizi, E S (2003). Homo- and heteroexchange of adenine nucleotides and nucleosides in rat hippocampal slices by the nucleoside transport system. *Br J Pharmacol* **139**(3): 623-633.
- Sperlagh, B & Vizi, E S (2007b). Extracellular interconversion of nucleotides reveals an ecto-adenylate kinase activity in the rat hippocampus. *Neurochem Res* **32**(11): 1978-1989.
- Stead, R H, Dixon, M F, Bramwell, N H, Riddell, R H & Bienenstock, J (1989). Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. *Gastroenterology* **97**(3): 575-585.
- Stefan, C, Jansen, S & Bollen, M (2006). Modulation of purinergic signaling by NPP-type ectophosphodiesterases. *Purinergic Signal* **2**(2): 361-370.
- Stefanovic, V, Mandel, P & Rosenberg, A (1975). Concanavalin A inhibition of ecto-5'-nucleotidase of intact cultured C6 glioma cells. *J Biol Chem* **250**(17): 7081-7083.
- Stolzenbach, F E & Kaplan, N O (1976). Immobilization of lactic dehydrogenase. *Methods Enzymol* **44**: 929-936.
- Strater, N (2006). Ecto-5'-nucleotidase: Structure function relationships. *Purinergic Signal* **2**(2): 343-350.

- Strohmeier, G R, Lencer, W I, Patapoff, T W, Thompson, L F, Carlson, S L, Moe, S J, Carnes, D K, Mrsny, R J & Madara, J L (1997). Surface expression, polarization, and functional significance of CD73 in human intestinal epithelia. *J Clin Invest* **99**(11): 2588-2601.
- Suzuki, T, Namba, K, Tsuga, H & Nakata, H (2006). Regulation of pharmacology by hetero-oligomerization between A₁ adenosine receptor and P2Y₂ receptor. *Biochem Biophys Res Commun* **351**(2): 559-565.
- Szentpali, K, Kaszaki, J, Tiszlavicz, L, Lazar, G, Balogh, A & Boros, M (2001). Bile-induced adenosine triphosphate depletion and mucosal damage during reflux esophagitis. *Scand J Gastroenterol* **36**(5): 459-466.
- Takagi, H, Kojima, M, Nagata, M & Kuromi, H (1970). On the site of action of hemicholinium-3 at the rat phrenic nerve-diaphragm preparation with special reference to its multiple presynaptic actions. *Neuropharmacology* **9**(4): 359-367.
- Takeuchi, T, Fujinami, K, Goto, H, Fujita, A, Taketo, M M, Manabe, T, Matsui, M & Hata, F (2005). Roles of M₂ and M₄ muscarinic receptors in regulating acetylcholine release from myenteric neurons of mouse ileum. *J Neurophysiol* **93**(5): 2841-2848.
- Takeuchi, T, Tanaka, K, Nakajima, H, Matsui, M & Azuma, Y T (2007). M₂ and M₃ muscarinic receptors are involved in enteric nerve-mediated contraction of the mouse ileum: Findings obtained with muscarinic-receptor knockout mouse. *Am J Physiol Gastrointest Liver Physiol* **292**(1): G154-164.
- Takeuchi, T, Toyoshima, M, Mukai, K, Hagi, K, Matsui, M, Nakajima, H, Azuma, Y T & Hata, F (2006). Involvement of M₂ muscarinic receptors in relaxant response of circular muscle of mouse gastric antrum. *Neurogastroenterol Motil* **18**(3): 226-233.
- Thompson, L F, Eltzschig, H K, Ibla, J C, Van De Wiele, C J, Resta, R, Morote-Garcia, J C & Colgan, S P (2004). Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med* **200**(11): 1395-1405.
- Thorn, J A & Jarvis, S M (1996). Adenosine transporters. *Gen Pharmacol* **27**(4): 613-620.
- Timmermans, J P, Hens, J & Adriaensen, D (2001). Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. *Anat Rec* **262**(1): 71-78.
- Tobin, G, Giglio, D & Lundgren, O (2009). Muscarinic receptor subtypes in the alimentary tract. *J Physiol Pharmacol* **60**(1): 3-21.
- Tomaru, A, Ina, Y, Kishibayashi, N & Karasawa, A (1995). Excitation and inhibition via adenosine receptors of the twitch response to electrical stimulation in isolated guinea pig ileum. *Jpn J Pharmacol* **69**(4): 429-433.

- Turnay, J, Olmo, N, Navarro, J M, Gavilanes, J G & Lizarbe, M A (1992). Isolation and characterization of the ecto-5'-nucleotidase from a rat glioblastoma cell line. *Mol Cell Biochem* **117**(1): 23-33.
- Uchiyama, T & Chess-Williams, R (2004). Muscarinic receptor subtypes of the bladder and gastrointestinal tract. *J Smooth Muscle Res* **40**(6): 237-247.
- Ungerer, J P, Oosthuizen, H M, Bissbort, S H & Vermaak, W J (1992). Serum adenosine deaminase: isoenzymes and diagnostic application. *Clin Chem* **38**(7): 1322-1326.
- Unno, T, Matsuyama, H, Sakamoto, T, Uchiyama, M, Izumi, Y, Okamoto, H, Yamada, M, Wess, J & Komori, S (2005). M₂ and M₃ muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. *Br J Pharmacol* **146**(1): 98-108.
- Valera, S, Hussy, N, Evans, R J, Adami, N, North, R A, Surprenant, A & Buell, G (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371**(6497): 516-519.
- van Calker, D, Muller, M & Hamprecht, B (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* **33**(5): 999-1005.
- Van Nassauw, L, Costagliola, A, Van Op den Bosch, J, Cecio, A, Vanderwinden, J M, Burnstock, G & Timmermans, J P (2006). Region-specific distribution of the P2Y₄ receptor in enteric glial cells and interstitial cells of Cajal within the guinea-pig gastrointestinal tract. *Auton Neurosci* **126-127**: 299-306.
- Vanderwinden, J M, Timmermans, J P & Schiffmann, S N (2003). Glial cells, but not interstitial cells, express P2X₇, an ionotropic purinergic receptor, in rat gastrointestinal musculature. *Cell Tissue Res* **312**(2): 149-154.
- Vieira, C, Duarte-Araújo, M, Adães, S, Magalhães-Cardoso, T & Correia-de-Sá, P (2009). Muscarinic M₃ facilitation of acetylcholine release from rat myenteric neurons depends on adenosine outflow leading to activation of excitatory A_{2A} receptors. *Neurogastroenterol Motil* **21**(10): 1118-e1195.
- Vieira, C, Ferreirinha, F, Silva, I, Duarte-Araújo, M & Correia-de-Sá, P (2011). Localization and function of adenosine receptor subtypes at the longitudinal muscle - Myenteric plexus of the rat ileum. *Neurochem Int*.
- Virginio, C, Robertson, G, Surprenant, A & North, R A (1998). Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X₁, P2X₃, and heteromeric P2X_{2/3} receptors. *Mol Pharmacol* **53**(6): 969-973.
- Vizi, E S, Sperlagh, B & Baranyi, M (1992). Evidence that ATP released from the postsynaptic site by noradrenaline, is involved in mechanical responses of guinea-pig vas deferens: cascade transmission. *Neuroscience* **50**(2): 455-465.

- Vlajkovic, S M, Thorne, P R, Seigny, J, Robson, S C & Housley, G D (2002). Distribution of ectonucleoside triphosphate diphosphohydrolases 1 and 2 in rat cochlea. *Hear Res* **170**(1-2): 127-138.
- von Kugelgen, I (2006). Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* **110**(3): 415-432.
- von Kugelgen, I & Wetter, A (2000). Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedebergs Arch Pharmacol* **362**(4-5): 310-323.
- Ward, S M, Beckett, E A, Wang, X, Baker, F, Khoyi, M & Sanders, K M (2000). Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neurosci* **20**(4): 1393-1403.
- Ward, S M & Sanders, K M (2001a). Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. I. Functional development and plasticity of interstitial cells of Cajal networks. *Am J Physiol Gastrointest Liver Physiol* **281**(3): G602-611.
- Ward, S M & Sanders, K M (2001b). Interstitial cells of Cajal: primary targets of enteric motor innervation. *Anat Rec* **262**(1): 125-135.
- Webb, T E, Simon, J, Krishek, B J, Bateson, A N, Smart, T G, King, B F, Burnstock, G & Barnard, E A (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett* **324**(2): 219-225.
- Westfall, T C & Westfall, D P (2005). Neurotransmission: the autonomic and somatic motor nervous systems. Goodman & Gilman's The Pharmacological Basis of Therapeutics Brunton, L L, J.; Parker, K., *McGraw-Hill Professional*.
- White, T D & Leslie, R A (1982). Depolarization-induced release of adenosine 5'-triphosphate from isolated varicosities derived from the myenteric plexus of the guinea pig small intestine. *J Neurosci* **2**(2): 206-215.
- Wiklund, N P & Gustafsson, L E (1986). Neuromodulation by adenine nucleotides, as indicated by experiments with inhibitors of nucleotide inactivation. *Acta Physiol Scand* **126**(2): 217-223.
- Williams, M & Jarvis, M F (2000). Purinergic and pyrimidinergic receptors as potential drug targets. *Biochem Pharmacol* **59**(10): 1173-1185.
- Wood, J D (2006a). The enteric purinergic P2Y₁ receptor. *Curr Opin Pharmacol* **6**(6): 564-570.
- Wood, J D (2006b). Integrative Functions of the Enteric Nervous System. Physiology of the Gastrointestinal Tract. Johnson, L R B, K.E.; Ghishan, F.K.; Merchant, J.L.; Said, H.M.; Wood, J.D., *Academic Press*: 665-683.

- Wunderlich, J E, Needleman, B J, Chen, Z, Yu, J G, Wang, Y, Grants, I, Mikami, D J, Melvin, W S, Cooke, H J & Christofi, F L (2008). Dual purinergic synaptic transmission in the human enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* **294**(2): G554-566.
- Wynn, G, Rong, W, Xiang, Z & Burnstock, G (2003). Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. *Gastroenterology* **125**(5): 1398-1409.
- Xiang, Z & Burnstock, G (2004). P2X2 and P2X3 purinoceptors in the rat enteric nervous system. *Histochem Cell Biol* **121**(3): 169-179.
- Xiang, Z & Burnstock, G (2005). Distribution of P2Y₂ receptors in the guinea pig enteric nervous system and its coexistence with P2X2 and P2X3 receptors, neuropeptide Y, nitric oxide synthase and calretinin. *Histochem Cell Biol* **124**(5): 379-390.
- Xiang, Z & Burnstock, G (2006). Distribution of P2Y₆ and P2Y₁₂ receptor: their colocalization with calbindin, calretinin and nitric oxide synthase in the guinea pig enteric nervous system. *Histochem Cell Biol* **125**(4): 327-336.
- Yan, H & Tsai, M D (1999). Nucleoside monophosphate kinases: structure, mechanism, and substrate specificity. *Adv Enzymol Relat Areas Mol Biol* **73**: 103-134, x.
- Yegutkin, G G (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* **1783**(5): 673-694.
- Yegutkin, G G & Burnstock, G (2000). Inhibitory effects of some purinergic agents on ecto-ATPase activity and pattern of stepwise ATP hydrolysis in rat liver plasma membranes. *Biochim Biophys Acta* **1466**(1-2): 234-244.
- Yegutkin, G G, Henttinen, T & Jalkanen, S (2001). Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. *FASEB J* **15**(1): 251-260.
- Yegutkin, G G, Henttinen, T, Samburski, S S, Spychala, J & Jalkanen, S (2002). The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem J* **367**(Pt 1): 121-128.
- Yoshioka, K, Saitoh, O & Nakata, H (2001). Heteromeric association creates a P2Y-like adenosine receptor. *Proc Natl Acad Sci U S A* **98**(13): 7617-7622.
- Zeng, F & Wess, J (2000). Molecular aspects of muscarinic receptor dimerization. *Neuropsychopharmacology* **23**(4 Suppl): S19-31.
- Zezula, J & Freissmuth, M (2008). The A_{2A}-adenosine receptor: a GPCR with unique features? *Br J Pharmacol* **153** Suppl 1: S184-190.

- Zhou, H, Das, S & Murthy, K S (2003). Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by M₃ and M₂ receptors. *Am J Physiol Gastrointest Liver Physiol* **284**(3): G472-480.
- Zhou, X & Galligan, J J (1996). P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. *J Physiol* **496** (Pt 3): 719-729.
- Zhou, X, Ren, J, Brown, E, Schneider, D, Caraballo-Lopez, Y & Galligan, J J (2002). Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther* **302**(3): 889-897.
- Zimmermann, H (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362**(4-5): 299-309.
- Zizzo, M G, Mule, F & Serio, R (2007). Evidence that ATP or a related purine is an excitatory neurotransmitter in the longitudinal muscle of mouse distal colon. *Br J Pharmacol* **151**(1): 73-81.

