Studies on a Putative Neuroprotective Role for Adenosine in the Enteric Nervous System

by

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ABSTRACT

Intact myenteric neural networks were isolated from the guinea-pig ileum and used in a parallel perifusion protocol to characterize the effects of altered pO_2 on the concentration of endogenous interstitial adenosine, as assessed by changes in the release of substance P-like immunoreactivity (SPLI). Increasing degrees of hypoxia were shown to diminish both basal and $\uparrow[K^+]_o$-evoked release of SPLI. Antagonism of the adenosine A_1 receptor under hypoxic conditions revealed tonic inhibition by endogenous adenosine. Adenosine levels increased exponentially as pO_2 fell below a slightly hyperoxic threshold value, a finding consistent with a neuroprotective role for the purine. A portion of the interstitial adenosine was shown to be derived from a tetrodotoxin-sensitive source (putatively neural). A nonselective P_{2X} purinoceptor antagonist produced a significant increase in evoked release of SPLI, indicating that ATP *per se* mediates inhibition of $\uparrow[K^+]_o$-depolarized networks via interaction with P_{2X} receptors. Evidence was obtained for the presence of excitatory P_{2X3} receptors on myenteric neurons. Finally, NMDA produced a decrease in evoked SPLI release, indicating that excitatory amino acids may mediate transmission in the enteric nervous system.

Keywords: adenosine, substance P, enteric nervous system, myenteric, hypoxia, pO_2, A_1 receptor, P_2 purinoceptor, excitatory amino acids.

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ABBREVIATIONS

α,β-MeATP:	α,β-methylene ATP
β,γ-MeATP:	β,γ-methylene ATP
2-MeSATP:	2-methylthio-ATP
5-HT:	5-hydroxytryptamine, serotonin
ACh:	acetylcholine
ADP:	adenosine 5'-diphosphate
AMP:	adenosine 5'-monophosphate
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOPCP:	α,β-methylene ADP
AP5:	D-2-amino-phosphonopentanoate
ASA:	acetylsalycylic acid
ATPγS:	adenosine 5'-O-(3-thiotriphosphate)
ATP:	adenosine 5'-triphosphate
cAMP:	cyclic adenosine 3',5'-monophosphate
CBR:	cumulative basal release (see Methods for calculation)
CCK:	cholecystokinin sulfate octapeptide
CER:	cumulative evoked release (see Methods for calculation)
CGRP:	calcitonin gene-related peptide
CGS 21680:	2-[*p*-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine
CHA:	N^6-cyclohexyladenosine
ChAT:	choline acetyltransferase
CNS:	central nervous system
CPA:	N^6-cyclopentyladenosine
CSC:	1,3,7-trimethyl-8-(3-chlorostyryl)xanthine
CV 1808	2-phenylaminoadenosine
DDA:	2', 5'-dideoxyadenosine
DPCPX:	1,3-dipropyl-8-cyclopentylxanthine
DYN:	dynorphin
EAA:	excitatory amino acid
EDTA:	ethylenediaminetetra-acetic acid, disodium salt
ei:	equilibrative, insensitive nucleoside transporter
ENK:	enkephalin
ENS:	enteric nervous system
EPSP:	excitatory postsynaptic potential
es:	equilibrative, sensitive nucleoside transporter
GABA:	γ-aminobutyric acid
GAD:	glutamic acid decarboxylase
GAL:	galanin
GI:	gastrointestinal
GRP:	gastrin releasing peptide
GTP-γS:	guanosine 5'-O-(3-thiotriphosphate)

GTP:	guanosine 5'-triphosphate
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC:	high pressure liquid chromatography
IB-MECA:	N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide
IP_3:	inositol trisphosphate
IPSP:	inhibitory postsynaptic potential
$\uparrow[K^+]_o$:	Locke's solution with elevated concentration of KCl
K_i:	binding constant for inhibition
LMMP:	longitudinal muscle-myenteric plexus preparation
LY 235959:	(-)3-SR,4a-RS,8a-SR-6-(phosphonomethyl)-1,2,3,4,4a,5,6,7,8,8a-decahy droisoquinoline-3-carboxylic acid
MMC:	migrating motor complex
MRS-1191:	3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1, 4-(+/-)-dihydropyridine-3,5-dicarboxylate
NBTI:	*S*-(4-nitrobenzyl)-6-thioinosine
NE:	noradrenaline
NECA:	5'-N-ethyl-carboxamidoadenosine
NKA:	neurokinin A
NKA-LI:	neurokinin A-like immunoreactivity
NMDA:	N-methyl-D-aspartate
NO:	nitric oxide
NPY:	neuropeptide Y
PACAP:	pituitary adenylate cyclase-activating peptide
PLCβ:	phospholipase C β
PNS:	peripheral nervous system
pO_2:	partial pressure of oxygen
PPADS:	pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
RIA:	radioimmunoassay
R-PIA:	N^6-(R-phenylisopropyl)-adenosine
SAH:	S-adenosyl-L-homocysteine
SAM:	S-adenosylmethionine
SP:	substance P
S-PIA:	N^6-(S-phenylisopropyl)-adenosine
SPLI:	substance P-like immunoreactivity
TK:	tachykinin
TK-IR:	tachykinin-like immunoreactivity
TTX:	tetrodotoxin
UTP:	uridine 5'-triphosphate
VIP:	vasoactive intestinal polypeptide
ZM 24135:	(4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-yl amino] ethyl) phenol)

INTRODUCTION

The studies described in this thesis address the hypothesis that purine nucleosides may provide a neuroprotective function in ischaemia and hypoxia. The work assesses the ability of adenosine, ATP analogues and selected excitatory amino acids to modulate the activity of nerves in the guinea-pig myenteric plexus, and characterizes the response of interstitial adenosine to changes in prevailing pO_2. Considerable evidence has accumulated over the past fifteen years to suggest that adenosine can indeed protect central neurons from the adverse functional and morphological consequences of ischaemia and hypoxia (Rudolphi et al., 1992). In cardiac tissue, the nucleoside demonstrates similar cytoprotective actions preceded by an increase in endogenous interstitial adenosine levels in response to an ischaemic insult (see Cook and Karmazyn, 1996). In CNS tissue, evidence for the protection afforded by endogenous adenosine has helped to define a role for the nucleoside as both an endogenous modulator of excitatory neurotransmission and a potential therapeutic agent under pathophysiological conditions. While such data is relatively abundant for central nerves, similar information at the enteric nervous system (ENS) is lacking. Perturbation of enteric neural function may cause or contribute to a variety of gastrointestinal disorders. Moreover, the morbidity associated with mesenteric atherosclerosis and intestinal ischaemia suggest that investigation of a putative neuroprotective role for adenosine in the enteric nervous system

would be worthwhile. To this end, the present studies sought to better characterize the actions of endogenous adenosine in the myenteric plexus under both normoxic and hypoxic conditions. The experiments described herein utilised isolated guinea-pig myenteric neural networks in a parallel perifusion protocol. Activity of the networks (and modulation of this activity by endogenous and exogenous agents) was assessed by quantitation of released Substance P (SP), one of the primary transmitters in the system. The objectives of this work, and the results obtained, should be viewed not only with respect to enteric neurophysiology, but also in light of evidence acquired at other tissues. This introduction will therefore review the current physiology of the enteric nervous system, and examine the neuromodulatory actions of adenosine (and related purinergic moieties) within both the ENS and the CNS under physiological and pathophysiological conditions.

The Enteric Nervous System

The major functions of the gastrointestinal (GI) tract, motility and secretion, are mediated by visceral smooth muscle and by the intestinal mucosa. The central nervous system (CNS) exerts relatively minor influence on GI function, the major areas of input being at the extremities of the tract (i.e. the proximal esophagus and the rectum). The remainder of the intestine is under the control of the enteric nervous system (ENS), which can regulate and maintain GI function in the absence of any CNS connection. The ENS is

comprised of a series of interconnected ganglionated plexuses and their supporting cells located within the wall of the gut (see Figure 1). The primary excitatory transmitters in the system are acetylcholine (ACh) and substance P (SP), while vasoactive intestinal polypeptide (VIP) is an abundant, potent inhibitory neuromediator (for extensive reviews of enteric neurophysiology, the reader is referred to Furness and Costa (1987) and Costa et al. (1996)). Transmission in the ENS can be modulated by purinergic compounds, such as adenosine, acting at P_1 and P_2 receptors (both pre- and post-synaptically), the evidence for which has uncovered a role for adenosine both as an endogenous mediator of GI function, and as a putative neuroprotective agent.

At an anatomical level the ENS contains a variety of neuronal elements, distributed throughout a series of interconnected neural networks, of which the major ones are the myenteric plexus (located between the longitudinal muscle layer and the circular muscle layer) and the submucous plexus (located between the circular muscle layer and the submucosal layers). The system is comprised of both excitatory and inhibitory motor neurons, enteric vasodilator neurons and secretomotor neurons, which together are responsible for the control of gastrointestinal muscle contraction, regulation of local blood flow, and absorption and secretion by the intestinal mucosa. The plexuses possess a reticular morphology and are composed of groups of cell bodies (ganglia) and varicose nerve processes.

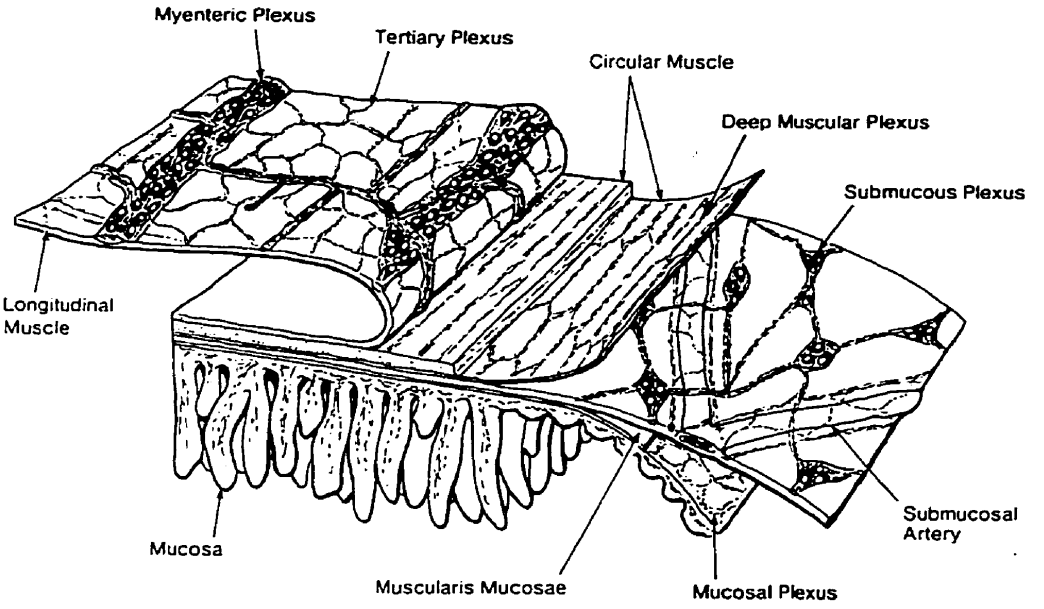


Figure 1: Diagrammatic representation of the enteric plexuses as seen on whole mounts of guinea-pig small intestine (Furness and Costa, 1987).

The varicosities along these processes are sites of neurotransmitter release, a morphological and functional feature which distinguishes enteric neurons from virtually all central neurons. Axons from neurons within the myenteric plexus may terminate within the plexus, or may project to the deep muscular plexus and/or the submucous plexus. In general, the myenteric plexus is responsible for control of GI motility, while the submucous plexus, which sends axons to the inner mucosa, regulates mucosal functions including blood flow and intestinal fluid and ion homeostasis.

Based upon their morphologies, enteric neurons have been classified into Types I, II and III (Furness et al., 1988). Electrophysiological evidence has resulted in somewhat different nomenclature; "S/Type 1" neurons are named for their ability to receive fast synaptic input and to fire multiple action potentials during a depolarizing pulse, while "AH/Type 2" neurons are identified by a long period of after-hyperpolarization (greater than five seconds) following an action potential (Hirst et al., 1972; Nishi and North, 1973). Excitatory postsynaptic potentials (EPSPs) in S/Type 1 neurons are quick, lasting less than 20 ms, and are followed by a short after-hyperpolarization. AH/Type 2 neurons generally respond to stimulation with slow EPSPs, lasting between 30 seconds and 5 minutes. Although slow EPSPs occur in both neuronal subsets, fast EPSPs are typically restricted to S/Type 1 neurons (Furness and Costa, 1987).

As might be expected in such a comprehensive system, a wide variety of neurotransmitters and neuromodulators have been identified as mediators

of enteric synaptic transmission. Immunohistochemical techniques have uncovered patterns of localization and co-localization for many of these transmitters including ACh, SP, dynorphin (DYN), galanin (GAL), enkephalin (ENK), neurokinin A (NKA), VIP, 5-hydroxytryptamine (5-HT), pituitary adenylyl cyclase-activating peptide (PACAP), nitric oxide synthase (which produces the transmitter nitric oxide), neuropeptide Y (NPY) and adenosine triphosphate (ATP) (Furness et al., 1995). There is a large body of evidence to support a role for ACh as the primary neurotransmitter of excitatory motor neurons innervating gastrointestinal muscle (Furness and Costa, 1980; Furness et al., 1983; Costa and Brookes, 1994). These same neurons have since been shown to release tachykinins (TKs), a family of related peptides that includes SP, NPY and NKA among others (Furness et al., 1995). While most attempts to determine co-localization of ACh and TKs using immunohistochemical approaches have been hampered by a relative lack of specific antibodies against choline acetyltranferase (ChAT), myenteric and circular muscle excitatory motor neurons in the guinea pig have been shown to contain tachykinin immunoreactivity (TK-IR) (Brookes et al., 1991; Steele et al., 1991). ACh and SP have thus come to be identified as the major excitatory mediators in the ENS.

Adenosine, ATP, and their Receptors

GI functions such as motility and secretion are subject to modulation by the purines adenosine and ATP (Westerberg and Geiger, 1989; Gil-Rodrigo et al., 1990; Cook, 1991; Ainz et al., 1993; Moneta et al., 1997; Christofi and Cook, 1997). They are released from enteric nerves, muscle tissue and various other cell types, and interact with specific cell-surface P_1 and P_2 purinoceptors in multiple systems to elicit a range of fairly well-characterized effects. ATP is a primary mediator of fast synaptic transmission (Silinsky et al., 1992; Galligan and Bertrand, 1994). In nerves it is packaged in neurotransmitter vesicles and released in response to an action potential in a calcium dependent manner. Typically, basal levels of endogenous ATP in the interstitium are very low, a consequence of the fact that the nucleoside is rapidly and efficiently metabolized by cell-surface ecto-ATPases (Zimmermann and Braun, 1996) and dephosphorylated to a diphosphate (ADP), a monophosphate (AMP) and, eventually, adenosine. Adenosine is thus a common moiety in the extracellular space of virtually every tissue, although it too is subject to relatively rapid uptake and metabolism to inosine. Adenosine has never been shown to exhibit properties associated with classical neurotransmitters (i.e. calcium-dependent exocytotic release in response to action potentials), but extracellular levels of adenosine do rise in response to increased synaptic transmission, and its neuromodulatory effect of reducing postsynaptic membrane depolarization is well documented. It has

been reported that in the brain of freely moving, unanaesthetised rats, the free concentration of adenosine is between 50 and 300 nm (Ballarin et al., 1991). However with a suggested biological half-life (in the brain) of only 3-6 seconds (Rudolphi et al., 1992), estimates of free adenosine concentration in the interstitium are difficult to obtain, and are severely restricted by the temporal resolution of the measurement protocol used. Extracellular dephosphorylation of ATP represents only a portion of the adenosine formation pathway (see Figure 2). An intracellular pathway exists involving the demethylation of S-adenosyl-L-methionine (SAM) to create S-adenosyl-L-homocysteine (SAH). SAH is hydrolysed by SAH-hydrolase to produce adenosine and L-homocysteine. Membrane-spanning equilibrative nucleoside transporters ensure that extracellular concentrations of adenosine stay closely matched with intracellular levels. In mammalian tissue, these transporters are divided into two broad categories: facilitative diffusion transporters and active-transporters (driven by an inwardly directed sodium gradient). Active transporters are widespread, and classified into N1, N2, N3, and N4 subtypes based on their substrate specificity, although adenosine appears to be a substrate for all of them (Thorn and Jarvis, 1996). Facilitative transporters are subclassified into two categories based upon their relative sensitivities to inhibition by the synthetic nucleoside analogue nitrobenzylthioinosine (NBTI). The *es* (equilibrative, sensitive) transporter is inhibited by NBTI with a K_i of 0.1 – 1 nM (Plagemann et al., 1988). In contrast, the *ei* (equilibrative, insensitive) transporter has been relatively

poorly characterized, but has been shown to maintain activity until exposed to NBTI concentrations greater than 1μM (Belt and Noel, 1985).

The purinoceptors involved in the mediation of synaptic transmission are widely distributed throughout the ENS and CNS. In 1978, after reviewing decades of research into the effects of purines on various systems and the compounds which appeared to either potentiate or inhibit these effects, Burnstock (1978) proposed a classification of purinoceptors into the P_1 and P_2 subtypes. This initial discrimination was based on four criteria: (1) the relative potencies of ATP, ADP, AMP and adenosine at the receptors, (2) antagonism of the receptors by methylxanthines, (3) modulation of intracellular adenylyl cyclase subsequent to ligand binding and (4) induction of prostaglandin synthesis. P_1 receptors were more sensitive to adenosine, while the P_2 subtype showed a greater sensitivity to ATP. The potency order at the P_1 receptor was adenosine > AMP > ADP > ATP. Caffeine, theophylline and other methylxanthines antagonized the P_1 receptor, resulting in intracellular modulation of adenylyl cyclase activity and cAMP levels. In contrast, P_2 receptors were not selectively antagonized by the methylxanthines, and there was no alteration of adenylyl cyclase activity, but P_2 activation did sometimes result in prostaglandin synthesis (Burnstock and Hoyle, 1985).

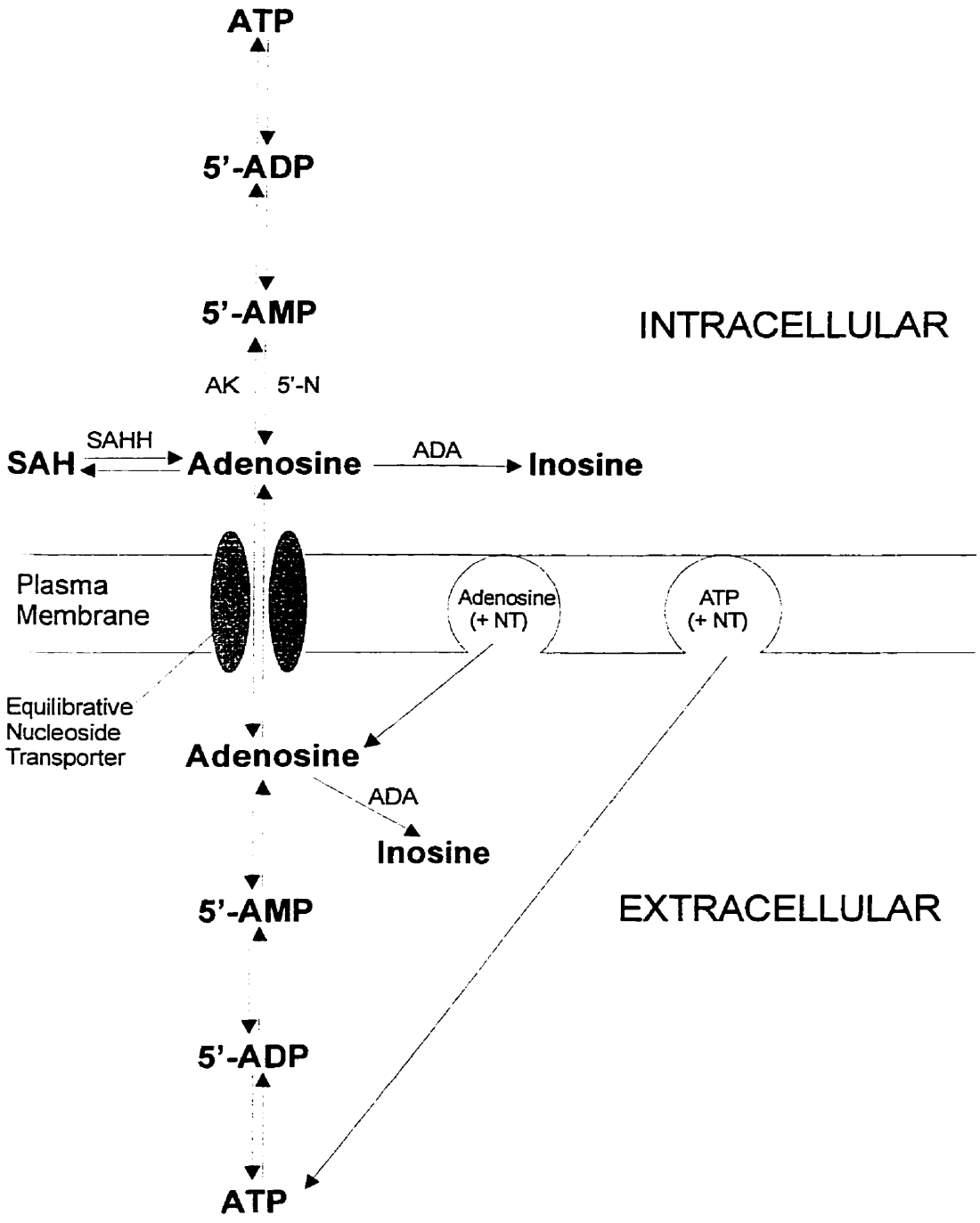


Figure 2: Diagrammatic representation of the metabolic pathways for adenosine. Abbreviations are as follows: (SAH) S-adenosyl homocysteine, (SAHH) S-adenosyl homocysteine hydrolase, (AK) adenosine kinase, (5'-N) 5'-nucleotidase, (ADA) adenosine deaminase, (NT) neurotransmitter.

The last few years have seen substantial attention paid to classification of the purinoceptors and the discovery of new subtypes within the adenosine/P_1 and ATP/P_2 families, such as a methylxanthine-insensitive P_1 receptor (known as the A_3 receptor). In addition, P_2 purinoceptors have recently been renamed P_2 receptors to accommodate a subclass sensitive to both purines and pyrimidines (Williams and Burnstock, 1997). For the purposes of this thesis, however, the terms P_2 receptor and purinoceptor will be used interchangeably. To date, the P_1 receptors have been divided into 3 types (A_1, A_2, and A_3), with A_{2a} and A_{2b} subtypes. The P_2 receptors are classified into P_{2X} and P_{2Y} types (the latter showing some sensitivity to pyrimidines such as UTP), with 7 X-subtypes and 8 Y-subtypes. The following will primarily discuss the pharmacology of the adenosine/P_1 receptors, with references to the P_2 family as they relate to the neuromodulatory effects of ATP.

The Adenosine (P_1) Receptors

Adenosine-sensitive P_1 receptors belong to the family of G protein-coupled receptors. It was recognized almost twenty years ago that activation of these receptors was linked to either a stimulation or inhibition of adenylyl cyclase activity (Londos and Wolff, 1977; van Calker et al., 1979). The initial receptor subclassification into the A_1 and A_2 types was therefore based on these characteristics; A_1 receptors mediated inhibition of adenylyl cyclase activity while A_2 receptors mediated stimulation of adenylyl cyclase activity.

However, it soon became clear that agonist binding at either receptor type was linked to *several* effector systems, such as activation of K^+ channels and inhibition of Ca^{2+} channels, for which changes in adenylyl cyclase and cAMP levels were not reliably predictive. As an example, it has been shown that an adenosine receptor-mediated increase in K^+ conductance is due to a coupling of the receptor with the ion channel via a pertussis toxin-sensitive G-protein. While intracellular GTP is required, cAMP is not (Trussell and Jackson, 1985) and in fact GTP-γS (a stable GTP analogue) can induce an increased K^+ conductance in the absence of an adenosine agonist. As a consequence, criteria for the subclassification of the P_1 purinoceptors was changed from reliance on adenylyl cyclase activation to relative agonist potencies of adenosine analogues at the receptors (Olah and Stiles, 1995). The rank order of potency for selected analogues at the A_1 receptor is N^6-[(R)-phenylisopropyl]adenosine (R-PIA) > 5'-N-ethylcarboxamidoadenosine (NECA) > N^6-[(S)-phenylisopropyl]adenosine (S-PIA). In addition, N^6-cyclohexyladenosine (CHA) and N^6-cyclopentyladenosine (CPA) have demonstrated potencies in the nanomolar range and are 300-800 times more selective for the A_1 receptor than the A_2 (Moos et al., 1985). The rank order of potency for agonists at the A_2 receptor is NECA > R-PIA > S-PIA (Stiles, 1997). In general, N^6-susbstituted adenosine analogues show more selectivity for the A_1 receptor, while 2- and 5'-substituted analogues do so for the A_2 receptor.

Classification of receptors using agonists is, however, not a preferred pharmacological method, due to the fact that apparent agonist potencies are linked to the efficiency of their signal transduction systems. Antagonists, having no efficacy, are therefore much better agents for this task. Attempts have been made to classify adenosine receptors using antagonists, but these efforts have been hampered by a relative lack of antagonists with a high degree of subtype selectivity. A common A_1 receptor antagonist is 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), which is 700 times more selective for the A_1 than the A_2 receptor type (Bruns et al., 1987). A new, non-xanthine compound ZM 24135 has shown high selectivity for the A_{2a} receptor compared to the A_1 (Poucher et al., 1995), but the selectivity of a more typical antagonist, (3-chlorostyryl)caffeine (CSC), is high in binding studies but relatively low in functional tests.

As mentioned earlier, the adenosine receptors are members of a G protein-linked superfamily of receptors. In the case of the A_1 receptor, *in vitro* studies have suggested a functional interaction with pertussis toxin-sensitive G_i and G_o proteins, mediating a decrease in adenylyl cyclase activation and an increase in K^+ channel conductance (Freissmuth et al., 1991). The A_1 receptor itself has been cloned from various tissues in several species (eg. rat, bovine, rabbit) with only slight differences in agonist binding affinities both within and across species (although no molecular evidence exists to suggest the existence of A_1 subtypes). On the other hand, observed differences in binding affinities for A_2 agonists across various tissues prompted a

subclassification into A_{2a} and A_{2b} subtypes (Daly et al., 1983). The A_2 agonists CV 1808 and CGS 21680 showed a high affinity for rat striatal A_2 receptors, but a low affinity for human fibroblast A_2 receptors (Bruns et al., 1986). Since the time of these observations, molecular cloning has confirmed the existence of two distinct receptor subtypes: the high affinity (eg. rat striatal) type is the A_{2a} and the low affinity (eg. human fibroblast) type the A_{2b}. The A_{2a} receptor is known to interact with G_s proteins, mediating the activation of adenylyl cyclase, and the A_{2b} receptor interacts with G_s proteins to stimulate adenylyl cyclase activity or open calcium channels (Stiles, 1997). In 1991, a G protein-coupled receptor clone with a 40-50% amino acid identity to the canine A_1 and A_2 receptors was identified (Meyerhof et al., 1991). In 1992, a similar clone was isolated from rat brain, transfected and characterized using various adenosinergic compounds (Zhou et al., 1992). This receptor was found to bind adenosine receptor ligands with high affinity, but agonist potencies were distinct from those at A_1 or A_2 receptors. Receptor activation caused inhibition of forskolin-induced adenylyl cyclase activation which was sensitive to pertussis toxin. The receptor was also insensitive to typical adenosine receptor antagonists such as the methylxanthines. The convergence of several such lines of functional and molecular evidence eventually led to the clone being termed the A_3 receptor. Due in part to the relative novelty of the A_3 receptor, few highly selective agonists and antagonists have been developed. One new and potent A_3 agonist, N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA) has

shown a 50-fold selectivity for the A_3 receptor over the A_1 and A_{2a} receptors *in vitro*, and is believed to be highly selective for the A_3 receptor *in vivo* (Gallo-Rodriguez et al., 1994). Recent exploration of flavonoids (phenolic compounds found in vascular plants) has yielded promising A_3 antagonist candidates (Ji et al., 1996). One such compound, MRS 1191, has shown 1300-fold selectivity for human A_3 receptors over rat A_1 receptors (Jiang et al., 1996), although its selectivity for the rat A_3 receptor is much lower.

Purinergic Modulation of Enteric Neural Function

Adenosine is a recognized neuromodulator in the ENS, mediating both overall neural excitability and release of neurotransmitters. Adenosine is capable of inhibiting electrically-mediated contractions of the guinea pig ileum and longitudinal muscle-myenteric plexus (LMMP), primarily via activation of the A_1 receptor (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976; Cook et al., 1978; Moody and Burnstock, 1982; Paul et al., 1982; Gustafsson et al., 1985). These contractions were originally thought to be the result of cholinergic neurotransmission, but it has been shown that approximately 30% of the response is not cholinergic, but rather mediated by the tachykinins (including SP) (Christofi et al., 1990). Contractions induced by the application of exogenous ACh are not attenuated by adenosine, suggesting that such inhibitory behaviour on electrically-stimulated preparations is mediated presynaptically (Dowdle and Maske, 1980). Good evidence exists to support the presence of presynaptic A_1 receptors and their functional role in inhibiting

ACh release (Christofi and Cook, 1986) and in suppressing fast and slow EPSPs in both S/Type 1 and AH/Type 2 neurons (Christofi and Wood, 1993; Kamiji et al., 1994). Furthermore, Christofi et al. (1990) showed that in preparations of guinea pig LMMP in which cholinergic transmission was blocked by atropine, contractions were inhibited by selective A_1 adenosine receptor antagonists. These contractions were abolished in the presence of tachykinin antagonists (Christofi et al., 1990). This strongly suggested a negative coupling between adenosine receptor activation and tachykinin release. Subsequent studies on isolated myenteric nerve varicosities and discreet ganglion networks have provided evidence to support the existence of a presynaptic A_1 receptor whose activation inhibits the release of evoked SP-like immunoreactivity (SPLI) and NKA-like immunoreactivity (NKA-LI) (Broad et al., 1992; Broad and Cook, 1993; Moneta et al., 1997). None of these studies, however, have provided conclusive evidence for the functional presence of A_2 receptors on myenteric nerves, and research into a possible functional role for A_3 receptors is ongoing.

ATP is known to inhibit both fast EPSPs in S/Type 1 neurons and slow EPSPs in S/Type 1 and AH/Type 2 neurons via interaction with P_2 purinoceptors. The stable ATP analogues ATPγS (a P_{2X2} and P_{2X4} receptor agonist) and α-β-methylene ATP (a P_{2X1} receptor agonist) also inhibit synaptic potentials in those neurons with a 10-fold increase in potency over adenosine. Interestingly, at concentrations greater than 1μM, ATP has been shown to potentiate ACh-induced nicotinic fast synaptic depolarizations in S/Type 1

neurons, while attenuating muscarinic and SP-induced depolarizations in S/Type 1 and AH/Type 2 neurons (Kamiji et al., 1994). There is therefore a possibility that, *in vivo*, ATP might bind at P_{2X} purinoceptors on smooth muscle, inducing release of ATP by smooth muscle cells which would then be dephosphorylated in the insterstitium to form adenosine. Adenosine in turn would act at A_1 receptors to inhibit cholinergic transmission (Katsuragi et al., 1993).

In addition to mediating cholinergic and tachykininergic transmission, adenosine has also been implicated in the modulation of noradrenergic signaling. A study conducted by Zafirov (1993) demonstrated that the amplitude and duration of inhibitory postsynaptic potentials (IPSPs) in submucous S/Type 1 neurons was increased by the application of forskolin (an adenylyl cyclase activator) and by membrane permeable analogues of cAMP. Suppression of these electrically-evoked IPSPs by α_2-adrenoceptor antagonists strongly suggested that the IPSPs were mediated by noradrenaline (NE) acting at α_2-adrenoceptors. Furthermore, application of selective adenosine A_1 receptor antagonists resulted in inhibition of both the IPSPs and the potentiating effects of forskolin. These data support a negative coupling between activation of the A_1 purinoceptor and adenylyl cyclase activity to functionally attenuate noradrenergic neurotransmission in the submucous plexus.

In the myenteric plexus, consistent demonstration of stable, robust IPSPs has been a difficult task (Wood, 1994). Christofi and Wood (1993)

have postulated that the reason for this may lie in the fact that electrical field stimulation sometimes induces both excitatory and inhibitory potentials, a phenomenon which could effectively mask slow IPSPs in myenteric AH/Type 2 neurons. Indeed, agonism of A_1 receptors (and thus inhibition of slow EPSPs) has revealed robust slow IPSPs in these neuronal populations (Christofi and Wood, 1993). Adenosine had no effect on IPSPs alone, demonstrating that the nucleoside is capable of attenuating excitatory transmission in the myenteric plexus without affecting inhibitory transmission. Conversely adenosine, acting at A_1 receptors in the submucous plexus, acts to suppress noradrenergic slow IPSPs and cholinergic fast EPSPs (Barajas-Lopez et al., 1991; Zafirov et al., 1993). This apparent dichotomy may well be due to differences in specialized function and neurotransmitter populations between the two plexuses.

Electrophysiological studies on AH/Type 2 neurons in the myenteric plexus have revealed that activation of the A_1 receptor results in a membrane hyperpolarization that is associated with decreased input resistance, enhanced postspike hyperpolarizing potentials and an overall decrease in neuronal excitability (Zafirov et al., 1985; Palmer et al., 1987b; Katayama and Morita, 1989). In addition, adenosine inhibits forskolin-induced excitability yet has no effect on excitation due to cAMP analogues or elevation of Mg^{2+} and reduction of Ca^{2+} in the medium, suggesting postsynaptic suppression of the catalytic activity of adenylyl cyclase. More evidence for purinergic postsynaptic neuromodulation has been acquired by Barajas-Lopez et al.

(1991) who showed that selective adenosine A_2 receptor agonists (eg. CGS 21680) caused hyperpolarization of AH/Type 2 neurons, but depolarization of S/Type 1 neurons in the submucous plexus. The depolarization of S/Type 1 neurons was insensitive to tetrodotoxin (TTX), a sodium channel blocker, and to cholinergic antagonists, evidence which strongly supported a postsynaptic site of action for the A_2 receptor agonists.

Application of certain ENS neurotransmitters to AH/Type 2 neurons of the guinea pig myenteric plexus can produce EPSPs which are also subject to modulation by adenosine (Palmer et al., 1987a; Christofi and Wood, 1993). In these neurons, slow EPSPs produced in response to PACAP were attenuated by adenosine acting at postsynaptic A_1 receptors (Christofi and Wood, 1993). In addition, slow EPSPs produced by gastrin-releasing peptide (GRP), cholecystokinin (CCK), VIP, bombesin and histamine were blocked by pretreatment of the neurons with adenosine, while slow EPSPs produced by SP, 5-HT and calcitonin gene-related peptide (CGRP) were enhanced. The implication is that GRP, CCK, VIP, bombesin and histamine can mimic slow EPSPs by activating adenylyl cyclase, and that adenosine, acting via postsynaptic A_1 purinoceptors, blocks the response to the mimetic agents by inhibiting adenylyl cyclase activation. The observed potentiating effects of adenosine on EPSPs produced by SP, 5-HT and CGRP can be explained in two ways: (1) that the effector systems for these mimetic agents do not involve adenylyl cyclase activation and (2) that the receptors for these agents are concentrated on a different subset of neurons. This putative subset could

preferentially express A_2 receptors, activation of which would mediate the activation of adenylyl cyclase and the enhancement of mimicked EPSPs. This possibility is consistent with the findings of Christofi et al. (1994) who showed that some AH/Type 2 neurons in the myenteric plexus of the guinea pig ileum demonstrate a concentration-dependent membrane depolarization and increased overall neuronal excitability in response to treatment with selective A_2 receptor agonists. These agonists also enhanced excitatory responses to forskolin, supporting the existence of high affinity A_2 purinoceptors mediating adenylyl cyclase activation and subsequent elevation of cAMP in a small subset of AH/Type 2 myenteric neurons.

Several groups have shown that ATP is also capable of postsynaptically modulating neurotransmission (Katayama and Morita, 1989; Barajas-Lopez et al., 1994; Kamiji et al., 1994). In most AH/Type 2 myenteric neurons, ATP-induced membrane hyperpolarization was only partially antagonized by the methylxanthines, while in most S/Type 1 myenteric neurons, this hyperpolarization was not significantly affected by the methylxanthines. Adenosine also induced similar hyperpolarizations, but was generally less effective at doing so than ATP. The results support the presence of P_2 purinoceptors on both types of neurons and suggest that ATP might exert its hyperpolarizing effects by activating, and its depolarizing effects by inhibiting, a Ca^{2+}-dependent K^+ conductance. Barajas-Lopez et al. (1994) showed that ATP inhibited K^+ conductance and opened a cationic conductance in S/Type 1 neurons. ATP also induced single fast

depolarizations in some AH/Type 2 neurons. These depolarizations were insensitive to TTX, suggesting that postsynaptic P_2 purinoceptors were mediating the response to ATP. In addition, superfusion of S/Type 1 neurons with ATP analogues resulted in a slow depolarization typical of a reduction in potassium conductance. The rank order of agonist potencies was 2-methylthioATP (2-MeSATP) > ATP > ATPγS, with α,β-MeATP and β,γ-MeATP inactive. This profile was indicative of the P_{2Y} purinoceptor. Whole cell recordings of S/Type 1 neurons revealed that ATP and its analogues were able to induce fast depolarizations typical of an enhanced cationic conductance. In these studies, the rank order of agonist potencies was ATP = ATPγS = 2-MeSATP >> α,β-MeATP = β,γ-MeATP. ATP was approximately 100-fold more potent in inhibiting K^+ conductance than in opening a cationic conductance. These data suggest that ATP may act via different P_{2Y} purinoceptor subtypes to depolarize neurons in the submucous plexus by inhibiting K^+ conductance and enhancing Ca^{2+} conductance. Indeed, P_{2Y} purinoceptors associated with cationic channels have been found in both S/Type 1 and AH/Type 2 neurons, while P_{2Y} purinoceptors associated with potassium channels have only been found in submucous S/Type 1 neurons (Barajas-Lopez et al., 1994).

Evidence for a Physiological Modulatory Tone

The kinetics of ATP metabolism argue against its role as a tonic neuromodulatory agent. However, the efficiency with which it is dephosphorylated to a relatively stable, ubiquitous nucleoside has wide-ranging implications for the role of adenosine as an endogenous modulator of neuronal activity. Extracellular adenosine concentrations have been shown to rise dramatically in response to evoked neural activity and metabolic stress brought on by seizures, hypoxia and ischaemia (Rudolphi et al., 1992). In these physiological states, the favoured pathway for adenosine formation is the intracellular dephosphorylation of ATP. Adenosine is then carried into the interstitial space by nucleoside transporters (Lloyd et al., 1993). A large proportion of the total extracellular adenosine is also formed from the extracellular breakdown of ATP during evoked nerve activity (MacDonald and White, 1985). Over the years, a large body of evidence has accumulated which suggests that endogenous adenosine exerts a tonic inhibitory tone on neural transmission in the CNS (Fredholm and Hedqvist, 1980; Dunwiddie et al., 1981; Phillis and Wu, 1983; Haas and Greene, 1988; Pak et al., 1994; Fredholm, 1995). Likewise in the enteric nervous system, many studies have provided quantitative evidence that adenosine is present in levels sufficient for functional inhibition of neurotransmission and neuronal excitability. Gustafsson et al. (1981) used HPLC to analyze the medium surrounding strips of guinea pig LMMP in an organ bath. They estimated the basal

extracellular adenosine concentration to be approximately 22 nM, although it was later discovered that significant inactivation of the nucleoside was likely to have occurred as a result of microbial contamination of the organ bath. In addition, measured adenosine concentrations were undoubtedly reflective of release from a variety of sources including muscle tissue and vasculature, and not solely of release from enteric nerves *per se*. Notwithstanding these caveats, transmural electrical stimulation of the LMMP strips resulted in a significant increase in the adenosine concentration in the extracellular medium. Addition of the nucleoside transport inhibitor dipyridamole caused a further increase in both basal and evoked release of adenosine. In a separate study, the use of adenosine receptor antagonists on myenteric neurons in the guinea pig ileum (Christofi and Wood, 1989; Christofi and Wood, 1993) and gastric antrum (Christofi et al., 1992) caused an enhancement in evoked EPSPs, while addition of adenosine deaminase (an adenosine-specific degradative enzyme) or dipyridamole resulted in attenuation of the EPSPs. Several groups have also demonstrated that the contractile response of guinea pig whole ileum or LMMP strips to transmural nerve stimulation is enhanced in the presence of adenosine antagonists or adenosine deaminase alone (Vizi and Knoll, 1976; Hayashi et al., 1978; Moritoki et al., 1978; Gustafsson et al., 1981; Wiklund et al., 1985; Wiklund and Gustafsson, 1987; Broad et al., 1993), and inhibited in the presence of dipyridamole alone (Gustafsson et al., 1981; Wiklund and Gustafsson, 1987). In addition, adenosine antagonists alone have been shown to augment both

the evoked release of ACh from guinea pig LMMP preparations (Sperlagh and Vizi, 1991) and the evoked release of SP from isolated guinea pig myenteric ganglia (Broad et al., 1993; Moneta et al., 1997). Thus there is compelling evidence that endogenous adenosine acts as a tonic inhibitory agent on neurotransmission and neuronal excitability in the ENS. For its part, ATP has been shown to be released directly from enteric nerve endings (White and Leslie, 1982; McConalogue et al., 1996). However, it is unlikely that ATP would accumulate in the interstitial space in concentrations large enough to effect tonic neuromodulation. A role for ATP as a mediator of fast synaptic transmission is supported by the presence of multiple mechanisms for rapid breakdown of the nucleotide at its extracellular receptor sites. Ecto-ATPases and ectophosphatases are ubiquitous enzymes which rapidly and efficiently hydrolyze ATP to adenosine, a relatively stable purinergic neuromodulator (Zimmermann and Braun, 1996).

A Neuroprotective Role for Adenosine

The characterization of adenosine as a tonic inhibitor of neural excitation has opened up many avenues of research into possible neuroprotective functions for the nucleoside. Pathophysiological conditions such as hypoxia and ischaemia, which increase metabolic demand relative to the availability of substrate, have been shown to significantly increase the concentration of adenosine in the interstitium (Van Wylen et al., 1986). Evidence is accumulating which suggests that this endogenous adenosine

may confer some degree of neuroprotection against excitotoxicity (Rudolphi et al., 1992; Rudolphi et al., 1992; von Lubitz et al., 1995). Studies using adenosine and its analogues under conditions of hypoxia, ischaemia, seizure and hypoglycemia support this hypothesis (Rudolphi et al., 1992; von Lubitz et al., 1995). In addition, adenosine kinase inhibition, which would preserve interstitial adenosine levels, has been shown to attenuate infarct size and to improve behavioural measures of neurological deficits in rats following transient focal ischaemia (Jiang et al., 1997; Tatlisumak et al., 1998). In clinical trials with dipyridamole (a nucleoside transport inhibitor) and acetylsalycylic acid (ASA), stroke risk in comparison to placebo was decreased by 37%, in part as a result of chronic elevation of endogenous adenosine levels (Picano and Abbracchio, 1998). Adenosine has also been shown to exert cardioprotective effects against cellular damage induced by ischaemia, free radicals and reperfusion (Karmazyn and Cook, 1992; Cook and Karmazyn, 1996). Several mechanisms, predominantly mediated by A_1 and A_2 receptors, underlie these effects (although recent research has begun to elucidate a role for A_3 receptors) (Fredholm, 1997; Schubert et al., 1997). During hypoxia or ischaemia, intracellular ATP is broken down to adenosine which is transported to the interstitium (Lloyd et al., 1993). There, it acts presynaptically to reduce Ca^{2+} influx, thus inhibiting the release of excitatory and potentially excitotoxic neurotransmitters such as glutamate. This is of particular importance in cerebral ischaemia, during which glutamate can cause cellular damage by impairing intracellular Ca^{2+} homeostasis via

activation of metabotropic receptors and by inducing uncontrolled membrane depolarization via activation of ionotropic N-methyl-D-aspartate (NMDA) receptors. In addition, adenosine acts postsynaptically to increase K^+ conductance, thereby alleviating excessive membrane depolarization which would otherwise lead to increased NMDA receptor-mediated Ca^{2+} influx and eventual excitotoxicity. Recent studies have examined the effects of the A_3 receptor agonist IB-MECA on neuronal survival following cerebral ischaemia and chemically induced seizures in the rat. Chronic administration of the A_3 agonist resulted in a significant increase in post-trauma neuronal survival, suggesting the A_3 receptor as an interesting new target for purinergic therapies (von Lubitz et al., 1995).

It is therefore generally accepted that adenosine is capable of exerting significant neuroprotective effects in the CNS. Ischaemic damage in brain tissue is caused in part by a pathologically elevated release of excitatory amino acids (EAA's) such as glutamate, which triggers excessive entry of Ca^{2+} into nearby cells, and, eventually, cell death. The mechanism for such excitotoxicity in central neurons has been well documented (Beal, 1992). Pharmacological studies have demonstrated the existence of four distinct classes of EAA receptors in mammalian brain: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, NMDA and a quisqualate-sensitive metabotropic site (Monaghan et al., 1989; Young and Fagg, 1990). Binding of an agonist to AMPA and kainate receptors triggers fast synaptic transmission via ionotropic channels. NMDA receptors are gated by Mg^{2+},

and mediate Ca^{2+} and Na^{+} ion flux. Finally, the metabotrobic EAA receptor appears to mediate release of intracellular calcium via activation of phospholipase C through a G-protein. Thus pathophysiological levels of glutamate achieved during cerebral ischaemia can trigger a cycle of neuronal hyperexcitability and Ca^{2+} release, the eventual consequence of which is cell death.

While the predominant neurotransmitter pathways in the ENS have traditionally been identified as cholinergic and tachykininergic, the potential for a mechanism for excitotoxicity to occur under ischaemic conditions arises. Early evidence that EAAs might play a role in enteric neurotransmission was obtained by Taniyama et al. (1982), who confirmed the endogenous presence of both γ-aminobutyric acid (GABA) and its synthesizing enzyme glutamic acid decarboxylase (GAD) in the myenteric plexus of cat colon. This suggested the possibility that GABA was synthesized and localized in the myenteric plexus of mammalian gut, and that it served as a neurotransmitter in the system. Furthermore, it generated speculation as to a more generalized function for EAAs in the mediation of overall excitability in the small intestine. The application of EAA receptor agonists to isolated strips of guinea pig LMMP in several subsequent studies produced significant muscle contraction, which could be blocked by application of NMDA receptor antagonists and Mg^{2+} (Moroni et al., 1986; Luzzi et al., 1988; Shannon and Sawyer, 1989; Moroni et al., 1989). Kainic acid and quisqualic acid were found to be inactive in these preparations (Luzzi et al., 1988). On the other hand, NMDA-

mediated contractions were potentiated by micromolar concentrations of glycine, and non-competitively antagonized by kynurenic acid, suggesting that both compounds act at the same site on the NMDA receptor, but produce opposite effects on ion channel function (Moroni et al., 1989). While these studies were crucial in identifying the functional presence of NMDA receptors in the myenteric plexus, they did not address the specific mechanism for apparent NMDA-mediated LMMP contraction. The possibility that contractions produced by L-Glutamate and NMDA were the result of activation of a cholinergic pathway was investigated by Wiley et al. (1991). This group confirmed that EAA-mediated contractions of LMMP were in fact blocked by atropine and TTX, suggesting interaction with a cholinergic pathway. Furthermore, addition of the adenylate cyclase inhibitor 2', 5' - dideoxyadenosine (DDA) antagonized the actions of L-Glutamate, supporting the possibility that EAAs induce release of acetylcholine via a cAMP-dependent pathway and that glutamate may act as an excitatory neurotransmitter in the myenteric plexus. A recent study conducted by Kirchgessner et al. (1997) demonstrated the presence of glutamate-immunoreactive neurons in cultured guinea pig myenteric ganglia, a subset of which expressed NMDA, AMPA and kainate receptor subunits. Prolonged exposure to glutamate induced necrosis and apoptosis in both acute and cultured preparations of enteric neurons, although the cultured neurons displayed a greater sensitivity to the EAA (presumably due to derangement of EAA transporter function). NMDA caused similar neurotoxicity, which was

blocked by the NMDA antagonist D-2-amino-phosphonopentanoate (AP5). Although the concentration of glutamate required to produce cell death was relatively high in the acute preparations (3mM), the study provides preliminary evidence for the existence of excitotoxicity in the ENS. A modulatory role for adenosine under these circumstances has not yet been characterized, but it is tempting to speculate that if the mechanism for excitotoxicity in the ENS mimics that demonstrated in the CNS, then perhaps the possibility of purinergic neuroprotection becomes an appropriate avenue for investigation.

Objectives

Although more attention has been devoted to studies in the CNS, there is growing evidence that adenosine may play a cytoprotective role in the small intestine (Grisham et al., 1989; Kaminski and Proctor, 1992). Transient intestinal ischaemia is a known cause of tissue edema, morphologic damage and diminished intestinal function. Furthermore, inflammatory mediators and cardiodepressive moieties released into circulation during reperfusion can cause additional injury (Schoenberg and Beger, 1993), leading to irreversible circulatory dysfunction and multi-organ failure (Thompson, 1995). Mesenteric ischaemia can be produced by atherosclerosis, especially in the elderly, and ischaemic derangement of enteric neural function has been implicated in the pathology of inflammatory bowel diseases such as Crohn's disease (Wakefield et al., 1989). Hebra et al. (1993) report that ischaemia produced

increases in duration, and decreases in cycling velocity of the piglet interdigestive migrating motor complex (MMC), a reliable index of intestinal motility and function. The neural mechanisms underlying such changes during ischaemia (and reperfusion) are of great importance during small intestinal transplantation. Quigley et al. (1990) have reported that jejuno-ileal autotransplantation in dogs produced abnormal myoelectric patterns in the transplanted segment which did not recover with time. Motor disruption following small intestinal transplantation in humans has also been reported (Sarr, 1996).

If ischaemia-induced toxicity occurs in enteric neurons under such circumstances, then the possibility exists that adenosine may exert neuroprotective effects in the ENS. For endogenous adenosine to serve such a function, it would necessarily have to be present in the enteric plexuses under ischaemic or hypoxic conditions in functionally relevant concentrations. Furthermore, the interstitial concentration of the nucleoside should be responsive to increasing degrees of ischaemic/hypoxic insults. Milusheva et al. (1990) showed that the hypoxia-induced reduction of [^{3}H]-ACh release could be partially reversed by theophylline, a non-selective adenosine receptor antagonist, suggesting that at least some of the original reduction in transmitter release was due to increased levels of endogenous adenosine. Studies in this laboratory have recently shown that interstitial adenosine is present in perifused guinea pig myenteric neural networks, as measured by the increased release of the excitatory transmitter Substance P (Substance P-

like immunoreactivity, SPLI) in the presence of the selective A_1 receptor antagonist DPCPX (Moneta et al., 1997). A primary objective of the present studies was therefore to determine whether the concentration of this interstitial endogenous adenosine varied with increasing degrees of hypoxia in the predicted inverse manner. In previous studies utilising this technique to examine the properties of adenosine in the ENS, the isolated ganglion networks were depolarized using an elevated extracellular K^+ concentration to evoke neurotransmitter release (Moneta et al., 1997). An additional objective of the present study was therefore to examine the changes occurring in the absence of any exogenous depolarizing agents, and determine the relationship between *spontaneous* release of SPLI and prevailing pO_2, since spontaneous release of transmitters and modulators might provide a more accurate model of neurotransmission and modulation in the myenteric plexus. Functional evidence was also sought in support of a neural source for the released endogenous adenosine.

Moneta (1996) had previously shown that, while ATP, β,γ-MeATP (a putative P_{2X} receptor agonist) and ATPγS (a selective $P_{2X2/4}$ receptor agonist) caused a reduction in evoked SPLI release, perifusion in the presence of the selective P_{2X3} agonist 2-MeSATP caused an increase in evoked SPLI. A further objective was therefore to expand this finding, and determine the nature of the 2-MeSATP-mediated effects. A final objective was to obtain preliminary data characterizing the functional effects of NMDA receptor

agonists and antagonists on isolated myenteric neural networks, and to assess putative modulation by endogenous interstitial adenosine.

METHODS

Preparation of isolated myenteric neural networks

The procedure used to obtain isolated myenteric neural networks from the guinea pig ileum was as described by Moneta et al. (1997). Briefly, the longitudinal muscle-myenteric plexus (LMMP) of one guinea pig (Hartley, 250-300 g, either sex, Charles River, Montreal, Quebec, Canada) was stripped and placed into Locke's solution of the following composition (in mM): 140 NaCl, 5 KCl, 5 $NaHCO_3$, 1 $MgCl_2$, 2.54 $CaCl_2$, 10 dextrose, 10 *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 0.1% bovine serum albumin, adjusted to pH 7.2 with NaOH. The LMMP strips were cut into 0.5cm lengths and placed in 30ml Locke's solution with the following composition (in mg/100 ml): 167 collagenase (Type IA), 133 protease (Type IX from Bacillus polymyxa), 16 deoxyribonuclease I, and 30 bovine serum albumin (fraction V). The mixture was shaken in a warm water bath (37°C) for 30 minutes, then centrifuged at 1000 x g for 10 minutes. The supernatant was removed and the pellet resuspended by gentle trituration with Locke's solution at 4°C. Ganglion networks were obtained by passing the solution through successive stainless steel mesh screens of 30, 50 and 150 mesh. The larger networks were trapped on the 50 mesh screen and the smaller networks on the 150 mesh screen. Muscle tissue and other large debris was trapped by the 30 mesh screen and discarded. The networks were washed

off the screens using Locke's solution, pooled and loaded onto 2.5 cm glass fibre filter disks (Whatman GF/F), placed in parallel perifusion chambers (Swinex filter holders, Millipore, Bedford, MA), and perifused with Locke's solution at 0.5 ml/min using a nonpulsatile roller pump (Gilson Minipuls 3, Mandel Scientific, Rockwood, ON, Canada). The temperature of the chambers, reservoirs and tubing was maintained at 37°C by immersion a large-volume water bath.

The perifusate collected during the first 28 minutes was discarded to allow for equilibration. Subsequently, 1ml samples of perifusate were collected every 2 minutes in 1.5 ml Eppendorf tubes using a fraction collector, were heated to 98°C for 4 minutes, and stored at -40°C until assayed for substance P-like immunoreactivity. In addition, total SPLI content of the tissue on each filter was measured by heating the filter disk to 98°C in 1 ml of water for 4 minutes. The tube was then centrifuged at 13, 500 g for 3 minutes, the supernatant removed and stored at -40°C until assayed.

Experimental protocols

Evoked release of SPLI was achieved by switching the perifusing solution from normal Locke's solution to one containing an isotonically elevated K^+ concentration (95 mM, $\uparrow[K^+]_o$). Perifusing solutions, either normoxic (ungassed) or maintained with altered partial pressures of dissolved

oxygen (pO_2), contained all necessary drugs appropriate for each experiment. Doses for DPCPX, TTX, 2-MeSATP, and PPADS were those demonstrated to have been efficacious in previous studies utilising this protocol (Moneta, 1996; Moneta et al., 1997). All experiments were performed using two chambers perifused in parallel, one serving as a control for the other.

Altered pO_2 was achieved using various O_2/N_2 gas mixtures. Locke's solutions were equilibrated by bubbling for 30 minutes prior to each experiment with the appropriate gas mixture which was also maintained in the headspace above each reservoir throughout each experiment. "Hypoxic" conditions were created by bubbling with 100% N_2, "Hyperoxic" conditions with 100% O_2, and intermediate oxygen tensions with 10% O_2, 20% O_2, 30% O_2 and 50% O_2. (the remainder being N_2). Solutions were equilibrated by bubbling with the gas(es) for 30 mins. using a glass wand fitted with a sintered glass disc and were then transferred to the appropriate reservoirs. The solutions were maintained at a constant pO_2 using a slow bleed of the relevant gas mixture into the headspace throughout the experiment. Direct measurement of the solution pO_2 was carried out after 1 hour of storage in the reservoir, a time corresponding approximately to the middle of the experimental period. pO_2 measurements were obtained by injecting a small sample of the perifusing medium (approximately 1ml) into an I-Stat (EG7+) Portable Clinical Analyzer cartridge and inserting the loaded cartridge into the analysis unit. The conditions prevailing during experiments using ungassed

Locke's solution were, for convenience, referred to as "Normoxic", notwithstanding the acknowledged small discrepancy between the pO_2 of such solutions and that of fully oxygenated blood.

Radioimmunoassay

The RIA procedure used in the present study has been outlined previously by Brodin et al. (1986). The SP antiserum SP-2 (kindly supplied by Dr. E. Brodin, Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden), at a final dilution of 1:35 000 detects 0.3 fmol per assay tube (10% drop from initial binding) and cross reacts with neurokinin A at 0.001% compared with a 100% reaction with SP.

The protocol used for SP RIA was as follows: Aliquots of perfusate (duplicates) and standard SP (triplicates; concentrations ranging from 3.125 – 1600 fmol) were incubated with SP-2 antibody and ^{125}I-labelled SP for 72 – 96 hr at 4°C in buffer of the following composition (in mM): Na_2HPO_4, 50; NaN_3, 50; EDTA, 10. Peptone (0.5%) was added to prevent peptide binding to glass surfaces. The assay also included standard blanks (buffer and tracer) and zeros (buffer and tracer and antiserum). After the incubation period, human plasma (final concentration 5% of total volume) and a mixture of alkaline charcoal (0.28%) and dextran (0.028%) made in peptone-free buffer were added, immediately vortexed and centrifuged at 1000 x g for 30 minutes at 4°C. The resulting pellet containing unbound (free) tracer and supernatant

containing antibody-bound tracer were separated by decanting and counted for gamma radiation (LKB-Wallac RackGamma II, Wallac Oy, Turku, Finland). The concentration of ^{125}I-labelled SP was adjusted to 2700 counts per min per assay tube to achieve a bound/free ratio approximately equal to one for the standard blanks.

RIA standard curves were analysed using a log-logit transformation. The logit of the bound-to-free ratios, (log[(B/F)/{K – (B/F)}]), was then plotted against the log of the concentration of standard peptide and a straight line fitted by least squares linear regression (K is a constant chosen to minimize deviation from linearity). Only standard curve data points which deviated from the regression line by less than 10% were accepted and used to interpolate the immunoreactivity present in the samples. Standards were assayed in triplicate, while samples were assayed in duplicate.

Data analysis

As described by Broad et al. (1993), evoked release of SPLI was determined by subtracting the mean SPLI obtained in the five fractions immediately preceding the measured period (i.e. mean unstimulated release) from the levels obtained during the measured period (41-53 minutes). The Cumulative Evoked Release (CER) of SPLI was determined by summing the net release and normalizing it to the total SPLI content on the filter disks. This permitted an integral of the area under the release profile for all points

above that of the mean basal release to be determined. Spontaneous SPLI release was calculated as the Cumulative Basal Release (CBR) which represented the integral of SPLI release obtained during the unstimulated period (29-39 minutes) prior to addition of $\uparrow[K^+]_o$, normalized to the total SPLI content. Algebraically, the CBR was calculated as:

$$C.B.R. = \sum_{t=i}^{j} \frac{R_t}{C_f} \times 100/10 \quad (\%.min^{-1})$$

where R_t is SPLI released at time t, i is 29 min, j is 39 min, and C_f is total content on the filter. As reported by Moneta et al. (1997), CER was calculated as:

$$C.E.R. = \sum_{t=i}^{j} \frac{\left(R_t - \bar{\upsilon}\right)}{C_f} \times 100/14 \quad (\%.min^{-1})$$

where R_t is SPLI released at time t, i = 41 min, j = 53 min, upsilon bar is mean unstimulated release, and C_f is total SPLI content on the filter. Each experiment was carried out using tissue from separate animals, and data from each experiment were pooled with equivalent data from at least three separate experiments. Statistical analysis of SPLI release under control and experimental conditions was performed using a two-tailed Student's t-test for single parameters (Graph Pad Instat, GraphPad Software, San Diego, CA, and Microsoft Excel '97, Microsoft Corporation, Seattle, WA). Paired

Student's t tests were also performed as appropriate. Statistical significance was accepted at $p<0.05$.

Materials

With the following exceptions, all chemicals and reagents were obtained from BDH (Toronto, ON). *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), collagenase (type I), protease (type IX), deoxyribonuclease I and tetrodotoxin (TTX) were obtained from Sigma Chemical (St. Louis, MO). 1,-3,-dipropyl-8-cyclopentylxanthine (DPCPX), 2-methylthio-adenosine 5'-triphosphate (2-MeSATP), pyridoxalphosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS), L-glutamate, and N-methyl-D-aspartate (NMDA) were obtained from Research Biochemicals (Natick, MA). LY 235959 was obtained from Tocris Cookson Inc. (St. Louis, MO). ^{125}I-substance P was purchased from DuPont (Markham, ON). All other reagents used for RIA were detailed by Brodin et al. (1986). Solution pO_2 measurements were obtained using an I-Stat (EG7+) Portable Clinical Analyzer (Hewlett Packard, Princeton, N.J.).

RESULTS

Release of SPLI from perifused myenteric ganglion networks under hypoxic and hyperoxic conditions in the absence and presence of DPCPX

Perifusion of isolated enteric ganglion networks with Locke's solution equilibrated with 100% N_2 yielded a significant reduction in both spontaneous and K^+-evoked SPLI release compared to control release. The CER and CBR values are given in Table 1 and the corresponding perifusion profiles are shown in Figure 3*A*. Perifusion of the networks with Locke's solution equilibrated with 100% N_2 in the additional presence of DPCPX (5 μM) resulted in a significant increase in both spontaneous and K^+-evoked SPLI release compared to that in the absence of the adenosine A_1 receptor antagonist. The CER and CBR values are shown in Table 1 and the corresponding perifusion profiles are shown in Figure 3*B*.

Perifusion of networks in Locke's solution equilibrated with 100% O_2 yielded a significant increase in K^+-evoked SPLI release while spontaneous release was not significantly altered. Perifusion in Locke's solution equilibrated with 100% O_2 in the additional presence of DPCPX (5 μM) did not alter either the spontaneous or K^+-evoked release of SPLI. The CER and CBR values are

given in Table 1 and the corresponding perifusion profiles are shown in Figure 8*A* and *B*.

Release of SPLI from perifused myenteric ganglion networks under conditions of increasing pO_2 in the absence and presence of DPCPX.

Perifusion of networks in Locke's solutions equilibrated with 10%, 20%, 30% or 50% O_2 yielded the spontaneous and K^+-evoked SPLI release shown by the appropriate CBR and CER values in Table 1. The perifusion profiles corresponding to 10% O_2, 20% O_2, 30% O_2 and 50% O_2 are shown in Figures 4*A*, 5*A*, 6*A*, and 7*A* respectively. The CBR and CER values obtained for perifusion under these conditions in the additional presence of DPCPX (5μM) are also shown in Table 1. The perifusion profiles corresponding to all these conditions in the additional presence of DPCPX are shown in Figure 4*B*, 5*B*, 6*B* and 7*B* respectively.

Comparison of spontaneous and K^+-evoked SPLI release values for each condition within the range of oxygen tensions and expressed as a percentage of a normoxic (ungassed) control is shown in Figure 9*A* and *B*. The histogram includes a linear regression analysis with 95% confidence intervals. Measured pO_2 values for Locke's solutions equilibrated with 100% N_2 , 10%,

20%, 30%, 50% and 100% O_2, and for ungassed solution are also shown in Figure 9*A inset.*

Comparison of the release values for spontaneous and $\uparrow[K^+]_o$-evoked SPLI release in the presence of DPCPX expressed as a percentage of control values, for each condition within the range of oxygen tensions used, is shown in Figure 10*A* and *B*. The scale of the abscissa has been reversed in order to present an increasing degree of hypoxia along the usual sense of the axis. A threshold for the increment in release is clearly present for networks perifused with Locke's solution equilibrated with between 30% and 20% O_2, corresponding to pO_2 values of between 260 and 146 mm Hg.

Release of SPLI from perifused myenteric ganglion networks under normoxic and hypoxic conditions in the absence and presence of tetrodotoxin and, under hypoxic conditions, with or without the additional presence of DPCPX.

Perifusion of the networks under normoxic conditions in the absence and presence of 0.1 μM TTX showed that TTX produced a significant increase in evoked SPLI release, while spontaneous release was unaffected. The CER and CBR values are given in Table 1 and the corresponding perifusion profiles are shown in Figure 11. Interestingly, perifusion of networks with

either normoxic Locke's solution or with Locke's solution equilibrated with 100% N_2 in the absence or presence of 1 μM TTX showed that TTX yielded a significant reduction in spontaneous SPLI release while K^+-evoked SPLI release was not significantly altered. The CER and CBR values are given in Table 1 and the corresponding perifusion profiles are shown in Figures 12 and 13. Perifusion with Locke's solution equilibrated with 100% N_2 and containing TTX (1 μM) in the absence or presence of DPCPX (5 μM) showed that the antagonist produced significant increases in the CER and CBR. The values are given in Table 1 and the corresponding perifusion profiles are shown in Figure 14. Graphical comparisons of these data are shown in Figures 15 and 16. The CBR and CER values for spontaneous and K^+-evoked SPLI release under normoxic and hypoxic conditions in the absence and presence of TTX are shown in Fig 15*A* and 15*B*. For the hypoxic condition, the CER values in the absence and presence of TTX (1 μM) and/or DPCPX (5 μM), are shown in Figure 16.

Release of SPLI from isolated myenteric ganglion networks in the presence and absence of the P_{2X3} receptor agonist 2-MeSATP, the nonselective P_{2X} antagonist PPADS, and DPCPX.

Perifusion of the networks in the presence of the selective P_{2X3} receptor agonist 2-MeSATP (1μM) resulted in a significant increase in evoked SPLI release, while spontaneous release was unaffected. The additional presence of 5 μM DPCPX yielded no significant change in evoked release of SPLI, and produced a small but significant increase in spontaneous release, relative to perifusion with 2-MeSATP alone. The CER and CBR values for both perifusion with 2-MeSATP alone, and in the additional presence of DPCPX, are given in Table 1, and the corresponding perifusion profiles are shown in Figures 17*A* and *B*, respectively.

Perifusion of the networks in the presence of the nonselective P_{2X} antagonist PPADS produced a significant increase in evoked release of SPLI, but did not significantly alter spontaneous release. The CER and CBR values are given in Table 1 and the perifusion profiles are shown in Figure 18. When the networks were perifused in the presence of 1 μM 2-MeSATP and in the additional presence of 10 μM PPADS, the antagonist yielded a significant reduction in evoked SPLI release and no significant change in spontaneous release relative to perifusion with the agonist alone. The CER and CBR

values are given in Table 1, and the corresponding perifusion profile is shown in Figure 19. Perifusion of the networks with 2-MeSATP (1μM) and PPADS (10μM) in the absence and presence of DPCPX (5μM) showed that the A_1 receptor antagonist produced a significant increase in evoked SPLI release, while spontaneous release remained unaffected. The CER and CBR values are given in Table 1 and the corresponding perifusion profiles are shown in Figure 20.

Release of SPLI from isolated myenteric ganglion networks perifused in the absence and presence of the excitatory amino acids NMDA and glutamate, the NMDA receptor antagonist LY 235959, and DPCPX.

Perifusion of the networks in the presence of the excitatory amino acid NMDA (10μM) produced a small but significant decrease in evoked SPLI release, but did not significantly alter spontaneous release. Perifusion in the additional presence of DPCPX (5μM) yielded significant increases in both evoked and spontaneous release of SPLI, relative to perifusion with NMDA alone. The CER and CBR values for both perifusion with NMDA alone, and in the additional presence of DPCPX, are given in Table 1, and the corresponding perifusion profiles are shown in Figure 21*A* and *B*, respectively.

Perifusion of the networks with the NMDA receptor antagonist LY 235959 (10μM) produced no significant change in SPLI release. Likewise, perifusion in the presence of the excitatory amino acid glutamate (100μM) did not effect any significant change on either evoked or spontaneous release of SPLI. The CER and CBR values for perifusion both in the presence of LY 235959 and in the presence of glutamate are given in Table 1, and the corresponding perifusion profiles are shown in Figures 22 and 23, respectively.

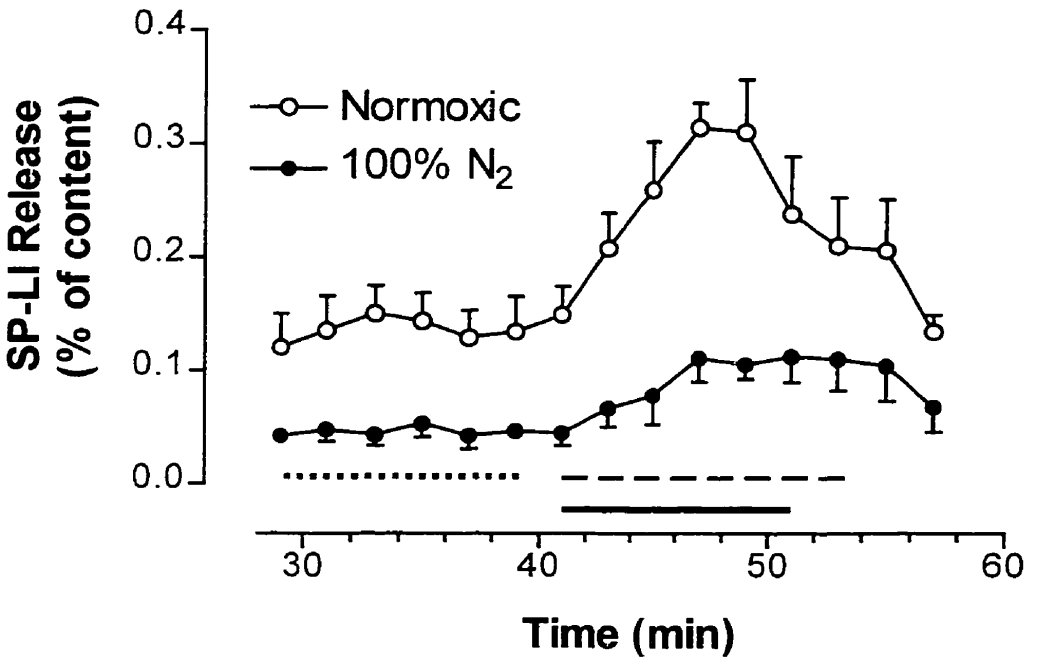


Figure 3*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 100% N_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=5 separate experiments. Perifusion with 100% N_2-Locke's solution resulted in a significantly lower cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.039$) and a significantly lower cumulative basal release (CBR) of SPLI ($p=0.038$) compared to the normoxic condition. Error bars are ±SEM.

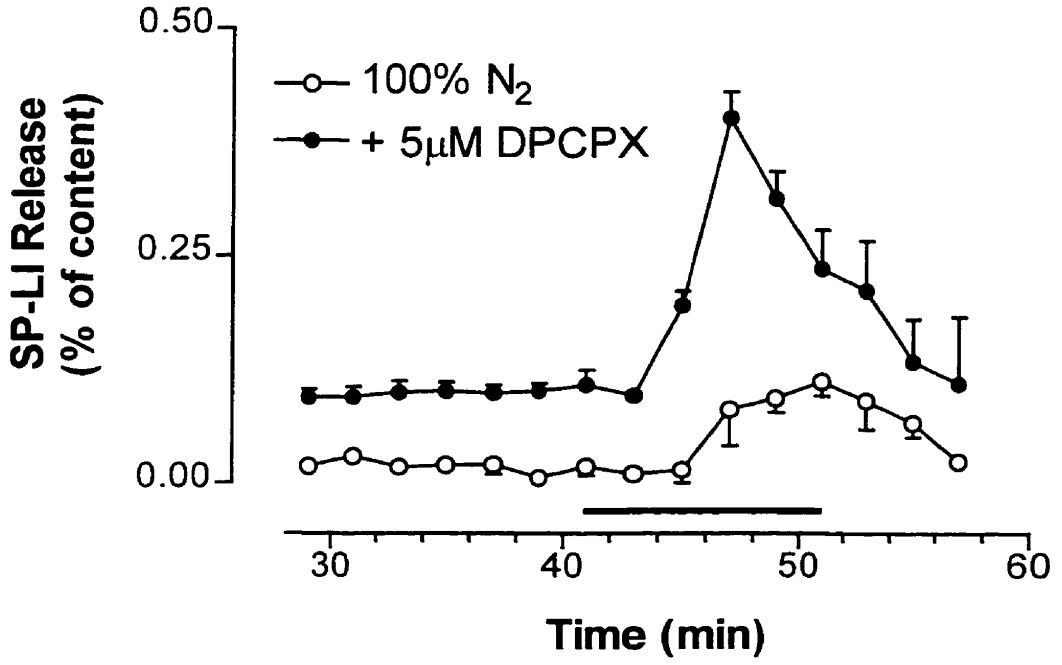


Figure 3*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 100% N_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX resulted in a significant increase in both CER ($p=0.038$) and CBR ($p=0.026$) compared to the control condition. Error bars are ±SEM.

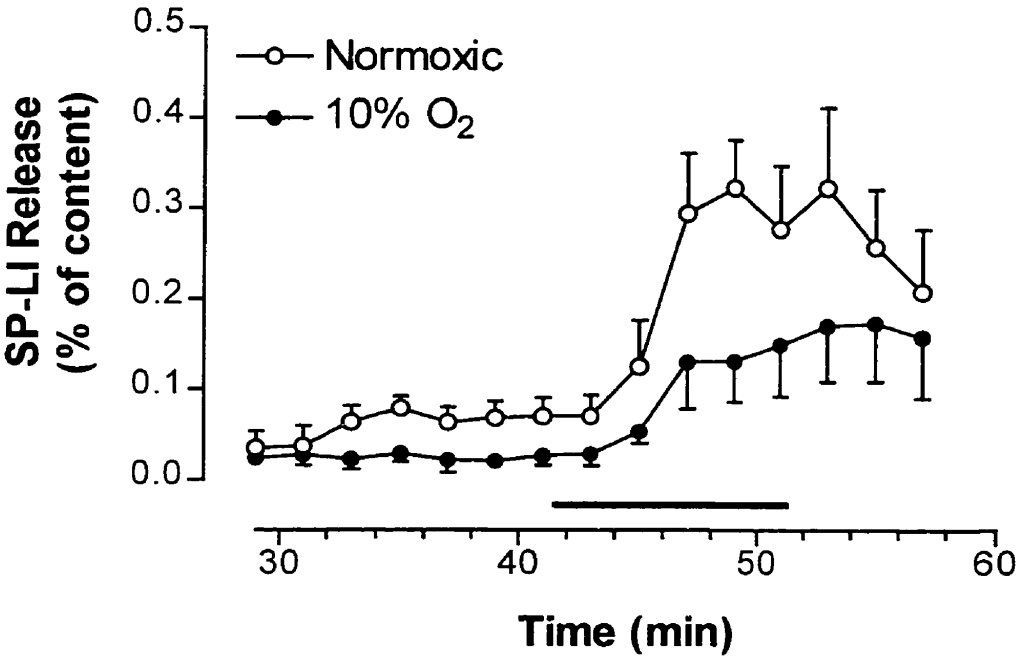


Figure 4A: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution, bubbled with 10% O_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=4 separate experiments. Perifusion with 10% O_2-Locke's solution resulted in a significantly increase in both CER (p=0.012) and CBR (p=0.019) compared to the normoxic condition. Error bars are ±SEM.

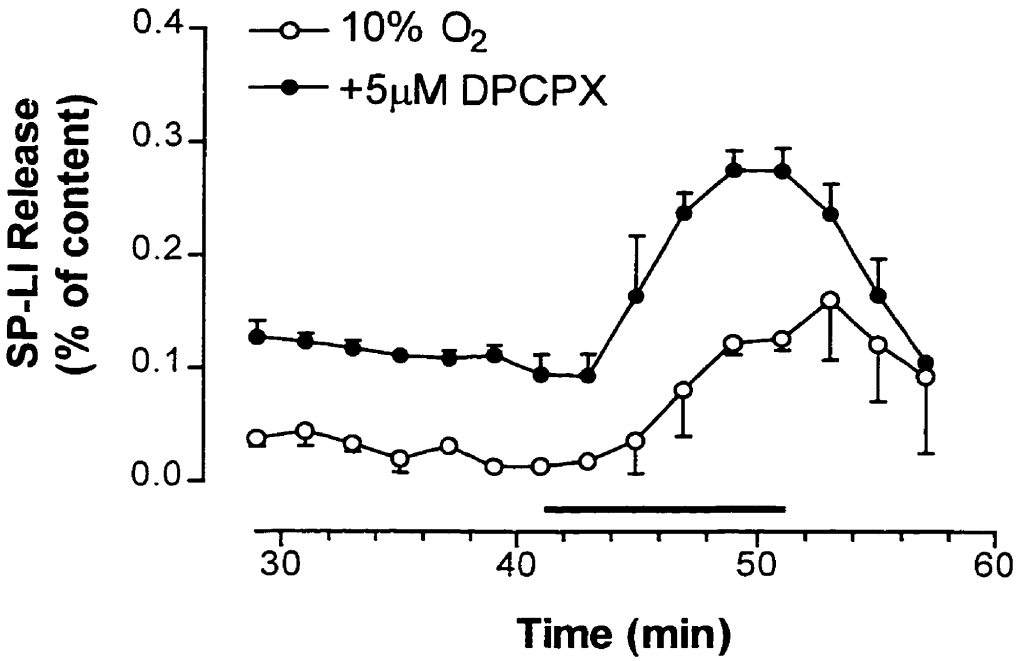


Figure 4*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 10% O_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX produced a significant increase in CBR (p=0.012), but no change in CER (p=0.244) compared to the control condition. Error bars are ±SEM.

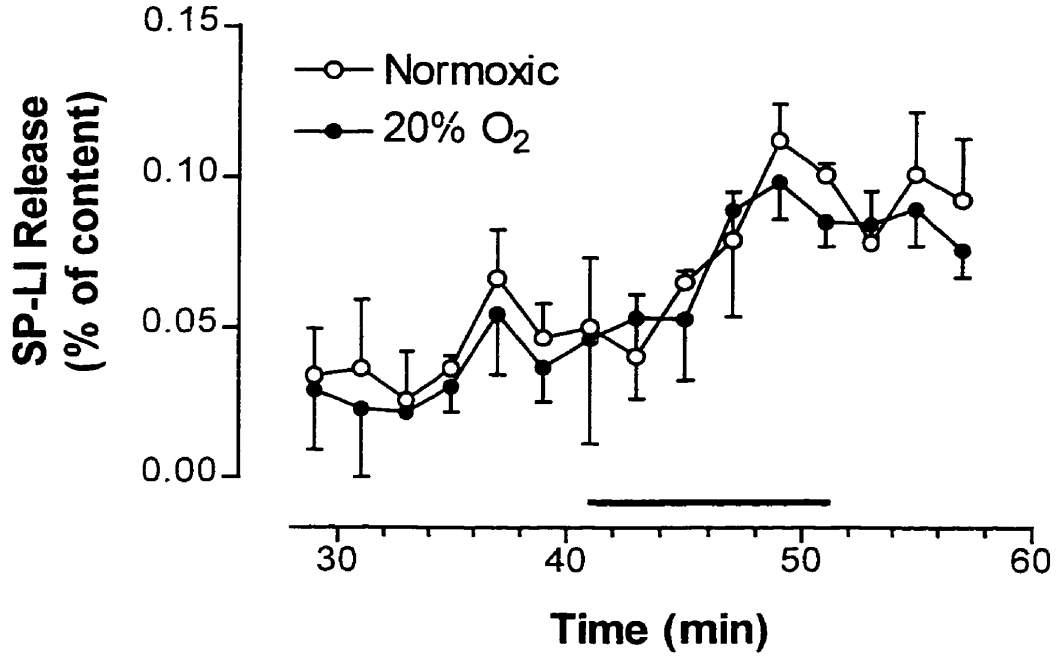


Figure 5*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 20% O_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=3 separate experiments. Perifusion with 20% O_2-Locke's solution produced no significant changes in either CER (p=0.672) or CBR (p=0.850) compared to the normoxic condition. Error bars are ±SEM.

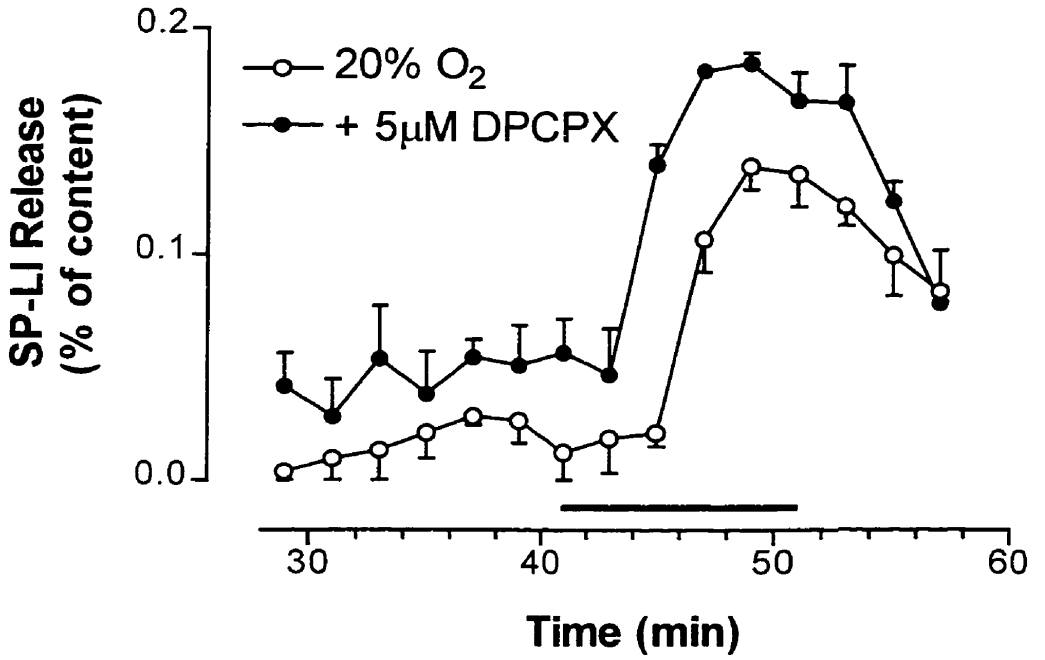


Figure 5*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 20% O_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX produced no significant changes in either CER ($p=0.111$) or CBR ($p=0.229$) compared to the control condition. Error bars are ±SEM.

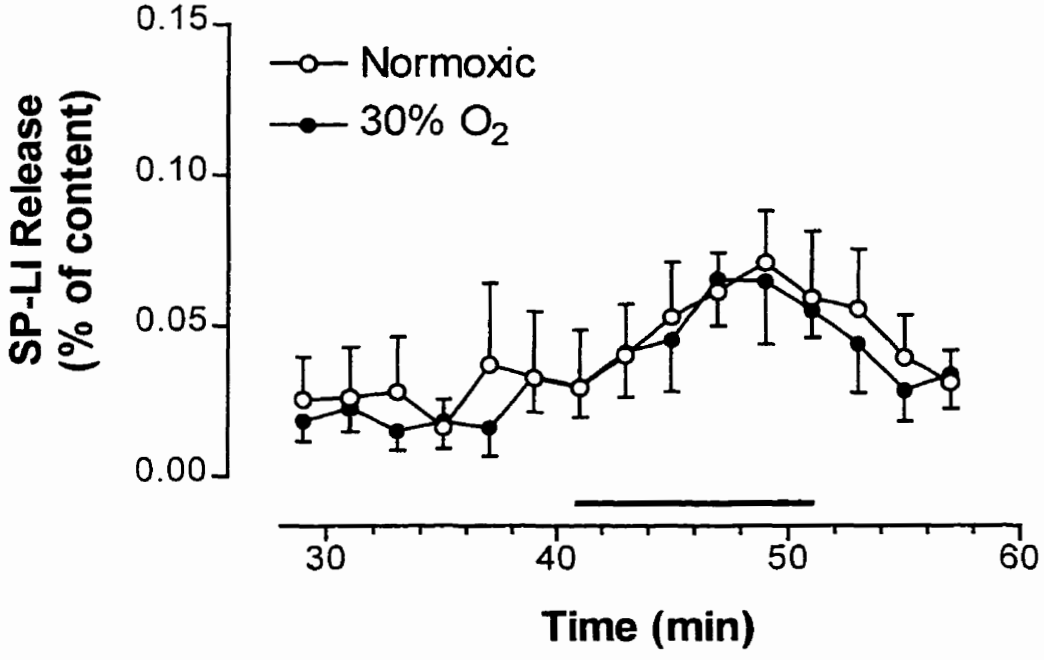


Figure 6*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution, bubbled with 30% O_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=4 separate experiments. Perifusion with 30% O_2-Locke's solution produced no significant changes in either CER (p=0.659) or CBR (p=0.577) compared to the normoxic condition. Error bars are ±SEM.

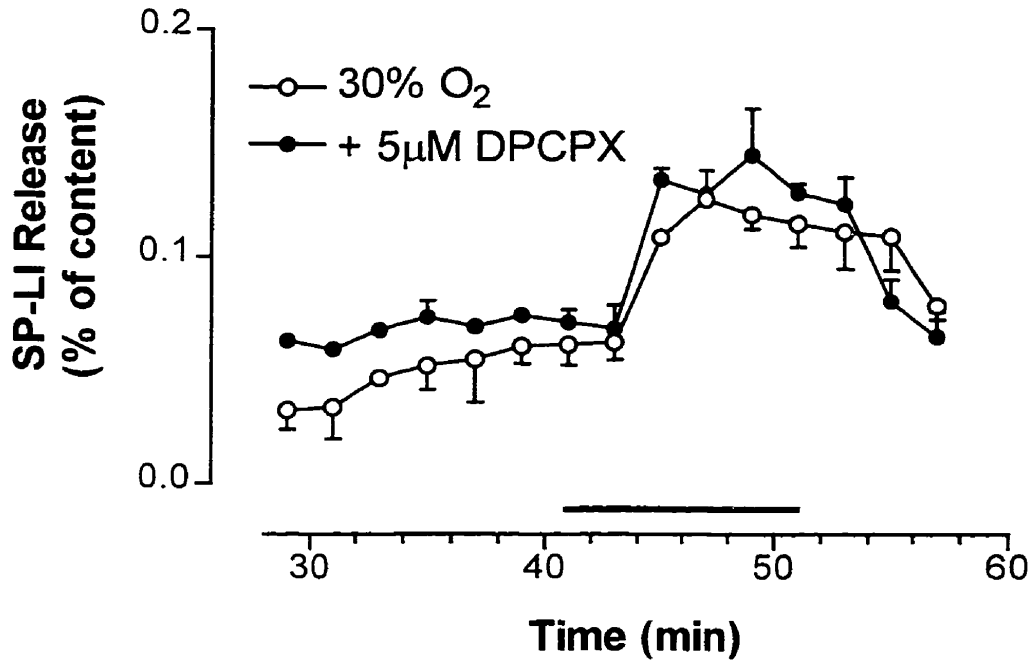


Figure 6*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 30% O_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX produced no significant changes in either CER ($p=0.163$) or CBR ($p=0.082$) compared to the control condition. Error bars are ±SEM.

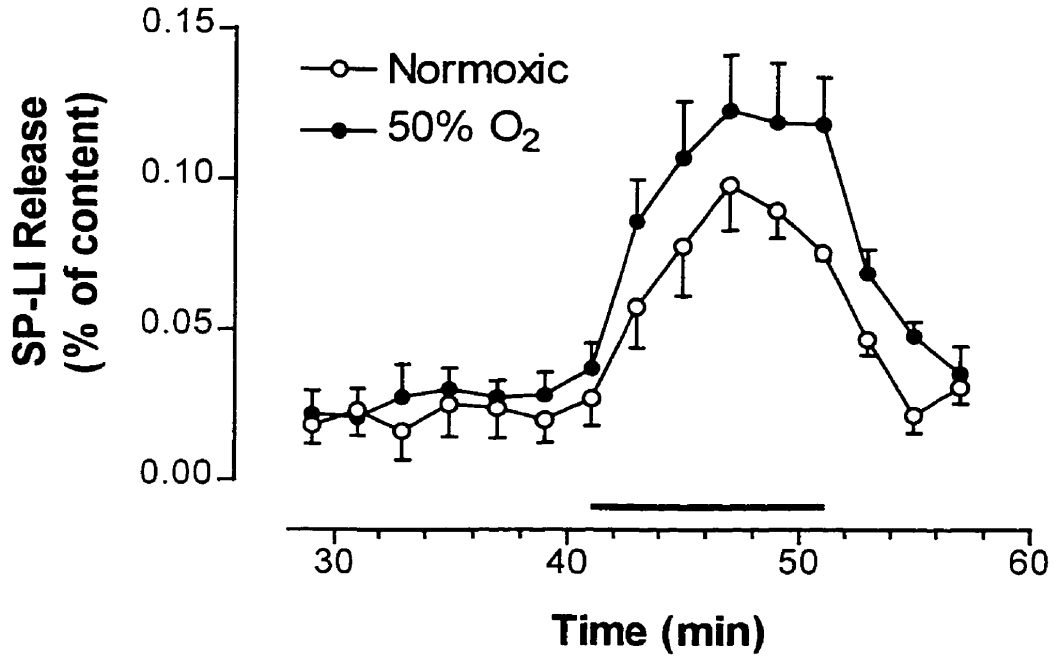


Figure 7*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution, bubbled with 50% O_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=3 separate experiments. Perifusion with 50% O_2-Locke's solution resulted in a significantly higher CER ($p=0.005$) compared to the normoxic condition. Error bars are ±SEM.

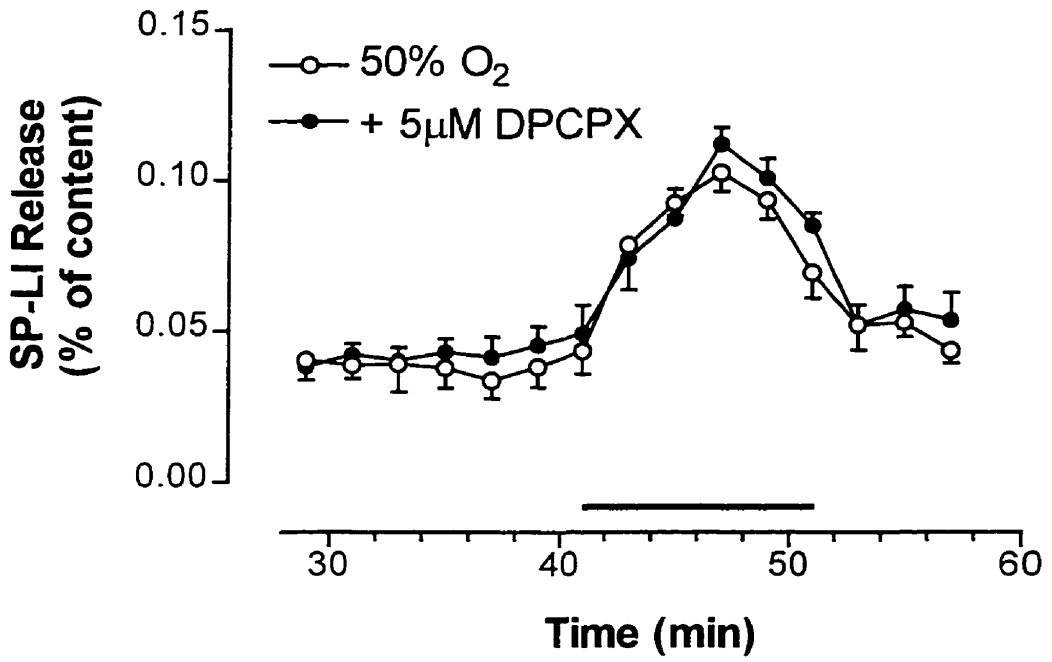


Figure 7*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$ -Locke's solution, bubbled with 50% O_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX produced no significant changes in either CER ($p=0.903$) or CBR ($p=0.342$) compared to the control condition. Error bars are ±SEM.

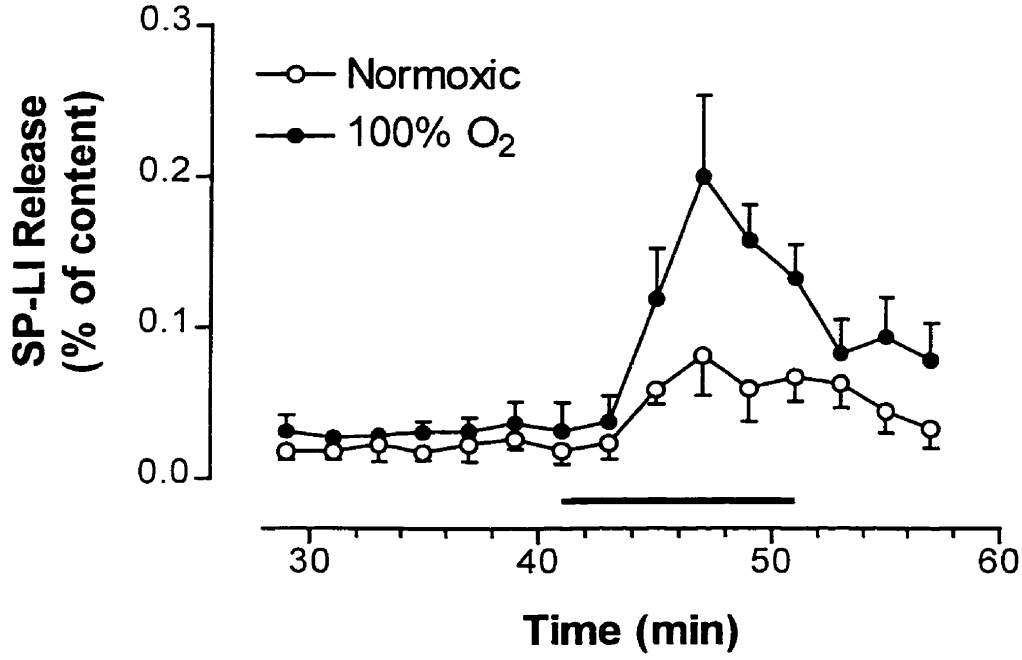


Figure 8A: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution, bubbled with 100% O_2. Solid line indicates the presence of $\uparrow[K^+]_o$. n=3 separate experiments. Perifusion with 100% O_2-Locke's solution resulted in a significantly higher CER (p=0.048) compared to the normoxic condition. Error bars are ±SEM.

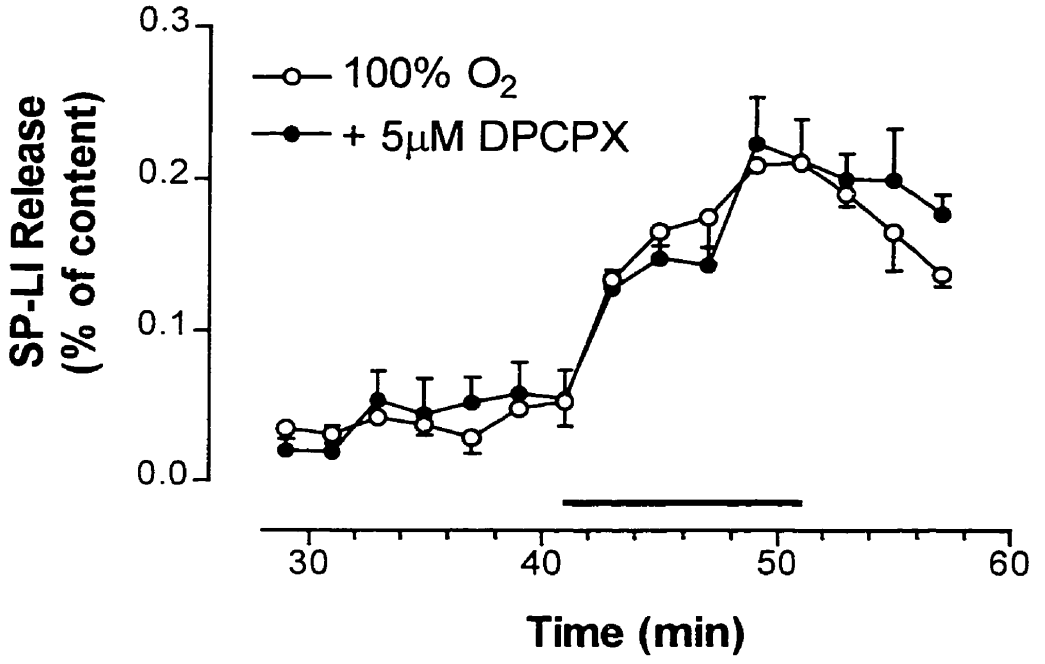


Figure 8*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution, bubbled with 100% O_2 in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion of the networks with DPCPX produced no significant changes in either CER ($p=0.482$) or CBR ($p=0.848$) compared to the control condition. Error bars are ±SEM.

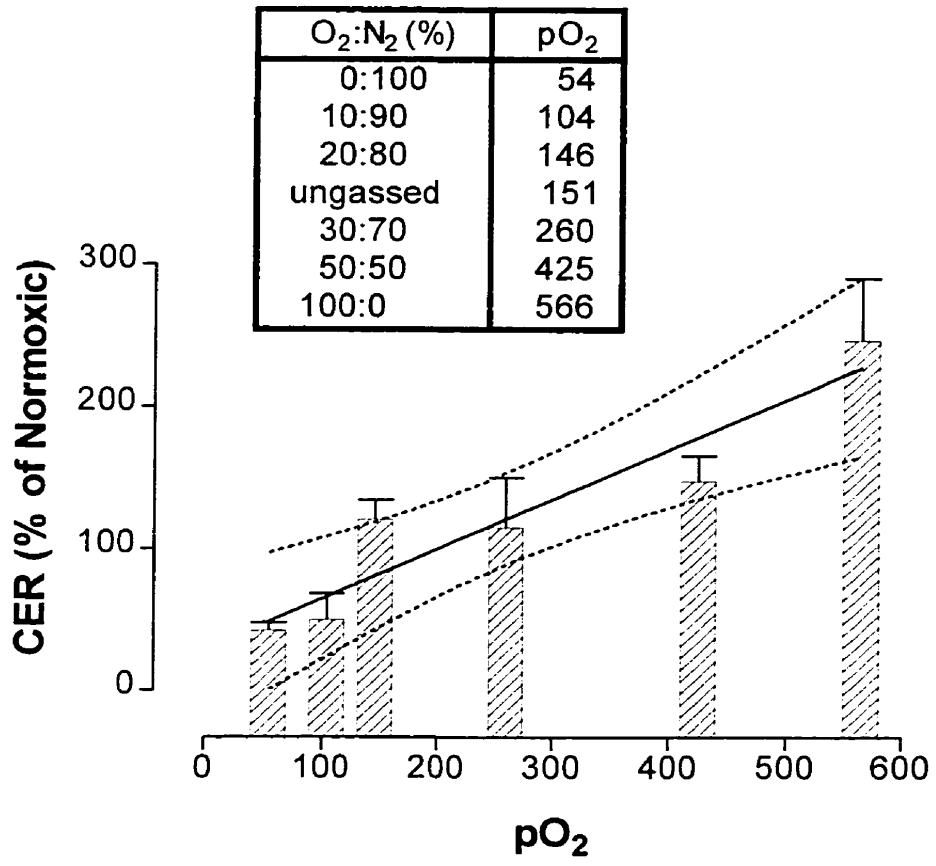


Figure 9*A*: Histogram showing the $\uparrow[K^+]_o$-evoked release (CER) of SPLI from myenteric neural networks perifused under varying oxygen tensions, expressed as percentages of $\uparrow[K^+]_o$-evoked SPLI release under normoxic conditions. The points were fitted by a positively-sloped linear regression line (with a 95% confidence interval). Note the clear correlation between pO_2 and evoked release of SP ($r^2 = 0.88$). Error bars are ±SEM.

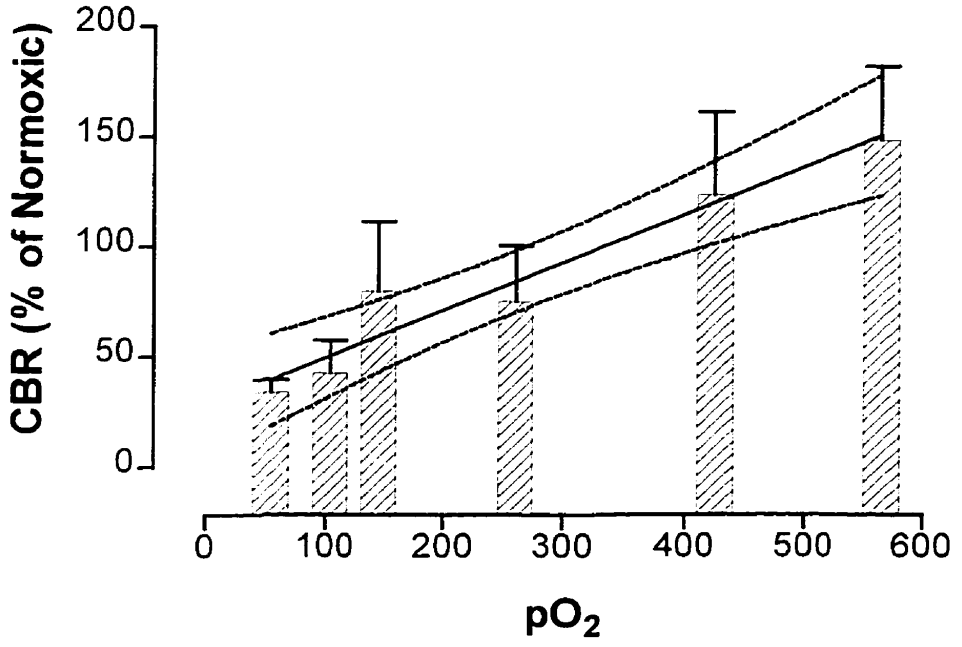


Figure 9*B*: Histogram showing the spontaneous release (CBR) of SPLI from myenteric neural networks perifused under varying oxygen tensions, expressed as percentages of spontaneous SPLI release under normoxic conditions. The points were fitted by a positively-sloped linear regression line (with a 95% confidence interval). Note the clear correlation between pO_2 and release of SP ($r^2 = 0.94$). Error bars are ±SEM.

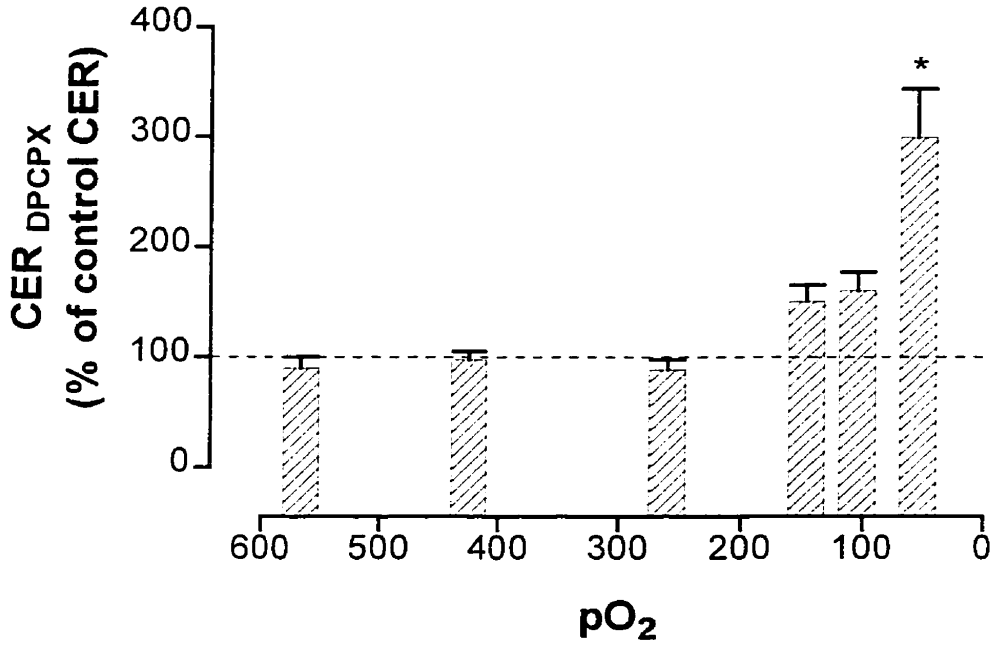


Figure 10*A*: Histogram showing the ↑$[K^+]_o$-evoked release (CER) of SPLI from myenteric neural networks perifused under varying oxygen tensions in the additional presence of 5μM DPCPX, expressed as percentages of ↑$[K^+]_o$-evoked SPLI release under control conditions. Note the inversion of the abscissa in order to display increasing hypoxia along the usual sense of the axis. * denotes significant difference from paired control in the absence of DPCPX ($p<0.05$). Error bars are ±SEM.

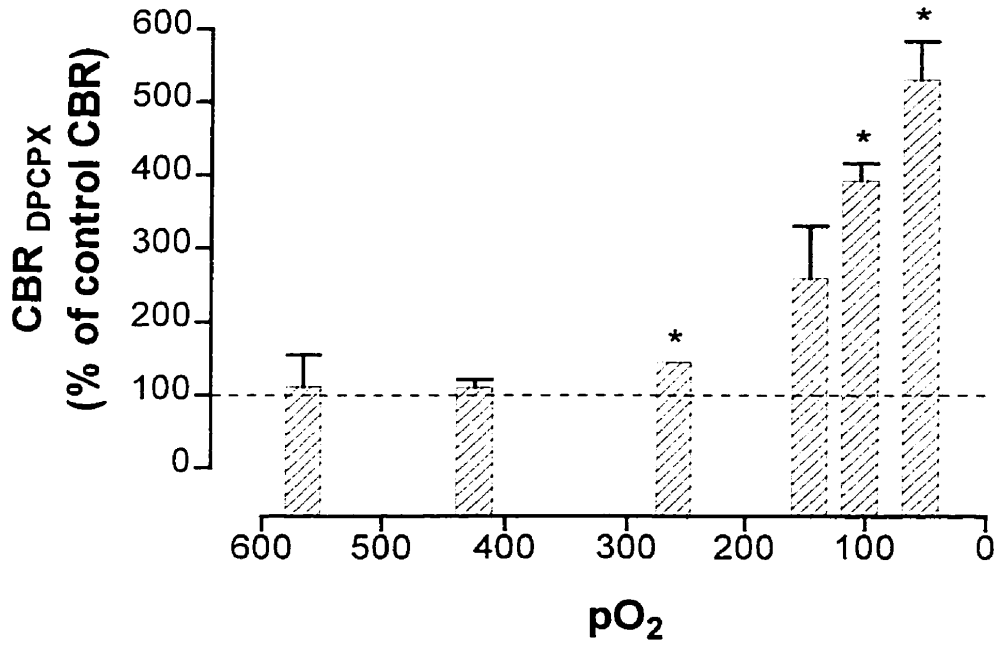


Figure 10*B*: Histogram showing the basal release (CBR) of SPLI from myenteric neural networks perifused under varying oxygen tensions in the additional presence of 5μM DPCPX, expressed as percentages of basal SPLI release under control conditions. Note the inversion of the abscissa in order to display increasing hypoxia along the usual sense of the axis. * denotes significant difference from paired control in the absence of DPCPX ($p<0.05$). Error bars are ±SEM.

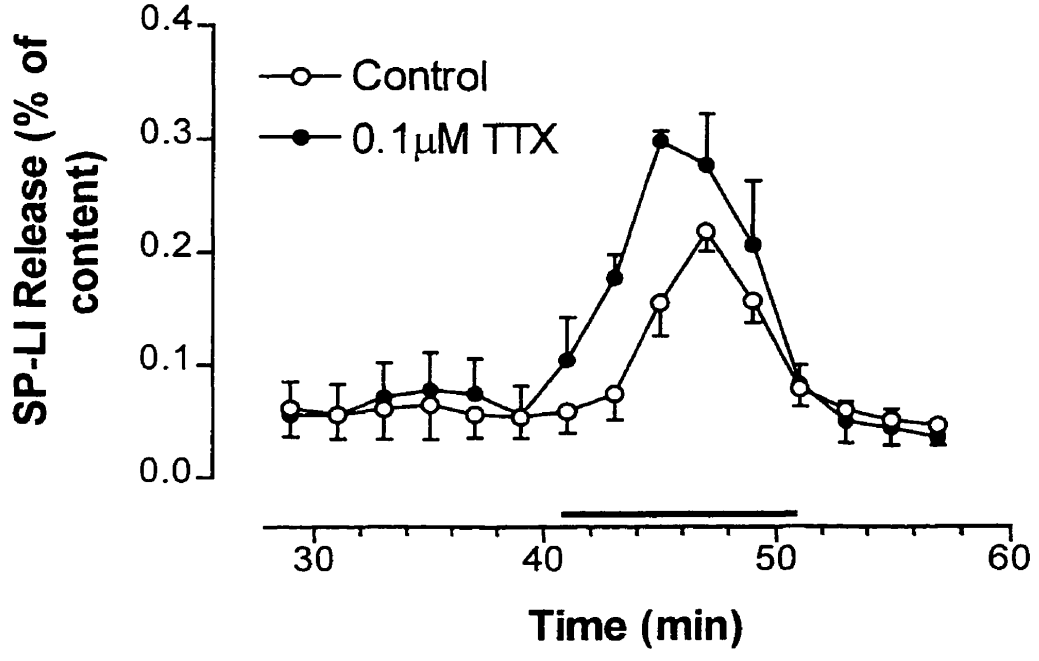


Figure 11: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the additional presence of 0.1μM tetrodotoxin (TTX). Solid line indicates the presence of $\uparrow[K^+]_o$. TTX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of TTX resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p<0.005$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.473$) compared to the control condition. Error bars are ±SEM.

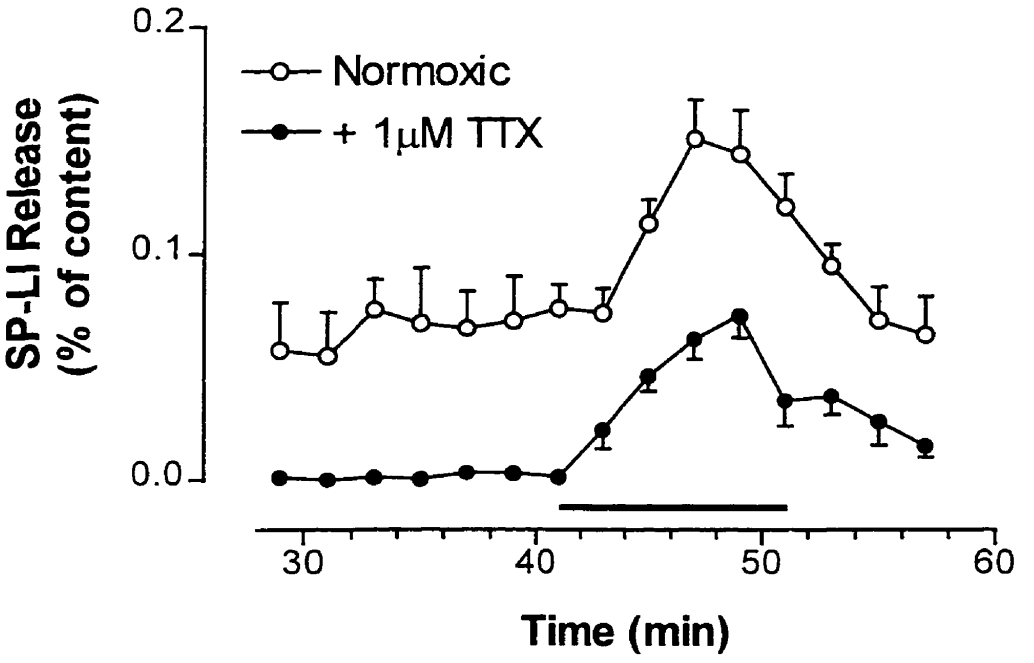


Figure 12: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the additional presence of 1μM tetrodotoxin (TTX). Solid line indicates the presence of $\uparrow[K^+]_o$. TTX was present for the entire perifusion period in the experimental chamber. n=4 separate experiments. Perifusion in the presence of TTX resulted in a significantly lower cumulative basal release (CBR) of SPLI ($p=0.044$), but produced no significant change in cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.769$) compared to the control condition. Error bars are ±SEM.

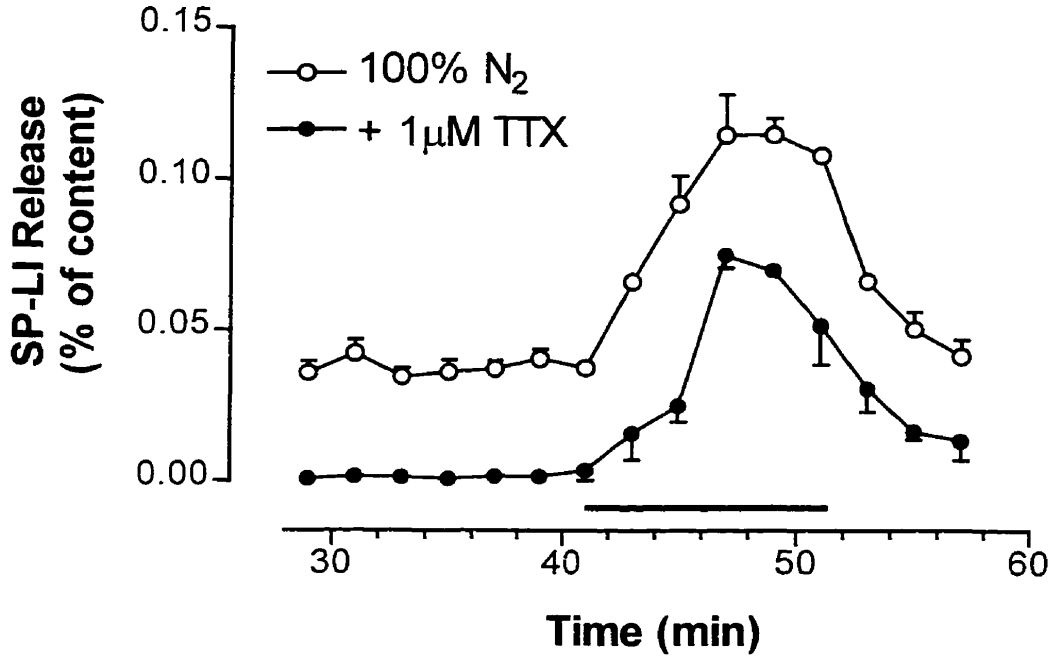


Figure 13: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution bubbled with 100% N_2, in the additional presence of 1μM tetrodotoxin (TTX). Solid line indicates the presence of $\uparrow[K^+]_o$. TTX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of TTX resulted in a significantly lower cumulative basal release (CBR) of SPLI ($p=0.003$), but produced no significant change in cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.118$) compared to the control condition. Error bars are ±SEM.

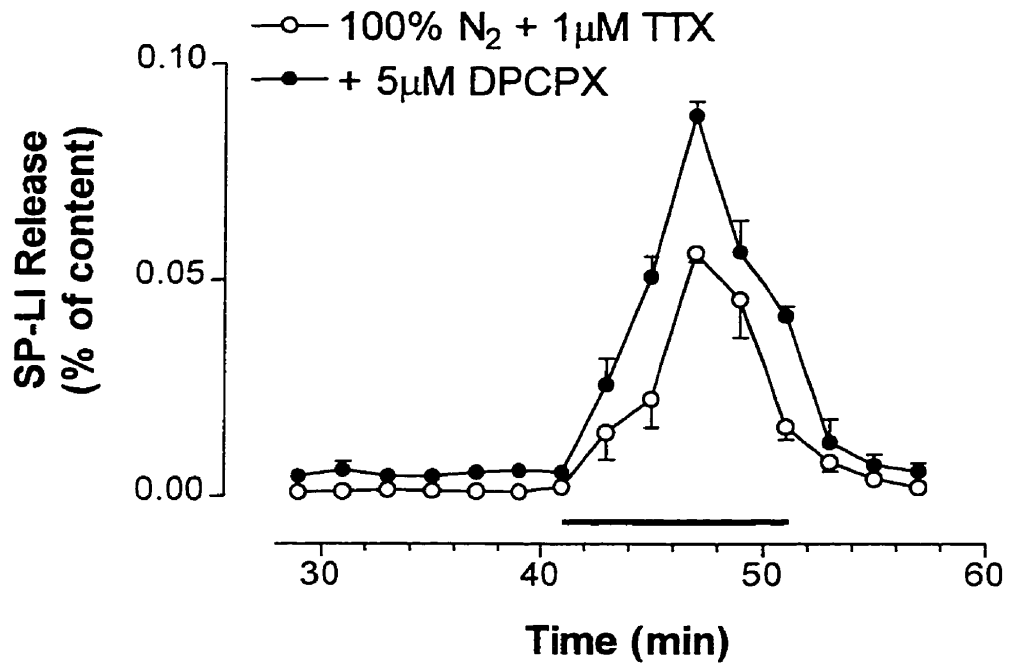


Figure 14: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution bubbled with 100% N_2 in the presence of 1μM tetrodotoxin (TTX), and in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the additional presence of DPCPX produced a non-significant increase in cumulative basal release (CBR) of SPLI ($p=0.005$) and a significant increase in cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.005$) compared to perifusion with TTX alone. Error bars are ±SEM.

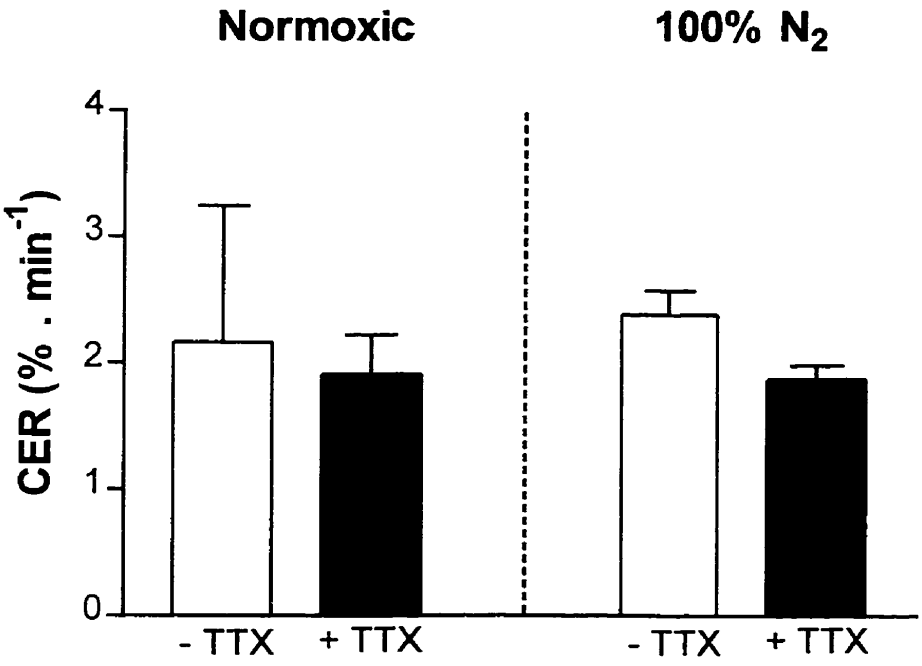


Figure 15*A*: Bar graph showing the changes in cumulative evoked release (CER) in the absence and presence of 1μM tetrodotoxin (TTX) under normoxic conditions (n=4 separate experiments) and hypoxic conditions (n=3 separate experiments). Error bars are ±SEM.

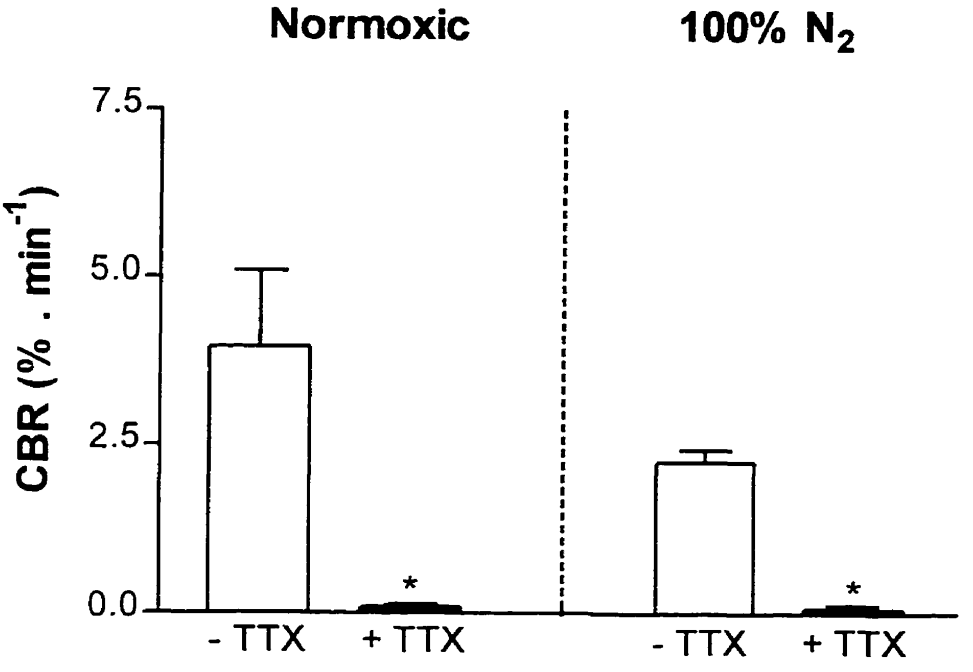


Figure 15*B*: Bar graph showing the changes in cumulative basal release (CBR) after treatment with 1μM TTX under normoxic conditions (n=4 separate experiments) and hypoxic conditions (n=3 separate experiments). * denotes significant difference from paired control in the absence of TTX ($p<0.05$). Error bars are ±SEM.

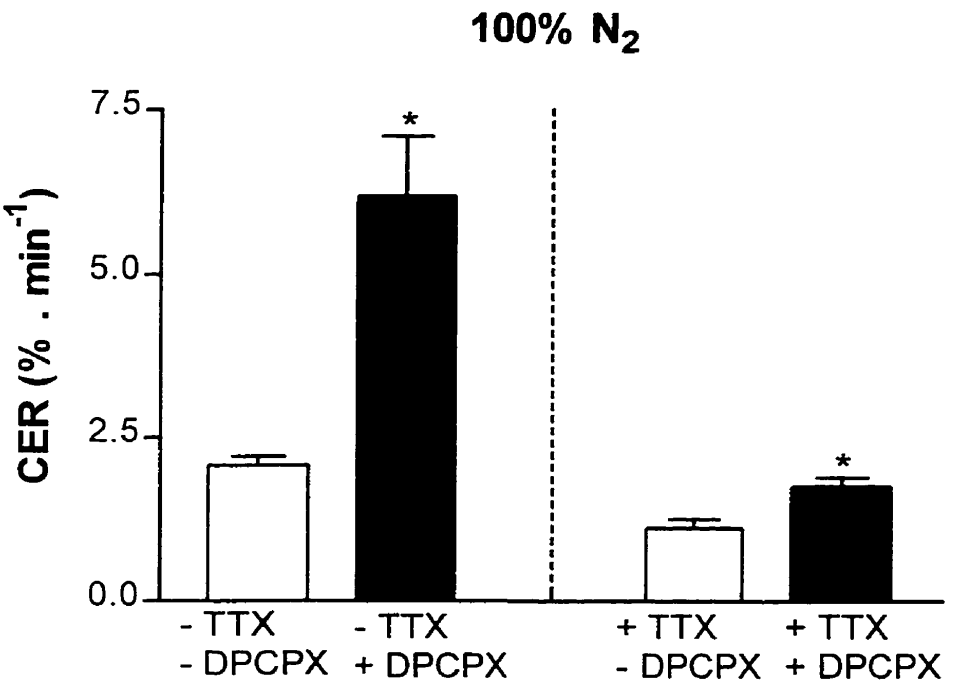


Figure 16: Bar graph showing the changes in cumulative evoked release (CER) of SPLI under hypoxic conditions in the absence (left) and presence (right) of 1μM TTX and, for each, in the additional absence and presence of 5μM DPCPX. For each condition, n=3 separate experiments. * denotes significant difference from paired control in the absence of DPCPX ($p<0.05$). Error bars are ±SEM.

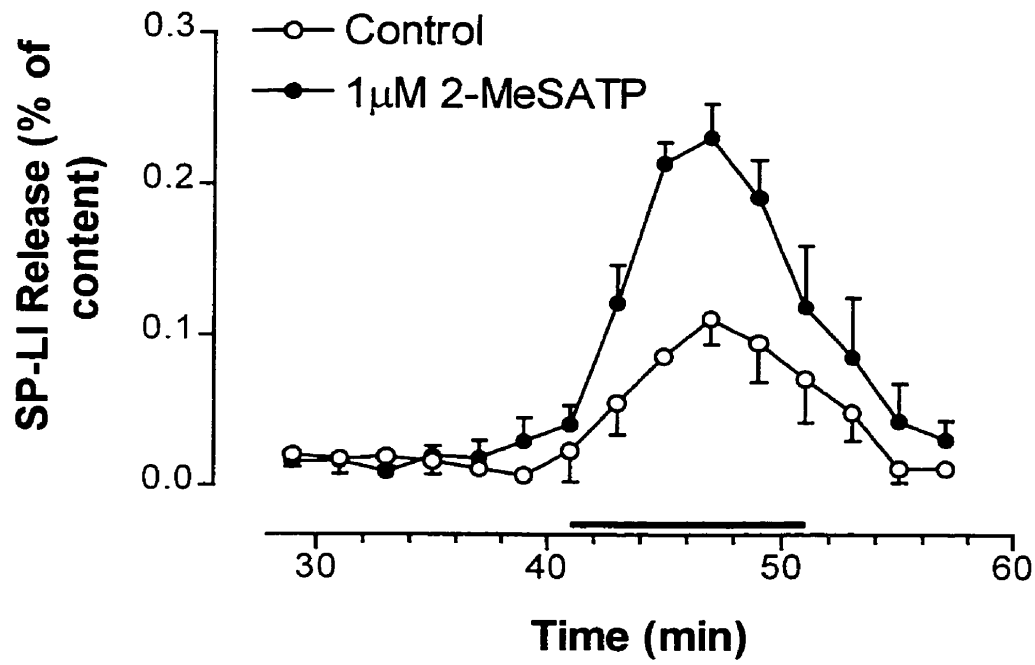


Figure 17*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 1μM 2-Methylthio ATP (2-MeSATP). Solid line indicates the presence of $\uparrow[K^+]_o$. 2-MeSATP was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of 2-MeSATP resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.012$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.568$) compared to the control condition. Error bars are ±SEM.

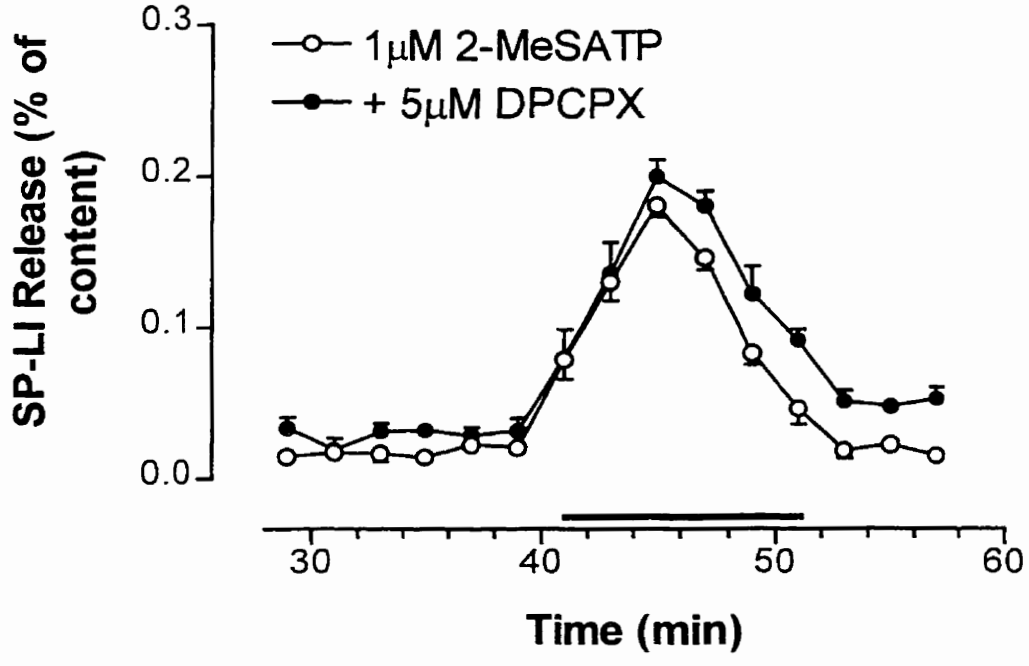


Figure 17*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 1μM 2-Methylthio ATP (2-MeSATP), and in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=4 separate experiments. Perifusion in the additional presence of DPCPX did not produce any significant change in cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.054$), but significantly increased cumulative basal release (CBR) of SPLI ($p=0.010$) compared to the control condition. Error bars are ±SEM.

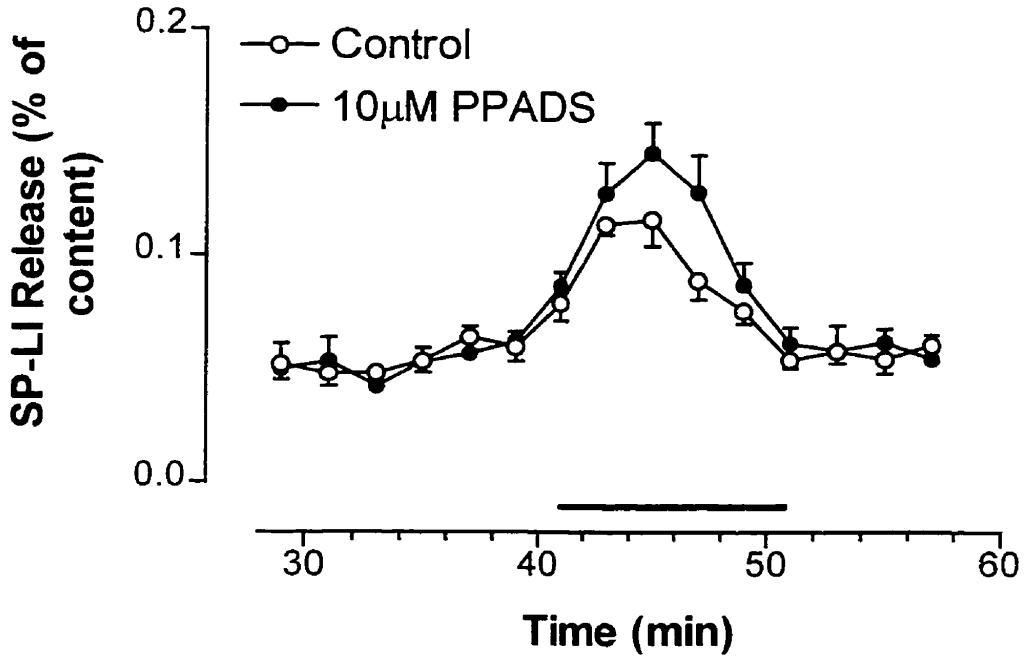


Figure 18: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 10μM PPADS. Solid line indicates the presence of $\uparrow[K^+]_o$. PPADS was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of PPADS resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.010$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.772$) compared to the control condition. Error bars are ±SEM.

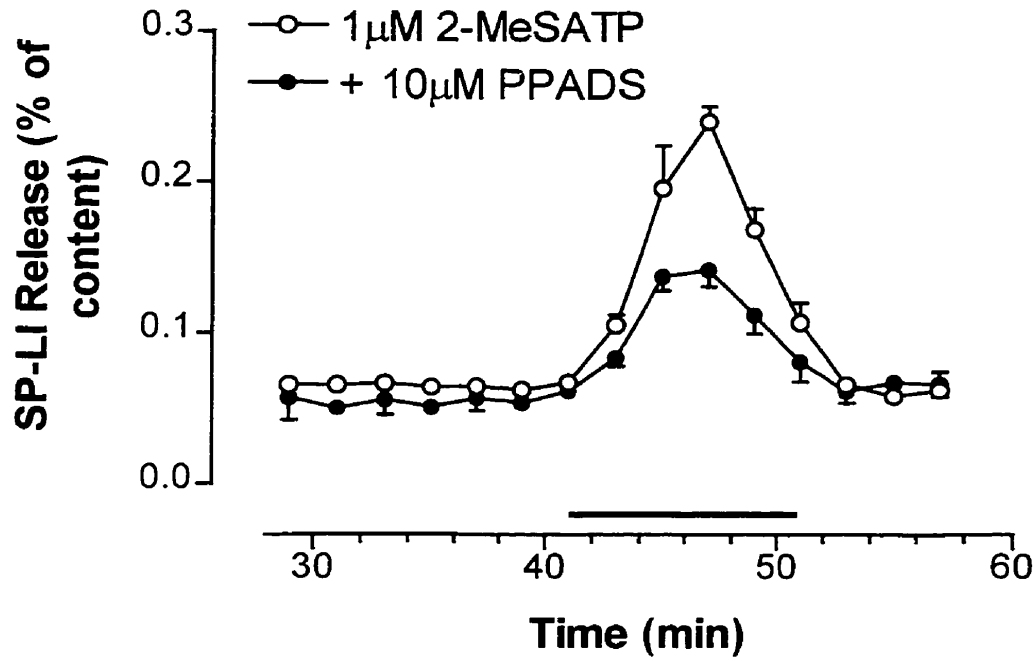


Figure 19: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 1μM 2-Methylthio ATP (2-MeSATP), and in the additional presence of 10μM PPADS. Solid line indicates the presence of $\uparrow[K^+]_o$. PPADS was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the additional presence of PPADS resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.024$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.117$) compared to the control condition. Error bars are ±SEM.

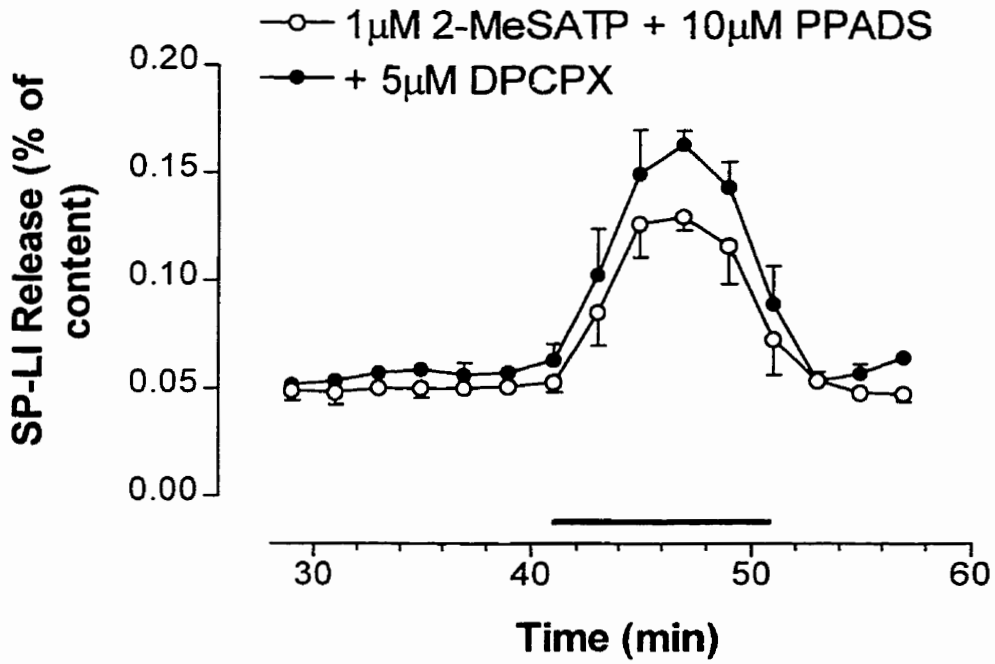


Figure 20: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 1μM 2-Methylthio ATP (2-MeSATP) and 10μM PPADS, and in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the additional presence of DPCPX resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.035$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.067$) compared to the control condition. Error bars are ±SEM.

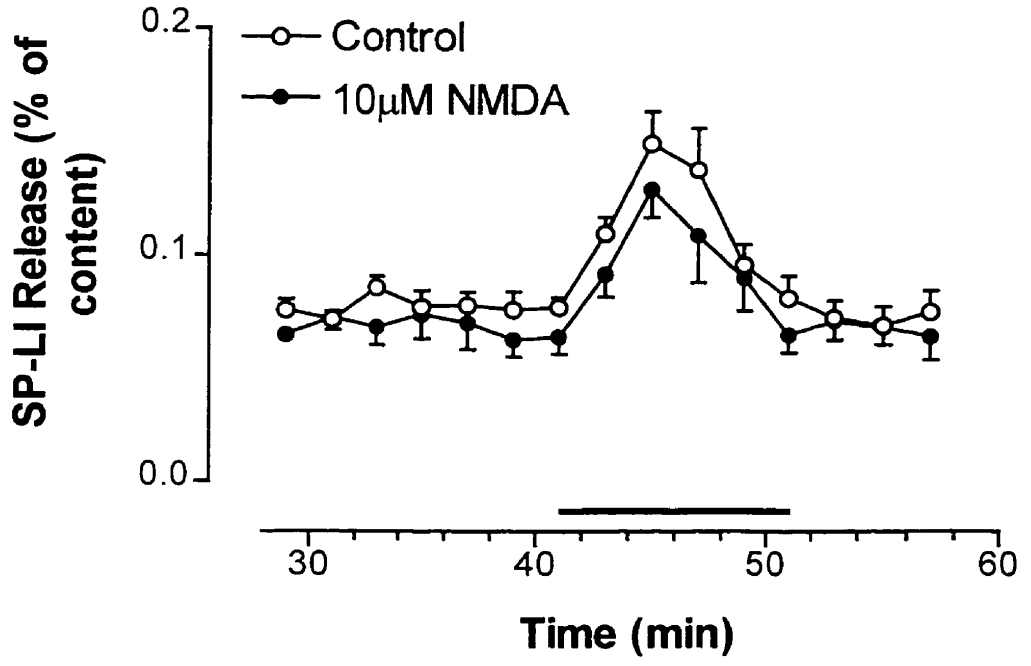


Figure 21*A*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 10μM N-methyl-D-aspartate (NMDA). Solid line indicates the presence of $\uparrow[K^+]_o$. NMDA was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of NMDA resulted in a significantly lower cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.039$), but produced no significant change in cumulative basal release (CBR) of SPLI ($p=0.060$) compared to the control condition. Error bars are ±SEM.

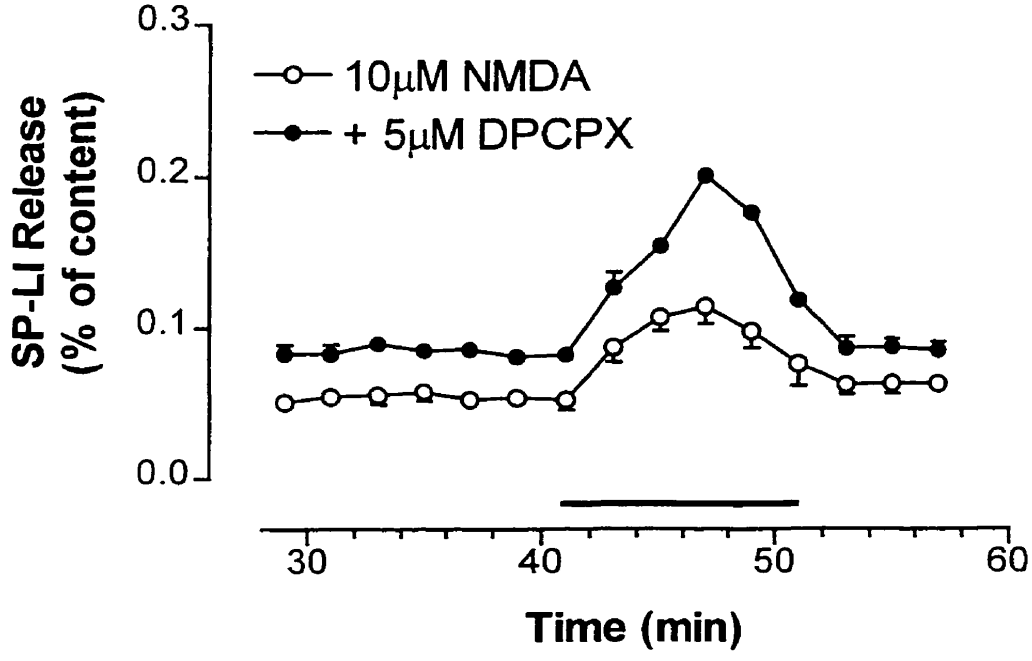


Figure 21*B*: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 10μM N-methyl-D-aspartate (NMDA), and in the additional presence of 5μM DPCPX. Solid line indicates the presence of $\uparrow[K^+]_o$. DPCPX was present for the entire perifusion period in the experimental chamber. n=4 separate experiments. Perifusion in the additional presence of DPCPX resulted in a significantly greater cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.037$), and a significantly greater cumulative basal release (CBR) of SPLI ($p=0.034$) compared to the control condition. Error bars are ±SEM.

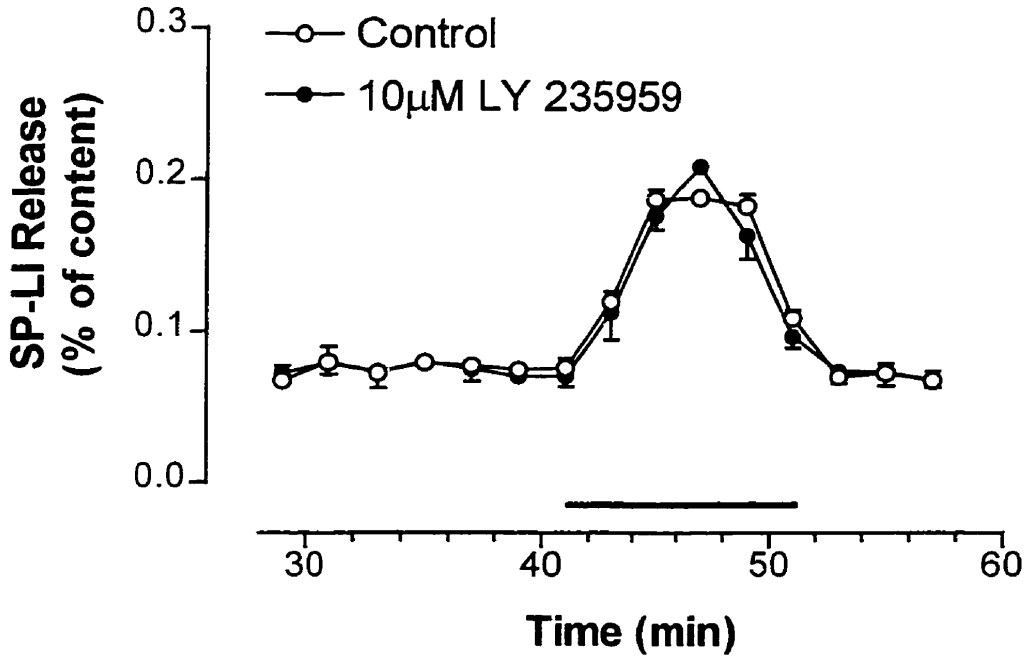


Figure 22: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of the NMDA receptor antagonist LY 235959 (10μM). Solid line indicates the presence of $\uparrow[K^+]_o$. LY 235959 was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of LY 235959 produced no significant change in either cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.532$), or in cumulative basal release (CBR) of SPLI ($p=0.954$) compared to the control condition. Error bars are ±SEM.

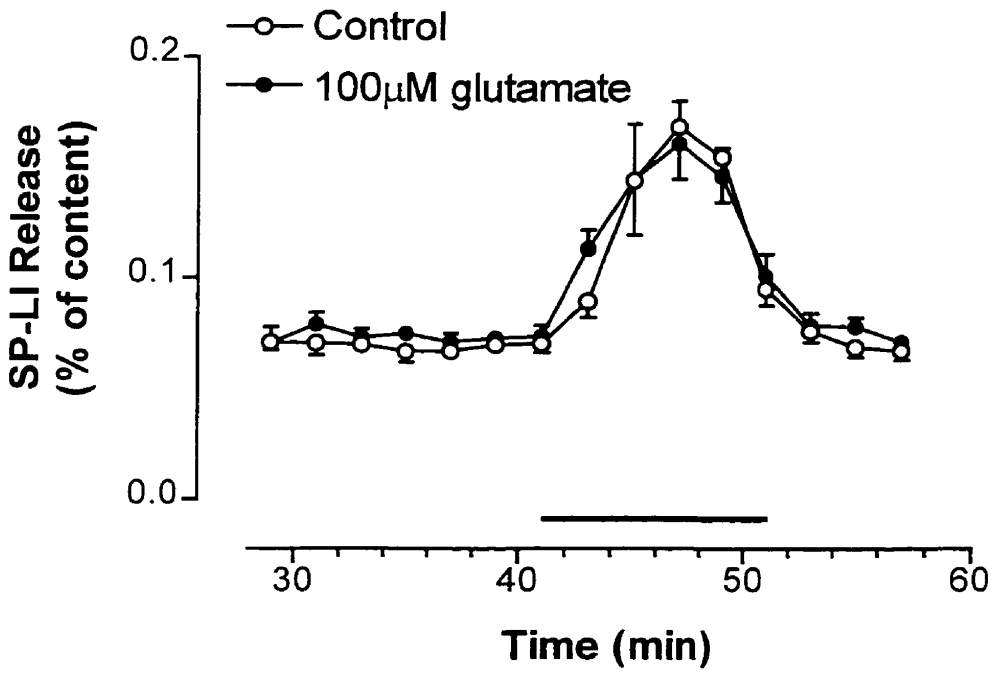


Figure 23: Perifusion profiles showing the release of SPLI from perifused myenteric neural networks evoked by $\uparrow[K^+]_o$-Locke's solution in the presence of 100μM glutamate. Solid line indicates the presence of $\uparrow[K^+]_o$. Glutamate was present for the entire perifusion period in the experimental chamber. n=3 separate experiments. Perifusion in the presence of glutamate produced no significant change in either cumulative $\uparrow[K^+]_o$-evoked release (CER) of SPLI ($p=0.497$), or in cumulative basal release (CBR) of SPLI ($p=0.471$) compared to the control condition. Error bars are ±SEM.

Table 1

Release of SPLI from perifused myenteric neural networks under all experimental conditions. (Cont'd overleaf)

CONTROL (n)		CER ± SEM	CBR ± SEM	TREATMENT	CER ± SEM	CBR ± SEM	Δ_{CER} †	Δ_{CBR}
Normoxic	(5)	5.16 ± 1.03	8.12 ± 1.57	100% N_2	2.16 ± 0.28*	2.74 ± 0.46*	- 2.39	- 2.96
100% N_2	(3)	2.07 ± 0.14	1.11 ± 0.21	+5μM DPCPX	6.20 ± 0.91*	5.90 ± 0.58*	+ 3.00	+ 5.32
Normoxic	(4)	7.48 ± 1.04	3.49 ± 0.90	10% O_2	3.70 ± 1.38*	1.48 ± 0.51*	- 2.02	- 2.36
10% O_2	(3)	2.57 ± 0.74	1.77 ± 0.23	+5μM DPCPX	4.13 ± 0.42	6.97 ± 0.41*	+ 1.61	+ 3.94
Normoxic	(3)	1.65 ± 0.58	2.46 ± 0.24	20% O_2	1.98 ± 0.22	1.95 ± 0.78	+ 1.20	- 1.26
20% O_2	(3)	2.98 ± 0.23	1.03 ± 0.47	+5μM DPCPX	4.49 ± 0.44	2.68 ± 0.73	+ 1.51	+ 2.60
Normoxic	(4)	1.24 ± 0.21	1.66 ± 0.94	30% O_2	1.41 ± 0.44	1.24 ± 0.42	+ 1.14	- 1.34
30% O_2	(3)	2.54 ± 0.14	2.81 ± 0.39	+5μM DPCPX	2.25 ± 0.24	4.08 ± 0.01	- 1.13	+ 1.45
Normoxic	(3)	2.29 ± 0.33	1.26 ± 0.45	50% O_2	3.36 ± 0.41*	1.55 ± 0.47	+ 1.47	+ 1.23
50% O_2	(3)	1.92 ± 0.42	2.28 ± 0.40	+5μM DPCPX	1.88 ± 0.14	2.51 ± 0.25	- 1.13	+ 1.10
Normoxic	(3)	1.58 ± 0.76	1.26 ± 0.43	100% O_2	3.88 ± 0.69*	1.86 ± 0.42	+ 2.46	+ 1.48
100% O_2	(3)	6.27 ± 0.21	2.21 ± 0.22	+5μM DPCPX	5.66 ± 0.61	2.46 ± 0.96	- 1.11	+ 1.11
95mM K^+	(3)	2.81 ± 0.57	3.48 ± 1.43	+0.1μM TTX	5.20 ± 0.53*	3.86 ± 1.76	+ 1.85	+ 1.11
Normoxic	(4)	2.16 ± 1.08	3.96 ± 1.13	+1μM TTX	1.91 ± 0.31	0.09 ± 0.04*	- 1.13	- 44.00
100% N_2	(3)	2.38 ± 0.19	2.26 ± 0.18	+1μM TTX	1.87 ± 0.11	0.07 ± 0.06*	- 1.27	- 32.39

CONTROL (n)	CER ± SEM	CBR ± SEM	TREATMENT	CER ± SEM	CBR ± SEM	Δ_{CER} †	Δ_{CBR}
100% N_2 +1μM TTX (3)	1.12 ± 0.13	0.06 ± 0.02	+5μM DPCPX	1.75 ± 0.14*	0.30 ± 0.05*	+ 1.56	+ 5.00
95mM K^+ (3)	2.79 ± 0.27	0.87 ± 0.39	+1μM 2-MeSATP	6.24 ± 0.65*	1.06 ± 0.364	+ 2.24	+ 1.22
1μM 2-MeSATP (4)	3.97 ± 0.05	1.07 ± 0.18	+5μM DPCPX	4.73 ± 0.28	1.77 ± 0.24*	+ 1.19	+ 1.65
95mM K^+ (3)	1.44 ± 0.29	3.25 ± 0.31	+10μM PPADS	2.27 ± 0.30*	3.17 ± 0.48	+ 1.58	- 1.02
1μM 2-MeSATP (3)	3.55 ± 0.08	3.90 ± 0.09	+10μM PPADS	2.18 ± 0.16*	3.26 ± 0.25	- 1.63	- 1.20
1μM 2-MeSATP +10μM PPADS (3)	2.05 ± 0.04	2.97 ± 0.18	+5μM DPCPX	2.63 ± 0.07*	3.34 ± 0.09	+ 1.28	+ 1.12
95mM K^+ (3)	1.29 ± 0.10	4.64 ± 0.30	+10μM NMDA	0.95 ± 0.15*	4.12 ± 0.40	- 1.35	- 1.13
10μM NMDA (4)	1.52 ± 0.29	3.26 ± 0.22	+5μM DPCPX	2.51 ± 0.11*	5.08 ± 0.18*	+ 1.65	+ 1.56
95mM K^+ (3)	2.81 ± 0.21	4.52 ± 0.33	+10μM LY 235959	2.65 ± 0.26	4.51 ± 0.32	- 1.06	- 1.00
95mM K^+ (3)	2.26 ± 0.44	4.16 ± 0.17	+100μM glutamate	2.13 ± 0.41	4.43 ± 0.23	- 1.06	+ 1.06

Table 1: (cont'd)

* denotes significant difference from control condition ($p<0.05$).
† denotes fold difference from control.

DISCUSSION

Perifusion of Neural Networks under Conditions of Altered pO_2

These studies were undertaken with an overall objective to characterize the possible changes in endogenous interstitial adenosine levels in response to changes in prevailing pO_2. The data presented here support the viability of the perifusion protocol as a means of assessing such functional changes, and demonstrate that an inverse exponential relationship exists between an index of endogenous interstitial adenosine concentration and oxygen tension. Furthermore, release of the excitatory neurotransmitter Substance P (SPLI) was shown to be graded with respect to prevailing pO_2, such that a linear relationship exists between oxygen tension and both $\uparrow[K^+]_o$-evoked and spontaneous release of SPLI. This finding confirms that measurement of basal SP release (CBR) is a reliable analytical tool, and that it may be a more accurate method for assessing functional purinergic activity in the myenteric plexus. Evidence is also presented to suggest that adenosine may be present in concentrations sufficient to provide a tonic inhibitory tone under normoxic conditions.

Previous studies in this laboratory utilising isolated, perifused ganglion networks restricted analysis of data to examination of changes in the $\uparrow[K^+]_o$-evoked release of SPLI, or the Cumulative Evoked Release (CER) (Moneta et

al., 1997). The present study addressed changes in oxygen tension, and initial results made apparent the fact that significant changes in spontaneous release of SPLI were occurring. In order to quantitate these changes, the Cumulative Basal Release (CBR) was calculated for all conditions as the integral of the release occurring spontaneously during the 10 minutes preceding addition of the depolarizing stimulus. Perifusion of the myenteric neural networks under all oxygen tensions tested provided detectable and quantifiable basal levels of SPLI, validating both the technique and the analysis. The findings show that changes in basal release of SPLI correspond well with changes in evoked release, and are often even larger in magnitude. It is tempting to speculate that the CBR may provide a more accurate picture of adenosinergic activity than CER. Adenosine is but one of many mediators released in the vicinity of myenteric nerves; transmission in the system is therefore dependent on the relative concentrations, the relative potencies, and the net effects of these excitatory and inhibitory moieties. Elevating the extracellular K^+ concentration, while augmenting the release of SPLI, undoubtedly effects the same change on the releasability of a multitude of neuromediators, and makes difficult an objective assessment of functional adenosinergic activity. The potential for this to occur is likely diminished in unevoked, isolated myenteric neural networks, where the inhibitory effects of spontaneously released adenosine may be more easily distinguished.

Previous studies in this laboratory have used a variety of techniques to determine that endogenous interstitial adenosine provides an inhibitory tone, in part by diminishing the release of SPLI (Moneta et al., 1997). Evidence for this includes the observations that release of SPLI from myenteric neural networks is enhanced by the addition of the selective adenosine A_1 receptor antagonist DPCPX, diminished by inhibition of equilibrative nucleoside transporters, and augmented by incubation with the metabolizing enzyme adenosine deaminase.

In the present study, perifusion under hypoxic conditions (100% N_2) yielded reductions in both spontaneous and evoked release (2 - 3 fold reduction in CER and CBR respectively, Fig 3*A*). In contrast, perifusion under hyperoxic conditions (100% O_2) yielded increases in both spontaneous and evoked SPLI release (2.5 and 1.5 fold increases for CER and CBR respectively, Fig 8*A*). In the additional presence of the A_1 antagonist DPCPX, the release of SPLI increased 3 and 5 fold for CER and CBR respectively (Fig 3*B*), unmasking considerable inhibition by endogenous interstitial adenosine acting at A_1 receptors. Perifusing the isolated myenteric ganglion networks under hyperoxic conditions produced no change in either spontaneous or evoked release (Fig 8*B*) indicating that functionally undetectable levels of interstitial adenosine were present. These findings offer clear evidence that the concentration of released endogenous adenosine is sensitive to the prevailing oxygen tension.

Perifusion of myenteric networks with solutions prepared at intermediate oxygen tensions (10% - 50% O_2) yielded responses which were intermediate to those obtained with the hypoxic and hyperoxic extremes. Perifusion with 10% O_2 yielded some inhibition of SPLI release, but perifusion with 20% and 30% O_2 did not alter the release profiles significantly from controls. At 50% O_2, a significant enhancement of SPLI release was observed (Fig 4*A*, 5*A*, 6*A* and 7*A*; see also Table 1 for values and increments/decrements). In the additional presence of DPCPX, clear increments in both spontaneous and evoked release of SPLI occurred at both 10% and 20% O_2. When perifused with 30% and 50% O_2, addition of the antagonist did not produce any functional changes; both the CER and CBR values remained essentially the same as those obtained with 100% O_2 (Fig 4*B*, 5*B*, 6*B* and 7*B*; Table 1). Figures 9*A* and *B* demonstrate that a clear relationship exists between pO_2 and both $\uparrow[K^+]_o$-evoked and spontaneous release of SPLI from myenteric neural networks. When the measured pO_2 was plotted against the CER and CBR values expressed as percentages of their respective normoxic controls, the relationship obtained was amenable to linear regression analysis yielding reasonable linearity and correlations for both. However, such mathematical linearity does not necessarily imply biological linearity and it is clear that the observed relationship arose as the net result of converging inhibitory and excitatory influences (Moneta et al., 1997). It is tempting to speculate that the higher slope exhibited by the

relationship between CER and pO_2 may have reflected an increased concentration of pO_2-sensitive inhibitory mediators in the interstitium, when compared to those present under resting conditions. These mediators would include nitric oxide (NO), VIP, PACAP and/or galanin (Murthy et al., 1996; Wang et al., 1998), when compared to those present under resting conditions. This contention is consistent with the substantially larger release of SPLI seen following depolarization. Notwithstanding this possibility, the contribution of adenosine to the overall pO_2-sensitive inhibitory tone could not be addressed by simply examining this relationship and therefore an assessment of the change in release of SPLI which occurred in the additional presence of adenosine receptor blockade was undertaken.

To assess the degree of inhibition which endogenous interstitial adenosine may have been eliciting, and to examine such effects in the presence of diminished pO_2, the isolated networks were perifused at the previously established range of oxygen tensions in the additional presence of the selective adenosine A_1 receptor antagonist DPCPX. When expressed as a percentage of control SPLI release in the absence of DPCPX, the release in its presence at each pO_2 revealed the magnitude of inhibition which had been due to the presence of interstitial adenosine alone. Figures 10*A* and *B* show the exponential increases in the incremented release obtained with decreasing pO_2 following the attainment of a threshold oxygen tension. The increment in spontaneous SPLI release caused by DPCPX at low pO_2 (Fig

10*B)* is greater than that observed for evoked release (Fig 10*A*), suggesting that endogenous adenosine contributed a greater proportion of the overall inhibitory tone present in the networks under resting conditions than it did to that which exists following general depolarization. This is consistent with the possibility that depolarization of the neural networks by elevated $[K^+]$ probably induced substantial neuromediator release, both excitatory and inhibitory. Under these conditions, the inhibition mediated solely by the action of endogenous adenosine at A_1 receptors would therefore impact less on the general state of excitation than it would under basal conditions. This was further supported by the observation that with declining pO_2 and in the absence of the A_1 antagonist, $\uparrow[K^+]_o$-evoked release of SPLI from the networks appeared to increase at a greater rate than did spontaneous release, suggesting an enhanced inhibitory tone for the latter (Figures 9*A* and *B*).

An obvious feature of the data presented in Figure 10*A* and *B* is the threshold for measurable adenosine A_1 receptor-mediated inhibition for both spontaneous and $\uparrow[K^+]_o$-evoked SPLI release. As pO_2 declined to somewhere between 260 mm Hg and 146 mm Hg, the degree of adenosinergic inhibition of SPLI release increased exponentially. When the oxygen tension of the perifusing medium was higher than this threshold, interstitial endogenous adenosine concentrations appeared to be insensitive to changes in pO_2. This threshold corresponds approximately to the slightly

hyperoxic oxygen tension achieved following treatment of solutions with between 30% and 20% O_2 in the perifusing medium. Of interest here is the fact that functionally detectable levels of adenosine were present in networks perifused under "normoxic" conditions (i.e. ungassed) (data not shown) and in networks perifused with a solution equilibrated at 20% O_2 (Figure 5*A*). This suggests that an adenosinergic inhibitory tone is present in the myenteric plexus under normal physiological conditions (i.e. when perfused with oxygenated blood). Furthermore, characterization of the nucleoside as a neuroprotective agent depends critically on its ability to respond to acute, pathophysiological insults. CNS studies have demonstrated that hypoxia induces significant elevations in endogenous adenosine concentrations. The data acquired in the present studies suggest that diminished pO_2 can elicit an increase in endogenous adenosine concentrations in the myenteric plexus, and that this increase is functionally linked to an inhibition of SPLI release. These are important factors, not only in the protection of enteric neurons from pathophysiological trauma, but also in the maintenance of normal physiological function. The sensitivity of endogenous adenosine concentrations to fluctuations in pO_2 may help to prevent enteric neural hyperexcitability during periods of transient ischaemia which may be associated with normal MMC cycling.

The existence of a threshold for elevated release of endogenous adenosine, and its occurrence at a relatively hyperoxic pO_2, is similarly consistent with both putative physiological and pathophysiological roles for

the nucleoside in the tonic regulation of neuromediator release. Measurement of pO_2 in the vicinity of the ENS *in vivo* is a parameter which appears to have escaped definitive scrutiny. However, one study, utilising small bowel tonometry and maintaining an arterial pO_2 of between 95 and 110 mmHg, determined that small gut *mucosal* pO_2 in the dog was between 30 and 45 mm Hg (Dawson et al., 1965). Using a similar method, another group found that pre-anaesthesia mucosal pO_2 in dog small bowel was as high as 57 mm Hg (Hamilton et al., 1968). Although neither of these studies address the oxygen tension in the ENS *per se*, it is certainly possible that common arterioles supply both intestinal mucosa and enteric nerves. By extension, the prevailing pO_2 within the ENS would not be expected to be very different and may, indeed, be considerably lower under those pathophysiological circumstances alluded to above. If the environment surrounding enteric neurons tends towards hypoxia under physiological conditions, the implication for pathophysiological states becomes evident. The magnitude of hypoxic insult required to push the system into a state of critical hypoxia may well be significantly smaller than that for CNS tissue. Indeed, Aranow and Fink (1996) have argued that biophysical considerations related to mesenteric arteriolar flow would suggest that the small intestinal mucosa is hypoxic relative to surrounding tissue in its *normal* physiological state. If similar arterioles are responsible for perfusion of the ENS, then it is conceivable that relatively minor occlusions of mesenteric arterial flow, or inflammatory reactions in the small intestine may produce an ischaemic condition sufficient

to cause derangement of enteric neural function. Contractions of isolated human small intestine are highly sensitive to hypoxia (Hayashi et al., 1986), and the mucosal architecture of isolated ileal segments shows significant morphologic damage after 6 - 10 hours of cold ischaemia and reoxygenation (Kawashima et al., 1996). Using transit time as an index of motility, one study has shown that intestinal motility in the rat decreases according to the duration of mesenteric ischaemia (Udassin et al., 1995). Furthermore, during hypoxia, mouse intestine exhibits a marked decrease in resting tension and spontaneous contractions (Bielefeldt and Conklin, 1997). Such findings are almost certainly the result of alterations of enteric neural function, evidenced in part by the fact that the response to transmural stimulation of nerves disappears after 60 minutes of hypoxia (Bielefeldt and Conklin, 1997). In the present study, the remarkable sensitivity of endogenous adenosine concentrations to changes in prevailing pO_2 demonstrates the capacity of the ENS to adapt to such acute pathophysiological insults.

The concentration of endogenous adenosine present in the vicinity of its receptors on enteric nerves is not known. However, estimates for interstitial adenosine levels within the myocardium and the CNS have been reported using several approaches (Van Wylen et al., 1986; Phillis et al., 1991; Cook and Karmazyn, 1996). The values determined for interstitial adenosine in the CNS are reported to be in the vicinity of 10-300 nM which are consistent with (unpublished) observations from this laboratory showing

an EC_{50} for adenosine on the isolated LMMP preparation, in the presence of nucleoside transport inhibition, of 5.2 nM. Pharmacologically, for adenosine to provide biologically useful regulation of mediator release in the ENS, its concentration in the vicinity of its receptors should ideally coincide with the steep portion of the sigmoid dose-response curve, permitting small changes in concentration to elicit the greatest change in response. The results presented here are consistent with this possibility.

The source of the adenosine released into the interstitium of myenteric networks was addressed in an additional series of experiments. The myenteric network preparation is acknowledged to be a mixed tissue in which smooth muscle and other contaminating cells are present together with enteric neural tissue, itself a mixed tissue consisting of neurons, glia and other support cells. All of these cells are potential sources of released adenosine although the tissue appears to be predominantly derived from the ENS (Moneta et al., 1997). We therefore used TTX in order to functionally assess the contribution of conducted nerve traffic to the release of endogenous adenosine.

Perifusion of networks in the presence of 1 μM TTX yielded a substantial reduction in spontaneous release with little effect on evoked release (Figs 12 and 13; see also Table 1 for values and fold changes). It should be noted that this result was at variance with our previous finding

showing an *increase* in CER in the presence of the sodium channel blocker. At first, the present finding was difficult to reconcile with that reported previously. However, upon repetition of the experiment with a lower concentration of TTX, it was revealed that the concentration reported in Moneta et al. (1997) as 1.0 μM was, in fact, 0.1 μM. Use of this lower concentration in the present experiments reproduced the previously reported enhanced release exactly. It is beyond the scope of this discussion to examine explanations for the clear difference in response to TTX other than to suggest that differential sensitivity of smaller diameter inhibitory nerve fibers, bearing lower densities of sodium channels, could be implicated. This apparent dose effect of TTX might therefore provide a useful approach to the functional investigation of distinct populations of enteric nerves, but will not be pursued here. Notwithstanding these considerations, the substantial reduction in SPLI release brought about by TTX was observed in four separate experiments and was not affected by perifusion under hypoxic (100% N_2) conditions (Figs 13 and 15*B*; Table 1). We had already shown that the inhibited spontaneous and evoked SPLI release under hypoxic conditions could be essentially restored by perifusion with DPCPX (Fig 3*B*; Table 1) and repetition of this experiment in the additional presence of TTX was undertaken. The results showed that, in the presence of TTX, the ability of DPCPX to restore the inhibited evoked release of SPLI was diminished, yielding a 1.5 fold increment in CER over its control value (Figure 16; Table 1). By contrast, in the absence of TTX, DPCPX incremented evoked release

approximately 3 fold (Figs 3*B* and 16; Table 1). While these values were both different from their corresponding controls, the biological significance of the differences is perhaps more important than the statistical differences. The $\uparrow[K^+]_o$-evoked release of SPLI from myenteric networks occurs by depolarization not only of nerve cell bodies but also directly at the nerve varicosities, a process which has long been recognized to be insensitive to TTX. Thus the small decrement in CER with TTX, and the similarly small increment in the additional presence of DPCPX, are expected findings. Since DPCPX was less able to disinhibit evoked release in the presence of TTX than in its absence, the interstitial adenosine which exerted the inhibition in the latter case must have been released by a process involving conducted action potentials and thus the source of such adenosine was most likely neural. It must be noted here that while adenosine released by contaminant muscle and vascular tissue would be insensitive to TTX, contaminant glial cells in the isolated preparation might also release adenosine in a manner sensitive to the actions of the sodium channel blocker (although neurons undoubtedly represent the majority of tissue in the preparation). The observation that there remains an increment in evoked release in the presence of both TTX and DPCPX indicates that some interstitial adenosine must have been inhibiting release and thus only a portion of the overall interstitial adenosine originated from those nerves (or glia) sensitive to TTX, at least under the conditions of these experiments.

While neural release of adenosine *per se* is implied from these studies, an additional mechanism is likely to be involved. We have previously shown that metabolism of ATP by *ecto*-ATPases yields adenosine which contributes to the overall endogenous inhibitory tone (Moneta et al., 1997) and the neural origin the majority of such ATP is supported by its role as a fast transmitter in the ENS (Galligan and Bertrand, 1994). Moreover, in addition to the known release of ATP from enteric synaptosomes (White and Leslie, 1982), McConalogue et al. have recently reported direct measurement of the release of the nucleotide from nerves in guinea pig taenia coli (McConalogue et al., 1996).

The findings reported here reflect changes in the release of mediator from myenteric networks under hypoxic, but not strictly ischaemic conditions. It is recognized that the conditions which may prevail following diminished mesenteric blood flow and/or increased intestinal metabolic demand are not necessarily modeled by simple alteration of pO_2. Nevertheless, tissue hypoxia is an important component of the changes brought about by pathophysiological processes in the environment of the ENS and the present findings show that, under such conditions, progressively diminishing tissue pO_2 will elicit exponentially increasing concentrations of interstitial adenosine. The presence of such adenosine in the vicinity of its receptors, and in functionally relevant concentrations, is compatible with a putative neuroprotective role for adenosine, the retaliatory metabolite (Newby et al.,

1985). It is in the right place, potentially at the right time, and in appropriate amounts.

The role of adenosine as a putative neuroprotective moiety in the ENS necessarily follows from the emerging evidence for such a function in the CNS (Rudolphi et al., 1992; Rudolphi et al., 1992; von Lubitz et al., 1995). In particular, the ability of the nucleoside to provide protection against acute excitatory amino acid toxicity is worthy of consideration. Glutamatergic transmission within the ENS is recognized (Wiley et al., 1991) and the excitotoxic effects of glutamate on both isolated enteric ganglia and enteric whole mount preparations have been reported (Kirchgessner et al., 1997). Interestingly, increased excitation following acute ischemia/hypoxia has been documented (Guisan et al., 1975; Corbett and Lees, 1997) and, while such excitation may not involve glutamate exclusively, the parallels between such acute responses at the CNS and those emerging at the ENS suggest that exploration of the possible protective role of endogenous adenosine at this locus may be useful.

P_2 Purinoceptor-mediated Neurotransmission in the Myenteric Plexus

If the consequence of A_1 receptor activation by endogenous adenosine is of such potential importance to neuronal survival under ischaemic or hypoxic conditions, then identification of the source and formative pathways for the nucleoside must also be a target for investigation. It is becoming clear that dephosphorylation of endogenous ATP is a significant pathway by which extracellular adenosine is created. McConalogue et al. (1996) have shown that ATP, ADP, AMP and adenosine are released from nerves (possibly enteric inhibitory neurons) in guinea pig taenia coli in response to electrical stimulation. Their results indicated a temporal shift, such that after electrical stimulation, peak ATP levels were achieved prior to the peak concentrations of its major metabolites, including adenosine. This finding strongly suggested ATP hydrolysis as a potential source for interstitial adenosine. Furthermore, a microdialysis study in fetal sheep has shown that increases in brain adenosine concentrations which accompany hypoxia can be prevented by a blocker of ecto-5'-nucleotidase, α,β-methylene-ADP (AOPCP), indicating that, under such pathophysiological conditions, increases in adenosine are dependent on the hydrolysis of extracellular 5'-AMP (Koos et al., 1997). The finding that AOPCP mediates inhibition of SPLI release from myenteric neurons lends further support to this hypothesis (Moneta et al., 1997). Earlier studies in this laboratory investigated the effects of various P_{2X} and P_{2Y} purinoceptor agonists in order to determine the presence and functional roles

of guinea pig enteric P_2 receptors (Moneta, 1996). These studies utilised the endogenous agonist, ATP, as well as the P_{2X} agonists β,γ-MeATP and ATPγS, and 2-MeSATP, believed at the time to be a selective P_{2Y} receptor agonist. The results showed that perifusion of isolated guinea pig myenteric neural networks with ATP and with the selective P_{2X} agonists yielded significant decreases in the evoked release of SPLI. Interestingly, however, perifusion in the presence of 2-MeSATP yielded the opposite effect - evoked release of SPLI was significantly enhanced. The conclusion was reached that in the guinea pig myenteric plexus, P_{2X} receptors appeared to be of an inhibitory nature, while P_{2Y} receptors were deemed to be excitatory. However, recent pharmacological studies have suggested that 2-MeSATP may be an agonist at the P_{2X3} purinoceptor subtype (Robertson et al., 1996). As a result, the present study sought to clarify the previous findings, and more fully investigate the nature of 2-MeSATP-mediated SPLI enhancement, considering the effects of the agonist on both $\uparrow[K^+]_o$-evoked and spontaneous release of SPLI.

Perifusion of the networks in the presence of 1μM 2-MeSATP produced a significant enhancement in the $\uparrow[K^+]_o$-evoked release of SPLI, but had no effect on spontaneous release (Fig. 17*A*), a finding consistent with that previously reported (Moneta, 1996). In order to address the possibility that elevation of basal SPLI release was being masked by the inhibitory actions of endogenous adenosine at A_1 receptors, the experiment was

repeated in the additional presence of 5μM DPCPX, the selective A_1 receptor antagonist (Fig. 17*B*). The antagonist elicited a small, yet significant, increase in CBR, but produced no significant change in CER when compared to the control condition (2-MeSATP alone). This suggests that the mechanism responsible for the elevation in SPLI mediated by 2-MeSATP is relatively insensitive to adenosinergic modulation, since blockade of endogenous A_1 receptor-mediated inhibition failed to significantly augment $\uparrow[K^+]_o$-evoked release. Furthermore, the results illustrate the curious inability of the P_{2X3} agonist alone to elicit any change in spontaneous release of SPLI. This finding implies either that receptors mediating the response to 2-MeSATP may be located primarily on nerve endings or that other mediators, modulators, or that combinations thereof released during $\uparrow[K^+]_o$-evoked depolarization of the ganglion networks potentiate the excitatory response of P_{2X3} receptors to the agonist. However, such potentiation of receptor function or depolarization-dependent changes in efficacy may also affect G protein-coupled systems, such as P_{2Y} receptors. It must be noted that true subtype-selectivity amongst purinoceptor agonists is a rare characteristic and, while 2-MeSATP is considered to be a selective P_{2X3} agonist, its efficacy at P_{2Y} receptors is also acknowledged (Barajas-Lopez et al., 1994; Schachter et al., 1996). Thus, in order to determine the type of receptor mediating the response to 2-MeSATP, guinea pig myenteric networks were perifused in the presence of 2-MeSATP and in the additional presence of the nonselective P_{2X} antagonist PPADS. Earlier controls in the presence of the antagonist alone

revealed that PPADS produced a significant increase in CER, but had no effect on the CBR (Fig. 18; Table 1). With 1μM 2-MeSATP in the perifusing medium, the additional presence of 10μM PPADS produced a significant *decrease* in $\uparrow[K^+]_o$-evoked SPLI release, while leaving spontaneous SPLI release relatively unaffected (Fig. 19; Table 1), suggesting that the agonist was not acting at an adenosine A_1 receptor. This argument was further supported by perifusion in the additional presence of the adenosine A_1 receptor antagonist DPCPX, which produced the expected disinhibition of the preparation and a significant increase in $\uparrow[K^+]_o$-evoked SPLI release compared to networks perifused with only a combination of 2-MeSATP and PPADS (Fig 20; Table 1).

These results demonstrate some interesting pharmacological properties of purinergic transmission in the ENS. The inability of PPADS to elicit any functional change in spontaneous SPLI release seems to indicate that endogenous P_2-mediated neurotransmission is not a significant contributor to the basal tone in myenteric networks. On the other hand, the disinhibition elicited by PPADS during $\uparrow[K^+]_o$-evoked depolarization suggests that, under these conditions, at least a portion of the net inhibitory tone is mediated by ATP *per se*. The possibility exists that, under basal conditions, low concentrations of endogenous ATP released into the interstitium might undergo such rapid hydrolysis to adenosine that functional activation of P_2 purinoceptors may not be possible. Alternately, the findings presented here

demonstrating that SPLI release following $\uparrow[K^+]_o$-evoked depolarization of myenteric neurons is not dramatically diminished by TTX suggest that spontaneous release of SPLI is likely due to axonal nerve traffic, while evoked release represents release from nerve endings (which is recognized to be TTX-insensitive). If this is the case, the receptors mediating the activity of 2-MeSATP may be located primarily on myenteric nerve varicosities and their activation during $\uparrow[K^+]_o$-evoked depolarization would lead to SPLI release directly. However, the nearly 2.5-fold increase in CER as a result of perifusion with 2-MeSATP also suggests that the functional effect of the agonist may somehow be potentiated during depolarization. P_{2Y} receptors (and their associated signal transduction mechanisms) may be candidates for such a type of modulation in addition to the ligand-gated cation channel P_{2X} receptors, for which 2-MeSATP is reportedly selective. It is acknowledged, however, that 2-MeSATP also activates P_{2Y1} receptors (Barajas-Lopez et al., 1994; Schachter et al., 1996), and therefore the possibility that P_{2Y} receptors were mediating the enhancement of SPLI was addressed by perifusion of the networks with PPADS. The P_{2X} antagonist was, however, able to significantly reduce 2-MeSATP-mediated increases in SPLI, suggesting that a majority of the observed response was due to interaction of the agonist with P_{2X} receptors. Potentiation of P_{2X} receptors has been investigated by various groups, and the findings demonstrate that both Zn^{2+} and Cu^{2+}, which are contained in vesicles and released from neurons in various brain regions, can allosterically increase the affinity of certain P_{2X} receptor subtypes for ATP by

interacting with a common site in the molecule (Li et al., 1996; Li et al., 1996; Garcia-Guzman et al., 1997). Furthermore, a study by King et al. (1996) has shown that acidification of the superfusing medium increases the activity of ATP at recombinant P_{2X2} receptors expressed in *Xenopus* oocytes. While acidification of the perifusing solution in the present study was unlikely (due to the presence of HEPES buffer), the extent of allosteric enhancement as a result of Zn^{2+} and/or Cu^{2+} release upon depolarization of the networks is unknown. The use of functional pharmacological techniques to assess the presence or absence of P_2 purinoceptors is made exceedingly difficult by the lack of true subtype-selective agents. Moreover, compounds that show a high affinity for particular purinoceptor subtypes in one tissue, often bind poorly with the same subtypes in other tissues (Soto et al., 1997). These problems seemingly necessitate the use of electrophysiological and molecular biological techniques to complete a more accurate picture of endogenous P_2-mediated neurotransmission. The data presented here support the functional presence of P_{2X} receptors under depolarized conditions in myenteric neural networks. Excitation due to exogenously applied 2-MeSATP notwithstanding, nonselective antagonism of P_{2X} receptors by PPADS augments SPLI release, suggesting that the consequence of endogenous ligand binding is *inhibition* of excitatory transmission. Nonetheless, these data do not rule out the functional presence of P_{2Y} receptors in guinea pig myenteric neural networks.

Glutamatergic Neurotransmission in the Myenteric Plexus

The dicarboxylic acid glutamate is now widely recognized as a primary excitatory neurotransmitter in the mammalian CNS (Watkins and Evans, 1981; Mayer and Westbrook, 1987; Shannon and Sawyer, 1989). Excessive stimulation of excitatory amino acid receptors can result in neuronal necrosis via two distinct contributing processes (Olney et al., 1971; Choi, 1987; Rothman and Olney, 1995). The first phase produces marked neuronal swelling, and is characterized by a depolarization of neuronal membranes, followed by Na^+ influx and secondary Cl^- and H_2O influx. The second phase involves calcium influx, mediated primarily by NMDA receptor activation of PLCβ/IP_3 second messenger systems, and release of intracellular calcium from stores. This activates intracellular lipases and proteases, generates free fatty acids and free radicals, produces mitochondrial dysfunction and depletes cellular energy stores (Choi, 1988), resulting in the eventual degradation of neuronal cytoskeletal and membrane components. Neurotoxicity as a result of this pathway can occur in response to a wide variety of insults, both physical and metabolic. In fact, under pathophysiological conditions such as hypoxia-ischaemia, the function of energy-dependent presynaptic high affinity EAA transporters can be impaired, leading to an accumulation of EAA's in the interstitium, prolonged activation of their receptors, and a severe exacerbation of the previously described cellular damage (Silverstein et al., 1986; McDonald and Johnston, 1990; O'Neill et al., 1994).

Hypoxic EAA-induced excitotoxicity is a fairly well characterized phenomenon in CNS tissue. NMDA receptor mRNA is expressed in both guinea pig and rat myenteric nerves (Burns et al., 1994; Broussard et al., 1994; Burns and Stephens, 1995), and exogenous EAA's have been shown to elicit increased intracellular calcium levels in cultured myenteric neurons (Kimball and Mulholland, 1995). Despite this, however, relatively little else is known about the function of glutamate and the potential for similar activity in the mammalian PNS (especially the ENS). The current study therefore sought to begin characterizing the effects of EAA agonists on myenteric neural function. Perifusion of the networks with 10μM NMDA produced a small but significant decrease in the magnitude of $\uparrow[K^+]_o$-evoked SPLI release, but had no appreciable effect on spontaneous release relative to control (Fig. 21*A*; Table 1). Networks perifused in the additional presence of 5μM DPCPX were predictably disinhibited, and displayed significantly greater spontaneous and $\uparrow[K^+]_o$-evoked release of SPLI (1.56 and 1.65-fold, respectively) relative to those perifused in the presence of NMDA alone. This degree of disinhibition for both basal and $\uparrow[K^+]_o$-evoked release, however, was less than that elicited by DPCPX on control networks in the absence of NMDA (2.80 and 3.57-fold, respectively; data not shown), suggesting that NMDA may have been inhibiting the preparation through a non-A_1 receptor-mediated pathway (putatively via interaction with an NMDA-type receptor). For its part, glutamate, perifused through the networks at a concentration of

100μM, was unable to elicit any change in the release profile for SPLI, either basally or during depolarization (Fig. 23; Table 1). This apparently negative finding may be due to rapid uptake of glutamate by endogenous transporters. Liu et al. (1997) report that immunoreactivity for three high-affinity glutamate transporters has been found in enteric ganglia, and that unless glutamate uptake is blocked, high concentrations of the excitatory amino acid are required to depolarize enteric neurons. Similarly, Kirchgessner et al. (1997) have found that glutamate can induce necrosis in isolated myenteric ganglia, but only at concentrations higher than 1mM. Therefore, the concentration of glutamate used in the present study may have been insufficient to exceed the capacity of endogenous EAA transporters to clear it from the interstitium. Studies utilising higher concentrations of the agonist and the addition of EAA uptake inhibitors are planned, but must take into account the buffering capacity of HEPES, and the potential for acidification of the system. In addition, further experiments are required to determine the functional presence of NMDA receptors in myenteric ganglion networks. Nearly all enteric neurons express NMDA R1 receptor subunits, which suggests that most enteric neurons may be susceptible to NMDA excitotoxicity (Kirchgessner et al., 1997). An initial set of experiments in the present study found that perifusion of isolated myenteric ganglion networks in the presence of the selective NMDA receptor antagonist LY 235959 (10μM) produced no significant change in either $\uparrow[K^+]_o$-evoked or spontaneous release of SPLI (Fig. 22; Table 1). Perifusion of the networks with NMDA in the additional

presence of the antagonist represents the next step in a series of ongoing experiments designed to better functionally characterize NMDA-mediated activity in the myenteric plexus. NMDA receptors, however, have been known to express different functional properties in different tissues, and so a direct correlation between observed phenomena in the CNS and in the ENS may not be appropriate. Inclusion of subunits from the NMDA R2 receptor subtype into NMDA R1 receptors may be one of the causes of functional diversity among these receptors. Studies have demonstrated that varying the NMDA R2 receptor subunit combined with NMDA R1 produces channels differing in glycine sensitivity and channel deactivation time (Monyer et al., 1992). An additional property of NMDA receptors that will require attention in future studies is the unique ability of Mg^{2+} to block the ion channel in a non-competitive voltage-dependent manner (Evans et al., 1977; Ault et al., 1980; Nowak et al., 1984). MgCl, which is present in Locke's solution, may therefore inactivate NMDA channels that would otherwise display sensitivity to the agonist in the isolated myenteric ganglion networks. Perifusion protocols may therefore require modification in future studies to eliminate Mg^{2+} from the perifusing medium in order to examine NMDA receptor function within the ENS. The data acquired thus far, however, support the presence of NMDA receptors within the myenteric plexus. Initial findings indicate that binding of exogenous NMDA with its receptor produces a functional decline in $\uparrow[K^+]_o$-evoked SPLI release. It is unclear at this time whether or not the receptors responsible for this effect are located on all enteric neurons, or

merely on certain subsets. The net inhibitory effect of the EAA on Substance P release may be an early indication that NMDA receptors are present on a subset of inhibitory of neurons within the myenteric plexus. This is supported by evidence that mRNA for the NMDA R1 receptor and the inhibitory transmitter VIP are co-expressed in rat enteric neurons (Burns and Stephens, 1995). However, further studies are clearly required.

CONCLUSIONS AND FUTURE STUDIES

The neuroprotective actions of endogenous adenosine in response to ischaemic and hypoxic insults in brain and cardiac tissue have been extensively characterized during the last twenty years. The studies presented here represent the first step in an attempt to extend such findings into the enteric nervous system. The results thus far are promising. Endogenous adenosine concentrations were shown to rise exponentially in response to declining pO_2 in the perifusing medium, with a net effect of increasing inhibition of the release the excitatory neurotransmitter Substance P. This inhibition was observed for both spontaneous and $\uparrow[K^+]_o$-evoked release of SPLI, and in fact was more pronounced in the former condition. This is consistent with the possibility that activity in spontaneously firing myenteric neurons may be under more significant inhibitory regulation by endogenous adenosine than $\uparrow[K^+]_o$-depolarized neurons as a consequence of the fact that the latter circumstance results in massive release of multiple mediators, both excitatory and inhibitory. A clear threshold pO_2 was found to exist between 260 mm Hg and 146 mm Hg, below which adenosine concentrations increased exponentially and above which there appeared to be little change in adenosine concentrations with respect to varying oxygen tension. The threshold corresponds to a slightly hyperoxic oxygen content of between 20% and 30% in the perifusing medium. This is of potential biological significance,

since it implies that inhibitory regulation of neurotransmission by detectable levels of endogenous adenosine may occur under normoxic conditions *in vivo* (i.e. with oxygenated blood). Moreover, several studies in the CNS have reported similar rises in endogenous adenosine concentrations in response to hypoxia, strengthening the argument for a neuroprotective role in the ENS. The diminished capacity of DPCPX to disinhibit SPLI release from myenteric networks in the presence of TTX revealed that at least some of the interstitial endogenous adenosine was derived from a neural source, in common with reported CNS findings. The ability to effectively culture intact myenteric ganglia will provide the groundwork for a series of future studies designed to identify putative neuroprotective effects of adenosine in the system. Firstly, long term incubation of the network preparation with anti-mitotic agents will allow for a more accurate assessment of neurally derived mediators and modulators (the current acute preparations contain unknown quantities of cellular debris from muscle tissue, connective tissue, vasculature, and glial cells - all of which are potential sources for interstitial adenosine). In addition, networks should be cultured under hypoxic/ischaemic conditions, and in the presence of excitatory neurotransmitters. This will allow for an analysis of the long term effects of oxygen deprivation on neuronal function and of the potential for excitotoxicity. Perifusion of the networks following incubation may permit an assessment of any functional changes, while measurement of lipid peroxidation and dye exclusion will provide indices of cellular damage induced by hypoxia and excitotoxicity, and of the putative protective effects of

adenosine. Future studies in this area should also include a characterization of nucleoside transporter function during hypoxia and hyperoxia. Blockade of the *es* nucleoside transporter with NBTI and dipyridamole may reveal a putative reversal of transport direction as the pO_2 in the perifusing medium increases from hypoxia towards hyperoxia, and the concentration of interstitial endogenous adenosine falls. This would be consistent with the exponential relationship between interstitial adenosine concentration and pO_2 demonstrated in this study.

With respect to the role of released ATP in myenteric neurotransmission, the present study has demonstrated that ATP *per se* mediates inhibition of $\uparrow[K^+]_o$-depolarized networks, via interaction at P_{2X} receptors. Furthermore, the study has shown that the P_{2X3} purinoceptor subtype may be present on enteric neurons, and that activation of this particular subtype may augment the $\uparrow[K^+]_o$-evoked release of SPLI from myenteric networks. The rapidity with which ATP is hydrolysed in the interstitial space argues against a tonic modulatory role for the nucleotide, but a recent study on brain stem neurons has concluded that ATP enhances the release of fast excitatory transmitters such as glutamate (Khakh and Henderson, 1998). The enhancement is abolished in the presence of PPADS, but persists in the presence of TTX, indicating a nerve ending locus for the P_{2X} receptor-mediated mechanism and illustrating the complexity of neurotransmitter interactions. Further studies in this area are clearly required,

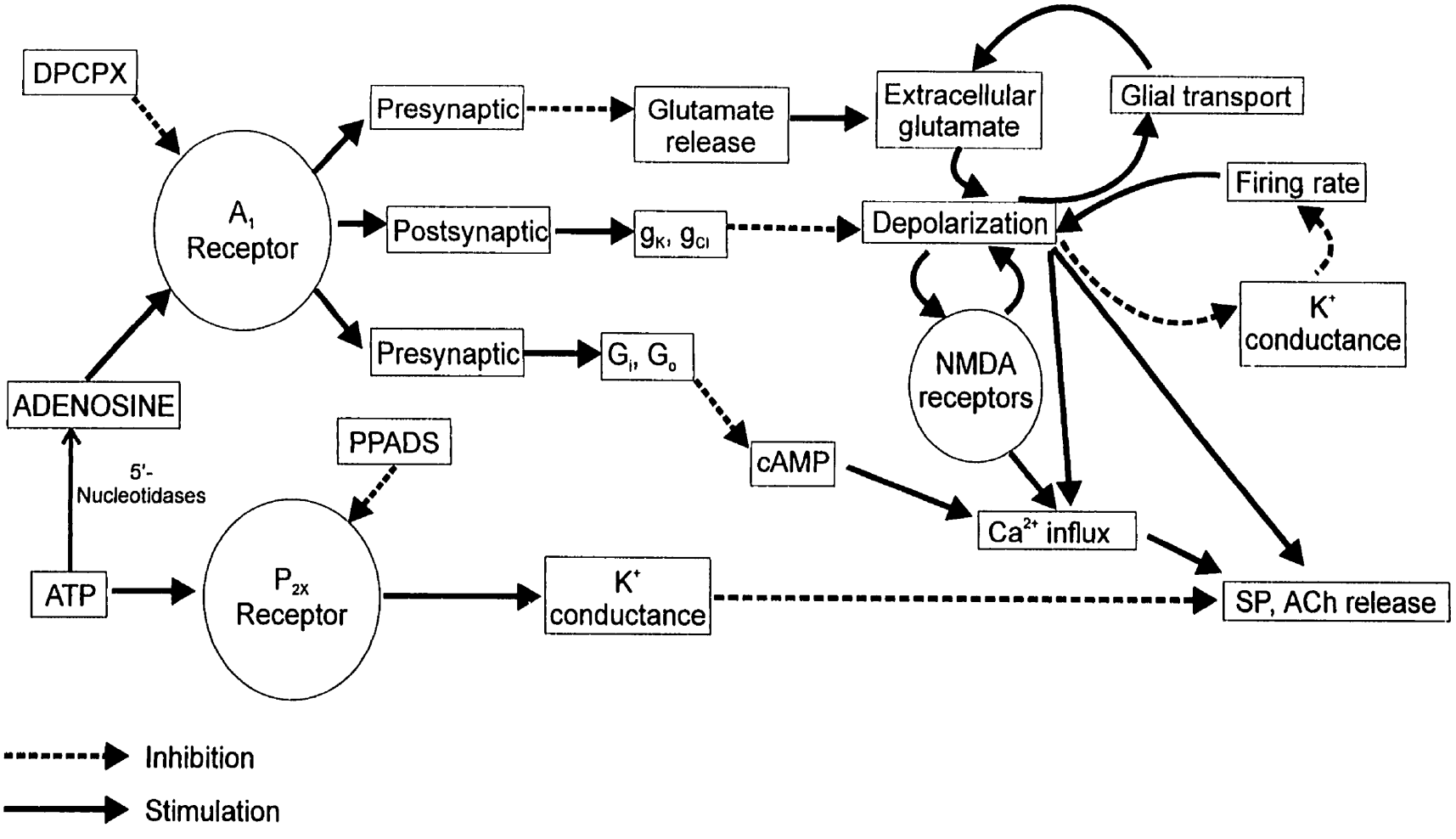
but functional pharmacological investigation will have to await the advent of more stable, selective P_2 agonists and antagonists. A more promising avenue of research may be an electrophysiological approach, using characteristic current traces to identify receptor subtypes on myenteric nerves.

Finally, establishing a functional pathway for excitatory amino acid neurotransmission in the myenteric plexus may prove to be the most important link to CNS studies. Glutamate has been shown to elicit ACh-induced, concentration-dependent contractions of ileal longitudinal muscle (Wiley et al., 1991). In addition, glutamate and NMDA can act as neurotoxins in acute and cultured preparations of enteric ganglia (Kirchgessner et al., 1997). Early indications from the present study are that NMDA-type receptors are present in myenteric neural networks. Application of exogenous NMDA elicited a small decrease in the magnitude of $\uparrow[K^+]_o$-evoked SPLI release, suggesting an inhibitory subset of neurons within the plexus as a possible locus for these receptors. A more rigorous investigation of these effects, however, should include conduction of all experiments in a Mg^{2+}-free medium, to facilitate NMDA channel opening. In addition, establishing a dose-response curve for NMDA agonists on isolated strips of LMMP, investigating the effects of adenosine A_1 receptor-mediated inhibition on this dose-response curve, and utilising electrophysiological techniques to assess the

specific NMDA receptor subtypes present should be priorities for future studies in this area.

This body of work represents the first stage in the characterization of adenosine as a putative neuroprotective agent in the ENS. The protection against ischaemia/reperfusion injury afforded to central neurons and cardiac tissue is well documented, but such evidence at the ENS is lacking. Ischaemic derangement of enteric neural function may ultimately underlie a variety of gastrointestinal disorders, and may contribute to the morbidity associated with intestinal transplantation. Altered MMC cycling following jejunoileal transplantation has been reported (Quigley et al., 1990), and ischaemia resulting from cocaine abuse (Van Thiel and Perper, 1992), inflammatory bowel disease (Wakefield et al., 1989), and mesenteric atherosclerosis undoubtedly produce changes in enteric neural activity. Interestingly, a recent study on rat nucleus accumbens determined that withdrawal from chronic cocaine use augmented uptake of extracellular adenosine, thus decreasing the ability of the nucleoside to inhibit glutamate release by activating A_1 receptors (Manzoni et al., 1998). Such a finding illustrates the complexity of interactions which may occur to exacerbate neuronal damage produced by ischaemia. The functional sequelae of adenosinergic inhibition of excitatory neurotransmission have yet to be defined in intestinal tissue. The putative cellular mechanisms by which adenosine and ATP may influence excitatory transmission in both central and

enteric neurons are illustrated in Appendix I. Studies with cardiac transplantation have shown that infusion of adenosine into the cardioplegic solution can protect donor hearts from cellular damage and prolong ischaemic storage time (Moneta, 1996; Katayama et al., 1997). Adenosine in blood cardioplegia has been shown to assist recovery of postischaemic left ventricular systolic performance (Hudspeth et al., 1994). As an adjunct to hyperkalemic cardioplegia, adenosine can attenuate K^+-induced intracellular calcium loading, and can improve postischaemic recovery (de Jong et al., 1990; Jovanovic et al., 1998). The mere potential for such therapeutic benefits in the ENS during intestinal transplantation and in the treatment of ischaemic bowel disorders underlines the need to better understand the functional role of adenosine in the enteric nervous system and the neuroprotection it may provide.



Appendix I: Diagram of the putative cellular effects of A_1, P_{2x}, and NMDA receptor activation on central and enteric neurons.

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