EXOGENOUS PURINES INDUCE DIFFERENTIAL RESPONSES IN THE PROXIMAL AND DISTAL REGIONS OF THE SPHINCTER OF ODDI: PARTIAL CHARACTERISATION OF THE PURINERGIC RECEPTOR SUB-TYPES INVOLVED

A thesis submitted for the degree of Doctor of Philosophy

By

Charmaine Michelle Woods

Bachelor of Biotechnology (Honours)

Pancreatobiliary Research Group

Department of General and Digestive Surgery

School of Medicine

Faculty of Health Sciences

Flinders University

Australia

TABLE OF CONTENTS

SUMMARY OF THESIS

The sphincter of Oddi (SO) is a neuromuscular structure located at the junction of the bile and pancreatic ducts with the duodenum. The primary functions of the SO are to regulate the delivery of bile and pancreatic juice into the duodenum, and to prevent reflux of duodenal contents into the biliary and pancreatic systems. Neural, hormonal or functional disturbances of biliary motility can lead to painful and sometimes life threatening clinical conditions, such as SO dysfunction and acute pancreatitis. Clearly understanding the regulation of biliary and duodenal motility patterns is necessary and may provide useful pharmacological sites for drug development to aid in the treatment of these diseases.

Spontaneous activity of the SO is regulated by complex interactions between the enteric nervous system, hormones, possibly interstitial cells of Cajal and other bioactive agents, together with modulation via neural reflexes between the duodenum, common bile duct/gallbladder, and stomach. Purines are one group of neurotransmitters/regulatory agents that have been shown to effect gastrointestinal motility, however their functions in the regulation of SO motility have not been elucidated.

The studies described in this thesis used *in vitro* organ bath techniques and *in vivo* preparations to determine the effects of exogenous purines on possum SO and duodenal motility. The possum SO has been extensively characterized and is an excellent model for motility studies. *In vitro*, exogenous adenosine was found to decrease spontaneous activity in both

the SO and duodenum. In contrast exogenous ATP induced both excitatory and inhibitory responses in the SO and duodenum. Interestingly, the adenosine and ATP-induced effects were predominantly exhibited by the proximal portion of the SO (proximal-SO), with no or little effect observed in the distal portion of the SO (distal-SO). These data support the hypothesis that the SO is comprised of different functional components that can act differently in response to certain stimuli, and highlights the importance of studying each of the SO components.

Agonists and antagonists, together with immunohistochemical studies, were used in an attempt to identify the P1 and P2 receptor sub-types responsible for mediating the adenosine- and ATP-induced responses. In the duodenum the adenosine-induced decrease in spontaneous activity was likely to be mediated by A_{2A} and A_3 receptors, but the receptors mediating the proximal-SO response could not be identified. In the duodenum ATP induced a complex non-neural response consisting of a $P2X_1$, and $P2Y_2$ and/or $P2Y_4$ mediated immediate inhibition. This was followed by a return to baseline activity or small excitation. The response concluded with a late inhibitory response, likely to be mediated by $P2Y_1$ receptors, but the effects of other P2Y receptors could not be excluded. In contrast, ATP application to the proximal-SO evoked a partially neurally mediated early excitation, likely via P2X receptors, followed by an inhibition of activity, likely via activation of non-neural $P2Y_2$ and/or $P2Y_4$ receptors.

In vivo studies with exogenous application of adenosine and ATP to the SO activated neural pathways to produce increased motor activity.

Characterisation of these neural pathways found ATP and/or adenosine to activate excitatory cholinergic motor neurons. ATP also activated an inhibitory nicotinic/nitrergic pathway.

This is the first comprehensive investigation of the possible involvement of purines in the regulation of SO motility. These studies demonstrate that exogenous purines influence SO and duodenal motility, inducing complex neural and non-neural responses, acting via multiple P1 and P2 receptors. It now remains to be determined if endogenously released purines induce similar responses, together with elucidation and location of the receptor sub-types involved.

PUBLICATIONS RESULTING FROM THE STUDIES IN THIS THESIS

Manuscripts

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2003) A_{2A} and A_3 receptors mediate adenosine-induced relaxation in spontaneously active possum duodenum *in vitro*. *British Journal of Pharmacology*, **138**:1333-1339.

Woods, C.M., Mawe, G.M., Toouli, J. and Saccone, G.T.P. (2005) The sphincter of Oddi: Understanding its control and function. *Neurogastroenterology and Motility*, **17** (Suppl 1): 31-40.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2006) Exogenous purines induce differential responses in the proximal and distal regions of the possum sphincter of Oddi. *Autonomic and Autacoid Pharmacology* (in press).

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2006) Exogenous adenosine triphosphate and adenosine stimulate proximal sphincter of Oddi motility via neural mechanisms in the anesthetized Australiam possum. *Digestive Diseases and Sciences* (in press).

Note: these publications are included at the end of this thesis

Abstracts and Conference Presentations

Woods, C.M. and Saccone, G.T.P. (2000) Exogenous adenosine has a biphasic effect on the spontaneous contractile activity of duodenal longitudinal muscle strips from the Australian brush-tailed possum. Purines 2000, Madrid, Spain, July 2000. *Drug Development Research*, **5**:79.

Woods, C.M. and Saccone, G.T.P. (2000) Exogenous adenosine inhibits spontaneous contractile activity in the Australian possum sphincter of Oddi *in vitro*. International Society of Autonomic Neuroscience, London, UK, July 2000. *Journal of Autonomic Nervous System*, **82**:82-83.

Woods, C.M. and Saccone, G.T.P. (2000) Exogenous adenosine triphosphate (ATP) has a complex effect on spontaneous contractile activity in the Australian possum sphincter of Oddi (SO) *in vitro*. International Society of Autonomic Neuroscience, London, UK, July 2000. *Journal of Autonomic Nervous System*, **82**:83.

Woods, C.M., Toouli, T. and Saccone, G.T.P. (2000) Effects of adenosine and adenosine triphosphate (ATP) on sphincter of Oddi spontaneous contractile activity. The Surgical Research Society of Australasia, Adelaide, Australia, August 2000. *ANZ Journal of Surgery*, **71**:A115.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2000) Effect of exogenous adenosine on sphincter of Oddi and duodenal contractile activity *in vitro*. The Physiological and Pharmacological Society, Melbourne, Australia, November 2000. *Proceedings of the Australian Physiological and Pharmacological Society*, **31**:81P

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2001) Inhibitory effect of adenosine on spontaneous duodenal activity in the Australian Brush-tailed possum. XXXIV International Congress of Physiological Sciences, Christchurch, New Zealand, August 2001.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2002) Pharmacological characterisation of purinergic P1 receptors in duodenum longitudinal smooth muscle of the Australian possum. Digestive Diseases Week, San Francisco, USA, May 2002. *Gastroenterology*, **122**:A258.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2002) Characterisation of P1 receptors in the duodenum. 7th International Symposium on Adenosine and Adenine Nucleotides, Gold Coast, Queensland, Australia, September 2002.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2002) Exogenous adenosine triphosphate increases sphincter of Oddi activity *in vivo*. Australian Gastroenterology Week, Adelaide, Australia, October 2002.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2003) Sphincter of Oddi (SO) motility is increased by topical application of adenosine triphosphate (ATP). Australian Neuroscience, Adelaide, Australia, January 2003. *Proceedings of the Australian Neuroscience Society*, **14**: ORAL-02-07.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2003) Exogenous ATP increases sphincter of Oddi motility, acting via cholinergic and nitrergic pathways. Enteric Nervous System, Banff, Canada, July 2003. *Neurogastroenterology and Motility*, **15**: 216.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2004) Exogenous adenosine increases sphincter of Oddi motility, acting via cholinergic motor neurons. Digestive Diseases Week, New Orleans, USA, May 2004. *Gastroenterology* **126**: A-278.

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2004) Exogenous adenosine increases sphincter of Oddi motility, acting via cholinergic motor neurons. 6th World Congress, International Hepato-Pancreato-Biliary Association, Washington, USA, June 2004.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

……..

Charmaine M. Woods

September 30th, 2005

ACKNOWLEDGEMENTS

I wish to take the opportunity to thank a number of people whose assistance has been instrumental in the studies associated with this thesis.

I am indebted to my supervisor Associate Professor Gino Saccone for his tireless support and encouragement during my candidature. I wish to thank Gino together with Dr Steve Johnson and Professor Jim Toouli for their useful discussions and comments regarding the preparation of this thesis, associated manuscripts and conference presentations.

I am most grateful for the technical expertise, nimble fingers, patience and friendship of Ann Schloithe, especially in teaching me the fine surgery required for these studies and for her preparation of the pico-manometry catheters. I am also grateful for Ann's help together with aid from the statistical consultant Lynne Giles that was essential for the design and interpretation of the statistical analyses associated with these studies. The assistance and friendship of staff and students in the Pancreatobiliary Research Laboratory and other research groups, both past and present, was especially helpful. In particular I wish to thank Marlene Grivell, Aaron Citti, Adrian Meedeniya, Lisa De Candia and Magali Chauvet.

There are a number of people who assisted with the immunohistochemical studies that all require my thanks for their time and efforts: Professor Marcello Costa, Associate Professor Sean Ward, Jim Brennan, and Dr Shiyong Yuan.

I wish to thank Dr Italo Biaggioni of Vanderbilt University, USA for the kind donation of the A_{2B} receptor antagonist IPDX used in the *in vitro* studies.

I would especially like to mention the smiling and helpful services of the staff in Medical Illustration and Media together with the staff in the Animal House, thank you all for your help.

Finally, I wish to thank my husband, family and friends for their patience, encouragement and tireless support during the time taken to complete these studies.

ABBREVIATIONS

The following abbreviations are used throughout the text, figures and figure legends of this thesis.

ATP	Adenosine triphosphate
CBD	Common bile duct
CCK-8	Cholecystokinin octapeptide
EFS	Electrical field stimulation
ENS	Enteric nervous system
EPSP	Excitatory post synaptic potential
GI	Gastrointestinal
IA	Intra-arterial
ICC	Interstitial cells of Cajal
IV	Intravenous
IPSP	Inhibitory post synaptic potential
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
SEM	Standard error of the mean
SO	Sphincter of Oddi
TTX	Tetrodotoxin
UTP	Uridine triphosphate

Note: abbreviations for purinergic drugs are listed in **Table 1.2a** and **Table 1.2b**

STRUCTURE OF THESIS

History of candidature

My candidature for this thesis commenced in May 1999. The literature was surveyed during 1999 and preliminary studies were performed, leading to the development of the overall hypothesis and specific hypotheses. Experimental studies were performed from 2000-2002 on a full-time basis. Subsequently during 2003-submission the thesis was compiled on a part-time basis whilst undertaking full-time employment. During my candidature there has been considerable progress in understanding the role of purines in the small and large intestine, with regard to both secretory and motility functions, and in the localization of purinergic receptor sub-types. However there have been very few developments regarding the understanding of purines in the biliary tree.

Thesis chapters

The structure of this thesis conforms to Flinders University guidelines. This thesis is presented in the following chapters.

Chapter 1 contains an overview of the relevant literature up to the time I commenced experimental studies (end 1999). This literature review has been updated to include key findings that aid in our understanding of biliary motility, but have bearing on the hypotheses generated or the experimental design. A major component of this chapter is a review of purinergic receptors, their agonists and antagonists. As information was limited regarding the use of these drugs in biliary or possum tissues, information

published prior to and during the period of experiments (pre1999-2002) is presented with regard to their use in the small intestine, specifically the guinea-pig ileum. Publications that directly relate to the interpretation of the data presented in this thesis that have been published since 2003 are included in the discussion section of the appropriate Results chapter.

Chapter 1 concludes with the presentation of the general hypothesis and specific hypotheses, followed by the research aims. It should be noted that technical limitations associated with the use of SO tissues resulted in the possum duodenum being used to evaluate drug concentration ranges. Therefore, the hypotheses and aims were expanded to incorporate a comparison between purinergic responses and receptors in the SO and duodenum.

Chapter 2 describes the methodology, experimental, analysis and statistical protocols used for the *in vitro* and *in vivo* studies.

Chapters 3-8 present the results of the experimental studies. Each chapter begins with a brief introduction, which builds on the information presented in the literature review and the findings presented in previous chapters. This is followed by the aims of the particular study, a brief methods section, and the results of the investigations. Each of these chapters contains an interpretation and comprehensive discussion of the data presented and refers to discussion in previous chapters to maintain continuity.

To aid interpretation of the data a number of summary diagrams are presented. Summary 1 summarises the *in vitro* investigations with adenosine, in both the SO and duodenum. Summary 2 summarises the *in vitro* investigations with ATP, in both the SO and duodenum. Summary 3 summarises the *in vivo* investigations of adenosine and ATP in the SO.

Chapter 9 contains a general discussion. As the previous chapters have included a comprehensive discussion of the data presented, the purpose of this final chapter is to relate the finings to the original hypotheses. This section concludes with suggestions for future research.

Appendix 1 contains the methodology and results of the immunohistochemical studies. These immunohistochemical studies were performed prior to the *in vitro* antagonist experiments in an attempt to identify the purine receptor sub-types present in the possum SO and duodenum, and their distribution. However due to non-specificity of the antibodies tested the results were equivocal and no conclusions could be drawn, but are presented for completeness.

The thesis concludes with a list of references to publications mentioned in the text.

Location of figures

For minimize disruption to the text, all figures and tables are located in a group near the end or prior to the discussion of each chapter.

Chapter 1

1 INTRODUCTION

This chapter provides a detailed literature review of the control and regulation of spontaneous activity of the sphincter of Oddi (SO). Activity of the SO should not be considered in isolation as the pressure present in both the common bile duct and the duodenum influences its function. For the studies described in this thesis the SO is considered part of a functional unit, which includes the duodenum, and therefore information regarding duodenal motility is also presented. Based on the control of intestinal motility, a model for SO motility is presented which integrates the involvement of the enteric nervous system (ENS) with interstitial cells of Cajal (ICC). The likelihood of the involvement of purines in the control of SO activity is raised. Information regarding purine receptors, agonists and antagonists is presented followed by a review of the limited literature regarding the role of purines in the regulation of SO motility. As little is known regarding the actions of purines on the function of the SO, or other sphincters, a brief review of the actions of purines on small intestinal motility is presented as a basis for drug concentration selection and recognition of the possible effects purines may evoke in the SO. This chapter concludes with a statement of the hypotheses and specific aims that will be evaluated and described in subsequent chapters of this thesis.

1.1 Overview

The SO is a neuromuscular structure located at the junction of the bile and pancreatic ducts with the duodenum. The primary functions of the SO are to regulate the delivery of bile and pancreatic juice into the duodenum, and to

prevent the reflux of duodenal contents into the biliary and pancreatic systems.

Spontaneous activity (motility) of the SO is primarily myogenic in nature, and like the duodenum is probably regulated by ICC. An extensive neural innervation exists within the SO to regulate its activity, and also co-ordinates activity between the SO and the duodenum, gallbladder and stomach. Changes in SO and duodenal motility are the result of the integration of signals from ICC, nerves and other bioactive agents at the level of the smooth muscle cells. SO and duodenal motility have been shown to be affected by a number of neurotransmitters, gut endocrine peptides and other bioactive agents. Purines are one group of neurotransmitters/regulatory agents that have been shown to effect gastrointestinal (GI) motility, however their actions on SO motility has not been elucidated.

Neural, hormonal or functional disturbances of biliary motility patterns can lead to painful clinical conditions, such as SO Dysfunction and acute pancreatitis, which may become life threatening. Clearly, understanding the regulation of biliary motility patterns and neural reflexes is necessary and will provide useful information for understanding SO Dysfunction and hence identify possible sites for future drug development to permit targeted pharmacological therapy.

1.2 Anatomy of the SO and duodenum

1.2.1 General extra-hepatic biliary anatomy

The extra-hepatic biliary tree consists of the gallbladder, common bile duct (CBD), and SO which joins the duodenum. The biliary system acts as a conduit for the delivery of bile into the duodenum. Bile is secreted from the hepatocytes into the bile canaliculi. The canaliculi drain into ductules, which enlarge to become the left and right hepatic ducts. These in turn form the common hepatic duct, which fuses with the cystic duct from the gallbladder (a pear-shaped organ under the liver which stores and concentrates bile) to form the CBD. The terminal portion of the CBD, usually together with the terminal pancreatic duct, pass through a muscular sphincter, the SO, which traverses the duodenal wall, entering into the duodenal lumen via the papilla of Vater (**Figure 1.1**).

1.2.2 Duodenal anatomy

Physiological studies have implicated the duodenum in modifying or modulating SO activity and it is now known that a neural circuit exists between the SO and the duodenum (see **Section 1.3.4**), therefore it is important to consider duodenal activity when investigating SO motility.

The duodenum is the first portion of the small intestine. It consists of mucosal, submucosal, circular muscle and longitudinal muscle layers and the serosa. The submucous plexus is found between the mucosa and circular muscle layers, and a myenteric plexus is located between the circular and longitudinal muscle layers (**Figure 1.2**). Duodenal anatomy appears to be consistent between species.

1.2.3 SO anatomy and function

The SO is an anatomically and functionally distinct organ, separate to the duodenum. The arrangement of the bile and pancreatic ducts in the SO and the length of the SO are both species dependent (reviewed by Dodds *et al.* 1989; Toouli and Baker 1991) (**Figure 1.3**). In humans the bile and pancreatic ducts usually unite forming a common channel, with the SO present within the duodenal wall. A similar arrangement is seen in cats. In dogs, pigs and rabbits the SO is also contained within the duodenal wall, however the pancreatic duct enters the duodenum separately to the bile duct. In the guinea-pig the bile and pancreatic ducts unite, forming a large ampulla, within the duodenal wall. Similar to the human SO, the Australian Brush-tailed possum and the American opossum SO encompasses both the bile and pancreatic ducts however they both have a prominent portion (approximately 1cm and 3cm, respectively) located outside the duodenal wall (extra-duodenal). This latter feature has lead to the possum and opossum being commonly used for biliary motility studies.

The possum SO has been used exclusively in the studies described in this thesis and therefore its anatomy is presented in more detail. The sphincter muscle envelopes the terminal portion of the bile and pancreatic ducts. The lumens of the bile and pancreatic ducts join to form a short ampulla within the duodenal wall, and enter into the duodenum via the papilla of Vater. The sphincter muscle contains four separate muscle layers (**Figure 1.2**). The outer longitudinal and outer circular muscle layers are a continuation of the duodenal muscularis externa. The inner sphincter muscle layer consists of inner circular and inner longitudinal muscle layers. The inner longitudinal

muscle surrounds the CBD and pancreatic duct lumens independently, whereas the circular muscle layer encases both ducts. As the sphincter passes obliquely through the duodenal wall the inner circular muscle layer continues beyond the duodenal muscularis externa and extends into the duodenal submucosa. The inner longitudinal muscle layer on the other hand continues beyond the circular muscle and is contiguous with the muscularis mucosae of the duodenum (Padbury *et al.* 1993a).

An extensive neural innervation also exists within the SO. In the possum SO, located between the outer longitudinal and outer circular muscle layers of the extra-duodenal portion of the SO is the external sphincteric plexus, which is continuous with the duodenal myenteric plexus (**Figure 1.2**). Located between the inner circular and inner longitudinal muscle layers is the internal sphincteric plexus, which traverses the entire length of the SO, and is continuous with the duodenal submucous plexus (Padbury *et al.* 1993a). Padbury *et al.* (1993a) also identified a third sphincteric plexus located between the two circular muscle layers termed the inter-sphincteric plexus, which may be continuous with the deep muscular plexus of the duodenum. The SO is a complex structure with multiple muscle and neural layers. This structural complexity may be responsible for functional differences within the SO.

1.2.3.1 Functional regions within the SO: proximal-SO and distal-SO

A number of regions within the SO have been identified using anatomical studies, however the presence of these regions is species dependent. The regions have been termed the sphincter choledochus, sphincter pancreaticus, sphincter ampullae and sphincter papillae (Boyden 1937;

Schwegler and Boyden 1937a; Boyden 1957; Boyden 1937; Schwegler and Boyden 1937c; Schwegler and Boyden 1937a; Schwegler and Boyden 1937b; Schwegler and Boyden 1937c) (**Figure 1.3**).

It has been suggested that these regions have the potential to act independently, however this view is controversial (Boyden 1957; Suarez 1982; Dodds *et al.* 1989). A manometric assessment of canine SO, using a multi-lumen catheter, suggested there were three functional sphincters: sphincter choledochus, sphincter papillae and sphincter pancreaticus (Hauge and Mark 1965). More recently, a number of functional and electrophysiological studies in American opossum, guinea-pig, rabbit and possum SO have indicated that the distal (juxta duodenum) and proximal segments of the SO (distal-SO and proximal-SO respectively) can display different activities in response to certain stimuli (Helm *et al.* 1985; Vogalis *et al.* 1989; Baker *et al.* 1990c; Hirose and Ito 1991; Lonovics *et al.* 1994; Longeville *et al.* 1997; Parkman *et al.* 1998; Woods *et al.* 2000; Saccone *et al.* 2000; Al-Jiffry *et al.* 2001). Furthermore, there is also controversy as to whether the bile duct and pancreatic duct components of the SO can also display independent activities (Ivy *et al.* 1933; Carr-locke *et al.* 1985; Saccone *et al.* 2000; Woods *et al.* 2000).

Two different zones of activity in the possum SO were first identified in manometric studies, and were located at the distal and proximal ends of both the bile and pancreatic components of the SO (Di Francesco *et al.* 1994; Saccone *et al.* 2000). It was proposed that the differences in activity might reflect differences in the neural circuitry of the SO. Further *in vitro* and *in vivo*

studies have revealed that different regions of the SO respond differently to certain stimuli, e.g. secretin, cholecystokinin octapeptide (CCK-8) or neural stimulation of the duodenum (Saccone *et al.* 2000; Woods *et al.* 2000; Al-Jiffry *et al.* 2001). Studies in the guinea-pig SO have suggested a greater inhibitory activity in the distal-SO (Vogalis *et al.* 1989; Hirose and Ito 1991), with electrical field stimulation (EFS) stimulating tonic contractions in rings of the choledochal sphincter and phasic contractions in rings from the ampulla region (Vogalis *et al.* 1989). Interestingly, immunohistochemical and receptor binding studies have found polarized distributions for substance P receptors and a gradient of nitric oxide synthase (NOS) innervation in the SO (Von Schrenck *et al.* 1991; Parkman *et al.* 1998; Simula *et al.* 2001f) which may explain different responses by different regions of the SO.

Some of theses differential responses may be explained by the SO anatomy. In the possum it is important to note that the outer SO muscle layers, which are continuous with the duodenal muscularis externa, are not incorporated into the papilla region of the SO (most distal-SO) (**Figure 1.2**). Therefore signals from the external sphincteric plexus of the proximal-SO or from the duodenal myenteric plexus may not be directly communicated to the distal-SO, and this may account for some of the observed regional differences in activity. Clearly, studies concerning SO motility need to consider the responses of the different SO regions to bioactive agents or neural stimulation to obtain a better understanding of SO function.

1.2.3.2 SO - pump or resistor?

The action of the SO in facilitating the flow of bile and pancreatic juice into the duodenum appears to be species dependent, and has been categorised

as acting like a pump (guinea-pig, American opossum, rabbit, prairie dog) or like a resistor (possum, pig, cat, human) (Sand *et al.* 1997). If the SO acts as a pump, an increase in phasic contractile activity (i.e. frequency and amplitude) is required to pump more bile into the duodenum. If the SO acts as resistor, a decrease in basal pressure and/or phasic contractile activity will decrease the resistance to bile flow through the SO therefore allowing more bile into the duodenum.

Recent studies in the possum SO raise doubt as to the validity of the pump and resistor categories. It has been found that the possum SO can act as either a pump or a resistor, with the function being dependent on both CBD and duodenal pressures (Grivell *et al.* 2004b). *In vitro* investigations demonstrated that the possum SO can act as a pump under low CBD pressure (<3.5mmHg), but has a more resistor-like function at higher (>3.5mmHg) CBD pressures. Similarly increasing duodenal pressure affected SO function. Importantly this study highlights the. These investigations highlight the influence of the organs adjacent to the SO in the regulation of SO motility. It is therefore important to consider the SO as part of a 'functional unit', incorporating both proximal-SO and distal-SO regions in conjunction with the adjacent CBD and duodenum.

1.3 Control of SO spontaneous activity

The generation of spontaneous activity in the SO has been poorly investigated. Therefore for the purposes of the studies presented in this thesis it is assumed that spontaneous activity of the SO is generated in a similar manner to the small intestine. Spontaneous activity of the small

intestine is myogenic in nature and modulated by ICC together with extrinsic and intrinsic neural inputs to the smooth muscle cells. Below is a brief summary of the actions of ICC and nerves in generating spontaneous activity based on the literature from the small intestine, and concludes with an integrated model for the generation of spontaneous activity in the SO.

1.3.1 ICC

ICCs generate cyclic depolarisation and repolarisation of smooth muscle cells (slow waves) that result in the spontaneous activity observed in the GI tract. The slow waves are believed to determine the contraction frequency in the GI tract.

Involvement of the c-kit protein in ICC function (Maeda *et al.* 1992) has lead to a number of immunohistochemical studies which found ICC to be located between nerve terminals and smooth muscle cells (Ward *et al.* 1994; Romert and Mikkelsen 1998; Young *et al.* 1996). Several classes of ICC are now recognized (Burns *et al.* 1997; Sanders *et al.* 1999b), some are pacemaker cells that generate and conduct electrical slow waves to smooth muscle cells, whereas other types receive, transduce, and conduct neural signals to the smooth muscle. Receptors for a number of neurotransmitters have now been located on ICC, including receptors for acetylcholine (M_2, M_3), tachykinins (NK_1, NK_3) and vasoactive intestinal polypeptide (VIP) ($VPAC_1$) (see Kunze and Furness 1999; Lecci *et al.* 2002a). Furthermore ICC of the lower oesophageal and pyloric sphincters generally express nitric oxide synthase (NOS) immunoreactivity suggesting ICC mediate inhibitory neurotransmission (Ward *et al.* 1998). Recently receptors for adenosine

triphosphate (ATP) ($P2X_2$, $P2X_5$) were identified on ICC (Burnstock and Lavin 2002a).

Since the development of suitable immunohistochemical tools for the identification of ICC, it is now believed that ICC play a pivotal roll, in conjunction with the enteric nervous system, in controlling and/or modulating GI. A recent review article supports this view by integrating the action of various neurotransmitters on ICC, with nerves and smooth muscle cells (Lecci *et al.* 2002b). The ICC literature has been reviewed extensively over the last few years (Farrugia 1999; Horowitz *et al.* 1999; Sanders *et al.* 1999a; Komuro 1999; Takayama *et al.* 2002; Vanderwinden 1999; Lecci *et al.* 2002c; Takayama *et al.* 2002).

1.3.1.1 ICC in SO

The presence of ICC in the SO, particularly in the pacemaker proximal-SO region, has been suspected, and a recent conference report has indicated the presence of ICC in the SO. In the guinea-pig SO and adjacent duodenum c-kit immunoreactive cells were detected closely associated with the ganglionated nerve plexus, suggesting the presence of ICC (Parr *et al.* 2003). Interestingly the presence of c-kit immunoreactive cells extended only a few micrometers into the distal CBD suggesting the CBD is devoid of ICC.

1.3.2 Innervation of SO

1.3.2.1 Extrinsic innervation of the biliary tree

The gallbladder, bile ducts and SO are supplied with extrinsic nerves via the anterior and posterior hepatic plexuses, consisting of postganglionic fibres

from the coeliac ganglion, and preganglionic sympathetic fibres from the left and right vagus (Burnett *et al.* 1964). The extrinsic innervation of the biliary tree has previously been reviewed (Toouli and Baker 1991).

Very little is known about afferent nerves in the biliary tree. Early studies found afferent fibres from the biliary tree passed along the sympathetic nerves and to a lesser extent along the right phrenic nerve, with a negligible presence of vagus afferent nerves (see Burnett *et al.* 1964).

1.3.2.2 Intrinsic innervation of the biliary tree

As a result of the embryological development of the biliary tree and pancreas from a diverticula of the small intestine, intrinsic ganglia in the gallbladder, CBD and SO are considered part of the ENS (see Furness and Bornstein 1995).

There are differences in the innervation of the SO between species, but there is a general agreement that a rich distribution of nerves and ganglia exist in the muscle and mucosa (Padbury *et al.* 1993a). The neural plexuses from the duodenum continue through the SO in guinea-pig and possum (Cai and Gabella 1983; Padbury *et al.* 1993a; Simula *et al.* 2001e) (**Section 1.2.3**; **Figure 1.2**).

In the biliary tree immunohistochemical studies have identified the presence of a number of neurotransmitters or neuromodulating agents (see Woods *et al.* 2003b). However only a few studies have identified specific neuronal classes (Kennedy and Mawe 1998a; Meedeniya *et al.* 2001; Meedeniya *et al.* 2003). It should be noted that there are substantial immunohistochemical

and electrophysiological differences between regions of the GI tract and between species (Bornstein *et al.* 2002), and therefore the functional neuron classes and the intrinsic neuronal reflexes characterized in the guinea-pig ileum (see reviews by Furness and Bornstein 1995; Wood 1999; Bornstein *et al.* 2002; Furness and Sanger 2002) should only be used as a guide to the innervation of other GI tissues and species. In fact, ganglia in the biliary system exhibit structural, neurochemical and physiological characteristics that are different to the neurons of the ENS (Balemba *et al.* 2004a). Furthermore, because the function of the SO is distinctly different to that of the small intestine one could argue that the intrinsic neural reflexes should be expected to be different.

1.3.3 Integrated model of spontaneous GI motility

Until recently the effects of ICC and the ENS on GI motility were considered in isolation. The following model was one of the first to integrate the actions of both the ICC and the ENS in generating and modifying GI motility (Horowitz *et al.* 1999). For the basis of discussion in this thesis it is assumed that both ICC and the ENS are involved in the generation and/or modulation of spontaneous SO activity (**Figure 1.5**).

ICC networks in pacemaker regions express the ionic mechanism to generate slow waves that actively propagate through the ICC network via gap junctions connecting the ICC. The slow waves electrotonically conduct into smooth muscle cells (which are coupled to the ICC but lack the mechanism to generate slow waves). The slow waves depolarise the smooth muscle cells and activate voltage-dependent (L-type) Ca^{2+} channels. If the

threshold depolarisation is achieved, Ca^{2+} action potentials result. If threshold is not achieved, the activation of an inward current in smooth muscle cells is seen as an increase in the plateau phase of the slow wave (i.e. no muscle contraction).

The coupling of slow waves to contractions can also be regulated by external stimuli such as neural inputs. Excitatory neurotransmitters activate non-selective cation channels that increase the effectiveness of a slow wave reaching the threshold potential. Inhibitory neurotransmitters activate potassium channels that decrease the probability of a slow wave reaching the threshold potential. Furthermore, intramuscular ICC (ICC-IM) are closely associated with enteric neurons and appear to act as receivers and transducers for some of the neural inputs. Neural inputs can be transmitted through ICC-IM to the smooth muscle cells because they are electrically coupled to the smooth muscle. In addition some neurotransmitters, particularly peptides, are likely to “spill over” and directly affect receptors expressed by the smooth muscle.

This model incorporates knowledge about ICC and slow wave generation in GI tissues and suggests that input from the ENS, either onto the ICC or directly onto the smooth muscle, results in changes in smooth muscle contraction. It should be noted that other models for the generation of spontaneous activity have also been presented in recent years, but all infer an interaction between ICC, nerves and smooth muscle cells.

The above model for the generation, propagation and regulation of spontaneous GI motility can be further extended to include a constant inhibitory neural drive to the smooth muscle. The inhibitory innervation of the GI tract has been shown to be very important in regulating the passage of slow waves to smooth muscle cells and regulating the resultant contraction or relaxation. It had been observed that circular muscle does not respond to all slow waves and that contractions do not spread via the smooth muscle syncytium throughout the entire length of the intestine every time they occur. It is believed that muscle can only respond to slow waves when the inhibitory motor neurons in the segment of the gut are turned off by input from other neurons, and similarly action potentials and contractions can only propagate into 'disinhibited' regions (Kunze and Furness 1999; Wood *et al.* 1999). Consequently, the inhibitory neurons control the initiation of slow wave derived-contraction and how far that contraction can propagate through the smooth muscle syncytium.

This theory of constant inhibition of GI muscle is important to consider when studying the peristaltic-like contractions observed in the SO. Studies in guinea-pig, pig and human SO have suggested that the inhibitory neurotransmitter NO is released tonically to inhibit contractile activity (Sand *et al.* 1997; Vogalis and Smith 2000b). Tonic inhibition of the SO by NO could have opposite physiological responses depending on the function of the SO. In a pump-like SO inhibition of excitatory innervation would decrease trans-sphincteric flow, whereas in a resistor-like SO trans-sphincteric flow would be increased (Sand *et al.* 1997).

1.3.4 Neural reflexes between the SO and other organs

SO motor activity is complex as there are many factors that can influence its activity including: slow wave activity presumably controlled by ICC, neural inputs from the ENS, extrinsic neural inputs and bioactive agents released in response to nutrients or inflammation, CBD and duodenal pressures. In addition, SO activity is also modulated by neural reflexes that exist between the SO and the stomach (Wyatt 1967; Webb *et al.* 1988), the duodenum (Padbury *et al.* 1993c; Saccone *et al.* 1994c; Simula *et al.* 1997; Kennedy and Mawe 1998b; Mawe and Kennedy 1999; Simula *et al.* 2001d; Konomi *et al.* 2002b), and the CBD/gallbladder (Wyatt 1969; Thune *et al.* 1986; Padbury *et al.* 1993b; Sonoda *et al.* 2005). Many of these reflexes were studied using distension or EFS. Furthermore extensive neural connections with the SO have been demonstrated by retrograde tracing techniques and immunohistochemistry. To further complicate our understanding of SO activity, the SO has distinct regions (distal-SO and proximal-SO) and intra-sphincteric reflexes may also exist between these regions (Vogalis *et al.* 1989; Hirose and Ito 1991; Saccone *et al.* 2000). In many of these reflexes the nicotinic and cholinergic components have been examined, but few studies have characterised other neurotransmitters that may be involved.

1.3.5 Possible involvement of purines in SO motility

There are many neurotransmitters and/or neuromodulators that could potentially control/modulate SO motility. ATP is a neurotransmitter in the GI tract and together with other purines and pyrimidines are implicated in modulating GI motility, particularly enteric reflexes (see review by Burnstock 2001b). As the SO neural plexuses are continuous with the duodenal

myenteric and submucous plexuses it is hypothesised that purines may also affect SO motility. However, very few studies have investigated the actions of purines on SO motility.

1.4 Purines

Purines are molecules such as ATP, adenosine diphosphate (ADP) and adenosine. Other than the well characterised intracellular functions of purines they also have very important extracellular functions, acting as extracellular signalling molecules. Purines act via cell surface receptors and the area of purinergic signalling has expanded to include pyrimidines (uridine triphosphate, UTP; uridine diphosphate, UDP) and dinucleoside polyphosphates, which also interact with purine receptors.

There are a number of sources of extracellular purines in the body and these include release from: cells on cell death, adrenal chromaffin granules, mast cells, erythrocytes, leukocytes, cardiac myocytes, fibroblasts and endothelial cells. Of particular interest in the GI tract purines are released from: nerves, epithelial cells, smooth muscle cells, enterochromaffin cells and are also found in bile and pancreatic juice (Katsuragi *et al.* 1991; Chari *et al.* 1996b; Bodin and Burnstock 2001).

Purines have been found to be involved in a number of biological systems including: smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, the immune response, inflammation, platelet aggregation, pain and cardiac function (see reviews by Ralevic and Burnstock 1998w; Burnstock 2001b). Today, purines are being investigated

regarding their role in the pathogenesis of disease, are involved in clinical trials, or used for the treatment of diseases such as: ischemia-reperfusion injury, stroke, cardiac arrest and hypertension, acute inflammation and wound healing, irritable bowel syndrome, cancer, asthma, cystic fibrosis, Parkinson's disease and erectile dysfunction to name a few (Burnstock 2002a; Jacobson *et al.* 2002; Mutlu *et al.* 2002a).

It was proposed that in the GI tract purine nucleotides and/or nucleosides mediated non-adrenergic non-cholinergic (NANC) inhibitory and excitatory neurotransmission (Burnstock *et al.* 1970a; Burnstock *et al.* 1972c). However the proposal of ATP as the NANC neurotransmitter was resisted for more than 20 years, as favour was given to VIP, and later to NO (Burnstock 2001b). Today it is recognised that ATP together with VIP, NO and pituitary adenyl cyclase activation peptide (PACAP) are co-transmitters of NANC inhibitory neurons, however the proportions of the co-transmitters vary between GI regions and species (Burnstock 2001b). Evidence for the involvement of purines in various GI functions has increased considerably especially with the advent of molecular biology techniques and improved agonist and antagonist development. The following sections review purine receptors, agonists and antagonists. As little was known regarding the actions of purines on the SO, or other sphincters, a brief review of the actions of purines on small intestinal motility is presented to provide the basis for discussion and interpretation of the data described in this thesis.

1.4.1 Purine receptors

Purines and pyrimidines mediate their effects by interacting with cell surface receptors. These receptors are divided into two major classes: P1 receptors where adenosine is the principal ligand and P2 receptors where ATP, ADP, UTP or UDP are the principal ligands (Burnstock 1978). The P2 receptors have been further divided into two subgroups: P2X receptors are ligand-gated cation channels and P2Y receptors are G protein-coupled receptors. Furthermore, both the P1 and the P2 receptors have a number of specific receptor sub-types. The current purine receptor nomenclature is summarized in **Table 1.1** (Fredholm *et al.* 1997c; Fredholm *et al.* 2001a).

1.4.1.1 P1 Receptors

There are four separate P1 receptors: A_1, A_{2A}, A_{2B} and A_3. The principal ligand for each of these receptors is adenosine. The separation of the A_2 receptor into two distinct sub-types is based on differences in agonist binding properties (A_{2A} high affinity; A_{2B} low affinity) (Olah and Stiles 1995). All P1 receptors are coupled to G proteins. Each of the P1 receptor sub-types has a distinct pharmacological profile (**Section 1.4.2**).

Each of the P1 receptors have been cloned and it is interesting to note that there is a considerable lack of amino acid sequence homology between cloned P1 receptors. Importantly, variation in the amino acid sequence of less than 10% (between canine, rat and bovine) or less than 5% (between human and bovine) may be sufficient to cause considerable interspecies differences in ligand binding or subtle differences in mechanisms regarding receptor desensitisation (Ralevic and Burnstock 1998x).

P1 receptors are expressed in a broad range of cell types and tissues. Specific to the GI tract, P1 mRNA or receptors have been localised: A_1 receptors in oesophagus, colon and antrum; A_{2A} receptors in blood vessels; A_{2B} receptors in caecum, colon and blood vessels; and low levels of A_3 receptors were found in the intestine (see Fredholm *et al.* 2001b). Immunohistochemical studies have recently characterised P1 receptors in a number of GI tissues (see **Appendix 1**). The P1 receptor sub-type distribution in the biliary tree has not been reported.

1.4.1.2 P2 Receptors

P2 receptors are defined as receptors for a purine or pyrimidine nucleotide or dinucleotide (Fredholm *et al.* 1997b). P2 receptors have been further categorised based on structural characteristics: P2X receptors are ligand-gated cation channels, and P2Y receptors are G protein-coupled receptors.

1.4.1.2.1 P2X Receptors

P2X receptors are ATP-gated ion channels. There are seven P2X receptor proteins ($P2X_1$-$P2X_7$). Each of the P2X receptor subunits have been cloned from mammalian tissue, however there can be as little as 27% homology between subunits within the same species, and alternative splice variants exist (King and Townsend-Nicholson 2003). P2X subunit proteins form either homomeric or heteromeric receptors consisting of either 3 or 6 subunits (King and Townsend-Nicholson 2003). However specific subunit combinations appear to be common ($P2X_{2/3}$, $P2X_{1/5}$, $P2X_{1/6}$; Burnstock 2004). It should be noted that the combination of receptor subunits in heteromeric P2X receptors results in different pharmacological properties. P2X receptors

have been reviewed recently (see Khakh *et al.* 2001; King and Townsend-Nicholson 2003; Burnstock 2004).

P2X receptors mediate the rapid non-selective passage of cations (Na^+, K^+, Ca^{2+}) across the cell membrane resulting in an increase in intracellular Ca^{2+} and depolarisation. This transduction mechanism does not depend on a second messenger system and results in a rapid response time. Consequently these receptors play an important role in fast neuronal signalling and regulation of muscle contractility. Cations can modulate the ATP-activated current, with Mg^{2+} and Ca^{2+} usually inhibiting the current, probably by decreasing the affinity of the ATP binding site by an allosteric change in the receptor (see Ralevic and Burnstock 1998a).

P2X receptors are found on excitable cells such as smooth muscle cells, neurons and glial cells and act as mediators of fast excitatory neurotransmission to ATP in the central and peripheral nervous systems (Ralevic and Burnstock 1998p). P2X receptor subunit mRNA is expressed in various tissues of the GI tract: $P2X_1$ in vascular smooth muscle; $P2X_2$ in intestine; $P2X_3$ in subset of sensory neurons and absent from smooth muscle; $P2X_4$ in sensory ganglia; other sub-types had not been characterised in the intestine (Collo *et al.* 1996; Collo *et al.* 1997 and Ralevic and Burnstock 1998s). Immunohistochemical studies have recently characterised P2X receptors in a number of GI tissues (see **Appendix 1**). The P2X receptor sub-type distribution in the SO has not been reported.

1.4.1.2.2 P2Y Receptors

P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins. Currently this includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ and each has a distinct pharmacological profile (Fredholm *et al.* 1997a; Khakh and Kennedy 1998b; Ralevic and Burnstock 1998r; King and Townsend-Nicholson 2003). Like the P2X receptors, a large range of heterogeneity exists between different P2Y receptors, and also between species for the same receptor (Communi and Boeynaems 1997; Khakh and Kennedy 1998a; Ralevic and Burnstock 1998c).

P2Y receptors, like P2X and P1 receptors, are also found on many cell types and in many tissues. P2Y receptor mRNA is expressed in various tissues of the GI tract: $P2Y_1$ is throughout the GI tract and vascular smooth muscle; $P2Y_2$ on epithelial cells; $P2Y_6$ in the small and large intestine; other sub-types had not been characterised in the intestine (Ralevic and Burnstock 1998d). Immunohistochemical studies have recently characterised P2Y receptors in a number of GI tissues (see **Appendix 1**). P2Y receptor sub-type distribution in the SO has not been reported.

1.4.2 Agonists and antagonists

Sub classification of P1 and P2 receptors has proven difficult due to the lack of subclass and sub-type specific agonists and antagonists. Furthermore extracellular ATP is rapidly metabolised to ADP and adenosine by ectonucleotidases, and tissues express multiple receptor sub-types. Historically these receptors have been categorised pharmacologically

according to agonist potency curves. The P1 receptors agonist rank potency is adenosine > AMP > ADP > ATP, where the P2 receptors are activated by ATP > ADP > AMP > adenosine. Agonists and antagonists for each of the P1 and P2 classes and subclasses are described below, including their limitations, but for more detail see reviews by King and Townsend-Nicholson 2003, Ralevic and Burnstock 1998e, Burnstock 2004. In addition refer to **Table 1.2a,b** for lists of chemical names and abbreviations of common purinergic drugs mentioned in the text or used in the investigations described in this thesis.

1.4.2.1 P1 Receptor Agonists

A number of adenosine analogues have been produced that have greater stability than adenosine, however they do not discriminate between P1 receptor subclasses. These non-specific adenosine agonists include NECA, APNEA, and ^{125}I-AB-MECA. ATP and stable derivatives such as ATPγS and β,γ-meATP can also act as P1 agonists in some tissues that have been treated with methylxanthines (sub-type non-specific P1 antagonists) (Ralevic and Burnstock 1998f).

Sub-type specific agonists have been developed for most P1 receptors, however they need to be used with care because at high concentrations their selectivity is limited (**Table 1.3**). It is important to note that the affinity, and hence selectivity, of a given agonist at a particular receptor sub-type can vary widely depending on species. For example: CCPA is the most selective agonist for the A_1 receptor where it is 50 fold selective for human A_3 and more than 600 fold selective for rat A_3. CGS21680 is the most selective

agonist for A_{2A} receptors and is 26 fold selective for rat A_{2A}, but less than 3 fold selective for human A_3 receptor. There is no selective A_{2B} agonist available. NECA is currently the most potent agonist to A_{2B} receptors but is not specific to this receptor. CGS 21680 has only very low affinity to the A_{2B} receptor and has been used to distinguish between A_{2A} and A_{2B} receptors, but may also bind A_3 receptors depending on concentration. CI-IB-MECA is 11 fold selective for human and 1400 fold selective for rat A_3 receptor (see King and Townsend-Nicholson 2003). Therefore the use of selective antagonists is more reliable to characterise endogenous P1 responses (King and Townsend-Nicholson 2003).

A further consideration with the use of P1 agonists is receptor desensitisation. A_1, A_{2A}, and A_3 receptors can desensitise within minutes to hours of agonist exposure. Due to a lack of A_{2B} receptor agonists A_{2B} receptor desensitisation has not been addressed. For more information regarding desensitisation mechanisms see Ralevic and Burnstock 1998g.

1.4.2.2 P1 Receptor Antagonists

Xanthine and xanthine derivatives, such as theophylline and caffeine, are non-selective P1 antagonists. Furthermore these antagonists are not universal, with xanthine resistant responses being recorded in a number of tissues (Ralevic and Burnstock 1998h). It is important to note that in some species A_3 receptors are characteristically insensitive to methylxanthines. Furthermore methylxanthines can act through other mechanisms e.g. phosphodiesterase inhibition (King and Townsend-Nicholson 2003). 8-PT and its water soluble analog 8-SPT along with DPSPX are more potent than theophylline, but are still sub-type insensitive (Daly *et al.* 1985).

DPCPX is an A_1 selective antagonist however it is only 2 fold selective over A_{2B} receptors. MRS1754 is a recently developed antagonist for A_{2B} receptors. SCH58261 is effective at human A_{2A} receptors but not at rat A_{2A} receptors. Similarly there are selectivity problems with A_3 receptor antagonists with regard to variable effectiveness between species. See **Table 1.3** for a listing of specific P1 receptor antagonists and their K_i values.

1.4.2.3 P2 Receptor Agonists

The natural ligands of P2 receptors include ATP, ADP, UTP, UDP and the diadenosine polyphosphates. Currently there are no agonists that are specific enough to differentiate between P2X and P2Y classes, or to distinguish between specific receptor sub-types. However, agonists that are more potent for a particular receptor class and weak or inactive for other receptor classes are particularly useful. Such agonists are the metabolically stable ATP analogs α,β-meATP and β,γ-meATP, which if active imply actions at P2X receptors (specifically $P2X_1$ and $P2X_3$ sub-types) and are generally inactive at P2Y receptors. Similarly ADP, ADPβS and UTP are agonists at some P2Y receptor sub-types but generally weak or inactive at P2X receptors (Ralevic and Burnstock 1998m). Other P2 agonists include 2MeSATP, ATPγS, BzATP, Ap_4A, ADPβF, ATPαS. See **Table 1.4** and **Table 1.5** for agonist pEC_{50} values at each P2X or P2Y receptor.

Agonist potency curves have been established for the different receptor sub-types. However the potency of ATP can vary greatly at recombinant P2X receptors, with EC_{50} values ranging from 50nM to 300μM, depending on

subunit composition of the receptor (King and Townsend-Nicholson 2003). Furthermore agonist potency can vary depending on the presence or suppression of ectonucleotidase activity requiring careful interpretation of the data. α,βmeATP is up to 100 fold more potent than ATP or 2MeSATP when ectonucleotidase activity is not suppressed, but under conditions where ectonucleotidase activity is controlled, or suppressed (with ectonucleotidase inhibitors or by removal of divalent cations) α,βmeATP is less potent than 2MeSATP or ATP (Ralevic and Burnstock 1998i).

1.4.2.4 P2 Receptor Antagonists

A number of P2 antagonists exist however many do not discriminate between P2X and P2Y receptor classes or their receptor sub-types. The lack of selective P2 antagonists has made receptor sub-type identification difficult based on pharmacological techniques (Ralevic and Burnstock 1998u). Outlined below are some of the more commonly used antagonists, see **Table 1.4** and **Table 1.5** for antagonist pIC_{50} values at each P2X or P2Y receptor.

Suramin is a P2 selective antagonist. However, suramin does not distinguish between P2X and P2Y receptors and is not a universal antagonist (active at $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and is weak or inactive at $P2X_4$, $P2X_6$, $P2X_7$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) (Lambrecht *et al.* 2002). PPADS is a slowly equilibrating and slowly reversible antagonist and is also non-selective and non-universal. PPADS does not distinguish between P2X and P2Y receptors as originally believed and past studies need to be re-evaluated keeping this in mind. PPADS is active at $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ receptors, is also active at $P2Y_1$, and is weak or ineffective at $P2X_4$, $P2X_6$, $P2X_7$, $P2Y_2$,

$P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ receptors (Lambrecht *et al.* 2002). When used at concentrations greater than 10μM PPADS has non-specific effects. Furthermore suramin and PPADS can complicate agonist potency curves due to G-protein inhibition or ATPase inhibition (Ralevic and Burnstock 1998l). Reactive blue 2 (also known as cibacron blue) is a non-selective P2 antagonist that also inhibits ectonucleotidase activity. NF023 is based on suramin however it is moderately selective for P2X receptors compared to P2Y receptors. It also inhibits ectonucleotidase activity but selectively favours $P2X_1$ receptors over ectonucleotidase. NF279 is another suramin analog and is approximately 10-fold more potent than NF023, and has the highest P2X versus P2Y selectivity (Damer *et al.* 1998).

1.4.2.5 Limitations for the characterisation of purinergic receptor sub-types

Research in the area of purinergic receptors has become somewhat difficult due to a number of findings that require past studies to be re-evaluated (Ralevic and Burnstock 1998j). These include:

1. Co-expression of multiple P1, P2X and/or P2Y receptors within a tissue.
2. P2X receptors are multi-subunit proteins that exist as heteromers and may have different pharmacology compared to homomers.
3. Cations can affect P2X channel activity.
4. Agonists previously believed to be P2X/P2Y selective have been found to be non-selective.
5. Ectonuclotidases can alter agonist potency.

6. Antagonists believed to be P2 selective have been found to be non-selective, can affect ectonucleotidase activity and may have allosteric effects on P2 receptors.
7. Affinity of P1 agonists for a particular receptor sub-type varies considerably between species and experimental conditions.

Other factors, including the lack of receptor specific agonists and antagonists, the coexistence of different receptor sub-types in tissues, and the use of 'mixed solutions' due to the rapid metabolism of ATP to ADP and adenosine have slowed the process of receptor classification.

To complicate matters further, ATP is known to be co-released with a number of other neurotransmitters. ATP is present in sympathetic nerves with noradrenaline and neuropeptide Y, in parasympathetic nerves with acetylcholine and VIP, in the central nervous system with glutamine, in the retina with GABA, in enteric inhibitory nerves with NO and VIP and in sensory-motor nerves with cGRP and substance P (see reviews by Ribeiro *et al.* 1996; Burnstock 1999a; Burnstock 1999b; Sneddon *et al.* 1999). Furthermore there is the possibility of peptides, such as CCK, being mediated via ATP release (Martin *et al.* 1998).

In the GI tract it is currently believed that ATP, NO, VIP and its related peptide PACAP are co-transmitters in NANC inhibitory nerves. However their proportions vary between species and between regions of the gut, with NO being the dominant transmitter in upper GI regions and ATP more prominent in the lower GI regions (Burnstock 2001b). Interestingly there are also reports of excitatory nerves which are believed to mediate their effects

via the actions of ATP (Burnstock *et al.* 1972b; Monro *et al.* 2002b; Storr *et al.* 2002c).

In summary, the co-release of ATP with other neurotransmitters such as acetylcholine or NO, the degradation of ATP to adenosine via ectonucleases, the involvement of ATP and adenosine in neural reflexes together with the existence of multiple receptor sub-types present in tissues make identifying the specific role/action of a particular purine very difficult.

1.4.3 Effect of purines on SO motility

There are only a few reports investigating the effects of ATP or adenosine on SO motor activity, and the responses reported vary depending on the species and preparation studied. Currently, there has been no comprehensive investigation of the actions of purines on SO motility, or identification of the purinergic receptor sub-types involved in mediating these responses.

1.4.3.1 ATP

Application of ATP to spontaneously active rabbit SO *in vitro* was shown to produce an immediate and short lasting relaxation, predominantly a decrease in basal tension (Azuma and Fujiwara 1973a). In the feline SO, exogenous ATP or ADP produced an inhibition of spontaneous activity with only a slight decrease in basal tension (Persson 1976b). This response was potentiated in the presence of dipyridamole, an adenosine uptake inhibitor, thereby suggesting that adenosine was involved in mediating this response. Conversely, studies on guinea-pig SO longitudinal muscle strips found ATP induced a non-cholinergic contractile response that was PPADS sensitive

(Patacchini *et al.* 1998g). However, in human SO muscle strips exogenous ATP was found to have no effect (McKirdy 1988).

A recent electrophysiological study of the NANC relaxation in rabbit SO found a significant portion of the IJP was suppressed by desensitization of P2 receptors with $\alpha\beta$-meATP (Imoto *et al.* 1998d). The authors concluded that ATP, together with PACAP, were the principal apamin-sensitive transmitters involved in NANC relaxation of rabbit SO. The specific P2 receptor sub-types mediating these responses have not been identified.

1.4.3.2 Adenosine

There are only a couple of reports investigating the effects of adenosine on SO motor activity. Exogenous adenosine was found to cause a moderate inhibition of EFS-induced contraction in guinea-pig SO, and it was suggested that cholinergic nerve activity in the SO was subject to an inhibitory mechanism mediated by purine receptors (Katsuragi *et al.* 1985c). The specific P1 receptor sub-type involved has not been identified.

Further evidence, although indirect, for a role of adenosine in regulating SO activity comes from an *in vivo* human study where the P1 antagonist theophylline was used to inhibit SO spasm provoked by morphine in patients with SO dysfunction (Pap and Forro 1998a), which suggests an excitatory action of adenosine on SO activity.

1.4.3.3 Possible down-stream effect of purines in bile

It is of interest to note that purines are released into bile and it remains to be determined if they have a down-stream effect by acting on purinergic

receptors in the SO or duodenum. Purines have been detected in human, rat and pig bile samples (Chari *et al.* 1996a).

1.4.3.4 Possible purinergic signalling in pain pathways in biliary tree

In the biliary tree distension of the bile/pancreatic ducts is believed to be associated with the sensation of pain. A number of P2X receptors have been localised to sensory neurons, however the discovery of $P2X_3$ receptors selectively being expressed on nociceptive sensory neurons has led to a number of studies investigating purinergic signalling involved in pain pathways (for reviews see Burnstock 2001a; Chizh and Illes 2001). A recent hypothesis (purinergic mechanosensory transduction) suggested that upon distension of tubes (e.g. ureter, bile duct and gut) and sacks (e.g. urinary gladder, gallbladder and lung), ATP is released from the epithelial cells lining the lumen, which acts on $P2X_3$ receptors located in the subepithelial plexus transmitting a signal to the central nervous system (Burnstock 1999e). Recent investigations in the urinary tract and colon appear to support this hypothesis (Knight *et al.* 2002; Rong *et al.* 2002; Wynn *et al.* 2003a). It remains to be determined if pain associated with distension of the CBD or gallbladder, the impaction of a gallstone or abnormal SO activity (i.e. SO dysfunction; Toouli and Baker 1991; Craig and Toouli 2002) also occurs via a similar mechanism.

1.4.4 Effect of purines on other sphincters

Similar to the SO, there are few investigations regarding the actions of purines on other sphincters in the GI tract. ATP together with NO has been implicated in the NANC inhibition of the lower oesophageal sphincter, pyloric sphincter and the internal anal sphincter motor function (Crema *et al.* 1983;

Nissan *et al.* 1984; Soediono and Burnstock 1994; Rae and Muir 1996; Imaeda *et al.* 1998; Yuan *et al.* 1998; DeLuca A. *et al.* 1999; Imaeda and Cunnane 2003). However characterisation of the specific P1 and P2 receptor sub-types involved still need to be determined.

1.4.5 Effects of purines on small intestinal motility

Accumulating evidence indicates that the actions of bioactive agents and the neural circuits characterised in the small intestine can be different to those in the SO. For example, ganglia in the biliary system exhibit structural, neurochemical and physiological characteristics that are different to the neurons of the enteric nervous system (Balemba *et al.* 2004b; Hillsley and Mawe 1998). Consequently motility patterns and control mechanisms described in the small intestine may not be relevant to biliary motility. However, as little is known regarding the actions of purines on the motor function of the SO, or other sphincters, a brief review of the actions of purines on small intestinal motility is presented below. This information was used as a basis for recognition and comparison of possible purine-evoked responses that may be displayed by the SO, and to provide a guide for drug concentrations required in the experiments described in this thesis.

A large body of work has been conducted to investigate the role of purines (and pyrimidines) in the GI tract. After the announcement of the 'Purinergic Hypothesis' (Burnstock *et al.* 1970b) there was a flurry of research activity in the gut field, however activity slowed considerably during the 1980s primarily due to a lack of specific receptor agonists and antagonists. Only recently have most of the receptor sub-types been cloned allowing molecular

approaches to be undertaken, especially regarding receptor sub-type distribution in different tissues and species. Recently a number of antibodies have been developed for immunohistological studies (refer to **Appendix 1**), and most importantly some receptor sub-type selective agonists and antagonists have become available. During the course of the investigations described in this thesis a number of advances have been made in understanding the role of purines (and pyrimidines) in the regulation of small intestinal motility. It now remains to be determined if similar purinergic mechanisms exist in the regulation of SO motility.

Pharmacological and electrophysiological studies in the small and large intestines of rodents have implicated the involvement of ATP in both increasing and inhibiting motor activity. However the actions of ATP, and the receptor sub-types characterized as being responsible, appear to depend on the species, tissue type and experimental design used. In pharmacological *in vitro* studies there are a number of inconsistencies regarding the effects (excitatory or inhibitory), and potential receptor sub-types characterized. The findings were dependent on the use of exogenous or the manipulation of endogenous ATP (Vanneste *et al.* 2004b; Van Crombruggen and Lefebvre 2004a; Manzini *et al.* 1985d; De Man *et al.* 2003c; Giaroni *et al.* 2002; Rozsai *et al.* 2001; Storr *et al.* 2000), or the use of pre-contracted (Vanneste *et al.* 2004a; Van Crombruggen and Lefebvre 2004b) or non-contracted muscle strips (Giaroni *et al.* 2002; Monro *et al.* 2002a; Ivancheva *et al.* 2001; Ivancheva *et al.* 2000; Sakai *et al.* 1979; Moody and Burnstock 1982; Kennedy and Humphrey 1994; Burnstock 2001b).

Similarly, electrophysiological studies in the small intestine have provided evidence for the involvement of ATP in reflex-activated excitatory post-synaptic potentials (EPSPs) *and* IJPs. The putative purinergic receptor sub-types involved differ between intestinal tissues and species (EPSPs: LePard *et al.* 1997; Monro *et al.* 2004; Nurgali *et al.* 2004; Ren *et al.* 2003; Nurgali *et al.* 2003; Hu *et al.* 2003. IJPs: Xue *et al.* 1999; Murr *et al.* 1999; Zagorodniuk and Shuba 1986; Fernandez *et al.* 1998; Bian *et al.* 2000).

Studies have also implicated a role for adenosine in modifying small intestinal motility. Pharmacological *in vitro* studies in rat and guinea-pig determined that adenosine decreases motor activity (Mule *et al.* 1989; Nicholls *et al.* 1992g), by acting at pre-synaptic A_1 receptors which inhibit acetylcholine release (Lee and Parsons 2000a; Lee *et al.* 2001a; Storr *et al.* 2002b). However further investigation found A_1 and A_2 receptors have post-junctional actions mediating relaxation of rat duodenum longitudinal muscle, but a contraction was observed by stimulation of A_1 and/or A_{2B} receptors of the duodenal muscularis mucosa (Nicholls *et al.* 1996h; Burnstock 2001b). The actions of adenosine appear to be dependent on the location of receptors (pre-synaptic versus post-synaptic) and the tissue under investigation (longitudinal muscle versus muscularis mucosa). These factors, together with species differences may account for the variance in responses observed in the purinergic literature.

Interestingly purinergic receptors have also been implicated in other neural pathways in the GI tract. P2X and nicotinic receptors are linked in a mutually inhibitory manner in guinea-pig myenteric neurons (Zhou and Galligan

1998b). ATP has been reported to regulate synaptic transmission by pre- and post-synaptic mechanisms, where ATP augmented nicotinic fast depolarisation produced by acetylcholine, and inhibited muscarinic and substance P-mediated depolarisation (Kamiji *et al.* 1994). Adenosine can also suppress nicotinic synaptic transmission in myenteric ganglia, presumably by acting at pre-synaptic P1 receptors located at cholinergic synapses (Christofi *et al.* 1992; Christofi and Wood 1993). It has also been postulated that adenosine is an important contributor to the inhibitory tone of myenteric networks (Moneta *et al.* 1997). Clearly purines are involved in numerous processes, both neural and non-neural, throughout the small intestine and characterizing these pathways is a challenge.

The role of purines in enteric neurotransmission during pathological conditions of the GI tract has only recently been addressed. Purines have been implicated in a mouse model of chronic intestinal inflammation (De Man *et al.* 2003b), inflammatory bowel disease (Yiangou *et al.* 2001a), post-operative ileus in rat colon (Kadowaki *et al.* 2003), and experimental colitis (Mabley *et al.* 2003). Purinergic receptors now represent a potential new therapeutic target for GI motility disorders (Burnstock 2002b; Marteau 2002; Mutlu *et al.* 2002b).

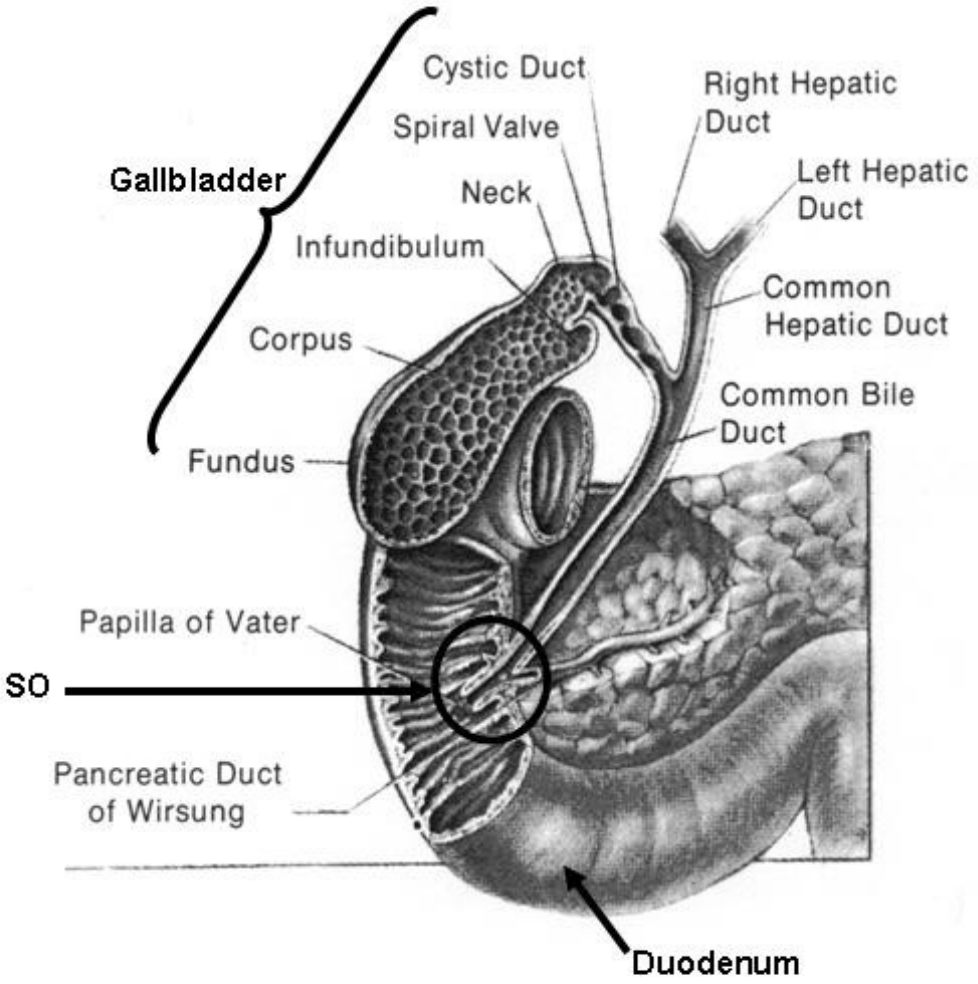
In summary, purines are involved in the regulation of small intestinal motility. The observed responses are likely to be due to a combination of factors including the location of receptors (pre-synaptic or post-synaptic), the tissue type, muscle layer, or neural plexus under investigation, and the animal species. Furthermore there are complex interactions between purines and

other neurotransmitters that need to be considered. Regardless of the complexities associated with unravelling the specific purinergic pathways and receptor sub-types involved in normal GI motility, it is important to note that recent investigations also point to the involvement of purines in pathological conditions of the intestine. The information presented above raises the possibility that purines may be important modulators of SO function. However, due to the variable results from studies investigating the effects of purines and purinergic pathways in different regions of the small intestine, caution should be used when extrapolating small intestinal data to the SO.

1.5 Summary

The control of SO activity is complex. Disturbances in SO motility are known to be related to painful clinical conditions such as SO dysfunction and acute pancreatitis. Understanding normal SO motility and comparing this to disturbed SO motility patterns may identify mechanisms that could be targeted for future pharmacological intervention. Purines are one class of chemicals/neurotransmitters identified to be involved in normal motility of the GI tract, and more recently also in pathophysiological conditions. Very few studies have investigated the involvement of purines in the regulation of SO motility. Therefore initial studies need to identify the actions of exogenous purines on SO motor activity. This information will then form a foundation for further studies defining the role of endogenous purines and elucidating the mechanisms of action.

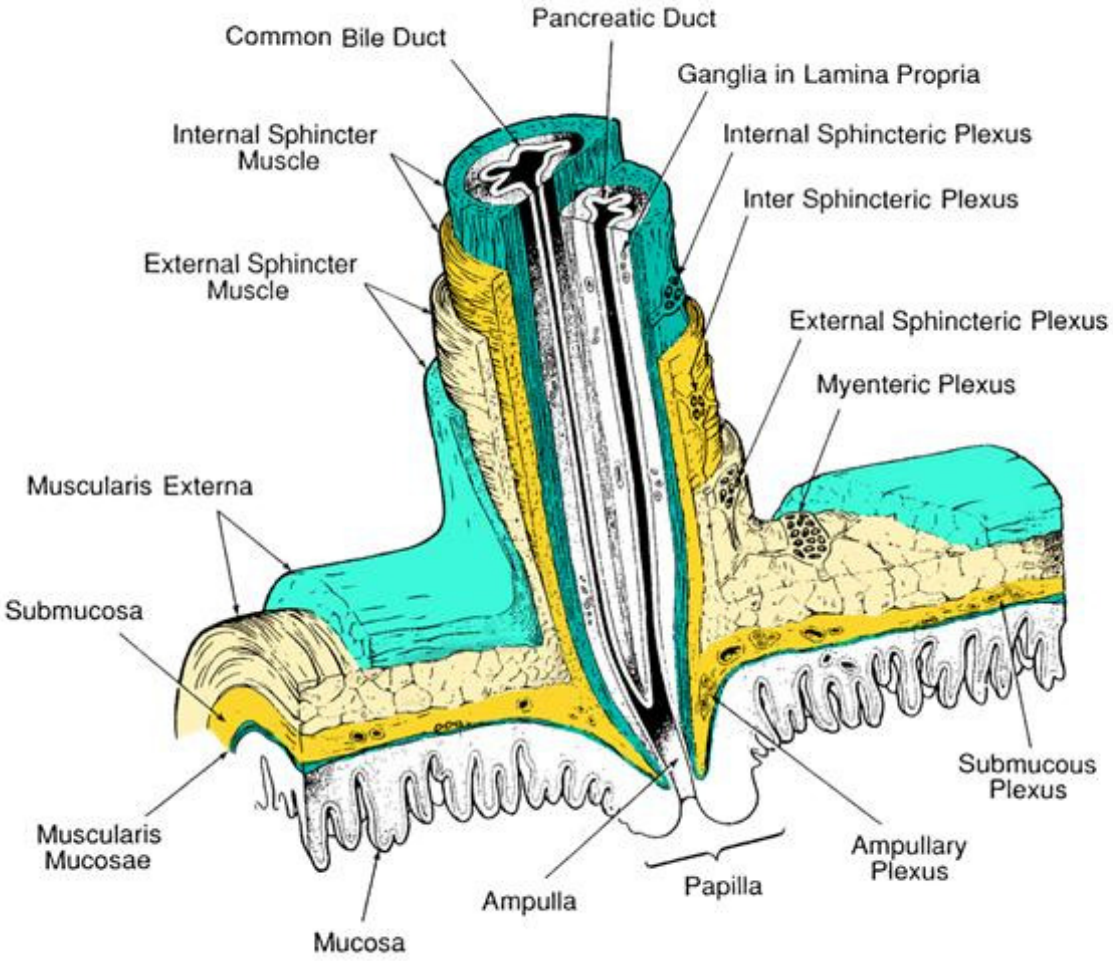
Figure 1.1: Schematic diagram of the human biliary tree



The SO is located at the junction of the terminal common bile duct and pancreatic ducts with the duodenum.

[Modified from: Sabiston DC Jr. Textbook of Surgery; The biological basis of modern surgical practice. 14th Edition; WB Saunders Company; Philadelphia; page 1043]

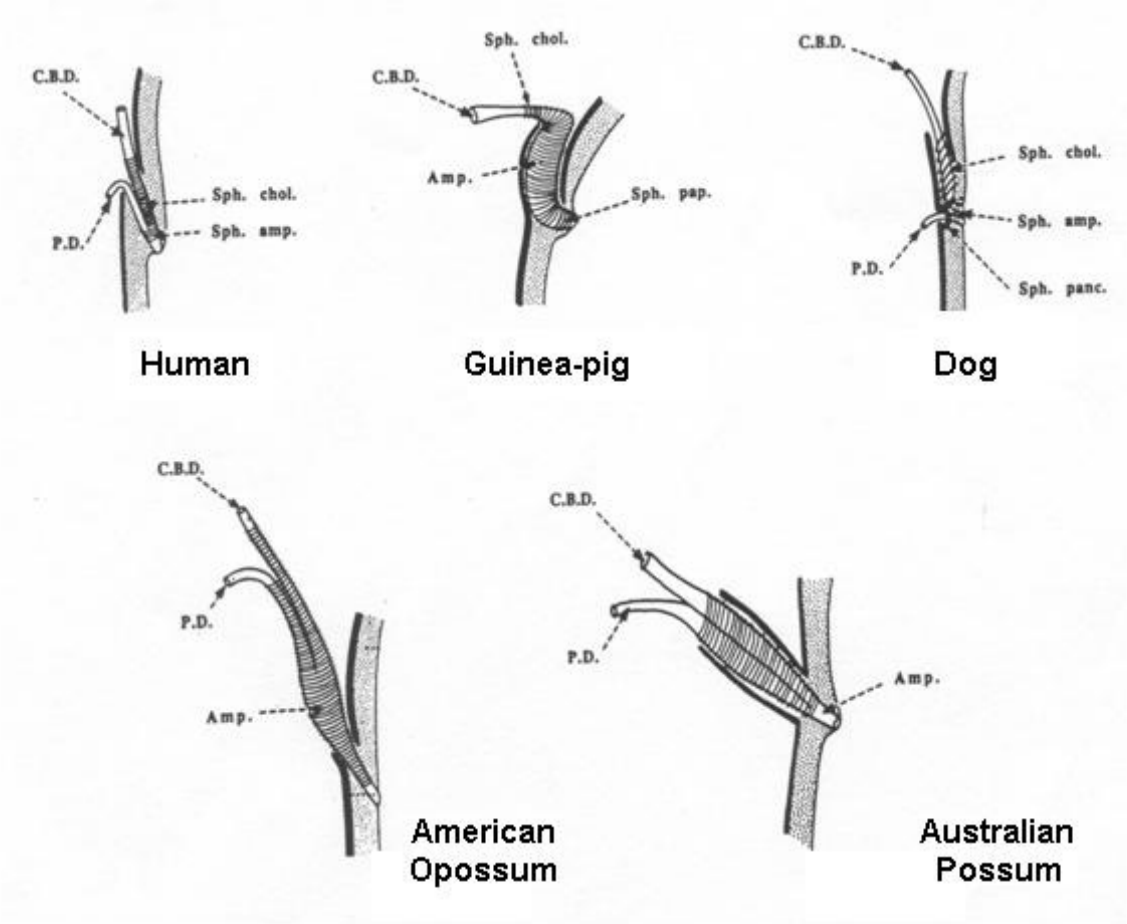
Figure 1.2: Schematic diagram of the structure of possum SO and adjacent duodenum, illustrating the muscle layers and neural plexuses



The muscle layers of the SO are continuous with those of the adjacent duodenum (represented by shading; circular muscle in yellow and longitudinal muscle in green). Similarly the SO neural plexuses are continuous with the duodenal myenteric and submucosal plexus.

[Modified from Padbury *et al* (1993a)]

Figure 1.3: Comparative anatomy of the SO illustrating the different arrangement of bile and pancreatic ducts

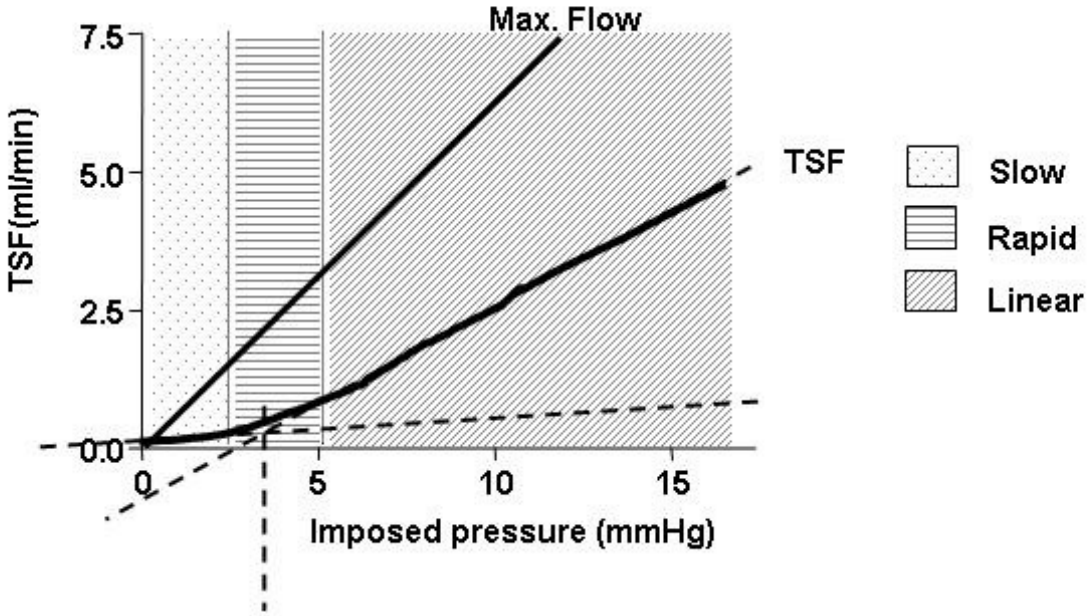


The heavy black line represents the muscle of the adjacent duodenum and the transverse lines represent the sphincter muscle. The SO of human, guinea-pig and dog is located predominantly within the duodenal wall whereas in the American opossum and the Australian possum it has a prodominant extra-duodenal portion. The arrangement of the common bile duct and the pancreatic duct in the possum and opossum is more similar to human than in other species.

C.B.D.	common bile duct
P.D.	pancreatic duct
Sph. chol.	sphincter choledochus
Sph. Amp.	sphincter ampullae
Sph. pap.	sphincter papilla
Sph. panc.	sphincter pancreaticus
Amp.	ampulla

[From Toouli & Baker (1991); adapted from Boyden (1937) The sphincter of Oddi in man and certain representative mammals. Surgery 1:25-37]

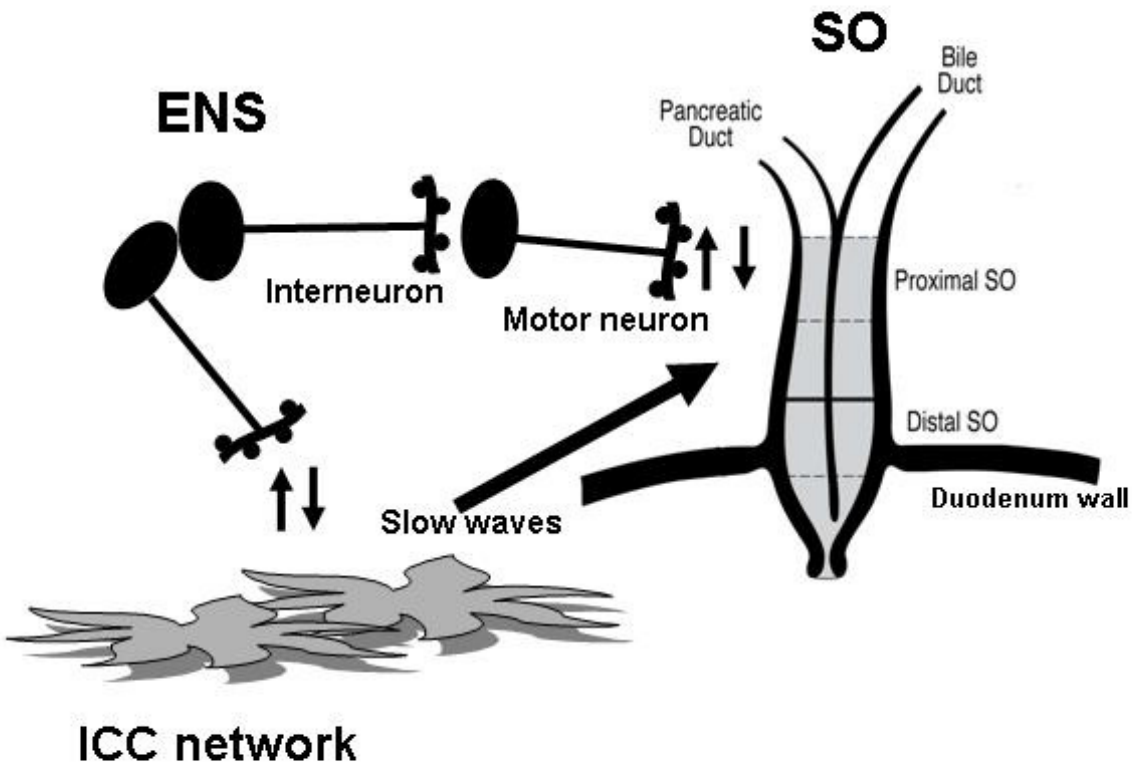
Figure 1.4: Possum SO acts as either a pump or a resistor depending on CBD and duodenal pressures



When CBD pressure is low (<3mmHg) trans-sphincteric flow (TSF) is slow and occurs via a pump-like mechanism. When CBD pressure is high (>5mmHg) TSF is fast and occurs via a resistor-like mechanism. This change in SO function occurs *in vitro* at a CBD pressure of 3.4mmHg and is referred to as 'opening pressure'. Furthermore the SO opening pressure increases if duodenal pressure increases (not shown).

[From Grivell et al (2003)]

Figure 1.5: Proposed model of SO motility



Similar to the duodenum, spontaneous SO activity is regulated by the generation of slow waves presumably by ICC, however this can be modified by excitatory or inhibitory neural inputs from the ENS either via the ICC or directly onto the SO smooth muscle. Therefore spontaneous SO activity is the cumulation of inputs from both the ICC and ENS.

Table 1.1: Purine receptor classes

	P1 receptors	**P2 receptors**	
Natural ligand	Adenosine	ATP ADP UTP UDP Adenine dinuceotides	
Subgroup	-	P2X	P2Y
Receptor type	G protein-coupled	Ion channel	G protein-coupled
Receptor Subtype	A_1 A_{2A} A_{2B} A_3	$P2X_1$ $P2X_2$ $P2X_3$ $P2X_4$ $P2X_5$ $P2X_6$ $P2X_7$	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$ $P2Y_{11}$ $P2Y_{12}$ $P2Y_{13}$ $P2Y_{14}$

Purine receptors are classified as P1 or P2 receptors. P2 receptors are further classified into P2X or P2Y receptor classes. Each of the P1, P2X and P2Y receptor classes have a number of specific receptor subtypes.

For comparison between old and more recent literature it is important to note that P_{2X}=$P2X_1$, P_{2Y}=$P2Y_1$, P_{2U}=$P2Y_2$ (or $P2Y_4$), P_{2T}=$P2Y_{ADP}$ and P_{2Z}=$P2X_7$

Table 1.2a: List of purinergic drugs and their abbreviations for P1 receptors

General P1 Agonists	
NECA	N-ethylcarboxamidoadenosine
APNEA	N-[2-(4-aminophenyl)ethyl] adenosine
^{125}I-AB-MECA	N^6-(3-[^{125}I]iodo-4-aminobenzyl)-5'-N-methylcarboxamidoadenosine
A_1 Receptor Agonists	
CPA	N6-cyclopentyladenosine
CHA	N6-cyclohexyladenosine
R-PIA	(R)N6-phenylisopropyl adenosine
CCPA	2-chloro-N6-cyclopentyladenosine
GR79236	N-[1S-, trans,2-hydroxycyclopentyl]adenosine
A_{2A} Receptor Agonists	
CGS 21680	2-[*p*-(2-carbonyl-ethyl)-phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine
APEC	2-[(2-aminoethylamino) carbonylethylphenylethylamino]-5'-N-ethyl-carboxamido adenosine
DITC-APEC	1,4-phenylene-diisothiocyanate, 4-isothiocyanatophenyl aminothiocarbonyl-APEC
PAPA-APEC	2-[4-(2-([4-aminophenyl]methylcarbonyl)-ethyl)-phenyl]ethylamino-5'-N-ethylcarboxamido adenosine
CV 1808	2-phenylaminoadenosine
DPMA	N^6-(2(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl)-adenosine
2He-NECA	2-Hexyl-5'-N-ethyl carboxamidoadenosine
WRC-0470	2-cyclohexyl-methylidenehydrazino adenosine
A_{2B} Receptor Agonists	
	No specific A_{2B} receptor agonists currently exist
A_3 Receptor Agonists	
IB-MECA	N^6-(3-iodo-benzyl)-5'-(N-methylcarbamoyl) adenosine
2Cl-IB-MECA	2-chloro-IB-MECA

P1 Receptor Antagonists	
theophylline	
caffeine	
8-PT	8-Phenyltheophylline
8-SPT	8-(p-sulfophenyl)theophylline
DPSPX	1,3-dipropyl-8-sulfophenylxanthine
A_1 Receptor Antagonists	
PACPX	1,3-dipropyl-8-phenyl(2-amino-4-chloro)xanthine
DPCPX	1,3-dipropyl-8-cyclopentyl xanthine
XAC	xanthine amine congener
KFM 19	(±)-8-(3-oxocyclopentyl)-1,3-dipropyl-xanthine
KW-3902	8-noradamant-3-yl-1,3-dipropylxanthine
ENX	1,3-dipropyl-8-[2-(5,6-epoxy)norbornyl]xanthine
N 0861	1,3-dipropyl-8-[2,(5,6-epoxy)norbornyl]
WRC 0571	8-(N-methylisopropyl)amino-N^6-(5'-endohydroxy-endonorbornyl-)9-methyl adenine
FK 453	(+)-(R)-[(E)-3-(2-phenylpyrazolol[1,5-α]pyridin-3yl)acryloyl]-2-peperidine ethanol
A_{2A} Receptor Antagonists	
CSC	8-(3-chlorostyryl)caffeine
KF-17837	1,3-dialklly-7-methyl-8-(3,4,5-trimethoxystyryl)xanthine
DMPX	3,7-dimethyl-1-propargylxanthine derivatives
ZM 241385	4-(2-[7-amino-2-(2-furyl)[1,2,4}-triazolo[2,3-α][1,3,5]triazin-5-yl amino]ethyl)phenol)
[^{3}H]SCH 58261	[^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolol[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
SCH 63390	5-amino-7-(3-phenylpropyl)-2-(2-furyl) pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
A_{2B} Receptor Antagonists	
enprofylline	
alloxazine	
IPDX	3-isobutyl-8-pyrrolidinoxanthine
MRS1754	8[4-[(4-Cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine
A_3 Receptor Antagonists	
I-ABOPX or BW-A522	3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetatephenyl-1-propylxanthine
L-268605	3-(4-methoxyphenyl)-5-amino-7-oxo-thiozolo[3,2]pyrimidine
L-249313	6-carboxymethyl-5,9-dihydor-9-methyl-2-phenyl-[1,2,4]-triazolo[5,1-a][2,7]naphthyridine
MRS 1067	3,6-dichoro-2'-isopropyloxy-4'methyl-flavonoid
MRS 1097	3,5-diethyl[2-methyl-6-phenyl-(*E*)vinyl]-1,4-(±)-dihydropyridine-3,5-dicarboxylate
MRS 1191	3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,4-dicarboxylate
MRS 1220	9-chloro-2-(2-furyl)-5-phenylacetylamino[1,2,4] triazolo[1,5-c]quinazoline

Table 1.2b: List of purinergic drugs and their abbreviations for P2 receptors

P2 Receptor Agonists		
ATP	Adenosine triphosphate	
ADP	Adenosine diphosphate	
UTP	Uridine triphosphate	
UDP	Uridine diphosphate	
α,β-meATP	α,β-methylene ATP	(non-metabolizable)
β,γ-meATP	β,γ-methylene ATP	(non-metabolizable)
ADPβS	adenosine 5'-O-(2-thiodiphosphate)	
2MeSATP	2-methylthio ATP	
ATPγS	3-thiotriphosphate	(non-metabolizable)
BzATP	3'-O-(4-benzoyl)benzoyl ATP	
Ap4A	P^1,P^4-diadenosine tetraphosphate	
ADPβF	adenosine 5'-O-(2-fluro)-diphosphate	
dATPαS	3'-deoxyATPαS	
P2 Receptor Antagonists		
Suramin	8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid	
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid	
reactive blue 2		
NF023	3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulfonic acid	
NF279	8,9'-(carbonylbis(imino-4,1-phenylene-carbonylimino-4,1-phenylenecarbonylimino))bis(1,3,5-naphthalenetrisulfonic acid)	

Table 1.3: Agonists and antagonists acting at P1 receptor subtypes

Receptor subtype	Agonists	pKi	Antagonists	pKi
hA_1	CCPA	9.1, *r 9.4-9.6	DPCPX	9.1, *r 9.5-9.7
	(R)-PIA	8.7, *r 8.9-9.3	CGS 15943	8.5, *r 7.7-8.2
	IAB-MECA	8.1, *r 7.7	XAC	7.5, *r 8.4-8.6
	NECA	7.9, *r 7.9-8.4	ZM 241385	6.6, *r 5.7
	Cl-IB-MECA	6.9, *r 6.1	SCH 58261	6.5, *r 6.9
	CGS 21680	6.5, *r 5.5	MRS 1754	6.4, *r 6.9
	Adenosine	pEC_{50} 6.5	MRE3008F20	6.0, *r 5.0
hA_{2A}	NECA	7.7, *r 7.7-8.0	SCH 58261	9.2, *r 8.6
	CGS 21680	7.6, *r 7.7	ZM 241385	9.1, *r 9.5
	IAB-MECA	6.3, *r 6.7	XAC	9.0, *r 7.3-7.6
	Adenosine	pEC_{50} 6.2	CGS 15943	8.4, *r 8.5-8.9
	(R)-PIA	6.1, *r 6.1-6.9	DPCPX	6.9, *r 6.5
	Cl-IB-MECA	5.7, *r 6.3	MRE3008F20	6.9, *r 5.7
	CCPA	5.6, *r 5.4	MRS 1754	6.3, *r 6.2
hA_{2B}	NECA	6.4-6.5	MRS 1754	8.7
	Adenosine	pEC_{50} 4.6	XAC	7.9
	(R)-PIA	4.5-5.4	CGS 15943	7.8
	CCPA	4.4	ZM 241385	7.5
	CGS 21680	3.4	DPCPX	7.3
			MRE3008F20	5.7
hA_3	IAB-MECA	9.2, *r 8.9	MRE3008F20	9.5, *r >5.0
	NECA	8.2, *r 6.6	CGS 15943	7.3, *r >4.0
	Cl-IB-MECA	8.0, *r 9.5	XAC	7.0, *r >4.0
	(R)-PIA	7.8, *r 6.7-6.8	MRS 1754	6.2
	CCPA	7.4, *r 6.6	DPCPX	5.4, *r >5.0
	CGS 21680	7.2, *r 6.2	SCH 58261	5.0
	Adenosine	pEC_{50} 6.5	ZM 241385	5.0, *r 3.8

Potency indices for agonists and antagonists at human adenosine receptor subtypes. Data for some rat receptors are also given (*r).

[From King and Townsend (2003)]

Table 1.4: Agonists and antagonists acting at homomeric P2X receptor subtypes

Receptor Subtype	Agonists	pEC_{50}	Antagonists	pIC_{50}
$rP2X_1$	ATP	7.0	NF 449	9.5
	2-MeSATP	7.0	NF 279	7.7
	ATPγS	6.2	NF 023	6.7
	Ap_6A	6.0	Suramin	5.7
	Ap_6G	5.7	Ip_5I	8.5
	α,β-meATP	5.5	TNP-ATP	9.0
	Bz-ATP	4.6	MRS 2257	8.3
	CTP	4.4	MRS 2159	8.0
			PPNDS	7.8
			PPADS	6.9
			RB-2	5.7
			h* A-317491	4.9
$rP2X_2$	ATP	5.3	RB-2	6.4
	2-MeSATP	5.1	NF 279	6.4
	ATPγS	5.1	BBG	5.9
	Ap_4A	4.8	TNP-ATP	5.9
	α,β-meATP	3	PPADS	5.8
			Suramin	5.0
			h* A-317491	4.3
$rP2X_3$	2-MeSATP	6.7	A-317491	7.6
	ATP	5.9	NF 279	5.8
	ATPγS	5.1	NF 449	5.6
	Ap_5A	5.5	Suramin	5.4
	Ap_5G	5.6	NF 023	5
	α,β-meATP	5.7	TNP-ATP	9.5
	UTP	4.0	MRS 2257	7.7
			MRS 2159	6.9
			PPADS	6.7
			Ip_5I	5.5
			RB-2	4.3
$rP2X_4$	ATP	5.4	TNP-ATP	4.8
	2-MeSATP	3.6	BBG	3.9
	CTP	3.5	h* BBG	5.5
	partial agonists:		h* PPADS	4.6
	Ap_4A	5.5		
	α,β-meATP	4.2		
$rP2X_5$	ATP	6.4	PPADS	6.7
	2-MeSATP	6.4	TNP-ATP	6.3
	ATPγS	6.5	Suramin	5.8
	Bz-ATP	5.9	RB-2	4.7
	α,β-meATP	6.0		
	Ap_4A	6.6		
	GTP	4.6		
	CTP	4.3		
$rP2X_6$	ATP	6.2		
$rP2X_7$	BzATP	5.2	BBG	8.0
	2-MeSATP	5	PPADS	4.3
	ATP	3.4	TNP-ATP	approx 4.3
	m* ADP	2.7	h* KN-62	7.4
	m* AMP	2.3	h* KN-04	approx 6.0

Potency indices for agonists and antagonists acting at rat P2Y receptor subtypes presented as $-\log_{10}$ EC_{50} (pEC_{50}) values. Data for human (*h) and mouse (*m) isoforms are given for some ligands.
[From King and Townsend (2003)]

Table 1.5: Agonists and antagonists acting at human P2Y receptor subtypes

Receptor subtype	Agonists	pEC_{50}	Antagonists	pIC_{50}
$hP2Y_1$	2-MeSADP	8.7-7.9	MRS 2279	7.3
	2-MeSATP	8.5-6.9	MRS 2179	6.5
	2-HT-AMP	6.4	A3P5P	6
	ADP	8.0-6.6	PPADS	5.4
	ATP	6.5-5.0	BzATP	pKi 5.4
	ATPγS	6.4	2-MeSATP	pKi 5.2
	ADPβS	6.1-6.0	ATP	pKi 5.3
	*r ADP	8.1		
	*r 2-MeSADP	9.2		
$hP2Y_2$	UTP	7.7-6.2	Suramin	pA_2 4.9-4.3
	UTPγS	6.6		
	5-BrUTP	5.7		
	ATP	7.1-6.6		
	ATPγS	6.2-5.8		
	GTP	4.9		
	Up_4U	7		
	dCp_4U	6.7		
	AP_4A	6.6-6.1		
$HP2Y_4$	UTPγS	7.6-5.6	PPADS	4.8
	Up_4U	6.4	ATP	pKb 6.1
	dCp_4U	6.1	*r RB-2	4.7
	UTPγS	5.8	*m RB-2	4.3
	5-BrUTP	4.8-43		
	ATP	5.4-4.4		
	GTP	5.2		
	ITP	5.1-4.0		
	*r ATP	5.9-5.7		
	*r Ap_4A	5.5		
	*m ATP	6.4-6.2		
$hP2Y_6$	UDPβS	7.6	RB-2	4.5
	UDP	7.0-6.5		
	Up_3U	6.7		
	5-BrUDP	6.1		
	UTP	5.2-5.0		
	IDP	4.5		
	ADP	4.5-4.2		
	*r UDP	8.2-9.7		
	*m ADP	7.4		
$hP2Y_{11}$	AR-C67085	8.5	Suramin	4.8
	ATPγS	5.5-4.6	AMPS	3.5-2.5
	dATP	5.1-5.0		
	BzATP	5.1-4.2		
	ATP	4.9-4.2		
	2-MeSATP	4.6-4.3		
	α,β-meATP	4.1-3.9		
	β,γ-meATP	3.7		

Receptor subtype	Agonists	pEC_{50}	Antagonists	pIC_{50}
$hP2Y_{12}$	2-MeSATP 2-MeSADP ADP ADPβS ATP *r 2-MeSADP *r ADP	10-8.5 10-7.9 7.4-6.5 7.0-6.4 6.2-5.9 8.7 7.1	AR-C69931 C-1330-7 RB-2 2-MeSAMP Suramin *r BzATP	7.6 7.4 5.9 5.9-5.3 5.4 5.3-5.0
$hP2Y_{13}$	ADP 2-MeSADP 2-MeSATP ADPβS ATP IDP *m ADP *m IDP	8 7.9 7.1 7.4 6.6 6.3 8.4 8		
$hP2Y_{14}$	UDP-glucose UDP-galactose UDP-N-acetylglucosamine *r UDP-glucose *m UDP-glucose	7.1 6.4 6.1 7.6 7.7		

Potency indices for agonists and antagonists acting at human P2Y receptor subtypes presented as $-\log_{10}$ EC_{50} (pEC_{50}). Data given as a range of reported values to make allowance for the effects of receptor reserve on drug potency. Data for mouse (*m), rat (*r) and canine (*c) isoforms are provided for some ligands for comparison. Note some ligands (eg ATP) have been reported as both agonists and antagonists at some P2Y receptor subtypes (eg $P2Y_1$ and $P2Y_4$).

[From King and Townsend (2003)]

Hypotheses

1.6 Hypothesis and aims

As summarised above, purines are known to mediate GI motility, however limited information is available regarding the effect of purines in SO motility. Based on a review of the literature (pre-2000) the following overall hypothesis was formulated, followed by a number of specific hypotheses and research aims.

1.6.1 Overall hypothesis

Purines are involved in the regulation of spontaneous SO motility, and are an important modulator of neural transmission within the SO.

1.6.1.1 Specific hypotheses

1. Exogenous purines modify spontaneous SO activity, and the effect of purines on the proximal-SO is different to the distal-SO.
2. The SO and duodenum respond differently to exogenous purines, mediated by different purine receptor sub-types.
3. The effect of exogenous purines on SO motility are mediated in part via nerves.
4. SO and/or duodenal responses to exogenous purines are mediated by receptors located on nerves, smooth muscle and/or ICC.
5. Endogenous purines are involved in the neural reflexes between the SO and duodenum or the SO and CBD/gallbladder.

1.6.2 Aims

The aims of the proposed studies were to:

1. Determine if exogenous adenosine or ATP modify spontaneous SO and duodenal motility, and if so compare the distal-SO and proximal-SO responses, and to determine if these responses are neurally mediated (**Chapter 3**).
2. Characterise the P1 receptors mediating the effects of exogenous adenosine on the spontaneous contractile activity displayed by duodenal muscle strips (**Chapter 4**).
3. Characterise the P1 receptors mediating the effects of exogenous adenosine on the spontaneous contractile activity displayed in proximal-SO muscle rings, and compare these to the P1 receptors mediating the duodenal response (**Chapter 5**).
4. Characterise the P2 receptors mediating the effects of exogenous ATP on the spontaneous contractile activity displayed by duodenal muscle strips (**Chapter 6**).
5. Characterise the P2 receptors mediating the effects of exogenous ATP on the spontaneous contractile activity displayed in proximal-SO muscle rings, and compare these to the P2 receptors mediating the duodenal response (**Chapter 7**).
6. Determine the effect of exogenous adenosine and ATP on SO motility *in vivo*, and characterise the neural pathways involved (**Chapter 8**).
7. Determine if endogenous adenosine or ATP is involved in modulating SO motility (**Future studies**)

8. Identify the location (smooth muscle, nerves or ICC) of P1 receptor sub-types in the SO and duodenum using immunohistochemical techniques (**Appendix 1**).

1.6.3 Research rationale

There is very little known about the effects of purines on SO motility, and the reported responses differ between preparations and species. Furthermore the effects of purines on the different SO regions has not been assessed, and may account at least in part, for some these disparate reports. The SO is anatomically and functionally associated with the duodenum and therefore the effects of purines on spontaneous duodenal motor activity were also examined.

Initial studies assess the effects of ATP and adenosine on SO and duodenal spontaneous contractile activity. *In vitro* muscle strip/ring preparations were used to minimise the confounding effects of extrinsic neural pathways, neural reflexes or humoral effects. These initial experiments provided a 'proof of concept' i.e. purines affect the spontaneous motility of SO regions differently.

Additional organ bath experiments using a neurotoxin were performed to ascertain if the adenosine or ATP responses were mediated by activation of receptors located on nerves in the SO or duodenum. However data from pharmacological experiments cannot be used to determine the specific cell types (nerves, ICC or smooth muscle) that mediate the responses to adenosine and ATP. Therefore an immunohistochemical approach was used to assess which P1 receptors were located on the various cell types that are

present in the SO and duodenum. This approach also involved searching for a suitable antibody that identified ICC in possum tissues.

Due to non-specificity of antibodies the immunohistochemistry studies were inconclusive (Appendix 1). Consequently, a pharmacological approach was used to define the purinergic receptor sub-types involved. Sub-type specific agonists and antagonists where available, together with non-specific, degradable and non-degradable agonists were used to characterise the purinergic receptor sub-types in the SO. However, due to the small size of the SO, and the labile spontaneous activity of SO muscle rings progress was slow. In contrast, the duodenum had the advantage of providing many muscle strips, which displayed spontaneous activity for many hours. Therefore duodenal muscle strip studies were used to determine the appropriate concentration ranges for the purine receptor agonists and antagonists. These drug concentrations were then used in the SO studies.

Subsequent studies were performed *in vivo* to describe the action of exogenous adenosine and ATP on SO motility in the anaesthetised possum. Purine antagonists were also tested *in vivo* to assess the role of endogenous purines on SO motility. However the antagonists used were ineffective and further studies with other antagonists are required.

Chapter 2

2 METHODS

2.1 Possums and animal ethics approval

All studies performed utilised either possum tissues (*in vitro* or immunohistochemistry) or anaesthetised possums (*in vivo*). All procedures related to the possums (e.g. housing, anaesthesia, euthanasia) were performed with approval from the Flinders University Animal Welfare Committee (project approval number 489/99).

Numerous studies relating to biliary motility have used the Australian Brush-tailed possum (*Trichosurus vulpecula*; for example Baker *et al.* 1993; Cox *et al.* 1998; Al-Jiffry *et al.* 2001). The possum is an excellent species to study biliary motility. As outlined in Chapter 1 the possum SO is predominantly extra-duodenal and therefore allows reliable measurement of SO motility.

Australian Brush-tailed possums were fasted overnight and anaesthetised by intramuscular injection of ketamine (20mg/kg; Ketamil Injection; Troy Laboratories Pty. Ltd., NSW, Australia) and xylazine (5mg/kg; Rompun; Bayer Australia Ltd., NSW, Australia). Tissues were then removed for *in vitro* preparations or immunohistochemical studies, or possums were used for *in vivo* preparations.

2.2 *In vitro* studies

2.2.1 Tissue harvest

In anaesthetised possums, following an abdominal incision the SO and/or proximal duodenum (from pylorus to SO) were removed. Then each animal was killed with a lethal dose of pentobarbitone sodium (Lethabarb ®; Virbac Pty. Ltd., NSW, Australia). The isolated tissues were used for *in vitro* pharmacological experiments.

2.2.2 Duodenal muscle strips

The isolated duodenum was immediately placed in oxygenated modified Krebs solution containing: NaCl 133.4mM, KCl 4.7mM, NaH_2PO_4 1.3mM, $NaHCO_3$ 16.3mM, D-glucose 7.7mM, $CaCl_2$ 2.12mM at room temperature (Baker *et al.* 1992; Woods *et al.* 2000). The duodenum was pinned to a Sylgard (Dow Corning Corporation, Michigan, USA) lined Petri dish and covered with oxygenated modified Krebs solution. The mesenteric blood vessels and connective tissue were removed by dissection and 8 full thickness longitudinal muscle strips (10mm x 2mm) were prepared from tissue immediately adjacent to the SO.

Muscle strips were secured in double-jacketed 7ml organ baths, containing continuously oxygenated modified Krebs solution at 37^{o}C (**Figure 2.1**). Each strip was attached to an isometric force-displacement transducer (FT03, Grass Instrument Co., Quincy, Mass., USA), which was then connected to a MacLab recording system (ADInstruments, Castle Hill, NSW, Australia) that utilised the software Chart v3.5.6/s (ADInstruments). Additionally, in some

experiments strips were passed through a ring electrode, which was used for EFS to confirm effective neural blockade by TTX.

Strips were equilibrated at a basal tension of 5mN for 60min, with fresh modified Krebs solution exchanged every 10min. Spontaneous contractile activity was recorded.

2.2.3 SO muscle rings

The isolated SO was immediately placed in oxygenated modified Krebs solution. The SO was pinned to a Sylgard lined Petri dish and covered with oxygenated modified Krebs solution. The mesenteric blood vessels, connective tissue and adjacent duodenum were carefully dissected from the SO. The SO was cut into four equal 'figure 8' muscle rings approx 2-3 mm wide, and allocated to either a distal-SO or proximal-SO group (**Figure 2.2**). Each SO muscle ring was secured in an organ bath and equilibrated to 5mN (Woods *et al.* 2000) for 30min as described above for duodenal muscle strips.

2.2.4 Reagents used

The concentrations of agonists and antagonists used are similar to those used in other published gastrointestinal studies or reviews (Giaroni *et al.* 2002; Ralevic and Burnstock 1998b; Burnstock 2004). A summary table is provided to show which of the P2 agonists and antagonists used in these experiments are selective against which particular purine receptor sub-types or sub-classes (**Table 2.1**).

The P1 receptor agonists used were: general P1 agonist adenosine (Sigma Chemical Company, St. Louis, MO, USA); A_1 receptor agonist 2-chloro-N^6-cyclopenyladenosine (CCPA, Tocris, Bristol, UK); A_{2A} receptor agonist 2-p-(2-Carboxyethyl)phenethylamino-5'-N-thylcarboxamidoadenosine hydrochloride (CGS21680, Sigma); A_3 receptor agonist 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (2Cl-IB-MECA, Tocris). Note that no suitable selective agonist exists for the A_{2B}R.

The P1 receptor antagonists used were: general P1 antagonist 1,3-Dipropyl-8-p-sulfophenylxanthine (DPSPX, RBI, Massachusetts, USA); A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10nM, RBI); A_{2A} receptor antagonist 8-(3-chlorostyryl) caffeine (CSC, 10μM, RBI); A_{2B} receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX, 10μM, gift from Dr Italo Biaggioni, Vanderbilt University, USA; Feoktistov *et al.* 2001); A_{2B} receptor antagonist 8[4-[((4-Cyanophenyl) carbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl)xanthine (MRS1754, Sigma); A_3 receptor antagonist 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline (MRS1220, RBI).

The non-hydrolysable P2 agonists used were: α,β-methyleneadenosine 5'-triphosphate lithium ($\alpha\beta$meATP, Sigma); adenosine 5'-[γ-thio]triphosphate tetralithium salt (ATPγS, Sigma); and the degradable P2 agonists used were: Adenosine 5'-triphosphate (ATP, Sigma); 2-methylthioadenosine 5'-triphosphate tetrasodium salt (2meSATP, Sigma); uridine 5'-triphosphate (UTP, Sigma).

Non-specific P2 antagonists used were: suramin (Sigma) or pyridoxal-phosphate-6-azophenyl,-2',4'-disulphonic acid tetrasodium (PPADS; RBI).

Other reagents used included the neurotoxin TTX (Alomone Laboratory, Jerusalem, Israel), and dimethyl sulfoxide (DMSO, BDH Merck Pty Ltd, Victoria, Australia), which was used as a solvent in some studies. Other general laboratory reagents were supplied by Sigma.

2.2.5 Experimental protocols

These *in vitro* experimental protocols were designed in consultation with the Flinders University Biostatistical Consultant Lynne Giles to ensure that the data generated could be analysed using appropriate statistical tests.

The protocols used in these studies were based on the following considerations and data from preliminary experiments. Possum duodenal muscle strips prepared for recording of circular muscle contractility typically have a cyclic burst-like pattern of spontaneous activity with a varying baseline that makes analysis very difficult. However, duodenal muscle strips prepared for recording of longitudinal muscle contractility have a very stable baseline with a very regular pattern of spontaneous activity that lasts for hours. Therefore longitudinal duodenal muscle strips were chosen for these studies. The SO muscle rings were mounted in organ baths to record circular muscle contractility. Spontaneous SO muscle ring activity is short-lived (lasting approximately 1-1.5h), particularly if the ring is from the distal-SO. Some SO muscle rings although viable, as determined by a contraction in

response to exogenous carbachol (100μM; Sigma), did not display spontaneous activity, and were excluded from the studies. The approach of pre-contracting muscle strips to study purine-induced inhibitory responses has been used by numerous investigators in the purine field. However, due to possible interactions between ATP and acetylcholine release (Ribeiro *et al.* 1996) or cholinergic receptors (Zhou and Galligan 1998a; Sawyer *et al.* 2000) this approach was not adopted. These studies investigate the actions of exogenous adenosine and ATP on spontaneous SO and duodenal activity.

A maximum of 4 SO rings, 2 from the proximal-SO and 2 from the distal-SO, or 8 duodenal muscle strips were prepared per animal. To evaluate concentration-response relationships, each muscle strip/ring was exposed only to a single concentration of agonist. Agonist concentrations were applied randomly, with muscle rings from the same SO region of one animal receiving different concentrations. Consequently, for the distal-SO and proximal-SO concentration–response curves each data point is the mean of at least 4 muscle rings, with 1 animal contributing 1 ring per concentration. For the duodenal concentration-response curves each point is the mean of a least 4 muscle strips, with 1 animal contributing 1 muscle strip per concentration.

To avoid possible receptor desensitization due to repeated application of agonists, and because of the relatively short period that the SO rings displayed spontaneous activity, only one concentration of ATP (1μM–1mM) or adenosine (0.5μm–1mM) was applied to each SO ring or duodenal muscle strip. Preliminary experiments demonstrated that after washout a second

application of the same concentration of adenosine/ATP produced a response comparable to that produced by the first application, suggesting minimal desensitization under these conditions.

To identify the purinergic receptors involved in mediating the ATP/adenosine induced responses, purinergic receptor antagonists were used in a paired protocol. In these experiments one concentration of adenosine/ATP was applied to the organ bath and the response recorded. The muscle strips/rings were then repeatedly washed (3x5min washes), a P1 (P1, DPSPX 10nM-100μM; A_1, DPCPX, 1nM-1μM; A_{2A}, CSC, 100nM-10μM; A_{2B}, IPDX, 100nM-10μM; A_3, MRS1220, 100nM-10μM) or P2 (suramin, 1μM-10mM; or PPADS, 10nM-10μM) receptor antagonist was added to the bath and 30min later the same concentration of agonist was reapplied. In separate experiments, to determine if the agonist-induced responses might involve a neural mechanism the same paired-protocol was used but after washout of the first adenosine/ATP concentration, the muscle strips/rings were pre-treated for 10min with TTX (1μM; effective neural blockade confirmed by EFS 70V, 30Hz, 0.2ms, for 10sec) and the same concentration of adenosine/ATP reapplied.

To confirm the P1 receptor antagonist results, experiments were then performed using specific P1 receptor agonists (A_1, CCPA, 0.1nM-10μM; A_{2A}, CGS21680, 10nM-100μM; A_3, 2Cl-IB-MECA, 1nM-100μM). A selective P1 receptor agonist was applied and then repeated in the presence of the appropriate antagonist. Initial concentrations of each agonist were based on the pKi values obtained in rat and human (**Table 1.3**), with the assumption

that 100% of the available receptors were binding the agonist (i.e. no receptor reserve). Importantly, concentrations were chosen to avoid agonists acting at other receptor sub-types. Preliminary experiments showed that the agonists at these lower concentrations failed to have an effect, therefore the concentration range was extended. It is important to note that at the high concentrations of agonists used may no longer be receptor sub-type selective, and data is only valid if the agonist response is blocked by the appropriate antagonist.

To further identify the possible P2 receptor sub-classes involved in the ATP-induced response muscle strips/rings were exposed to non-hydrolysable or metabolisable P2 agonists ($\alpha\beta$meATP, 1nM-100μM; ATPγS, 1nM-10μM; 2meSATP, 0.1nM-10μM; UTP, 10nM-1mM) using a non-cumulative protocol (a randomized order was used with SO rings) with a minimum of 3x5min washes between drug applications. To test that there was no desensitization, the magnitude of an initial response (high concentration) was compared to the same high concentration at the end of the non-cumulative protocol, and no significant difference was found.

Preliminary experiments investigated the possibility of the ATP-induced late inhibitory component being due to metabolism of ATP to adenosine. The above paired protocol was used, and incorporated a 15min pre-treatment with an ecto-ATPase inhibitor (ARL67156; 30μM).

2.2.6 Analysis of recordings

Muscle ring/strip contractile activity was quantified by measuring changes in basal tension (mN), contraction amplitude (mN) and frequency (contraction/min) (**Figure 2.3**). Changes in contractile were also quantified for area under curve (mN.s). Area under curve is a combined measure of contraction amplitude and frequency, and is used as an indicator of motility Konomi *et al.* 2002b; Saccone *et al.* 1994b). Area under curve has the advantage that significant changes in this parameter can be observed where no significant change in contraction amplitude or contraction frequency is evident, as these individual changes may not be statistically significant. Note: for SO muscle rings, exogenous adenosine or ATP did not induce any significant change in basal tension, therefore this data is not presented for any of the *in vitro* experiments.

To normalise muscle strip/ring activity (i.e. take into consideration the variation in magnitude of spontaneous activity displayed by different muscle strips/rings) the agonist response was calculated as the change from baseline activity. The initial agonist response was calculated by taking a 1min period immediately prior to agonist application (baseline), and a 1min period representing the peak response to the agonist, and determining the change in activity. For quantitation of the ATP-induced early inhibitory response in duodenum an additional 5s period was analysed for changes in basal tension and contraction amplitude. In the SO, assessment of the ATP-induced early excitatory response was performed over a 30s period as SO contraction frequency is slower than the duodenum. Note the 'peak response' was assessed visually and was dependent on the concentration of agonist.

However the early ATP response occurred within 30s, whereas the inhibitory response to ATP/adenosine occurred 2-3min after agonist application.

When muscle strips/rings were pre-treated with an antagonist or TTX, a 1min period immediately prior to the second agonist application (i.e. 30min after antagonist application) was determined as the baseline activity. The appropriate periods representing the peak response to the agonist (as above) in the presence of the antagonist or TTX were analysed and the change in activity from baseline determined. The magnitude of the agonist responses, before and after treatment, was compared to establish if the antagonist or TTX had an effect on the agonist-induced response. Therefore, any change in spontaneous activity due to time, or the antagonist or TTX application alone is accounted for and the actual change in the agonist-induced response is determined. Note: it is not possible with this SO preparation to accurately determine if the P1 or P2 antagonists alone affected spontaneous SO activity as spontaneous activity often decays slowly over time. The effect of an antagonist on basal duodenal activity was performed in duodenal muscle strips.

Group data is expressed as the change from baseline activity, or as percentage of baseline activity, mean ± SEM. Because more than one muscle ring/strip was prepared from each animal but each muscle ring/strip was only used for one concentration of agonist, the number of muscle rings/strips used at each concentration is stated in the figure legend of each graph. The number of animals used in the experiment is also stated. Graphs were prepared using GraphPad Prism (GraphPad Software Inc, San Diego,

CA, USA). The non-linear regression function of Prism was used to generate curves of best fit. For the duodenal data EC_{50} values were determined before and after pre-treatment with P1 antagonists (note this function extrapolates from the data to generate maximum response values provided the response has started to plateau within the tested concentration range).

2.2.7 Statistical analysis

The SO group data was subjected to statistical analysis in two steps. Initially agonist responses, irrespective of SO region, were pooled and analysed for concentration-response relationships. Then the proximal-SO and distal-SO groups were tested separately to determine if responses were region-specific. Statistical analyses on SO and duodenal data were performed according to advice from the Flinders University Biostatistical Consultant, performed using the statistical package SPSS (version 11.5; SPSS inc., Chicago, Ill. USA) and utilized non-parametric paired or unpaired tests as appropriate: Kruskal-Wallis, Mann-Whitney, Wilcoxon, and Friedman Tests. P values less than 0.05 were considered to be significant.

2.3 *In vivo* studies

2.3.1 Anaesthesia

Possums were fasted overnight and anaesthesia was induced as previously described for *in vitro* experiments (**Section 2.1**). Anaesthesia was maintained with an intravenous infusion of pentobarbitone sodium (20-30mg/hr; Nembutal; Boehringer Ingelheim Pty. Ltd., NSW, Australia). At the conclusion of the experiment each animal was euthanased as previously described (**Section 2.1**).

2.3.2 Surgical preparation

In each animal, the femoral artery and veins were cannulated to enable the monitoring of blood pressure and the infusion of fluid (3.0ml/hr of 0.9% NaCl). Body temperature was maintained at 37^{o}C with a heating blanket (Harvard Apparatus Limited, Edenbridge, Kent). A tracheotomy was performed and the possum was artificially ventilated with a small animal respirator (Phipps and Bird Inc., Virginia, USA). A laparotomy was performed to enable access to the common bile duct, duodenum, pancreas and SO.

A ligature was loosely placed at the pylorus-duodenal junction to prevent the flow of gastric juices into the duodenum. A drain was inserted retrogradely into the duodenum anal to the SO-duodenal junction to prevent duodenal distension. The splenic artery was cannulated to allow close intra-arterial injection or infusion of drugs directly to the biliary tree. The SO-duodenal area was stabilised by gently pinning the duodenum to a Sylgard covered platform (2.0cm x 3.5cm) attached to a retort stand.

A multi-lumen pico-manometry catheter (5-lumen catheter, outer diameter 0.90mm, lumen diameter 0.2mm; Putnam Plastics, Dayville, CT, USA) was positioned in the biliary component of the SO via a small incision in the common bile duct (**Figure 2.4**). Each lumen was connected to a pressure transducer (Sorenson Transpac disposable transducer; Abbot Critical Care Systems, Illinios, USA) and perfused with saline via a low compliance pneumohydraulic capillary infusion system (Dentsleeve Pty. Ltd., Wayville, SA, Australia). The perfusion rate of 0.02ml/min/lumen had been determined as optimal for measurement of SO pressure waves with a pico-manometric assembly (Craig *et al.* 2000). To minimise movement of the manometry catheter and respiration artefact in the manometry recording, the manometry catheter was attached to a Sylgard covered platform positioned under the common bile duct. The manometry catheter was positioned within the SO to record proximal-SO, body-SO and distal-SO activity (**Figure 2.5**). A saline filled catheter (1.2mm outer diameter x 0.8mm internal diameter) was inserted retrogradely into the common bile duct and diverted bile directly to the jejunum, by-passing the SO. All pressure transducers were connected to a MacLab recording system (MacLab/8S; ADInstruments, Castle Hill, Australia) and the software Chart v3.6/s (ADInstruments) was utilised.

At the end of the operative procedure the abdomen was filled with warm saline (37^{o}C) and covered with plastic wrap to minimise evaporation. An equilibration period of at least 30min was allowed for the blood pressure and SO activity to stabilise.

2.3.3 Experimental protocols

These *in vivo* experimental protocols were designed in consultation with the Flinders University Biostatistical Consultant Lynne Giles to ensure that the data generated could be analysed using appropriate statistical tests.

ATP or adenosine was freshly prepared in saline for each experiment and the pH adjusted to 7-8. A topical route of administration of purines was chosen for these studies. Topical application of a small volume directly applied to the SO allowed interpretation of the effects of ATP/adenosine on SO motility, without the confounding effects resulting from the actions of purines on the SO vasculature which could be produced when purines are administered via a close intra-arterial route (i.e. splenic artery). Therefore, aliquots (10μl; 1μM–1mM) were slowly applied to the exposed surface of the extra-duodenal portion of the SO, in a manner that prevented it from running off the SO. Preliminary studies showed that if successive concentrations were applied too rapidly a decrease in the magnitude of response occurred, presumedly due to receptor desensitisation. Therefore a period of 5-10min was allowed to elapse between successive applications to ensure SO activity had returned to baseline levels. Preliminary experiments demonstrated that after rinsing the SO with warm saline and allowing SO activity to return to baseline levels (approx 30min) a second dose-response curve could be generated that produced a response comparable to the first dose-response curve, suggesting minimal desensitization under these conditions.

To assess if the responses to ATP or adenosine were neurally mediated, the concentration-response studies were repeated after pre-treatment with the

neurotoxin TTX (9-18μg/kg bolus 300μl injection i.a.). Elimination of the SO-duodenal reflex, evoked by EFS (70V, 30Hz, 0.1ms duration for 10s) of the adjacent duodenum 10min after TTX injection (Saccone *et al.* 1994a), was used to confirm neural blockade within the SO. To assess if the responses to topical ATP or adenosine were mediated by cholinergic nerves, the concentration-response studies were repeated in the presence of hexamethonium, to block nicotinic receptors (infusion 3mg/kg/min i.a.; modified from Baker *et al.* 1990b) or atropine, to block muscarinic receptors (infusion 3μg/kg/min i.a.; modified from Baker *et al.* 1990a). Confirmation of muscarinic receptor blockade was determined by topical application of carbachol (1-10μM) to the SO. Although there was a substantial decrease in SO activity indicating that atropine was having an effect, in a few animals the atropine infusion was not sufficient to completely block the response to carbachol. Therefore in these few animals the atropine infusion represented only a partial block of muscarinic receptors, and this accounts for the slight response observed to 1mM ATP (see **Section 8.33**). Confirmation of nicotinic receptor blockade by hexamethonium was difficult as the nicotinic receptor agonist 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP; 10μl 1mM or 50μg/kg bolus close i.a.; Sigma) had no obvious consistent effect on SO motility. However considerable changes in the adenosine and ATP-induced responses indicated that hexamethonium was having an effect. To assess if the responses to topical ATP or adenosine were mediated by nitrergic nerves, the concentration-response studies were repeated in the presence of the non-selective NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 15mg/kg bolus followed by 6mg/kg/h infusion i.v.; Konomi *et al.* 2001). Confirmation of NOS inhibition was provided by the observed increase in

blood pressure, and spontaneous SO motility as described previously (Konomi *et al.* 2001).

2.3.4 Analysis of recordings

Similar to the *in vitro* experiments changes in contractile activity were quantified as area under curve (mmHg.s), basal pressure (mmHg), contraction amplitude (mmHg) and contraction frequency (contraction/min). The response to ATP and adenosine was observed most prominently in the proximal-SO recording site, therefore only recordings from this site were analysed (note that direct application to the most distal-SO recording site was not possible as it is contained within the duodenal wall).

The analysis for ATP or adenosine application before and after treatment with each of the antagonists was performed as described for the *in vitro* protocol (**Section 2.2.6**). Group data is presented as the change from baseline activity, mean ± SEM. Graphs were prepared using GraphPad Prism as for *in vitro* data (**Section 2.2.6**).

2.3.5 Statistical analysis

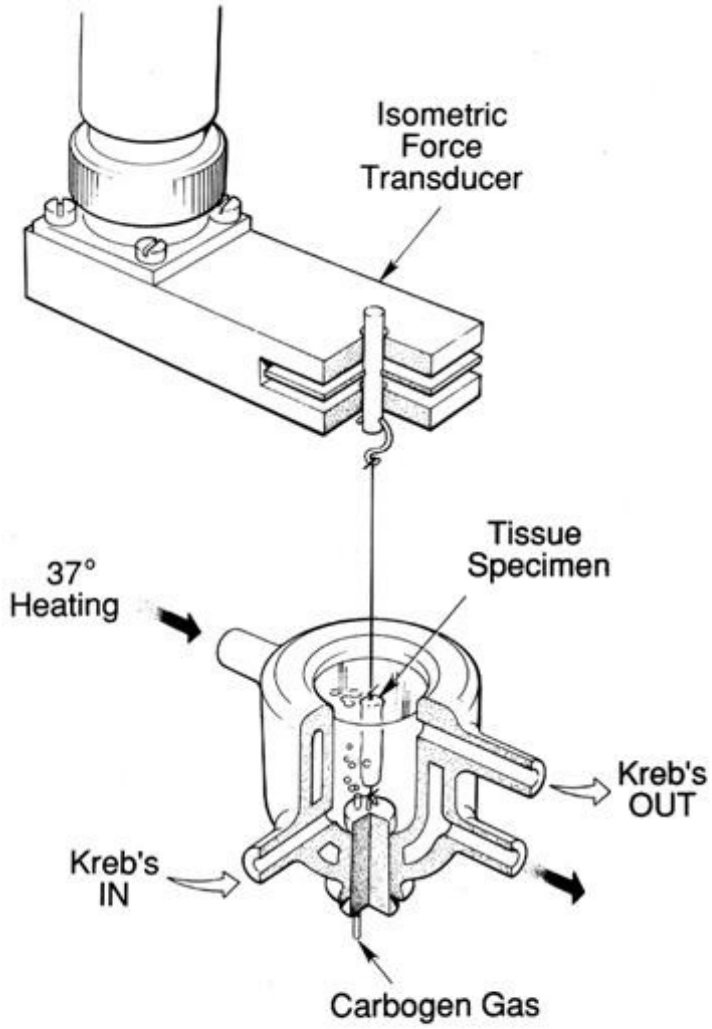
Statistical analyses were performed using the statistical package SPSS. To determine if adenosine or ATP had an effect on SO activity an ANOVA was performed. Pair wise comparisons were then performed using post-hoc tests with Bonferroni correction to determine which concentrations of adenosine/ATP were significantly different. To determine if atropine, hexamethonium or L-NAME treatment had an effect on the adenosine/ATP concentration-response a repeated measures ANOVA was performed. P

values less than 0.05 were considered to be significant. For simplicity all P values are reported as being less than or greater than 0.05 as appropriate.

2.4 Immunohistochemical Studies

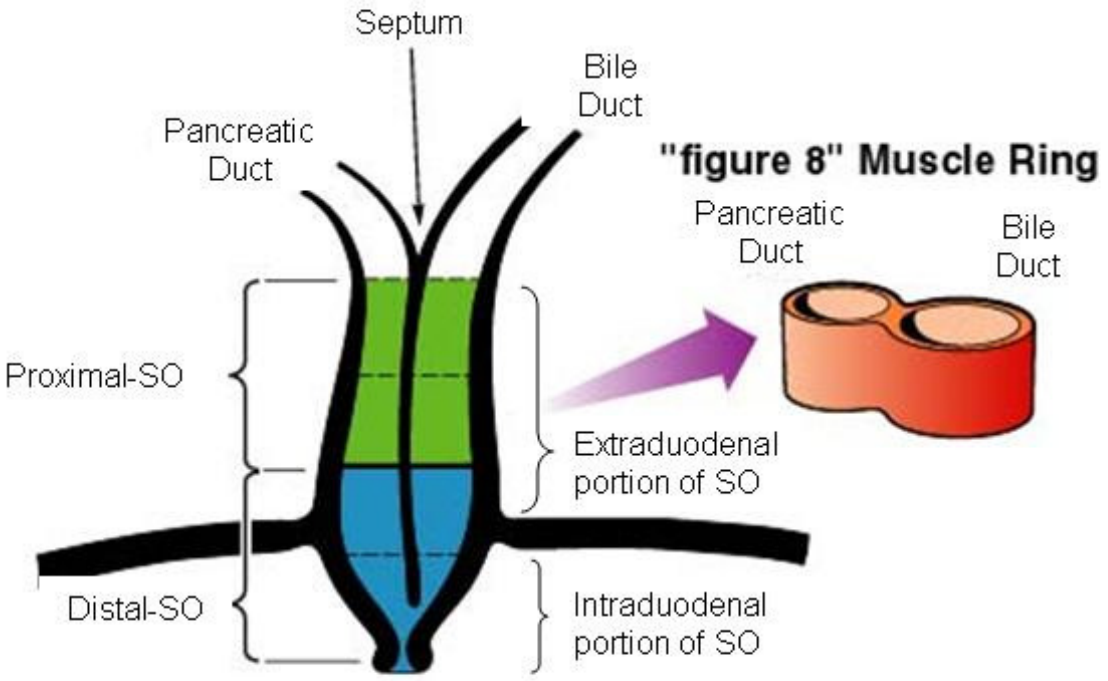
The methodology used for the immunohistochemical studies is presented in **Appendix 1**.

Figure 2.1: Schematic diagram of organ bath assembly



Muscle strips/rings were attached to the base of the organ bath and to an isometric force transducer, which in turn was connected to a MacLab recording system, which allowed measurement of spontaneous contractile activity. The organ bath has ports to allow exchange of Krebs' solution in the bath and the outer jacket is perfused with warm water to maintain the Krebs' solution at 37°C. The Krebs' solution was continually bubbled with carbogen gas (95% O_2/5% CO_2).

Figure 2.2: Preparation of proximal-SO and distal-SO muscle rings



Schematic diagram illustrating that the possum SO is predominantly extraduodenal, and the location of the proximal-SO and distal-SO regions. For preparation of SO muscle rings, the attached duodenal wall was first removed by dissection. The SO was then cut into 4 "figure 8" muscle rings and each ring was allocated to either the proximal-SO or distal-SO group, as appropriate.

Table 2.1: Receptor sub-type selectivity of P2 agonists and antagonists used in the experiments described in this thesis

Drug	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$	$P2X_{2/3}$	$P2X_{1/5}$	$P2X_{1/3}$	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
Agonists																		
ATP	✓✓✓	✓✓	✓✓✓	✓✓	✓✓	✓	✓	✓✓✓	✓✓✓	✓✓	✓	✓✓✓	✓✓	–	✓			
αβmeATP	✓✓✓	–	✓✓✓	✓	✓	✓	–	✓✓✓	✓✓	✓			–					
2meSATP	✓✓✓	✓✓	✓✓✓	✓✓	✓✓	✓	✓				✓✓		✓	✓	✓	✓	✓✓	
ATPγS	✓✓	✓✓	✓✓	✓	✓✓	✓	–				✓	✓	✓		✓✓✓	✓		
UTP	–	–	–	–	✓							✓✓✓	✓✓✓	✓				
Antagonists																		
PPADS	✓✓	✓✓	✓✓	–	✓✓	✓	✓					✓	✓	✓✓				
Suramin	✓✓	✓	✓	–	✓✓	–	–				✓	✓	–	–	✓✓	✓		

Number of ticks indicates relative potency with respect to agonist/antagonist concentration for mammalian P2 receptors.
Agonists: ✓✓✓ <1μM, ✓✓ <1-10μM, ✓ >10μM, – virtually inactive.
Antagonists: ✓✓✓ <10nM, ✓✓ <10-300nM, ✓ >300nM, – virtually inactive

(Extract from Table 1 from:Burnstock G. *Current Topics in Medicinal Chemistry* 2004, **4**:793-803)

Figure 2.3: Analysis of SO and duodenal muscle strip/ring recordings

Chart recording from SO

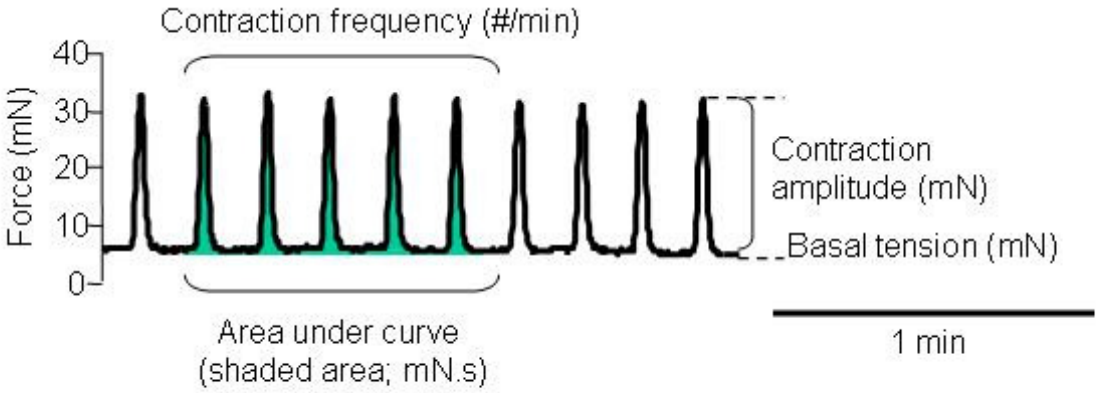
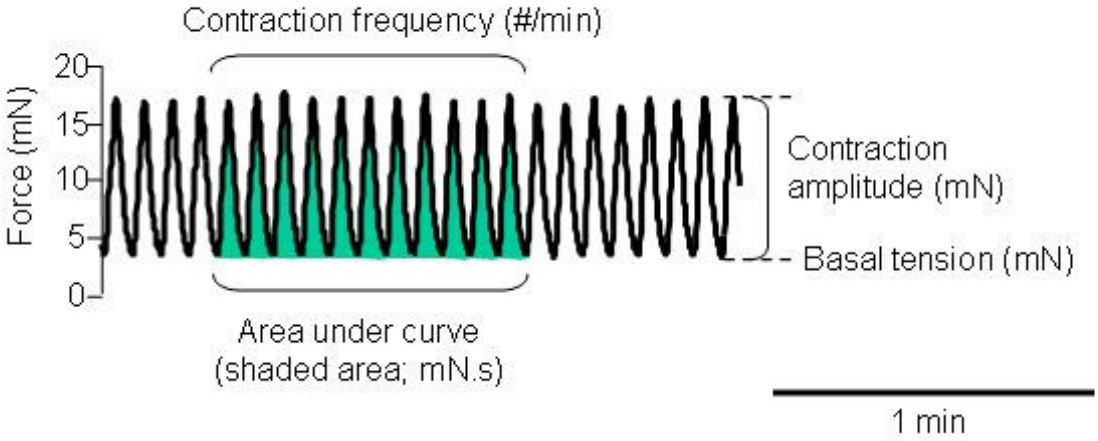


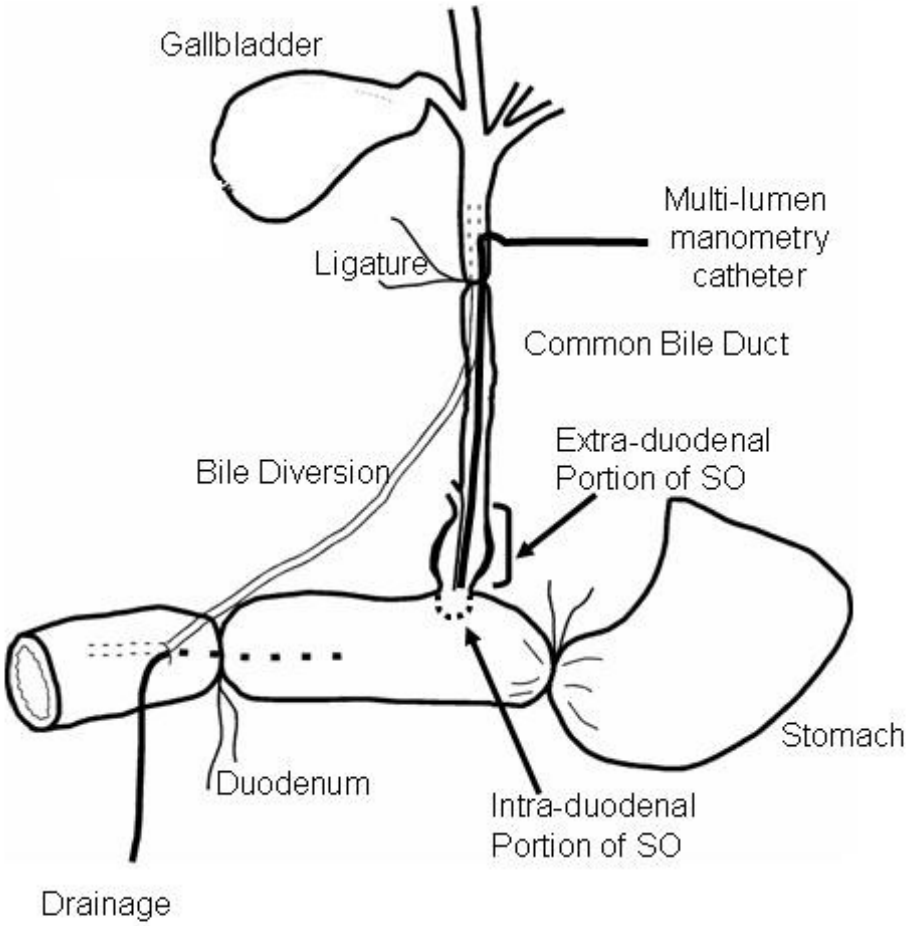
Chart recording from duodenum



Analysis of SO and duodenal muscle strip/ring recordings from *in vitro* experiments. For each muscle strip/ring contraction amplitude (height of contraction), basal tension (resting tension between contractions), contraction frequency (number of contractions) and area under curve (combination of contraction amplitude and frequency) were analysed for 1min periods, unless otherwise stated.

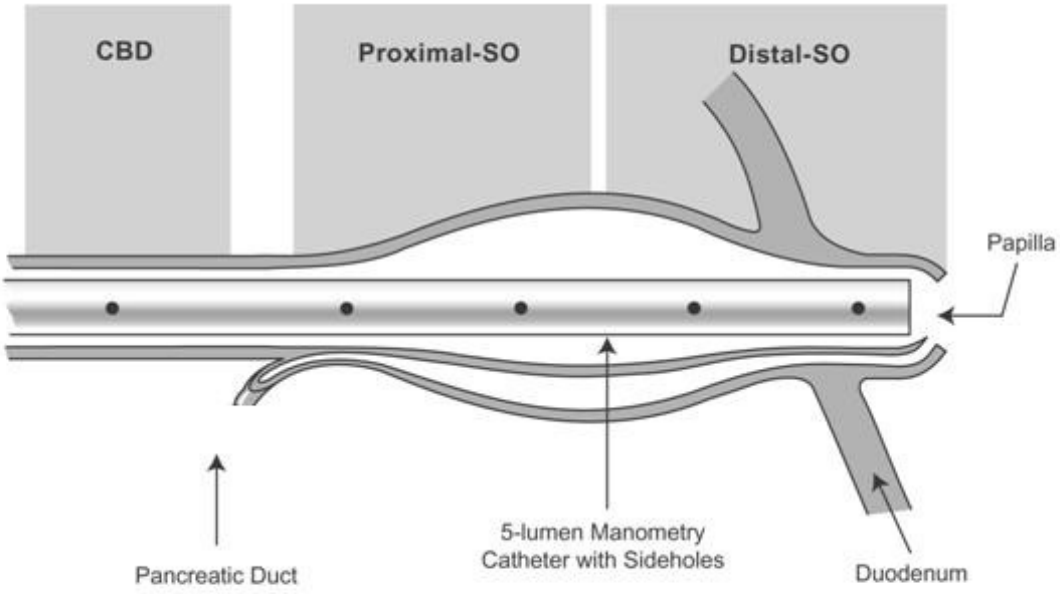
A similar analysis of *in vivo* recordings was used. However instead of basal tension, basal pressure was determined, and units are in mmHg instead of mN.

Figure 2.4: Schematic diagram of possum *in vivo* preparation for manometric measurement of SO activity



Schematic diagram of SO and adjacent duodenum illustrating the position of the multi-lumen manometry catheter. A ligature was placed around the pyloric sphincter to prevent flow of stomach contents. A ligature was placed around the distal duodenum and a drainage tube positioned distal to the SO papilla to prevent duodenal distention. A ligature around the proximal CBD anchored the bile diversion tube and the multi-lumen manometry catheter. To prevent movement of the manometry catheter due to respiration, platforms (not shown) were positioned under the duodenum and under the CBD and the tissues were gently pinned to it.

Figure 2.5: Schematic diagram of pico-manometry assembly used in *in vivo* preparations



The multi-lumen pico-manometry catheter, consisting of 5 recording ports, was positioned within the possum SO and CBD. Ideal recording positions were considered to be from the most distal-SO near the papilla and the most proximal-SO near the junction with the CBD, with one or two recording sites in the body of the SO and another in the CBD. Due to varying SO lengths between animals and the fixed side-hole positions on the catheter, obtaining recordings from all of these positions was not always possible. It was considered to be essential for these experiments to have the catheter positioned to record from the most proximal-SO region, which was often at the expense of recording from the most distal-SO region.

Chapter 3

3 EFFECT OF EXOGENOUS ATP AND ADENOSINE ON SPONTANEOUS SO AND DUODENAL CONTRACTILE ACTIVITY *IN VITRO*

3.1 Introduction

The studies described in this thesis focus on the effects of purines on SO motility. However SO motility is modulated by duodenal motility, via neural reflexes and as such SO motility should not be considered in isolation. Furthermore it cannot be assumed that agents that affect duodenal motility also affect SO motility in the same manner. Therefore the *in vitro* studies outlined in this thesis will determine the effects of purines on spontaneous contractile activity of both the duodenum and the SO.

As reviewed in Chapter 1 few studies have established a role for purines in the regulation of SO motility, and these responses vary depending on the species and preparation studied (**Section 1.4.3**). In comparison a number of studies addressing the actions of adenosine and ATP on spontaneous duodenal contractile activity have been performed in rabbit and rat. Exogenous ATP elicits a rapid relaxation followed by excitation (Manzini *et al.* 1985c; Johnson and Hourani 1994b; Windscheif *et al.* 1995c), whereas adenosine induced an inhibitory response (Small and Weston 1979b; Gaion *et al.* 1988b; Mule *et al.* 1989; Serio *et al.* 1990c; Nicholls *et al.* 1992e; Nicholls *et al.* 1996g). The specific purinergic receptors mediating these responses have not been well characterized.

The investigation of the effects of purines on possum duodenal motility will provide a basis for comparison of purine responses/receptors between the SO and the duodenum. Furthermore the data from studies with duodenal muscle strips will be used as a guide for comparable studies with SO muscle rings.

The aims of the studies described in this chapter were to determine:

1. The effect of exogenous adenosine and ATP on spontaneous contractile activity of possum duodenum and SO.
2. If the proximal-SO and distal-SO regions respond differently to exogenous adenosine and ATP.
3. If the adenosine/ATP induced responses are neurally mediated.

3.2 Methods

Possums were anaesthetised and the SO with the adjacent duodenum was harvested as described in **Section 2.1**.

Organ bath pharmacology techniques were used. Full thickness muscle rings were prepared from the SO, separated into distal-SO and proximal-SO regions. Full thickness muscle strips were prepared from a segment of the duodenum from each animal as outlined in **Section 2.2**.

The protocols described in **Section 2.2.5** were used. Briefly, a single concentration of adenosine or ATP was applied to each muscle ring/strip. A paired protocol was used to determine if the response was neurally

mediated. Refer to **Section 2.2** for drug preparation, analysis of recordings and statistical analysis.

3.3 Results

3.3.1 SO

As expected, a difference was observed in muscle ring spontaneous contractile activity, predominantly contraction frequency. The proximal-SO group displayed a higher contraction frequency as previously noted (Baker *et al.* 1990c; Woods *et al.* 2000).

3.3.1.1 Effect of ATP on spontaneous SO motility

Exogenous ATP induced a bi-phasic response, consisting of a short excitatory component, followed by a longer lasting inhibitory component (n=23-41 strips/concentration, from 55 animals) (**Figure 3.1**).

3.3.1.1.1 ATP-induced excitatory component

Analysis of the data, irrespective of SO region showed that the early excitatory component of the ATP-induced response consisted of a concentration-dependent increase in contractile activity, represented as area under curve ($P<0.05$) and contraction frequency ($P<0.05$) (**Figure 3.2**). ATP also increased contraction amplitude ($P<0.05$), but not in a concentration-dependent manner (**Figure 3.2**).

Analysis of the separate proximal-SO and distal-SO regions showed that the ATP-induced response was more pronounced in the proximal-SO, with an increase in contraction amplitude only evident in the proximal-SO rings ($P<0.05$) (n=13-24 muscle rings/concentration; from 55 possums) (**Figure**

3.2). The ATP-induced increase in contraction frequency was observed in both the proximal-SO and distal-SO rings (n=13-19 muscle rings/concentration; from 55 possums) with no difference between the two groups (**Figure 3.2**).

3.3.1.1.2 ATP-induced inhibitory component

Analysis of the pooled SO data (n=23-41 strips/concentration, from 55 animals) showed that the ATP-induced late inhibitory response consisted of decreases in area under curve ($P<0.05$), contraction amplitude ($P<0.05$), and contraction frequency ($P<0.05$), but only the decrease in contraction frequency was concentration-dependent ($P<0.05$) (**Figure 3.3**).

Analysis of the separate SO regions revealed that only the proximal-SO (n=13-24 muscle rings/concentration; from 55 possums) responded to exogenous ATP. The proximal-SO response consisted of a concentration-dependent decrease in area under curve ($P<0.05$), contraction amplitude ($P<0.05$) and contraction frequency ($P<0.05$) (**Figure 3.3**). No effect was evident in the distal-SO (n=13-19 muscle rings/concentration; from 55 possums).

3.3.1.1.3 Effect of TTX pre-treatment on the ATP-induced response

As the data described above indicated that the ATP-induced response predominated in the proximal-SO muscle rings further experiments evaluating the effect of TTX pre-treatment are presented separately for the two SO groups.

Pre-treatment with TTX (1μM) decreased the early excitatory component of the ATP response. As expected the TTX-effect was only evident in the proximal-SO data for contraction amplitude ($P<0.05$) (n=3-4 muscle rings/concentration; from 13 possums) (**Figure 3.4**), implicating a neural component to the response. In contrast, TTX pre-treatment significantly inhibited the ATP-induced increase in contraction frequency displayed by the distal-SO ($P<0.05$) (n=3-5 muscle rings/concentration; from 13 possums), but not by the proximal-SO. TTX pre-treatment had no significant effect on the ATP-induced excitatory response expressed as area under curve ($P>0.05$) for either SO region (**Figure 3.4**).

Pre-treatment with TTX did not modify the ATP-induced inhibitory response displayed by either SO region (**Figure 3.5**).

3.3.1.2 Effect of adenosine on spontaneous SO motility

Exogenous adenosine decreased spontaneous SO contractile activity (**Figure 3.6**). Concentration-dependent decreases (n=34-42 muscle rings/concentration; from 67 possums) in area under curve, contraction amplitude and contraction frequency were observed ($P<0.05$ for all) (**Figure 3.7**).

Separate analysis of the data from the two SO regions found the adenosine-induced inhibition of contractile activity was more pronounced in the proximal-SO rings. This response was evident as concentration-dependent decreases in area under curve, contraction amplitude and contraction frequency ($P<0.05$ for all) (n=17-20 muscle rings/concentration; from 67 possums) (**Figure 3.7**). In comparison, the data from the distal-SO rings

(n=17-22 muscle rings/concentration; from 67 possums) shows that adenosine decreased area under curve, contraction amplitude and contraction frequency ($P<0.05$ for each), but these changes were not concentration-dependent ($P>0.05$ for each) (**Figure 3.7**).

3.3.1.2.1 Effect of TTX pre-treatment on the adenosine-induced response

As the data described above indicated that the adenosine-induced response predominated in the proximal-SO muscle rings further experiments evaluating the effect of TTX pre-treatment are presented separately for the two SO groups.

Pre-treatment with TTX (1μM) had no significant effect on the adenosine-induced response for any parameter measured ($P>0.05$), irrespective of the SO region (**Figure 3.8**).

3.3.2 Duodenum

3.3.2.1 Effect of ATP on spontaneous duodenal motility

Exogenous ATP induced a complex tri-phasic response consisting of an immediate decrease in contractile activity followed by a recovery to baseline activity or increased contractile activity, and concluded with a reduction in contractile activity (**Figure 3.9A**). The immediate inhibitory response consisted of a very rapid, short-lasting, concentration-dependent decrease in basal tension ($P<0.05$) and contraction amplitude ($P<0.05$) (n=18-20 muscle strips/concentration; from 20 possums) (**Figure 3.9B**). Due to the short duration of the immediate inhibitory response it was not possible to reliably quantify area under curve or contraction frequency. This inhibitory response

was followed by a return to baseline activity, or at higher ATP concentrations an excitatory response. The magnitude and duration of this excitatory response was quite variable between muscle strips and could not be analysed accurately. Finally, a long-lasting inhibitory response was observed consisting of concentration-dependent decreases in area under curve ($P<0.05$), contraction amplitude ($P<0.05$), basal tension ($P<0.05$) and contraction frequency ($P<0.05$) (**Figure 3.9C**). Due to the minor effect of adenosine on contraction frequency this data is not presented in further experiments.

3.3.2.1.1 Effect of TTX pre-treatment on the ATP-induced response

Pre-treatment with TTX (1μM) alone did not modify spontaneous contractile activity ($P>0.05$ for all parameters). TTX pre-treatment had no statistically significant effect on the ATP-induced early or late inhibitory responses for any parameter measured (n=5-6 muscle strips/concentration; from 6 possums) (**Figure 3.10**).

3.3.2.2 Effect of adenosine on spontaneous duodenal motility

Exogenous adenosine decreased spontaneous longitudinal duodenal smooth muscle activity (**Figure 3.11A**). The response consisted of a long-lasting, concentration-dependent decrease in area under curve ($P<0.05$), contraction amplitude ($P<0.05$) and basal tension ($P<0.05$) (n=34 muscle strips/concentration; from 36 possums) (**Figure 3.11B**). A minor decrease in contraction frequency ($P<0.05$) (**Figure 3.11B**) was also observed, but this was not concentration-dependent. Due to the minor effect of adenosine on contraction frequency this data is not presented in further experiments.

3.3.2.2.1 Effect of TTX pre-treatment on the adenosine-induced response

TTX pre-treatment had no significant effect on the adenosine-induced response (n=5 muscle strips/concentration; from 5 animals) (**Figure 3.12**).

Figure 3.1: Effect of ATP on spontaneous SO motility

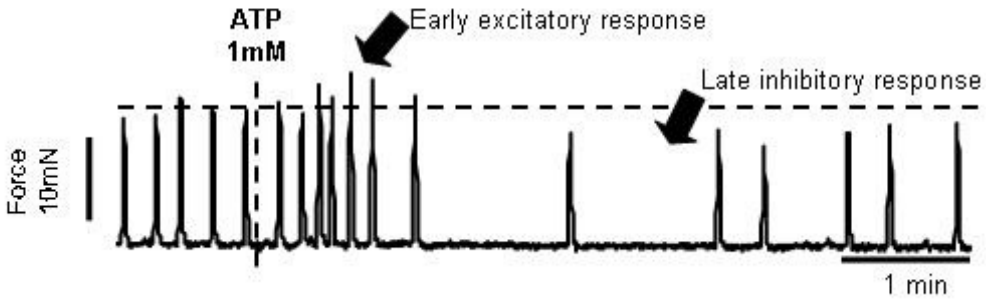
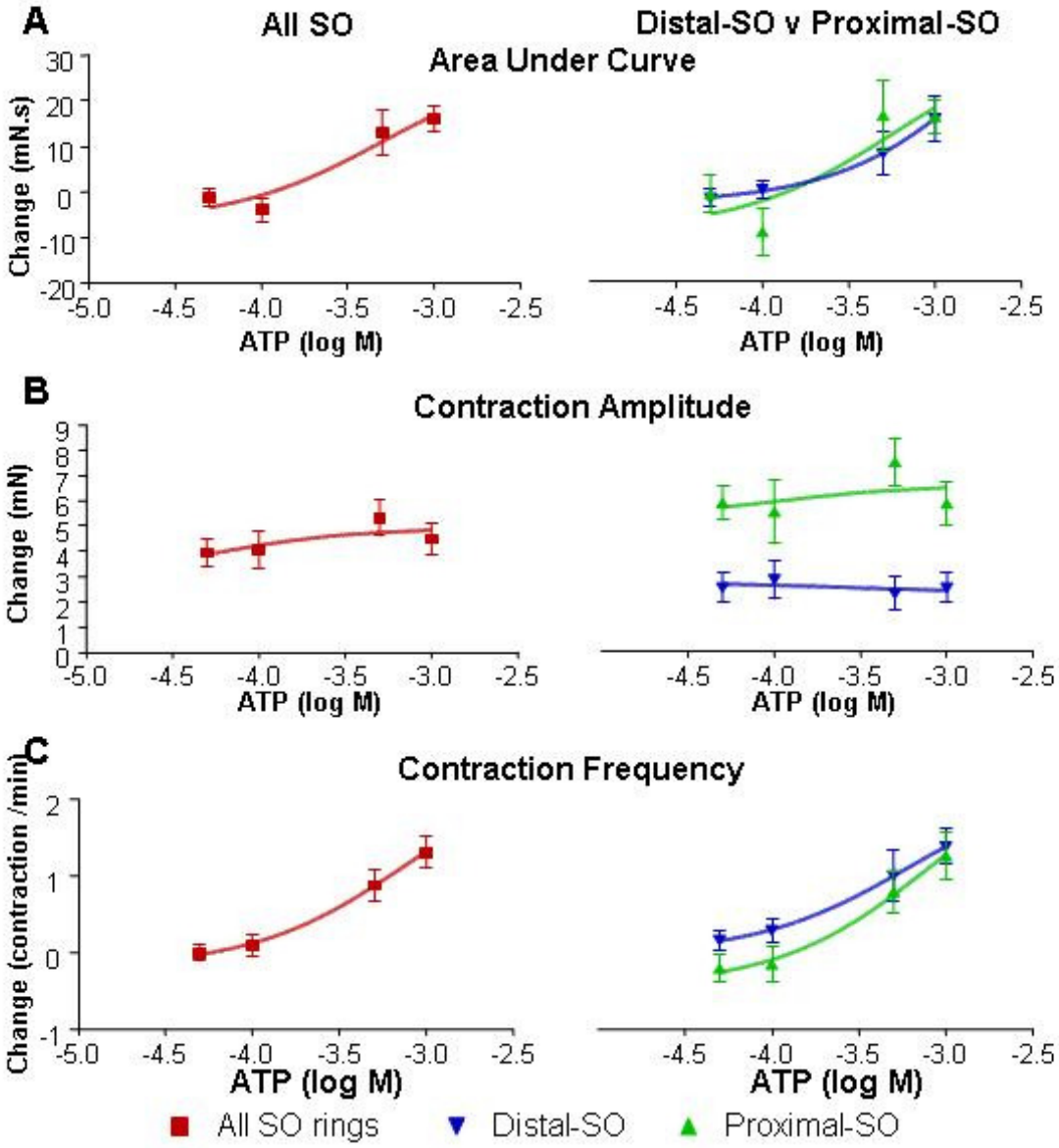


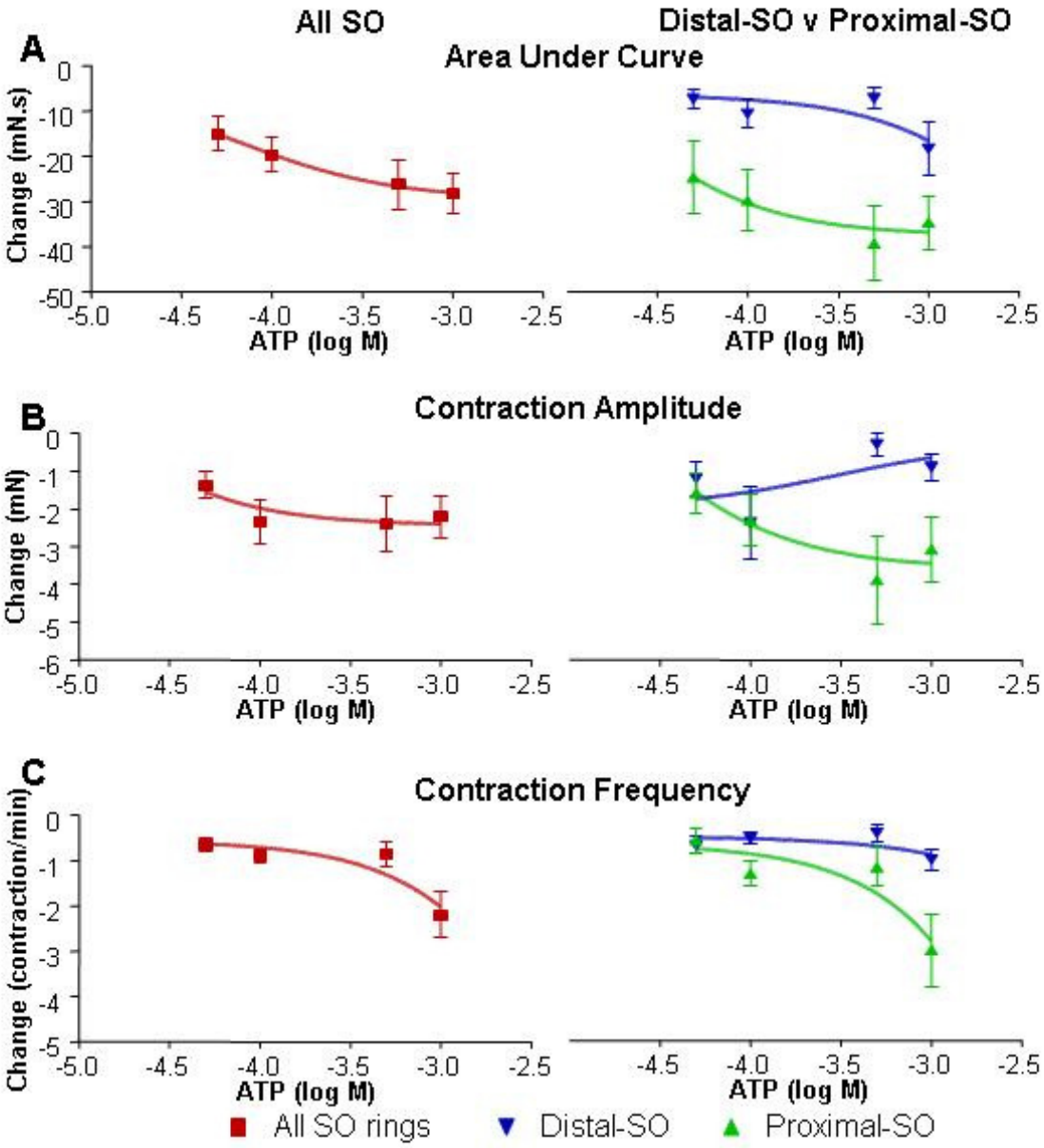
Chart recording illustrating the complex action of exogenous ATP on a spontaneously active proximal-SO muscle ring. A bi-phasic response was evident consisting of an excitatory component followed by a longer-lasting inhibitory component. Horizontal dashed line indicates average contraction amplitude prior to ATP application. Dashed vertical line indicates time of ATP application to the organ bath.

Figure 3.2: Excitatory component of the ATP-induced change in SO motility with comparison of distal-SO and proximal-SO data



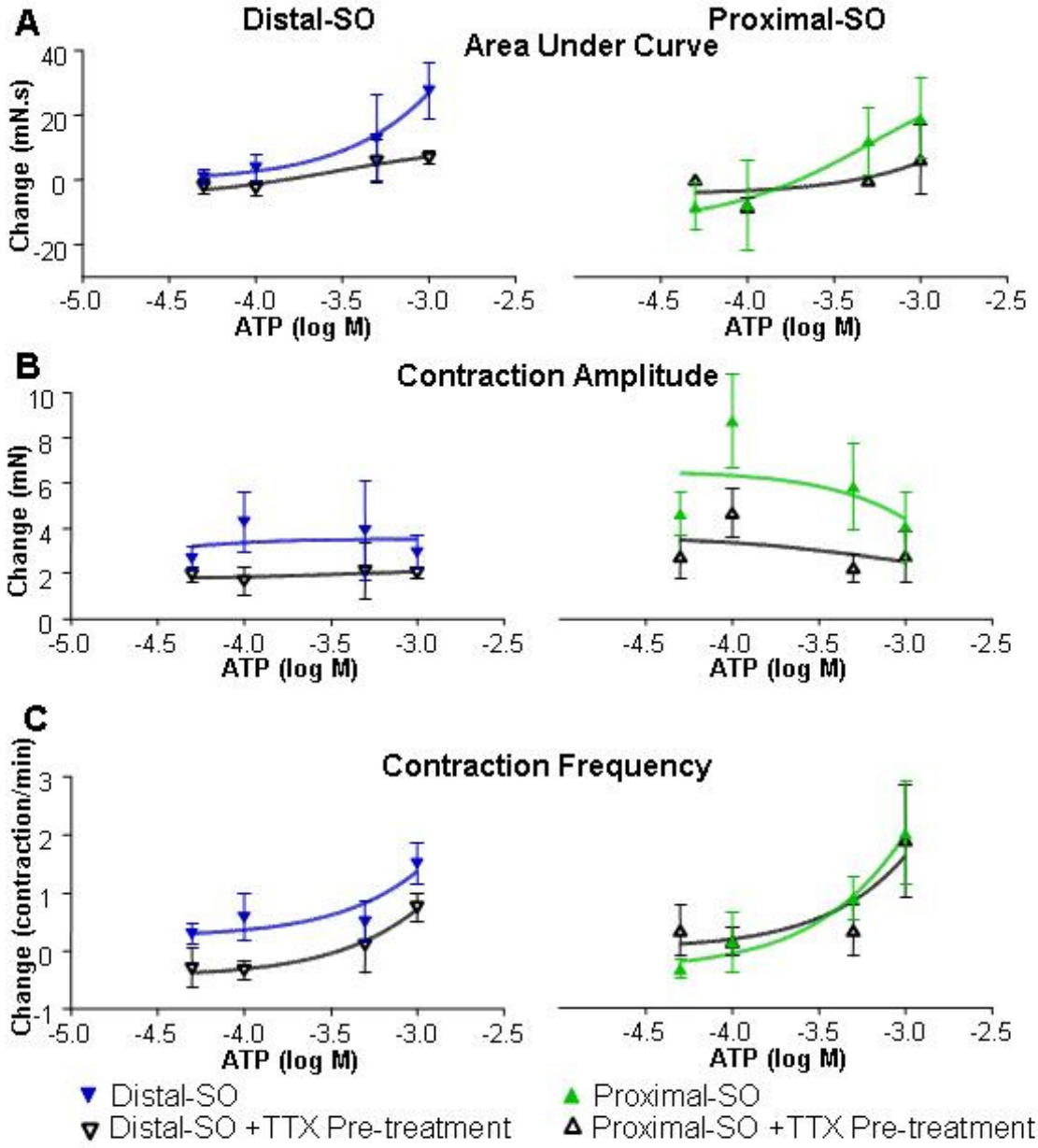
Group data (red; n=23-41 muscle rings/concentration; from 55 possums), as a change from baseline activity, illustrating the excitatory ATP component of the response to exogenous ATP. Concentration-dependent increases were observed for area under curve (**A**) and contraction frequency (**C**) (both $P<0.05$), but not for contraction amplitude (**B**) ($P>0.05$). Comparison of the distal-SO (blue; n=13-19 muscle rings/concentration; from 55 possums) and proximal-SO (green; n=13-24 muscle rings/concentration; from 55 possums) data found the two regions responded differently with respect to contraction amplitude (**B**) ($P<0.05$) with only the proximal-SO displaying a response. There was no difference between SO regions with respect to the other parameters.

Figure 3.3: Inhibitory component of the ATP-induced change in SO motility with comparison of distal-SO and proximal-SO data



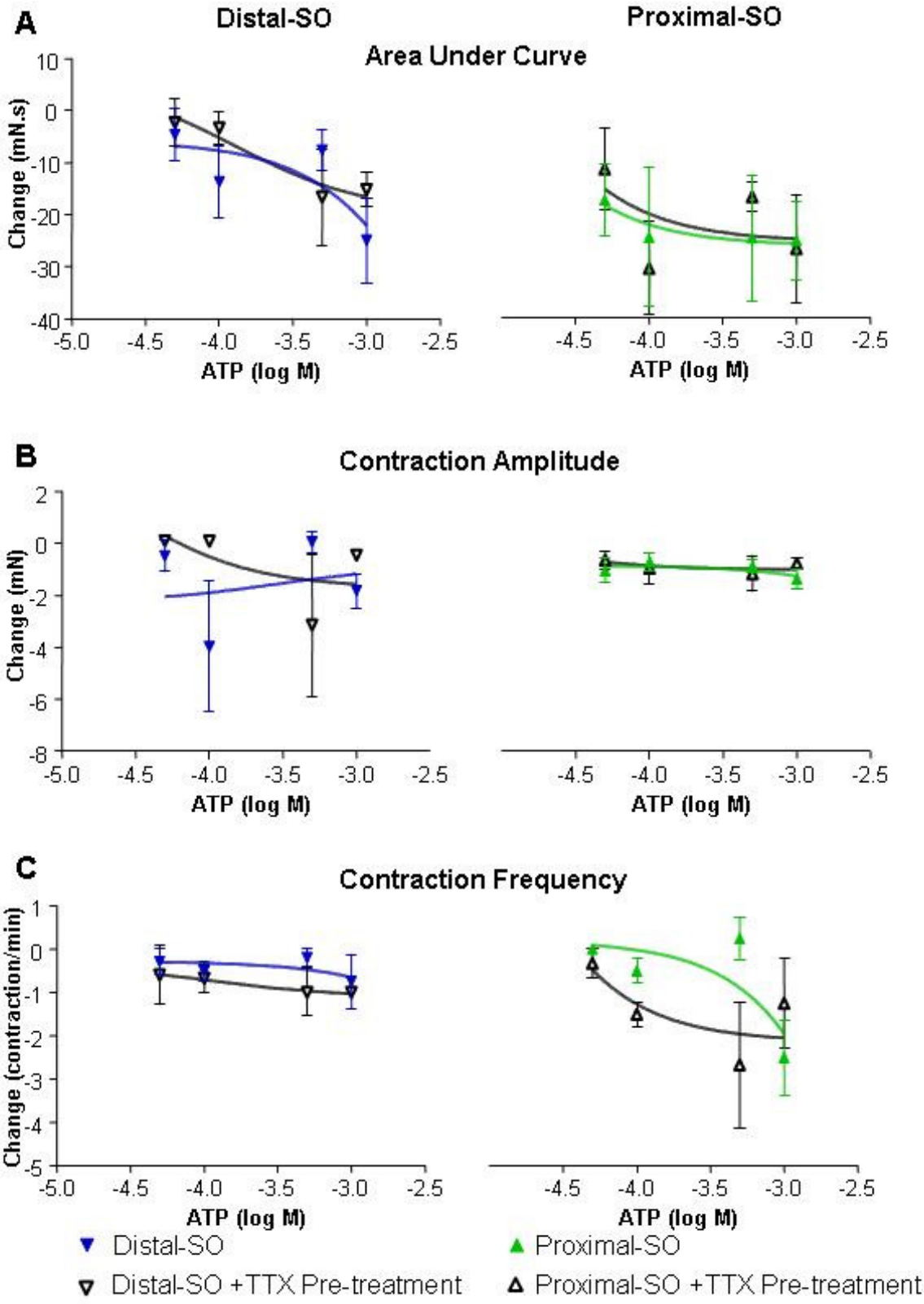
Group data (red; n=23-41 muscle rings/concentration; from 55 possums), as a change from baseline activity, illustrating the inhibitory ATP component of the response to exogenous ATP. A decrease in contractile activity was observed for area under curve (**A**), contraction amplitude (**B**), and contraction frequency (**C**) ($P<0.05$ for all). Comparison of the distal-SO (blue; n=13-19 muscle rings/concentration; from 55 possums) and proximal-SO (green; n=13-24 muscle rings/concentration; from 55 possums) data found the response was predominantly exhibited in the proximal-SO with respect to area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P<0.05$ for all).

Figure 3.4: Effect of TTX pre-treatment on the excitatory component of the ATP-induced changes in distal-SO and proximal-SO motility



Pre-treatment with the TTX (1μM) decreased aspects of the ATP-induced excitatory response. TTX suppressed the ATP-induced increase for contraction amplitude (**B**) ($P<0.05$) displayed by the proximal-SO muscle rings (green; n=3-4 muscle rings/concentration; from 13 possums), and contraction frequency (**C**) ($P<0.05$) displayed by the distal-SO muscle rings (blue; n=3-5 muscle rings/concentration; from 13 possums), but had no effect on the increase in area under curve (**A**).

Figure 3.5: Effect of TTX pre-treatment on the inhibitory component of the ATP-induced change in distal-SO and proximal-SO motility



Pre-treatment with TTX (1μM) did not have a significant effect on the ATP-induced inhibitory response expressed as area under curve (**A**), contraction amplitude (**B**) or contraction frequency (**C**) for either proximal-SO (green; n=3-4 muscle rings/concentration; from 13 possums) or distal-SO (blue; n=3-5 muscle rings/concentration; from 13 possums) muscle rings (all $P>0.05$).

Figure 3.6: Effect of adenosine on spontaneous SO motility

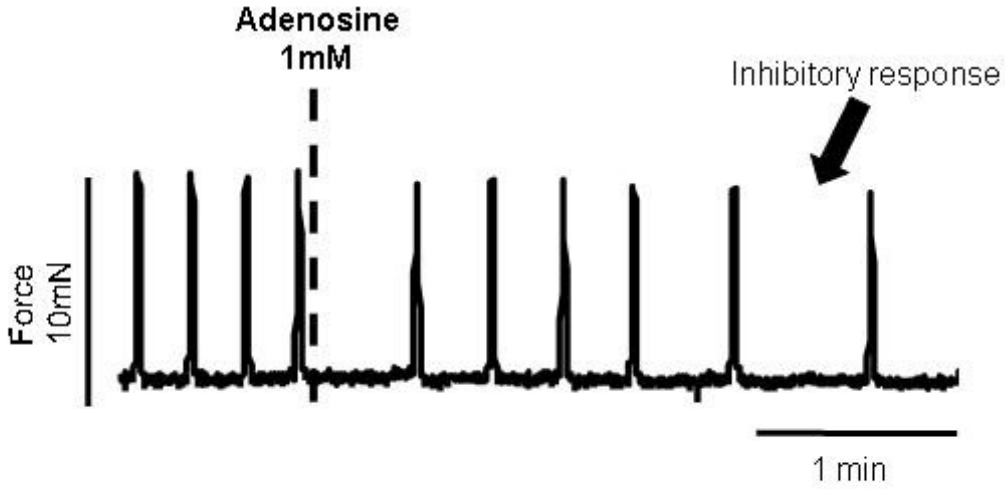
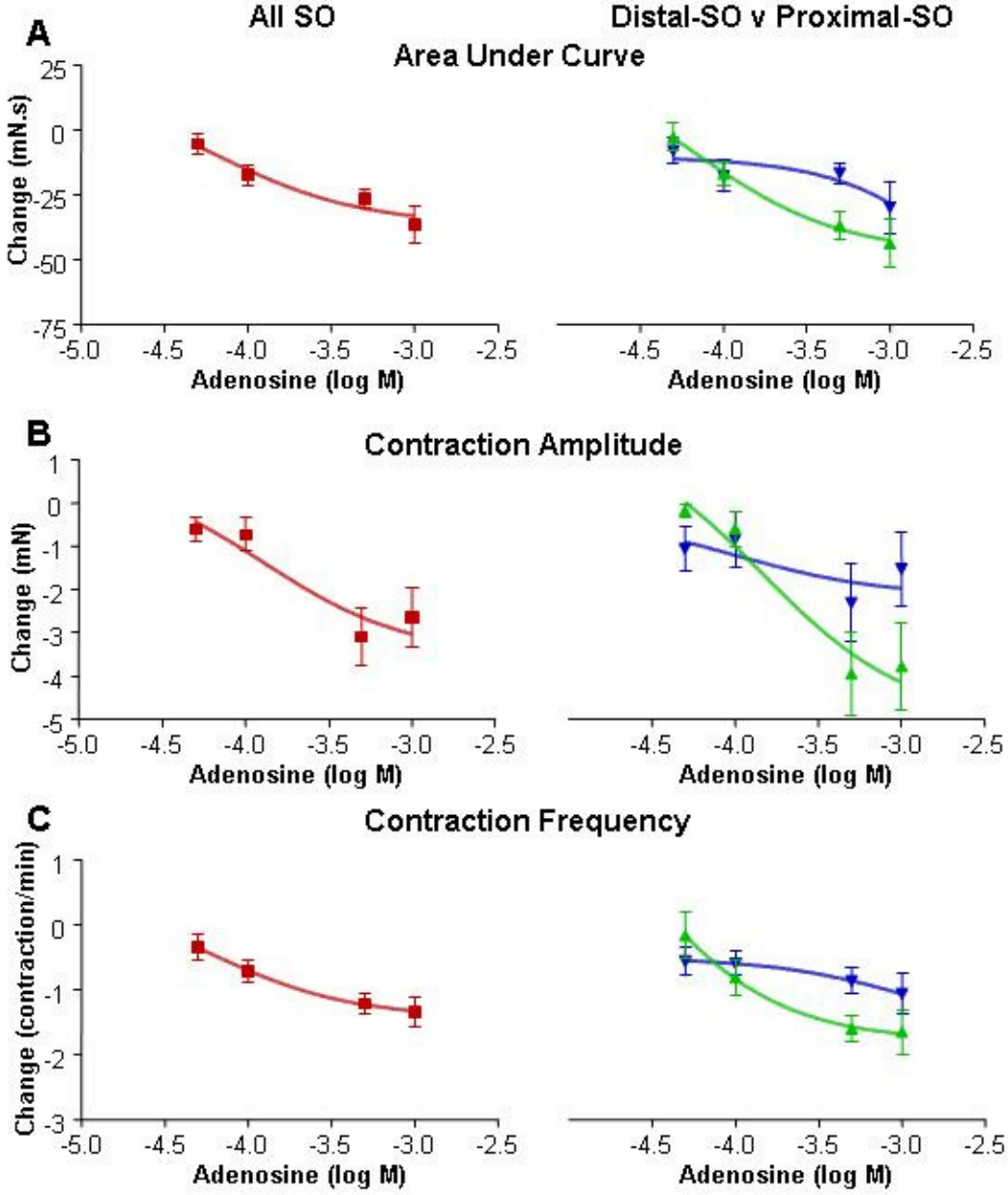


Chart recording illustrating the inhibitory action of exogenous adenosine on a spontaneously active proximal-SO muscle ring, evident here as a decrease in contraction frequency. Dashed vertical line represents time of adenosine application to organ bath.

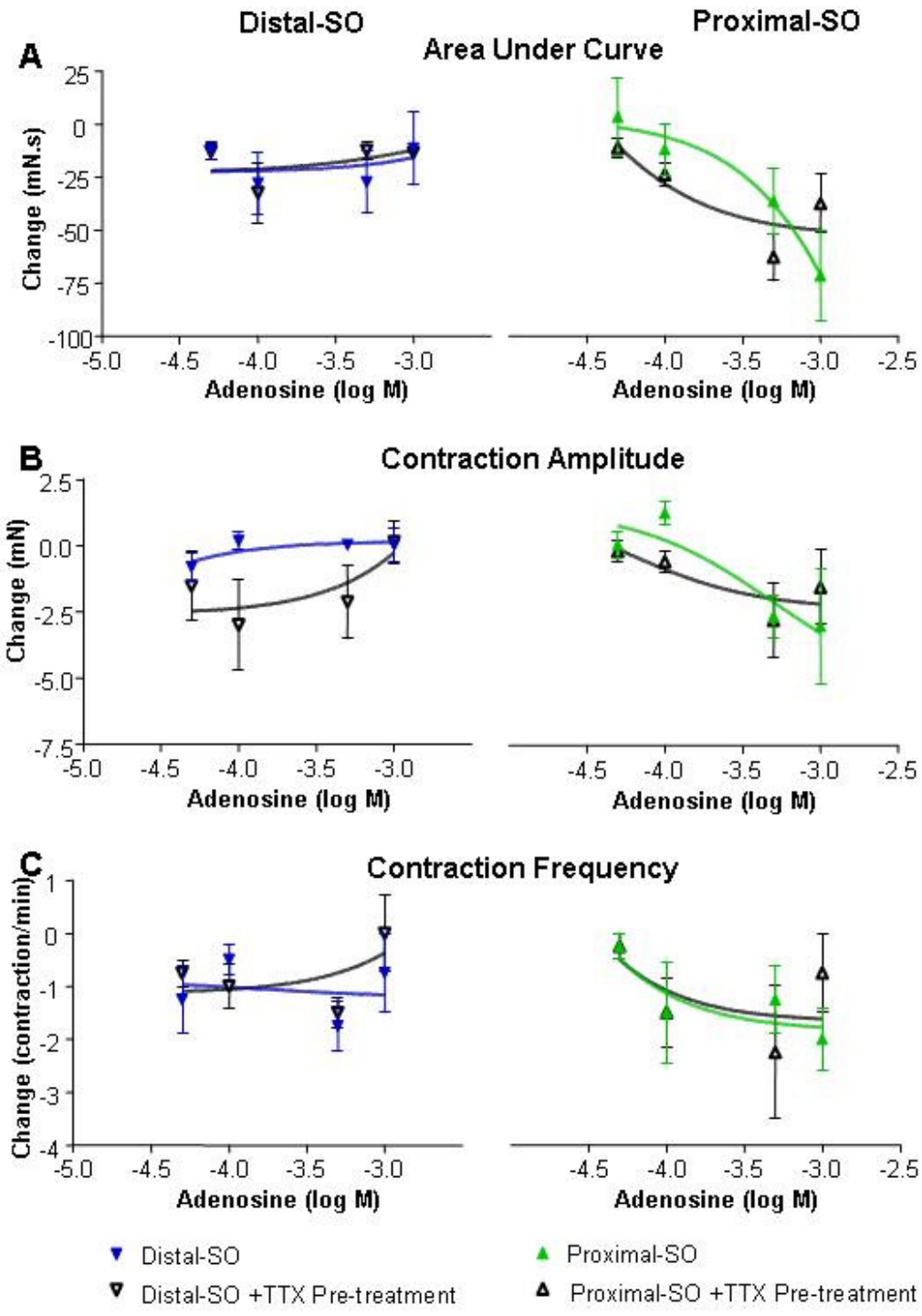
Figure 3.7: Effect of adenosine on SO motility with comparison of distal-SO and proximal-SO data



Group data (shown in red; n=34-42 muscle rings/concentration, from 67 possums) as a change from baseline activity, illustrating the concentration-dependent decrease in contractile activity in response to exogenous adenosine application expressed as area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P<0.05$ for all). Separate analysis of data found that the adenosine-induced response was predominant in the proximal-SO muscle rings (green; n=17-20 muscle rings/concentration, from 67 possums) for area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P<0.05$ for all). Minor concentration-independent changes in contractile activity was displayed by the distal-SO muscle rings (blue; n=17-22 muscle rings/concentration, from 67 possums).

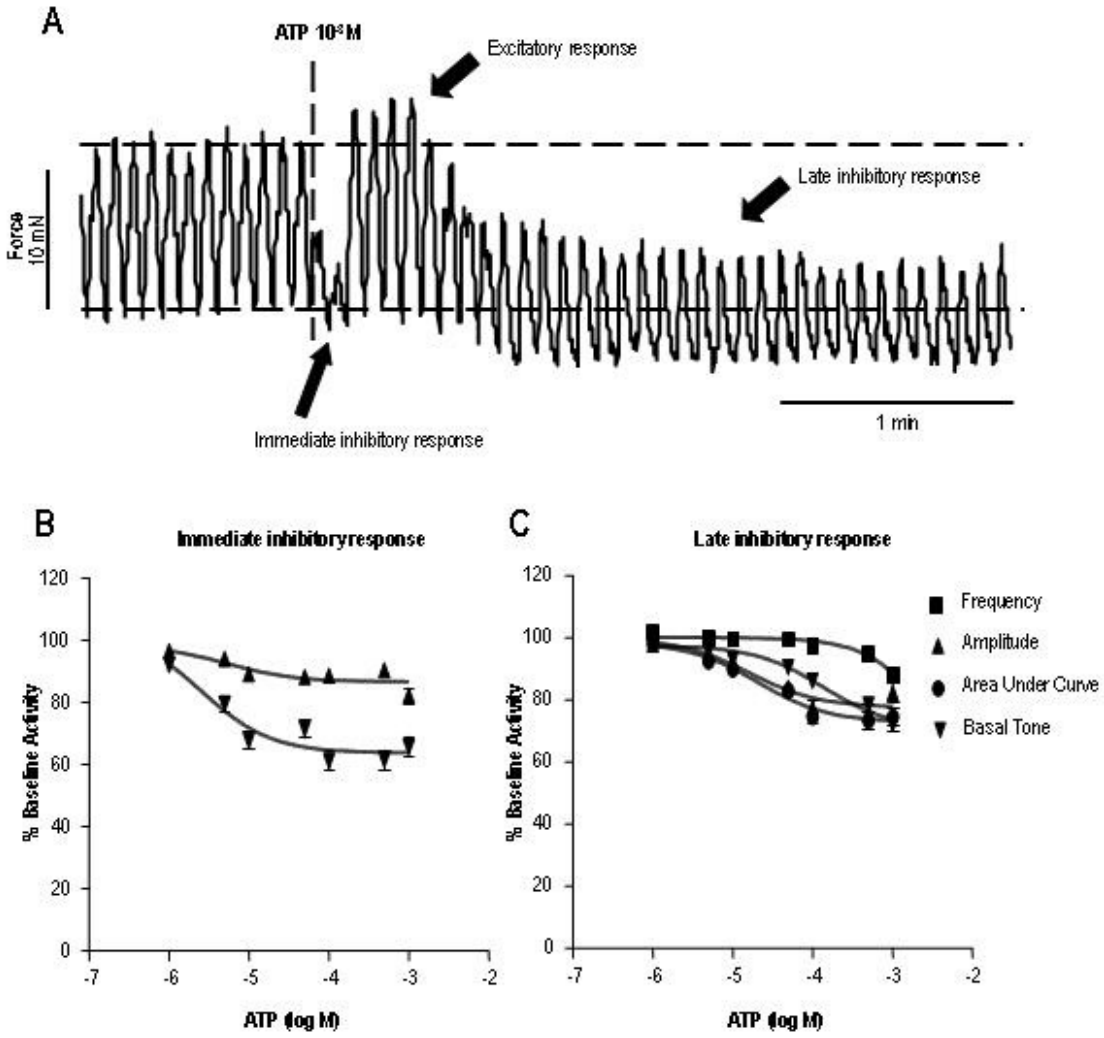
107

Figure 3.8: Lack of effect of TTX pre-treatment on the adenosine-induced changes in distal-SO and proximal-SO motility



Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment with TTX (1μM) on the adenosine-induced response displayed by the distal-SO (n=16 muscle rings; from 11 possums) and the proximal-SO muscle rings (n=16 muscle rings; from 11 possums). Contractile activity expressed as area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) (distal-SO and proximal-SO $P>0.05$ for all).

Figure 3.9: Effect of ATP on spontaneous duodenal motility

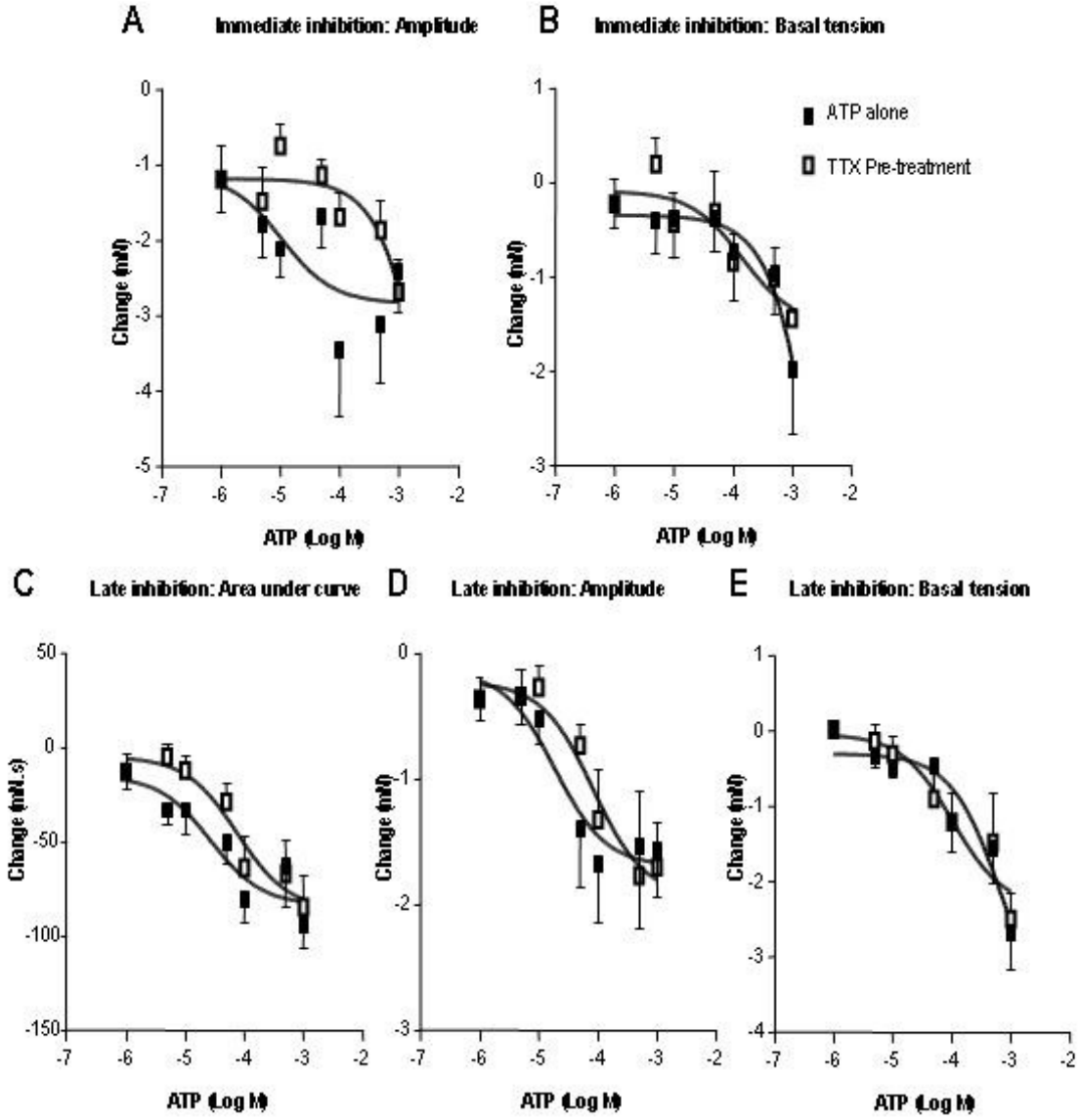


A: Representative chart recording of a spontaneously active longitudinal duodenal muscle strip illustrating the tri-phasic response to ATP application (1mM) consisting of an immediate inhibition, followed by a brief excitatory response, and concluding with a late inhibition. The horizontal dashed lines indicate baseline contraction amplitude, and basal tension (lower). The vertical dashed line indicates the time of ATP application to the organ bath.

B: Group data, as a percentage of baseline activity, illustrating the concentration-dependent ATP-induced immediate reduction in basal tension ($P<0.05$) and contraction amplitude ($P<0.05$) (n=18-20 muscle strips/concentration, from 20 possums). Area under curve and contraction frequency were not quantified due to the short duration of this response. Some of the error bars (SEM) are within the size of the symbols.

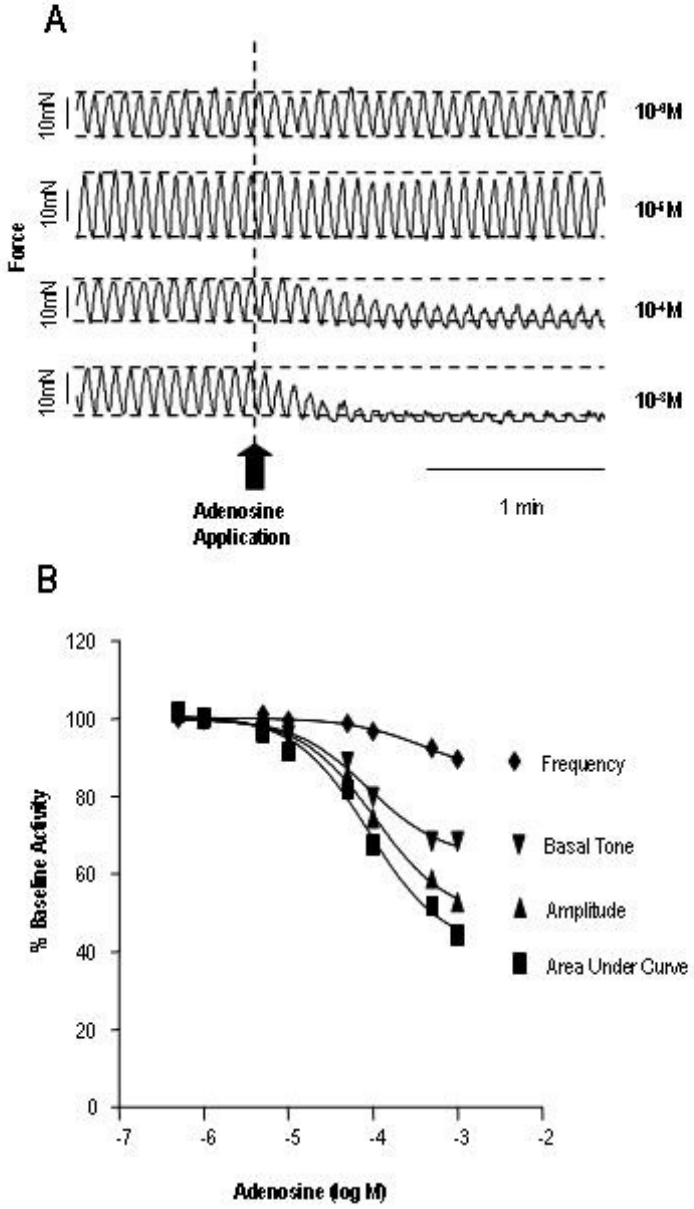
C: Group data, as a percentage of baseline activity, illustrating the concentration-dependent ATP-induced late inhibition expressed as area under curve, contraction amplitude, basal tension and contraction frequency ($P<0.05$ for all) (n= 18-20 muscle strips/concentration, from 20 possums). Some error bars (SEM) are within the size of the symbols.

Figure 3.10: Effect of TTX pre-treatment on the ATP-induced decrease in duodenal motility



Group data, as a change from baseline activity, illustrating the lack of significant effect of pre-treatment of longitudinal duodenal muscle strips with TTX (1μM) on the ATP-induced immediate inhibition expressed as contraction amplitude (**A**) and basal tension (**B**), or on the ATP-induced late inhibition expressed as area under curve (**C**), contraction amplitude (**D**) and basal tension (**E**) ($P>0.05$ for all). (n=5-6 muscle rings/concentration; from 6 possums). Note: TTX pre-treatment did appear to minimise the magnitude of the ATP-induced immediate decrease in contraction amplitude (**A**), but due to the variability in the magnitude of this response (possible due to the onset of the secondary component), this did not reach statistical significance.

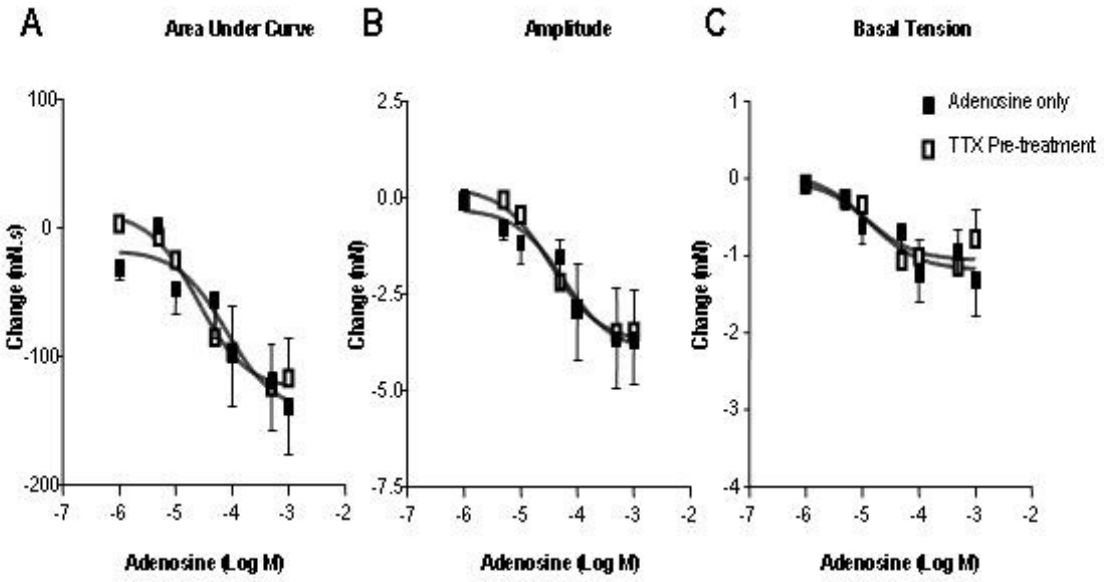
Figure 3.11: Effect of adenosine on spontaneous duodenal motility



A: Representative chart recordings from four different muscle strips, from the same animal, illustrating responses to various concentrations of adenosine. Horizontal dashed lines indicate baseline activity for contraction amplitude and basal tension in each strip. Vertical dashed line indicates the time of adenosine application to the organ baths.

B: Group data, as a percentage of baseline activity, illustrating the concentration-dependent adenosine-induced decrease in motility expressed as area under curve, contraction amplitude, basal tension and contraction frequency ($P<0.05$ for each) (n=34 muscle strips/concentration, from 36 possums). Error bars (SEM) are within the size of the symbols.

Figure 3.12: Effect of TTX pre-treatment on the adenosine-induced decrease in duodenal motility



Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment of longitudinal duodenal muscle strips with TTX (1μM) on the adenosine-induced decrease in motility, expressed as area under curve (**A**), contraction amplitude (**B**) and basal tension (**C**) ($P>0.05$ for each) (n=5 muscle strips/concentration, from 5 possums).

Table 3.1: Summary of effects of exogenous ATP or adenosine on spontaneous contractile activity of SO muscle rings and duodenal muscle strips

	SO			Duodenum
	All SO	**Proximal-SO**	**Distal-SO**	
ATP	Bi-phasic response	Prominent response	Negligible response	Tri-phasic response
TTX sensitivity	-	TTX-sensitive excitatory component	-	TTX-insensitive
Adenosine	Inhibitory response	Prominent response	Negligible response	Inhibitory response
TTX sensitivity	-	TTX-insensitive	-	TTX-insensitive

3.4 Discussion

Exogenous application of adenosine and ATP affected spontaneous motor activity of both the SO and the duodenum. The results are summarized in **Table 3.1**.

3.4.1 Spontaneous SO motility and ATP

These initial investigations with spontaneously active SO muscle rings revealed that exogenous ATP induced a bi-phasic response, consisting of an excitatory component followed by an inhibitory component. This response predominated in the proximal-SO, with only minor effects observed in the distal-SO. The difference in the ATP-induced responses between the proximal-SO and distal-SO muscle rings provides further evidence to support the hypothesis that the SO is composed of different functional components (see **Section 3.4.5**).

There are conflicting reports in the literature concerning the SO motor response to exogenous ATP. Interestingly the possum proximal-SO response demonstrated both excitatory and inhibitory components, with very little response from the distal-SO muscle rings. The proximal-SO data is at variance with a report using human SO muscle strips which found ATP to have no effect (McKirdy *et al.* 1987). However, the distal-SO data support this finding in human SO, thus highlighting the importance of distinguishing the SO region under investigation.

In support of an excitatory effect of ATP, studies in guinea-pig SO reported that exogenous ATP evoked contractions (Patacchini *et al.* 1998f). In the

possum SO, exogenous ATP induced TTX-sensitive contractions, suggesting the P2 receptors are located on nerves, and provides some evidence to suggest that ATP may be an excitatory neurotransmitter in the SO. Patacchini *et al.* 1998e) provided further evidence for ATP to be an excitatory neurotransmitter in guinea-pig SO when the ATP-antagonist PPADS, in combination with tachykinin antagonists, blocked EFS-induced contractions.

In the possum exogenous ATP also induced an inhibitory response, which was TTX-insensitive, indicating that the receptors are likely to be on the smooth muscle or ICC (see **Section 3.4.7**). Other studies have found ATP to cause a relaxation (i.e. decrease in basal tension), as opposed to the decrease in contractile activity seen here with possum SO muscle rings. Studies in rabbit SO found ATP to cause a relaxation (Azuma and Fujiwara 1973b), and subsequent investigations found that ATP acting at P2X receptors, was involved in the NANC inhibitory junction potential (Imoto *et al.* 1998c).

Further experiments are required to clarify which P2 receptors are responsible for mediating these responses to exogenous ATP. ATP may mediate the excitatory and inhibitory effects by interacting with multiple P2 receptor sub-types. Alternatively ATP may be metabolized by endogenous ectonucleases to adenosine with the interaction of P1 receptors. In support of this mechanism a study in the cat SO (Persson 1976a) found ATP to inhibit spontaneous SO activity, but the response was increased in the presence of dipyridamole, an adenosine uptake inhibitor, thereby suggesting adenosine was involved in the response. Investigations of the P2 and/or P1

receptors involved in mediating the ATP-induced response are presented in **Chapter 5** of this thesis.

3.4.2 Spontaneous SO motility and adenosine

These initial investigations with possum SO muscle rings found exogenous adenosine decreased spontaneous SO motor activity. The adenosine-induced response was elicited predominantly by the proximal-SO.

The inhibitory effect of exogenous adenosine on possum SO motility shown here concurs with a study in guinea-pig SO where exogenous adenosine inhibited EFS-induced contractions (Katsuragi *et al.* 1985b). However, these authors further suggested that the adenosine receptors are located on cholinergic nerves of the SO and act to decrease acetylcholine release (Katsuragi *et al.* 1985a). Experiments with TTX described here found the adenosine-induced inhibition of possum SO motor activity was TTX-insensitive, which suggests the response is mediated by receptors located on smooth muscle and/or ICC (refer to **Section 3.4.7**). On the other hand, the *in vitro* possum data described here contrasts with a human study that suggested adenosine may have an excitatory action, and was implicated in SO spasm associated with SO dysfunction (Pap and Forro 1998b). Further experiments were required to determine which P1 receptors are responsible for mediating the adenosine-induced response, and are presented in **Chapter 7** of this thesis.

3.4.3 Spontaneous duodenal motility and ATP

These initial investigations using possum duodenal muscle strips found that exogenous ATP induced a complex tri-phasic response, consisting of an

immediate, very short-lasting relaxation followed by a recovery to baseline activity or an increase in contractile activity, and concluded with an inhibition of contractile activity. It is interesting to note that the duodenal response to exogenous ATP is quite different to that observed in studies with SO muscle rings, and infers that the SO may not have the same receptor sub-types as the duodenum.

Other reports have shown a bi-phasic response to ATP (Burnstock *et al.* 1972a; Maggi *et al.* 1984d; Serio *et al.* 1990b), however in many cases the ATP was only applied to the tissue for 20-30s therefore potentially limiting the possibility of observing a late inhibitory component. In the possum duodenum the early relaxation was TTX-insensitive, which concurs with other investigations (Maggi *et al.* 1984c; Manzini *et al.* 1985b).

The second phase of the ATP response was inconsistent, consisting of a recovery to baseline activity or an excitation of motor activity. Similar to the guinea-pig taenia coli (Burnstock *et al.* 1975), studies in the rat duodenum found that this response was indomethacin sensitive and therefore due to the production of prostaglandins (Maggi *et al.* 1984b). In the present study the inconsistent nature of the response, probably due to competition between the putative prostaglandin-induced excitation and the ATP-induced late inhibition, precluded further investigation.

The late inhibitory component of the ATP response has not previously been reported. This inhibition may be in part due to the metabolism of ATP to adenosine with the activation of P1 receptors. The kinetics of the ATP-

induced late inhibition was similar to that of the exogenous adenosine response, and like the adenosine response it also was TTX-insensitive. Alternatively this late inhibition of activity may be due to ATP interacting with multiple P2 receptor sub-types. Further experiments were required to ascertain the mediator of the early excitatory and late inhibitory components of the ATP-induced response, and these are described in **Chapter 4** of this thesis.

3.4.4 Spontaneous duodenal motility and adenosine

These initial investigation with duodenal muscle strips found that exogenous adenosine acts to reduce longitudinal muscle motor activity.

These findings support previous studies in rabbit and rat duodenum where exogenous adenosine also induced a decrease in contraction amplitude or basal tension (Small and Weston 1979a; Gaion *et al.* 1988a; Mule *et al.* 1989; Serio *et al.* 1990a). Interestingly, the adenosine-induced reduction in duodenal motor activity was different to that observed with the SO muscle rings, where no change in basal tension was observed. Similar to the actions of adenosine on SO motility, the adenosine-induced decrease in duodenal motility was also TTX-insensitive. Other investigators have also found the adenosine-induced duodenal relaxation to be TTX-insensitive (Mule *et al.* 1989; Serio *et al.* 1990d). Further investigations were required to determine the P1 receptors involved in mediating the adenosine-induced response in the duodenum and are described in **Chapter 6** of this thesis.

3.4.5 SO regional selectivity of purine responses

The observation that the responses to exogenous ATP and adenosine were more pronounced in the proximal-SO suggests that purines may play a significant role in modulating the propagation of SO contractions and hence SO function i.e. trans-sphincteric flow. The proximal region of the SO is believed to contain a pacemaker, which controls the frequency of propagation of the peristaltic like contractions displayed by the intact SO. The nature of the pacemaker however is unclear. Recently interstitial cells of Cajal (ICC) were localized in guinea-pig SO (Parr *et al.* 2003), but the role of ICC in the regulation of SO motility remains ill-defined. The regional selectivity demonstrated here could also be explained by a gradient of purinergic receptors. In the SO, a gradient was observed with substance P receptors and nitric oxide synthase immunoreactivity (Von Schrenck *et al.* 1991; Simula *et al.* 2001c). The existence of a receptor gradient would be consistent with the preferential expression of the purine-induced changes in motility exhibited by the proximal-SO, but the distribution of purine receptors in the SO is unknown (see **Section 3.4.6**). Furthermore a number of studies investigating the SO response to a variety of bioactive agents have found regional differences (reviewed by Woods *et al.* 2003a; Woods *et al.* 2003c). This possum data provides further evidence that regions of the SO can respond independently to certain stimuli. The experiments described in the following chapters present data for the proximal-SO only.

3.4.6 Localisation of purinergic receptors

We found that the late inhibitory response to ATP and the adenosine-induced response were both TTX-insensitive. These findings suggest that purinergic

receptors are located on the smooth muscle and/or ICC, but nerves are not involved. TTX-insensitive nerves have been described in the small intestine and characterized as AH neurons (Galligan and North 2004). However the guinea-pig SO has very few AH neurons (Wells and Mawe 1993). Assuming the possum SO has few AH neurons, this supports non-neural mechanisms underlying the ATP/adenosine-induced responses in question. This highlights the difference between SO and other gastrointestinal tissues and illustrates that mechanisms characterized in the intestines may not be relevant to SO motility. Only the ATP excitatory response was TTX-sensitive, and this is consistent with the relevant P2 receptors being located on nerves.

Immunohistochemistry can provide information concerning receptor sub-type presence and their location in tissues. During the course of my PhD candidature immunohistochemical studies have localized purinergic receptor sub-types on nerves and smooth muscle in several gastrointestinal tissues (for example, Christofi *et al.* 2001d; Castelucci *et al.* 2002; Nurgali *et al.* 2003; Poole *et al.* 2002a). The possibility that purine receptors are present on ICC has only recently been considered with some P2X receptor proteins localised on ICC of guinea-pig and mouse ileum (Burnstock and Lavin 2002d). This discovery has led to the speculation that the release of ATP from enteric nerves, glial cells or contracting smooth muscle may provide a feedback mechanism for pacemaker activity in the intestine (Burnstock and Lavin 2002c), and a similar mechanism may exist for adenosine and adenosine receptors. The minor decrease in contraction frequency observed with exogenous adenosine/ATP in possum duodenum may be consistent with activation of purinergic receptors on ICC. I attempted to define the

distribution of ICC and adenosine receptor sub-types in possum duodenum and SO, but this was unsuccessful due to a lack of suitable antibodies (see **Appendix 1**).

In summary, attempts to localise specific purine receptor sub-types in the possum SO and duodenum were unsuccessful. The receptors mediating the TTX-insensitive responses to ATP and adenosine in the SO are likely to be on smooth muscle and/or ICC. No firm conclusions can be drawn with regard to the location of the P1 or P2 receptors mediating the responses in the duodenum, other than to say these were TTX-insensitive which usually infers the receptors are located on smooth muscle and/or ICC, but does not rule out the possibility of receptors being located on nerve terminals.

3.5 Conclusions

Exogenous adenosine and ATP modify possum spontaneous SO and duodenal motility. Importantly, the adenosine and ATP-induced responses in the SO predominated in the proximal-SO, with little response observed in the distal-SO, thereby providing further evidence supporting the hypothesis that the SO is composed of different functional components that can respond differently to certain stimuli. As different responses were observed between the SO and duodenum, especially in regard to exogenous ATP, it is unwise to assume that the mechanisms underlying the regulation of SO motility are similar to those characterized in the small intestine.

These studies raised a number of questions that required further investigation, and these are addressed in the subsequent chapters of this thesis.

1. What receptors mediate the responses to adenosine and ATP in each of the SO regions and the duodenum?
2. Are the adenosine and ATP-induced responses in the SO and duodenum mediated by the same or different receptor sub-types?
3. Are the complex responses induced by ATP in the SO and duodenum due to the activation of different P2 receptor sub-types and/or due to metabolism of ATP to adenosine and activation of P1 receptors?
4. What are the physiological consequences of the distal-SO and proximal-SO responding differently to adenosine and ATP?

Chapter 4

4 PHARMACOLOGICAL CHARACTERISATION OF P2 RECEPTORS MEDIATING THE ATP-INDUCED TRI-PHASIC RESPONSE IN SPONTANEOUS DUODENAL MOTOR ACTIVITY *IN VITRO*

4.1 Introduction

In the possum exogenous ATP was found to have a TTX-insensitive tri-phasic response consisting of an immediate relaxation, followed by a recovery to baseline activity or an increased contractile activity, and concluded with a reduction in contractile activity (**Chapter 3**). The receptors mediating the ATP-induced relaxation in other species are not well defined. In separate studies the relaxation in rat duodenum is reported to be sensitive to reactive blue 2, suramin, and PPADS (Manzini *et al.* 1985a; Johnson and Hourani 1994c; Windscheif *et al.* 1995b). Agonist potency curves suggested that $P2Y_1$ receptors were involved (Johnson and Hourani 1994d), but another study was unable to identify a particular receptor sub-type responsible (Serio *et al.* 1990e). However, a recent study using murine precontracted duodenal muscle strips suggested the ATP-induced relaxation is mediated via $P2Y_1$ and perhaps $P2Y_2$ receptors (Giaroni *et al.* 2002).

Unlike for the P1 receptor sub-types, good pharmacological reagents have not been developed for characterisation of P2 receptors. When the experiments described here were performed there were no available agonists or antagonists with suitable selectivity to discriminate between P2X and P2Y receptors, or to specific receptor sub-types. However, the activity of certain

P2 agonists or antagonists is often used to imply actions at particular receptor classes or sub-types (**Section 1.4.2**; **Tables 1.4, 1.5 and 2.1**).

The aims of the experiments in this chapter were to:

1. Identify the P2 receptor sub-classes involved in mediating the tri-phasic response to exogenous ATP in spontaneously active possum duodenal muscle strips.
2. Determine if the late inhibitory component of the ATP-induced response was due to activation of different P2 receptors or due to the metabolism of ATP to adenosine with activation of P1 receptors.

These data provided basic information regarding appropriate agonist and antagonist concentrations to be used in subsequent studies with SO muscle rings.

4.2 Methods

Possums were anesthetised and a segment of duodenum, between the pylorus and SO, was removed prior to euthanasia (see **Section 2.1**). Eight full thickness muscle strips were prepared from the duodenum of each animal (**Section 2.2.2**).

Organ bath pharmacology studies were then undertaken using the protocols described in **Section 2.2.5**. Briefly, a paired protocol was used to assess if the magnitude of the ATP-induced response changed in the presence of an antagonist. To further identify the P2 receptors present a number of degradable and non-degradable P2 agonists were also used. To determine if ATP was being metabolized to adenosine the paired protocol used the ectoATPase inhibitor ARL67156.

Refer to **Section 2.2** for drug preparation and analysis of recordings. The results from **Chapter 3** demonstrated that the second component of the ATP response was quite variable and therefore was not analysed in these experiments. Furthermore the minor changes in contraction frequency observed in the third component of the ATP-induced response was investigated with the antagonists and agonists below but where there was no significant change the data is not presented.

4.3 Results

4.3.1 Non-selective P2 receptor antagonists

Pre-treatment with PPADS alone (n=66 muscle strips, from 7 possums) had no effect on spontaneous duodenal contractile activity or basal tension (P>0.05) (**Table 4.1**). PPADS pre-treatment did not significantly inhibit the ATP-induced immediate or late responses (P>0.05) (**Figure 4.1**).

Pre-treatment with suramin alone (n=39 muscle strips, from 6 possums) did not significantly influence spontaneous duodenal contractile activity (P>0.05) (**Table 4.1**). Suramin pre-treatment did not significantly inhibit the ATP-induced immediate or late responses (P>0.05; **Figure 4.2**).

4.3.2 P2 receptor agonists

As neither of the P2 antagonists affected the early relaxation or the late inhibitory components of the ATP-induced response, a number of P2 agonists were tested to determine if they could mimic all or part of the ATP-induced response, and to suggest which receptor sub-types may be involved.

Application of the non-degradable P2X agonist αβmeATP induced an early inhibitory response followed by a rebound excitatory response (**Figure 4.3A**). The early inhibitory response consisted of a decrease in contraction amplitude ($P<0.05$), but no change in basal tension (n=4-6 muscle strips/concentration, from 6 possums) (**Figure 4.3B**). Unlike the ATP-induced response, no late inhibition of contractile activity was evident (**Figure 4.3C**).

Similar to ATP, application of the non-degradable P2 agonist ATPγS induced a tri-phasic response consisting of an early inhibitory component, followed by a recovery to baseline, then a late inhibitory component (**Figure 4.4A**). The early inhibitory response consisted of a decrease in contraction amplitude, however due to variability in the magnitude of this response between strips it did not reach statistical significance ($P>0.05$) (n=4-6 muscle strips/concentration, from 6 possums) (**Figure 4.4B**). This was followed by a short-lasting recovery, before a concentration-dependent late inhibitory component (contraction amplitude $P<0.05$) (**Figure 4.4C**).

The metabolisable P2 agonist 2meSATP induced an early inhibitory response (**Figure 4.5A**) consisting of a concentration-dependent decrease in contraction amplitude ($P<0.05$) (n=5 muscle strips/concentration, from 5 possums) (**Figure 4.5B**). 2meSATP did not induce the rebound excitatory or late inhibitory components characteristic of the ATP-induced response (**Figure 4.5C**).

Application of the P2Y agonist UTP induced an early inhibitory response followed by a rebound excitatory response, but no late inhibitory response was observed (**Figure 4.6A**). The early inhibitory response consisted of a concentration-dependent decrease in contraction amplitude ($P<0.05$) (n=5 muscle strips/concentration, from 5 possums) (**Figure 4.6B**). Like the ATP-induced response, this was followed by a rebound excitatory component, but no late inhibitory component (area under curve $P>0.05$; **Figure 4.6C**).

4.3.3 Preliminary experiments with Ecto-ATPase inhibitor: ARL67156

It is well known that ATP is metabolized to adenosine via endogenous ectonucleases. To determine if ATP metabolism to adenosine and subsequent activation of P1 receptors could be responsible for the ATP-induced late inhibitory component, preliminary experiments were performed using the ecto-ATPase inhibitor ARL67156.

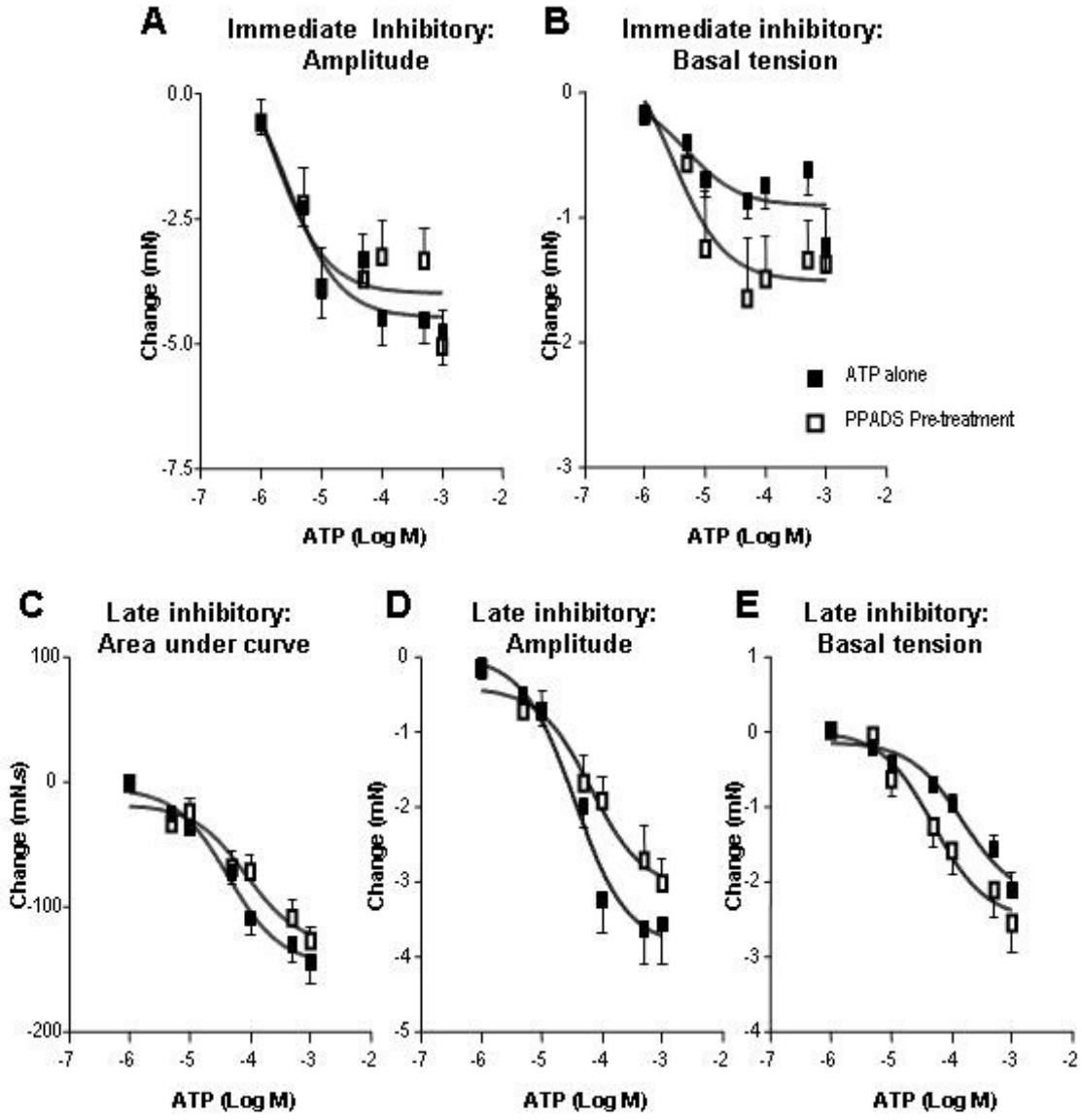
Initially ATP-induced concentration-response curves before and after ARL67156 treatment was attempted, but due to considerably variable responses between strips this approach was abandoned. Therefore only the data generated with the highest concentration of ATP used was analysed. Addition of the ecto-ATPase inhibitor ARL67156 (30μM) had no obvious effect on the ATP-induced early inhibition, but variable effects on the late inhibitory component of the response were observed. Due to the variable nature of the response and the expense of ARL67156 no further experiments were performed.

Table 4.1: Effect of purinergic P2 antagonist treatment on spontaneous duodenal activity.

Antagonist	Area Under Curve	Contraction Amplitude	Basal Tension	Contraction Frequency
PPADS (10μM, n=66)	101.0 ±1.0%	100.3±1.0%	97.0 ±1.0%	101.6 ±1.0%
Suramin (100μM, n=39)	101.3±1.1%	102.7±1.1%	95.0±2.1%	101.5±1.1%

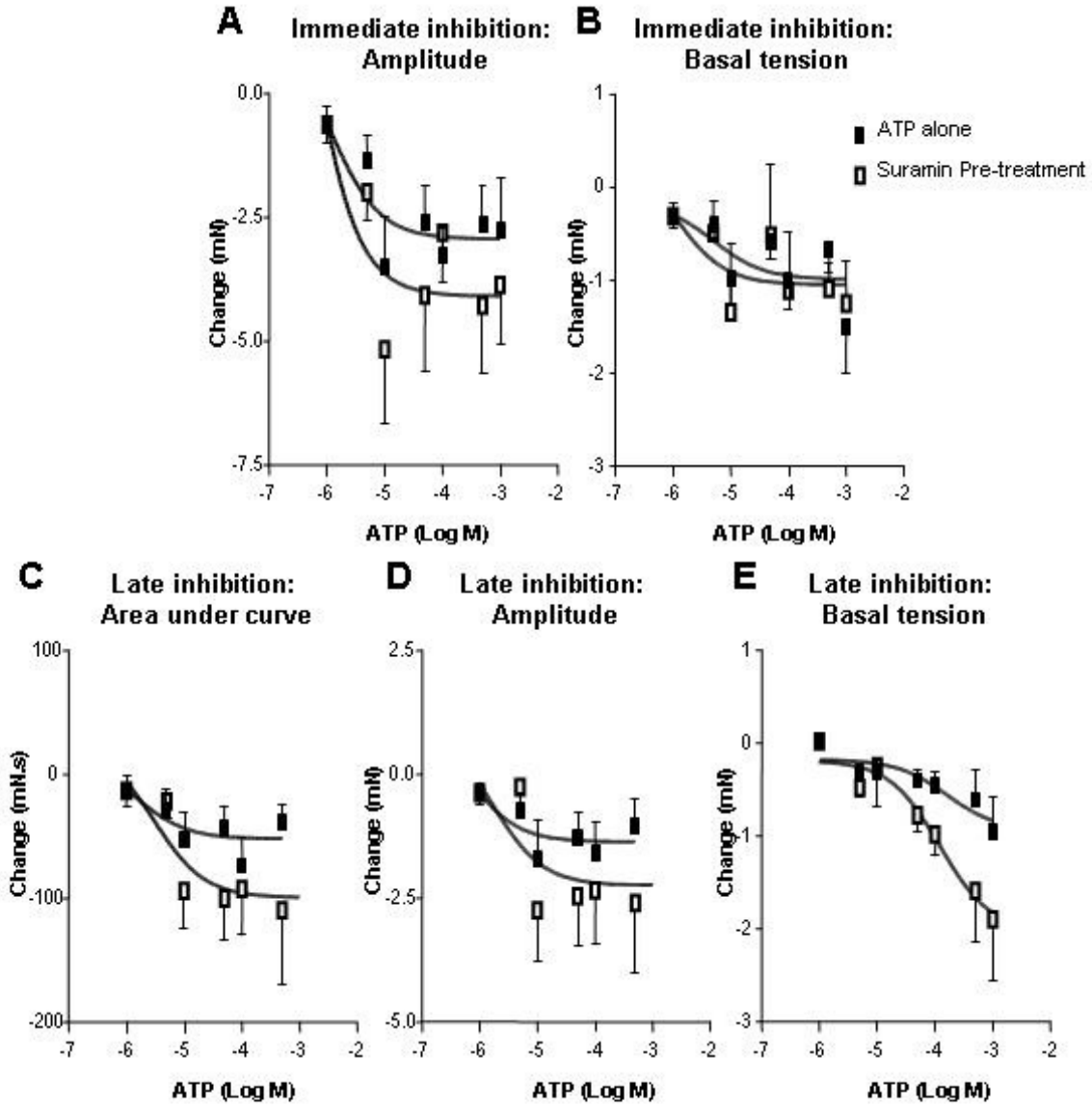
Data presented as % of baseline activity.
n = number of muscle strips from 7 possums (PPADS) or 6 possums (Suramin)
P>0.05

Figure 4.1: Lack of effect of the P2 antagonist PPADS on the ATP-induced changes in spontaneous duodenal contractile activity



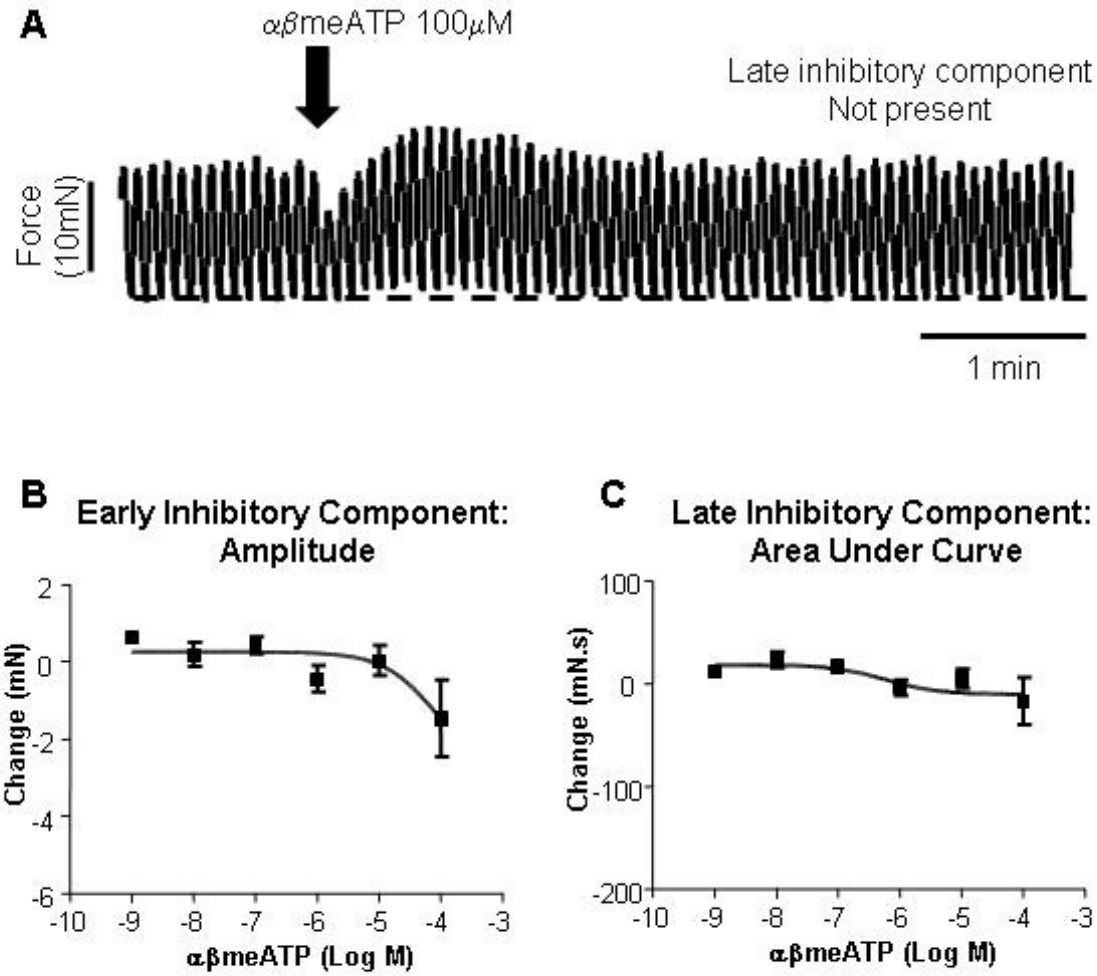
Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment of longitudinal duodenal muscle strips with the P2 antagonist PPADS (10μM) on the ATP-induced immediate inhibition of contraction amplitude (**A**) and basal tension (**B**), and the ATP-induced late inhibition expressed as area under curve (**C**), contraction amplitude (**D**) and basal tension (**E**), ($P>0.05$ for all). Each point is the mean ± SEM of 6-7 muscle strips, from 7 possums.

Figure 4.2: Effect of the P2 antagonist suramin on the ATP-induced changes in spontaneous duodenal contractile activity



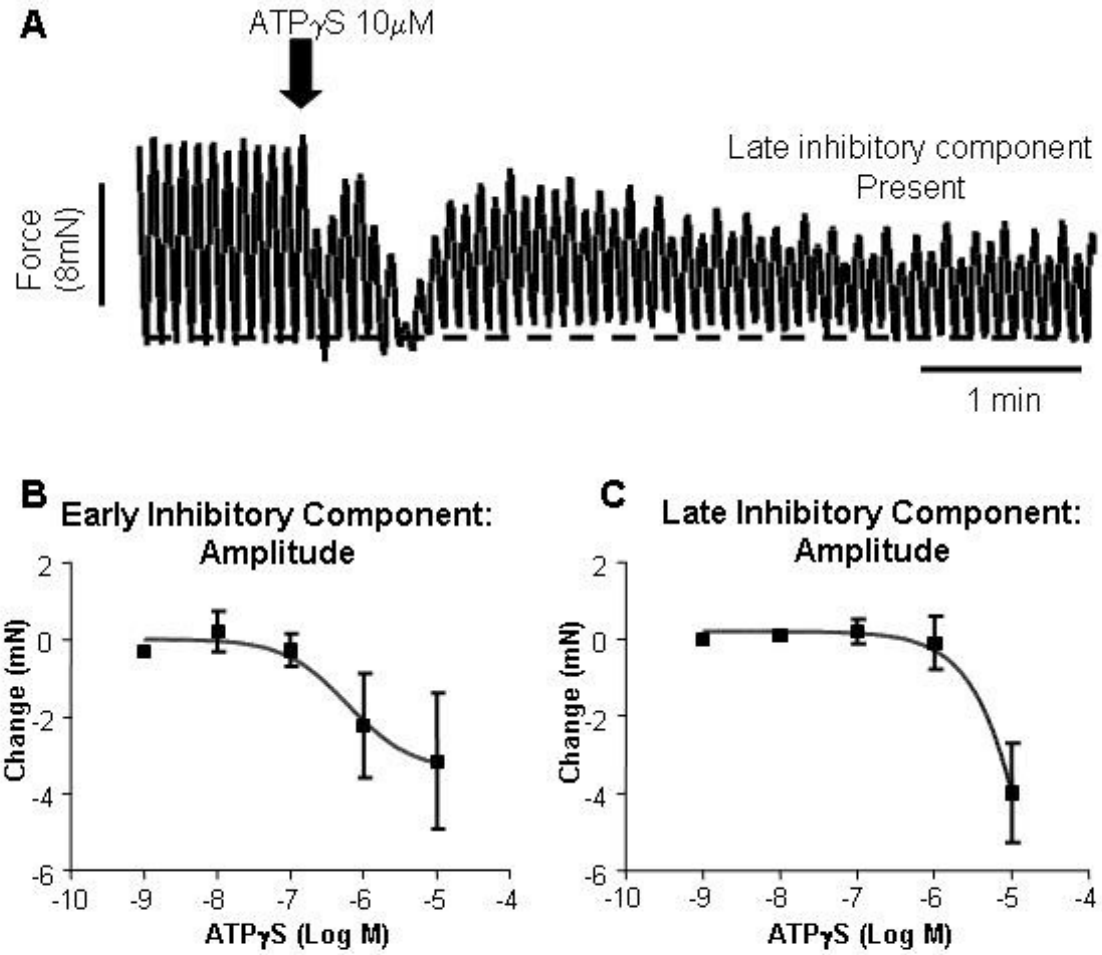
Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment of longitudinal duodenal muscle strips with the P2 antagonist suramin (100μM) on the ATP-induced immediate inhibtion of contraction amplitude (**A**) and basal tension (**B**), and the ATP-induced late inhibition expressed as area under curve (**C**), contraction amplitude (**D**) and basal tension (**E**) ($P>0.05$ for all). Note: suramin pre-treatment increased the magnitude of the late inhibitory componet in a number of strips, however because of the inconsistent nature of this effect, it was not statistically significant. Each point is the mean ± SEM of 5-6 muscle strips, from 6 possums.

Figure 4.3: Effect of the non-degradable P2X agonist αβmeATP on spontaneous duodenal contractile activity



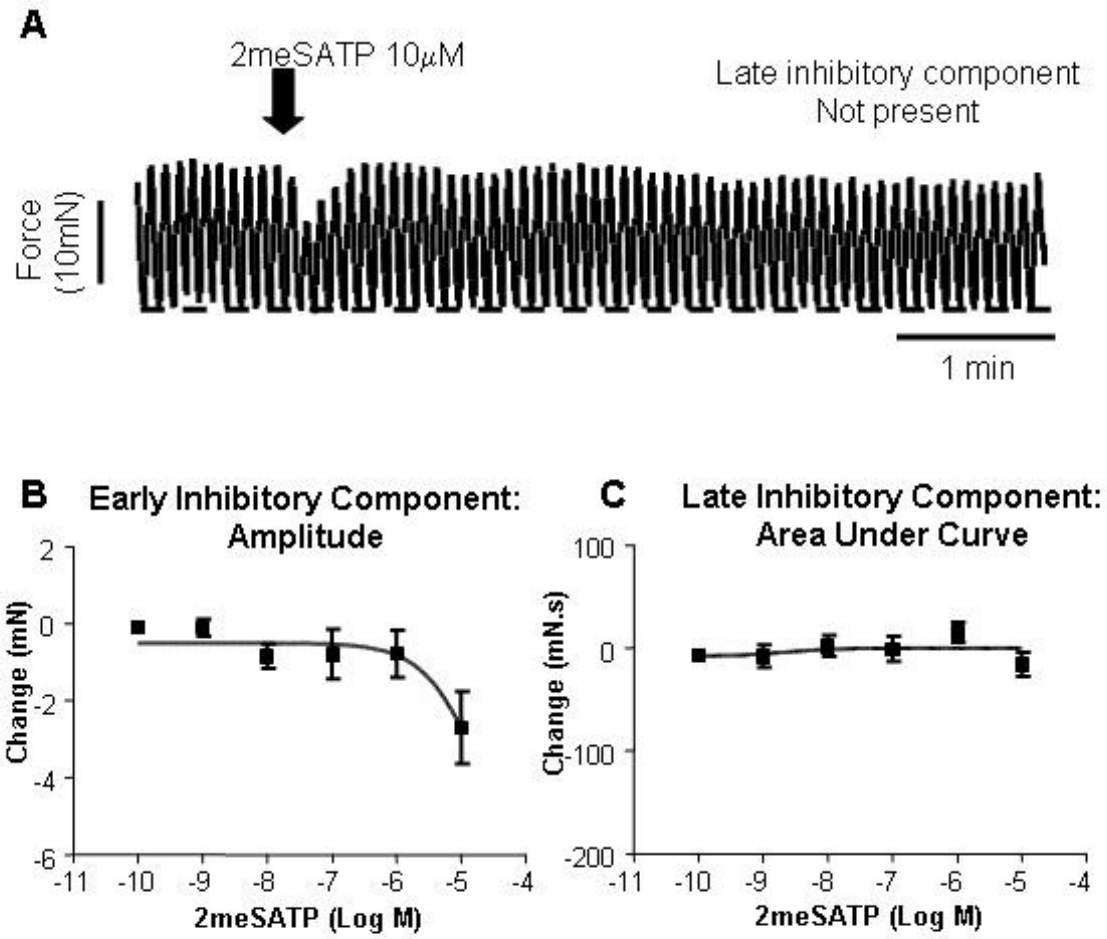
A chart recording illustrating the change in spontaneous contractile activity displayed by duodenal longitudinal muscle strips induce by αβmeATP (100μM) (**A**). The early inhibition and rebound excitatory components are present but the late inhibitory component is not present. Dashed line represents average basal tension before αβmeATP application. Arrow indicates time of αβmeATP application to the organ bath. Group data, as a change from baseline activity demonstrating the decrease in contraction amplitude during the early inhibitory component (**B**) ($P<0.05$), and the lack of a late inhibitory component expressed as area under curve (**C**) ($P>0.05$). Each point is the mean ± SEM of 4-6 muscle strips from 6 possums.

Figure 4.4: Effect of the non-degradable P2 agonist ATPγS on spontaneous duodenal contractile activity



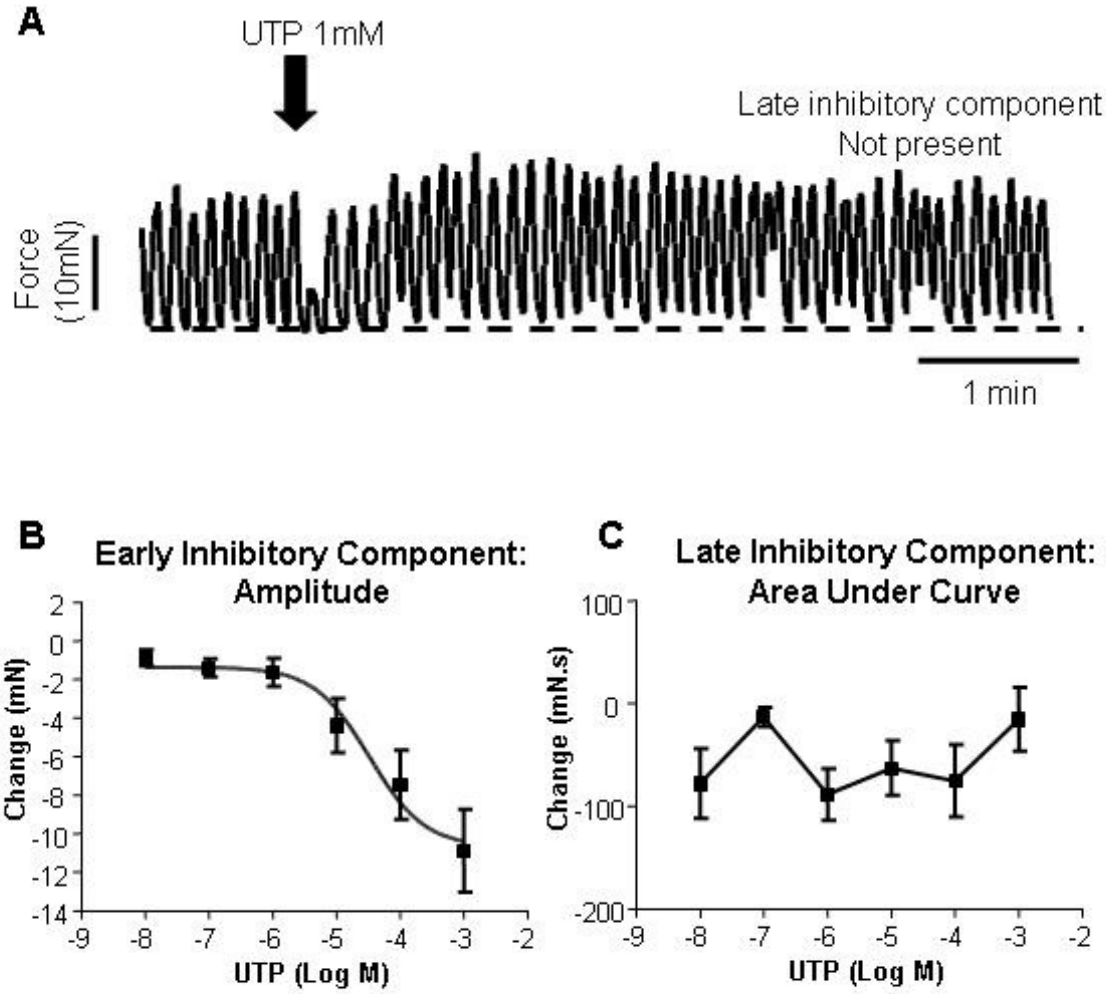
A chart recording illustrating the change in spontaneous contractile activity displayed by duodenal longitudinal muscle strips induce by ATPγS (10μM) (**A**). The early inhibition and rebound/excitatory components are present, together with the late inhibitory component. Dashed line represents average basal tension before ATPγS application. Arrow indicates time of ATPγS application to the organ bath. Note that for the early inhibitory component considerable variability in the magnitude of the response existed between muscle strips. Group data, as a change from baseline activity, demonstrating a trend to decreased contraction amplitude during the early inhibitory component (**B**) ($P>0.05$), and the concentration-dependent decrease in contraction amplitude during the late inhibitory component (**C**) ($P<0.05$). Each point is the mean ± SEM of 4-6 muscle strips from 6 possums.

Figure 4.5: Effect of the P2 agonist 2meSATP on spontaneous duodenal contractile activity



A chart recording illustrating the change in spontaneous contractile activity displayed by duodenal longitudinal muscle strips induce by 2meSATP (10μM) (**A**). The early inhibition component is present, but the rebound excitatory and the late inhibitory components are not present. Dashed line represents average basal tension before 2meSATP application. Arrow indicates time of 2meSATP application to the organ bath. Group data, as a change from baseline activity demonstrating the concentration-dependent decrease in contraction amplitude during the early inhibitory component (**B**) ($P<0.05$), and the lack of a late inhibitory component expressed as area under curve (**C**) ($P>0.05$). Each point is the mean ± SEM of 5 muscle strips from 5 possums.

Figure 4.6: Effect of the P2Y agonist UTP on spontaneous duodenal contractile activity



A chart recording illustrating the change in spontaneous contractile activity displayed by duodenal longitudinal muscle strips induce by UTP (1mM) (**A**). The early inhibitory and the rebound excitatory components are present, but the late inhibitory component is not present. Dashed line represents average basal tension before UTP application. Arrow indicates time of UTP application to the organ bath. Group data, as a change from baseline activity demonstrating the concentration-dependent decrease in contraction amplitude during the early inhibitory component (**B**) ($P<0.05$), and the lack of a late inhibitory component expressed as area under curve (**C**) ($P>0.05$). Each point is the mean ± SEM of 5 muscle strips from 5 possums.

Table 4.2: Summary of effects of P2 agonists and antagonists on spontaneous duodenal activity.

Agonist or Antagonist	Drug Characteristics and Receptor Selectivity	Early Inhibition	Rebound Excitation	Late Inhibition
ATP	P2X and P2Y	✓	✓	✓
PPADS	P2X and P2Y	Did not block ATP response	N/A	Did not block ATP response
Suramin	P2X and P2Y	Did not block ATP response	N/A	Did not block ATP response
αβmeATP	P2X Non-metabolisable	✓	✓	x
ATPγS	P2X and P2Y Non-metabolisable	✓ but variable	x	✓
2meSATP	P2X and P2Y Metabolisable	✓	x	x
UTP	P2Y Metabolisable	✓	✓	x
ARL67156	Ecto-ATPase Inhibitor	No effect	No effect	Reduced magnitude but variable

✓ Response evident
X Response not evident
N/A Not assessed

4.4 Discussion

The experiments described in this chapter aimed to define the P2 receptors involved in mediating the ATP-induced tri-phasic response exhibited by spontaneously contracting longitudinal duodenal smooth muscle strips. The results are summarized in **Table 4.2**. Note: agonist and antagonist receptor sub-type selectivity was presented in **Table 2.1**.

Due to the lack of universal or selective P2X or P2Y antagonists it is difficult to determine which receptors are involved in mediating the tri-phasic response induced by ATP. In rat duodenum the initial inhibition was shown to be suramin-sensitive (Johnson and Hourani 1994e), reactive blue 2-sensitive (Manzini *et al.* 1985e; Serio *et al.* 1990f), and PPADS-sensitive (Windscheif *et al.* 1995a) but TTX-insensitive (Maggi *et al.* 1984a ;Serio *et al.* 1990g). In possum duodenal muscle strips the ATP-induced tri-phasic response was also found to be TTX-insensitive (**Chapter 3**). But in contrast to the rat duodenal data the initial relaxation and the late inhibition were not significantly altered in the presence of PPADS or suramin. A likely explanation for this difference may be the experimental preparation. The studies described here used spontaneously contracting duodenal muscle strips and decreases in amplitude predominated, whereas the rat studies used pre-contracted muscle strips to specifically measure changes in basal tension. Perhaps higher concentrations of suramin (1mM) or PPADS (30-100μM) may have been effective in the possum duodenum studies described here, but were not undertaken because of concerns of likely non-specific actions of these antagonists (reported to occur at 300μM suramin and concentrations greater than 10μM PPADS; see Ralevic and Burnstock

1998n). It should be noted that interpretation of data generated using some P2 antagonists can be complicated due to interactions with ectonucleotidase activity, which in turn can modify the observed concentration response curves (**Section 1.4.2.5**).

Due to the lack of effect of these P2 antagonists, a number of P2 agonists were tested on spontaneously contracting possum duodenal muscle strips. An EC_{50} value for each agonist could not be determined, as the responses had not begun to plateau within the concentration ranges tested. Therefore agonist potency curves could not be generated and analysed to suggest the specific receptor sub-types responsible. However, the ATP-induced immediate inhibitory component was observed after application of $\alpha\beta$meATP, ATPγS, 2meSATP and UTP. This data suggests that both P2X and P2Y receptors mediate the ATP-induced immediate inhibitory component observed in the possum duodenal muscle strips (refer to **Tables 2.1, 1.5 and 1.6**). A response elicited by $\alpha\beta$meATP infers activity at $P2X_1$ and/or $P2X_3$ receptor sub-units, which are also activated by 2meSATP and ATPγS (note that $P2X_3$ subunits are located on sensory neurons and not smooth muscle and therefore would not be involved here, refer to **Section 1.4.1.2.1**), whereas a response induced by UTP infers activity at $P2Y_2$ and/or $P2Y_4$ sub-types. The likely presence of a number of P2X and P2Y receptor sub-types could account for the disparate reports in the literature relating to the identity of the P2 receptors mediating the duodenal relaxation (Serio *et al.* 1990h; Hourani *et al.* 1991; Johnson and Hourani 1994a; Giaroni *et al.* 2002).

The late inhibitory component of the ATP-induced response described here with possum duodenal muscle strips has not previously been reported. Experiments with the non-hydrolysable P2 agonist ATPγS clearly demonstrated a late inhibitory component, which suggests ATP is acting on P2 receptors. However these P2 receptors are not activated by αβmeATP, therefore they are not likely to be P2X receptors. This late inhibitory component was also not mimicked by UTP or 2meSATP suggesting $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, or $P2Y_{13}$ could be involved. The $P2Y_1$ receptor sub-type has been reported to mediate inhibitory responses in other GI smooth muscle (Giaroni *et al.* 2002; De Man *et al.* 2003a) and may be responsible for the ATP-induced late inhibitory component observed in possum duodenal muscle strips.

Alternatively the ATP-induced late inhibitory component may be due in part to the metabolism of ATP to adenosine with the activation of P1 receptors. The ATP-induced late inhibitory component displayed similar kinetics to the response induced by exogenous adenosine, and like the adenosine response it also was TTX-insensitive (**Chapter 3**). In preliminary experiments inhibition of ecto-ATPase activity, thereby preventing the metabolism of ATP to adenosine, had variable effects. Interestingly in one of three preparations the ATP-induced late inhibitory component was blocked, and this is consistent with ATP metabolism to adenosine with subsequent activation of P1 receptors in some strips. Further experiments involving application of ATP to duodenal muscle strips after pre-treatment with a P1 antagonist were not attempted, as none of the P1 antagonists previously tested (specifically DPSPX and CSC; see **Section 6.3**) were capable of inhibiting all the effects

of adenosine. However the possibility that ATP metabolism to adenosine with the resultant activation of P1 receptors (A_{2A} and A_3 receptors, see **Chapter 6**) should not be completely dismissed.

4.5 Conclusion

In conclusion, ATP induces a tri-phasic response in spontaneously contracting duodenal muscle strip. The immediate inhibitory component likely involves $P2X_1$, and $P2Y_2$ and/or $P2Y_4$ receptors. The rebound excitatory component, when present, is probably mediated by ATP-induced prostaglandin release as demonstrated in guinea-pig and rat, but was not investigated here. The late inhibitory component is likely to be mediated by $P2Y_1$ receptors, but other P2Y receptors may be involved. Furthermore the possible metabolism of ATP to adenosine with activation of P1 receptors is possible and may also be partially responsible for the ATP-induced late inhibitory component.

Chapter 5

5 PHARMACOLOGICAL CHARACTERISATION OF P2 RECEPTORS MEDIATING THE ATP-INDUCED BI-PHASIC RESPONSE IN SO MOTILITY *IN VITRO*

5.1 Introduction

The data presented in **Chapter 3** showed that SO muscle rings exposed to exogenous ATP displayed a bi-phasic response, consisting of a neural excitatory component followed by a TTX-insensitive inhibitory component. This response predominated in proximal-SO muscle rings, with only minor effects observed with the distal-SO muscle rings. ATP-induced SO contractions have been reported to be PPADS-sensitive (Patacchini *et al.* 1998d). Whereas ATP-induced inhibitory junction potentials were inhibited by desensitisation with $\alpha\beta$meATP (Imoto *et al.* 1998b), implying the involvement of $P2X_1$ and/or $P2X_3$ receptors. The specific P2 receptors involved in mediating the ATP-induced response in SO have not been clearly established.

The aims of the experiments described in this chapter were to:

1. Define the P2 receptor sub-classes responsible for mediating the ATP-induced response exhibited by possum proximal-SO muscle rings.
2. Determine if the late inhibitory component of this response is due to activation of P2 receptors or due to metabolism of ATP to adenosine and activation of P1 receptors.

3. Determine if the same receptor sub-types that mediate the proximal-SO muscle ring response to exogenous ATP mediate the duodenal muscle strip response to exogenous ATP.

5.2 Methods

Possums were anaesthetised and the SO and adjacent duodenum was removed prior to euthanasia (**Section 2.1**). Four full thickness muscle rings were prepared from the SO of each animal, and separated into distal-SO and proximal-SO groups (**Section 2.2.3**).

Organ bath pharmacology studies were then undertaken using the protocols described in **Section 2.2.5**. Briefly, a paired protocol was used to assess if the magnitude of the ATP-induced response was altered by pre-treatment with a P2 antagonist. To identify the P2 receptor sub-type present, a single concentration of either degradable or non-degradable P2 agonists were also used. The concentrations of agonists and antagonists used were based on those that were effective with possum duodenal muscle strips (Chapter 4), and are similar to those used in other published gastrointestinal studies or reviews (Giaroni *et al.* 2002; Ralevic and Burnstock 1998k; Burnstock 2004; refer to **Table 2.1** for receptor sub-type specificity of P2 agonists and antagonists). To provide evidence that ATP was being metabolized to adenosine by ectonucleases the paired protocol used the ectoATPase inhibitor ARL67156.

Refer to **Section 2.2** for drug preparation and analysis of recordings.

5.3 Results

For simplicity only the data generated using agonists and antagonists with the proximal-SO muscle rings is presented, as ATP evoked little response with distal-SO muscle rings (**Chapter 3**).

5.3.1 Non-selective P2 receptor antagonists

Pre-treatment with PPADS (10μM) decreased the ATP-induced early excitatory component exhibited by proximal-SO muscle rings. ATP-induced changes in contraction amplitude ($P<0.05$) were significantly altered by pre-treatment with PPADS, whereas area under curve and contraction frequency was unaltered ($P>0.05$) (**Figure 5.1**) (n=4-7 muscle rings/concentration, from 18 possums). PPADS pre-treatment antagonised the ATP-induced late inhibitory component when expressed as area under curve ($P<0.05$), but had no significant effect on other parameters (**Figure 5.2**).

Pre-treatment with suramin (100μM) affected the ATP-induced excitatory component exhibited by proximal-SO muscle rings. ATP-induced changes in contraction amplitude ($P<0.05$) were altered by suramin pre-treatment, but other parameters were unaltered ($P>0.05$) (**Figure 5.3**) (n=5-8 muscle rings/concentration, from 24 possums). Suramin pre-treatment also antagonised the ATP-induced late inhibitory component when expressed as area under curve ($P<0.05$) or contraction amplitude ($P<0.05$), but not contraction frequency ($P>0.05$) (**Figure 5.4**).

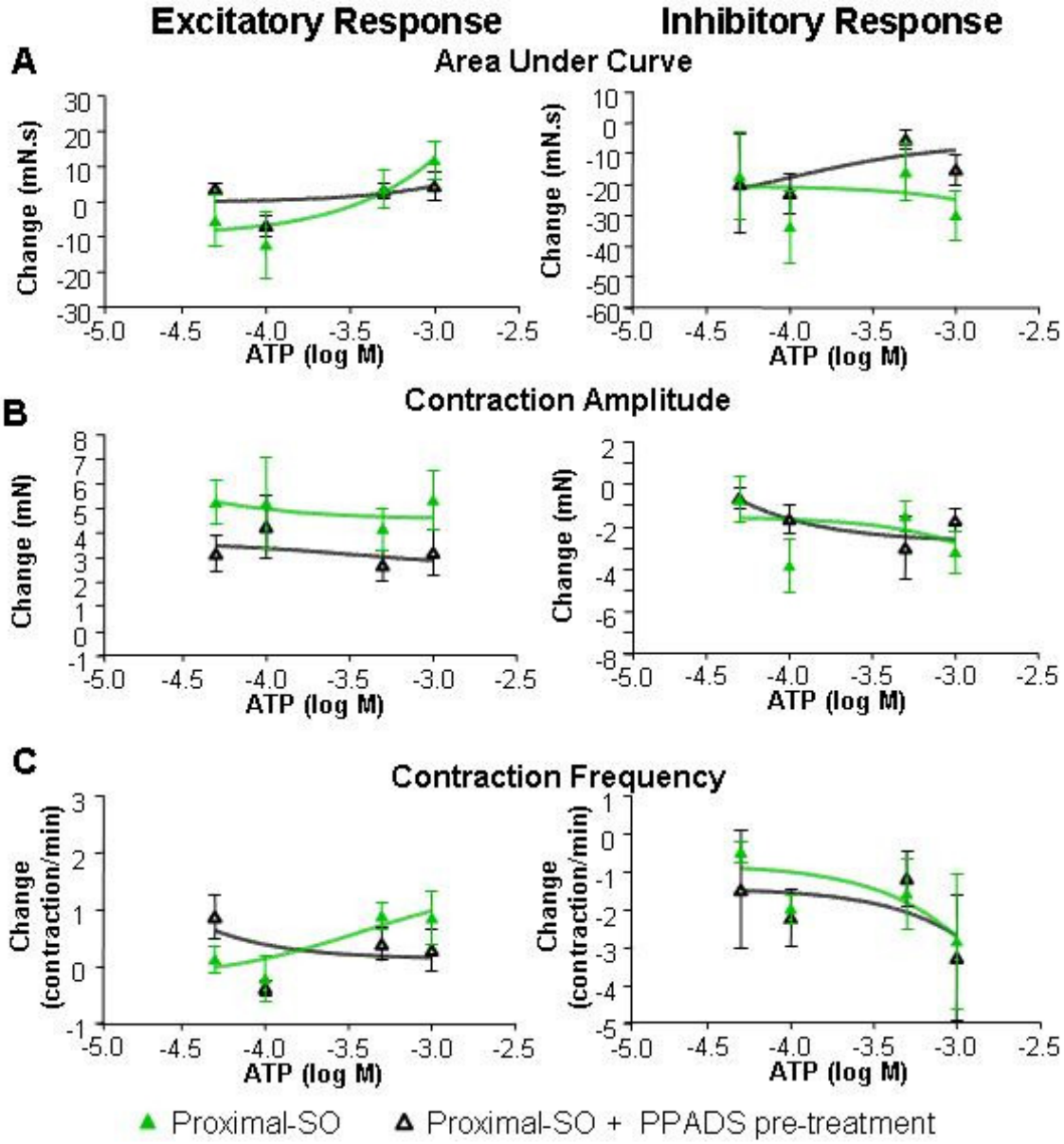
5.3.2 P2 receptor agonists

To further characterize which P2 receptors could be involved in mediating the ATP-induced biphasic response in proximal-SO, a number of P2 agonists were separately applied to proximal-SO muscle rings. Application of the non-degradable P2X agonist $\alpha\beta$meATP (100μM) induced a long lasting increase in contraction amplitude ($P<0.05$) and area under curve ($P<0.05$), but not frequency, and no inhibitory response was evident (n=7 muscle rings, from 7 possums) (**Figure 5.3**). Application of the non-degradable ATP agonist ATPγS (10μM) to proximal-SO muscle rings produced an early excitatory response in only 1/7 muscle rings, but all muscle rings displayed a late inhibitory response consisting of a decrease in contraction amplitude ($P<0.05$) and area under curve ($P<0.05$), but not frequency (**Figure 5.3**). Application of the metabolisable P2 agonist 2meSATP (10μM) to proximal-SO muscle rings did not produce an excitatory response, but a late inhibitory response was observed consisting of a decrease in contraction amplitude ($P<0.05$) and area under curve ($P<0.05$), but not contraction frequency (n=7 muscle rings from 7 possums) (**Figure 5.3**). Application of UTP (1mM) to proximal-SO muscle rings induced an inhibitory response consisting of a decrease in contraction amplitude ($P<0.05$), contraction frequency ($P<0.05$) and area under curve ($P<0.05$) (n=7 muscle rings, from 7 possums) (**Figure 5.3**). No excitatory response was evident. These data suggest that multiple P2 receptor sub-types are involved in the bi-phasic response to ATP in proximal-SO rings.

5.3.3 Preliminary experiments with Ecto-ATPase inhibitor: ARL67156

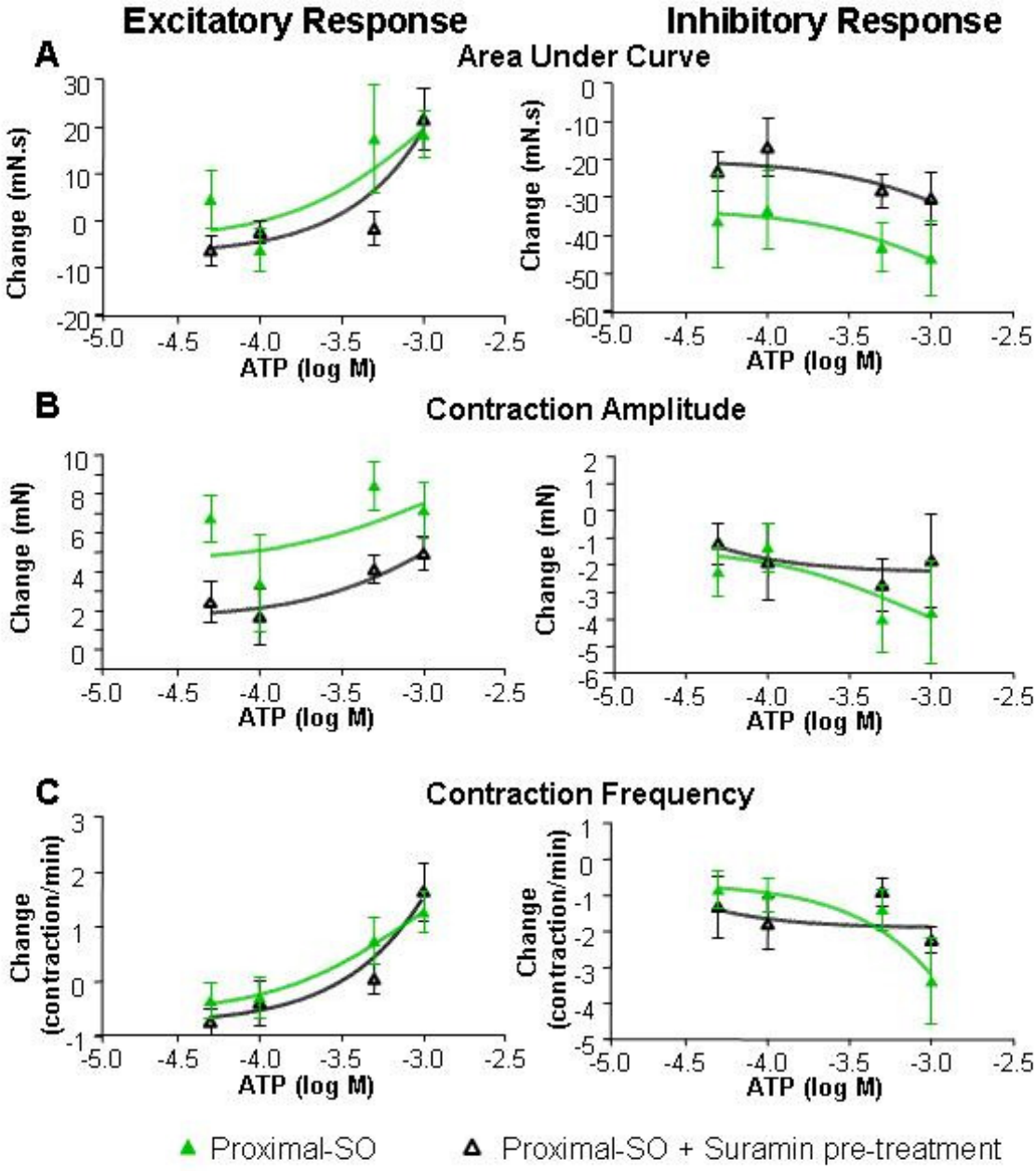
To determine if ATP metabolism to adenosine and subsequent activation of P1 receptors could be responsible for the ATP-induced late inhibitory component, preliminary experiments were performed using the ecto-ATPase inhibitor ARL67156. Pre-treatment of proximal-SO muscle rings with the ecto-ATPase inhibitor ARL67156 (30μM) produced variable effects (n=6 muscle rings, from 4 possums). The ATP-induced late inhibitory component was reduced in 2/6 proximal-SO muscle rings, but had no obvious effect in 4/6 muscle rings (**Figure 5.4**). Furthermore in 2/6 proximal-SO rings the excitatory response to ATP (1mM) was increased in magnitude after pre-treatment with ARL67156. In summary, the variable nature of the effects of pre-treatment with ARL67156 does not allow definitive conclusions to be drawn regarding possible metabolism of ATP to adenosine and activation of P1 receptors. However these preliminary data do suggest that this possibility should not be ignored. Further experiments to test the possible involvement of adenosine derived from ATP metabolism were not attempted as none of the P1 antagonists tested blocked the adenosine-induced inhibitory response (**Chapter 7**).

Figure 5.1: Effect of PPADS pre-treatment on the excitatory and inhibitory components of the ATP-induced response in proximal-SO muscle rings



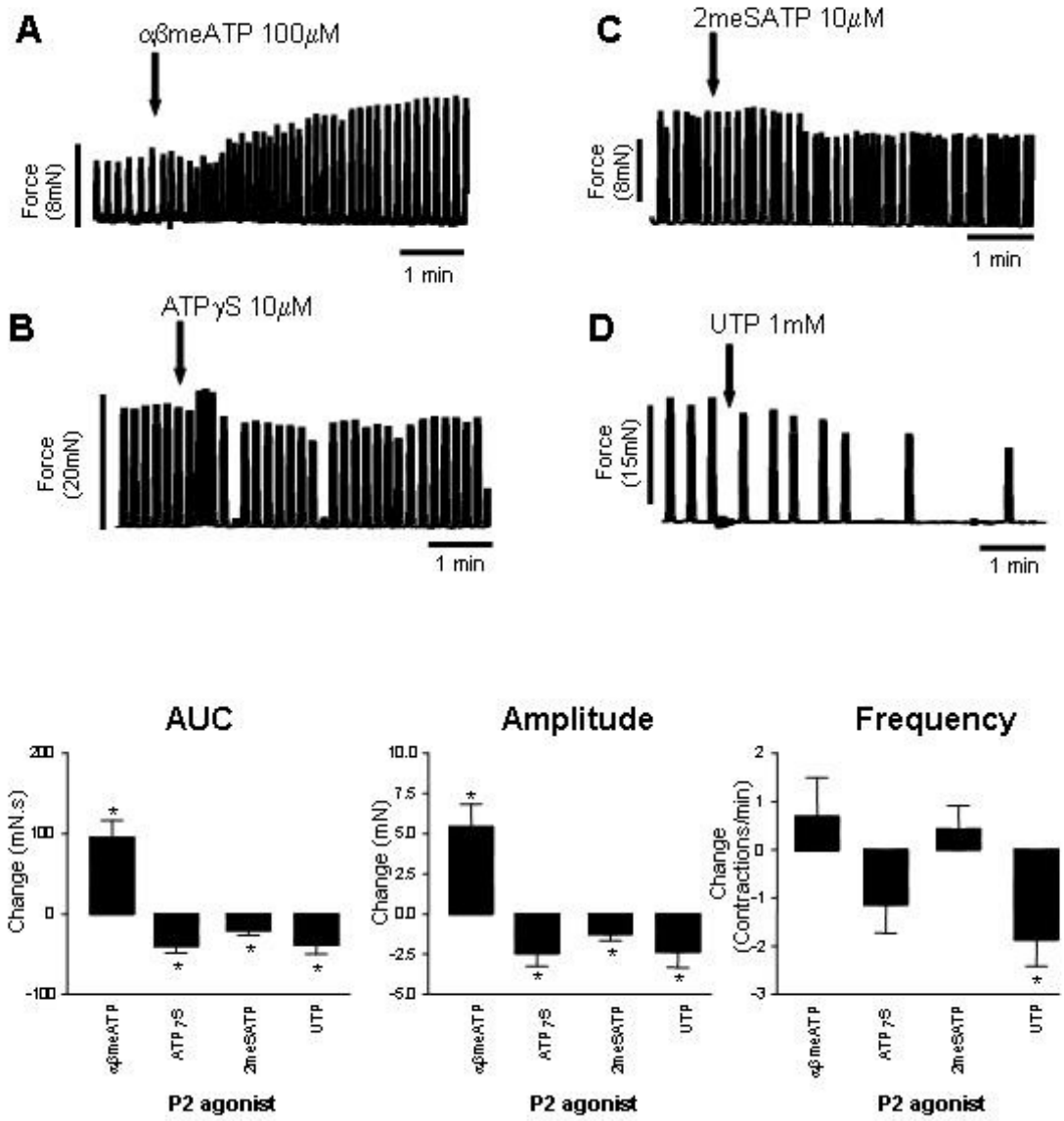
Pre-treatment of proximal-SO muscle rings with PPADS decreased the ATP-induced excitatory component. PPADS produced significant changes in contraction amplitude ($P<0.05$) (**B**), but not area under curve (**A**) or contraction frequency (**C**) (both $P>0.05$) (n=4-7muscle rings/concentration, from 18 possums). PPADS pre-treatment decreased the ATP-inhibitory component expressed as area under curve (**A**) ($P<0.05$), but had no effect on the other parameters.

Figure 5.2: Effect of suramin pre-treatment on the excitatory and inhibitory components of the ATP-induced response in proximal-SO rings



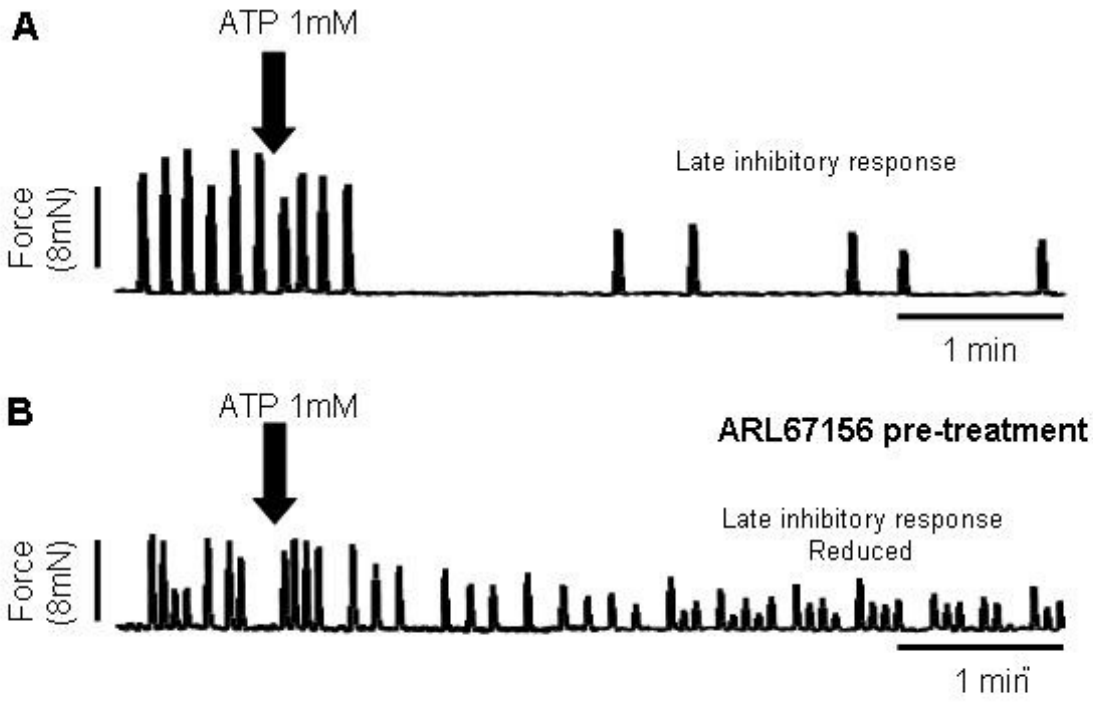
Pre-treatment of proximal-SO muscle rings with suramin significantly affected the ATP-induced excitatory component. Suramin pretreatment produced significant changes in contraction amplitude (**B**) ($P<0.05$), but not area under curve (**A**) ($P>0.05$) or contraction frequency (**D**) ($P>0.05$) (n=5-8 muscle rings/concentration, from 24 possums). Suramin pre-treatment antagonised the ATP-induced late inhibitory component for area under curve ($P<0.05$) and contraction frequency ($P<0.05$), but not contraction amplitude (**C**) ($P>0.05$).

Figure 5.3: Effect of P2 agonists on proximal-SO rings



Representative Chart recordings illustrating the proximal-SO response to the P2 agonists $\alpha\beta$meATP (100μM; **A**), ATPγS (10μM, **B**), 2meSATP (10μM, **C**), and UTP (1mM, **D**). Arrows indicate time of agonist application to the organ bath. Group data (**E**), presented as change from baseline, expressed as area under curve (left), contraction amplitude (middle) and contraction frequency (right). (For each agonist n=7 proximal-SO muscle rings, with each ring from a different possum). Data is presented as mean ± SEM. * = P<0.05, response activity compared to baseline activity (Wilcoxin signed ranks test).

Figure 5.4: Effect of pre-treatment with the ecto-ATPase inhibitor ARL67156 on the ATP-induced response on a spontaneous proximal-SO muscle ring



Representative Chart recordings demonstrating the response induced by addition of ATP (1mM) to a spontaneous active proximal-SO muscle ring (**A** and **B**) before (**A**) and after (**B**) pre-treatment with the ectoATPase inhibitor ARL67156 (30μM). Arrow indicates time of addition of ATP to organ bath. Note pre-treatment with ARL67156 had variable effects on the ATP-induced response in proximal-SO muscle rings, with most muscle rings not affected by ARL67156 pre-treatment. The response displayed by the muscle ring illustrated here after ARL67156 pre-treatment consisted of a decrease in the magnitude of the ATP-induced late inhibitory component, but had no effect on the excitatory component.

Table 5.1: Summary of effects of P2 agonists and antagonists on spontaneous proximal-SO muscle ring activity.

Agonist or Antagonist	Drug Characteristics and Receptor Selectivity	Early Excitation	Late Inhibition
ATP	P2X and P2Y	✓	✓
PPADS	P2X and P2Y	Inhibit ATP response	Inhibit ATP response
Suramin	P2X and P2Y	Inhibit ATP response	Inhibit ATP response
αßmeATP	P2X Non-metabolisable	✓	x
ATPγS	P2X and P2Y Non-metabolisable	✓ variable	✓
2meSATP	P2X and P2Y Metabolisable	x	✓
UTP	P2Y Metabolisable	x	✓
ARL67156	Ecto-ATPase Inhibitor	Increased response, but variable	Reduced response, but variable

✓ Response evident
X Response not evident

5.4 Discussion

The experiments described in this chapter aimed to characterize the P2 receptors involved in the ATP-induced bi-phasic response elicited by proximal-SO muscle rings, and to determine if the late inhibitory component of the response could be mediated by metabolism of ATP to adenosine with activation of P1 receptors. The responses induced by the P2 agonists and the effects of the P2 antagonists on the ATP-induced responses exhibited by the proximal-SO and duodenum will be compared.

The particular P2 receptor sub-types, or sub-classes that mediate the ATP-induced responses in the proximal-SO muscle rings are unclear (data is summarized in **Table 5.1**). The commonly used P2 antagonists suramin and PPADS are not universal P2 antagonists and do not distinguish between P2X and P2Y receptor sub-classes (Burnstock 2004; Lambrecht *et al.* 2002; Ralevic and Burnstock 1998o) (refer to **Section 1.4.2.4**, **Table 1.4** and **Table 1.5**). Both the early excitatory and late inhibitory components of the ATP-induced response observed with possum proximal-SO were found to be suramin and PPADS-sensitive, suggesting the involvement of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, or $P2Y_2$ receptors (**Table 2.1**). The early excitatory component, which was also TTX-sensitive, is in accord with studies in guinea-pig SO which also reported that exogenous ATP evoked a PPADS-sensitive contraction (Patacchini *et al.* 1998c). Further investigations found EFS induced contractions were blocked by PPADS, in combination with tachykinin antagonists, suggesting that ATP is an excitatory neurotransmitter (Patacchini *et al.* 1998b). Electrophysiological experiments are required to determine if ATP is an excitatory neurotransmitter in the possum SO.

The use of a number of P2 agonists, which are degradable, non-degradable or sub-type selective can be useful to characterize the P2 receptor sub-types mediating the ATP-induced response. Ideally concentration-response curves with comparison of EC_{50} values should be performed to determine activation of particular receptor sub-types. This approach is not feasible for the SO muscle ring preparations described here due to the relatively short duration of spontaneous activity displayed by the SO muscle rings. However the P2 agonist experiments that were undertaken do provide some insight to potential receptor sub-types that may be involved.

The excitatory action of the non-degradable agonist $\alpha\beta$meATP suggests that the ATP-induced early excitation likely occurs via activation of $P2X_1$ receptors. Other P2X receptor sub-types may also be involved, as ATPγS occasionally produced an early excitatory response, but 2meSATP did not (**Table 1.4, Table 2.1**). Currently the P2X receptor sub-type responsible for the ATP-induced early excitatory component is unclear.

The ATP-induced late inhibitory component is likely to occur via a number of different P2 receptor sub-types. UTP decreased contraction frequency in proximal-SO rings indicating the presence of $P2Y_2$ and/or $P2Y_4$ receptors. Furthermore the induction of an inhibitory response with ATPγS and 2meSATP implies the activation of a number of P2X and/or P2Y receptor sub-types, but the lack of an inhibitory response with $\alpha\beta$meATP makes the involvement of a P2X receptor unlikely (**Tables 1.4, 1.5, and 2.1**). In contrast to these findings, the possible involvement of P2X receptors in the inhibitory

modulation of SO motility is supported by an electrophysiological study in the rabbit which found ATP, acting at P2X receptors, was involved in the NANC inhibitory junction potential (Imoto *et al.* 1998a).

It is also possible that ATP could be metabolized by endogenous ectonucleases, and therefore the late inhibitory response may be partly due to adenosine acting at P1 receptors. In the proximal-SO muscle rings the ATP-induced late inhibitory component occurred over a similar time course to the adenosine-induced inhibition, and both responses were TTX-insensitive (**Chapter 3**). Due to the inability of any of the P1 antagonists to block the adenosine-induced response (see **Chapter 7**) it was not possible to use these antagonists in the presence of ATP to clarify this possibility. In the preliminary experiments described in this chapter pre-treatment of the proximal-SO muscle rings with an ectoATPase inhibitor produced variable responses. In some muscle rings the inhibitor did appear to partially block the ATP-induced late inhibitory component, suggesting some involvement of adenosine. In support of this possibility, studies in the cat SO (Persson 1976c) found ATP inhibited spontaneous SO activity and this effect was increased in the presence of dipyridamole (an adenosine uptake inhibitor) thereby suggesting the involvement of adenosine in this response. The preliminary data presented here suggests that ATP metabolism to adenosine with activation of P1 receptors may be partially responsible for the late inhibitory component of the ATP-induced response in possum proximal-SO muscle rings, but further experiments are required for verification.

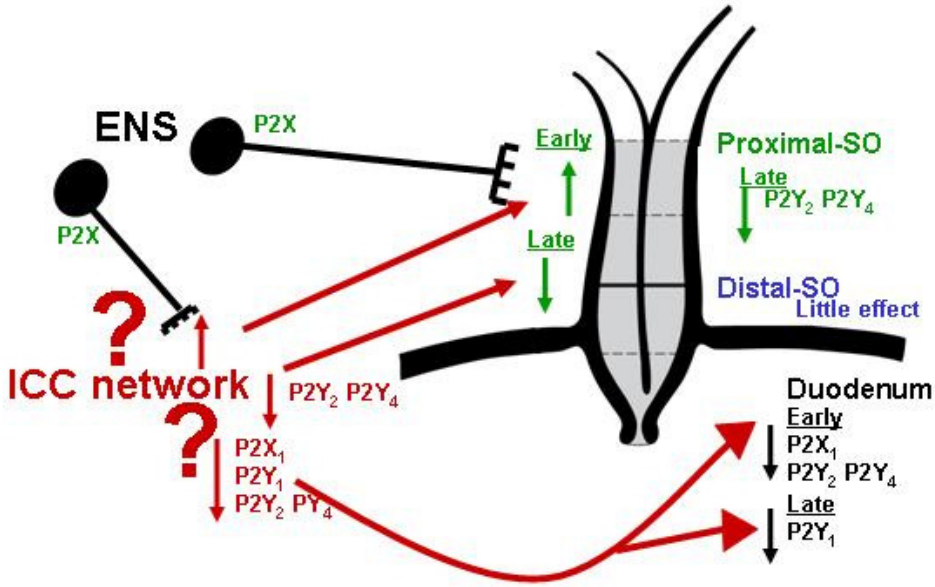
Comparison of the ATP-induced responses in the proximal-SO muscle rings and duodenal muscle strips suggests that different purine receptor sub-types mediate the responses in the different tissues. Firstly the nature of the ATP-induced responses was different, i.e. tri-phasic response in the duodenum compared to a biphasic response in the SO, and also the sensitivity to TTX (**Chapter 3**). Secondly the P2 agonists produced different responses in the two tissues also indicating the activation of different receptor sub-types. For example the actions of UTP were considerably different between the two tissues. In the duodenal muscle strips UTP induced an immediate relaxation followed by an excitation, whereas in the proximal-SO muscle rings only a late inhibitory response was observed. Clearly different P2Y receptor sub-types are mediating these different responses. However the specific P2 receptor sub-types involved cannot be clarified until better sub-type selective agonists are available, or alternatively until successful immunohistochemical studies can be performed. These data demonstrate that in the possum ATP evokes different responses via different receptor sub-types in the duodenum and the SO.

5.5 Conclusion

In conclusion exogenous ATP likely acts via excitatory neural P2X receptors to cause contraction of proximal-SO muscle rings. ATP then inhibits proximal-SO motor activity, likely via activation of non-neural $P2Y_2$ and/or $P2Y_4$ receptors, possibly in conjunction with its metabolism to adenosine and activation of non-neural P1 receptors. The receptors mediating the response induced by exogenous ATP in proximal-SO muscle rings are different to

those mediating the response induced by exogenous ATP in duodenal muscle strips.

SUMMARY 1: EXOGENOUS ATP INDUCES A COMPLEX RESPONSE ON SPONTANEOUS SO AND DUODENAL MOTILITY *IN VITRO*



Application of exogenous ATP induced complex tri-phasic responses in duodenal motility and bi-phasic responses in SO motility *in vitro*. Interestingly the effects of ATP predominated in proximal-SO muscle rings, with little effect observed in distal-SO muscle rings. The early excitatory component of the response was found to be neurally mediated, and most likely involves activation of P2X receptors. However stimulation of these nerves may result in effects directly on the smooth muscle or have an indirect effect via ICC. The late inhibitory component of the response was found to be non-neural and is likely the result of activation of $P2Y_2$ and/or $P2Y_4$ receptors.

The bi-phasic response observed with duodenal muscle strips was non-neural suggesting direct activation of receptors on smooth muscle or perhaps via activation of receptors on ICC resulting in a change in slow wave activity. In the duodenal muscle strips the early inhibitory component is likely to be mediated by $P2X_1$ and $P2Y_2$, $P2Y_4$ receptors, whereas the late inhibitory component is mostly likely due to activation of $P2Y_1$ receptors.

Metabolism of ATP to adenosine with activation of P1 receptors remains a potential mechanism partly responsible for the late inhibitory component of the response observed in both the proximal-SO muscle rings and the duodenal muscle strips. The possible activation of P2 receptors on ICC, resulting in changes to SO and duodenal spontaneous activity due to modification of slow wave activity, remains to be addressed.

Chapter 6

6 PHARMACOLOGICAL IDENTIFICATION OF P1 RECEPTORS MEDIATING THE INHIBITORY EFFECTS OF EXOGENOUS ADENOSINE ON SPONTANEOUS DUODENAL ACTIVITY *IN VITRO*

6.1 Introduction

The identification of the P1 receptor sub-types involved in mediating the possum duodenal response to exogenous adenosine remains to be determined. An immunohistochemical approach to establish the potential P1 receptor sub-types that may be involved and their locations in possum tissues proved unsuccessful (**Appendix 1**). Therefore a pharmacological approach was used to define the P1 receptor sub-types involved.

A number of agonists and antagonists have recently been developed for P1 receptor sub-types, with the exception of agonists for the A_{2B} receptor (**Section 1.4.2**, **Table 1.3**). Studies with specific agonists/antagonists have attempted to determine which P1 receptor sub-types mediate the adenosine response in rat duodenum. Using pre-contracted rat duodenal muscle strips sub-type selective antagonists revealed the presence of A_1 receptors (Nicholls *et al.* 1992a). However, non-selective concentrations of DPCPX were required to inhibit the effect of exogenous adenosine, thus raising the possibility that A_2 receptors may also be involved. Furthermore the A_{2A} receptor agonist CGS21680 had no effect, thereby suggesting that A_{2B} receptors could be involved (Nicholls *et al.* 1992i). The location of various P1 receptors within the layers of the rat duodenal wall was examined

pharmacologically to a limited extent with studies separating the tissue into longitudinal muscle strips and muscularis mucosae strips (Nicholls *et al.* 1996f). An inhibitory response was observed in the longitudinal muscle strips mediated by A_1 and possibly A_{2B} receptors. In contrast, a contractile response was identified in the muscularis mucosae muscle strips, possibly mediated by A_{2B} receptors due to the low potency of the A_{2A} receptor agonist CGS21680. However the responses of the longitudinal muscle were found to dominate when full thickness muscle strips were used (Nicholls *et al.* 1992d). Due to the use of non-selective concentrations of the A_1 receptor antagonist and the lack of an appropriate A_{2B} receptor agonist/antagonist this finding requires further study, particularly as the possible involvement of A_3 receptors has not been addressed.

The aim of the experiments described in this chapter was to pharmacologically identify the P1 receptor sub-types involved in mediating the response to exogenous adenosine in spontaneously active possum duodenal muscle strips. These data then form the basis for comparable studies in the SO by providing appropriate agonist and antagonist concentration ranges that are active in possum tissues.

6.2 Methods

Possums were anaesthetised and a segment of duodenum, between the pylorus and SO, was removed prior to euthanasia (**Section 2.1**). Eight full thickness muscle strips were prepared from the duodenum of each animal (**Section 2.2.2**).

Organ bath pharmacology studies were then undertaken using the protocols described in **Section 2.2.5**. Briefly, a paired protocol was used to assess if the magnitude of the adenosine-induced response was altered by pre-treatment with selective P1 receptor antagonists. These results were then verified with the use of selective P1 receptor sub-type agonists. Refer to **section 2.2.5** for details regarding the use of P1 sub-type selective antagonists and agonists and appropriate concentrations with regard to their selectivity.

Refer to **Section 2.2** for drug preparation and analysis of recordings. Where possible EC_{50} values were determined for the adenosine-induced response before and after pre-treatment with the antagonist. Note that only the responses to effective concentrations of antagonists, or the highest concentration used, are presented below. The results from **Chapter 3** demonstrated that adenosine had a minor affect on duodenal contraction frequency. Therefore contraction frequency has been analysed in response to each of the drugs tested, but where there was no significant effect of adenosine, or the antagonist, the data is not presented.

6.3 Results

6.3.1 P1 receptor antagonists

Pre-treatment with the general P1 antagonist DPSPX (100μM) did not modify spontaneous duodenal activity ($P>0.05$) (n=30 muscle strips, from 5 possums) (**Table 6.1**). DPSPX pre-treatment inhibited the adenosine-induced decrease in activity expressed as area under curve ($P<0.05$) and contraction amplitude ($P<0.05$), but had no effect on basal tension ($P>0.05$)

(n=5 muscle strips/concentration, from 5 possums) (**Figure 6.1** and **Table 6.2**).

Pre-treatment with the A_1 receptor antagonist DPCPX (10nM) produced a small increase in the basal tension ($P<0.05$) but did not influence the amplitude or frequency of spontaneous contractions ($P>0.05$ for both) (n=56 muscle strips, from 9 possums) (**Table 6.1**). DPCPX pre-treatment had no effect on the response induced by adenosine for any parameter measured ($P>0.05$ for all) (n=7-9 muscle strips/concentration, from 9 animals) (**Figure 6.2** and **Table 6.3**). A higher, but non-selective, concentration of DPCPX (1μM) also failed to have a significant effect on the response to adenosine (data not shown).

Pre-treatment with the A_{2A} receptor antagonist CSC (10μM) produced a minor increase in basal tension ($P<0.05$) but did not significantly alter the amplitude or frequency of spontaneous duodenal contractions (n=48 muscle strips, from 6 possums) (**Table 6.1**). Pre-treatment with CSC completely blocked the adenosine-induced decrease in contraction amplitude ($P<0.05$) and decreased the adenosine response expressed as on area under curve ($P<0.05$), but had no effect on the adenosine-induced decrease in basal tension ($P>0.05$) (n=6 muscle strips/concentration, from 6 possums) (**Figure 6.3**, **Table 6.4**).

Pre-treatment with the A_{2B} receptor antagonist IPDX (10μM) produced a significant decrease in spontaneous contractile activity expressed as area under curve ($P<0.05$), contraction amplitude ($P<0.05$) and basal tension

(P<0.05) (n=41 muscle strips, from 6 possums) (**Table 6.1**). IPDX pre-treatment had no significant effect on the adenosine-induced response for any parameter measured (P>0.05 for all) (n=4-6 muscle strips/concentration; from 6 possums) (**Figure 6.4**, **Table 6.5**).

Pre-treatment with the A_3 receptor antagonist MRS1220 (10μM) reduced spontaneous contractile activity expressed as area under curve (P<0.05) and contraction amplitude (P<0.05), but had no effect on basal tension (P>0.05) (n=40 muscle strips, from 7 possums) (**Table 6.1**). MRS1220 pre-treatment also reduced the adenosine-induced decrease in contraction amplitude (P<0.05) and area under curve (P<0.05) (n=5-7 muscle strips/concentration, from 7 possums). Furthermore at the higher adenosine concentrations a slight enhancement of the adenosine-induced decrease in basal tension was observed after MRS1220 pre-treatment (P<0.05) (**Figure 6.5**, **Table 6.6**).

6.3.2 P1 receptor agonists

Application of the A_1 agonist CCPA (0.1nM-10μM) did not significantly modify spontaneous duodenal activity for any parameter (all P>0.05) (n=3-4 muscle strips, from 4 possums) (**Figure 6.6**).

Application of the A_{2A} agonist CGS21680 (10nM-100μM) concentration-dependently decreased spontaneous duodenal contractile activity expressed as area under curve (P<0.05; EC_{50}=1.16μM), contraction amplitude (P<0.05; EC_{50}=6.14 μM) and basal tension (P<0.05; EC_{50}=12.7mM), but not contraction frequency (P>0.05) (n=3-4 muscle strips, from 4 possums) (**Figure 6.7**). To confirm that CGS21680 was acting at A_{2A} receptors at the

high concentrations (100μM) CGS21680 application was repeated in the presence of the A_{2A} antagonist CSC (10μM). CSC pre-treatment antagonised the CGS21680-induced response expressed as area under curve ($P<0.05$) at 100μM CGS21680 ($P<0.05$) (n=3-4 muscle strips for each CGS21680 concentration, from 4 possums) (**Figure 6.8**). Furthermore there was a trend for CSC pre-treatment to inhibit the CGS21680-induced decrease in contraction amplitude and basal tension, but this did not reach statistical significance ($P>0.05$) (**Figure 6.8**). This data suggests that A_{2A} receptors are involved in the adenosine-induced response.

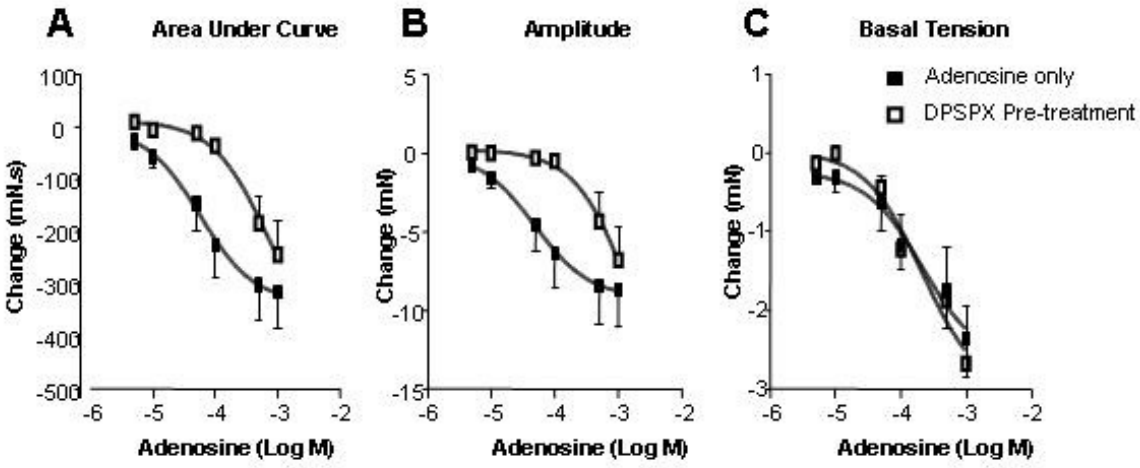
Application of the A_3 agonist 2Cl-IB-MECA (1nM-100μM) did not significantly modify spontaneous duodenal muscle strip contractile activity for any parameter (all $P>0.05$) (n=3-4 muscle strips, from 4 possums) (**Figure 6.9**).

Table 6.1: Effect of P1 receptor antagonists on spontaneous duodenal muscle strip contractile activity

Receptor Selectivity	Antagonist	Area Under Curve	Contraction Amplitude	Basal Tension	Contraction Frequency
Non-selective	DPSPX (100μM, n=30)	94.5± 4.2%	94.3± 4.1%	95.5± 2.6%	101.8± 3.2%
A_1	DPCPX (10nM, n=56)	98.3±1.0%	98.1±1.0%	105.7±1.8% *	101.1±0.8%
A_{2A}	CSC (10μM, n=48)	97.7±1.5%	97.1±1.2%	108.5±1.6% *	100.9±0.7%
A_{2B}	IPDX (10μM, n=41)	77.9±2.4% *	86.0±2.4% *	79.1±3.8% *	97.7±1.3%
A_3	MRS1220 (10μM, n=40)	90.4±2.4% *	92.4±1.0% *	104.4±2.2%	101.7±0.9%

Data expressed as % of baseline activity and is presented as mean ± SEM. * $P<0.05$ as determined by Wilcoxon matched pairs test. n = number of muscle strips, from 4-9 possums.

Figure 6.1: Effect of P1 antagonist DPSPX pre-treatment on adenosine-induced inhibition of spontaneous duodenal muscle strip contractile activity

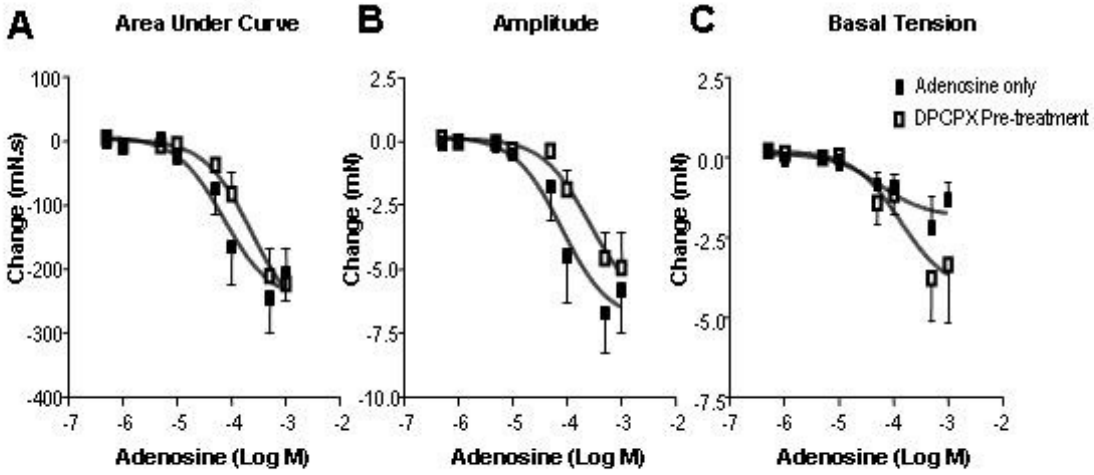


Group data, as a change from baseline activity, illustrating the effect of pre-treatment of longitudinal duodenal muscle strips with the P1 receptor antagonist DPSPX (100μM) on the adenosine-induced inhibition of contractile activity expressed as area under curve (**A**) ($P<0.05$) and contraction amplitude (**B**) ($P<0.05$). The adenosine-induced decrease in basal tension was not affected by DPSPX pre-treatment (**C**) ($P>0.05$). Each point is the mean ± SEM of 5 muscle strips, from 5 possums.

Table 6.2: Change in EC_{50} values for duodenal muscle strip response to adenosine application in the presence of P1 receptor antagonist DPSPX (100μM).

	Adenosine (EC_{50}; μM)	**DPSPX (EC_{50}; μM)**
Area under curve	56.1	665
Contraction amplitude	47.8	1635
Basal tension	215	210

Figure 6.2: Effect of A_1 receptor antagonist DPCPX pre-treatment on the adenosine-induced inhibition of spontaneous duodenal muscle strip contractile activity

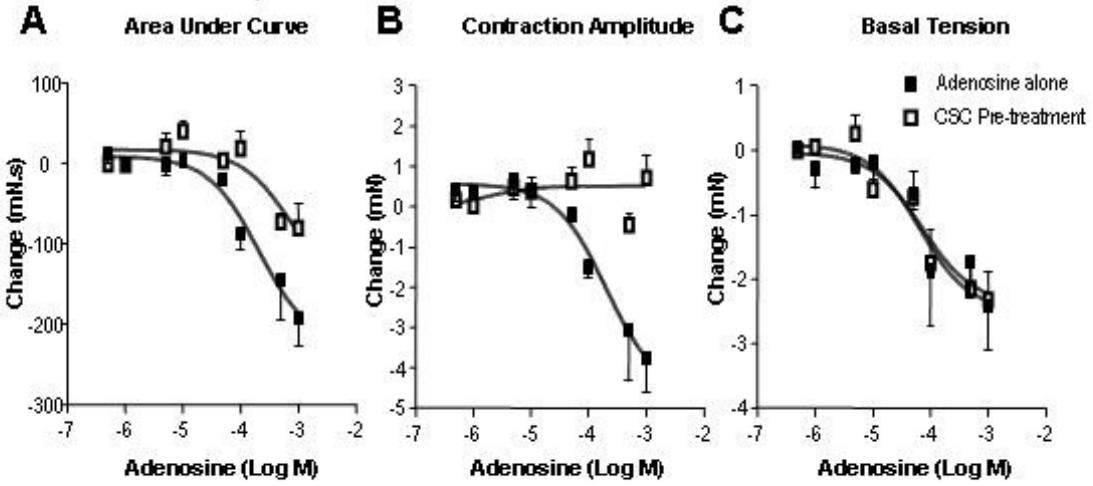


Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment of longitudinal duodenal muscle strips with the A_1 receptor antagonist DPCPX (10nM) on the adenosine-induced inhibition of contractile activity expressed as area under curve (**A**), contraction amplitude (**B**) and basal tension (**C**) (all $P>0.05$). Each point is the mean ± SEM of 7-9 muscle strips, from 9 possums.

Table 6.3: Change in EC_{50} values for duodenal muscle strip response to adenosine application in the presence of A_1 receptor antagonist DPCPX (10nM).

	Adenosine (EC_{50}; μM)	**DPCPX (EC_{50}; μM)**
Area under curve	72	114
Contraction amplitude	81	131
Basal tension	57	79

Figure 6.3: Effect of A_{2A} receptor antagonist CSC pre-treatment on the adenosine-induced inhibition of spontaneous duodenal muscle strip contractile activity

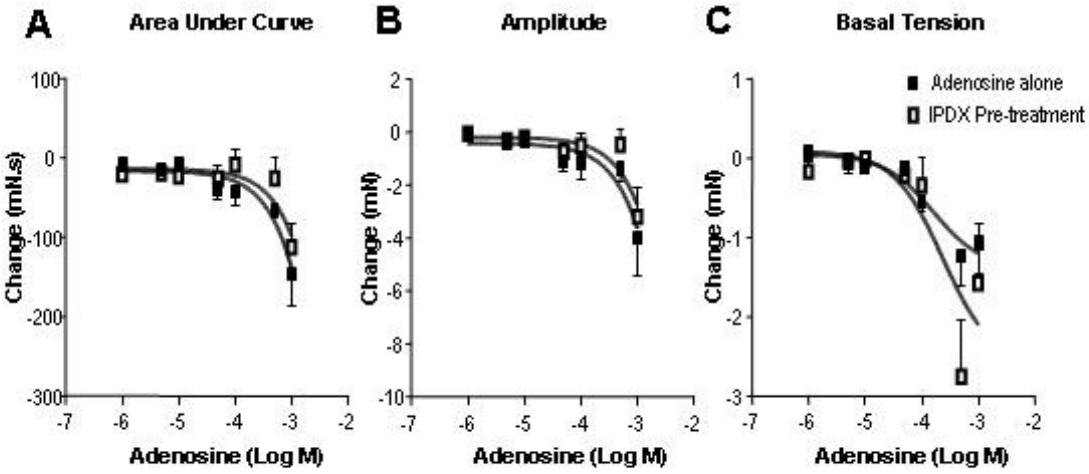


Group data, as a change from baseline activity, illustrating the effect of pre-treatment of longitudinal duodenal muscle strips with the A_{2A} receptor antagonist CSC (10μM) on the adenosine-induced inhibition. CSC pre-treatment antagonised the adenosine-induced response expressed as area under curve (**A**) ($P<0.05$), blocked the decrease in contraction amplitude (**B**) ($P<0.05$), but did not effect basal tension (**C**) ($P>0.05$). Each point is the mean ± SEM of 6 muscle strips, from 6 possums.

Table 6.4: Change in EC_{50} values for duodenal muscle strip response to adenosine application in the presence of A_{2A} receptor antagonist CSC (10μM).

	Adenosine (EC_{50}; μM)	**CSC (EC_{50}; μM)**
Area under curve	205	754
Contraction amplitude	199	3325
Basal tension	72	14

Figure 6.4: Effect of A_{2B} receptor antagonist IPDX pre-treatment on the adenosine-induced inhibition of spontaneous duodenal muscle strip contractile activity



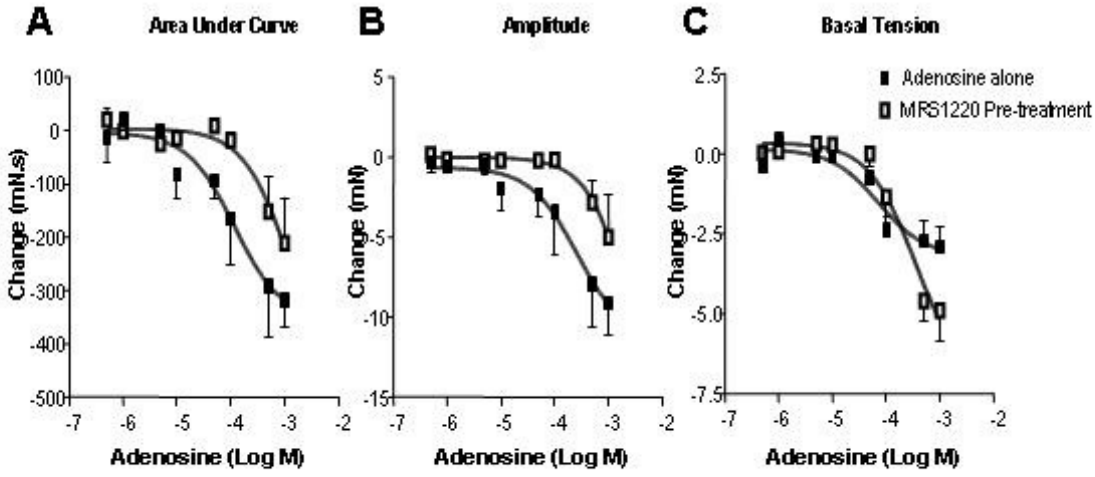
Group data, as a change from baseline activity, illustrating the lack of effect of pre-treatment of longitudinal duodenal muscle strips with the A_{2B} receptor antagonist IPDX (10μM) on the adenosine-induced inhibition. IPDX pre-treatment did not effect the response to adenosine when expressed as area under curve (**A**), contraction amplitude (**B**) or basal tension (**C**) (all P>0.05). Each point is the mean ± SEM of 4-6 muscle strips, from 6 possums.

Table 6.5: Lack of change in EC_{50} values for duodenal muscle strip response to adenosine application in the presence of A_{2B} receptor antagonist IPDX (10μM).

	Adenosine (EC_{50}; μM)	**IPDX (EC_{50}; μM)**
Area under curve	N/A	N/A
Contraction amplitude	N/A	N/A
Basal tension	160	N/A

EC_{50} values not able to be determined by Prism as concentration curves had not started to plateau within the adenosine concentration range.

Figure 6.5: Effect of A_3 receptor antagonist MRS1220 pre-treatment on the adenosine-induced inhibition of spontaneous duodenal muscle strip contractile activity



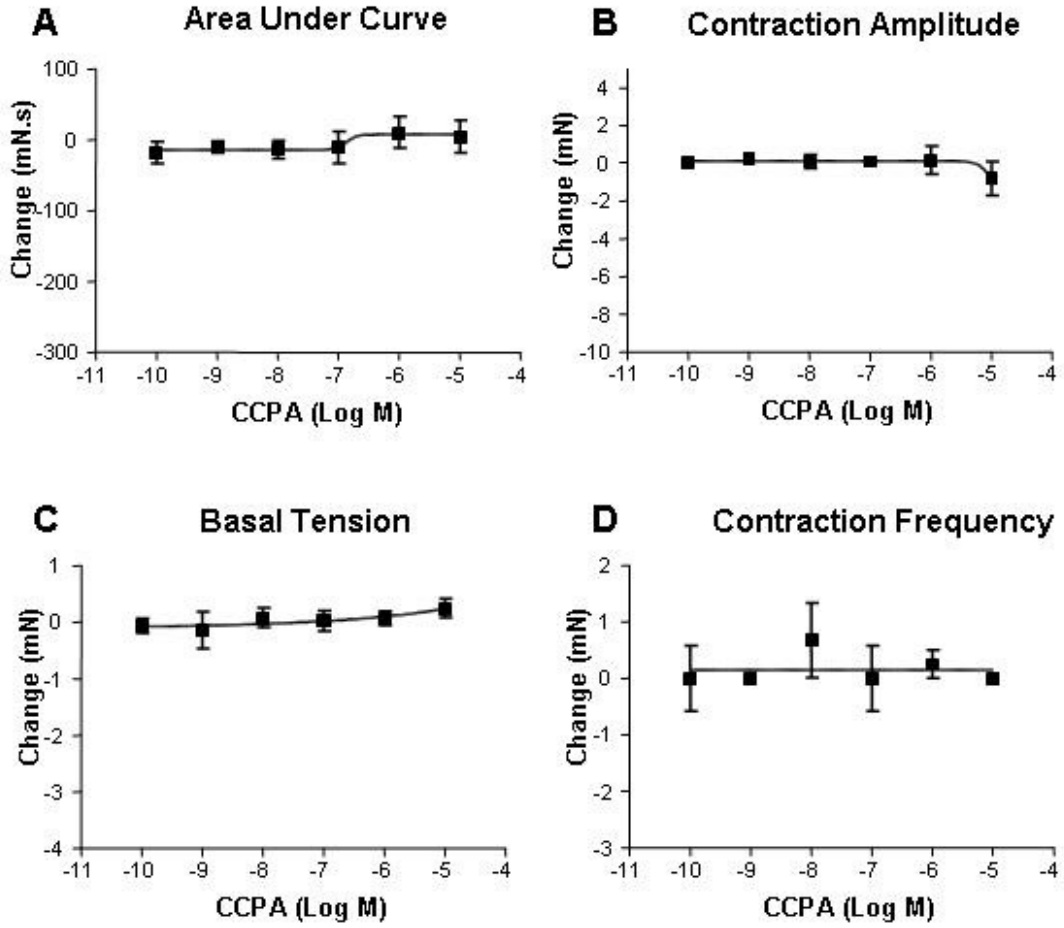
Group data, as a change from baseline activity, illustrating the effect of pre-treatment of longitudinal duodenal muscle strips with the A_3 receptor antagonist MRS1220 (10μM) on the adenosine-induced inhibition. MRS1220 pre-treatment antagonised the adenosine-induced response expressed as area under curve (**A**) ($P<0.05$) and contraction amplitude (**B**) ($P<0.05$). MRS1220 pre-treatment increased the adenosine-induced decrease in basal tension (**C**) ($P<0.05$) at the higher adenosine concentrations. Each point is the mean ± SEM of 5-7 muscle strips, from 7 possums.

Table 6.6: Change in EC_{50} values for duodenal muscle strip response to adenosine application in the presence of A_3 receptor antagonist MRS1220 (10μM)

	Adenosine (EC_{50}; μM)	**MRS1220 (EC_{50}; μM)**
Area under curve	117	1279
Contraction amplitude	260	N/A
Basal tension	71	346

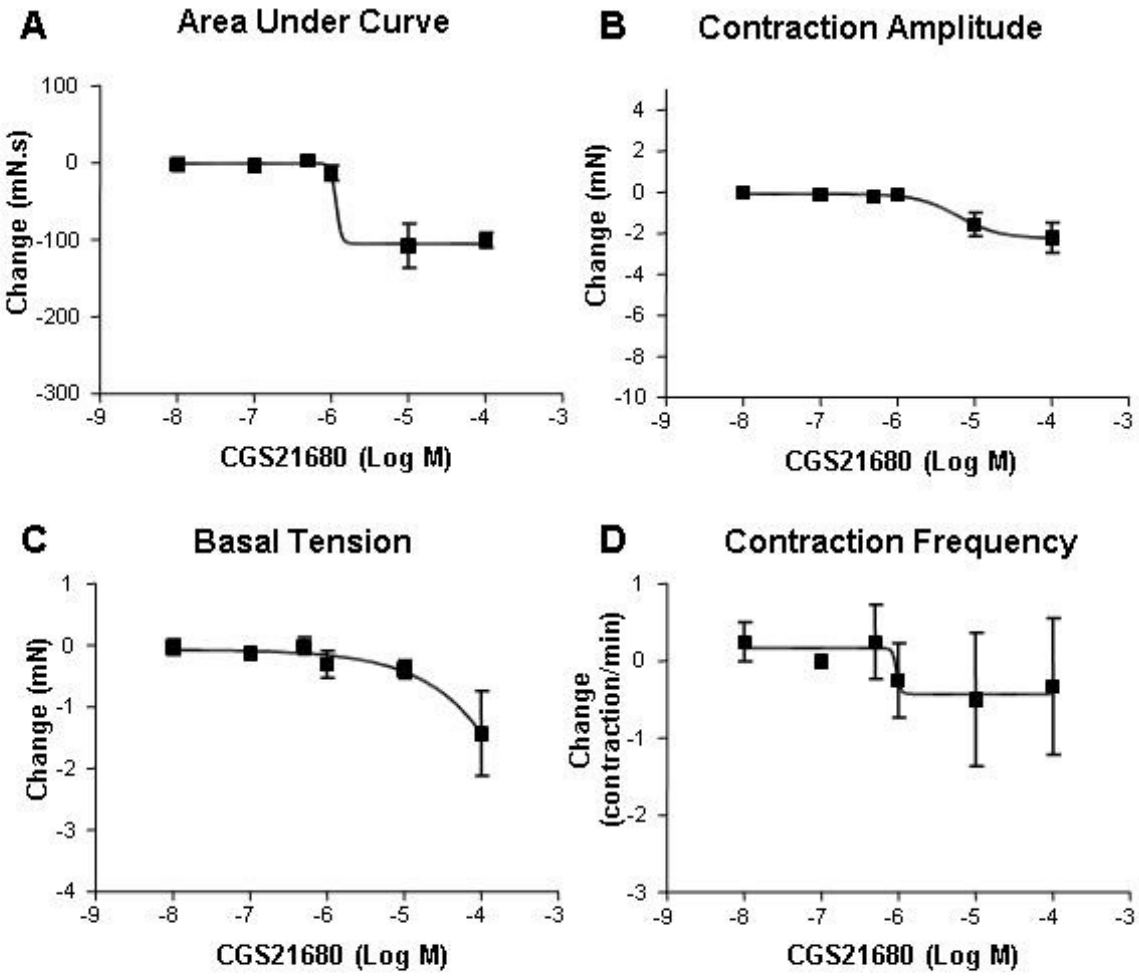
EC_{50} values not able to be determined by Prism for some parameters as concentration curves had not started to plateau within the adenosine concentration range.

Figure 6.6: Lack of effect of A_1 agonist CCPA on spontaneous duodenal muscle strip contractile activity.



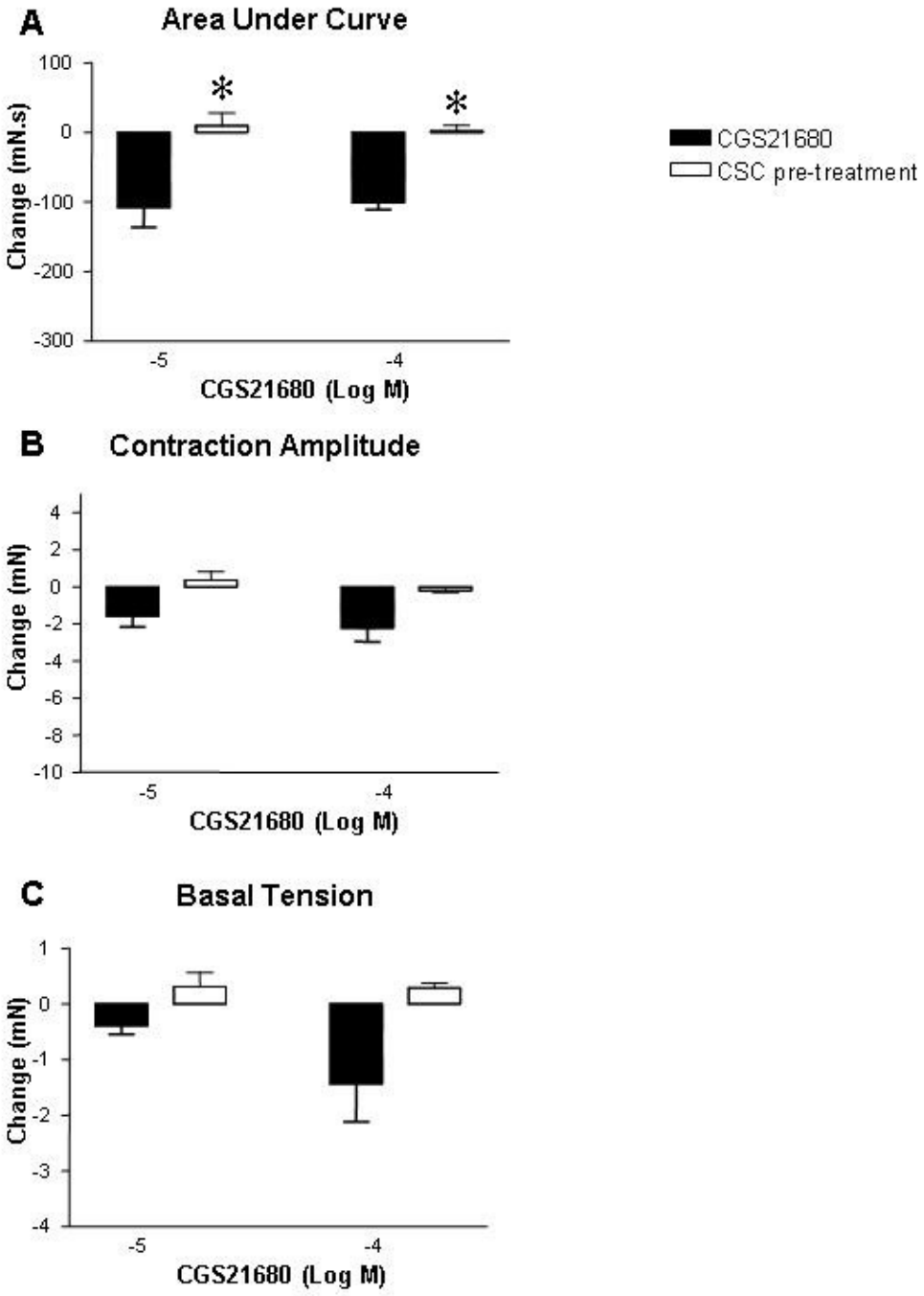
Group data, as a change from baseline activity, illustrating the lack of effect of the A_1R agonist CCPA on spontaneous longitudinal duodenal muscle strip contractile activity. CCPA did not effect activity expressed as area under curve (**A**), contraction amplitude (**B**), basal tension (**C**), or contraction frequency (**D**) ($P>0.05$ for all). Each point is the mean ± SEM of 3-4 muscle strips, from 4 possums.

Figure 6.7: Effect of A_{2A} agonist CGS21680 on spontaneous duodenal muscle strip contractile activity.



Group data, as a change from baseline activity, illustrating the effect of the A_{2A}R agonist CGS21680 on spontaneous longitudinal duodenal muscle strip contractile activity. CGS21680 concentration-dependently decreased activity expressed as area under curve (**A**), contraction amplitude (**B**) and basal tension (**C**) ($P<0.05$ for all), but not contraction frequency (**D**) ($P>0.05$). Each point is the mean ± SEM of 3-4 muscle strips, from 4 possums.

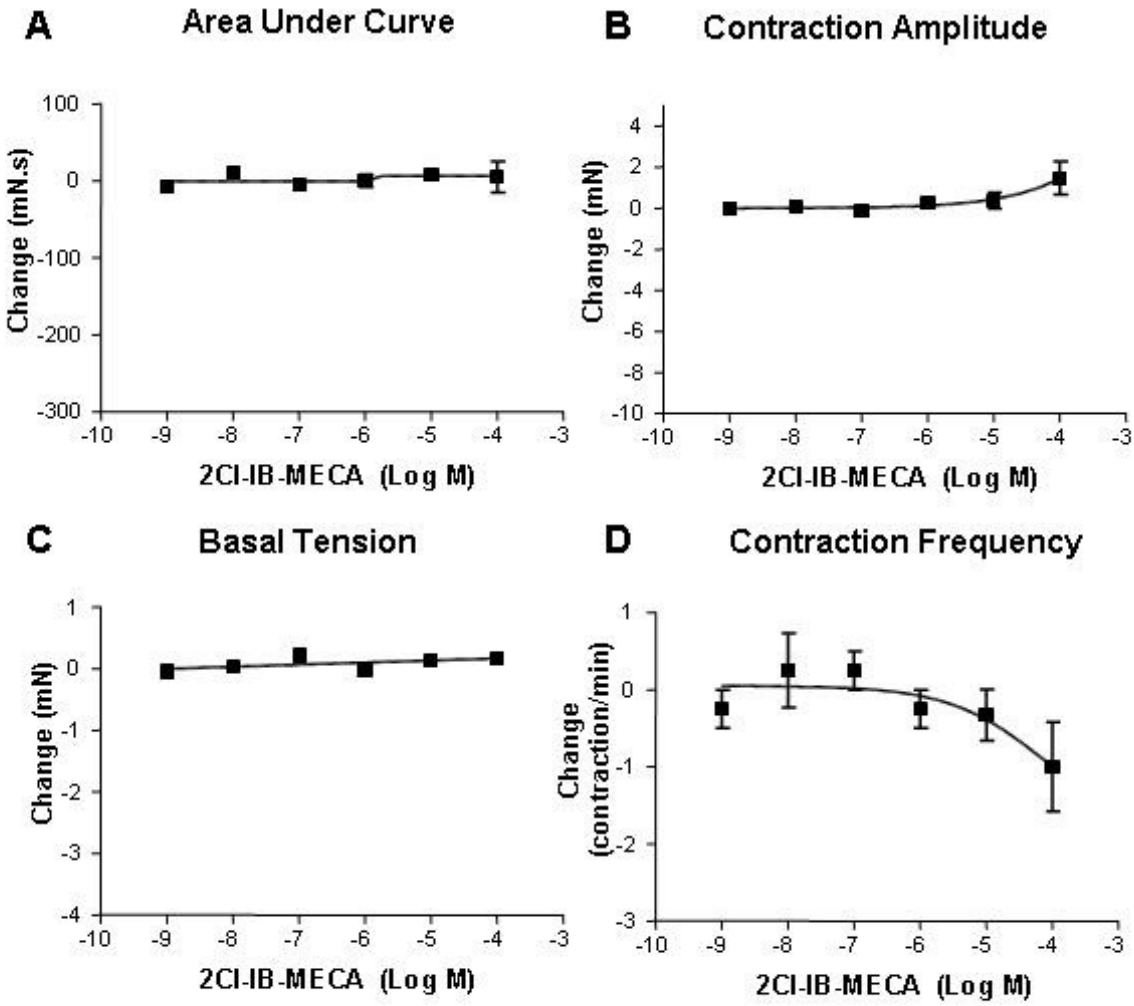
Figure 6.8: CGS21680-induced response on spontaneous duodenal muscle strip contractile activity is antagonised by CSC pre-treatment.



Group data, as a change from baseline activity, illustrating the specificity of the $A_{2A}R$ agonist CGS21680-induced response on spontaneous longitudinal duodenal muscle strip activity. The CGS21680-induced decrease in contractile activity was blocked by pre-treatment with the $A_{2A}R$ antagonist CSC (10μM) expressed as area under curve (**A**) (*P<0.05). There was a trend for CSC pre-treatment to antagonise the CGS21680-induced response in contraction amplitude (**B**) and basal tension (**C**) but this did not reach significance (P>0.05 for both). Each point is the mean ± SEM of 3-4 muscle strips, from 4 possums.

173

Figure 6.9: Lack of effect of A_3 agonist 2Cl-IB-MECA application on spontaneous duodenal muscle strip contractile activity.



Group data, as a change from baseline activity, illustrating the lack of effect of application of the A_3R agonist 2Cl-IB-MECA on longitudinal duodenal muscle strip activity. 2Cl-IB-MECA did not effect contractile activity expressed as area under curve (**A**), contraction amplitude (**B**), basal tension (**C**) or contraction frequency (**D**) ($P>0.05$ for all). Each point is the mean ± SEM of 3-4 muscle strips, from 4 possums.

Table 6.7: Summary of effects of P1 agonists and antagonists on spontaneous duodenal muscle strip contractile activity

Receptor Selectivity	Antagonist	Effect on spontaneous contractile activity	Antagonised adenosine-induced response	Agonist	Produced inhibition of contractile activity
Non-selective	DPSPX	X	X	adenosine	✓
A_1	DPCPX	↑	X	CCPA	X
A_{2A}	CSC	↑	✓	CGS21680	✓
A_{2B}	IPDX	↓	X	N/A	-
A_3	MRS1220	↓	✓	2Cl-IB-MECA	X

↓ Decreased spontaneous activity
↑ Increased spontaneous activity
X No effect of antagonist/agonist
✓ Effect of antagonist/agonist
N/A Not available

6.4 Discussion

The experiments described in this chapter found that exogenous adenosine acts via receptor sub-types A_{2A} and A_3 to reduce spontaneous possum duodenal longitudinal muscle contractile activity, predominantly affecting contraction amplitude. Furthermore, these data suggest the involvement of endogenous adenosine in the maintenance of spontaneous contractile activity. The results are summarized in **Table 6.7**.

These findings were somewhat unexpected compared to those previously reported in the rat duodenum. Interestingly the possum data presented here does support the rat duodenal data (Nicholls *et al.* 1992h; Nicholls *et al.* 1996e) when the specificity and availability of P1 receptor agonists and antagonists used in the rat experiments is considered. In agreement with the rat duodenal data (Nicholls *et al.* 1992b; Nicholls *et al.* 1996d) the current possum data also found that nanomolar concentrations of DPCPX did not affect the adenosine-induced response, thereby suggesting that the A_1 receptor is not likely to be involved in mediating the adenosine-induced response. Nicholls *et al*. (1992) found higher but non-selective concentrations of DPCPX were required to influence adenosine-induced response, thereby suggesting the presence of A_2 receptors. Due to the lack of available A_2 receptor specific antagonists at the time of the rat study it was suggested that A_{2B} receptors were involved. This was based on the lack of response to an A_{2A} receptor agonist CGS12680, which has low potency for the A_{2B} receptors (Nicholls *et al.* 1992c; Nicholls *et al.* 1996c). The finding that the A_{2A} receptor antagonist blocked the adenosine-induced decrease in possum duodenal contraction amplitude but had no effect on basal tension, contrasts with the

findings of Nicholls *et al*. (1992) who found an A_{2A} receptor agonist to have little effect. This anomaly is likely to be due to technical differences, as pre-contracted rat duodenal muscle strips were used to demonstrate a decrease in basal tension only, and changes in contraction amplitude would not be observable. The rat studies (Nicholls *et al.* 1992f; Nicholls *et al.* 1996b) did not investigate the possible involvement of A_3 receptors in mediating the adenosine-induced response. In summary, the antagonist experiments using spontaneously active possum duodenal muscle strips have provided evidence implicating the A_{2A} and A_3 receptors as mediators for the inhibitory response induced by exogenous adenosine. Further investigation will be required to determine the receptor sub-types that mediate the adenosine-induced decrease in basal tension.

The antagonist data presented here is further supported by experiments with specific receptor agonists. The A_1 receptor agonist had no effect, supporting the lack of effect of the A_1 receptor antagonist on the adenosine-induced response. Similar to the effects of exogenous adenosine, the A_{2A} receptor agonist CGS21680 also decreased duodenal contractile activity. However the concentration required was considered very high compared to that used in human and rat studies (**Table 1.3**), which raises questions regarding agonist specificity. Therefore in a limited number of experiments the high concentrations of CGS21680 were applied to duodenal muscle strips pre-treated with the A_{2A} receptor antagonist CSC. Pre-treatment with the antagonist blocked the CGS21680-induced decrease in contractile activity expressed as area under curve. Therefore it can be assumed that this high concentration of CGS21680 is specific for A_{2A} receptors. No specific A_{2B}

receptor agonists were available for testing. The effect with the A_3 receptor antagonist could not be verified with an A_3 receptor agonist, which may be due to the concentration range tested or the specificity of the agonist in possum tissue (it is well known that A_3 agonist specificity is species dependent). It should be noted that the affinity of a given P1 receptor agonist at a particular receptor sub-type can vary widely depending on experimental conditions, and as a consequence the use of selective antagonists is regarded as more reliable endogenous P1 receptor characterisation (King and Townsend-Nicholson 2003).

The reasons for the use of possum tissue rather than rat tissue for these studies were outlined previously. Consequently it was assumed that the adenosine antagonists behaviour in possum tissue is similar to that described in rat and human tissues. To confirm this attempts were made to estimate and compare the antagonists' dissociation constant (K_B). The Schild equation can be used to estimate K_B values for simple competitive antagonism (one-receptor systems), but the present data implicates the involvement of both A_{2A} and A_3 receptors in the adenosine-induced response. When two receptors are involved, the agonist will encounter the second receptor, which is not blocked by the antagonist, and this results in the generation of a response which is greater than that predicted by a competitively antagonised single receptor population. This results in a deviation from the Schild regression and an erroneous estimation of K_B (Kenakin 1992). Therefore, Schild equations and K_B estimations could not be determined (and are not included in other chapters of this thesis).

The findings from the antagonist experiments described here also provide some evidence for the role of endogenous purines in the maintenance of spontaneous duodenal contractile activity. The regulation of spontaneous activity is complex, involving myogenic, neural and non-neural mechanisms. Application of the A_1 and A_{2A} receptor antagonists increased basal tension suggesting that endogenous adenosine may modulate duodenal motility. In contrast, antagonism of the A_{2B} and A_3 receptors decreased spontaneous activity. These conflicting responses suggest that activation of these receptors at various sites within the tissue (i.e. muscle, myenteric neurons, epithelial cells etc.) by endogenous adenosine most likely produces complex interactions, which are then expressed as a net reduction in spontaneous activity.

6.5 Conclusion

In conclusion the possum duodenum *in vitro* data suggests that endogenous adenosine modulates spontaneous contractile activity, and exogenous adenosine decreases contraction amplitude acting via A_{2A} and A_3 receptors, but the receptors mediating the adenosine-induced decrease in basal tension remain to be identified.

Chapter 7

7 PHARMACOLOGICAL CHARACTERISATION OF P1 RECEPTORS MEDIATING THE INHIBITORY EFFECTS OF EXOGENOUS ADENOSINE ON SO MOTILITY *IN VITRO*

7.1 Introduction

There are only two reports of the actions of adenosine on SO motility. One reports that adenosine had an inhibitory effect on EFS-induced contraction, whereas a clinical study suggests adenosine may be involved in morphine induced SO spasm (**Section 1.4.3**).

The possum studies described in **Chapter 3** found that adenosine decreased SO contractile activity. This response was TTX-insensitive and much more pronounced in the proximal-SO muscle rings. *In vitro* studies in the possum duodenum found adenosine reduced spontaneous activity, likely acting via A_{2A} and A_3 receptors (**Chapter 6**). As the SO and duodenum have distinctly different functions different receptors may be involved in mediating the adenosine-induced response. This was demonstrated by the SO and duodenal responses to exogenous ATP (**Chapter 5**).

The aims of this study were to:

1. Identify the P1 receptor sub-types mediating the inhibitory response induced by exogenous adenosine in the proximal-SO.
2. Compare the P1 receptor sub-types mediating the adenosine-induced response in proximal-SO muscle rings with those identified in the duodenum.

7.2 Methods

Possums were anaesthetised and the SO and adjacent duodenum were removed prior to euthanasia (**Section 2.1**). Four full thickness muscle rings were prepared from the SO of each animal, and separated into distal-SO and proximal-SO groups (**Section 2.2.3**).

Organ bath pharmacology studies were then undertaken using the protocols described in **Section 2.2.5**. Briefly, a paired protocol was used to assess if the magnitude of the adenosine-induced response was altered by pre-treatment with selective P1 receptor antagonists. These results were then verified with the use of selective P1 receptor sub-type agonists. The concentration of antagonists and agonists used were based on those that were effective in possum duodenum (**Chapter 6**), or the highest doses used in the literature. Refer to **section 2.2.5** for details regarding the use of P1 sub-type selective antagonists and agonists and appropriate concentrations with regard to their selectivity.

Refer to **Section 2.2** for drug preparation and analysis of recordings. The results from **Chapter 3** demonstrated that adenosine had a very minor effect on basal tension. This parameter was analysed in the studies reported in this chapter, but where there was no significant effect of the antagonist treatment the data is not presented. Due to the variable responses obtained with the P1 agonists on spontaneous SO activity, and the high concentrations required, only preliminary experiments were performed and these are reported here in a qualitative manner for completeness.

7.3 Results

For simplicity only the data generated using agonists and antagonists with the proximal-SO muscle rings is presented, as little response was evoked by adenosine with distal-SO muscle rings (**Chapter 3**).

7.3.1 P1 receptor antagonists

Pre-treatment with the A_1 receptor antagonist DPCPX (10nM) did not antagonise the adenosine-induced response in proximal-SO contractile activity expressed as area under curve, contraction amplitude or contraction frequency ($P>0.05$ for all) (n=4-7 muscle rings/concentration, from 15 possums) (**Figure 7.1**).

Pre-treatment of proximal-SO muscle rings with the A_{2A} receptor antagonist CSC (10μM) did not significantly modify the adenosine-induced decrease contractile activity expressed as area under curve, contraction amplitude or contraction frequency (all $P>0.05$) (n=4 muscle rings/concentration, from 11 possums) (**Figure 7.2**).

The A_{2B} receptor antagonist IPDX used in the studies with duodenal muscle strips (**Chapter 6**) could not be used in these SO muscle ring experiments due to an inhibitory effect of the solvent DMSO. Other suitable solvents were tested and also inhibited spontaneous SO muscle ring contractile activity. Another A_{2B} receptor antagonist, MRS1754 (Kim *et al.* 2000) was tested (the concentration required to block all A_{2B} receptors was recommended by Dr Jacobson, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, USA). Pre-treatment of proximal-SO muscle

rings with MRS1754 (1μM) did not modify the adenosine-induced decrease in contractile activity expressed as area under curve, contraction amplitude or contraction frequency (all P>0.05) (n=4-6 muscle rings/concentration, from 18 possums) **Figure 7.3**).

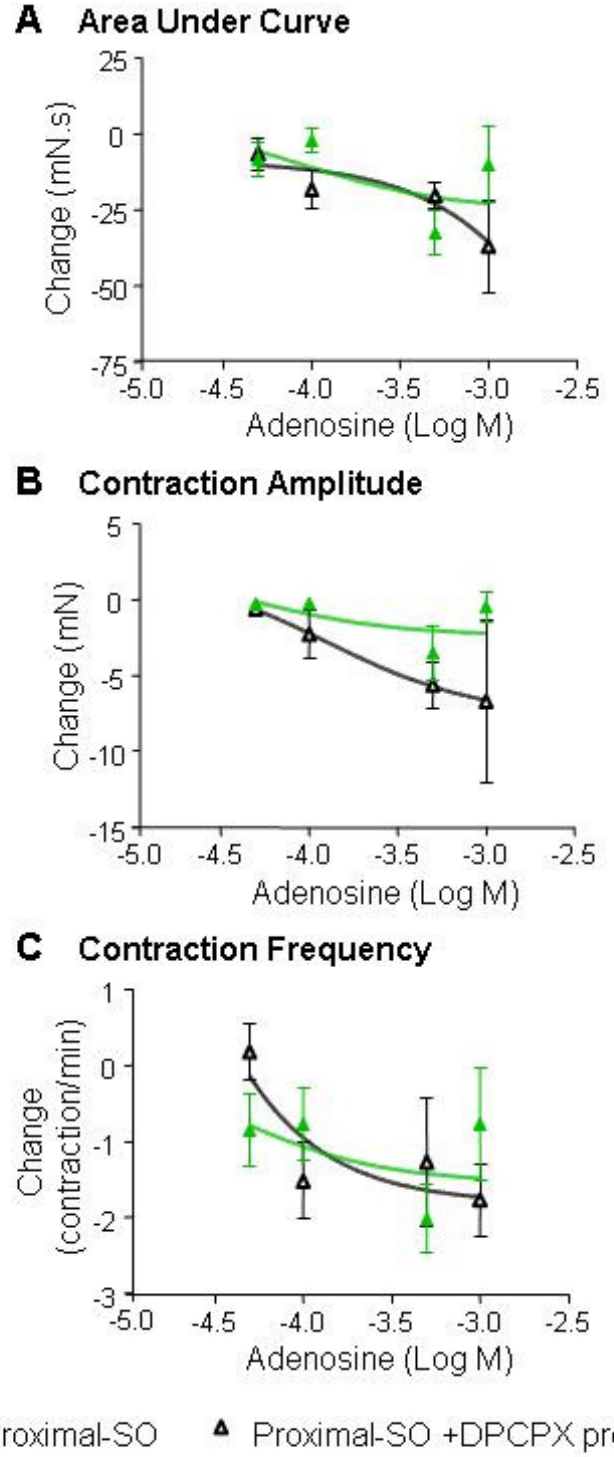
Pre-treatment of proximal-SO muscle rings with the A_3 receptor antagonist MRS1220 (10μM) also failed to modify the adenosine-induced decrease in contractile activity expressed as area under curve, contraction amplitude or contraction frequency (P>0.05) (n=4 muscle rings/concentration, from 12 possums) (**Figure 7.4**).

7.3.2 Preliminary experiments with P1 receptor agonists

As none of the antagonists tested antagonised the adenosine-induced decrease in proximal-SO motility, in separate experiments single concentrations of selective agonists were applied to proximal-SO muscle rings. The A_1 receptor agonist CCPA (10μM) had no obvious effect on the spontaneous contractile activity displayed by proximal-SO muscle rings (n=4 muscle rings, from 4 possums) (**Figure 7.5**). The A_{2A} receptor agonist CGS21680 (100μM) had no obvious effect on 3/4 proximal-SO muscle ring spontaneous activity (from 4 possums), with the exception of one preparation that displayed a bi-phasic response consisting of an excitatory response followed by an inhibitory response (**Figure 7.5**). The A_3 receptor agonist 2Cl-IB-MECA (100μM) had a variable effect on proximal-SO muscle rings where in 3 muscle rings (from 3 possums) a decrease in contraction amplitude was observed (**Figure 7.5**), but in another 3 muscle rings (from 3 possums) no response was observed. However, the high concentrations of agonists

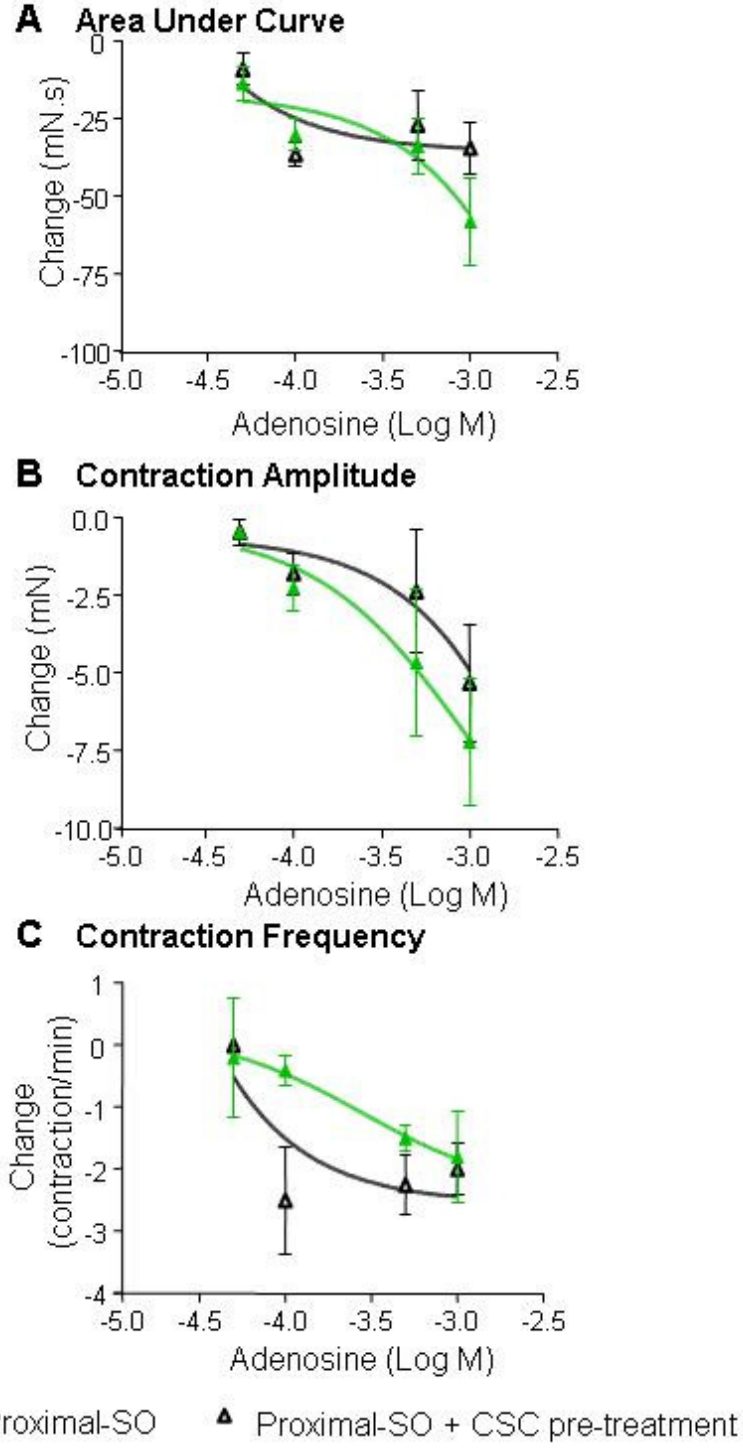
required to elicit a response raised concerns regarding receptor sub-type selectivity of each agonist. Due to these difficulties no further experiments were performed.

Figure 7.1: Lack of effect of A_1 receptor antagonist DPCPX pre-treatment on the adenosine-induced decrease in proximal-SO muscle ring contractile activity



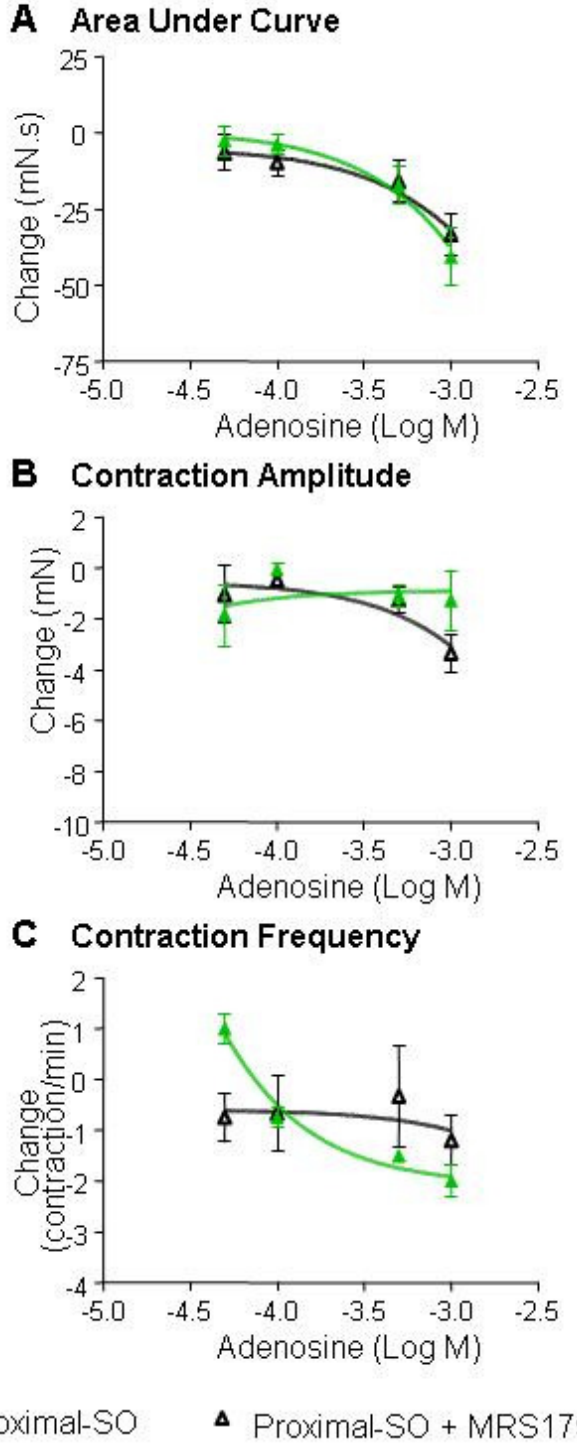
Group data showing the lack of effect of pre-treatment with the A_1 antagonist DPCPX (10nM), on the adenosine-induced decrease in proximal-SO muscle ring activity expressed as area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P>0.05$ for all) (n=4-7 muscle rings/concentration, from 15 possums)

Figure 7.2: Lack of effect of A_{2A} receptor antagonist CSC pre-treatment on the adenosine-induced decrease in proximal-SO muscle ring contractile activity



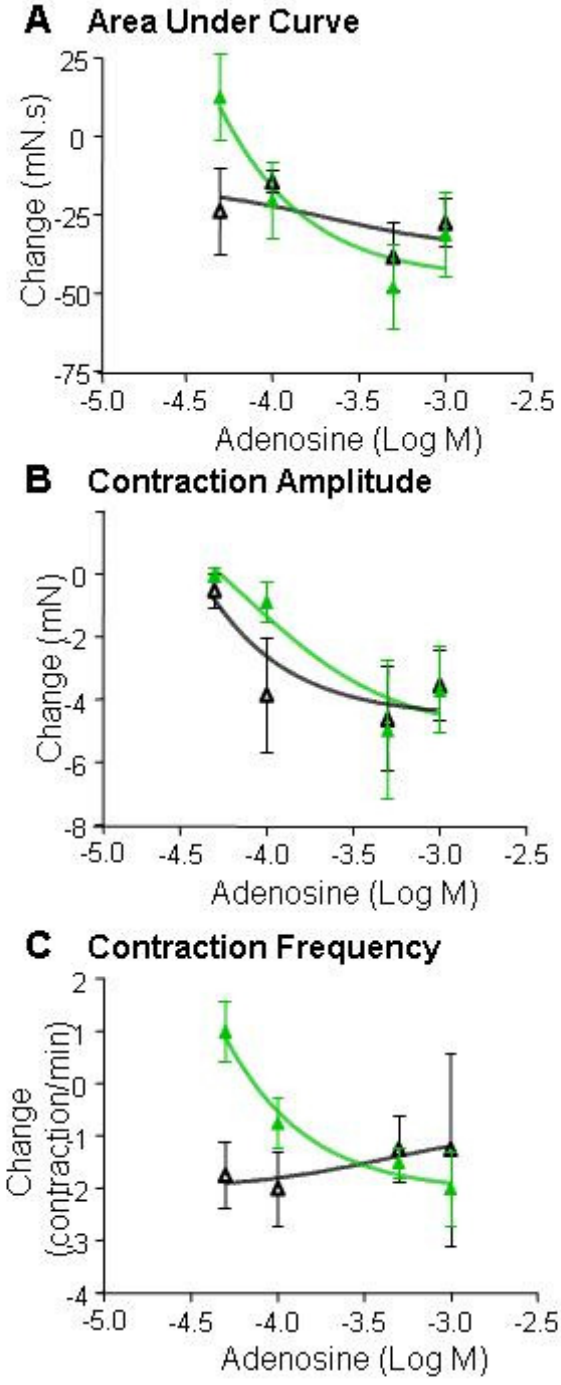
Group data showing the lack of effect of pre-treatment with the A_{2A} receptor antagonist CSC on the adenosine-induced decrease in proximal-SO muscle ring contractile activity expressed as for area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P>0.05$ for all) (n=4 muscle rings/concentration, from 11 possums).

Figure 7.3: Lack of effect of A_{2B} receptor antagonist MRS1754 pre-treatment on the adenosine-induced decrease in proximal-SO muscle ring contractile activity



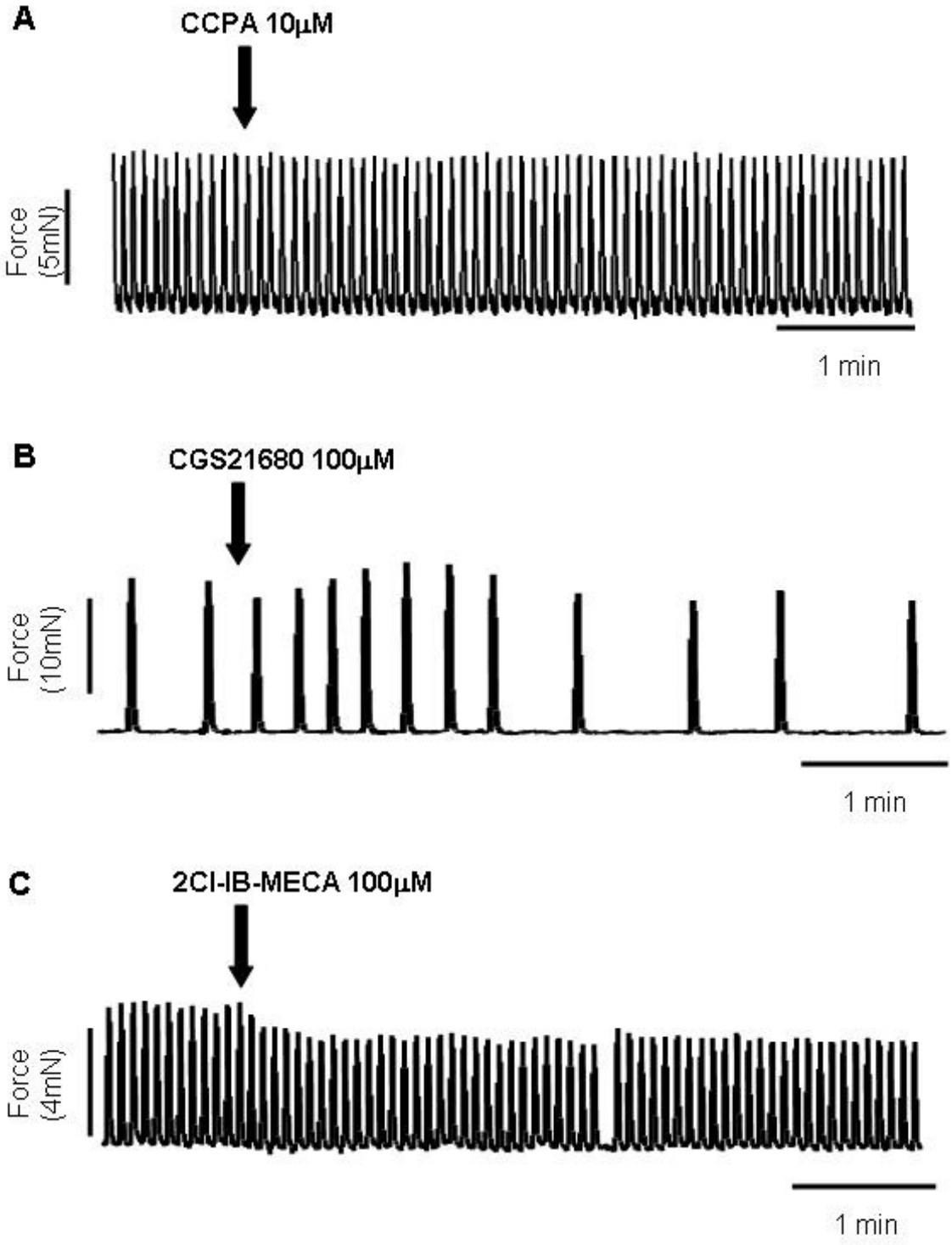
Group data showing the lack of effect of pre-treatment with the A_{2B} receptor antagonist MRS1754 on the adenosine-induced decrease in proximal-SO muscle ring contractile activity expressed as for area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) ($P>0.05$ for all) (n=4-6 muscle rings/concentration, from 18 possums).

Figure 7.4: Lack of effect of A_3 receptor antagonist MRS1220 pre-treatment on the adenosine-induced decrease in proximal-SO muscle ring contractile activity



Group data showing the lack of effect of pre-treatment with the A_3 receptor antagonist MRS1220 on the adenosine-induced decrease in proximal-SO muscle ring contractile activity expressed as for area under curve (**A**), contraction amplitude (**B**) and contraction frequency (**C**) (all $P>0.05$) (n=4 muscle rings/concentration, from 12 possums).

Figure 7.5: Effect of P1 receptor sub-type selective agonists on spontaneous proximal-SO muscle ring contractile activity



Representative Chart recordings illustrating the lack of effect of the A_1 agonist CCPA (10μM) (**A**), a bi-phasic response to the A_{2A} agonist CGS21680 (**B**), and an inhibitory response to the A_3 agonist 2Cl-IB-MECA (**C**) in proximal-SO muscle rings. Note: variable responses were observed with CGS21680 and 2Cl-IB-MECA. Arrow indicates time of agonist application to the organ bath.

Table 7.1: Summary of effects of P1 agonists and antagonists on spontaneous proximal-SO muscle ring contractile activity

Receptor Selectivity	Antagonist	Antagonised adenosine-induced response	Agonist	Produced inhibition of contractile activity
Non-selective			adenosine	✓
A_1	DPCPX	x	CCPA	x
A_{2A}	CSC	x	CGS21680	Inconsistent bi-phasic response
A_{2B}	MRS1754	x	N/A	-
A_3	MRS1220	x	2Cl-IB-MECA	Inconsistent

x No effect of antagonist/agonist
✓ Effect of antagonist/agonist
N/A Not available

Note: the high concentration of P1 agonists required to obtain a response in proximal-SO muscle rings likely results in loss of receptor selectivity.

7.4 Discussion

Surprisingly the data presented in this chapter found that each of the A_1, A_{2A}, A_{2B} and A_3 antagonists failed to block the adenosine-induced decrease in proximal-SO motility. Studies with selective agonists also failed to clearly define the receptor sub-types involved due to inconsistent responses at very high concentrations. The data is summarized in **Table 7.1**.

The current study used antagonists that have been previously characterised with rat and human tissues, and previously tested in possum duodenum (**Chapter 6**). Interestingly none of these antagonists tested antagonized the adenosine-induced response. This is somewhat surprising as similar experiments using possum duodenal longitudinal muscle strips found the adenosine-induced decrease in contractile activity was blocked by CSC and inhibited by MRS1220, with DPCPX and IPDX having no effect. This difference between the SO and duodenal data may represent true differences in the P1 receptor sub-types mediating the adenosine-induced inhibition of motility in these two tissues.

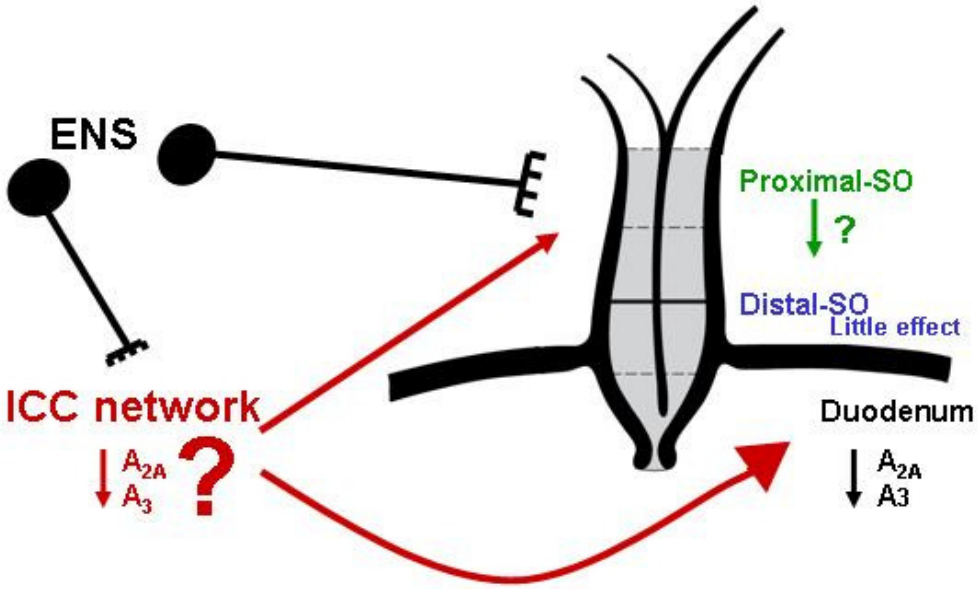
Specific receptor sub-type agonists were evaluated in order to define the particular receptor sub-type(s) involved in the adenosine-induced proximal-SO response. Application of these agonists at concentrations effective in duodenal muscle strip preparations (**Chapter 6**) failed to elicit a response in proximal-SO muscle rings. The use of higher concentrations produced variable responses with the A_{2A} and A_3 agonists, but likely results in loss of receptor selectivity. No selective A_{2B} receptor agonist was available to test and the common practice of using NECA to activate both A_{2A} and A_{2B}

receptors with simultaneous blockade of A_{2A} receptors with CSC was not possible, as the effectiveness of CSC as an antagonist in the proximal-SO was uncertain. Therefore, further experiments were not performed, and the receptor sub-types involved cannot be identified until pharmacological agents effective in possum tissues are available.

7.5 Conclusion

In conclusion, it was not possible to definitively identify the receptors that mediate the adenosine-induced decrease in spontaneous proximal-SO motility. Further experiments with more potent and selective P1 agonists and antagonists are required.

SUMMARY 2: EXOGENOUS ADENOSINE DECREASES SPONTANEOUS SO AND DUODENAL MOTILITY *IN VITRO*



Application of exogenous adenosine decreased SO and duodenal motility *in vitro*. Interestingly the effects of adenosine predominated in the proximal-SO, with little effect at the distal-SO. The decrease in contractile activity was not neurally mediated in either the proximal-SO or duodenum. This suggests adenosine activates P1 receptors located on smooth muscle or on ICC, which could then have an indirect effect by decreasing slow wave activity. The adenosine-induced inhibitory responses in the duodenum are mediated by A_{2A} and A_3 receptors. The receptors responsible for the adenosine-induced inhibitory response in the proximal-SO could not be clearly identified.

Chapter 8

8 EFFECT OF EXOGENOUS ADENOSINE AND ATP ON SO MOTILITY *IN VIVO*

8.1 Introduction

In vitro studies with possum SO muscle rings found exogenous ATP induced a neurally mediated excitation followed by a TTX-insensitive inhibition of spontaneous activity. This response was predominant in proximal-SO muscle rings, and experiments were performed to identify the P2 receptor sub-types involved (**Chapters 3 and 5**). In contrast, exogenous adenosine only induced a TTX-insensitive inhibition of spontaneous SO motility. This response was also predominant in the proximal-SO muscle rings, but the P1 receptor sub-types involved were unable to be identified (**Chapters 3 and 7**). SO motility is modulated by intrinsic and extrinsic neural activity, together with inputs from neural reflexes between the SO and stomach, duodenum, and CBD/gallbladder (**Section 1.3.4**). However few studies have characterised the neurotransmitters involved in these reflexes with the SO, and none have determined if purines are involved.

Before experiments can be designed to specifically determine if purines are involved in inter-sphincteric reflexes, it is first necessary to determine:

1. The effect of exogenous adenosine/ATP on SO motility *in vivo* – are the *in vivo* responses the same as the *in vitro* responses?
2. If the SO response to exogenous adenosine/ATP is neurally mediated, and if so to characterise the pathway(s) involved.

8.2 Methods

To examine these aims *in vivo* preparation was used, where intra-sphincteric reflexes, along with other SO neural reflexes remained functional. Possums were anaesthetized and ventilated as described in **section 2.3**. A multi-lumen manometry catheter was inserted into the bile duct component of the SO to measure SO motility (**Figure 2.4** and **Figure 2.5**).

The experimental protocol used was described in **section 2.3.3**. Briefly, increasing doses of exogenous ATP or adenosine were applied topically (10μl) to the entire extra-duodenal surface of the SO, allowing 5-10min between applications (**Section 2.3.3**). The dose-response curve was repeated after pre-treatment with the neurotoxin TTX, the nicotinic receptor antagonist hexamethonium, the muscarinic receptor antagonist atropine, or the NOS inhibitor L-NAME (**Section 2.3.3**).

Refer to **Section 2.3** for drug preparation and analysis of recordings. As direct application of adenosine/ATP to the most distal-SO was not possible, only recordings from the proximal-SO recording site were analysed.

8.3 Results

8.3.1 Effect of exogenous ATP or adenosine on SO motility *in vivo*

All preparations displayed spontaneous SO contractile activity. Topical application of ATP (1μM-1mM; n=14) to the SO increased proximal-SO contractile activity, which persisted for up to 170±16s with the 1mM concentration (**Figure 8.1**). ATP concentration-dependently increased

proximal-SO activity expressed as area under curve (P<0.05), contraction amplitude (P<0.05), and frequency (P<0.05), but there was no significant change in SO basal pressure (P>0.05) (**Figure 8.2**). Similarly exogenous adenosine (1μM-10mM; n=17) increased proximal-SO contractile activity, which persisted for up to for up 255±102s with the 10mM concentration (**Figure 8.3**). Adenosine concentration-dependently increased SO motility expressed as area under curve (P<0.05), contraction amplitude (P<0.05), and frequency (P<0.05), but there was no significant change in SO basal pressure (**Figure 8.4**). As neither ATP nor adenosine application influenced SO basal pressure, this parameter is not reported in the following experiments.

8.3.2 TTX pre-treatment

To assess if the ATP- or adenosine-induced responses were neurally mediated, the influence of TTX pre-treatment was assessed. TTX pre-treatment blocked the ATP-induced excitation for area under curve (P<0.05), contraction amplitude (P<0.05) and contraction frequency (P<0.05) (n=6) (**Figure 8.5**). Similarly TTX pre-treatment blocked the adenosine-induced excitation for area under curve (P<0.05), contraction amplitude (P<0.05) and contraction frequency (P<0.05) (n=5) (**Figure 8.6**).

8.3.3 Atropine and hexamethonium pre-treatment

Atropine pre-treatment inhibited the ATP-induced increase in proximal-SO activity, abolishing the concentration-response relationship (all parameters P<0.05) (n=6) (**Figure 8.7**). However, pre-treatment with atropine did not completely block the response induced by the highest concentration of ATP.

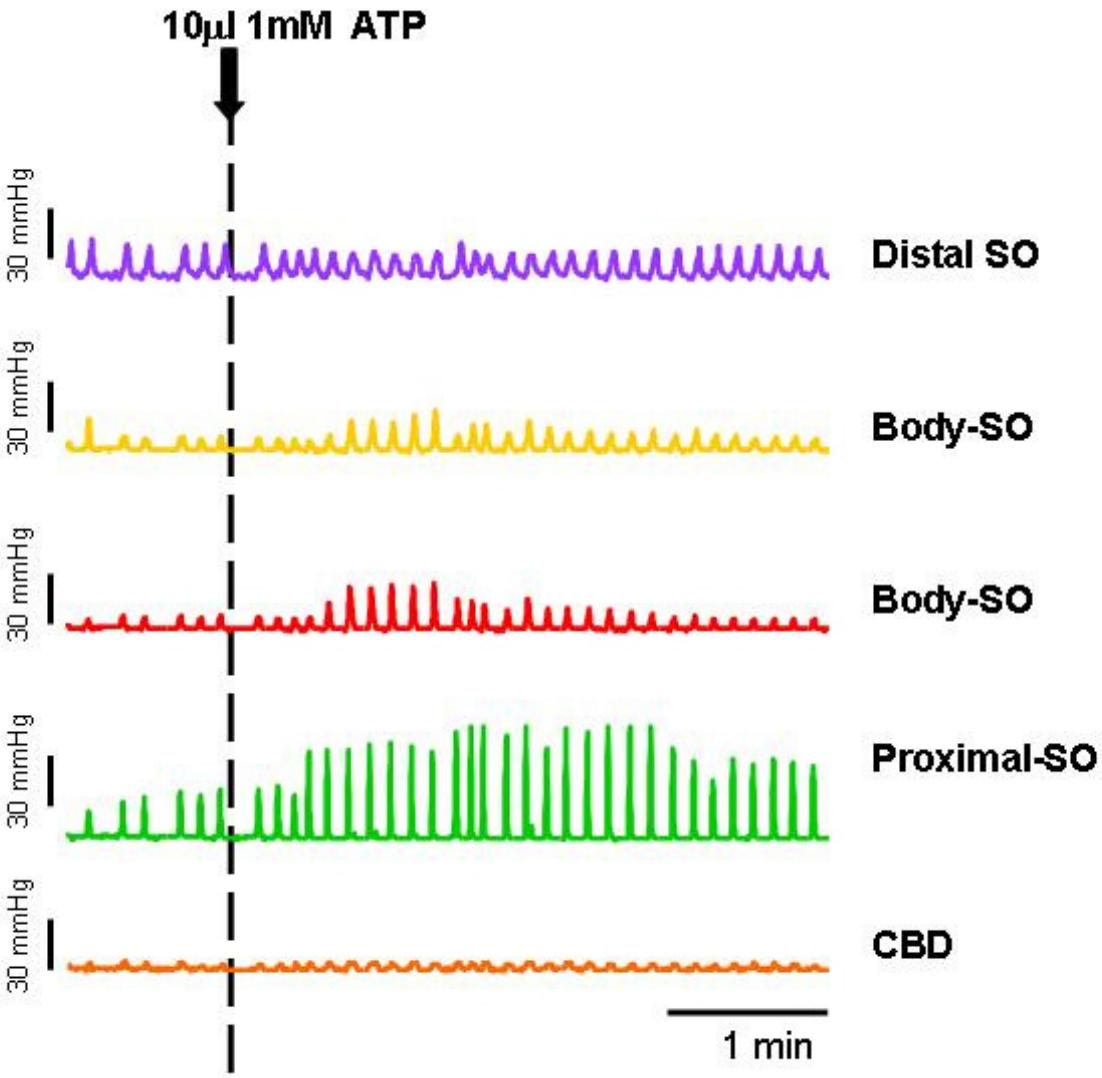
Atropine pre-treatment completely blocked the adenosine-induced increase in contractile activity (all parameters $P<0.05$) (n=4) (**Figure 8.8**).

Interestingly, pre-treatment with hexamethonium enhanced the ATP-induced proximal-SO response (n=6). Increases in motility were observed for area under curve ($P<0.05$) and contraction amplitude ($P<0.05$), but not contraction frequency (**Figure 8.9**). In contrast hexamethonium pre-treatment did not significantly modify the adenosine-induced proximal-SO response (all parameters $P>0.05$) (n=5) (**Figure 8.10**).

8.3.4 Inhibition of NOS: L-NAME

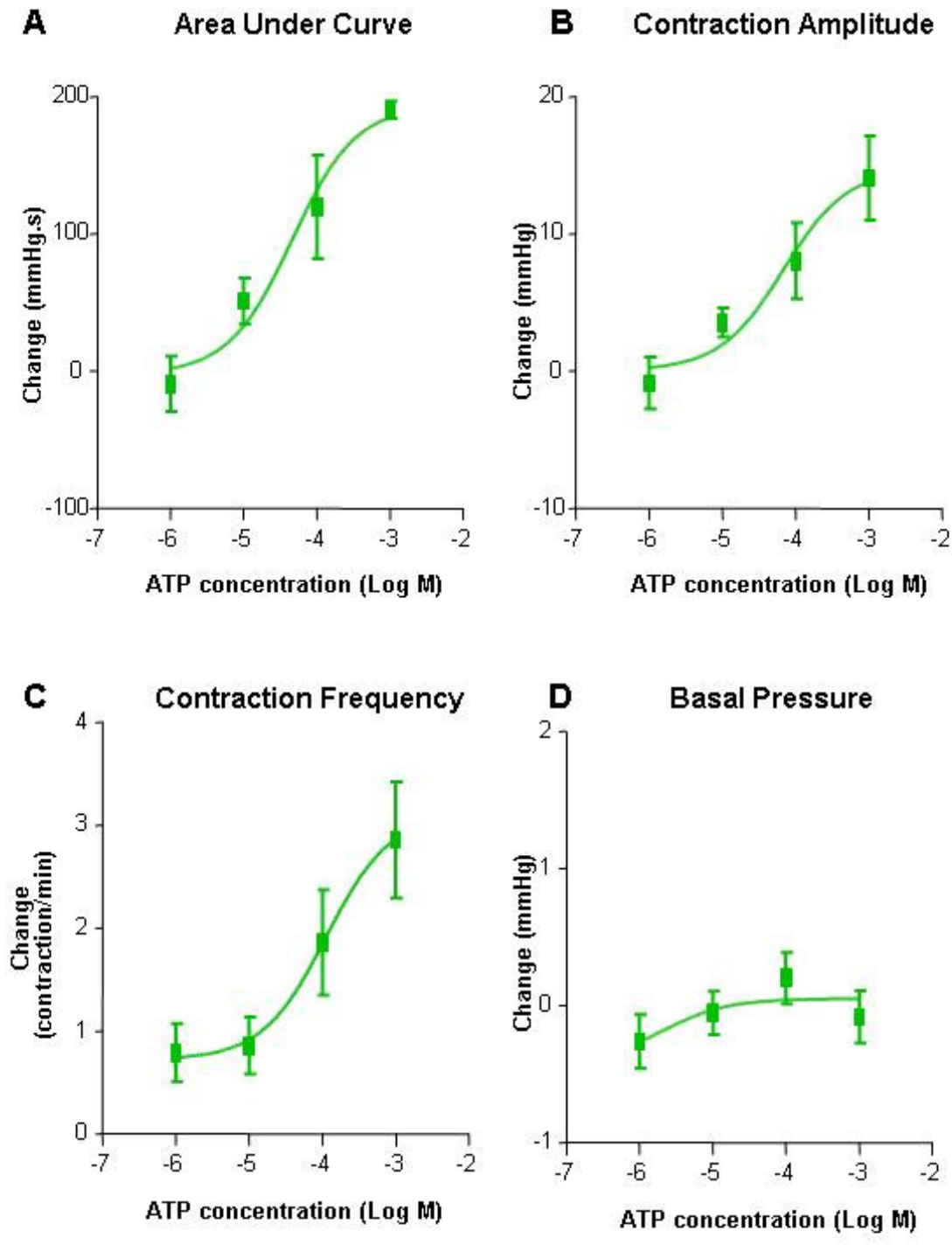
Inhibition of NOS activity with L-NAME pre-treatment did not affect the magnitude of the ATP-induced excitation of proximal-SO activity ($P>0.05$) (n=6) (**Figure 8.11**). However, analysis of the duration of the ATP-induced response found that it increased from 170±16s to 203 ± 15s with 1mM ATP after L-NAME pre-treatment ($P<0.05$) (**Figure 8.11**). In contrast, L-NAME pre-treatment did not significantly modify the adenosine-induced increase in proximal-SO activity for any parameter measured ($P>0.05$ for all) (n=6) (**Figure 8.12**).

Figure 8.1: Topical ATP application increases SO activity



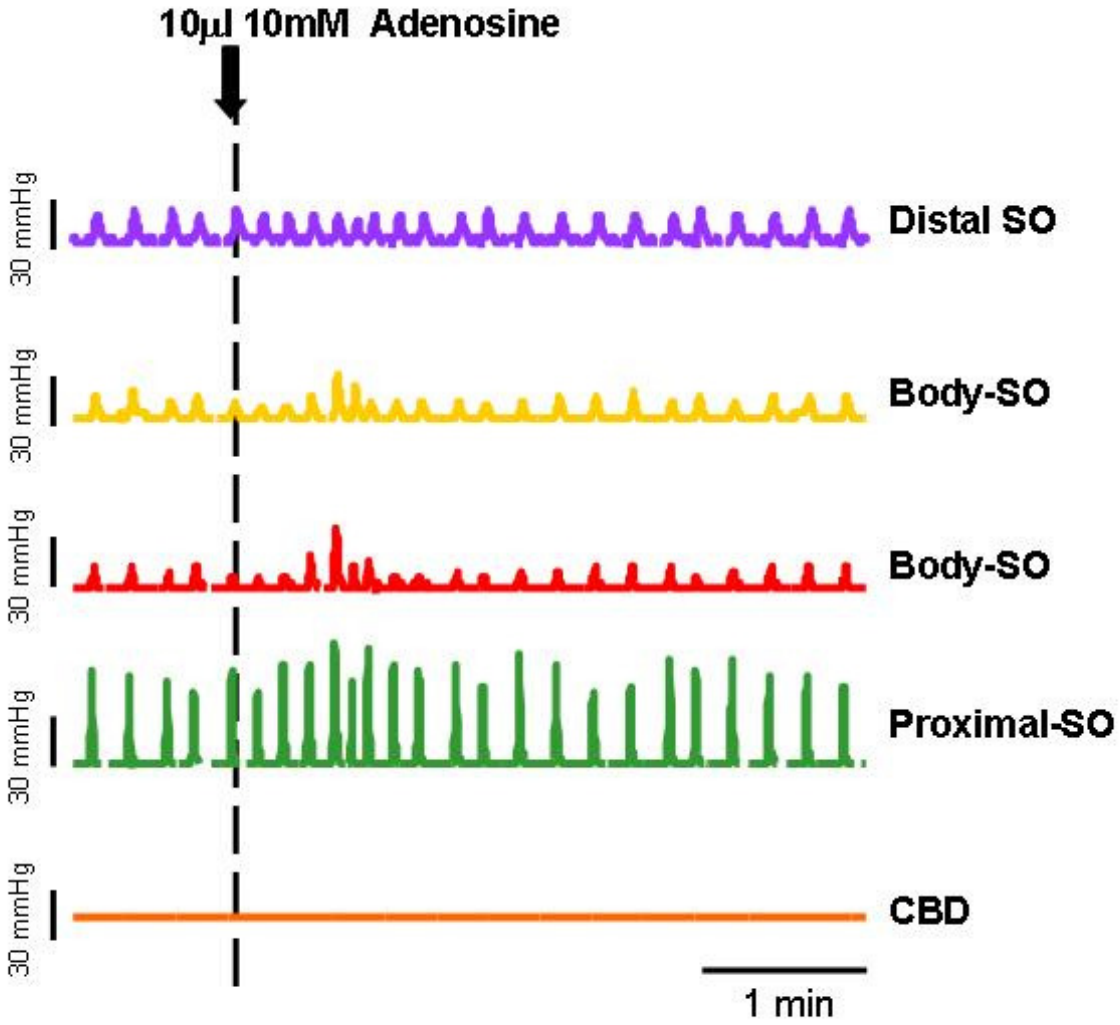
Representative Chart recording showing four simultaneous recordings from within the SO and CBD. Increases in SO activity were recorded in all channels, however the response was greatest in the proximal-SO region. Note: ATP could not be directly applied to the most distal-SO region as it is intra-duodenal.

Figure 8.2: Effect of ATP on proximal-SO motility *in vivo*



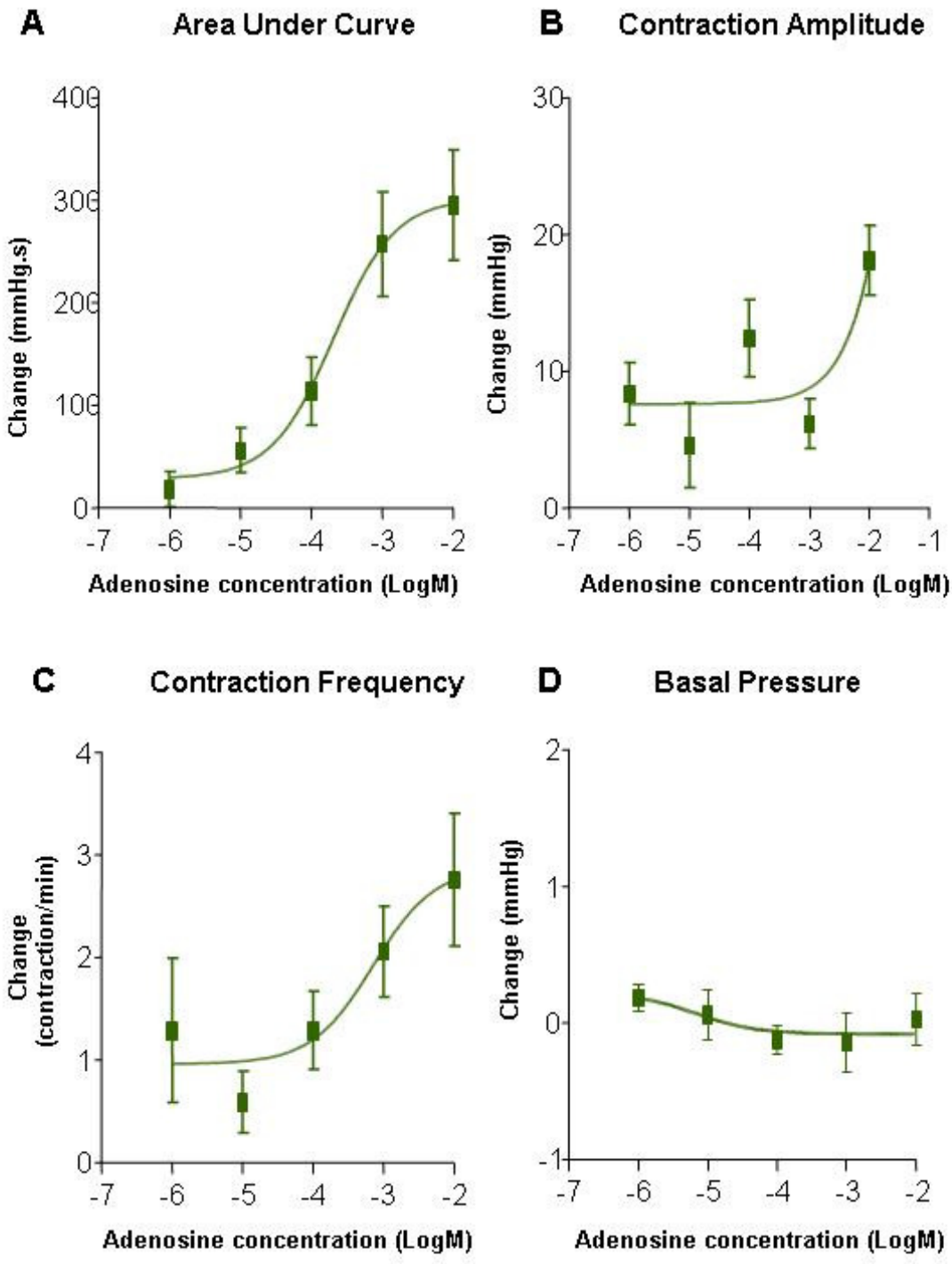
ATP (n=14) dose-dependently increased proximal-SO activity for area under curve (**A**) ($P<0.05$), contraction amplitude (**B**) ($P<0.05$) and contraction frequency (**C**) ($P<0.05$). There was no significant change in basal pressure (**D**) in response to ATP application.

Figure 8.3: Topical Adenosine application increases SO activity



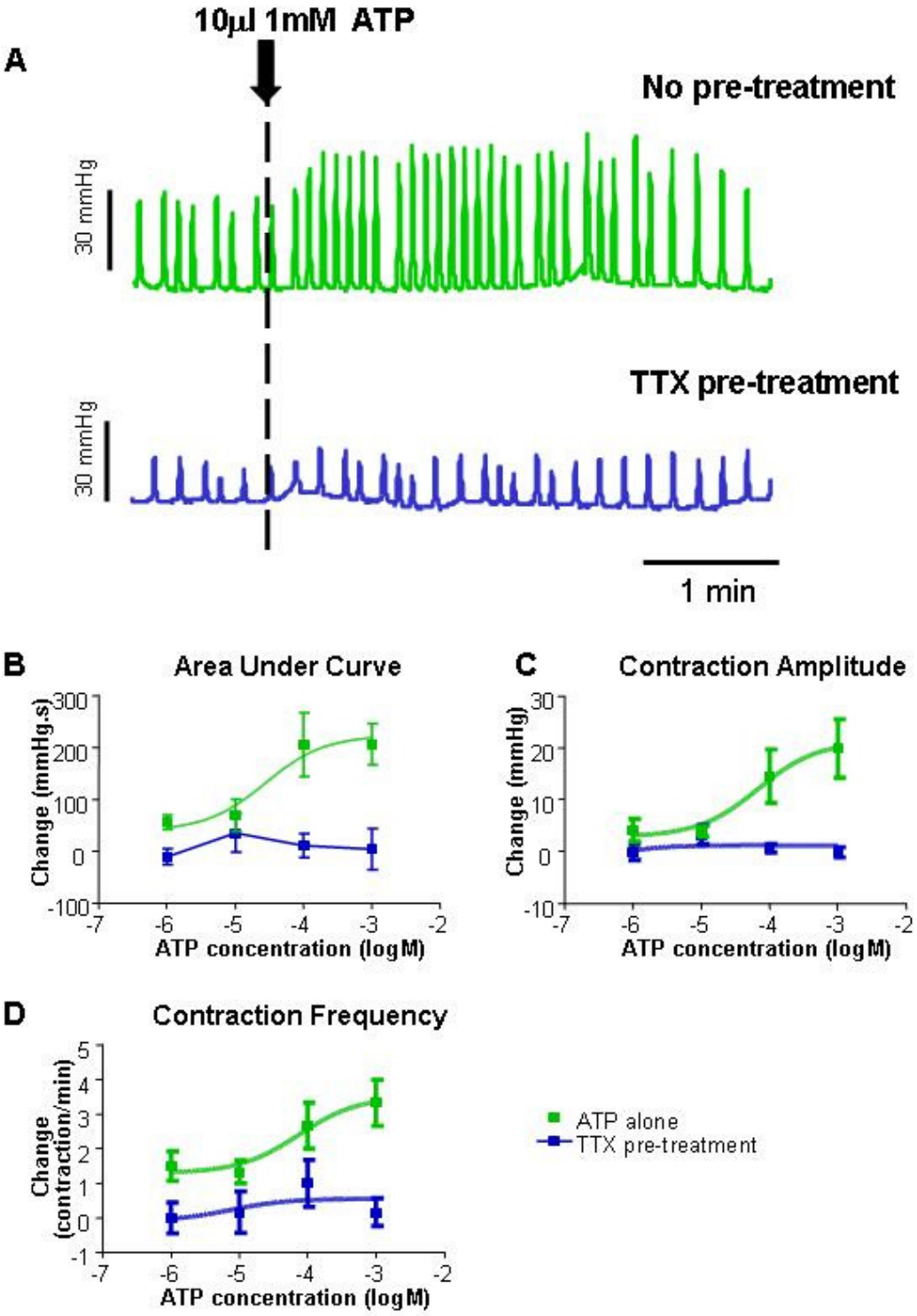
Representative Chart recording showing four simultaneous recordings from within the SO and CBD. Increases in SO activity were recorded in all channels, however the response was greatest in the proximal-SO region. Note: adenosine could not be directly applied to the most distal-SO region as it is intra-duodenal.

Figure 8.4: Effect of adenosine on proximal-SO motility *in vivo*



Adenosine (n=17) dose-dependently increased proximal-SO activity for area under curve (**A**) ($P<0.05$), contraction amplitude (**B**) ($P<0.05$) and contraction frequency (**C**) ($P<0.05$). There was no significant change in basal pressure (**D**) in response to adenosine application.

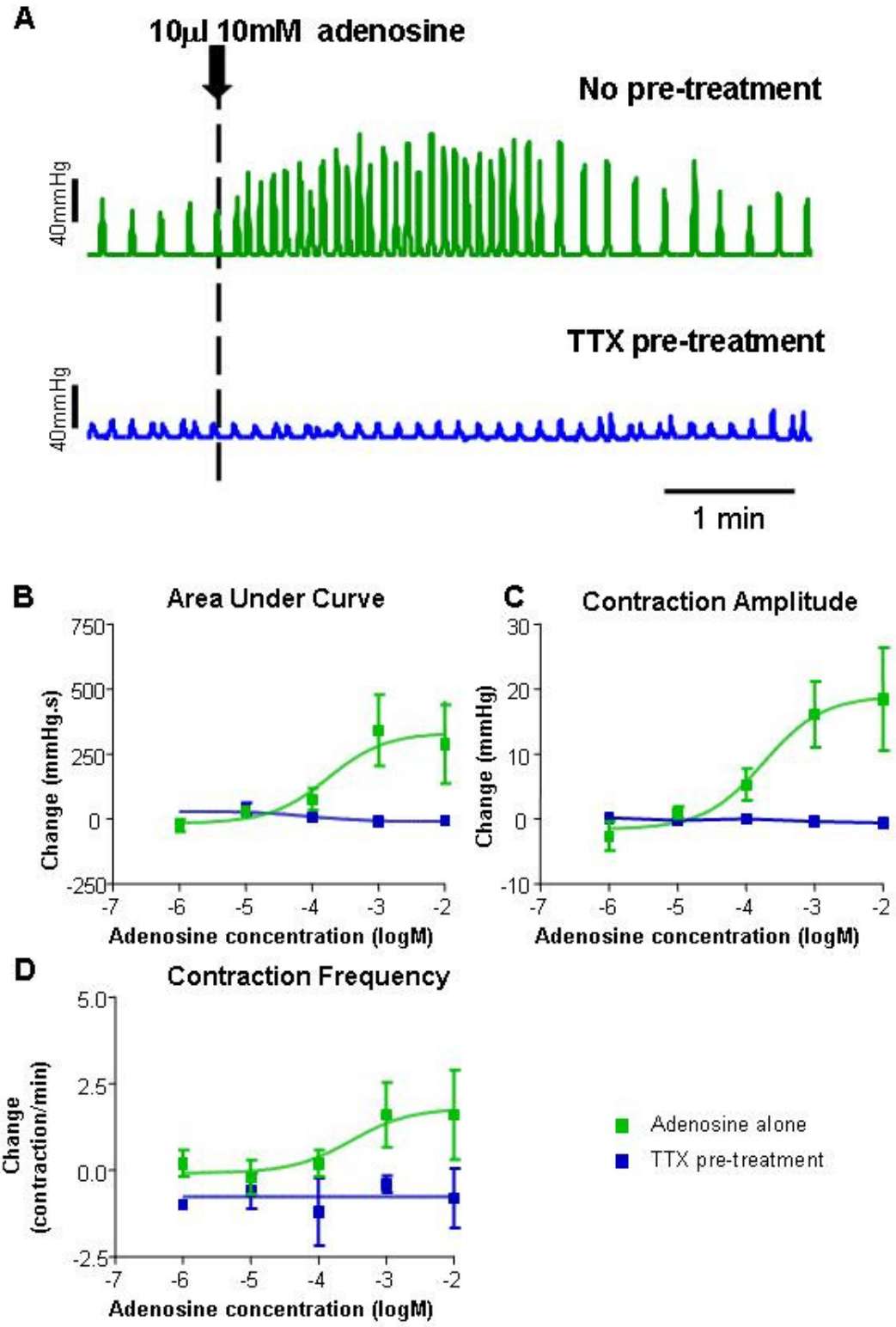
Figure 8.5: Neural blockade inhibits ATP-induced proximal-SO activity



Representative Chart recording from one preparation illustrating the response to ATP (1mM) before and after TTX pre-treatment (**A**). TTX (n=6) blocks the ATP-induced increase in SO activity for area under curve (**B**) ($P<0.05$), contraction amplitude (**C**) ($P<0.05$) and contraction frequency (**D**) ($P<0.05$).

204

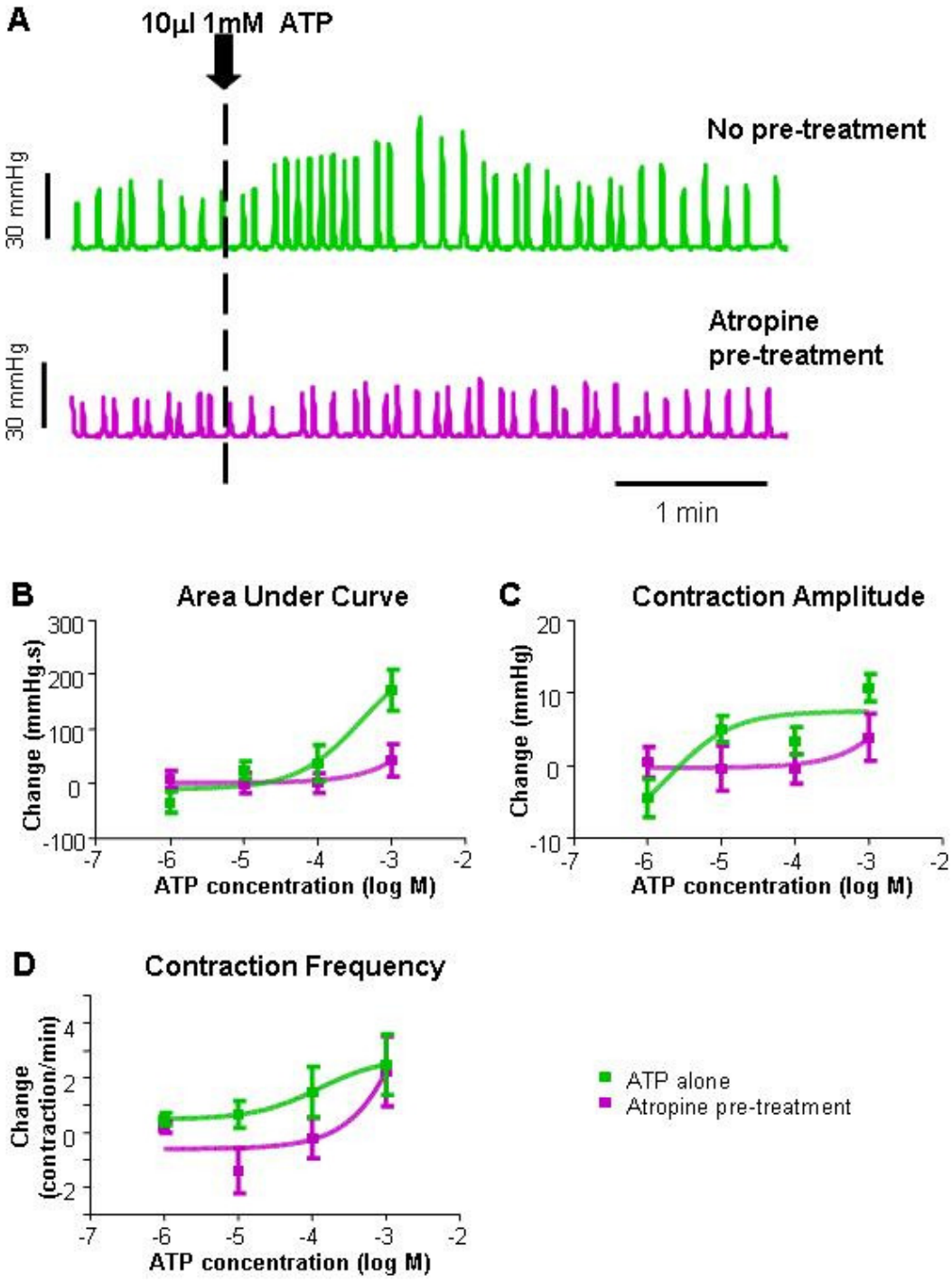
Figure 8.6: Neural blockade inhibits adenosine-induced proximal-SO activity



Representative Chart recording from one preparation illustrating the response to adenosine (10mM) before and after TTX pre-treatment (**A**). TTX (n=5) blocks the adenosine-induced increase in SO activity for area under curve (**B**) ($P<0.05$), contraction amplitude (**C**) ($P<0.05$) and contraction frequency (**D**) ($P<0.05$).

205

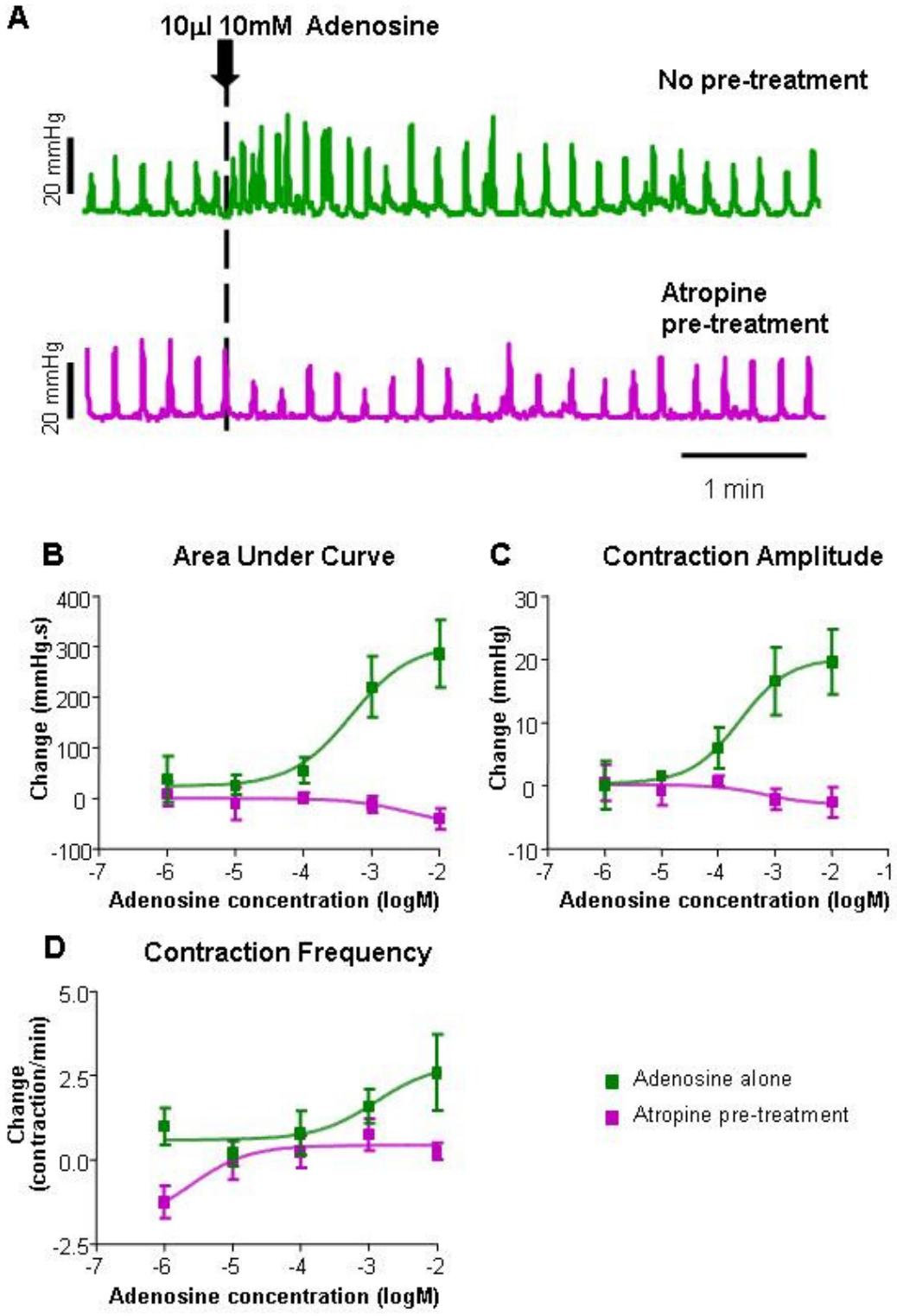
Figure 8.7: Blockade of muscarinic receptors inhibits ATP-induced proximal-SO activity



Representative Chart recording from one preparation illustrating the response to ATP (1mM) before and after atropine pre-treatment (**A**). Atropine (n=6) inhibits the ATP-induced increase in SO activity for area under curve (**B**), contraction amplitude (**C**) and contraction frequency (**D**). For each parameter the dose-response was abolished in the presence of atropine ($P<0.05$), however treatment with atropine did not block the response induced by the highest concentration of ATP.

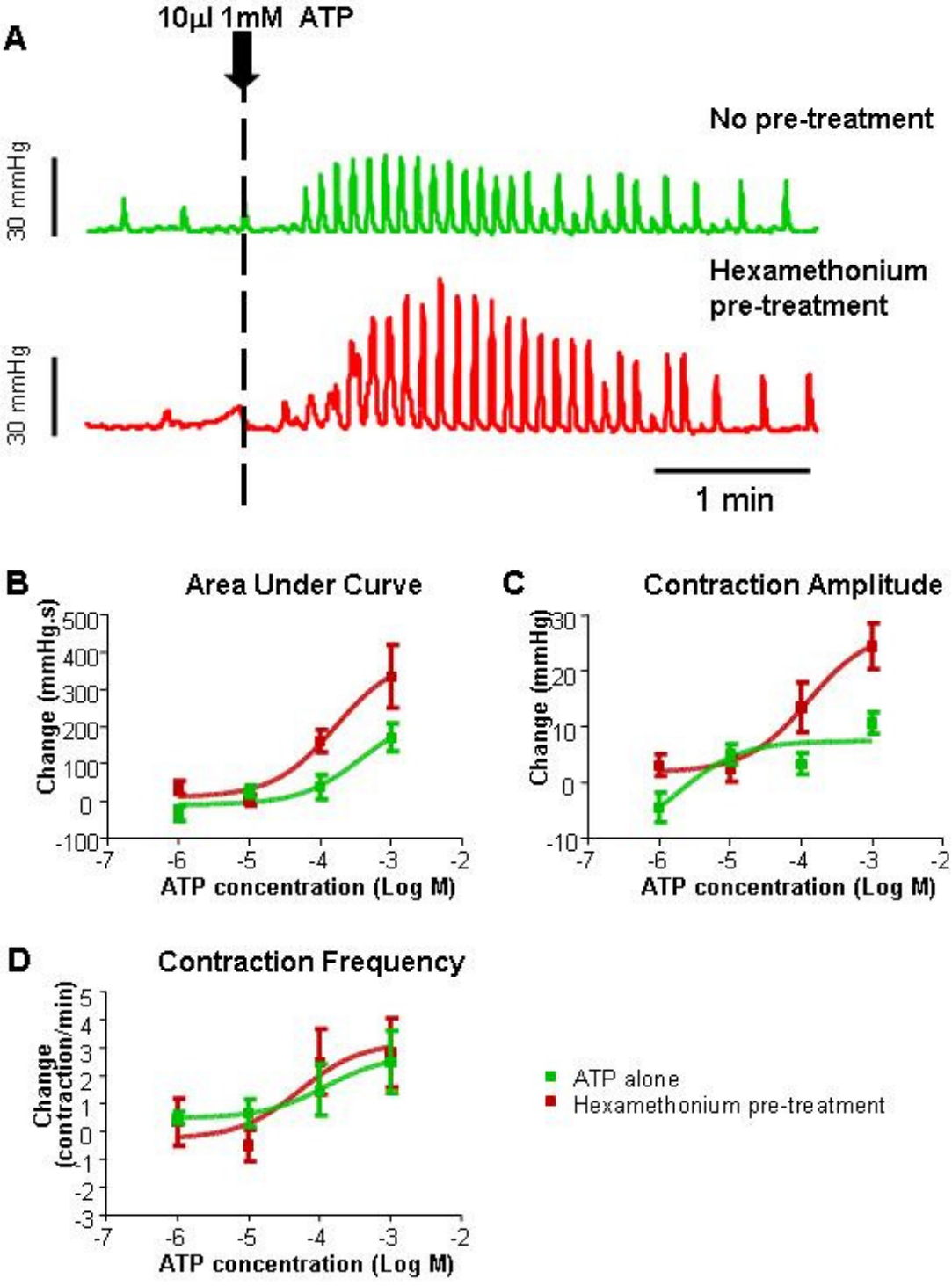
206

Figure 8.8: Blockade of muscarinic receptors inhibits adenosine-induced proximal-SO activity



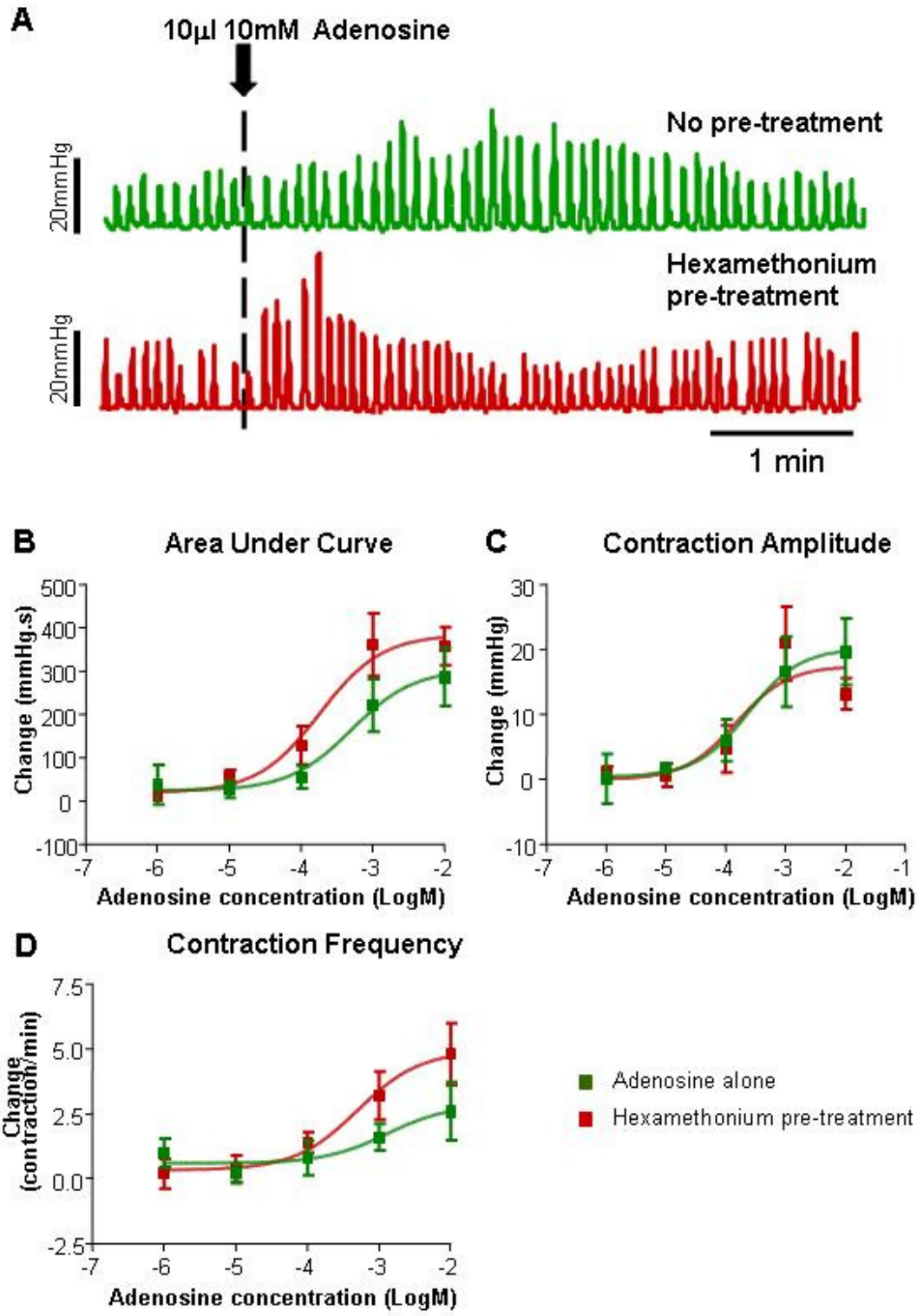
Representative Chart recording from one preparation illustrating the response to adenosine (10mM) before and after atropine pre-treatment (**A**). Atropine (n=4) blocks the adenosine-induced increase in SO activity for area under curve (**B**) ($P<0.05$), contraction amplitude (**C**) ($P<0.05$) and contraction frequency (**D**) ($P<0.05$).

Figure 8.9: Blockade of nicotinic receptors enhances ATP-induced proximal-SO activity



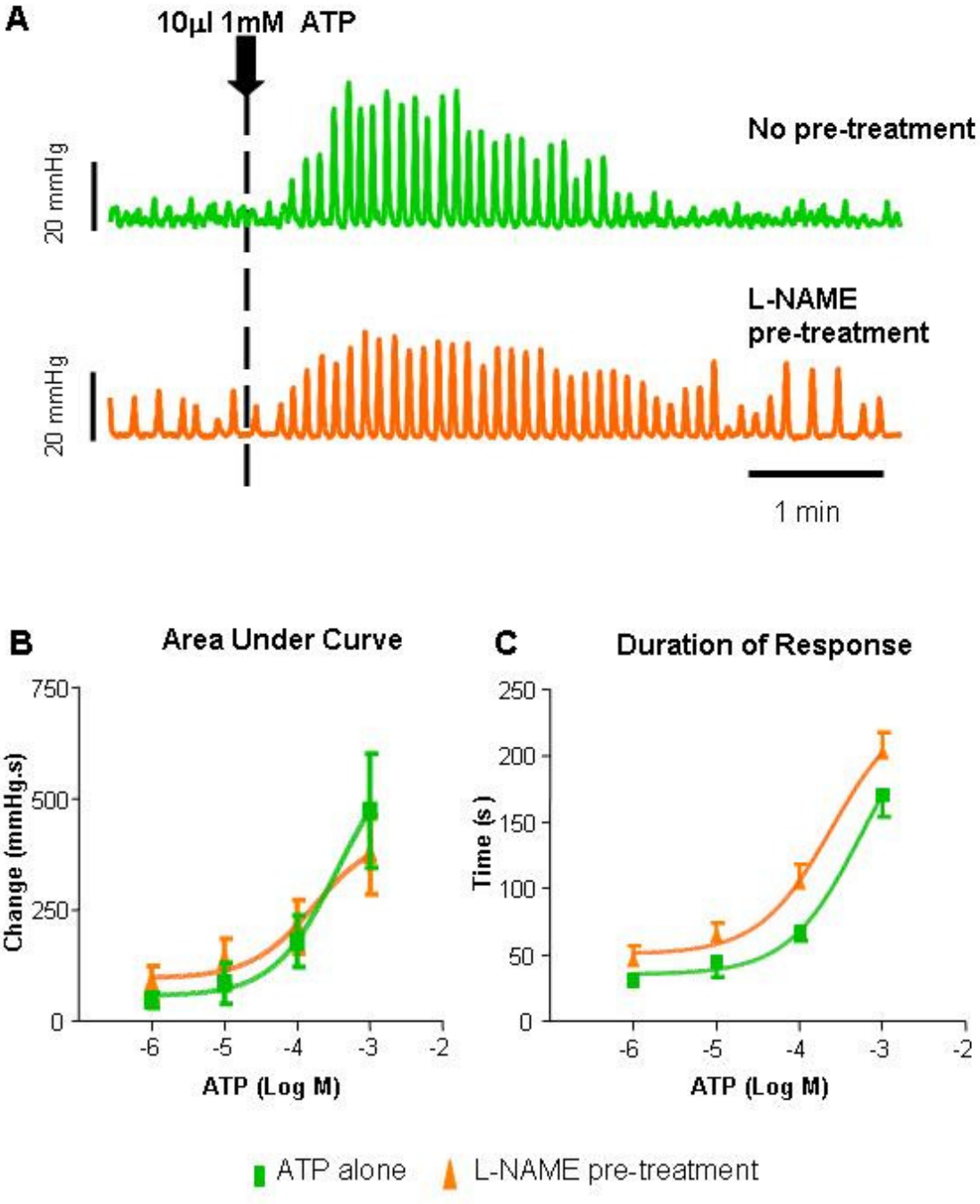
Representative Chart recording from one preparation illustrating the response to ATP (1mM) before and after hexamethonium pre-treatment (**A**). Hexamethonium (n=6) enhances the ATP-induced increase in SO activity for area under curve (**B**) ($P<0.05$) and contraction amplitude (**C**) ($P<0.05$), but does not affect contraction frequency (**D**).

Figure 8.10: Blockade of nicotinic receptors enhances adenosine-induced proximal-SO activity



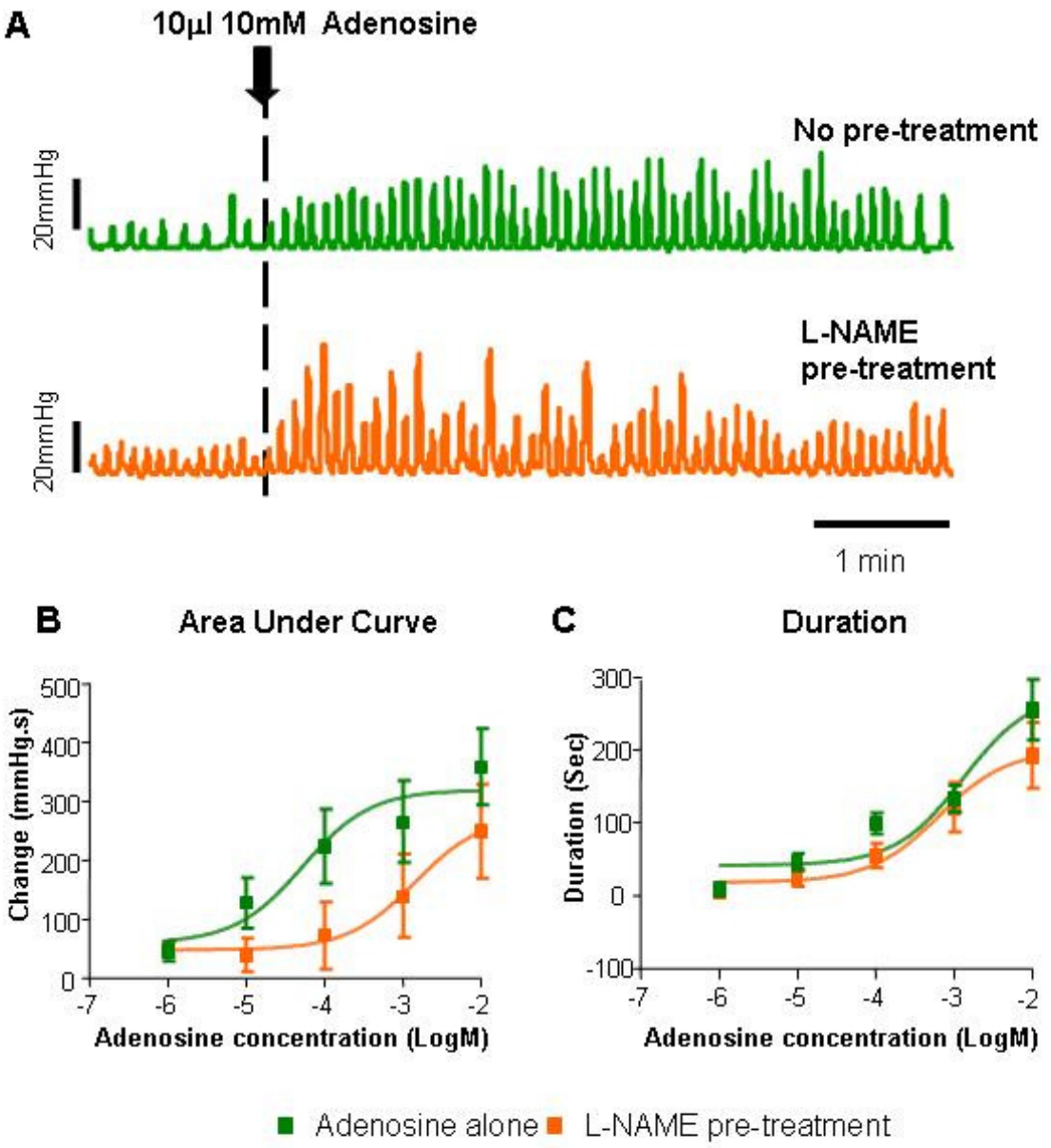
Representative Chart recording from one preparation illustrating the response to adenosine (10mM) before and after hexamethonium pre-treatment (**A**). Hexamethonium (n=5) did not significantly modify the adenosine-induced increase in SO activity for area under curve (**B**), contraction amplitude (**C**), or contraction frequency (**D**) (P>0.05 for all).

Figure 8.11: Inhibition of NOS enhances the duration of the ATP-induced proximal-SO response



Representative Chart recording from one preparation illustrating the response to ATP (1mM) before and after L-NAME pre-treatment (**A**). L-NAME (n=6) did not significant increase in the peak response for area under curve (**B**) ($P>0.05$), however it did enhance the duration of the ATP-induced response (**C**) ($P<0.05$).

Figure 8.12: Blockade of NOS does not affect the adenosine-induced proximal-SO response

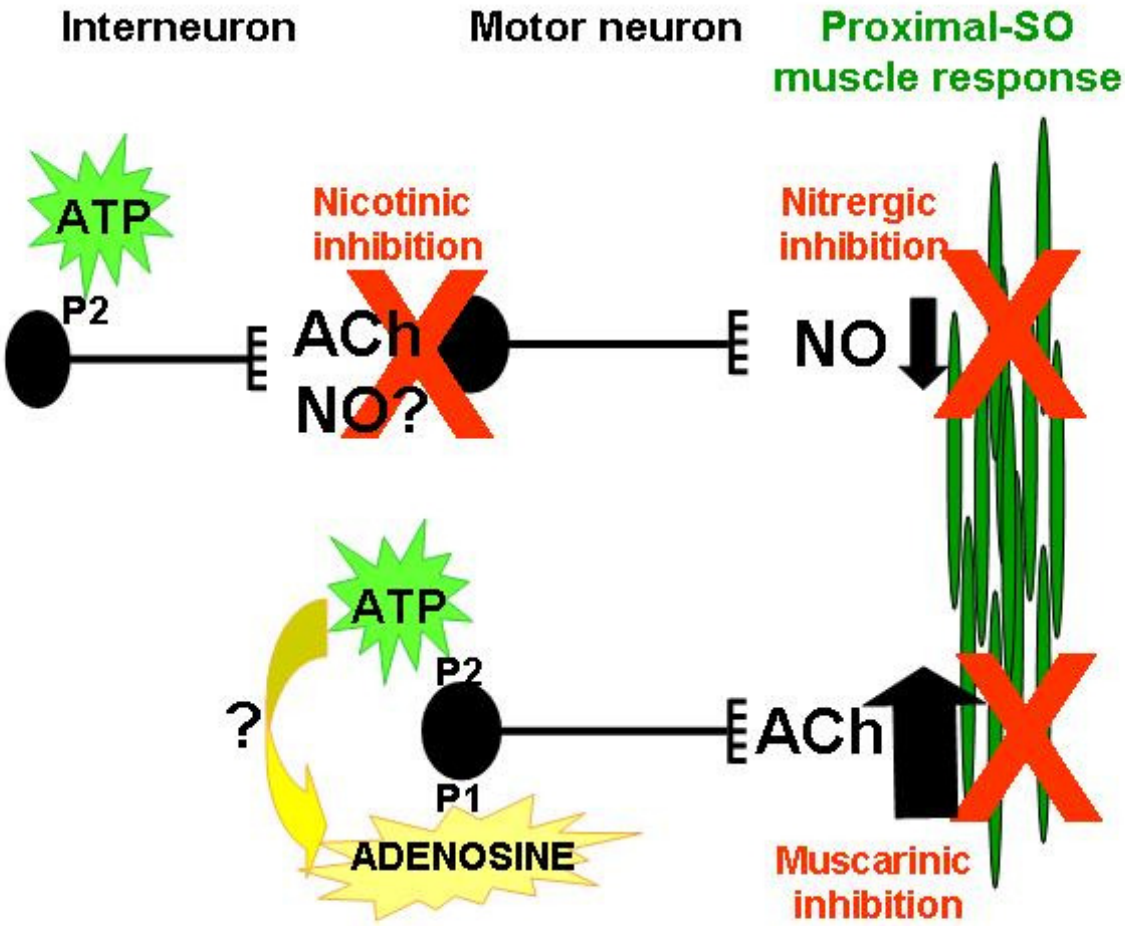


Representative Chart recording from one preparation illustrating the response to adenosine (10mM) before and after L-NAME pre-treatment (**A**). There was a trend for L-NAME pre-treatment (n=6) to decrease the magnitude of the adenosine-induced response, especially with the middle adenosine concentrations, but this was not statistically significant for area under curve (**B**), or other parameters (data not shown), or the duration of the adenosine-induced response (**C**) (all P>0.05). Note some error bars are within the size of the symbol.

Table 8.1: Summary of effects of neural, cholinergic and nitric oxide blockers on the ATP- and adenosine-induced responses on proximal-SO activity *in vivo*

Agonist	Antagonist/Blocker	Proximal-SO Response
ATP	Alone	Excitation
	TTX	Blocked excitation
	Atropine	Blocked excitation (except at 1mM ATP)
	Hexamethonium	Enhanced excitation
	L-NAME	Enhanced duration of ATP-response
Adenosine	Alone	Excitation
	TTX	Blocked excitation
	Atropine	Blocked excitation
	Hexamethonium	No significant effect on adenosine-response
	L-NAME	No significant effect on adenosine-response

Figure 8.13: Proposed mechanisms underlying the action of exogenous ATP and adenosine on the proximal-SO *in vivo*



Exogenous ATP acts on neural P2 receptors, or maybe degraded to adenosine with activation of neural P1 receptors, to increase proximal-SO activity. Primarily proximal-SO activity is increased by activation of P2 and/or P1 receptors located on cholinergic motor neurons. An inhibitory pathway also exists where ATP activates P2, but not P1, receptors located on interneurons leading to the release of nitric oxide from inhibitory motor neurons, however this pathway may only observed when the stimulatory cholinergic motor neurons are blocked. The proximal-SO muscle response is the sum of the effects of excitatory and inhibitory transmitters.

ACh, acetylcholine; NO, nitric oxide

8.4 Discussion

The experiments described in this chapter have demonstrated that exogenous ATP and adenosine increase proximal-SO activity via a complex mechanism. The excitatory effect of ATP involved muscarinic receptors. Furthermore, ATP's action also involves inhibitory neural pathways utilizing nitrergic and nicotinic mechanisms. Exogenous adenosine also increases proximal-SO activity via a neural pathway activating muscarinic receptors, but unlike ATP does not involve nitrergic or nicotinic mechanisms (data summarized in **Table 8.1**). The overall response of the proximal-SO to exogenous ATP is dependent on the integration of excitatory and inhibitory signals at the smooth muscle cells, some of which may be mediated by adenosine (**Figure 8.13**). These data suggests that endogenous ATP and adenosine may be involved in the neural regulation of possum SO function by modulating proximal-SO motor activity.

Excitatory effects of exogenous ATP on SO motility have been previously reported. *In vitro* studies in guinea-pig SO reported exogenous ATP evoked SO contraction, and furthermore EFS-induced contractions were blocked by the P2 antagonist PPADS, in combination with tachykinin antagonists (Patacchini *et al.* 1998a). The data in this chapter is consistent with the hypothesis that ATP is involved as an excitatory neurotransmitter/neuromodulator in the SO (Patacchini *et al.* 1998h). Interestingly, it was also found that exogenous ATP affects inhibitory neural pathways, involving nicotinic receptors and nitrergic nerves. In support of the presence of an inhibitory purinergic pathway in the SO, an electrophysiological study using rabbit SO found ATP acting via P2X

receptors, together with PACAP, was involved in the NANC inhibitory junction potential (Imoto *et al.* 1998e).

A clinical study found that theophylline, a P1 antagonist, inhibited morphine induced SO spasm, thereby suggesting adenosine has an excitatory effect on SO motility (Pap and Forro 1998c). This possum data supports an excitatory role for adenosine in modulating SO motility, and further suggests its involvement is via the activation of P1 receptors located on cholinergic motor neurons. These results are similar to those obtained with ATP, and are consistent with ATP metabolism to adenosine, which in turn activates cholinergic motor neurons (**Figure 8.13**). It remains to be determined if specific P2 receptor activation is also involved in the activation of these cholinergic motor neurons.

The SO is known to have a major excitatory cholinergic drive. The current investigations demonstrate that exogenously applied adenosine and ATP activate cholinergic motor neurons. Of particular interest are the inhibitory effects of ATP, which are indirectly demonstrated in this study. The proposed pathways for the action of ATP (**Figure 8.13**) suggest that when muscarinic receptors are blocked an inhibitory response would be observed. In these studies an inhibitory response was not evident in the presence of atropine, which is most likely due to an incomplete block of all muscarinic receptors with the atropine infusion (see **Section 2.3.3**). However, from the increased responses observed in the presence of hexamethonium and L-NAME, the data suggests that ATP activates interneuron transmission, involving nicotinic receptors probably activating a nitrergic pathway, which may involve nitrergic

interneurons or motor neurons. Immunohistochemistry studies have shown NOS containing nerves are abundant in the SO (Simula *et al.* 2001b; Wells *et al.* 1995). Studies in guinea-pig, pig and human SO have suggested that nitric oxide is released tonically to inhibit contractile activity (Vogalis and Smith 2000a; Sand *et al.* 1997), therefore ATP may be involved in maintaining the tonic release of nitric oxide in the SO. Clearly, the observed effects of exogenous ATP on SO motility *in vivo* are the integration of these complex excitatory and inhibitory neural pathways.

The finding that exogenous ATP and adenosine act via neural mechanisms *in vivo* contrasts with the *in vitro* data (**Chapter 3**), where inhibitory TTX-insensitive responses were observed. This disparity may be explained by the neural purinergic receptors being more sensitive to ATP/adenosine than those located on the smooth muscle cells and/or ICC. The activation and resultant excitatory response may mask the possible inhibitory response due to activation of the non-neural purinergic receptors. A similar disparity was reported in rat ileum where endogenously released adenosine increased acetylcholine release via activation of A_{2A} receptors, but exogenous adenosine decreased acetylcholine release (Duarte-Araujo *et al.* 2004b). The interaction between excitatory and inhibitory responses may be dependent on the extracellular concentration of adenosine, which is influenced by extracellular adenosine generation and inactivation (via cellular uptake and/or deamination mechanisms) (Duarte-Araujo *et al.* 2004a). It remains to be determined if a similar interaction between ATP/adenosine generation and inactivation occurs in the SO *in vitro* and *in vivo* preparations.

This data in the proximal-SO demonstrates that ATP acts via excitatory and inhibitory pathways. The involvement of ATP in both excitatory and inhibitory processes has been implicated by pharmacological and electrophysiological studies in the jejunum, ileum and colon. However, results and the predicted receptor sub-types involved vary between investigators, often dependent on species and experimental design (tissue type, *in vivo* vs *in vitro*, pre-contracted muscle strips vs non-contracted muscle strips, EFS-induced excitations or relaxations in the presence of particular blockers or antagonists etc) (refer to **Section 1.4.5**). In comparison the adenosine-induced excitatory response observed in the proximal-SO was somewhat unexpected. A number of pharmacological *in vitro* studies in guinea-pig ileum have determined that adenosine acts at pre-synaptic A_1 receptors to inhibit acetylcholine release (Lee and Parsons 2000b; Lee *et al.* 2001b; Storr *et al.* 2002a), thereby decreasing activity. However, there is a report of adenosine mediating contraction in rat duodenal muscularis mucosa (Nicholls *et al.* 1996a). Again, a difference between species and tissue-type is evident. Accumulating evidence indicates that the actions of bioactive agents and the neural circuits characterised in the small intestine can be different to those in the SO. Consequently motility patterns and control mechanisms described in the small intestine may not be relevant to biliary motility.

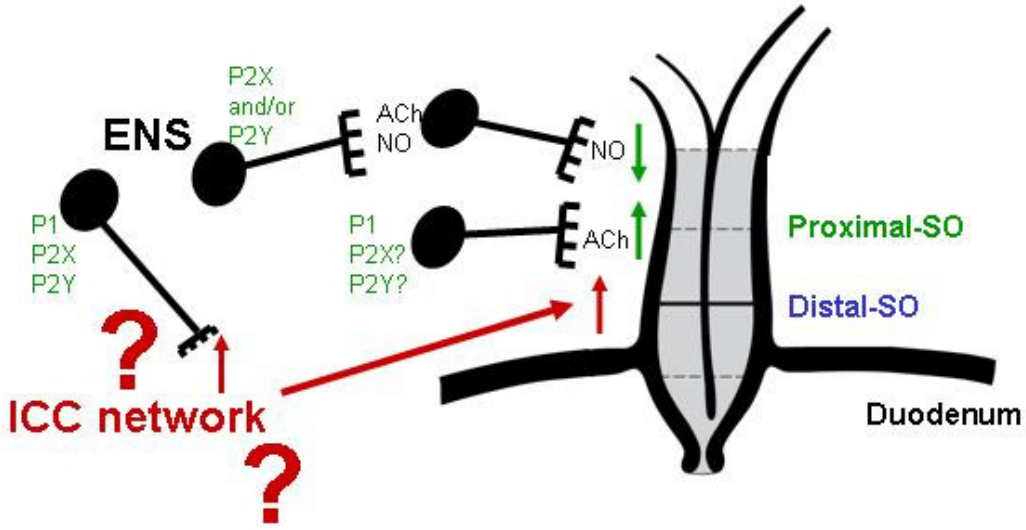
One major difference between the *in vitro* and *in vivo* SO a preparation is the existence of an intact neural system and neural reflexes. It remains to be determined if the ATP/adenosine-induced increase in SO activity is due to activation of SO intrinsic neurons or if extrinsic pathways are being activated. Further studies (not undertaken due to time constraints) involving stimulation

of the SO-CBD/gallbladder or SO-duodenum reflexes, via electrical field stimulation or physiological stimuli, demonstrating the involvement of endogenous purines on SO motility are warranted. This approach requires the use of P1 and P2 antagonist infusions. Preliminary experiments were unsuccessful in obtaining suitable levels of P1 or P2 receptor blockade with DPSPX, DPCPX, PPADS or suramin via i.v. or i.a. administration (n=10; data not presented). Similarly the effects of P1 and P2 agonists were not tested in these experiments. Their effects on SO motility *in vivo* will shed some light on the purinergic receptor subtypes involved in mediating the responses to exogenous and/or endogenous ATP and adenosine. Retrograde tracing studies in conjunction with sub-type specific P1 and P2 receptor antibodies would also be a useful technique to determine the location of the purinergic receptors and characterization of the neuronal classes (**Appendix 1**).

8.5 Conclusion

In conclusion exogenous ATP and adenosine increase proximal-SO motility acting via neural pathways, involving cholinergic motor neurons. Additionally, ATP modulates nicotinic and nitrergic pathways within the proximal-SO to decrease SO motility. It remains to be determined if endogenous purines are involved in the regulation of SO motility, or SO neural reflexes, and to identify the purinergic receptor sub-types activated.

SUMMARY 3: EXOGENOUS ADENOSINE AND ATP INCREASE SO MOTILITY *IN VIVO*, VIA STIMULATION OF PURINERGIC RECEPTORS ON NERVES



Application of exogenous ATP or adenosine induced a neurally mediated net increase in SO motility *in vivo*. Exogenous ATP increased SO motility by activating cholinergic motor neurons. However metabolism of ATP to adenosine by endogenous ectonucleases and activation of P1 receptors may be the source of this response, as exogenous adenosine also increased SO motility by activating cholinergic motor neurons. Furthermore exogenous ATP was also found to activate an inhibitory nicotinic/nitrergic pathway. The possible activation of P1 and P2 receptors located on ICC, resulting in changes to slow wave activity, remains to be addressed.

Chapter 9

9 GENERAL DISCUSSION

As each preceding chapter has included a detailed discussion, this chapter provides an overview of the findings and a discussion of their broader ramifications.

9.1 Overview of findings

The studies reported in this thesis have established that exogenous purines modulate spontaneous SO and duodenal motility. The actions of exogenous purines on SO motility are complex. A number of different purinergic receptor sub-types are involved in mediating inhibitory and excitatory effects via neural and non-neural pathways. The change in spontaneous SO motility is a result of the integration of the inhibitory and excitatory signals received at the smooth muscle.

As described in **Chapter 1**, SO and duodenal motility are modulated by a number of external factors including hormones, the extrinsic nervous system and neural reflexes. Therefore, to avoid these confounding factors the initial studies described in this thesis used *in vitro* techniques to determine the effects of purines on spontaneous SO and duodenal motility. Due to the relatively short duration of spontaneous activity exhibited by SO muscle rings, separate studies were first performed with duodenal muscle strips to establish: (1) if the possum duodenum displayed similar responses to exogenous purines acting via the same receptor sub-types as found in other species; (2) if the available agonists and antagonists were effective in possum tissues; (3) appropriate concentration ranges for agonists and antagonists to be later used in possum SO muscle ring preparations. This

information would permit the first comparison of the purinergic receptor sub-types in SO and duodenum of the same species mediating the responses to exogenous ATP and adenosine.

The results of these duodenal *in vitro* studies have been presented and discussed in **Chapters 3, 4 and 6**. The information generated from these studies was used in subsequent SO *in vitro* studies, presented in **Chapters 3, 5 and 7**. The relevant findings are summarized in **Tables 3.1, 4.2, 5.1, 6.7 and 7.1** and **Summary Figures 1 and 2**. These *in vitro* pharmacological studies provide evidence to support a number of the original hypotheses stated in Chapter 1.

Hypothesis 1: Exogenous purines modify spontaneous SO activity, and the effect of purines on the proximal-SO is different to the distal-SO.

The data reported in Chapter 3 support this hypothesis. There is considerable debate in the literature regarding the number of components comprising the SO and particularly whether the proximal and distal regions of the sphincter can function independently. The *in vitro* studies established that exogenous purines do modify spontaneous SO activity, and this occurs predominantly in the proximal-SO, suggesting that purines may play a significant role in modulating SO function. This regional difference can be explained by purines acting on ICC. A pacemaker region is believed to exist in the proximal-SO, which is responsible for controlling the frequency of propagating peristaltic-like contractions displayed by the intact SO. Whether such a pacemaker utilizes ICCs is unknown. Alternatively there may be a polarized distribution of purinergic receptors within the SO, possibly reflecting

the different origins of the muscle in the different regions of the SO. A number of studies investigating the effects of a range of bioactive agents on SO motility in the possum and other species have also found regional differences in responses or the distribution of receptors (**Section 1.2.3.1**). These possum data further support the hypothesis that regions of the SO can act independently in response to certain stimuli. Clearly future experiments need to be designed that investigate the actions of bioactive agents in both SO regions. This in turn may have important ramifications regarding the interpretation of SO function (is it a pump, a resistor, or both), or more importantly SO malfunction (i.e. spasm, SO dysfunction).

Hypothesis 2: The SO and duodenum respond differently to exogenous purines, mediated by different purine receptor sub-types.

The data reported in **Chapters 3-7** support this hypothesis with respect to the ATP-induced responses in the proximal-SO and duodenum and the P2 receptor sub-types involved. However this hypothesis could not be completely tested with respect to the P1 receptor sub-types involved in mediating the adenosine-induced response, as they could not be identified in the proximal-SO.

It is often assumed by other investigators in the GI field that the SO will respond to bioactive agents in the same way as the duodenum. This is based on embryological and anatomical studies demonstrating that the SO musculature and neural plexuses are continuous with the duodenum. However, I believe that as the SO has distinctly different physiological functions to the duodenum it is unwise to assume that the SO utilizes the

same mechanisms that have been characterized in the small intestine. In support of this, investigations of the extrahepatic biliary tree found that ganglia exhibit structural, neurochemical and physiological characteristics that are distinct from those of the ENS (Balemba *et al.* 2004c).

As indicated in summary 1, in proximal-SO muscle rings exogenous ATP likely acts via neural P2X receptors, probably via non-neural $P2Y_2$ and/or $P2Y_4$ receptors and possibly as a result of its metabolism to adenosine with activation of non-neural P1 receptors. In comparison, in duodenal muscle strips exogenous ATP induced a tri-phasic response. The immediate inhibitory component likely involves $P2X_1$, and $P2Y_2$ and/or $P2Y_4$ receptors. The rebound excitatory component was not investigated in these studies. The late inhibitory component is likely to be mediated by $P2Y_1$ receptors, but other P2Y receptors may be involved, in conjunction with the possible metabolism of ATP to adenosine and activation of non-neural P1 receptors.

Comparison of these ATP-induced responses suggests that different purine receptor sub-types mediate the responses in the different tissues. Firstly there were differences between the two preparations with regard to the nature of the ATP-induced response (i.e. a bi-phasic versus a tri-phasic response), and the sensitivity of the different components of the responses to TTX. Secondly the P2 agonists produced different responses in the two tissues, indicating the activation of different receptor sub-types. For example the action of UTP was considerably different between the two tissues. In the duodenal muscle strips UTP induced an immediate relaxation followed by an

excitation, whereas in the proximal-SO muscle rings only a late inhibitory response was observed.

The differences in the purine-induced responses in these two tissues could also be due (at least in part) to the coupling of these receptors to different intracellular mechanisms. P2Y receptors are coupled to G proteins involving second messenger systems. Activation of the same receptor sub-type could cause opposite responses if the intracellular mechanisms are different between the two tissues. The signal transduction mechanisms involve activation of phospholipase C followed by formation of inositol triphosphate and mobilization of intracellular Ca^{2+}, which can stimulate a number of signalling pathways including protein kinase C, phospholipase A_2, Ca^{2+} dependent K^+ channels, NOS and formation of endothelium derived relaxing factor or endothelium-derived hyperpolarizing factor (Harden *et al.* 1995; Ralevic and Burnstock 1998t). Other signalling mechanisms include inhibition of adenylate cyclase, leading to decreased levels of cyclic AMP (Harden *et al.* 1995). However, $P2Y_{11}$ is believed to be unique compared to the other P2Y receptors as it couples to both the phosphoinositide and the adenylate cyclase pathways (Ralevic and Burnstock 1998q). There is no evidence to suggest $P2Y_{11}$ is involved in mediating the observed responses, therefore it is more likely that different P2Y receptor sub-types are involved in mediating these different responses in the proximal-SO and duodenum, but the intracellular mechanisms have not been studied.

In vitro pharmacological experiments identified A_{2A} and A_3 receptor sub-types as mediating the adenosine-induced decrease in duodenal motility.

Unfortunately experiments using P1 sub-type specific agonists and antagonists could not identify the receptors involved in mediating the adenosine-induced decrease in proximal-SO motility. Therefore a direct comparison between the receptors mediating the adenosine-induced decrease in duodenal and proximal-SO motility could not be made.

In summary, the differences in the characteristics of the responses, the involvement of nerves, and the different receptor sub-types likely to mediate the ATP-induced response provides some support for this hypothesis. Furthermore, these data suggest that future studies need to address the actions of bioactive agents on SO motility, with its different functional components, separately to its actions on duodenal motility.

Hypothesis 3: The effect of exogenous purines on SO motility are mediated in part via nerves.

The *in vitro* studies (**Chapters 3-7**) clearly demonstrate that exogenous purines modulate spontaneous motility of the SO and duodenum. However, as effects observed in simplified *in vitro* systems are not necessarily duplicated *in vivo*, further studies were performed to assess the actions of exogenous purines on SO motility *in vivo*. The findings support this hypothesis and are summarized in **Table 8.1**, **Figure 8.13** and **Summary 3**.

The ATP-induced bi-phasic response observed with proximal-SO muscle rings *in vitro* was the first indication that the activation of purinergic receptors located on nerves may be involved (**Chapter 3**). *In vivo* studies found that topical application of ATP or adenosine to the extraduodenal SO

unexpectedly resulted in increased SO motility. This response was blocked by TTX.

Further characterisation of the neural pathways found the adenosine-induced excitation was mediated by P1 receptors located on cholinergic motor neurons. The ATP-induced excitation also involved cholinergic motor neurons. However metabolism of ATP to adenosine with subsequent activation of P1 receptors is possible and requires further investigation. Interestingly inhibitory nicotinic and/or nitrergic pathways are also involved in the ATP-induced response. The overall response of the proximal-SO to exogenous ATP is dependent on the integration of excitatory and inhibitory signals to the smooth muscle cells, some of which may be mediated by adenosine. Further investigations are required to ascertain the purinergic receptor sub-types involved in these neural pathways.

Hypothesis 4: SO and/or duodenal responses to exogenous purines are mediated by receptors located on nerves, smooth muscle and/or ICC.

This hypothesis could not be adequately tested. The *in vitro* and *in vivo* investigations demonstrated that exogenous adenosine and ATP mediated their actions via both neural and non-neural receptors. Using a pharmacological approach neural purinergic receptors were located on cholinergic motor neurons and on nicotinic and/or nitrergic interneurons *in vivo* (**Chapter 8**). However the location of the receptors mediating the TTX-insensitive responses identified *in vitro* (**Chapter 3**) remains unresolved. These receptors may be located on smooth muscle (mediate ATP/adenosine effects directly), or alternatively they may be located on ICC (mediate effects

indirectly via modification of slow wave activity), or they may be on pre-junctional nerve terminals (activation modifies neural transmission to smooth muscle and/or ICC). In an attempt to identify the purinergic receptor sub-types and their distribution within the SO and duodenum immunohistochemical studies were undertaken but were unsuccessful (**Appendix 1**). Attempts to visualize ICC in the possum SO and duodenum using immunohistochemistry were also unsuccessful (**Appendix 1**).

Hypothesis 5: Endogenous purines are involved in the neural reflexes between the SO and duodenum or the SO and CBD/gallbladder.

This hypothesis could not be tested. SO activity is modulated by neural reflexes that exist between the SO and the stomach, the duodenum, and the CBD/gallbladder (**Section 1.3.4**), but the neural circuitry involved has not been adequately characterized. To determine if purines are involved in mediating these reflexes, infusions of purinergic antagonists are required. Preliminary *in vivo* experiments were unsuccessful in obtaining suitable levels of P1 or P2 receptor blockade with DPSPX, DPCPX, PPADS or suramin via i.v. or i.a. administration (n=10; data not presented). Due to the lack of purinergic *in vivo* investigations in the GI tract, it is possible that these antagonists are not always effective, probably due to their rapid degradation and clearance in this setting. Due to time constraints this hypothesis was not further tested.

9.2 Functional significance of activation of purinergic receptors in the SO and further studies

The findings presented in this thesis demonstrate that exogenous application of purines modifies SO motility. Specifically, motility of the proximal-SO was affected, with negligible effects observed at the distal-SO. As the pacemaker region of the SO is believed to exist in the proximal-SO these findings imply that there is a significant modulatory role for purines in regulating SO function.

One of the complications that arise in attempting to incorporate the experimental findings described above into our understanding of SO function is the quantitation of SO motor function. Measurement of motility can be used to determine if the net SO motor activity has increased or decreased. Historically SO activity in response to bioactive agents or other stimuli has been characterized as changes in basal tension, contraction amplitude and contraction frequency, and as yet there has been no formal investigation relating the interaction between these parameters with SO function. Unfortunately these measures are not easily extrapolated to changes in SO function, specifically trans-sphincteric flow. Caution should be exercised with the interpretation of motility data until *in vivo* functional studies using trans-sphincteric flow as a direct measure of SO function are performed.

9.2.1 Potential effects of purine-evoked responses on SO function: theoretical considerations

Recent studies have shown that the possum SO can act as both a pump and as a resistor, with the function dependent on CBD and duodenal pressures

(Grivell *et al.* 2004a). Therefore an increase in proximal-SO activity, as demonstrated in response to exogenous ATP or adenosine, could have different effects on SO function.

9.2.1.1 SO function at low CBD pressures: pump

Grivell *et al* (2004) have recently demonstrated that at CBD pressures <3.5 mmHg, the possum SO displays peristaltic-like pumping. If endogenous purines produce the same effects as observed with exogenous purines (*in vivo*) then enhanced proximal SO contraction frequency would result in faster pumping. In addition, increased contraction amplitude could equate to a stronger contraction (more effective pumping). However if proximal-SO activity increased to such an extent as to cause SO spasm (i.e. too high a contraction frequency and/or amplitude such that the proximal-SO muscle could not 'relax' sufficiently to allow filling of the proximal-SO lumen), then trans-sphincteric flow would decrease considerably, or even cease. This in turn would increase CBD pressure. In humans an increase in CBD pressure is believed to cause the pain associated with SO Dysfunction. Furthermore spasm of the pancreatic component of the SO may lead to acute pancreatitis.

9.2.1.2 SO function at high CBD pressures: resistor

At CBD pressure >3.5mmHg trans-sphincteric flow progressively changes to predominantly passive "resistor"-type flow as the CBD pressure exceeds the pressure generated by the proximal-SO. If purines enhance the proximal-SO contraction amplitude at these moderate CBD pressures, pumping would continue until the CBD pressure increased further resulting in only resistor-type function of the SO. It should be noted that the distal-SO/papilla region is primarily responsible for the resistor function of the SO. The *in vitro* studies

outlined in this thesis showed that the distal-SO motility was little affected by exogenous purines, which suggests that SO resistor function is not likely to be altered by purines. However the effects of exogenous purines on the activity of the distal-SO/papilla region have not been studied *in vivo*. These considerations are based on the assumption that the coordination of the proximal-SO and distal-SO activity required for pumping is not influenced by purines, but the effect of purines on this coordination has not been studied.

Grivell *et al* (2004) also observed that progression from pump to resistor function commenced when CBD pressure was 2-4mmHg greater than duodenal pressure. Thus the effects of purines on duodenal motility are also relevant to SO function.

9.2.1.3 SO function influenced by duodenal pressure

High duodenal pressures increase the resistance to flow (i.e. increases the SO opening pressure). The mechanism responsible is believed to involve the SO–duodenal reflex and serves to prevent reflux of the duodenal contents into the SO. The effects of exogenous purines on duodenal motility have not been investigated *in vivo*. However exogenous purines do affect duodenal motility *in vitro*, but as the results between *in vitro* and *in vivo* experiments can differ considerably the effects of purines on duodenal motility *in vivo* needs to be established. A purine-induced increase in duodenal activity is likely to modify SO function via activation of the SO-duodenal reflex. The possible regulation of this reflex by purines requires further investigation (**Section 1.3.4**).

The complexity of the regulation of SO function is such that future *in vivo* experiments need to address the actions of purines on trans-sphincteric flow, in combination with changes in CBD and duodenal pressures.

9.2.1.4 The CBD/SO reflex

Possum SO motility has also been shown to be influenced via a CBD/SO reflex. Studies using electrical field stimulation found very complex SO responses that were bi- or tri-phasic (Sonoda *et al.* 2005). Interestingly these responses are similar to the ATP-induced response. Further studies using effective specific P2 antagonists may unearth a potential purinergic component in this and other reflexes. Due to the ineffectiveness of P2 antagonists used in *in vivo* possum experiments these future investigations could utilize an *in vitro* approach where the SO remains attached to the CBD/gallbladder and the duodenum. However comprehensive testing would be required to establish that the purine mediated neural excitatory and inhibitory pathways identified in **Chapter 8** are still present, as the extrinsic nervous system may be integral for the response.

9.2.2 CBD distension and pain perception

An interesting development in the field of purine research in the hypothesis that distension of tubes and sacs releases ATP from epithelial cells that act on $P2X_{2/3}$ receptors located on sensory neurons involved in the perception of pain (Burnstock 1999d). In the biliary tree, distension of the CBD is often associated with SO spasm or the passage gallstones through the SO/CBD, and is thought to cause CBD distension and pain. It would be interesting to determine if endogenous ATP released in response to CBD distension can also induce SO spasm and pain. If so, then investigations leading to the

development of pharmaceutical treatments for biliary pain could also include a purine antagonist.

9.2.3 Summary

The studies described herein have all used exogenous application of purines to investigate changes in SO motility, with an attempt to characterize the purinergic receptor sub-types involved. The *in vitro* and *in vivo* studies have demonstrated disparate findings suggesting that the nervous system plays an important role in regulating SO activity. Similarly, one cannot assume that the effects of exogenous purines will mirror the effects of endogenously released purines. A critical question remaining to be addressed is: Do endogenous purines modify spontaneous SO activity *in vivo*? To answer this question several considerations need to be addressed. Firstly, it is essential to identify an endogenous source(s) of extracellular purines in the SO and duodenum. Purines are available from a number of sources throughout the GI tract (including bile and pancreatic juice, nerves, muscle etc), but it needs to be determined if they are available in the concentrations required to elicit similar responses to those observed in these studies. Secondly, demonstration that these endogenous purines are released by physiological stimuli is required. Activation of neural reflexes or stimulation of muscle contraction using EFS or distension (by fluid or a balloon) may be useful triggers for the release of endogenous purines at the appropriate location and at appropriate concentrations. It is noted that the studies described in this thesis have used high concentrations of purines to elicit responses, but these concentrations are not dissimilar to those used by other investigators in the GI purinergic field. Furthermore due to biological degradation of purines the actual

concentration of the applied purine at the receptor site that evokes a response is not known.

9.3 Conclusions

The findings presented in this thesis form the first comprehensive investigation of the effects of exogenous purines on SO motility and provides a foundation for future studies (as outlined above). These studies have demonstrated that exogenous purines modify SO and duodenal motility in a complex manner via multiple receptor sub-types. These data implicate purines as important modulators of SO function. It now remains to be determined if endogenous purines are involved in the regulation of SO motility and to identify the receptor sub-types involved.

Appendix 1

10 APPENDIX 1: ATTEMPTED LOCALISATION OF P1 RECEPTORS IN SO AND DUODENUM

Immunohistochemical studies were undertaken to visualize the location of P1 receptors and ICC in possum SO and duodenum, however this approach was unsuccessful. The rationale, methodology, results and discussion of these studies are presented below for completeness. The labelling produced by the antibodies to the P1 receptors proved to be non-specific, and the antibody for ICC did not produce a suitable signal for use in possum flat sheet preparations. During my PhD candidature a number of antibodies were developed and used to map the location of P1, P2X and P2Y receptors in numerous GI tissues. These reports are briefly reviewed after the discussion in this appendix.

A1.1 Introduction

In an attempt to characterise the specific purine receptor sub-types present in tissues, immunohistochemical studies are commonly undertaken. Quinacrine histochemistry, a marker for vesicular ATP, has demonstrated numerous ATP containing nerve cell bodies, nerve bundles and fibres in GI tissues (Crowe and Burnstock 1981; Belai and Burnstock 1994). As antibodies to the specific P1, P2X and P2Y receptor sub-types become available, the distributions of receptors are being mapped in various tissues.

Pharmacological studies in possum duodenum and SO suggested the presence of P1 and P2 receptors (**Chapter 3**). To narrow the search for potential receptor sub-types that could be mediating the response to exogenous adenosine/ATP an immunohistochemical approach was

undertaken with the additional aim of distinguishing which specific cell type/s express the receptors. Furthermore as this thesis addresses the role of purines on spontaneous activity, which could be generated by the actions of ICC, the possible interaction of purines with ICC could not be ignored. Available antibodies for each of the P1 receptor sub-types and ICC were used in immunohistochemical studies to:

1. Determine the distribution of P1 receptor sub-types in the possum SO and adjacent duodenum.
2. Identify a marker for ICC in possum tissue.

A1.2 Methods

A1.2.1 Tissue collection

Duodenum and SO were collected from anaesthetised possums (**Section 2.1**) as described for *in vitro* studies (**Section 2.2.1**) and placed into phosphate buffered saline (PBS; 0.15M sodium chloride in 0.01M sodium phosphate, pH7.2).

A1.2.1.1 Duodenum

The duodenum was opened longitudinally along the mesenteric border and pinned flat in a Sylgard lined dish and fixed overnight with modified Zamboni's fixative (4^{o}C; 2% formaldehyde and 15% saturated picric acid in 0.1M phosphate buffer, pH7). The submucosal layer was removed by sharp dissection. Circular muscle strands were then peeled off the preparation, exposing the myenteric plexus (Simula *et al.* 2001a; Konomi *et al.* 2002a).

A1.2.1.2 SO

The attached pancreas, connective tissue and mesenteric blood vessels were dissected from the SO and adjoining duodenum. The duodenum was opened longitudinally along the anti-mesenteric border and pinned flat, mucosal surface uppermost. The SO was opened by cutting along the bile and pancreatic ducts, near the adjoining septum, to produce a flat sheet. This whole-mount preparation was fixed overnight in modified Zamboni's fixative (4^{o}C). The submucosal layer of the SO and adjoining duodenum was then removed by dissection. This process removes the papilla-SO leaving the outer circular and outer longitudinal muscle layers, with the myenteric plexus as a continuous preparation with the adjacent duodenum. The circular muscle of the SO and adjoining duodenum was then peeled away, exposing the myenteric plexus. A similar SO-duodenum preparation has been used previously for immunohistochemical studies (Simula *et al.* 2001g).

A1.2.2 Adenosine receptor immunohistochemistry

To enhance tissue penetration of the antisera, all whole-mount preparations were permeabilised with DMSO (3x10min exposures at room temperature), and then rinsed with PBS. Indirect immunofluorescence techniques with primary antisera combined with fluorescent-labelled secondary antisera were used. Adenosine receptor (AR) antisera to each receptor sub-type (rabbit anti-A_1R, A_{2A}R, A_{2B}R and A_3R antisera; Chemicon International, Temecula, CA, USA) were used in this study.

Preliminary investigations using different antibody dilutions and incubation periods determined 1:200 dilutions with 48h incubations to be optimal for

each of the antibodies. In addition, to determine the nerve cell body population, whole-mounts were double labelled with mouse anti-Hu antisera (Ultraclone, Cambridge, UK; Lin *et al.* 2002). Alternatively, whole-mounts were double or triple labelled with sheep anti-NOS antisera (K205, gift from Dr Emson, Babraham Institute, Cambridge, UK) as a marker for most inhibitory neurons, or with rat anti-substance P (Harlan Sera-Lab Limited, Loughborough, England, UK) as a marker for some excitatory neurons.

Preparations were incubated with anti-AR antisera (1:200) and anti-Hu antisera (1:500), or anti-NOS antisera (1:800) or anti-SP antisera (1:20), prepared in 10% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) to block non-specific binding sites, for 48h at room temperature. After several washes with PBS, preparations were left overnight at room temperature in species-specific fluorescent secondary antisera: CY^{TM}3-conjugated AffiniPure donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (1:100, Jackson ImmunoResearch); CY^{TM}5-conjugated AffiniPure donkey anti-rat IgG (1:20 Jackson ImmunoResearch Labs), CY^{TM}2-conjugated AffiniPure donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Labs). Preparations were then washed in PBS, processed through 50%, 70% and 100% bicarbonate-buffered glycerol (pH 8.6) baths, and then mounted on glass slides. Omission of the primary antibody was used to determine background staining due to secondary antibodies alone.

A1.2.2.1 Control peptide absorption experiments

To test that the immunostaining observed was specific, experiments were performed where the antibody was pre-adsorbed with the appropriate control

peptide. These experiments were performed in consultation with Professor Costa (Department of Human Physiology, Flinders University, Australia). Control peptides (Chemicon International) were prepared similarly to antibody stock solutions, by dissolving in 100μl glycerol/sodium azide (0.1%) solution to give a final concentration of 500mg/ml, and stored at –70^{o}C. A method similar to Christofi *et al.* 2001c) was used where the antigenic peptide for A_1R, $A_{2A}R$, $A_{2B}R$ or A_3R were pre-absorbed with the respective primary antibody overnight, and used to show blockade of adenosine receptor immunoreactivity. The control peptide was in excess of the antibody concentration (1:1, 1:5 or 1:10 antibody:peptide (μg/ml)). The immunohistochemistry protocol was then performed in parallel where paired pieces of tissue were incubated with the normal antibody or the control peptide absorbed antibody. This was performed in duodenal tissues.

A1.2.3 ICC immunohistochemistry

To determine if the adenosine receptors were present on ICC, a number of markers for ICC were tested in possum tissue. These studies were performed in conjunction with a number of collaborators. Preliminary investigations using acetone fixation (10min ice-cold acetone) or modified Zamboni's fixation were unsuccessful with the ACK-2 antibody (Gibco-BRL; performed by Associate Professor Ward, Department of Physiology and Cell Biology, University of Nevada, USA). Similarly acetone fixation and modified Zamboni's fixation were unsuccessful with the C-19 antibody (Santa Cruz, California, USA; performed by Dr Yuan, Department Human Physiology, Flinders University, Australia). These antibodies are validated ICC markers

in mouse and guinea-pig tissues and have been routinely used by the respective collaborators.

Further reviews of the literature identified another antibody used for clinical diagnosis of GI stromal tumours, CD117 polyclonal rabbit anti-human c-kit (DAKO, Kyoto, Japan). Paraffin sections of possum duodenum (tissue fixed in 10% buffered formalin, then paraffin blocked) with heat-induced epitope retrieval (2ml 0.01M citrate buffer pH6.0 applied to tissue, boiled for 10-15min, simmered for further 20min, washed with PBS) proved useful for this antibody (1:100 concentration; performed by Mr Brennan, Department of Anatomical Pathology, Flinders University, Australia). The antibody signal was visualised using diaminobenzidine tetrahydrochloride (DAB). However standard whole mount preparations, with modified Zamboni's or 10% buffered formalin fixation, with or without heat-induced epitope retrieval were unsuccessful.

A1.2.4 Microscopy and image analysis

Paraffin sections were viewed with a standard light microscope. Flat-sheet preparations for immunofluorescence were viewed with an Olympus AX70 epifluorescent microscope (Tokyo, Japan) fitted with epifluorescent filters (Chroma Technology, Brattleboro, Vt., USA). CY3 was viewed using filter set 31002 (excitation 515/550nm; dichroic 565nm; emission 575/615nm), FITC and CY2 were viewed using filter set 31001NB (excitation 470/490nm; dichroic 505nm; emission 515/545nm), CY5 was viewed with filter set 41008 (excitation 590/650nm, dichroic 660nm, emission 665-740nm). All images

were captured using a Macintosh computer using NIH Image software (NIH, Bethesda, Md., USA).

A1.3 Results

P1 receptor immunohistochemistry performed on duodenal tissues is presented in **Figures A1.1** and **A1.2**. Immunofluorescence for A_1R was very faint and difficult to distinguish from background levels. $A_{2B}R$ and A_3R immunofluorescence was not blocked by the control peptide and is therefore termed 'pseudo'-immunoreactivity, whereas immunofluorescence for $A_{2A}R$ was blocked by the control peptide and is therefore termed 'like'-immunoreactivity. Data from the control peptide blocking experiments are presented in **Figure A1.2**. Immunohistochemistry for NOS, Hu and substance P was successful in the same tissues. Results with substance P are not presented as NOS and Hu clearly illustrated the neurons in ganglia. Due to the non-specificity of P1 antibodies in possum tissues it was not necessary to perform the co-localisation of P1 receptors with markers of excitatory and inhibitory neurons. Due to non-specificity of the antibodies further experiments using SO tissue were not carried out. For completeness the following observations were made in the duodenum.

A1.3.1 P1 immunohistochemistry in the duodenum

A_1R immunofluorescence was very faint and difficult to distinguish from background levels. $A_{2A}R$-like immunoreactivity was observed on the nucleus of all neurons, smooth muscle cells and cells near the serosa, but not on nerve fibres. A 'glittery' immunofluorescence was present in the muscle layers. Immunofluorescence for $A_{2B}R$ was very bright. Faint nuclear pseudo-$A_{2B}R$ immunoreactivity was observed in some neurons. Pseudo-$A_{2B}R$

immunoreactivity was present on internodal strands and surrounding myenteric ganglia. Cells in the circular muscle were also pseudo-A_{2B}R immunoreactive, however these cells were not immunoreactive for Hu indicating they are not neural. A large number of unidentified cells near the serosa also showed pseudo-A_{2B}R immunoreactivity. Pseudo-A_3R immunoreactivity was observed in a sub-population of neurons with a granular-type cytoplasmic fluorescence in many but not all neurons. No A_3R immunofluorescence was observed in the circular or longitudinal muscle (**Figure A1.1**).

A1.3.2 P1 control peptide experiments

To confirm that the P1 staining patterns were specific each antibody was pre-absorbed with an excess of the appropriate control peptide overnight. Pre-absorption of the A_{2A}R antibody blocked the nuclear-type staining pattern, but did not affect the 'glittery' immunofluorescence observed in the muscle layers, indicating it was not specific. Pre-absorption of the A_{2B}R antibody did not reduce or block the immunoreactivity pattern previously observed. Pre-absorption of the A_3R antibody slightly reduced the granular type cytoplasmic immunofluorescence but did not block it. Higher concentrations of the control peptide in repeated experiments did not have any further effect on the immunoreactivity patterns observed for each of the antibodies (**Figure A1.2**).

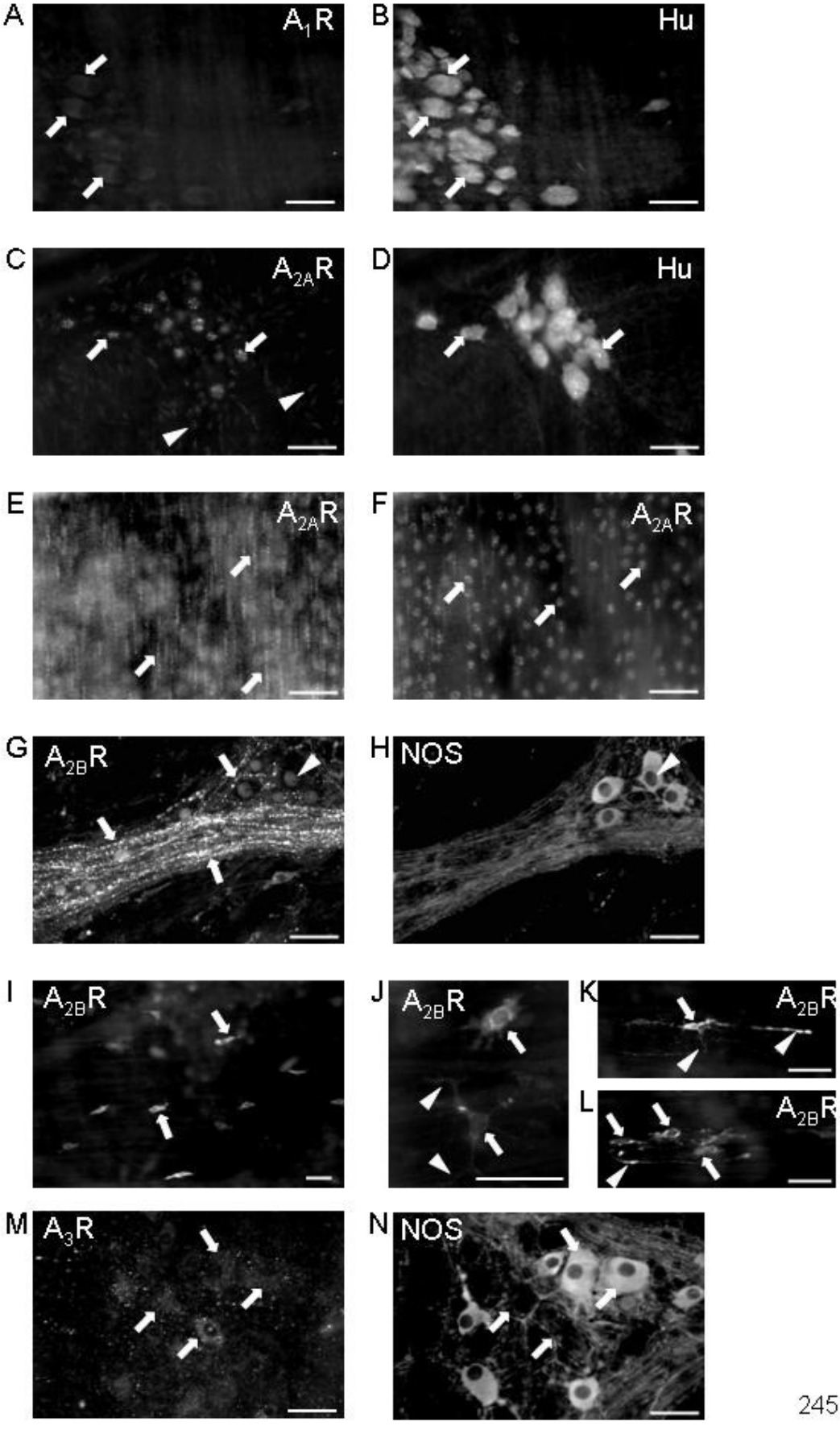
A1.3.3 ICC immunohistochemistry

C-Kit positive cells were only observed in formalin fixed paraffin sections of possum duodenum, after heat-induced epitope retrieval, using the CD117 antibody to c-kit. C-Kit immunoreactive cell bodies were observed in the myenteric plexus. Faint staining was observed throughout the circular

muscle, with the additional presence of darker processes and cell bodies. An occasional immunoreactive cell was observed in the longitudinal muscle (**Figure A1.3**).

An attempt to co-localise putative P1 receptors, NOS or substance P with ICC was made in whole-mount preparations, but was unsuccessful. Technical difficulties resulted with the necessary heat-induced epitope retrieval process in the whole-mount preparations. No further attempts were made as other antibodies for ICC did not produce immunoreactivity in possum tissues and the P1 receptor antibodies were non-specific.

Figure A1.1: Immunofluorescence to P1 receptor antibodies in possum duodenal muscle layer



245

Figure A1.1: Immunofluorescence to P1 receptor antibodies in possum duodenum muscle layer

Varying intensities of immunofluorescence with each of the P1 antibodies was observed in the duodenum muscle layer, containing the myenteric plexus and circular and longitudinal muscle layers. Note that the terms 'like immunoreactivity' and 'pseudo immunoreactivity' refer to immunofluorescence that was or was not blocked by the control peptide, respectively (as demonstrated in **Figure A1.2**). For comparison some ganglia are shown with NOS or Hu immunoreactivity.

(A) A_1R immunofluorescence (arrow) in myenteric ganglia was faint and difficult to distinguish from background fluoresecence.

(B) Same ganglia as in (A) with Hu immunoreactivity for comparison.

(C) A_{2A}R-like immunoreactivity was present on cell nucleus of neurons (arrow) and other cells (arrow head).

(D) Same ganglia as in (C) with Hu immunoreactivity for comparison.

(E) A_{2A}R-like immunoreactivity was present in longitudinal muscle (arrow) and circular muscle (not shown).

(F) A population of unidentified cells near the serosa were also A_{2A}R-like immunoreactive (arrow).

(G) Bright pseudo-A_{2B}R immunoreactivity was observed on inter- and intra-ganglionic fibers (arrows), and faint immunoreactivity was observed on the nucleus of some neurons (arrowhead).

(H) Same ganglia as in (G) with NOS immunoreactivity for comparison.

(I) A network of unidentified cells near the serosa were psuedo-A_{2B}R immunoreactive (arrow), these cells were Hu negative.

(J) Larger magnification of psuedo-A_{2B}R immunoreactive cells near serosa (arrow) illustrating cell processes (arrowhead).

(K) Cells in the circular muscle were psuedo-A_{2B}R immunoreactive (arrow) and also Hu negative. These cells had very long processes (arrowhead) running parallel with the circular muscle strands.

(L) Psuedo-A_{2B}R immunoreactive cells (arrow) and fibers (arrowhead) in the circular muscle appeared to form a network.

(M) Pseudo-A_3R immunoreactivity (arrow) was evident in the cell body of a sub-population of neurons in the myenteric ganglia.

(N) Same ganglia as (M) with NOS immunoreactivity for comparison.

Bar = 50μm

Figure A1.2: P1 receptor control peptide binding experiment in possum duodenum

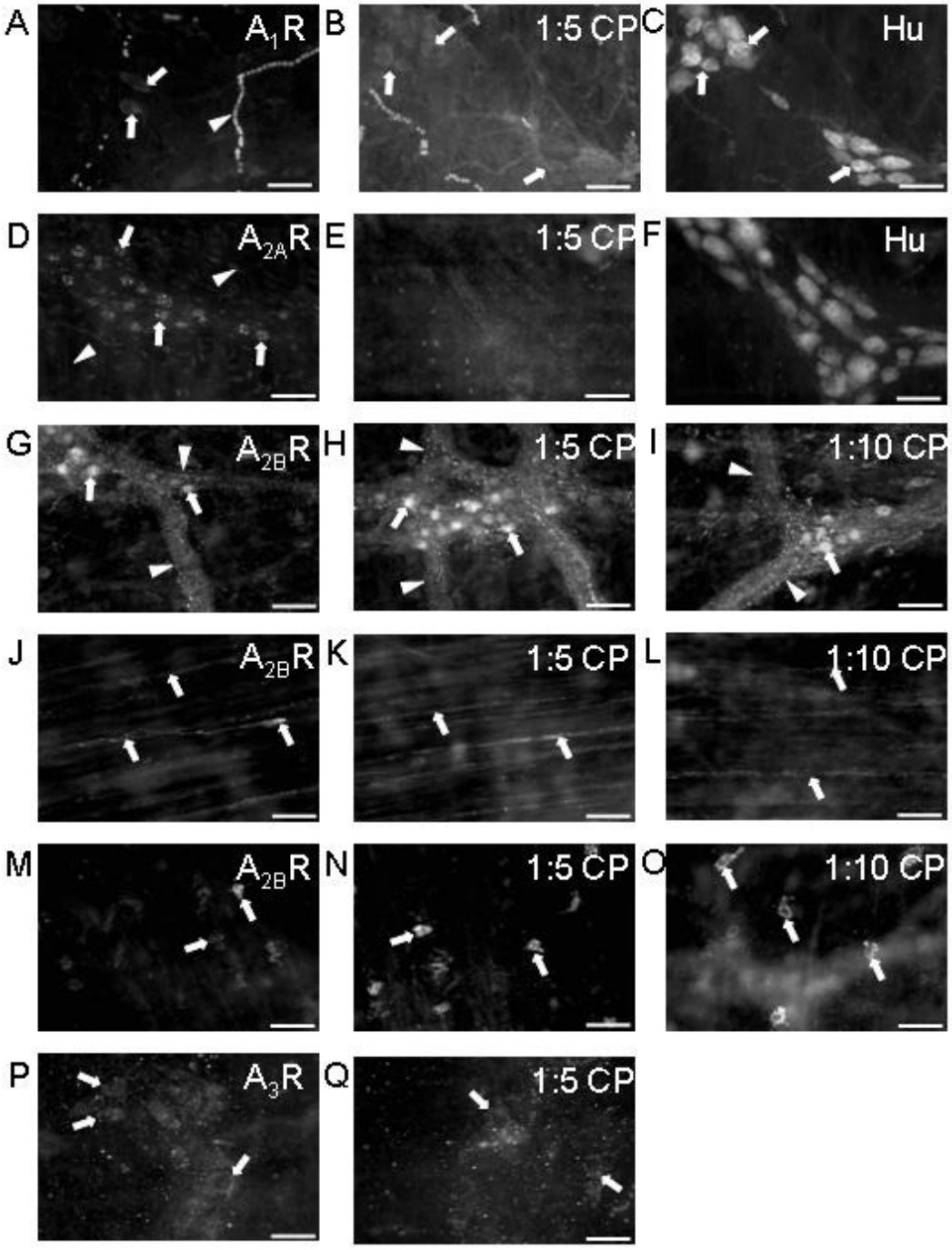


Figure A1.2: P1 receptor control peptide binding experiment in possum duodenum

Each P1 receptor antibody was pre-absorbed with its appropriate control peptide (CP; at 1:5 or 1:10, antibody:CP in μg/ml) overnight. Adjacent sections of tissue were processed in parallel with absorbed and unabsorbed antibody. For comparison some ganglia are shown with Hu immunoreactivity.

(A) A_1R immunofluorescence in myenteric ganglia (arrow). Note autofluorescence of erythrocytes in arterioles (arrowhead).

(B) A_1R immunofluorecence was not blocked by pre-absorption with the CP.

(C) Same ganglia as (B) with Hu immunoreactivity for comparison.

(D) A_{2A}R immunofluorescence in myenteric ganglia (arrow) and other cells (arrow head).

(E) A_{2A}R immunofluorescence was blocked by pre-absorption with the CP.

(F) Same ganglia as (E) with Hu immunoreactivity for comparison.

(G) A_{2B}R immunofluorescence in myenteric ganglia cell nucleus (arrow) and fibers (arrowhead).

(H) A_{2B}R immunofluorescence in myenteric ganglia cell nucleus (arrow) and fibers (arrowhead) was not blocked by pre-absorption with the CP.

(I) A_{2B}R immunofluorescence in myenteric ganglia cell nucleus (arrow) and fibers (arrowhead) was not blocked by pre-absorption with higher concentrations of the CP (1:10).

(J) A_{2B}R immunofluorescence of cells in circular muscle (arrow).

(K) A_{2B}R immunofluorescence of cells in circular muscle (arrow) was not blocked by pre-absorption with the CP.

(L) A_{2B}R immunofluorescence of cells in circular muscle (arrow) was not blocked by pre-absorption with higher concentrations of the CP (1:10).

(M) A_{2B}R immunofluorescence of unidentified cells near serosa (arrow).

(N) A_{2B}R immunofluorescence of unidentified cells near serosa was not blocked by pre-absorption with the CP (arrow).

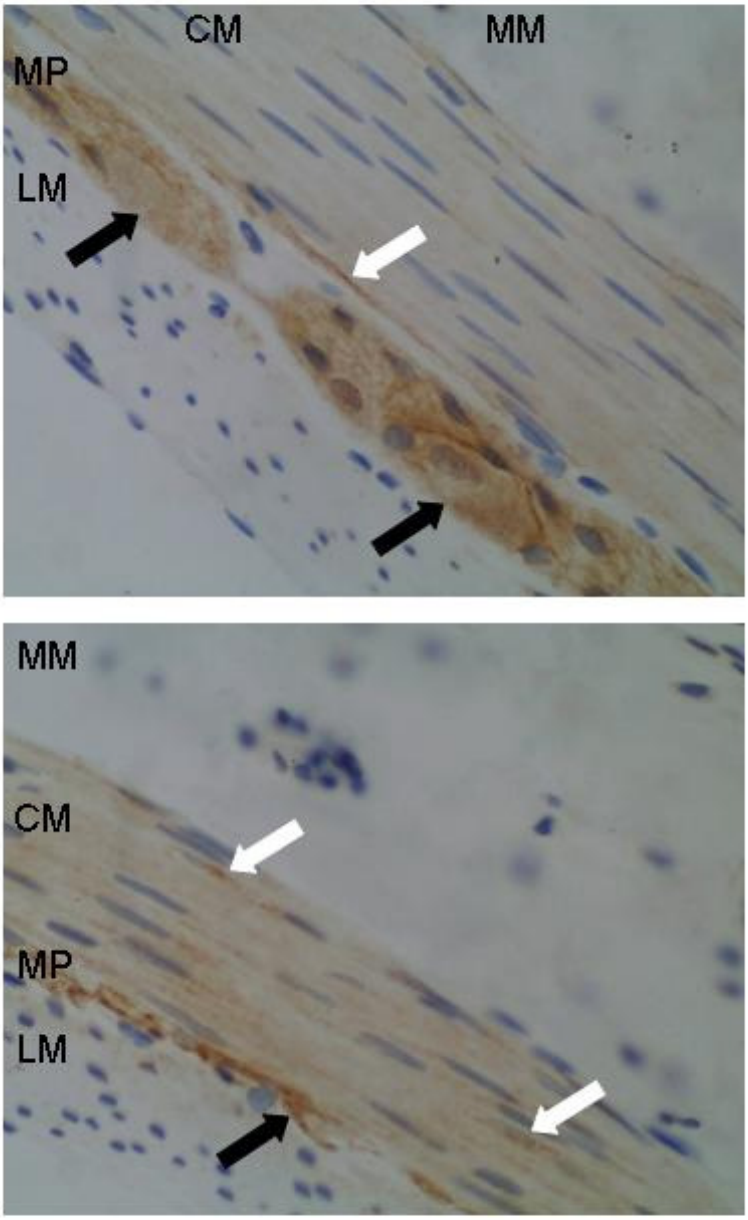
(O) A_{2B}R immunofluorescence of unidentified cells near serosa (arrow) was not blocked by pre-absorption with higher concentrations the CP (1:10).

(P) A_3R immunofluorescence of myenteric neurons (arrow).

(Q) A_3R immunofluorescence was not blocked by pre-absorption of the CP (arrow).

Bar = 50μm

Figure A1.3: ICC in the possum duodenum



C-Kit positive cells were observed in formalin fixed paraffin sections using the CD117 antibody after heat-induced epitope retrieval. C-Kit immunoreactive (brown staining) cell bodies were observed in the myenteric plexus (black arrow). Faint staining was observed throughout the circular muscle, with the additional presence of darker processes and cell bodies (white arrow).

Magnification x40. MM, muscularis mucosa; CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle.

A1.4 Discussion

The immunoreactivity of these P1 antibodies is not specific for possum tissues with either no immunofluorescence (A_1R), the immunofluorescence not blocked by the control peptide and therefore non-specific ($A_{2B}R$ and A_3R), or the immunofluorescence is associated with every cell nucleus ($A_{2A}R$). Consequently no conclusion can be drawn from this study using these antibodies.

The immunoreactivity associated with the $A_{2A}R$ antibody indicates that the epitope/s recognised by the antibody are present on the nucleus of possibly every cell. A similar nuclear staining pattern was observed with a different $A_{2A}R$ antibody used in brain preparations (Rosin *et al.* 1998). However this staining pattern is most likely not relevant to GI motility and was therefore not further investigated.

The most interesting immunofluorescence patterns were obtained with the $A_{2B}R$ and A_3R antibodies, however the immunofluorescence was not blocked by pre-absorption of the antibody with the appropriate control peptide, thereby indicating that the staining observed was non-specific. Consequently further studies with the SO were not performed.

An exhaustive study describing the expression and localization of P1 receptors in the ENS of human GI tissues has been published (Christofi *et al.* 2001b). A different suite of antibodies was used compared to those reported here. Immunoreactivity for the P1 receptors was different between the jejunum and colon. Immunoreactivity to each of the P1 receptors was

observed in different sub-populations of neurons of the submucous and/or myenteric plexuses indicating a presence on interneurons, secretomotor, motor neurons and intrinsic primary afferent neurons. Similarly some P1 receptors were variably expressed on smooth muscle cells, glial cells and mucosal epithelial cells (Christofi *et al.* 2001a). Of interest, the nuclear distribution of $A_{2A}R$ observed in possum tissues was not reported in human tissues, and suggests that the possum $A_{2A}R$ immunofluorescence may be an artefact.

The difficulties encountered with this immunohistochemical study may be a result of species differences at the molecular level of the P1 receptors. It is known that there is a considerable lack of homology between cloned P1 receptors and it is believed that as little as a 5% variability in amino acid sequence may be sufficient to cause considerable interspecies differences in ligand binding (Ralevic and Burnstock 1998v). It is therefore possible that the possum P1 receptor sequences are different to the rabbit antigens used to develop the antibodies and would account for the antibody/antigen binding difficulties encountered in this study.

The presence of $A_{2B}R$ positive cells in the longitudinal and circular muscle, that were Hu negative and therefore not neural, prompted studies to find a marker for ICC in possum tissue to determine if P1 receptors are present on ICC. A further aim was to determine if ICC are present in SO. A number of antibodies were tested on whole-mount preparations of possum duodenum, as mouse and guinea-pig duodenum contain ICC. However the possum duodenum studies were unsuccessful. One antibody was found to work

under extreme conditions (CD117) but this prevented it being useful for whole-mount preparations and co-localisation studies.

It has since been reported that ICC are present in guinea-pig SO, but not in guinea-pig gallbladder (Parr *et al.* 2003). Of particular interest is the distribution of ICC in the SO, but this has not been investigated. P2 receptors have recently been localized on ICC. The first report indicated $P2X_2$ receptors were present on ICC of vas deferens (Burton *et al.* 2000), followed shortly by a report demonstrating $P2X_2$ receptors on ICC of guinea-pig ileum, and $P2X_5$ receptors on ICC of mouse ileum (Burnstock and Lavin 2002b). The presence of purine receptors on ICC leads to the speculation that ATP released from enteric nerves or contracting smooth muscle, or perhaps ATP metabolism to adenosine, may be involved in a feedback mechanism regulating GI slow wave activity.

A1.5 Summary and conclusion

Organ bath experiments utilizing both SO and duodenum tissues demonstrated an effect of exogenous adenosine and ATP on spontaneous activity (**Chapter 3**). To determine which purine receptor sub-types and which cell types (neurons, smooth muscle cells or ICC) might mediate these responses immunohistochemical studies were performed. A_1R immunoreactivity was difficult to distinguish from background levels suggesting the antibody is not specific in possum tissues. The nuclear distribution of $A_{2A}R$ on all cells indicates that it is probably not relevant to GI motility. The $A_{2B}R$ and A_3R antibodies were not specific in possum tissues as evidenced from the control peptide studies. A suitable marker for ICC in

possum tissues was not found. Therefore these immunohistochemical studies were not continued. Further studies with other purinergic receptor antibodies were not attempted, and instead a pharmacological approach was used to determine which receptors are responsible for mediating the responses to adenosine and ATP.

A1.6 Recent immunohistochemical studies using purinergic receptor antibodies in GI tissues

At the outset of this study (1999) there was very limited information on purine receptor distributions in GI tissues. There were no reports of immunohistochemical studies assessing the presence and distribution of purinergic receptors in the biliary tree. However during the course of this project in addition to the publications mentioned in the discussion, the following reports were published and highlight differences in purine receptor distribution between species and GI tissues.

Immunoreactivity to $P2X_1$, $P2X_5$ and $P2X_7$, but not $P2X_2$, $P2X_3$, $P2X_4$ or $P2X_6$ receptor subunits were observed in distinct regions of the rat duodenal villus suggesting an involvement with the uptake and transport of metabolites, the synthesis and release of mucins, and apoptotic events in enterocytes and goblet cells (Groschel-Stewart *et al.* 1999).

In the guinea-pig ileum $P2X_2$ and $P2X_7$, but not $P2X_1$ or $P2X_4$ immunoreactivity was abundant in myenteric and submucosal ganglia. $P2X_7$ immunoreactivity was also associated with blood vessels and circular muscle (Hu *et al.* 2001). Co-localisation studies in guinea-pig stomach, small and

large intestine found $P2X_2$ receptor subunits were expressed on specific sub-types of enteric neurons, specifically inhibitory motor neurons, non-cholinergic secretomotor neurons and intrinsic primary afferent neurons, and also on the endings of vagal afferent fibres in the stomach (Castelucci *et al.* 2002).

An immunohistochemical study was performed with mouse ileum and colon using antibodies to $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_2$ receptors (Giaroni *et al.* 2002). No staining was observed for $P2Y_4$ receptors. The immunohistochemical findings were supported by pharmacological data suggesting that $P2Y_1$ receptors on myenteric and submucosal NANC inhibitory neurons, co-localised with NOS, mediated relaxation of longitudinal muscle through production of NO and by ATP acting at $P2Y_1$ receptors on muscle. $P2Y_2$ receptors were also present on smooth muscle however due to the lower potency of UTP the $P2Y_1$ receptors dominated. $P2X_2$ receptors located on the smooth muscle produced a contractile response in the mouse colon (Giaroni *et al.* 2002).

$P2X_{2/3}$ receptors are hypothesized to be located on sensory neurons (Burnstock 1999c). Many immunohistochemistry studies have now investigated the presence of $P2X_2$ and/or $P2X_3$ receptors in a number of GI tissues and the gallbladder (Ma *et al.* 2005; Furness *et al.* 2004; Ruan and Burnstock 2004; Wang and Neuhuber 2003; Wynn *et al.* 2003b; Castelucci *et al.* 2003; Nurgali *et al.* 2003; Poole *et al.* 2002b; Facer *et al.* 2001; Yiangou *et al.* 2001b) and ATP, via activation of $P2X_{2/3}$ receptors, is believed to be involved in mediating painful sensations in the GI tract.

References

11 REFERENCES

Al-Jiffry BO, Jobling JM, Schloithe AC, Toouli J, Saccone GTP (2001) Secretin induces variable inhibition of motility in different parts of the Australian possum sphincter of Oddi. *Neurogastroenterology* **13,** 449-455.

Azuma H, Fujiwara M (1973b) Analysis of contractions induced by transmural stimulation in the isolated sphincter of Oddi. *Japanese Journal of Pharmacology* **23,** 723-732.

Azuma H, Fujiwara M (1973a) Analysis of contractions induced by transmural stimulation in the isolated sphincter of Oddi. *Japanese Journal of Pharmacology* **23,** 723-732.

Baker RA, Saccone GT, Brookes SJ, Toouli J (1993) Nitric oxide mediates nonadrenergic, noncholinergic neural relaxation in the Australian possum. *Gastroenterology* **105,** 1746-1753.

Baker RA, Saccone GT, Costi D, Thune A, Toouli J (1992) Motilin and erythromycin enhance the in vitro contractile activity of the sphincter of Oddi of the Australian brush-tailed possum. *Naunyn-Schmiedebergs Archives of Pharmacology* **345,** 71-77.

Baker RA, Saccone GT, Toouli J (1990a) Cisapride inhibits motility of the sphincter of Oddi in the Australian possum. *Digestive Diseases & Sciences* **35,** 711-715.

Baker RA, Saccone GT, Toouli J (1990b) Cisapride inhibits motility of the sphincter of Oddi in the Australian possum. *Digestive Diseases & Sciences* **35,** 711-715.

Baker RA, Saccone GTP, Toouli J (1990c) Contractile gradient in sphincter of Oddi muscel strips in vitro: Is there a pacemaker? *Proceedings of the Australian Physiological and Pharmacological Society* **21,** 134P.

Balemba OB, Salter MJ, Mawe GM (2004a) Innervation of the extrahepatic biliary tract. *Anat.Rec.A Discov.Mol.Cell Evol.Biol.* **280,** 836-847.

Balemba OB, Salter MJ, Mawe GM (2004c) Innervation of the extrahepatic biliary tract. *Anat.Rec.A Discov.Mol.Cell Evol.Biol.* **280,** 836-847.

Balemba OB, Salter MJ, Mawe GM (2004b) Innervation of the extrahepatic biliary tract. *Anat.Rec.A Discov.Mol.Cell Evol.Biol.* **280,** 836-847.

Belai A, Burnstock G (1994) Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and anococcygeus muscle. *Cell & Tissue Research* **278,** 197-200.

Bian X, Bertrand PP, Bornstein JC (2000) Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. *J.Physiol* **528,** 551-560.

Bodin P, Burnstock G (2001) Purinergic signalling: ATP release. *Neurochem.Res.* **26,** 959-969.

Bornstein JC, Furness JB, Kunze WA, Bertrand PP (2002) Enteric reflexes that influence motility. In 'Innervation of the gastrointestinal tract'. (Eds SJ Brookes and M Costa) pp. 1-55. (Taylor and Francis: London)

Boyden EA (1937) The spincter of Oddi in man and certain representative mammals. *Surgery* **1,** 25-37.

Boyden EA (1957) The anatomy of the choledochoduodenal junction in man. *Surg Gynecol Obstet* **104,** 641-652.

Burnett W, Gairns FW, Bacsich P (1964) Some observations on the innervation of the extrahepatic biliary system in man. *Ann.Surg.* **159,** 8-26.

Burns AJ, Herbert TM, Ward SM, Sanders KM (1997) Interstitial cells of Cajal in the guinea-pig gastrointestinal tract as revealed by c-Kit immunohistochemistry. *Cell Tissue Res.* **290,** 11-20.

Burnstock G (1978) A basis for distinguishing two types of purinergic receptor. In 'Cell membrane receptors for drugs and hormones: a multidisciplinary approach'. (Eds RW Straub and L Bolis) pp. 107-118. (Raven Press: New York)

Burnstock G (1999a) Current status of purinergic signalling in the nervous system. *Progress in Brain Research* **120,** 3-10.

Burnstock G (1999b) Purinergic cotransmission. *Brain Research Bulletin* **50,** 355-357.

Burnstock G (1999d) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *Journal of Anatomy* **194,** 335-342.

Burnstock G (1999c) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *Journal of Anatomy* **194,** 335-342.

Burnstock G (1999e) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *Journal of Anatomy* **194,** 335-342.

Burnstock G (2001a) Purine-mediated signalling in pain and visceral perception. *Trends Pharmacol.Sci.* **22,** 182-188.

Burnstock G (2001b) Purinergic signalling in gut. In 'Handbook of Experimental Pharmacology, Vol. 151/II, Purinergic and Pyrimidinergic Signalling II, Cardiovascular, Respiratory, Immune, Metabolic and Gastrointestinal Tract Function'. (Eds MP Abbracchio and M Williams) pp. 141-237. (Springer-Verlag: Berlin Heidelberg)

Burnstock G (2002b) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin.Med.* **2,** 45-53.

Burnstock G (2002a) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin.Med.* **2,** 45-53.

Burnstock G (2004) Introduction: P2 receptors. *Curr.Top.Med.Chem.* **4,** 793-803.

Burnstock G, Campbell G, Satchell D, Smythe A (1970b) Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *British Journal of Pharmacology* **40,** 668-688.

Burnstock G, Campbell G, Satchell D, Smythe A (1970a) Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *British Journal of Pharmacology* **40,** 668-688.

Burnstock G, Cocks T, Paddle B, Staszewska-Barczak J (1975) Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. *European Journal of Pharmacology* **31,** 360-362.

Burnstock G, Lavin S (2002a) Interstitial cells of Cajal and purinergic signalling. *Autonomic Neuroscience-Basic & Clinical* **97,** 68-72.

Burnstock G, Lavin S (2002d) Interstitial cells of Cajal and purinergic signalling. *Autonomic Neuroscience-Basic & Clinical* **97,** 68-72.

Burnstock G, Lavin S (2002b) Interstitial cells of Cajal and purinergic signalling. *Autonomic Neuroscience-Basic & Clinical* **97,** 68-72.

Burnstock G, Lavin S (2002c) Interstitial cells of Cajal and purinergic signalling. *Autonomic Neuroscience-Basic & Clinical* **97,** 68-72.

Burnstock G, Satchell DG, Smythe A (1972b) A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *British Journal of Pharmacology* **46,** 234-242.

Burnstock G, Satchell DG, Smythe A (1972c) A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *British Journal of Pharmacology* **46,** 234-242.

Burnstock G, Satchell DG, Smythe A (1972a) A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *British Journal of Pharmacology* **46,** 234-242.

Burton LD, Housley GD, Salih SG, Jarlebark L, Christie DL, Greenwood D (2000) P2X(2) receptor expression by interstitial cells of Cajal in vas deferens

implicated in semen emission. *Autonomic Neuroscience-Basic & Clinical* **84,** 147-161.

Cai WQ, Gabella G (1983) Innervation of the gallbladder and biliary pathways in the guinea pig. *Journal of Anatomy* **136,** 97-109.

Carr-locke DL, Gregg JA, Chey WY (1985) Effects of exogenous secretin on pancreatic and biliary ductal and sphincteric pressures in man demonstrated by endoscopic manometry and correlation with plasma secretin levels. *Digestive Diseases and Sciences* **30,** 909-917.

Castelucci P, Robbines HL, Poole DP, Furness JB (2002) The distribution of purine P2X2 receptors in the guinea-pig enteric nervous system. *Histochemical Cell Biology* **117,** 415-422.

Castelucci P, Robbins HL, Furness JB (2003) P2X(2) purine receptor immunoreactivity of intraganglionic laminar endings in the mouse gastrointestinal tract. *Cell Tissue Res.* **312,** 167-174.

Chari RS, Schutz SM, Haebig JE, Shimokura GH, Cotton PB, Fitz JG, Meyers WC (1996a) Adenosine nucleotides in bile. *American Journal of Physiology* **270,** G246-G252.

Chari RS, Schutz SM, Haebig JE, Shimokura GH, Cotton PB, Fitz JG, Meyers WC (1996b) Adenosine nucleotides in bile. *American Journal of Physiology* **270,** G246-G252.

Chizh BA, Illes P (2001) P2X receptors and nociception. *Pharmacol.Rev.* **53,** 553-568.

Christofi FL, Tack J, Wood JD (1992) Suppression of nicotinic synaptic transmission by adenosine in myenteric ganglia of the guinea-pig gastric antrum. *European Journal of Pharmacology* **216,** 17-22.

Christofi FL, Wood JD (1993) Presynaptic inhibition by adenosine A1 receptors on guinea pig small intestinal myenteric neurons. *Gastroenterology* **104,** 1420-1429.

Christofi FL, Zhang HM, Yu JG, Guzman J, Xue JJ, Kim MS, Wang YZ, Cooke HJ (2001a) Differential gene expression of adenosine A1, A2a, A2b, and A3 receptors in the human enteric nervous system. *Journal of Comparative Neurology* **439,** 46-64.

Christofi FL, Zhang HM, Yu JG, Guzman J, Xue JJ, Kim MS, Wang YZ, Cooke HJ (2001b) Differential gene expression of adenosine A1, A2a, A2b, and A3 receptors in the human enteric nervous system. *Journal of Comparative Neurology* **439,** 46-64.

Christofi FL, Zhang HM, Yu JG, Guzman J, Xue JJ, Kim MS, Wang YZ, Cooke HJ (2001c) Differential gene expression of adenosine A1, A2a, A2b, and A3 receptors in the human enteric nervous system. *Journal of Comparative Neurology* **439,** 46-64.

Christofi FL, Zhang HM, Yu JG, Guzman J, Xue JJ, Kim MS, Wang YZ, Cooke HJ (2001d) Differential gene expression of adenosine A1, A2a, A2b, and A3 receptors in the human enteric nervous system. *Journal of Comparative Neurology* **439,** 46-64.

Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North, RA, Buell G (1997) Tissue distribution of the P2X7 receptor. *Neuropharmacology* **36,** 1277-1283.

Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart, Surprenant A, Buell G (1996) Cloning OF P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *Journal of Neuroscience* **16,** 2495-2507.

Communi D, Boeynaems JM (1997) Receptors responsive to extracellular pyrimidine nucleotides. *Trends in Pharmacological Sciences* **18,** 83-86.

Cox MR, Padbury RT, Snelling TL, Schloithe AC, Harvey JR, Toouli J, Saccone GT (1998) Gastrin-releasing peptide stimulates gallbladder motility but not sphincter of Oddi motility in Australian brush-tailed possum. *Digestive Diseases & Sciences* **43,** 1275-1284.

Craig A, Toouli J (2002) Sphincter of Oddi dysfunction: is there a role for medical therapy? *Curr.Gastroenterol.Rep.* **4,** 172-176.

Craig AG, Omari TI, Saccone GTP, Toouli J, Dent J (2000) Evaluation of multiple-point measurement of sphincter of Oddi motility in the Australian brush-tailed possum. *American Journal of Physiology* **279,** G837-G843.

Crema A, Frigo GM, Lecchini S, Manzo L, Onori L, Tonini M (1983) Purine receptors in the guinea-pig internal anal sphincter. *British Journal of Pharmacology* **78,** 599-603.

Crowe R, Burnstock G (1981) Comparative studies of quinacrine-positive neurones in the myenteric plexus of stomach and intestine of guinea-pig, rabbit and rat. *Cell & Tissue Research* **221,** 93-107.

Daly JW, Padgett W, Shamim MT, Butts-Lamb P, Waters J (1985) 1,3-Dialkyl-8-(p-sulfophenyl)xanthines: potent water-soluble antagonists for A1- and A2-adenosine receptors. *Journal of Medicinal Chemistry* **28,** 487-492.

Damer S, Niebel B, Czeche S, Nickel P, Ardanuy U, Schmalzing G, Rettinger J, Mutschler E, Lambrecht G (1998) NF279: a novel potent and selective antagonist of P2X receptor-mediated responses. *European Journal of Pharmacology* **350,** R5-R6.

De Man JG, Seerden TC, De Winter BY, Van Marck EA, Herman AG, Pelckmans PA (2003b) Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation. *British Journal of Pharmacology* **139,** 172-184.

De Man JG, Seerden TC, De Winter BY, Van Marck EA, Herman AG, Pelckmans PA (2003c) Alteration of the purinergic modulation of enteric

neurotransmission in the mouse ileum during chronic intestinal inflammation. *British Journal of Pharmacology* **139,** 172-184.

De Man JG, Seerden TC, De Winter BY, Van Marck EA, Herman AG, Pelckmans PA (2003a) Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation. *British Journal of Pharmacology* **139,** 172-184.

DeLuca A., Li CG, Rand MJ (1999) Nitrergic and purinergic mechanisms and their interactions for relaxation of the rat internal anal sphincter. *Journal of Autonomic Pharmacology* **19,** 29-37.

Di Francesco V, Saccone GTP, Schloithe A, Harvey JR, Carbone A, Toouli J (1994) Manometric profile of the pancreatic duct duct and bile duct components of the sphincter of Oddi in the Australian brush-tailed possum. *Proceedings of the Australian Physiological and Pharmacological Society* **25,** 13P.

Dodds WJU, Hogan WJ, Geenen JE (1989) Motility of the biliary system. *Handbook of Physiology, The Gastrointestinal Tract* **1 Chap 28,** 1055-1101.

Duarte-Araujo M, Nascimento C, Alexandrina TM, Magalhaes-Cardoso T, Correia-de-Sa P (2004a) Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A(2A) and extrajunctional inhibitory A(1) receptors. *British Journal of Pharmacology* **141,** 925-934.

Duarte-Araujo M, Nascimento C, Alexandrina TM, Magalhaes-Cardoso T, Correia-de-Sa P (2004b) Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A(2A) and extrajunctional inhibitory A(1) receptors. *British Journal of Pharmacology* **141,** 925-934.

Facer P, Knowles CH, Tam PK, Ford AP, Dyer N, Baecker PA, Anand P (2001) Novel capsaicin (VR1) and purinergic (P2X3) receptors in Hirschsprung's intestine. *J.Pediatr.Surg.* **36,** 1679-1684.

Farrugia G (1999) Ionic conductances in gastrointestinal smooth muscles and interstitial cells of Cajal. *Annual Review of Physiology* **61,** 45-84.

Feoktistov I, Garland EM, Goldstein AE, Zeng D, Belardinelli L, Wells JN, Biaggioni I (2001) Inhibition of human mast cell activation with the novel selective adenosine A(2B) receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX)(2). *Biochemical Pharmacology* **62,** 1163-1173.

Fernandez E, Guo X, Vergara P, Jimenez M (1998) Evidence supporting a role for ATP as non-adrenergic non-cholinergic inhibitory transmitter in the porcine ileum. *Life Sciences* **62,** 1303-1315.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson, KA, Schwabe U, Williams M (1997c) Towards a revised nomenclature for P1 and P2 receptors. *Trends in Pharmacological Sciences* **18,** 79-82.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson, KA, Schwabe U, Williams M (1997a) Towards a revised nomenclature for P1 and P2 receptors. *Trends in Pharmacological Sciences* **18,** 79-82.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson, KA, Schwabe U, Williams M (1997b) Towards a revised nomenclature for P1 and P2 receptors. *Trends in Pharmacological Sciences* **18,** 79-82.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001b) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol.Rev.* **53,** 527-552.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001a) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol.Rev.* **53,** 527-552.

Furness JB, Bornstein JC (1995) The enteric nervous system and its extrinsic connections. In 'Textbook of Gastroenterology'. (Ed. T Yamada) pp. 2-25. (JB Lippincott Company: Philadelphia)

Furness JB, Robbins HL, Xiao J, Stebbing MJ, Nurgali K (2004) Projections and chemistry of Dogiel type II neurons in the mouse colon. *Cell Tissue Res.* **317,** 1-12.

Furness JB, Sanger GJ (2002) Intrinsic nerve circuits of the gastrointestinal tract: identification of drug targets. *Current Opinion in Pharmacology* **2,** 612-622.

Gaion RM, Dorigo P, Trolese B, Borin E, Adami R, Gambarotto L (1988b) Involvement of P1-purinoreceptors in the relaxing effect of adenosine in rat duodenum. *Journal of Autonomic Pharmacology* **8,** 135-140.

Gaion RM, Dorigo P, Trolese B, Borin E, Adami R, Gambarotto L (1988a) Involvement of P1-purinoreceptors in the relaxing effect of adenosine in rat duodenum. *Journal of Autonomic Pharmacology* **8,** 135-140.

Galligan JJ, North RA (2004) Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol.Motil.* **16 Suppl 1,** 64-70.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, Burnstock G (2002) P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43,** 1313-1323.

Grivell MB, Woods CM, Grivell AR, Neild TO, Craig AG, Toouli J, Saccone GT (2004a) The possum sphincter of Oddi pumps or resists flow depending on common bile duct pressure: a multilumen manometry study. *J.Physiol* **558,** 611-622.

Grivell MB, Woods CM, Grivell AR, Neild TO, Craig AG, Toouli J, Saccone GT (2004b) The possum sphincter of Oddi pumps or resists flow depending

on common bile duct pressure: a multilumen manometry study. *J.Physiol* **558,** 611-622.

Groschel-Stewart U, Bardini M, Robson T, Burnstock G (1999) P2X receptors in the rat duodenal villus. *Cell & Tissue Research* **297,** 111-117.

Harden TK, Boyer JL, Nicholas RA (1995) P2-purinergic receptors: subtype-associated signaling responses and structure. *Annual Review of Pharmacology & Toxicology* **35,** 541-579.

Hauge CW, Mark JBD (1965) Common bile duct motility and sphincter mechanism: pressure measurements with multiple-lumen catheter in dogs. *Annals of Surgery* **162,** 1028-1038.

Helm JF, Dodds WJU, Christensen J, Sarna SK (1985) Control mechanism of spontaneous in vitro contractions of the opossum sphincter of Oddi. *American Journal of Physiology* **249,** G572-G579.

Hillsley K, Mawe GM (1998) Correlation of electrophysiology, neurochemistry and axonal projections of guinea-pig sphincter of Oddi neurones. *Neurogastroenterol.Motil.* **10,** 235-244.

Hirose T, Ito Y (1991) Excitatory and inhibitory responses of Oddi's sphincter in guinea pigs. *American Physiological Society* **260,** G615-G624.

Horowitz B, Ward SM, Sanders KM (1999) Cellular and molecular basis for electrical rhythmicity in gastrointestinal muscles. *Annual Review of Physiology* **61,** 19-43.

Hourani SM, Bailey SJ, Nicholls J, Kitchen I (1991) Direct effects of adenylyl 5'-(beta,gamma-methylene)diphosphonate, a stable ATP analogue, on relaxant P1-purinoceptors in smooth muscle. *British Journal of Pharmacology* **104,** 685-690.

Hu HZ, Gao N, Lin Z, Gao CY, Liu SM, Ren J, Xia Y, Wood JD (2001) P2X(7) receptors in the enteric nervous system of guinea-pig small intestine. *Journal of Comparative Neurology* **440,** 299-310.

Hu HZ, Gao N, Zhu MX, Liu S, Ren J, Gao C, Xia Y, Wood JD (2003) Slow excitatory synaptic transmission mediated by P2Y1 receptors in the guinea-pig enteric nervous system. *J.Physiol* **550,** 493-504.

Imaeda K, Cunnane TC (2003) Electrophysiological properties of inhibitory junction potential in murine lower oesophageal sphincter. *J.Smooth Muscle Res.* **39,** 119-133.

Imaeda K, Joh T, Yamamoto Y, Itoh M, Suzuki H (1998) Properties of inhibitory junctional transmission in smooth muscle of the guinea pig lower esophageal sphincter. *Japanese Journal of Physiology* **48,** 457-465.

Imoto A, Inoue R, Tanaka M, Ito Y (1998b) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits - a possible role of PACAP. *Journal of the Autonomic Nervous System* **70,** 189-199.

Imoto A, Inoue R, Tanaka M, Ito Y (1998a) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits - a possible role of PACAP. *Journal of the Autonomic Nervous System* **70,** 189-199.

Imoto A, Inoue R, Tanaka M, Ito Y (1998e) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits - a possible role of PACAP. *Journal of the Autonomic Nervous System* **70,** 189-199.

Imoto A, Inoue R, Tanaka M, Ito Y (1998d) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits - a possible role of PACAP. *Journal of the Autonomic Nervous System* **70,** 189-199.

Imoto A, Inoue R, Tanaka M, Ito Y (1998c) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits - a possible role of PACAP. *Journal of the Autonomic Nervous System* **70,** 189-199.

Ivancheva C, Itzev D, Radomirov R (2000) Functional antagonism between nitric oxide and ATP in the motor responses of guinea-pig ileum. *Journal of Autonomic Pharmacology* **20,** 147-156.

Ivancheva C, Rahamimoff R, Radomirov R (2001) Apamin-sensitive nitric oxide- and ATP-mediated motor effects on the guinea pig small intestine. *Gen.Physiol Biophys.* **20,** 97-108.

Ivy AC, Voegtlin WL, Greengard H (1933) The physiology of the common bile duct. *Journal of the American Medical Association* **100,** 1319-1320.

Jacobson KA, Jarvis MF, Williams M (2002) Purine and pyrimidine (P2) receptors as drug targets. *J.Med.Chem.* **45,** 4057-4093.

Johnson CR, Hourani SM (1994d) Contractile effects of uridine 5'-triphosphate in the rat duodenum. *British Journal of Pharmacology* **113,** 1191-1196.

Johnson CR, Hourani SM (1994e) Contractile effects of uridine 5'-triphosphate in the rat duodenum. *British Journal of Pharmacology* **113,** 1191-1196.

Johnson CR, Hourani SM (1994c) Contractile effects of uridine 5'-triphosphate in the rat duodenum. *British Journal of Pharmacology* **113,** 1191-1196.

Johnson CR, Hourani SM (1994b) Contractile effects of uridine 5'-triphosphate in the rat duodenum. *British Journal of Pharmacology* **113,** 1191-1196.

Johnson CR, Hourani SM (1994a) Contractile effects of uridine 5'-triphosphate in the rat duodenum. *British Journal of Pharmacology* **113,** 1191-1196.

Kadowaki M, Nagakura Y, Tokita K, Hanaoka K, Tomoi M (2003) Adenosine A1 receptor blockade reverses experimental postoperative ileus in rat colon. *European Journal of Pharmacology* **458,** 197-200.

Kamiji T, Morita K, Katayama Y (1994) ATP regulates synaptic transmission by pre- and postsynaptic mechanisms in guinea-pig myenteric neurons. *Neuroscience* **59,** 165-174.

Katsuragi T, Inoue T, Furukawa T (1985a) Antagonism by clonidine of the adenosine-induced inhibition of the twitch contraction of the guinea-pig Oddi sphincter. *European Journal of Pharmacology* **108,** 151-156.

Katsuragi T, Inoue T, Furukawa T (1985b) Antagonism by clonidine of the adenosine-induced inhibition of the twitch contraction of the guinea-pig Oddi sphincter. *European Journal of Pharmacology* **108,** 151-156.

Katsuragi T, Inoue T, Furukawa T (1985c) Antagonism by clonidine of the adenosine-induced inhibition of the twitch contraction of the guinea-pig Oddi sphincter. *European Journal of Pharmacology* **108,** 151-156.

Katsuragi T, Tokunaga T, Ogawa S, Soejima O, Sato C, Furukawa T (1991) Existence of ATP-evoked ATP release system in smooth muscles. *Journal of Pharmacology & Experimental Therapeutics* **259,** 513-518.

Kenakin TP (1992) Tissue response as a functional discriminator of receptor heterogeneity: effects of mixed receptor populations on Schild regressions. *Molecular Pharmacology* **41,** 699-707.

Kennedy AL, Mawe GM (1998a) Duodenal sensory neurons project to sphincter of Oddi ganglia in guinea pig. *Journal of Neuroscience* **18,** 8065-8073.

Kennedy AL, Mawe GM (1998b) Duodenal sensory neurons project to sphincter of Oddi ganglia in guinea pig. *Journal of Neuroscience* **18,** 8065-8073.

Kennedy I, Humphrey PP (1994) Evidence for the presence of two types of P2 purinoceptor in the guinea-pig ileal longitudinal smooth muscle preparation. *European Journal of Pharmacology* **261,** 273-280.

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela P, Voigt M, Humphrey PP (2001) International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacological Reviews* **53,** 107-118.

Khakh BS, Kennedy C (1998a) Adenosine and ATP: progress in their receptors' structures and functions. *Trends in Pharmacological Sciences* **19,** 39-41.

Khakh BS, Kennedy C (1998b) Adenosine and ATP: progress in their receptors' structures and functions. *Trends in Pharmacological Sciences* **19,** 39-41.

Kim YC, Ji X, Melman N, Linden J, Jacobson KA (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *Journal of Medicinal Chemistry* **43,** 1165-1172.

King BF, Townsend-Nicholson A (2003) Nucleotide and Nucleoside Receptors. *Tocris Reviews* **23,** 1-11.

Knight GE, Bodin P, De Groat WC, Burnstock G (2002) ATP is released from guinea pig ureter epithelium on distension. *Am.J.Physiol Renal Physiol* **282,** F281-F288.

Komuro T (1999) Comparative morphology of interstitial cells of Cajal: ultrastructural characterization. *Microsc.Res.Tech.* **47,** 267-285.

Konomi H, Meedeniya AC, Simula ME, Toouli J, Saccone GT (2002a) Characterization of circular muscle motor neurons of the duodenum and distal colon in the Australian brush-tailed possum. *Journal of Comparative Neurology* **443,** 15-26.

Konomi H, Simula ME, Meedeniya ACB, Toouli J, Saccone GTP (2002b) Induction of duodenal motility activates the sphincter of Oddi (SO)-duodenal reflex in the Australian possum *in vitro*. *Autonomic and Autacoid Pharmacology* **22,** 109-117.

Konomi H, Woods CM, Meedeniya AC, Giles LC, Toouli J, Saccone GTP (2001) Effects of diaspirin cross-linked hemoglobin on motor function of the duodenum and biliary system in the Australian brush-tailed possum in vivo. *Journal of Pharmacology & Experimental Therapeutics* **296,** 1067-1073.

Kunze WA, Furness JB (1999) The enteric nervous system and regulation of intestinal motility. *Annual Review of Physiology* **61,** 117-142.

Lambrecht G, Braun K, Damer M, Ganso M, Hildebrandt C, Ullmann H, Kassack MU, Nickel P (2002) Structure-activity relationships of suramin and pyridoxal-5'-phosphate derivatives as P2 receptor antagonists. *Curr.Pharm.Des* **8,** 2371-2399.

Lecci A, Santicioli P, Maggi CA (2002c) Pharmacology of transmission to gastrointestinal muscle. *Curr.Opin.Pharmacol.* **2,** 630-641.

Lecci A, Santicioli P, Maggi CA (2002b) Pharmacology of transmission to gastrointestinal muscle. *Curr.Opin.Pharmacol.* **2,** 630-641.

Lecci A, Santicioli P, Maggi CA (2002a) Pharmacology of transmission to gastrointestinal muscle. *Curr.Opin.Pharmacol.* **2,** 630-641.

Lee JJ, Parsons ME (2000a) Signaling mechanisms coupled to presynaptic A(1)- and H(3)-receptors in the inhibition of cholinergic contractile responses of the guinea pig ileum. *J.Pharmacol.Exp.Ther.* **295,** 607-613.

Lee JJ, Parsons ME (2000b) Signaling mechanisms coupled to presynaptic A(1)- and H(3)-receptors in the inhibition of cholinergic contractile responses of the guinea pig ileum. *J.Pharmacol.Exp.Ther.* **295,** 607-613.

Lee JJ, Talubmook C, Parsons ME (2001a) Activation of presynaptic A1-receptors by endogenous adenosine inhibits acetylcholine release in the guinea-pig ileum. *Journal of Autonomic Pharmacology* **21,** 29-38.

Lee JJ, Talubmook C, Parsons ME (2001b) Activation of presynaptic A1-receptors by endogenous adenosine inhibits acetylcholine release in the guinea-pig ileum. *Journal of Autonomic Pharmacology* **21,** 29-38.

LePard KJ, Messori E, Galligan JJ (1997) Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea pig: distribution and pharmacology. *Gastroenterology* **113,** 1522-1534.

Lin Z, Gao N, Hu HZ, Liu S, Gao C, Kim G, Ren J, Xia Y, Peck OC, Wood JD (2002) Immunoreactivity of Hu proteins facilitates identification of myenteric neurones in guinea-pig small intestine. *Neurogastroenterology & Motility* **14,** 197-204.

Longeville JH, Schloithe AC, Simula ME, Giles L, Toouli J (1997) Bile and pancreatic duct components of the possum sphincter of Oddi can display independent activity. *Gastroenterology* **112,** A779.

Lonovics J, Jakab I, Szilvassy J, szilvassy Z (1994) Regional differences in nitric oxide-mediated relaxation of the rabbit sphincter of Oddi. *European Journal of Pharmacology* **255,** 117-122.

Ma B, Ruan HZ, Burnstock G, Dunn PM (2005) Differential expression of P2X receptors on neurons from different parasympathetic ganglia. *Neuropharmacology* **48,** 766-777.

Mabley JG, Pacher P, Liaudet L, Soriano FG, Hasko G, Marton A, Szabo C, Salzman AL (2003) Inosine reduces inflammation and improves survival in a murine model of colitis. *Am.J.Physiol Gastrointest.Liver Physiol* **284,** G138-G144.

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K, Nishikawa S (1992) Requirement of c-kit for development of intestinal pacemaker system. *Development* **116,** 369-375.

Maggi CA, Manzini S, Meli A (1984a) Evidence that GABAA receptors mediate relaxation of rat duodenum by activating intramural nonadrenergic-noncholinergic neurones. *Journal of Autonomic Pharmacology* **4,** 77-85.

Maggi CA, Manzini S, Meli A (1984b) Evidence that GABAA receptors mediate relaxation of rat duodenum by activating intramural nonadrenergic-noncholinergic neurones. *Journal of Autonomic Pharmacology* **4,** 77-85.

Maggi CA, Manzini S, Meli A (1984c) Evidence that GABAA receptors mediate relaxation of rat duodenum by activating intramural nonadrenergic-noncholinergic neurones. *Journal of Autonomic Pharmacology* **4,** 77-85.

Maggi CA, Manzini S, Meli A (1984d) Evidence that GABAA receptors mediate relaxation of rat duodenum by activating intramural nonadrenergic-noncholinergic neurones. *Journal of Autonomic Pharmacology* **4,** 77-85.

Manzini S, Maggi CA, Meli A (1985d) Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *European Journal of Pharmacology* **113,** 399-408.

Manzini S, Maggi CA, Meli A (1985e) Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *European Journal of Pharmacology* **113,** 399-408.

Manzini S, Maggi CA, Meli A (1985a) Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *European Journal of Pharmacology* **113,** 399-408.

Manzini S, Maggi CA, Meli A (1985b) Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *European Journal of Pharmacology* **113,** 399-408.

Manzini S, Maggi CA, Meli A (1985c) Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *European Journal of Pharmacology* **113,** 399-408.

Marteau P (2002) Inflammatory bowel disease. *Endoscopy* **34,** 63-68.

Martin MT, Gonalons E, Fernandez E (1998) Contribution of inhibitory neurotransmitters to the CCK induced relaxation of the circular muscle of avian ileum. *Life Sciences* **62,** 937-946.

Mawe GM, Kennedy AL (1999) Duodenal neurons provide nicotinic fast synaptic input to sphincter of Oddi neurons in guinea pig. *American Journal of Physiology* **40,** G226-G234.

McKirdy HC (1988) Novel autonomic neurotransmitters and upper gastrointestinal function. *Pharmacology & Therapeutics* **38,** 429-452.

McKirdy HC, Marshall RW, Griffin P (1987) Effect of drugs, hormones and electrical field stimulation on isolated muscle strips from human choledochoduodenal junction. *Quarterly Journal of Experimental Physiology* **72,** 215-225.

Meedeniya AC, Schloithe AC, Toouli J, Saccone GT (2003) Characterization of the intrinsic and extrinsic innervation of the gall bladder epithelium in the Australian Brush-tailed possum (*Trichosurus vulpecula*). *Neurogastroenterology & Motility* **15,** 383-392.

Meedeniya ACB, Al-Jiffry BO, Konomi H, Schloithe AC, Toouli J, Saccone GTP (2001) Inhibitory motor innervation of the gall bladder musculature by intrinsic neurones containing nitric oxide in the Australian Brush-tailed possum (*Trichosurus vulpecula*). *Gut* **49,** 692-698.

Moneta NA, McDonald TJ, Cook MA (1997) Endogenous adenosine inhibits evoked substance P release from perifused networks of myenteric ganglia. *American Journal of Physiology* **272,** G38-G45.

Monro RL, Bertrand PP, Bornstein JC (2002a) ATP and 5-HT are the principal neurotransmitters in the descending excitatory reflex pathway of the guinea-pig ileum. *Neurogastroenterol.Motil.* **14,** 255-264.

Monro RL, Bertrand PP, Bornstein JC (2002b) ATP and 5-HT are the principal neurotransmitters in the descending excitatory reflex pathway of the guinea-pig ileum. *Neurogastroenterol.Motil.* **14,** 255-264.

Monro RL, Bertrand PP, Bornstein JC (2004) ATP participates in three excitatory postsynaptic potentials in the submucous plexus of the guinea pig ileum. *J.Physiol* **556,** 571-584.

Moody CJ, Burnstock G (1982) Evidence for the presence of P1-purinoceptors on cholinergic nerve terminals in the guinea-pig ileum. *European Journal of Pharmacology* **77,** 1-9.

Mule F, Serio R, Postorino A (1989) Evidence that adenosine is not involved in the non-adrenergic non-cholinergic relaxation in the rat duodenum. *Archives Internationales de Physiologie et de Biochimie* **97,** 149-154.

Murr MM, Balsiger BM, Farrugia G, Sarr MG (1999) Role of nitric oxide, vasoactive intestinal polypeptide, and ATP in inhibitory neurotransmission in human jejunum. *Journal of Surgical Research* **84,** 8-12.

Mutlu EA, Farhadi A, Keshavarzian A (2002a) New developments in the treatment of inflammatory bowel disease. *Expert.Opin.Investig.Drugs* **11,** 365-385.

Mutlu EA, Farhadi A, Keshavarzian A (2002b) New developments in the treatment of inflammatory bowel disease. *Expert.Opin.Investig.Drugs* **11,** 365-385.

Nicholls J, Brownhill VR, Hourani SM (1996h) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996a) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996b) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996c) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996d) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996e) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996f) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Brownhill VR, Hourani SM (1996g) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *British Journal of Pharmacology* **117,** 170-174.

Nicholls J, Hourani SM, Kitchen I (1992f) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992c) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992b) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992e) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992g) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992d) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992a) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992h) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nicholls J, Hourani SM, Kitchen I (1992i) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *British Journal of Pharmacology* **105,** 639-642.

Nissan S, Vinograd Y, Hadari A, Merguerian P, Zamir O, Lernau O, Hanani (1984) Physiological and pharmacological studies of the internal anal sphincter in the rat. *Journal of Pediatric Surgery* **19,** 12-14.

Nurgali K, Furness JB, Stebbing MJ (2003) Analysis of purinergic and cholinergic fast synaptic transmission to identified myenteric neurons. *Neuroscience* **116,** 335-347.

Nurgali K, Stebbing MJ, Furness JB (2004) Correlation of electrophysiological and morphological characteristics of enteric neurons in the mouse colon. *J.Comp Neurol.* **468,** 112-124.

Olah ME, Stiles GL (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. *Annual Review of Pharmacology & Toxicology* **35,** 581-606.

Padbury RT, Baker RA, Messenger JP, Toouli J, Furness, JB (1993a) Structure and innervation of the extrahepatic biliary system in the Australian possum, *Trichosurus vulpecula*. *HPB Surgery* **7,** 125-139.

Padbury RT, Furness JB, Baker RA, Toouli J, Messenger, JP (1993b) Projections of nerve cells from the duodenum to the sphincter of Oddi and gallbladder of the Australian possum. *Gastroenterology* **104,** 130-136.

Padbury RT, Furness JB, Baker RA, Toouli J, Messenger, JP (1993c) Projections of nerve cells from the duodenum to the sphincter of Oddi and gallbladder of the Australian possum. *Gastroenterology* **104,** 130-136.

Pap A, Forro G (1998a) The effect of theophylline preparations on morphine-induced spasm of Oddi's sphincter in man. *Orvosi Hetilap* **139,** 1411-1414.

Pap A, Forro G (1998b) The effect of theophylline preparations on morphine-induced spasm of Oddi's sphincter in man. *Orvosi Hetilap* **139,** 1411-1414.

Pap A, Forro G (1998c) The effect of theophylline preparations on morphine-induced spasm of Oddi's sphincter in man. *Orvosi Hetilap* **139,** 1411-1414.

Parkman HP, Pagano AP, Thomas RM, Ryan JP (1998) Regional differences in myogenic and neurogenic properties of rabbit sphincter of Oddi smooth muscle. *Gastroenterology* **114,** A819.

Parr EJ, Kennedy AI, Mawe GM (2003) Lack of evidence for the existence of interstitial cells of Cajal in the gallbladder. *Gastroenterology* **124,** A-347.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998h) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998a) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998b) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998c) Evidence that tachykinins are the main NANC excitatory neurotransmitters in

the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998d) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998e) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998f) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Patacchini R, De GR, Bartho L, Barbara G, Corinaldesi R, Maggi CA (1998g) Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. *British Journal of Pharmacology* **124,** 1703-1711.

Persson CG (1976c) Inhibitory innervation of cat sphincter of Oddi. *British Journal of Pharmacology* **58,** 479-482.

Persson CG (1976a) Inhibitory innervation of cat sphincter of Oddi. *British Journal of Pharmacology* **58,** 479-482.

Persson CG (1976b) Inhibitory innervation of cat sphincter of Oddi. *British Journal of Pharmacology* **58,** 479-482.

Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB (2002b) The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. *Auton.Neurosci.* **101,** 39-47.

Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB (2002a) The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. *Auton.Neurosci.* **101,** 39-47.

Rae MG, Muir TC (1996) Neuronal mediators of inhibitory junction potentials and relaxation in the guinea-pig internal anal sphincter. *Journal of Physiology* **493,** 517-527.

Ralevic V, Burnstock G (1998j) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998i) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998m) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998g) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998f) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998e) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998d) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998c) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998r) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998a) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998w) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998k) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998t) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998h) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998n) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998o) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998p) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998l) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998s) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998v) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998x) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998q) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998u) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ralevic V, Burnstock G (1998b) Receptors for purines and pyrimidines. *Pharmacological Reviews* **50,** 413-492.

Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, Galligan JJ (2003) P2X2 subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. *J.Physiol* **552,** 809-821.

Ribeiro JA, Cunha RA, Correia-de-Sa P, Sebastiao AM (1996) Purinergic regulation of acetylcholine release. *Progress in Brain Research* **109,** 231-241.

Romert P, Mikkelsen HB (1998) c-kit immunoreactive interstitial cells of Cajal in the human small and large intestine. *Histochem.Cell Biol.* **109,** 195-202.

Rong W, Spyer KM, Burnstock G (2002) Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *J.Physiol* **541,** 591-600.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998) Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *Journal of Comparative Neurology* **401,** 163-186.

Rozsai B, Lazar Z, Benko R, Bartho L (2001) Inhibition of the NANC relaxation of the guinea-pig proximal colon longitudinal muscle by the purinoceptor antagonist PPADS, inhibition of nitric oxide synthase, but not by a PACAP/VIP antagonist. *Pharmacol.Res.* **43,** 83-87.

Ruan HZ, Burnstock G (2004) P2X2 and P2X3 receptor expression in the gallbladder of the guinea pig. *Auton.Neurosci.* **111,** 89-96.

Saccone GT, Harvey JR, Baker RA, Toouli J (1994c) Intramural neural pathways between the duodenum and sphincter of Oddi in the Australian brush-tailed possum *in vivo*. *Journal of Physiology* **481,** 447-456.

Saccone GT, Harvey JR, Baker RA, Toouli J (1994a) Intramural neural pathways between the duodenum and sphincter of Oddi in the Australian brush-tailed possum *in vivo*. *Journal of Physiology* **481,** 447-456.

Saccone GT, Harvey JR, Baker RA, Toouli J (1994b) Intramural neural pathways between the duodenum and sphincter of Oddi in the Australian brush-tailed possum *in vivo*. *Journal of Physiology* **481,** 447-456.

Saccone GTP, Woods CM, Schloithe AC, Simula ME, Al-Jiffry B, Toouli J (2000) Proximal and distal segments of the bile and pancreatic components of possum sphincter of Oddi can respond differently to neural and hormonal stimulation. In 'Falk Symposium 112: Neurogastroenterlology - from the

Basics to the Clinics'. (Eds H-J Krammer and MV Singer) pp. 338-349. (Kluwer Academic Publishers: Dordrecht)

Sakai K, Akima M, Matsushita H (1979) Analysis of the contractile responses of the ileal segment of the isolated blood-perfused small intestine of rats to adenosine triphosphate and related compounds. *European Journal of Pharmacology* **58,** 157-162.

Sand J, Arvola P, Jantti V, Oja S, Singaram C, Baer G, Pasricha PJ, Nordback I (1997) The inhibitory role of nitric oxide in the control of porcine and human sphincter of Oddi activity. *Gut* **41,** 375-380.

Sanders KM, Ordog T, Koh SD, Torihashi S, Ward SM (1999a) Development and plasticity of interstitial cells of Cajal. *Neurogastroenterology & Motility* **11,** 311-338.

Sanders KM, Ordog T, Koh SD, Torihashi S, Ward SM (1999b) Development and plasticity of interstitial cells of Cajal. *Neurogastroenterology & Motility* **11,** 311-338.

Sawyer GW, Lambrecht G, Ehlert FJ (2000) Functional role of muscarinic M(2) receptors in alpha,beta-methylene ATP induced, neurogenic contractions in guinea-pig ileum. *British Journal of Pharmacology* **129,** 1458-1464.

Schwegler RA, Boyden EA (1937a) The development of the pars intestinalis of the common bile duct in the human fetus with special reference to the origin of the ampulla of Vater and sphincter of Oddi: III: the composition of the musculus proprius. *Anatomical Record* **68,** 193-219.

Schwegler RA, Boyden EA (1937b) The development of the pars intestinalis of the common bile duct in the human fetus with special reference to the origin of the ampulla of vater and the sphincter of Oddi: I: the involution of the ampulla. *Anatomical Record* **67,** 441-468.

Schwegler RA, Boyden EA (1937c) The development of the pars intestinalis of the common bile duct in the human fetus with special reference to the origin of the ampulla of vater and the sphincter of Oddi: II: the early development of the musculus proprius. *Anatomical Record* **68,** 17-41.

Serio R, Mule F, Adamo EB, Postorino A (1990c) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990a) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990d) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990e) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990f) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990g) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990h) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Serio R, Mule F, Adamo EB, Postorino A (1990b) Evidence against purines being neurotransmitters of non-adrenergic, non-cholinergic nerves in rat duodenum. *European Journal of Pharmacology* **182,** 487-495.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001g) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001a) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001b) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001c) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001d) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001e) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Brookes SJ, Meedeniya AC, Toouli J, Saccone GT (2001f) Distribution of nitric oxide synthase and vasoactive intestinal polypeptide

immunoreactivity in the sphincter of Oddi and duodenum of the possum. *Cell & Tissue Research* **304,** 31-41.

Simula ME, Harvey JR, Costi D, Baker RA, Toouli J, Saccone GT (1997) *In vitro* characterisation of intramural neural pathways between the duodenum and the sphincter of Oddi of the brush-tailed possum. *Journal of the Autonomic Nervous System* **63,** 77-84.

Small RC, Weston AH (1979a) Theophylline antagonizes some effects of purines in the intestine but not those of intramural inhibitory nerve stimulation. *British Journal of Pharmacology* **67,** 301-308.

Small RC, Weston AH (1979b) Theophylline antagonizes some effects of purines in the intestine but not those of intramural inhibitory nerve stimulation. *British Journal of Pharmacology* **67,** 301-308.

Sneddon P, Westfall TD, Todorov LD, Mihaylova-Todorova S, Westfall DP, Kennedy C (1999) Modulation of purinergic neurotransmission. *Progress in Brain Research* **120,** 11-20.

Soediono P, Burnstock G (1994) Contribution of ATP and nitric oxide to NANC inhibitory transmission in rat pyloric sphincter. *British Journal of Pharmacology* **113,** 681-686.

Sonoda Y, Takahata S, Jabar F, Schloithe A, Grivell MB, Woods CM, Simula ME, Toouli J, Saccone GTP (2005) Electrical activation of common bile duct nerves modulate sphincter of Oddi motility in the Australian possum. *HPB* **in press**.

Storr M, Franck H, Saur D, Schusdziarra V, Allescher HD (2000) Mechanisms of alpha,beta-methylene atp-induced inhibition in rat ileal smooth muscle: involvement of intracellular Ca2+ stores in purinergic inhibition. *Clin.Exp.Pharmacol.Physiol* **27,** 771-779.

Storr M, Thammer J, Dunkel R, Schusdziarra V, Allescher HD (2002b) Modulatory effect of adenosine receptors on the ascending and descending neural reflex responses of rat ileum. *BMC Neuroscience* **3,** 21.

Storr M, Thammer J, Dunkel R, Schusdziarra V, Allescher HD (2002c) Modulatory effect of adenosine receptors on the ascending and descending neural reflex responses of rat ileum. *BMC Neuroscience* **3,** 21.

Storr M, Thammer J, Dunkel R, Schusdziarra V, Allescher HD (2002a) Modulatory effect of adenosine receptors on the ascending and descending neural reflex responses of rat ileum. *BMC Neuroscience* **3,** 21.

Suarez CV (1982) Structure of the major duodenal papilla of Vater. *Mt Sinai Journal of Medicine* **49,** 31-37.

Takayama I, Horiguchi K, Daigo Y, Mine T, Fujino MA, Ohno S (2002) The interstitial cells of Cajal and a gastroenteric pacemaker system. *Arch.Histol.Cytol.* **65,** 1-26.

Thune A, Thornell E, Svanvik J (1986) Reflex regulation of flow resistance in the feline sphincter of Oddi by hydrostatic pressure in the biliary tract. *Gastroenterology* **91,** 1364-1369.

Toouli J, Baker RA (1991) Innervation of the sphincter of Oddi: physiology and considerations of pharmacological intervention in biliary dyskinesia. *Pharmacology & Therapeutics* **49,** 269-281.

Van Crombruggen K, Lefebvre RA (2004b) Nitrergic-purinergic interactions in rat distal colon motility. *Neurogastroenterol.Motil.* **16,** 81-98.

Van Crombruggen K, Lefebvre RA (2004a) Nitrergic-purinergic interactions in rat distal colon motility. *Neurogastroenterol.Motil.* **16,** 81-98.

Vanderwinden JM (1999) Role of Interstitial Cells of Cajal and their relationship with the enteric nervous system. *Eur.J.Morphol.* **37,** 250-256.

Vanneste G, Robberecht P, Lefebvre RA (2004a) Inhibitory pathways in the circular muscle of rat jejunum. *British Journal of Pharmacology* **143,** 107-118.

Vanneste G, Robberecht P, Lefebvre RA (2004b) Inhibitory pathways in the circular muscle of rat jejunum. *British Journal of Pharmacology* **143,** 107-118.

Vogalis F, Bywater RAR, Taylor GS (1989) Nerve-mediated contractile and electrical activity of the guinea-pig choledocho-duodenal junction. *Journal of the Autonomic Nervous System* **29,** 19-28.

Vogalis F, Smith TK (2000a) Functional innervation of the biliary sphincter of the guinea-pig revealed by anti-autonomic drugs. *Journal of Autonomic Pharmacology* **20,** 177-183.

Vogalis F, Smith TK (2000b) Functional innervation of the biliary sphincter of the guinea-pig revealed by anti-autonomic drugs. *Journal of Autonomic Pharmacology* **20,** 177-183.

Von Schrenck T, Rusch B, Mirau S, Leschig P, Sievers J, Raedler A, Greten H (1991) Characterization of substance P (SP) receptors in the sphincter of Oddi (SO). *Gastroenterology* **100,** A344.

Wang ZJ, Neuhuber WL (2003) Intraganglionic laminar endings in the rat esophagus contain purinergic P2X2 and P2X3 receptor immunoreactivity. *Anat.Embryol.(Berl)* **207,** 363-371.

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *J.Physiol* **480 (Pt 1),** 91-97.

Ward SM, Morris G, Reese L, Wang XY, Sanders KM (1998) Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* **115,** 314-329.

Webb TH, Lillemoe KD, Pitt HA (1988) Gastrosphincter of Oddi reflex. *American Journal of Surgery* **155,** 193-198.

Wells DG, Mawe GM (1993) Physiological and morphological properties of neurons in sphincter of Oddi region of the guinea pig. *Am.J.Physiol* **265,** G258-G269.

Wells DG, Talmage EK, Mawe GM (1995) Immunohistochemical identification of neurons in ganglia of the guinea pig sphincter of Oddi. *J.Comp Neurol.* **352,** 106-116.

Windscheif U, Pfaff O, Ziganshin AU, Hoyle CH, Baumert HG, Mutschler E, Burnstock G, Lambrecht G (1995a) Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum. *British Journal of Pharmacology* **115,** 1509-1517.

Windscheif U, Pfaff O, Ziganshin AU, Hoyle CH, Baumert HG, Mutschler E, Burnstock G, Lambrecht G (1995b) Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum. *British Journal of Pharmacology* **115,** 1509-1517.

Windscheif U, Pfaff O, Ziganshin AU, Hoyle CH, Baumert HG, Mutschler E, Burnstock G, Lambrecht G (1995c) Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum. *British Journal of Pharmacology* **115,** 1509-1517.

Wood JD (1999) Enteric nervous control of motility in the upper gastrointestinal tract in defensive states. *Digestive Diseases & Sciences* **44,** 44S-52S.

Wood JD, Alpers DH, Andrews PL (1999) Fundamentals of neurogastroenterology. *Gut* **45 Suppl 2,** II6-II16.

Woods CM, Mawe GM, Shaffer EA, Toouli J, Saccone TP (2003c) Effects of bioactive agents on biliary motor function. *Curr.Gastroenterol.Rep.* **5,** 154-159.

Woods CM, Mawe GM, Shaffer EA, Toouli J, Saccone TP (2003a) Effects of bioactive agents on biliary motor function. *Curr.Gastroenterol.Rep.* **5,** 154-159.

Woods CM, Mawe GM, Shaffer EA, Toouli J, Saccone TP (2003b) Effects of bioactive agents on biliary motor function. *Curr.Gastroenterol.Rep.* **5,** 154-159.

Woods CM, Schloithe AC, Simula ME, Toouli J, Saccone, GTP (2000) Proximal and distal segments of the possum sphincter of Oddi respond differently to neural and cholecystokinin octapeptide stimulation *in vitro.* *Digestive Surgery* **17,** 241-249.

Wyatt AP (1967) The relationship of the sphincter of Oddi to the stomach duodenum and gallbladder. *Journal of Physiology* **193,** 225-243.

Wyatt AP (1969) Effect of gastrectomy on biliary dynamics. *Gut* **10,** 91-93.

Wynn G, Rong W, Xiang Z, Burnstock G (2003a) Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. *Gastroenterology* **125,** 1398-1409.

Wynn G, Rong W, Xiang Z, Burnstock G (2003b) Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. *Gastroenterology* **125,** 1398-1409.

Xue L, Farrugia G, Sarr MG, Szurszewski JH (1999) ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *American Journal of Physiology* **276,** G1373-G1379.

Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, Anand P (2001a) ATP-gated ion channel P2X(3) is increased in human inflammatory bowel disease. *Neurogastroenterol.Motil.* **13,** 365-369.

Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, Anand P (2001b) ATP-gated ion channel P2X(3) is increased in human inflammatory bowel disease. *Neurogastroenterol.Motil.* **13,** 365-369.

Young HM, Ciampoli D, Southwell BR, Newgreen DF (1996) Origin of interstitial cells of Cajal in the mouse intestine. *Dev.Biol.* **180,** 97-107.

Yuan S, Costa M, Brookes SJ (1998) Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115,** 661-671.

Zagorodniuk VP, Shuba MF (1986) Nature of non-adrenergic inhibition in the smooth muscles of the human intestine. *Neirofiziologiia* **18,** 373-381.

Zhou X, Galligan JJ (1998a) Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *Journal of Physiology* **513,** 685-697.

Zhou X, Galligan JJ (1998b) Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *Journal of Physiology* **513,** 685-697.