

**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	1
Publications from the studies in this thesis	4
Manuscripts.....	4
Abstracts and Conference Presentations.....	4
Declaration	7
Acknowledgements	8
Abbreviations	10
Structure of Thesis	11
History of candidature	11
Thesis chapters.....	11
Location of figures.....	13
1 Introduction	Error! Bookmark not defined.
1.1 Overview	Error! Bookmark not defined.
1.2 Anatomy of the SO and duodenum ..	Error! Bookmark not defined.
1.2.1 General extra-hepatic biliary anatomy.....	Error! Bookmark not defined.
1.2.2 Duodenal anatomy	Error! Bookmark not defined.
1.2.3 SO anatomy and function.....	Error! Bookmark not defined.
1.3 Control of SO spontaneous activity ..	Error! Bookmark not defined.
1.3.1 ICC.....	Error! Bookmark not defined.
1.3.2 Innervation of SO	Error! Bookmark not defined.
1.3.3 Integrated model of spontaneous GI motility ..	Error! Bookmark not defined.
1.3.4 Neural reflexes between the SO and other organs.....	Error! Bookmark not defined.
1.3.5 Possible involvement of purines in SO motility	Error! Bookmark not defined.
1.4 Purines	Error! Bookmark not defined.
1.4.1 Purine receptors	Error! Bookmark not defined.
1.4.2 Agonists and antagonists	Error! Bookmark not defined.
1.4.3 Effect of purines on SO motility ..	Error! Bookmark not defined.
1.4.4 Effect of purines on other sphincters.....	Error! Bookmark not defined.
1.4.5 Effects of purines on small intestinal motility ..	Error! Bookmark not defined.
1.5 Summary	Error! Bookmark not defined.
1.6 Hypothesis and aims	Error! Bookmark not defined.
1.6.1 Overall hypothesis.....	Error! Bookmark not defined.
1.6.2 Aims	Error! Bookmark not defined.
1.6.3 Research rationale	Error! Bookmark not defined.
2 Methods	Error! Bookmark not defined.
2.1 Possums and animal ethics approval	Error! Bookmark not defined.
2.2 <i>In vitro</i> studies	Error! Bookmark not defined.
2.2.1 Tissue harvest.....	Error! Bookmark not defined.
2.2.2 Duodenal muscle strips	Error! Bookmark not defined.
2.2.3 SO muscle rings	Error! Bookmark not defined.
2.2.4 Reagents used	Error! Bookmark not defined.
2.2.5 Experimental protocols.....	Error! Bookmark not defined.
2.2.6 Analysis of recordings	Error! Bookmark not defined.

2.2.7	Statistical analysis	Error! Bookmark not defined.
2.3	<i>In vivo</i> studies.....	Error! Bookmark not defined.
2.3.1	Anaesthesia	Error! Bookmark not defined.
2.3.2	Surgical preparation	Error! Bookmark not defined.
2.3.3	Experimental protocols.....	Error! Bookmark not defined.
2.3.4	Analysis of recordings	Error! Bookmark not defined.
2.3.5	Statistical analysis	Error! Bookmark not defined.
2.4	Immunohistochemical Studies.....	Error! Bookmark not defined.
3	Effect of exogenous ATP and adenosine on spontaneous SO and duodenal contractile activity <i>in vitro</i>	Error! Bookmark not defined.
3.1	Introduction.....	Error! Bookmark not defined.
3.2	Methods.....	Error! Bookmark not defined.
3.3	Results	Error! Bookmark not defined.
3.3.1	SO.....	Error! Bookmark not defined.
3.3.2	Duodenum.....	Error! Bookmark not defined.
3.4	Discussion	Error! Bookmark not defined.
3.4.1	Spontaneous SO motility and ATP.....	Error! Bookmark not defined.
3.4.2	Spontaneous SO motility and adenosine ..	Error! Bookmark not defined.
3.4.3	Spontaneous duodenal motility and ATP ..	Error! Bookmark not defined.
3.4.4	Spontaneous duodenal motility and adenosine.....	Error! Bookmark not defined.
3.4.5	SO regional selectivity of purine responses ...	Error! Bookmark not defined.
3.4.6	Localisation of purinergic receptors.....	Error! Bookmark not defined.
3.5	Conclusions.....	Error! Bookmark not defined.
4	Pharmacological characterisation of P2 receptors mediating the ATP-induced tri-phasic response in spontaneous duodenal motor activity <i>in vitro</i>	Error! Bookmark not defined.
4.1	Introduction.....	Error! Bookmark not defined.
4.2	Methods.....	Error! Bookmark not defined.
4.3	Results	Error! Bookmark not defined.
4.3.1	Non-selective P2 receptor antagonists.....	Error! Bookmark not defined.
4.3.2	P2 receptor agonists	Error! Bookmark not defined.
4.3.3	Preliminary experiments with Ecto-ATPase inhibitor: ARL67156	Error! Bookmark not defined.
4.4	Discussion	Error! Bookmark not defined.
4.5	Conclusion.....	Error! Bookmark not defined.
5	Pharmacological characterisation of P2 receptors mediating the ATP-induced bi-phasic response in SO motility <i>in vitro</i>	Error! Bookmark not defined.
5.1	Introduction.....	Error! Bookmark not defined.
5.2	Methods.....	Error! Bookmark not defined.
5.3	Results	Error! Bookmark not defined.
5.3.1	Non-selective P2 receptor antagonists.....	Error! Bookmark not defined.
5.3.2	P2 receptor agonists	Error! Bookmark not defined.

5.3.3	Preliminary experiments with Ecto-ATPase inhibitor: ARL67156	Error! Bookmark not defined.
5.4	Discussion	Error! Bookmark not defined.
5.5	Conclusion.....	Error! Bookmark not defined.
Summary 1: Exogenous ATP induces a complex response on spontaneous SO and duodenal motility <i>in vitro</i>		Error! Bookmark not defined.
6	Pharmacological identification of P1 receptors mediating the inhibitory effects of exogenous adenosine on spontaneous duodenal activity <i>in vitro</i>	Error! Bookmark not defined.
6.1	Introduction.....	Error! Bookmark not defined.
6.2	Methods.....	Error! Bookmark not defined.
6.3	Results	Error! Bookmark not defined.
6.3.1	P1 receptor antagonists	Error! Bookmark not defined.
6.3.2	P1 receptor agonists	Error! Bookmark not defined.
6.4	Discussion	Error! Bookmark not defined.
6.5	Conclusion.....	Error! Bookmark not defined.
7	Pharmacological characterisation of P1 receptors mediating the inhibitory effects of exogenous adenosine on SO motility <i>in vitro</i>	Error! Bookmark not defined.
7.1	Introduction.....	Error! Bookmark not defined.
7.2	Methods.....	Error! Bookmark not defined.
7.3	Results	Error! Bookmark not defined.
7.3.1	P1 receptor antagonists	Error! Bookmark not defined.
7.3.2	Preliminary experiments with P1 receptor agonists.....	Error! Bookmark not defined.
7.4	Discussion	Error! Bookmark not defined.
7.5	Conclusion.....	Error! Bookmark not defined.
Summary 2: Exogenous adenosine decreases spontaneous SO and duodenal motility <i>in vitro</i>.....		Error! Bookmark not defined.
8	Effect of exogenous adenosine and ATP on SO motility <i>in vivo</i> ...	Error! Bookmark not defined.
8.1	Introduction.....	Error! Bookmark not defined.
8.2	Methods.....	Error! Bookmark not defined.
8.3	Results	Error! Bookmark not defined.
8.3.1	Effect of exogenous ATP or adenosine on SO motility <i>in vivo</i>	Error! Bookmark not defined.
8.3.2	TTX pre-treatment.....	Error! Bookmark not defined.
8.3.3	Atropine and hexamethonium pre-treatment ..	Error! Bookmark not defined.
8.3.4	Inhibition of NOS: L-NAME.....	Error! Bookmark not defined.
8.4	Discussion	Error! Bookmark not defined.
8.5	Conclusion.....	Error! Bookmark not defined.
Summary 3: Exogenous adenosine and ATP increase SO motility <i>in vivo</i>, via stimulation of purinergic receptors on nerves		Error! Bookmark not defined.
9	General discussion	Error! Bookmark not defined.
9.1	Overview of findings	Error! Bookmark not defined.
9.2	Functional significance of activation of purinergic receptors in the SO and further studies.....	Error! Bookmark not defined.
9.2.1	Potential effects of purine-evoked responses on SO function: theoretical considerations.....	Error! Bookmark not defined.

9.2.2	CBD distension and pain perception	Error! Bookmark not defined.
9.2.3	Summary	Error! Bookmark not defined.
9.3	Conclusions	Error! Bookmark not defined.
Appendix 1: Attempted localisation of P1 receptors in SO and duodenum		
A1.1	Introduction	Error! Bookmark not defined.
A1.2	Methods	Error! Bookmark not defined.
A1.2.1	Tissue collection	Error! Bookmark not defined.
A1.2.2	Adenosine receptor immunohistochemistry	Error! Bookmark not defined.
A1.2.3	ICC immunohistochemistry	Error! Bookmark not defined.
A1.2.4	Microscopy and image analysis ...	Error! Bookmark not defined.
A1.3	Results	Error! Bookmark not defined.
A1.3.1	P1 immunohistochemistry in the duodenum	Error! Bookmark not defined.
A1.3.2	P1 control peptide experiments	Error! Bookmark not defined.
A1.3.3	ICC immunohistochemistry	Error! Bookmark not defined.
A1.4	Discussion	Error! Bookmark not defined.
A1.5	Summary and conclusion	Error! Bookmark not defined.
A1.6	Recent immunohistochemical studies using purinergic receptor antibodies in GI tissues	Error! Bookmark not defined.
10	References	Error! Bookmark not defined.

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/nitregic 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 subtypes involved.

PUBLICATIONS RESULTING FROM THE STUDIES IN THIS THESIS

Manuscripts

Woods, C.M., Toouli, J. and Saccone, G.T.P. (2003) A_{2A} and A₃ 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 Australian 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 nitrenergic 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	<i>N</i> ^o -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 findings 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.