ADENOSINE A_1 AND A_{2A} RECEPTORS IN THE RAT STOMACH: BIOLOGICAL ACTIONS, CELLULAR LOCALIZATION, STRUCTURE AND GENE EXPRESSION

by

LINDA YIP

B.Sc., The University of British Columbia, 1997

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Physiology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2004

Abstract

Adenosine has been shown to inhibit gastric acid secretion in the rat stomach, likely by regulating gastrin and somatostatin release. The current study demonstrates that A_1 and A_{2A} receptors may play significant roles in this action. Specifically, in the isolated vascularly perfused rat stomach, the effect of various selective adenosine agonists and antagonists suggests that the inhibition of immunoreactive gastrin (IRG) and somatostatin-like immunoreactivity (SLI) release is mediated by A_1 receptors, while the stimulation of SLI release is mediated by A_{2A} receptors. To determine the site at which adenosine acts to elicit these actions, immunohistochemistry was performed. Both A_1 receptor immunoreactivity (A_1R-IR) and A_{2A} receptor immunoreactivity (A_{2A}R-IR) were observed on mucosal somatostatin-containing D-cells. A_1R-IR was also observed on gastrin-containing G-cells, while neither A_1R-IR nor A_{2A}R-IR was observed on parietal cells. These results suggest that adenosine does not act directly on the parietal cells to inhibit gastric acid secretion. Instead, adenosine may act on A_1 receptors of G-cells and D-cells to inhibit IRG and SLI release, respectively, and may act on A_{2A} receptors of D-cells to stimulate SLI release. The localization of A_1R-IR and A_{2A}R-IR in the gastric plexi suggests that adenosine may also act indirectly on the gastric plexi by altering enteric neural transmission to regulate IRG and SLI release. The coding regions of the gastric A_1 and A_{2A} receptors were also examined and found to be structurally identical to those in the rat brain, but expressed in extremely low levels. Gene expression was measured using Real-Time reverse-transcriptase polymerase chain reaction (RT-PCR) assays developed in the current study. Using these assays, A_1 and A_{2A} receptor gene expression was found to be altered by fasting and omeprazole treatment.

These treatments produced reciprocal changes in A_1 receptor and gastrin gene expression, and similar changes in A_{2A} receptor and somatostatin gene expression. Further studies demonstrate that omeprazole treatment also altered adenosine agonist-induced changes in SLI release, suggesting that changes in adenosine receptor gene expression may result in actual changes in receptor expression. These results suggest that changes in the gastric state may alter A_1 and A_{2A} receptor expression, which in turn regulates the synthesis and release of gastrin and somatostatin, and consequently gastric acid secretion.

Table of contents

List of tables

List of figures

Acknowledgements

I am most grateful to my supervisor, Dr. Kenny Y. N. Kwok for his many years of guidance and support, and for his extraordinary kindness and generosity. The academic skills I've acquired from him will certainly serve me well throughout my career. My sincerest gratitude to Dr. Ross MacGillivray for his support and encouragement throughout my Ph.D. Many thanks also to Jeff Hewitt for teaching me the techniques of molecular biology that have figured so prominently in my work, and for useful technical discussion. I would also like to acknowledge Dr. Alison Buchan for her patience and guidance during our collaboration, through which I acquired my knowledge of immunohistochemistry. Thanks also to Dr. Ray Pederson for carefully reading over this thesis and making graduate studies in this department a more social experience. I am also grateful to other members of my supervisory committee, Dr. Steve Vincent and Dr. Chris McIntosh, for direction in preparation for my comprehensive exam. In addition, I would like to express my appreciation for the students who have worked in my laboratory, particularly Henry Leung, who contributed to the immunohistochemistry and perfusion studies. Thanks also to Zaira Khan for her administrative support, and finally to Stephan Gudmundson, my family, and friends, who supported me throughout this experience. Financial support from The University of British Columbia, in the form of several University Graduate Fellowship awards, from the Cordula and Gunter Paetzold Fellowship, and from the Wah-Sheung Prize was greatly appreciated.

Chapter 1: Introduction

Adenosine is found ubiquitously throughout the body, and has been shown to act as a neurotransmitter, neuromodulator, and local mediator in various biological systems (Ralevic and Burnstock 1998). Adenosine exerts its effect by activating purinergic receptors belonging to the P1 receptor family. These receptors are classified into the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes (Fredholm et al., 2001).

In the stomach, adenosine has been shown to protect against stress-, indomethacin-, and ethanol-induced ulcerative formations (Glavin et al., 1987; Cho and Ogle, 1990; Cho et al., 1991, 1995; Bozkurt et al., 1998). This gastroprotective action is likely due to the inhibitory effect of adenosine on gastric acid secretion. Although adenosine has been shown to exert its acid-inhibitory effect in dogs and guinea pigs by acting directly on parietal cells (Gerber et al., 1985; Heldsinger et al., 1986), this does not occur in the rat (Puurunen et al., 1987). Instead, adenosine inhibits the release of gastrin, a major secretagogue of gastric acid, and stimulates the release of somatostatin, a potent inhibitor of acid secretion (Kwok et al., 1990). Adenosine elicits these actions by activating adenosine receptors, but the receptor subtypes involved have not been determined. This thesis examines the adenosine receptor subtypes involved in regulating gastrin and somatostatin secretion in the rat stomach, and characterizes the cellular localization, abundance, and structure of these receptors.

Various physiological states of the stomach have been shown to alter gastric acid secretion by regulating the release and synthesis of gastrin and somatostatin. Another objective of this thesis is to examine whether adenosine may be involved in this regulatory process. Thus, changes in adenosine receptor gene expression were examined under altered

physiological states of the stomach that are known to alter gastrin and somatostatin synthesis and release.

This chapter provides background information pertinent to the current study, and is organized into three sections. The first section discusses the regulation of endogenous adenosine levels by processes controlling the synthesis, release, reuptake and metabolism of adenosine. The second section describes the classification and pharmacological and molecular characterization of each adenosine receptor subtype. This section also reviews the regulation of adenosine receptor gene and protein expression. Since this thesis focuses on the A_1 and A_{2A} receptors, emphasis is placed on background literature related to these two subtypes. The third section discusses the gastric actions of adenosine, with particular attention given to the role of adenosine in regulating gastric secretion. This section also describes key experiments that this study is based upon. This chapter concludes by presenting the experimental hypothesis, rationale and specific aims of the thesis research.

I. Background

1. Regulation of endogenous adenosine levels

Since adenosine is the endogenous ligand for all four adenosine receptor subtypes, it is essential to understand how the level of this nucleoside is regulated. Several processes are involved in the production, release, reuptake and metabolism of adenosine. In the stomach, adenosine is produced by intracellular and extracellular salvage pathways that produce adenosine from sources already containing an intact purine ring. *De novo* synthesis occurs mainly in cells of the liver, and is responsible for the complete formation of adenosine from sources that do not contain a purine ring. Diet is not a source of significant amounts of

adenosine since most purines absorbed by the intestines are catabolized by xanthine oxidase in the esophageal, gastric, small intestinal and large intestinal epithelium (Moriwaki et al., 1996).

When adenosine is present in the extracellular fluid, it is rapidly removed by cellular reuptake through specialized nucleoside transporters (discussed in section 1.4), degraded to inosine by adenosine deaminase (ADA) or converted to AMP by adenosine kinase (AK). The next section reviews the regulation of endogenous adenosine levels by the synthesis, release, reuptake and metabolism of adenosine.

1.1 The *de novo* synthesis pathway

Although *de novo* synthesis of adenosine has not been shown to occur in the stomach, this pathway is included in this overview to illustrate the biosynthetic origin of the purine ring. A simplified pathway for the *de novo* synthesis and metabolism of adenosine is described in Fig. 1. Components of the purine ring are donated by glutamine, glycine, formyl-tetrahydrofolic acid, and aspartate during this process (see Fig. 2). *De novo* synthesis occurs mainly in the liver. In other tissues, including the stomach, adenosine is produced through intracellular and extracellular salvage pathways.

1.2 Intracellular and extracellular salvage

Under normoxic conditions, intracellular adenosine is formed mainly by the conversion of S-adenosyl homocysteine (SAH) to adenosine (Lloyd et al., 1988) (See Fig. 3). SAH is formed when S-adenosyl methionine (SAM) donates a methyl group to methylate DNA, mRNA, RNA, and rRNA. SAH is hydrolyzed by the enzyme S-adenosyl

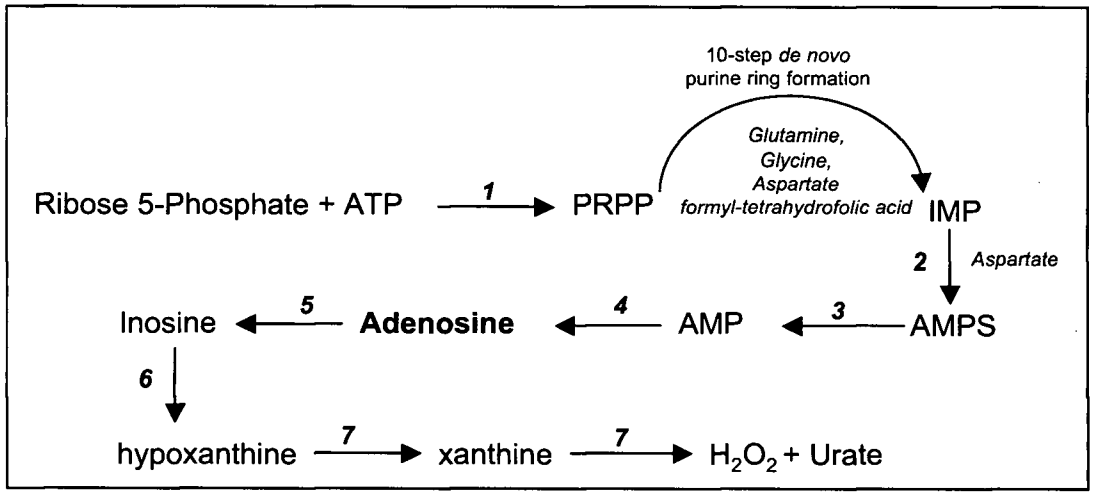


Fig. 1. The *de novo* synthesis and metabolism of adenosine. The *de novo* synthesis of adenosine occurs intracellularly through the actions of the following enzymes: 1) PRPP synthase; 2) adenylosuccinate synthetase ; 3) adenylosuccinate lyase; and 4) endo-5' nucleotidase. Intracellular metabolism of adenosine utilize the following enzymes: 5) adenosine deaminase; 6) phosphorlase; and 7) xanthine oxidase. In the initial step, two phosphates from ATP are transferred to ribose 5-phosphate to form PRPP. The purine ring is formed by a 10-step reaction which converts PRPP to IMP. IMP is converted to AMPS by the addition of aspartate. AMP is hydrolyzed by 5'endonucleotidase to form adenosine (Stone and Simmonds, 1991). [Abbreviations: 5'phospho-α-D-riobsyl 1 pyrophosphate (PRPP), inosine monophosphate (IMP), adenylosuccinate (AMPS), and adenosine monophosphate (AMP)].

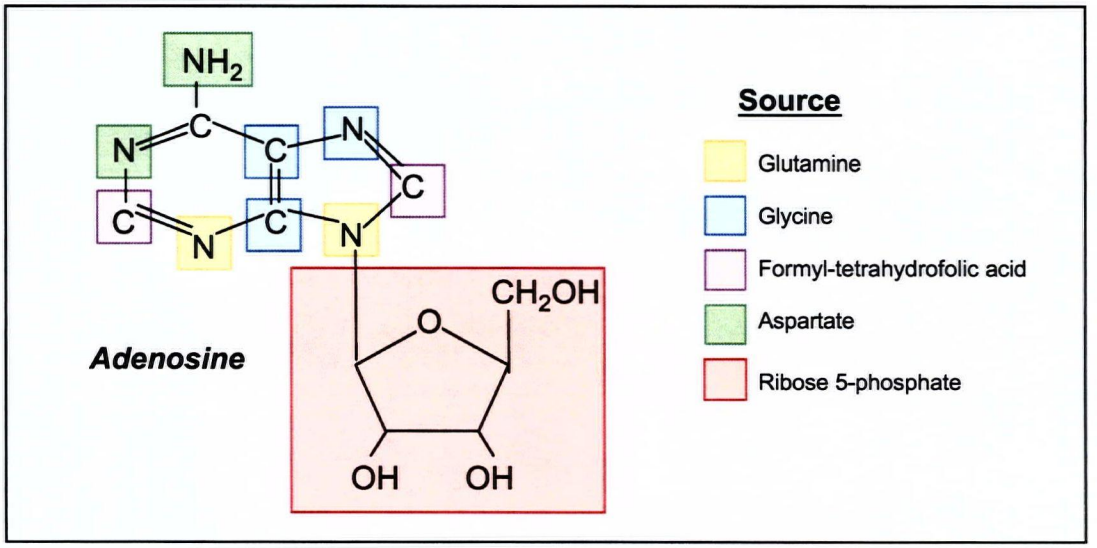


Fig. 2. The molecular structure of adenosine. The ribose moiety is donated by ribose 5-phosphate. The purine ring is formed by *de novo* synthesis and consists of components donated by glutamine, glycine, formyl-tetrahydrofolic acid and aspartate.

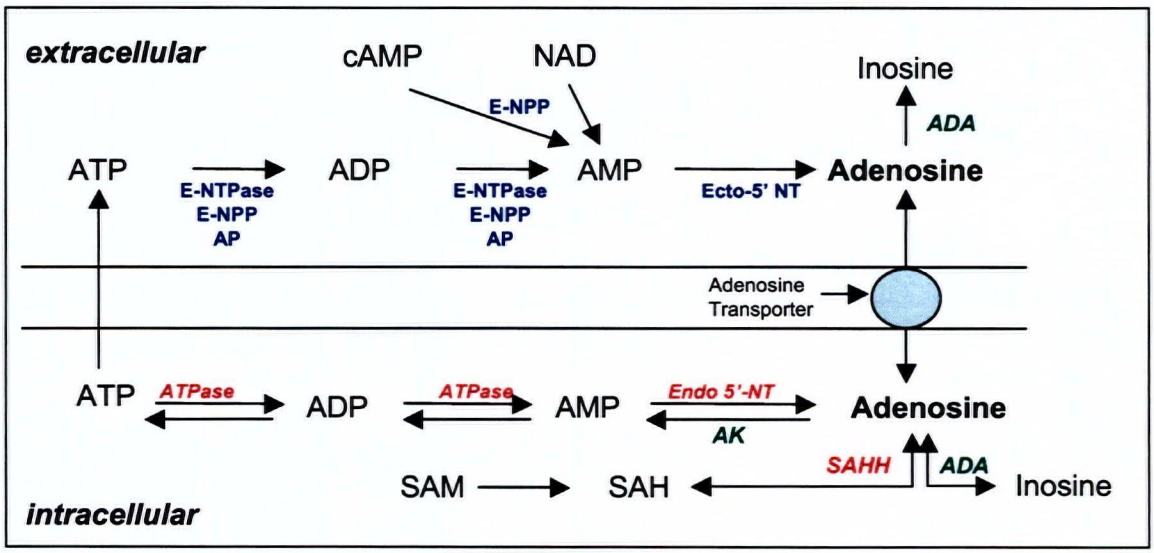


Fig. 3. The intracellular and extracellular synthesis and metabolism of adenosine. Adenosine is produced intracellularly and extracellularly through salvage pathways using sources which already contain an intact purine ring. Enzymes responsible for the extracellular synthesis (blue), intracellular synthesis (red), and metabolism (green) of adenosine are shown. Cellular release and reuptake of adenosine occur through the adenosine transporters. [Abbreviations: ecto-nucleoside triphosphate diphoshohydrolase (E-NTPase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase (AP), ecto-5' nucleotidase (ecto-5' NT), adenosine deaminase (ADA), adenosine kinase (AK), cyclic AMP (cAMP), nicotinamide adenine dinucleotide (NAD), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH), S-adenosyl homocysteine hydrolase (SAHH)]

homocysteine hydrolase (SAHH) to form adenosine (Lloyd et al., 1988). Under hypoxic or ischemic conditions, the contribution of SAH to adenosine levels is negligible (Deussen et al., 1989). Instead, intracellular adenosine is formed primarily by the dephosphorylation of adenosine triphosphate (ATP) (Geiger et al., 1997). ATP is converted to adenosine diphosphate (ADP) and then adenosine monophosphate (AMP) by ATPase, and AMP is then dephosphorylated by either cytosolic AMP-specific or IMP-specific 5' nucleotidase to produce adenosine (Sala-Newby et al., 1999).

In the stomach, extracellular adenosine may be synthesized though the hydrolysis of AMP by 5' nucleotidase. The membrane-bound form of 5' nucleotidase has been localized in the corpus and antrum of the stomach (Sakai et al., 1981; Grover et al., 1983, 1985; Borkje et al., 1986). The stomach is divided into three morphological and functionally distinct regions, the fundus, corpus and antrum, as shown in Fig. 36. The activity of 5' nucleotidase was found on plasma membrane enriched fractions extracted from canine antral and corporeal smooth muscle cells (Sakai et al., 1981; Grover et al., 1983), and rat corporeal smooth muscle cells (Grover et al., 1985). The activity of 5' nucleotidase has also been measured in biopsy samples extracted from human antral and corporeal gastric mucosa (Borkje et al., 1986). Thus, the activity of this enzyme may regulate gastric adenosine levels.

The state of the stomach has been shown to alter 5' nucleotidase activity. The activity of this enzyme is reduced in the gastric juice of patients suffering from gastric cancer, gastric ulcers, and gastritis (Durak et al., 1994). This may be due to changes in gastric acidity since 5' nucleotidase activity has been shown to be altered by changes in acidity in the rat skeletal muscle. In particular, increased acidity was shown to increase extracellular adenosine levels by stimulation of 5' nucleotidase activity (Cheng et al., 2000).

Extracellular adenosine is synthesized from AMP as shown in Fig. 3. AMP levels are regulated by a number of enzymes which hydrolyze ATP, ADP, nicotinamide adenine dinucleotide (NAD^+) and cyclic AMP (cAMP). These enzymes are classified into the ecto-nucleoside triphosphate diphoshohydrolase (E-NTPase) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, and the alkaline phosphatase family. The characteristics of these enzymes have been reviewed by Zimmerman (Zimmermann, 2000). The enzymes are not as important as 5' nucleotidase in regulating extracellular adenosine levels since the dephosphorylation of AMP to adenosine is the rate-limiting step in the production of extracellular adenosine (Dunwiddie et al., 1997). Therefore, 5' nucleotidase activity may be an important regulator of extracellular adenosine production in the stomach.

1.3 Presynaptically and postsynaptically released ATP as a source of adenosine

Extracellular adenosine can also be derived from the degradation of ATP released from purinergic nerve terminals (Burnstock et al., 1970; Burnstock, 1975). Purinergic nerves were proposed by Burnstock based on studies performed in the guinea pig and toad stomach (Burnstock et al., 1970). In the stomach, the cell bodies of purinergic nerves are found in the myenteric plexus, and the axons of these nerves project to the circular and longitudinal muscles (Burnstock, 1975). ATP is released from these nerves and acts as a neurotransmitter (Su et al., 1971; Burnstock, 1975). ATP is stored in synaptic vesicles with other neurotransmitters, and is released by nerve depolarization (Su et al., 1971). In the brain, ATP is released presynaptically from a number of neurons, including cholinergic, glutamatergic and catecholaminergic neurons, and from glial cells (Ralevic and Burnstock, 1998). In the gut, ATP was also suggested to be co-released with nitric oxide (Selemidis et al., 1997),

pituitary adenylate cyclase-activating polypeptide (Furness et al., 1995; Imoto et al., 1998), and vasoactive intestinal peptide (VIP) (Furness et al., 1995).

Vizi and colleagues have also suggested that acetylcholine and norepinephrine may elicit ATP release from postsynaptic sites through a process that they referred to as cascade transmission (Vizi et al., 1992). This type of release has been observed in the gut. Specifically, acetylcholine was shown to induce ATP release from smooth muscle cells of the guinea pig ileum via activation of postsynaptic M_3 receptors (Nitahara et al., 1995). Thus, in the stomach, adenosine may be derived from the hydrolysis of presynaptically or postsynaptically released ATP. Although cAMP may also be released from neurons and can be metabolized to form adenosine (see Fig. 3), cAMP was not shown to significantly alter extracellular adenosine levels unless there is continuous release over an extended period of time (Brundege et al., 1997).

1.4 Cellular release and reuptake of adenosine by adenosine transporters

Adenosine transporters are responsible for the cellular release and reuptake of intracellular and extracellular adenosine, respectively (Geiger et al., 1997). Adenosine transporters are classified into two groups, the equilibrative or facilitated-diffusion carriers and the concentrative or sodium-dependent nucleoside transporters (Thorn and Jarvis, 1996). The equilibrative nucleoside transporters are found in most cell types and are divided into two groups, the es (equilibrative, sensitive) or ei (equilibrative, insensitive) carriers, based on whether they are sensitive to blockade by nitrobenzylthioinosine, a synthetic nucleoside analogue (Lee and Jarvis, 1988). Both classes translocate adenosine bidirectionally across the plasma membrane, and are blocked by inhibitors, such as dipyridamole, dilazep and lidoflazine (Thorn and Jarvis, 1996). Our laboratory has suggested that these transporters

may be involved in regulating the endogenous level of extracellular adenosine in the rat stomach (Kwok et al., 1990). The administration of dipyridamole was shown to significantly enhance basal and adenosine-induced augmentation of gastric somatostatin-like immunoreactivity (SLI) release in the isolated perfused stomach preparation (Kwok et al., 1990). Other studies have also suggested that the ei transporters function as an adenosine-scavenging system to maintain the extracellular adenosine level at the basolateral membrane of the intestinal epithelia (Tally et al., 1996).

Concentrative nucleoside transporters have not been studied extensively in the stomach, but have been detected on epithelial cells of the intestines (Ritzel et al., 2001). Currently, four concentrative nucleoside transporters (N1-N4) have been identified and characterized (Thorn and Jarvis, 1996). All four can transport adenosine bidirectionally across the membrane, but are generally responsible for the influx of adenosine. Currently, no selective inhibitors exist for the concentrative nucleoside transporters (Geiger et al., 1997).

Like 5' nucleotidase, the expression of equilibrative adenosine transporters may be altered to regulate endogenous adenosine concentrations (Lorbar et al., 1999). Although this has not been examined in the stomach, alterations in adenosine transporter activity have been observed in the hearts of aged rats. A decrease in adenosine transporter expression was observed with increased levels of extracellular adenosine in these animals (Lorbar et al., 1999).

1.5 Metabolism of adenosine

Extracellular adenosine can be regulated by the action of ADA, an enzyme that converts adenosine to inosine (See Fig. 3). ADA is expressed in the cytoplasm and on cell membranes of all tissues (Franco et al., 1997), and can be inhibited by erythro-9-(2-hydroxy-

3-nonyl)adenine hydrochloride (EHNA) (Mendelson et al., 1983). ADA is highly expressed in the thymus and spleen in humans (Moriwaki et al., 1999), and the lung, spleen, and intestines in rats (Centelles et al., 1987). The expression of the ADA gene has also been detected in the epithelia of the mouse esophagus, forestomach and duodenum (Witte et al., 1991).

The high expression of ADA throughout the gastrointestinal tract suggests that much of the adenosine that is absorbed through the gut is metabolized. In ADA-deficient mice, a significantly elevated level of adenosine was observed in various regions of the gastrointestinal tract, including the forestomach, hindstomach, esophagus, duodenum, jejunum and ileum (Xu and Kellems, 2000). Thus, ADA plays a significant regulatory role in modulating endogenous adenosine levels in the gastrointestinal tract.

In humans, changes in gastric ADA activity have been shown to occur with changes in gastric acidity (Namiot et al., 1990, 1991, 1993). In particular, hypersecretors of acid were shown to exhibit increased ADA activity, while patients suffering from achlorhydria, a condition characterized by a lack of gastric acid secretion, were shown to exhibit lowered ADA activity (Namiot et al., 1990). Thus, ADA may play a role in regulating gastric acid secretion by modulating endogenous adenosine levels. Mucosal ADA activity has also been shown to decrease in the vicinity of a healed ulcer (Namiot et al., 1993) and in the gastric mucosa after treatment with ranitidine, a gastric acid inhibitor used for treatment of ulcers (Namiot et al., 1991). These findings suggest that increased adenosine levels may be involved in ulcer healing.

As shown on Fig. 3, the cytosolic enzyme adenosine kinase (AK) is responsible for catalyzing the phosphorylation of adenosine to AMP (Geiger et al., 1997), and is found in

various gastrointestinal tissues, including the stomach, duodenum, ileum, and large intestine (Andres and Fox, 1979). This enzyme has been shown to regulate endogenous adenosine levels. For example, inhibition of AK activity in the hippocampus has been shown to increase intracellular adenosine levels (Pak et al., 1994). During oxidative and metabolic stress, decreased AK activity has also been shown to significantly increase adenosine levels in DDT1 MF-2 smooth muscle cells (Sinclair et al., 2000).

1.6 Endogenous adenosine levels

As described in the previous sections, the endogenous adenosine level is tightly regulated by processes controlling its synthesis, release, reuptake and metabolism. Although adenosine can be synthesized intracellularly and can be produced extracellularly, under well oxygenated conditions, the majority of adenosine (92%) is produced intracellularly and is released into the extracellular space, as illustrated in the isolated guinea pig heart (Deussen, 2000).

In plasma, adenosine has a short half-life of only a few seconds because it is rapidly degraded by ADA or removed by cellular reuptake (Geiger et al., 1997). Under normoxic conditions, basal adenosine levels are consistently found in the nanomolar range (see below). Although the adenosine concentration has not been measured in the stomach, the venous adenosine concentration in the rat jejunum has been shown to be 62 nM (Sawmiller and Chou, 1990). This concentration is consistent with levels measured elsewhere, such as in the rat cerebrospinal fluid (160 nM) (Meno et al., 1991), cerebral cortex (30-50 nM) (Phillis et al., 1987), and striatum (41- 210 nM) (Ballarin et al., 1991; Pazzagli et al., 1995; Melani et al., 1999). In whole blood and plasma of rats, adenosine concentrations were found to be 79 nM (Phillis et al., 1992) and 320 nM (Conlay et al., 1997), respectively. Arterial adenosine

concentrations have also been measured in the rat and found to be 59 nM (Sawmiller and Chou, 1990). In human blood, the concentration was shown to be 35 nM (Geiger et al., 1997).

During periods of stress, adenosine levels can rise to micromolar concentrations. In rats, ischemia induced by medial cerebral artery occlusion was shown to increase adenosine concentrations in the striatum and hippocampus to 3 μM (Melani et al., 1999) and 30 μM (Latini et al., 1999), respectively. The increase in adenosine concentration is due to enhanced adenosine production caused by the degradation of ATP. The adenosine production rate in the rat heart has been shown to increase by approximately 70-fold during ischemia, from a normoxic production rate of 6.4-7.7 nmol min^{-1} g^{-1} (Lorbar et al., 1999) to an ischemic production rate of 410 nmol min^{-1} g^{-1} (Meghji et al., 1988). The plasma adenosine level has also been shown to increase to micromolar concentration after chronic blockade of adenosine receptors (Conlay et al., 1997). In rats treated with caffeine for two weeks, the plasma adenosine concentration was found to increase from 320 nM to 3.2 μM (Conlay et al., 1997). Thus, endogenous adenosine levels are clearly elevated by oxidative stress and by treatment with adenosine antagonists.

The concentration of adenosine exposed to target tissues may be important because adenosine has been shown to elicit concentration-dependent physiological effects. For example, our laboratory has shown that gastric somatostatin release is inhibited by low concentrations of adenosine (0.01 μM), but is stimulated by higher concentrations (≥0.6 μM) (Kwok et al., 1990). The physiological actions of adenosine are determined, in part, by the adenosine receptor subtype stimulated. These subtypes are described in detail in the next section. However, it is worth mentioning here that the activation of each adenosine receptor

subtype depends on the local extracellular adenosine concentration. The high affinity adenosine A_1 and A_{2A} receptor subtypes may be stimulated by relatively low concentrations of adenosine, while the low affinity A_{2B} and A_3 receptors may be activated by extremely high concentrations of adenosine, such as those present during oxidative stress (Ralevic and Burnstock, 1998). This concentration-dependent activation of adenosine receptors is suggested by studies showing that adenosine elicits A_1-, A_{2A}-, A_{2B}-, and A_3- mediated effects with EC_{50} values of 73, 150, 5100, and 6500 nM, respectively (Daly and Padgett, 1992; Zhou et al., 1992; Peakman and Hill, 1994). In certain tissues, adenosine has been shown to elicit distinct effects depending on the concentration in which it is administered, and the adenosine receptor subtype that is activated. For example, in the renal vasculature, the administration of low micromolar concentrations of adenosine was shown to vasoconstrict renal afferent arterioles via activation of A_1 receptors, while higher concentrations of adenosine (>10 μM) resulted in vasodilation of these vessels via activation of A_{2A} receptors (Tang et al., 1999; Tabrizchi and Bedi, 2001). In the lung, low adenosine concentrations mediate anti-inflammatory actions by activating the A_{2A} receptors, while high adenosine concentrations mediate pro-inflammatory actions by activation of the A_3 receptors (Blackburn, 2003). These observations raise the possibility that the concentration-dependent effect of adenosine on somatostatin release in the isolated rat stomach is mediated by the activation of different adenosine receptor subtypes.

2. The adenosine receptors

2.1 Classification of the adenosine receptors

The concept of purinergic receptors was introduced in the late 1970s by Burnstock (Burnstock, 1978). These receptors were classified into the P_1 and P_2 purinoceptors, based mainly on their binding preference for adenosine or ATP, respectively. The P1 receptors (or adenosine receptors) were initially characterized by the rank order of binding preference (adenosine $\geq$ AMP > ADP > ATP), by their sensitivity to blockade by methylxanthines, and by their ability to alter cAMP levels through the modulation of adenylate cyclase activity (Burnstock, 1978). The A_1 and A_2 receptor subtypes were proposed in 1979 by van Calker to describe the two distinct plasma membrane receptors on cultured brain cells that were capable of inhibiting and stimulating adenylate cyclase activity, respectively (van Calker et al., 1979). Two A_2 receptor subtypes were later identified: a high-affinity A_2 receptor (A_{2A}) on rat striatal membranes, and a low affinity A_2 receptor (A_{2B}) on rat cerebral cortical membranes (Daly, 1983). After binding studies demonstrated that these receptors represented distinct receptors subtypes (Bruns et al., 1986), the A_{2A} and A_{2B} nomenclature was proposed by Bruns to refer to these high and low affinity A_2 receptors, respectively. While the A_1, A_{2A} and A_{2B} receptors were initially identified and named based on pharmacological studies, the fourth adenosine receptor subtype, the A_3 receptor, was cloned and its molecular structure was determined before it was identified as a distinct subtype using pharmacological means (Zhou et al., 1992).

Currently, the nomenclature and classification of the adenosine receptors adhere to the principles of receptor nomenclature adopted by the International Union of Pharmacology (IUPHAR). Each distinct receptor subtype is identified based on both molecular and

pharmacological evidence. The existence of the four adenosine receptor subtypes is now well established (Ralevic and Burnstock, 1998; Fredholm et al., 2001), and the gene expression of all four subtypes has been demonstrated in various tissues, including the rat stomach (Dixon et al., 1996). Although additional adenosine receptor subtypes have been proposed, the structural and pharmacological evidence required by the IUPHAR to support their existence is lacking (Cornfield et al., 1992).

2.2 Pharmacological characterization of the adenosine receptors

The adenosine receptor subtypes can be characterized pharmacologically by the rank order of potency of various adenosine analogs in eliciting certain responses. In the past two decades, numerous adenosine agonists and antagonists have been developed. Nearly all known agonists are based on the structure of adenosine, while antagonists are based on both xanthine and non-xanthine compounds (Fredholm et al., 2001). Due to the large number of ligands available for these receptors, the following section focuses on ligands that are commonly used to characterize the four adenosine receptors subtypes, and on the ligands which are used in the present study.

Adenosine agonists are based on the structure of adenosine, and require an intact ribose moiety (Fredholm et al., 2001; Jacobson et al., 2001). Certain structural modifications have been shown to increase the metabolic stability of adenosine, resulting in longer half lives and/or resistance to degradation and cellular reuptake (Pavan 1998). Adenosine can be modified at three sites to increase metabolic stability and confer receptor subtype selectivity (Jacobson, 1996; Klotz, 2000): the 5' position of the ribose ring, the C2 position and the N^6 position of the purine ring (see Fig. 4). Substitutions at the N^6 position generally increase A_1 and A_3 receptor selectivity, while substitutions at the C2 position generally increase A_{2A}

receptor selectivity (Bruns et al., 1986; Klotz, 2000). Agonists containing modifications at more than one site are generally more potent (Jacobson, 1996). The binding affinity and the structure of some of the most common adenosine receptor agonists are shown in Fig. 4.

Analogs containing bulky substitutions at the N^6 position, such as N^6-cyclopentyladenosine (CPA), 5'-N^6-cyclohexyladenosine (CHA), R(-)-N^6-(2-phenylisopropyl)adenosine (R-PIA), and (S)-N^6-(2-phenylisopropyl)adenosine (S-PIA), preferentially bind to the A_1 receptor (Bruns et al., 1986; Klotz et al., 1998). CPA is one of the most potent and selective A_1 receptor agonists (see Fig. 4), and has been used extensively to characterize A_1 receptor-mediated responses in the gut. In the gastric antrum of the guinea pig, CPA was used to demonstrate the involvement of the A_1 receptor in the inhibition of fast excitatory postsynaptic potentials (Christofi et al., 1992). CPA has also been used to demonstrate the presence of A_1 receptors in the rat duodenum (Nicholls et al., 1992; Brownhill et al., 1996; Peachey et al., 1996), jejunum (Hancock and Coupar, 1995), and colon (Peachey et al., 1999).

A_{2A} receptors prefer agonists with bulky substitutions at the C2 position, such as 2-phenylaminoadenosine (CV1808) and the potent A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'N-ethylcarboxamidoadenosine (CGS 21680) (Hutchison et al., 1989; Lupica et al., 1990). CGS 21680 is highly selective for A_{2A} receptors, and exhibits a 140-fold selectivity for A_{2A} receptors over A_1 receptors (Hutchison et al., 1989). CGS 21680 has low affinity for the A_{2B} and A_3 receptors (Klotz et al., 1998) (see Fig. 4), and was used to characterize the first A_{2A} receptors cloned from the rat striatum (Fink et al., 1992). In the rat isolated perfused superior mesenteric arterial bed, CGS 21680 was used to demonstrate A_{2A} receptor-mediated vasodilation (Hiley et al., 1995). In our laboratory, CGS

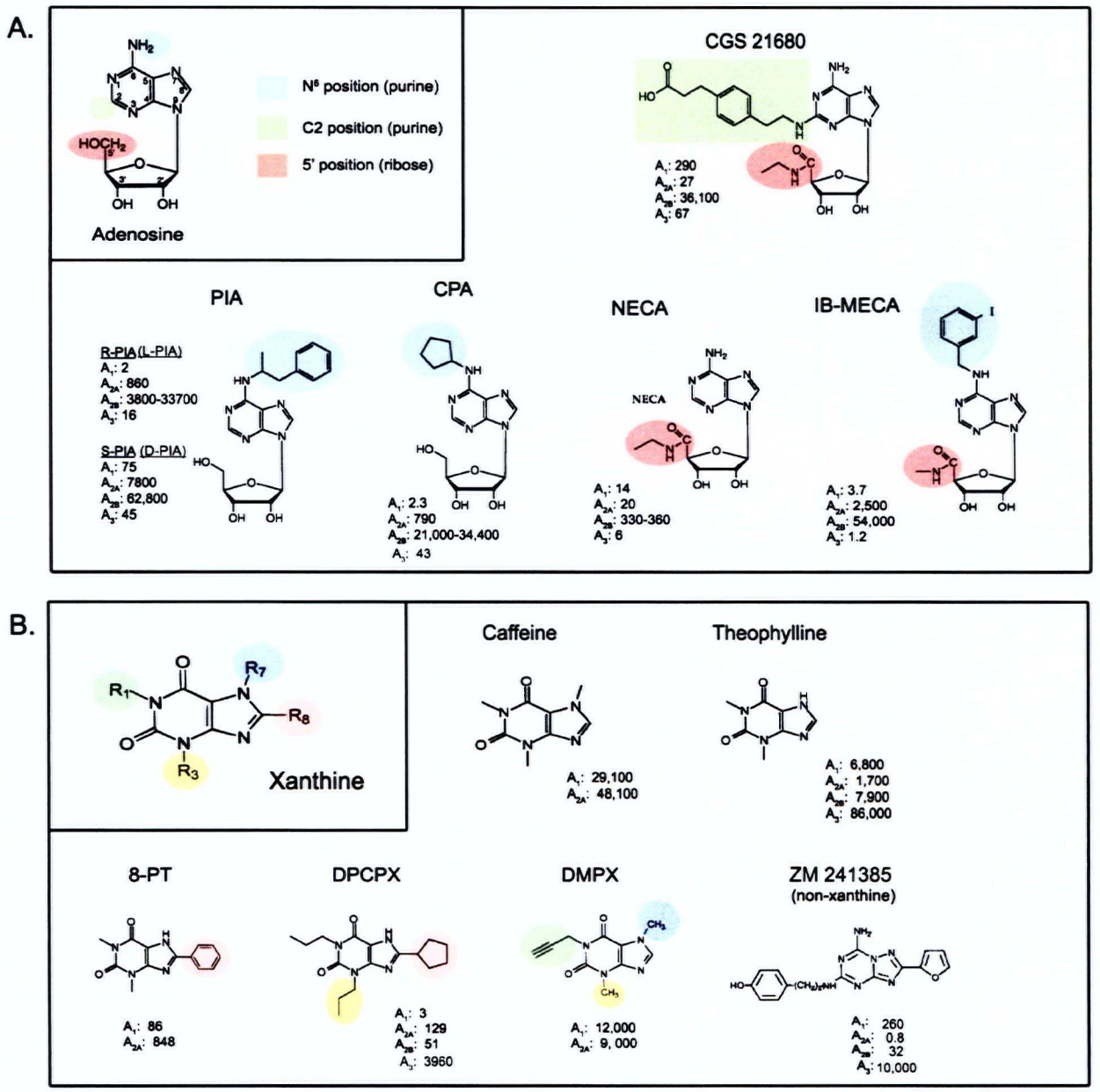


Fig. 4. Adenosine agonists (A) and antagonists (B). Adenosine agonists are based on the structure of adenosine. These compounds contain an intact ribose moiety, and may be modified at the N^6 and C2 position of the purine ring or at the 5' position of the ribose moiety. The Ki values (nM), which indicate the relative binding affinities at different receptor subtypes, are shown (Bruns et al., 1986; Klotz et al., 1998;Ralevic and Burnstock, 1998; Fredholm et al., 2001). Many adenosine antagonists are xanthine-based, containing possible modifications at the 1,3, 7, or 8 position of the xanthine ring. Caffeine and theophylline are naturally occurring xanthines. 8-PT, DPCPX, and DMPX are xanthine-based derivatives, and ZM 241385 is a non-xanthine A_{2A} receptor-selective antagonist

21680 was also shown to decrease perfusion pressure in the isolated vascularly perfused rat stomach in a concentration-dependent manner (Yip and Kwok, 2004).

Selective agonists have not been developed for the A_{2B} receptor, despite intense efforts (Cristalli et al., 1998; de Zwart et al., 1998). Currently, one of the most potent agonists for this receptor is the non-selective 5' modified agonist N-ethylcarboxamidoadenosine (NECA; see Fig. 4) (Ralevic and Burnstock, 1998). NECA and CGS 21680 bind to A_{2A} receptors with similar affinity, but NECA binds to the A_{2B} receptors with higher affinity than CGS 21680 (Brackett and Daly, 1994; Klotz et al., 1998). Thus, in various tissues, including the gut, A_{2A} and A_{2B} receptor-mediated effects are often distinguished by the relative potency of NECA and CGS 21680. In the rat duodenum (Brownhill et al., 1996; Nicholls et al., 1996) and distal colon (Peachey et al., 1999), A_{2B} receptor-mediated actions were demonstrated by the potent effect of NECA and by the lack of an effect by CGS 21680. Actions mediated by A_{2A} and A_{2B} receptors can also be distinguished by the EC_{50} values of NECA and CGS 21680 (Brackett and Daly, 1994). In particular, in PC12 cells that express A_{2A} receptors, activation of adenylyl cyclase elicited by CGS 21680 and NECA were characterized by EC_{50} values of 0.07 and 0.13 μM, respectively (Brackett and Daly, 1994). In fibroblast 3T3 cells expressing A_{2B} receptors, the activation of adenylyl cyclase activity elicited by NECA was characterized by an EC_{50} of 1.9 μM, and CGS 21680 was unable to elicit a maximal response (Brackett and Daly, 1994).

Although CPA, CGS 21680, and NECA can also bind to the A_3 receptors, N^6-substituted adenosine-5'-uronamides, such as 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-ß-D-ribofuranuronamide (IB-MECA), are more selective for this receptor subtype (Gallo-Rodriguez et al., 1994) (see Fig. 4). In the gut, IB-MECA has been

used to examine the involvement of A_3 receptors in the relaxation of the guinea pig taenia caeci (Prentice and Hourani, 1997). This agonist has also been used to demonstrate the involvement of A_3 receptors in protecting against colitis in the mouse (Mabley et al., 2003).

The adenosine receptor involved in eliciting a specific effect can be determined pharmacologically by comparing the relative potency of various adenosine agonists. Based on the selectivity and binding affinity of various agonists at each adenosine receptor subtype (see Fig. 4), the following rank order of potency can be used to characterize A_1, A_{2A}, A_{2B} and A_3 receptor mediated actions: **A_1:** CPA > CHA ≥ R-PIA > NECA > IB-MECA > CGS 21680; **A_{2A}:** CGS 21680 ≅ NECA ≅ CV1808 > CPA > IB-MECA; **A_{2B}:** NECA > CGS 21680 ≅ CV1808 > CPA > IB-MECA; **A_3:** IB-MECA > NECA > CPA > CGS 21680.

Since adenosine agonists are not specific, and are only selective for a given adenosine receptor subtype, the use of selective antagonists is also necessary to characterize the adenosine receptor subtype involved in eliciting various actions. Adenosine receptors, with the exception of the A_3 receptor in some species, are blocked by methylxanthines such as caffeine and theophylline (Zhou et al., 1992; Klotz, 2000). A number of selective xanthine-based antagonists have been developed by modification of the 1, 3, 7, and/or 8 positions of the xanthine ring. Unlike human A_3 receptors, rodent A_3 receptors are not blocked by xanthine derivatives (Zhou et al., 1992; Salvatore et al., 1993; van Galen et al., 1994). Several classes of non-xanthine derivatives have also been developed as A_1, A_{2A}, A_{2B} and A_3 receptor-selective antagonists. The characteristics of each class have been reviewed by Baraldi (Baraldi et al., 2000). The structure and binding affinities of several common xanthine and non-xanthine antagonists are shown in Fig. 4.

One of the most potent and widely used A_1 receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Lohse et al., 1987; Klotz, 2000; Muller, 2001), is produced by the substitution of a cycloalkyl group at position 8 of the xanthine ring (See Fig. 4). This antagonist has previously been used to demonstrate the involvement of A_1 receptors in various actions in the gastrointestinal tract. For example, DPCPX was shown to abolish adenosine-induced inhibition of gastrin release from canine antral G-cells (Schepp et al., 1990), inhibit CPA-induced contraction in the rat colonic muscularis mucosae (Bailey et al., 1992; Reeves et al., 1993), and inhibit CPA-induced relaxation in the rat duodenal longitudinal muscle (Nicholls et al., 1996).

The first A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), was generated by substitutions at the 1, 3, and 7 positions of the xanthine ring (Seale et al., 1988). Although this compound does not exhibit extremely high selectivity or affinity for the A_2 receptors, it remains one of the most widely used A_2 receptor antagonists due to its high solubility in water (Muller et al., 1997). More recently, a potent and highly selective non-xanthine A_{2A} receptor antagonist, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), was developed (Palmer et al., 1995b; Poucher et al., 1995, 1996). This antagonist, shown in Fig. 4, binds with high affinity to membranes expressing the A_{2A} receptor (Palmer et al., 1995b; Poucher et al., 1995), but binds with low affinity to membranes expressing the A_1 and A_3 receptors (Poucher et al., 1995). ZM 241385 was also shown not to antagonize A_{2B} receptor-mediated actions in the guinea pig aorta (Poucher et al., 1995).

While selective A_{2B} receptor agonists are not available, several selective A_{2B} antagonists have been developed. These include 1,3-dialkylxanthine derivatives, and 8-

phenylxanthine carboxylic congener derivatives (Kim et al., 2000). Some A_{2B} receptor antagonists demonstrate species-dependent differences in ligand binding affinity (Fozard et al., 2003). However, these differences are not as apparent as in the A_3 receptor. In humans, A_3 receptors are blocked by high concentrations of xanthine derivatives such as DPCPX (Salvatore et al., 1993). In rats, these receptors were not blocked by xanthine derivatives, including DPCPX (Zhou et al., 1992), but instead were blocked by a number of non-xanthine derivatives such as 9-chloro-2(2-furyl)[1,2, 4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943) (Baraldi and Borea, 2000). Since a number of selective adenosine agonists and antagonists are available, a pharmacological approach may be used to characterize the adenosine receptor subtype(s) involved in mediating various physiological actions.

2.3 Molecular characterization of the adenosine receptors

The four adenosine receptors subtypes have been cloned and characterized in various tissues of different species, including the human brain (Furlong et al., 1992; Libert et al., 1992; Pierce et al., 1992; Salvatore et al., 1993) and rat brain (Reppert et al., 1991; Fink et al., 1992; Stehle et al., 1992; Zhou et al., 1992). In this section, the cloning and characterization of the human and rat adenosine receptor genes are examined. In addition, the regulation of adenosine receptor gene and receptor expression is also reviewed. Emphasis is placed on studies related to the A_1 and A_{2A} receptors since these two receptors are the focus of the current study.

Both the A_1 and A_{2A} receptors were first cloned from a canine thyroid library (Libert et al., 1989), and thought to represent new members of the G protein-coupled family before they were recognized as clones of the A_1 and A_{2A} receptor (Furlong et al., 1992; Libert et al., 1992). The human A_1 receptor is localized on chromosome 1q32.1 (Townsend-Nicholson et

al., 1995), and contains six exons and five introns (Libert et al., 1992). All introns are present in the 5' untranslated region (UTR) except for one, which interrupts the coding region at a position corresponding to the second intracellular loop. The coding region of the human A_1 receptor consists of 987 bp (Libert et al., 1992). The coding region of the rat brain receptor was similar and consists of 981 bp (Mahan et al., 1991; Reppert et al., 1991). Two distinct A_1 receptor transcripts, a 3.5 kb and a 5.5 kb transcript, were revealed by Northern blot analysis in rat tissues (Reppert et al., 1991). Both transcripts are expressed throughout the body in various tissues, including the brain and stomach. Another study by Mahan and co-workers has also identified two distinct transcripts, 3.1 kb and 5.6 kb in size, which have been detected in various tissues, including the rat brain and stomach (Mahan et al., 1991). The 3.5 and 3.1 kb species were found to be the predominant forms in the stomach (Mahan et al., 1991; Reppert et al., 1991). Two human A_1 receptor transcripts have also been demonstrated in the brain (Ren and Stiles, 1994a). These transcripts differ in the 5' untranslated region (UTR) by 54 bp, and were initially thought to occur through alternative splicing (Ren and Stiles, 1994a). However, two different promoters, promoters A and B (Ren and Stiles, 1995), were later found to control the synthesis of the two transcripts. For the rat A_1 receptor, Reppert has suggested that the two transcripts are generated by posttranscriptional modifications (Reppert et al., 1991). However, the promoter regions and 5' UTR of the rat A_1 receptor gene have not been identified. Thus, the origin of the two rat A_1 receptor transcripts remains unclear.

The human and rat A_{2A} receptor genes exhibit some similarities with each other (Chern et al., 1992; Fink et al., 1992; Furlong et al., 1992). The human A_{2A} receptor gene is 9-10 kb in size, localized on chromosome 22q11.2 (Le et al., 1996), and contains 2 exons separated

by a 6-6.4 kb intron, localized at a position corresponding to the second intracellular loop (Le et al., 1996; Peterfreund et al., 1996). Northern blot analysis reveals the expression of only one human A_{2A} transcript that is 2.6 kb in size (Peterfreund et al., 1996). The rat A_{2A} receptor gene was also shown to contain 2 exons, separated by a 7.2 kb intron (Chu et al., 1996). The 5' UTR of the rat A_{2A} receptor has also been examined, and contains two independent promoters situated 338 bp apart (Chu et al., 1996). These two promoters were shown to drive the synthesis of two distinct transcripts that differ in the 5' UTR but encode for the same polypeptide sequence.

The rat A_{2B} receptor was first isolated from a rat brain cDNA library (Stehle et al., 1992). Extremely high levels of two distinct A_{2B} receptor transcripts, a 1.8 kb and a 2.2 kb transcript, were found by Northern blot analysis and were expressed in the large intestine and caecum in rats (Stehle et al., 1992). In humans, two A_{2B} receptor transcripts, a 1.8 kb and a 2.4 kb transcript, were also shown to be expressed in VA 13 fibroblast cells (Rivkees and Reppert, 1992). The origin of the two human and rat A_{2B} receptor transcripts is unclear since the promoter regions and 5' UTR of these genes have yet to be determined. The human A_{2B} receptor has been shown to contain a single intron at a position corresponding to the second intracellular loop (Pierce et al., 1992; Jacobson et al., 1995), and is localized on chromosome 17p12 (Jacobson et al., 1995).

The A_3 receptor was first discovered by its cloning and characterization in the rat brain (Zhou et al., 1992). This receptor also contains a single intron at a position corresponding to the second intracellular loop. A variant of the rat A_3 receptor, the A_3i, has also been cloned and characterized (Sajjadi et al., 1996). This transcript is identical to the A_3 receptor cloned by Zhou, except for a 57 bp insertion in the coding region, which occurs from alternative

splicing of the intron. This variant was detected in a number of tissues, including the heart, brain, liver and testis (Sajjadi et al., 1996). However, it is unclear whether this variant is also present in the stomach. Evidence suggests that alternative splicing does not occur in the human A_3 receptor gene (Murrison et al., 1996) and only one transcriptional start site was identified when the 5' UTR of this gene was analyzed (Murrison et al., 1996). The human A_3 receptor gene is localized on chromosome 1p13.3 (Atkinson et al., 1997), and like the other adenosine receptor genes, it contains an intron (~2.2 kb) in the second intracellular loop. Of the four adenosine receptor subtypes, only the rat A_3 receptor has been shown to be alternatively spliced within its coding region. The presence of a variant A_3 receptor may have functional implications.

2.4 Regulation of adenosine receptor gene and protein expression

The synthesis and expression of adenosine receptors can be regulated at a transcriptional or post-transcriptional level, and may be altered by changes in the physiological environment such as during periods of stress (see Table 1). The genomic structure of the A_1, A_{2A}, and A_3 receptor has been examined and the promoter regions of these genes contain putative binding sites for various transcription factors. A number of binding sites have been identified in the two promoters of the human A_1 receptor gene (Ren and Stiles, 1995, 1998; Rivkees et al., 1999). Some of these sites were shown to interact with glucocorticoids in the regulation of A_1 receptor gene transcription (Ren and Stiles, 1999). The promoter region of the rat A_1 receptor gene has not been defined. However, as in humans, the transcription of this gene is regulated by glucocorticoids (Svenningsson and Fredholm, 1997), suggesting that the promoter region of the rat A_1 receptor may also contain binding sites for glucocorticoids. The A_{2A} receptors also respond to glucocorticoid

treatment. Dexamethasone, a synthetic glucocorticoid, has been shown to attenuate the action of NECA in stimulating cAMP production (Gerwins and Fredholm, 1991), suggesting that glucocorticoids decrease A_{2A} receptor expression. However, it is unclear if glucocorticoids act at a transcriptional or post-transcriptional level to regulate A_{2A} receptor expression. A transcriptional repressor of the A_{2A} receptor gene has recently been identified in the rat and has been shown to bind to specific nuclear factor-1 proteins to repress gene transcription (Lee et al., 2003). The A_{2A} receptor has also been shown to regulate the expression of its own mRNA (Saitoh et al., 1994). Activation of the A_{2A} receptor has been shown to transiently increase and then decrease A_{2A} receptor gene and protein expression in PC12 cells (Saitoh et al., 1994). The translation of A_1 and A_{2A} receptor mRNA can also be regulated by the presence of out-of-frame AUG codons in the 5' UTR (Ren and Stiles, 1994a; Chu et al., 1996; Lee et al., 1999). Translational regulation of the A_{2A} receptor may occur in other species as well, since the 5' UTR of the rat A_{2A} receptor gene exhibits high interspecies homology (Chu et al., 1996).

Stress-induced changes in A_1 and A_{2A} receptor gene and protein expression have also been demonstrated. During periods of oxidative stress, A_1 and A_{2A} receptor gene and/or protein expression is upregulated (Nie et al., 1998; Kobayashi and Millhorn, 1999). A_1 receptor mRNA and receptor expression were increased in DDT MF-2 cells during oxidative stress. This was mediated by enhanced transcription of the A_1 receptor by the transcription factor nuclear factor κB (NFκB) (Nie et al., 1998). In the gut, oxidative stress induced by chronic ileitis has also been shown to increase A_1 receptor gene expression (Sundaram et al., 2003). In PC12 cells, stress induced by hypoxia was shown to increase A_{2A} receptor mRNA and to increase A_{2A} receptor protein expression through the synthesis of new A_{2A} receptors

(Kobayashi and Millhorn, 1999). These changes were mediated by PKC and may result from an increase in A_{2A} receptor transcription. This is consistent with another study showing that the transcription of A_{2A} receptors was enhanced in human neuroblastoma SH-SY5Y cells by the activation of PKC (Peterfreund et al., 1997).

Other forms of stress have also been shown to alter A_1 receptor expression in the rat. Stress induced by sustained performance, such as repeated restraint stress, was shown to exhibit increased A_1 receptor agonist binding in the rat hypothalamus (Anderson et al., 1988). Rats suffering from acute renal failure were also shown to exhibit increased A_1 receptor binding in renal membranes and increased A_1 receptor mRNA expression (Gould et al., 1997). Gould has suggested that the increased A_1 receptor expression was due to enhanced A_1 receptor transcription. Stress induced by sleep deprivation has also been shown to increase A_1 receptor mRNA expression in rats (Basheer et al., 2000). Prolonged wakefulness was shown to increase extracellular adenosine levels, which increased A_1 receptor activation. A_1 receptor activation was then shown to enhance the activity of the transcription factor NFκB, which initiates transcription of the A_1 receptor (Basheer et al., 2001).

Changes in A_1 and A_{2A} receptor expression can also be induced pharmacologically. Treatment of DDT1 MF-2 cells with R-PIA was shown to decrease A_1 receptor expression in membrane preparations within hours (Ramkumar et al., 1991). Chronic treatment with R-PIA has also been shown to decrease A_1 receptor expression in isolated adipocytes (Green et al., 1992). In the presence of caffeine, an adenosine antagonist, both A_1 and A_{2A} receptor expression are increased. Chronic daily intraperitoneal treatments with caffeine also increase A_1 receptor expression (Fredholm, 1982), although long-term oral treatment with caffeine did not alter A_1 receptor mRNA or receptor expression (Johansson et al., 1993). Similarly, in the

mouse brain, striatal A_{2A} receptor expression was upregulated by chronic intraperitoneal caffeine treatment (Traversa et al., 1994), while long-term oral caffeine treatment did not alter A_{2A} receptor mRNA or receptor expression (Johansson et al., 1993). These contradictory findings may result from the different route of drug administration and the preferential exposure of these compounds to different sites of the body.

The regulation of A_{2B} receptor gene and protein expression is not as well understood. A study in chicken melanocytes has shown that A_{2B} receptor mRNA expression can be upregulated by c-MyB (Worpenberg et al., 1997), a transcription factor that is encoded by the c-myb proto-oncogene. In human melanocytes, cell damage induced by 4-tertiary butylphenol, a phenolic agent, was shown to upregulate A_{2B} receptor expression (Le Poole et al., 1999). In mouse bone marrow-derived macrophages, A_{2B} mRNA expression can also be upregulated by IFN-γ, a cytokine involved in activating macrophages (Xaus et al., 1999).

The genomic structure of the A_3 receptor has been examined, and a number of binding motifs have been identified in the 5' UTR of the human A_3 gene (Murrison et al., 1996; Atkinson et al., 1997). These transcriptional binding sites may be important in regulating the promoter activity of the human A_3 receptor. Possible binding sites have also been identified in the promoter of the mouse A_3 receptor gene (Zhao et al., 1999; Yaar et al., 2002). In particular, the GATA-6 and CRE binding sites are involved in enhancing and inhibiting A_3 receptor promoter activity, respectively (Yaar et al., 2002). The regulation of A_3 receptor gene expression has some similarities to that of the A_1 receptor. As with the A_1 receptor, glucocorticoids and oxidative stress upregulate A_3 receptor mRNA expression (Ramkumar et al., 1995; Sundaram et al., 2003). A_3 receptor mRNA expression was significantly increased by dexamethasone treatment (Ramkumar et al., 1995) and by oxidative stress during chronic

ileitis (Sundaram et al., 2003). Alterations in A_3 receptor expression can also be induced pharmacologically. For example, the exposure of CHO cells to NECA for 24 h has been shown to significantly decrease A_3 receptor expression (Palmer et al., 1995a).

The studies presented in this section demonstrate that adenosine receptor gene and protein expression may be regulated through transcriptional and post-transcriptional mechanisms. In addition, the expression of these receptors can be altered by stress-induced changes and by treatment with various adenosine analogs.

2.5 Structural characterization of the adenosine receptors

All four adenosine receptor subtypes belong to the G protein-coupled receptor family, which signal by activating various heterotrimeric G proteins (Olah and Stiles, 1995). The adenosine receptors are characterized by seven hydrophobic transmembrane domains, an extracellular N-terminal, an intracellular C-terminal, and three intracellular and three extracellular loops (Ralevic and Burnstock, 1998). In the rat, the A_1, A_{2A}, A_{2B} and A_3 receptors are comprised of 326-7, 410, 332 and 320 amino acids, respectively (Mahan et al., 1991; Reppert et al., 1991; Stehle et al., 1992; Zhou et al., 1992; Chu et al., 1996). The large size of the A_{2A} receptor results from its extended carboxyl tail (see Fig. 5). Each transmembrane domain consists of approximately 21-28 amino acids and all four receptors are glycosylated by asparagine (N)-linked glycoproteins at the second extracellular loop.

Presently, the precise three-dimensional conformation of the adenosine receptors is unknown. Structural analysis identified receptor domains that participate in ligand recognition and binding. Most of these studies concentrate on the A_1, A_{2A}, and A_3 receptors, due to the lack of highly selective A_{2B} receptor analogs. Molecular modeling of the A_1

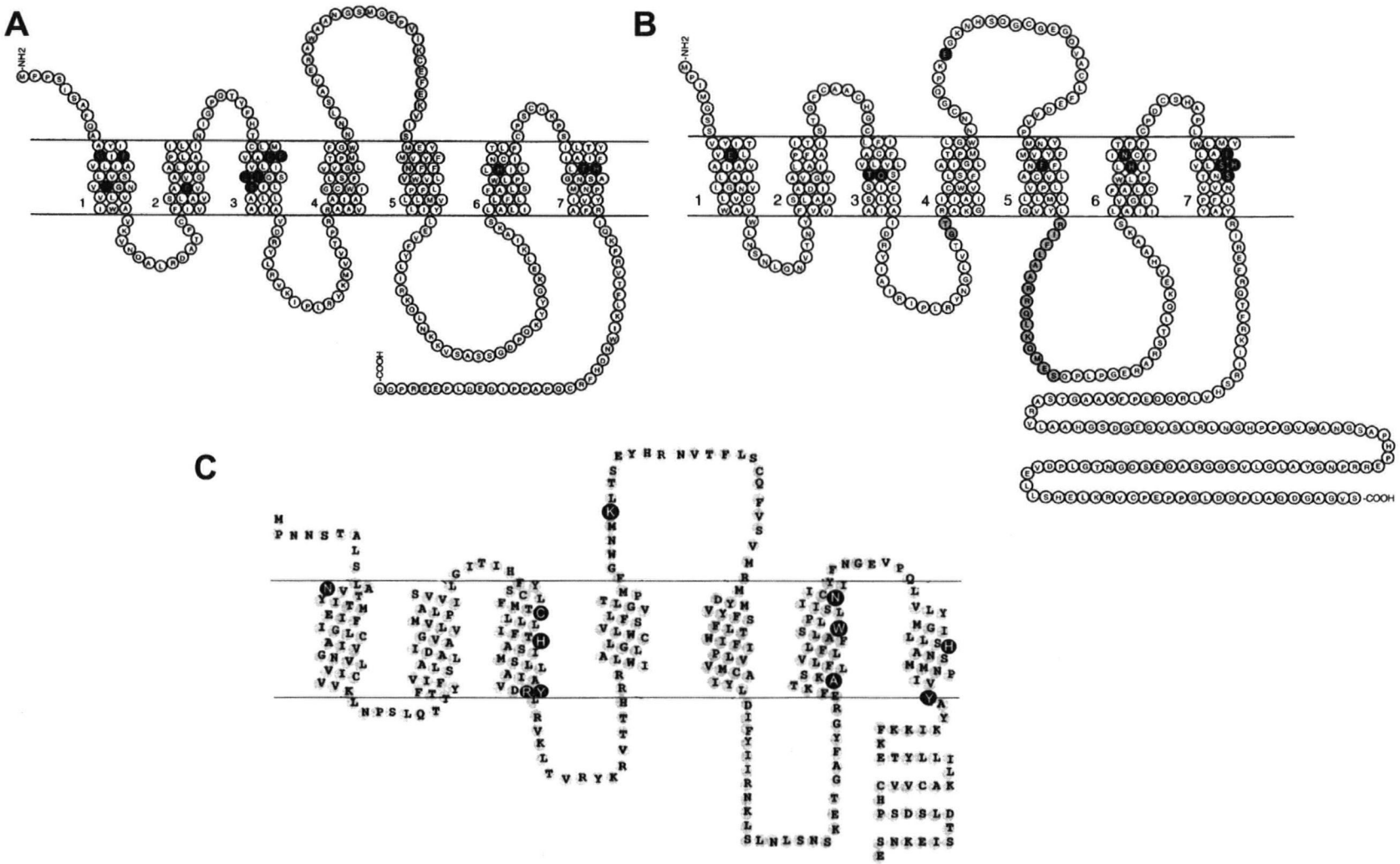


Fig. 5. Schematic representation of the human adenosine A_1 (A), A_{2A} (B), and A_3 (C) receptors. Transmembrane domains are numbered from 1 to 7, and amino acids shaded in black have been demonstrated, by site-directed mutagenesis, to participate in ligand binding. (figures are adapted from Olah and Stiles, 2000, and Gao et al., 2002).

(IJzerman et al., 1992), A_{2A} (IJzerman et al., 1994; Kim et al., 1995) and A_3 (van Galen et al., 1994) receptors has also been performed based on the structure of bacteriorhodopsin and/or rhodopsin, two structurally well characterized 7 transmembrane-spanning receptors. Based on these modeling studies and additional site-directed mutation studies, several amino acids were found to be involved in ligand binding at the A_1, A_{2A} and A_3 receptors (See Fig. 5).

2.6 Signal transduction mechanism

Table 1 shows that multiple signal transduction mechanisms and second messengers are involved in adenosine receptor signaling (Ralevic and Burnstock, 1998; Fredholm et al., 2001). The A_1 receptor couples to the G_i/G_o family of G proteins (Freissmuth et al., 1991; Jockers et al., 1994). Stimulation of A_1 receptors inhibits adenylate cyclase activity through the release of $G_{\alpha i}$, which decreases cAMP production and modulates cAMP-dependent protein kinase activity (Londos et al., 1980; Ralevic and Burnstock, 1998). This receptor also signals through a number of other messengers, such as phospholipase C (PLC), PKC, Ca^{2+} channels, K^+ channels, and mitogen activated protein kinases (MAPK), as described in Table 1. The A_{2A} receptor is coupled to the G_s protein in most tissues (Olah, 1997), and to G_{olf}, a member of the G_s subfamily of G proteins, in the striatum (Kull et al., 2000). Activation of the A_{2A} receptor can increase adenylate cyclase activity and cAMP production (Ralevic and Burnstock, 1998). However, these receptors can also mediate changes in the activity of other second messengers such as Ca^{2+} channels, K_{ATP} channels, and MAPK (Table 1). Like the A_{2A} receptors, the A_{2B} receptors also stimulate adenylate cyclase activity and increase cAMP production (Ralevic and Burnstock, 1998) through activation of the G_s protein (Pierce et al., 1992). The A_{2B} receptors can also couple to G_q/G_{11} to regulate PLC

Table 1: Adenosine receptor G protein coupling, signal transduction, and regulation of expression

Receptor Subtype	G protein Coupling	Effectors	Second Messengers	Receptor Expression
A_1	G_i, G_o	↓AC ↑ PLC ↓ PLC	↓ cAMP (van Calker et al., 1979) ↑ IP3, DAG, PKC (Gerwins and Fredholm, 1995) ↓ IP3, DAG, PKC (Delahunty et al., 1988) ↑ PLD (Gerwins and Fredholm, 1995) ↑K^+ channels (Belardinelli et al., 1995) ↓Ca^{2+} channels (Dolphin et al., 1986) ↑ MAPK (ERK1/2, p42/p44, p38) (Dickenson et al., 1998; Schulte and Fredholm, 2000; Robinson and Dickenson, 2001)	**upregulated by** glucocorticoid treatment (Svenningsson and Fredholm, 1997), oxidative stress (Nie et al., 1998), nuclear factor κB binding (Nie et al., 1998), caffeine treatment (i.p.) (Fredholm, 1982), and stress [sustained performance stress (Anderson et al., 1988), acute renal failure (Gould et al., 1997), sleep deprivation (Basheer et al., 2000)]
				suppressed by agonist treatment (PIA) (Green et al., 1992), and out of frame AUG codons (Ren and Stiles, 1994)
A_{2A}	G_s, G_{olf}	↑ AC	↑ cAMP (Furlong et al., 1992) ↑ Ca^{2+} channels (Gubitz et al., 1996) ↑ K^+ channels (Akatsuka et al., 1994) ↑ MAPK (ERK1/2) (Sexl et al., 1997; Schulte and Fredholm, 2000)	**upregulated by** oxidative stress (Kobayashi et al., 1998), PKC activation (Peterfreund et al., 1997), and treatment with caffeine (i.p.) (Traversa et al., 1994).
				suppressed by glucocorticoid treatment (Gerwins and Fredholm, 1991), binding of nuclear factor-1 proteins (Lee et al., 2003), A_{2A} receptor activation (Saitoh et al., 1994), and out of frame AUG codons (Chu et al., 1996).
A_{2B}	G_s, G_q/G_{11}	↑AC ↑PLC	↑cAMP (Feoktistov and Biaggioni, 1995) ↑ IP3, DAG, PKC (Feoktistov and Biaggioni, 1995) ↑MAPK (ERK 1/2, JNK, p38) (Feoktistov et al., 1999; Schulte and Fredholm, 2000)	**upregulated by** c-MyB transcription factor (Worpenberg et al., 1997), cell damage (Le Poole et al., 1999), and IFN-γ (Xaus et al., 1999).
A_3	G_i, G_q/G_{11}	↓AC ↑PLC	↓ cAMP (Zhou et al., 1992) ↑ IP3, DAG, PKC (Abbracchio et al., 1995) ↑MAPK (ERK 1/2) (Schulte and Fredholm, 2000)	**upregulated by** GATA-6 binding (Yaar et al., 2002), oxidative stress (Sundaram et al., 2003), and glucocorticoid treatment (Ramkumar et al., 1995).
				suppressed by CRE binding (Yaar et al., 2002), and treatment with agonist (NECA)(Palmer et al., 1995)

and MAPK activity (Yakel et al., 1993; Feoktistov and Biaggioni, 1995; Schulte and Fredholm, 2000) (Table 1). The A3 receptor is linked to the inhibition of adenylate cyclase activity (Zhou et al., 1992) through activation of the G_i protein (Palmer et al., 1995a), and can alter PLC and MAPK activity via activation of the G_q/G_{11} protein (Abbracchio et al., 1995; Schulte and Fredholm, 2000), as shown in Table 1. Since each adenosine receptor subtype is coupled to specific signal transduction mechanisms, adenosine can mediate specific actions throughout the body by binding to different adenosine receptor subtypes.

3. Biological actions of adenosine in the gastrointestinal tract

In the gastrointestinal tract, adenosine has been shown to modulate neurotransmitter release (Christofi, 2001), to alter gastric mucosal blood flow (Nagata et al., 1996; Burnstock, 2001), and to regulate gastric endocrine and exocrine secretion (Burnstock, 2001). The objectives of this thesis were to examine the role of the adenosine receptors in modulating gastrin and somatostatin secretion and to examine adenosine receptor gene expression under different physiological states of the stomach. A detailed review of the gastrointestinal actions of adenosine is presented in this section with emphasis on the role of adenosine in regulating gastric hormone secretion.

Adenosine modulates gastrointestinal motility, neurotransmission, and secretion in various species by acting on different adenosine receptors (Burnstock, 2001). Although all four adenosine receptors have been localized in the enteric nervous system of humans (Christofi et al., 2001), few biological studies have studied the gastrointestinal effects of adenosine in humans. The majority of biological studies have been performed in animal models such as the guinea pig and rat (Burnstock, 2001; Christofi, 2001), where adenosine modulates gastrointestinal neurotransmitter release and motility. In the guinea pig ileum,

adenosine has been shown to inhibit peristalsis (Shinozuka et al., 1985) and to inhibit cholinergic (Wiklund and Gustafsson, 1987) and adrenergic neurotransmission (Barajas-Lopez et al., 1991) via activation of presynaptic A_1 receptors (Barajas-Lopez et al., 1991; Nitahara et al., 1995). Adenosine also depolarizes submucosal neurons of the ileum through activation of A_{2A} receptors (Barajas-Lopez et al., 1991). In the guinea pig colon, adenosine was shown to inhibit contraction through activation of A_{2B} receptors on smooth muscle cells (Kadowaki et al., 2000). Thus, adenosine can act on different adenosine receptor subtypes to exert distinct actions on various regions of the guinea pig gastrointestinal tract.

In the rat, adenosine also modulates gastrointestinal neurotransmission, motility, and secretion. Adenosine was shown to induce relaxation of the gastric fundus (Matharu and Hollingsworth, 1992), enhance lower gastrointestinal propulsion (Suzuki et al., 1995), and inhibit peristalsis in the jejunum (Coupar and Hancock, 1994). These actions may be mediated by the activation of various adenosine receptor subtypes. In the rat ileum, the A_1 subtype was shown to modulate the myenteric reflex response by eliciting ascending contraction and descending relaxation (Storr et al., 2002). In the rat duodenum, A_1 and A_{2B} receptors were found to inhibit contraction of the longitudinal muscle and stimulate contraction of the muscularis mucosae, respectively (Nicholls et al., 1996).

In the rat stomach, adenosine has been shown to alter gastric mucosal blood flow (Cho and Ogle, 1990; Cho et al., 1991; Cho et al., 1995), and to regulate ethanol-induced gastric dilation in submucosal arterioles (Nagata et al., 1996). Our laboratory demonstrated that the administration of the A_{2A} receptor agonist, CGS 21680, significantly decreases perfusion pressure in the isolated vascularly perfused rat stomach (Yip and Kwok, 2004), suggesting that the A_{2A} receptor subtype may be involved in mediating gastric vasodilation. Adenosine

also regulates gastric endocrine and exocrine secretion. In particular, adenosine and its analogs can inhibit (Gerber et al., 1984; Glavin et al., 1987; Scarpignato et al., 1987), stimulate (Puurunen et al., 1986), or have no effect (Puurunen et al., 1987; Puurunen and Huttunen, 1988) on gastric acid secretion, depending on the route of drug administration. This nucleoside can also inhibit gastrin release (DeSchryver-Kecskemeti et al., 1981; Harty and Franklin, 1984; Kwok et al., 1990) and stimulate or inhibit gastric somatostatin release, depending on the concentration administered (Kwok et al., 1990). The gastric secretory actions of adenosine are discussed in section 3.2.

3.1 Gastroprotective actions of adenosine

In humans, caffeine has been implicated in promoting gastric ulcer formation (Cooke, 1976). Although the mechanism remains unclear, adenosine antagonists such as caffeine and theophylline were shown to stimulate gastric acid secretion (Krasnow and Grossman, 1949), possibly by inhibiting the action of adenosine. Studies have suggested that the gastric juice of ulcer patients contains decreased levels of adenosine (Durak et al., 1994) since xanthine oxidase activity and 5'nucleotidase activity in these patients were shown to be increased and decreased, respectively. As reviewed in Section 1, 5' nucleotidase is responsible for the synthesis of adenosine (see Fig. 3), while xanthine oxidase is involved in the metabolism of adenosine (see Fig. 1). Ulcer patients also exhibit decreased ADA activity in the vicinity of a healed ulcer after ranitidine treatment (Namiot et al., 1993) and increased ADA activity in the area surrounding an unhealed ulcer (Namiot et al., 1993). ADA is a metabolic enzyme of adenosine (see Fig. 3). Thus, it is likely that increased and decreased adenosine concentrations are also found surrounding a healed and unhealed ulcer, respectively. These

observations suggest that adenosine may be involved in the healing process and may be important in protecting the stomach against ulcer formation.

In rats, adenosine can act as a gastroprotective agent. Adenosine protects against indomethacin- (Bozkurt et al., 1998), ethanol- (Cho and Ogle, 1990; Cho et al., 1991), cold restraint stress- (Geiger and Glavin, 1985; Westerberg and Geiger, 1987), and ischemia/reperfusion- (Konturek et al., 2001; Pajdo et al., 2001) induced gastric ulcer formation. The ability of the non-selective adenosine antagonists 8-phenyltheophylline (8-PT) or 8-(p-sulfophenyl)theophylline (8-SPT) to block the protective action of adenosine in some of these studies confirms that adenosine receptors are involved (Geiger and Glavin, 1985; Westerberg and Geiger, 1987; Konturek et al., 2001; Pajdo et al., 2001). In indomethacin- and ethanol-treated rats, adenosine was also shown to inhibit indomethacin-induced gastric acid secretion (Bozkurt et al., 1998) and basal acid secretion (Cho and Ogle, 1990), respectively. In ethanol- and ischemia-induced ulcer studies, an increase in gastric mucosal blood flow was also observed (Cho et al., 1991; Konturek et al., 2001; Pajdo et al., 2001). Thus, the ability of adenosine to inhibit gastric acid secretion and to increase gastric mucosal blood flow may play an important role in the gastroprotective action of adenosine.

Although the action of adenosine is mainly protective, studies by Ushijima have shown that adenosine may also exacerbate gastric mucosa damage (Ushijima et al., 1985, 1992). Contrary to Westerberg (Westerberg and Geiger, 1987), the administration of adenosine analogs increased restraint stress-induced gastric ulcer formation in rats. The cause of this functional difference is currently undetermined.

3.2 Effect of adenosine on gastric acid secretion

In humans, indirect evidence suggests that adenosine is involved in regulating gastric acid secretion. Studies have shown that fundic ADA activity increases in parallel with gastric acid output (Namiot et al., 1990). Since ADA is a metabolic enzyme of adenosine, an increase or decrease in the activity of this enzyme should decrease or increase adenosine levels, respectively. Patients suffering from hypersecretion of acid exhibited higher fundic ADA activities (Namiot et al., 1990), while those suffering from the absence of gastric acid secretion, a condition known as achlorhydria, exhibited lower ADA activities. The inhibition of gastric acid secretion by ranitidine also decreased ADA activity (Namiot et al., 1991). Thus, in humans, it is possible that gastric acid secretion is regulated, in part, by ADA activity, and consequently by the adenosine level. The role of adenosine in regulating gastric acid secretion is also supported by the gastric action of caffeine and theophylline, two non-selective adenosine receptor antagonists. Both of these compounds stimulate gastric acid secretion (Krasnow and Grossman, 1949; Cohen and Booth, 1975), and may mediate this action by blocking the inhibitory effect of endogenously released adenosine.

Studies using experimental animal models indicate that the effect of adenosine on gastric acid secretion is likely to be species-specific, and may depend on the site(s) at which adenosine acts. Gastric acid is secreted by parietal cells which reside in the corporeal mucosa of the stomach (Sachs et al., 1997). The regulation of gastric acid secretion is complex and is controlled by neural, paracrine, and endocrine pathways that modulate the release of stimulatory mediators such as gastrin, acetylcholine, and histamine, and the release of inhibitory compounds such as somatostatin and prostaglandins (Hersey and Sachs, 1995).

Adenosine has been shown to inhibit gastric acid secretion in the dog, guinea pig, and rat, but in rabbits, adenosine stimulates gastric acid secretion. In isolated gastric glands (Gil-Rodrigo et al., 1990) and enriched parietal cells (Ota et al., 1989; Ainz et al., 1993) of the rabbit, adenosine and its analogs were shown to significantly stimulate basal and potentiate histamine-stimulated aminopyrine uptake. [^{14}C]aminopyrine uptake was used as an index of gastric acid secretion since it can become protonated and trapped in the acidic canaliculi of the parietal cell (Berglindh et al., 1976). The rank order of potency of different agonists in stimulating acid secretion was shown to be NECA > 2-chloroadenosine (2-CA) > adenosine, which suggests the involvement of adenosine A_2 receptor (Ota et al., 1989). The stimulatory action of adenosine on isolated gastric glands (Gil-Rodrigo et al., 1990) and NECA on enriched parietal cells (Ainz et al., 1993) were also inhibited by theophylline. Since activation of A_2 receptors increases cAMP production, this proposal is consistent with findings demonstrating the ability of adenosine to increase cAMP levels in the enriched rabbit parietal cell preparation (Ota et al., 1989). In these experiments, the effect of adenosine was not altered by the endogenous release of the acid secretagogue histamine (Ainz et al., 1993) or by the production of the acid inhibitor prostaglandin E_2 (Ota et al., 1992). Thus, in the rabbit, adenosine may act directly on the A_2 receptors of parietal cells to stimulate gastric acid secretion. In this species, ATP was shown to inhibit histamine-stimulated aminopyrine uptake in the isolated gastric gland (Gil-Rodrigo et al., 1990). Further experiments demonstrated that this is likely due to the activation of ATP receptors, of the P_{2Y} subtype, on the parietal cells (Gil-Rodrigo et al., 1996). The inhibitory action of ATP is consistent with the localization of P_{2Y} receptors on gastric glands of the rabbit fundus (Vallejo et al., 1996). Thus, in rabbits, purinergic compounds are able to directly alter gastric

acid secretion. In particular, adenosine can act on A_2 receptors and ATP can act on P_{2Y} receptors to stimulate and inhibit gastric acid secretion, respectively, from parietal cells.

In other species, such as the dog, adenosine has been shown to inhibit histamine- and methacholine-stimulated gastric acid secretion in vivo (Gerber et al., 1984). This inhibitory effect may be due to the direct action of adenosine on the parietal cells since 2-chloroadenosine (2-CA) and R-PIA have been shown to inhibit histamine-stimulated aminopyrine uptake from isolated canine parietal cells (Gerber et al., 1985). Activation of A_1 receptors is likely involved in this action since the A_1 selective analog R-PIA was more potent than 2-CA in inhibiting histamine-stimulated cAMP accumulation. Additional studies have also shown that the inhibitory effect of adenosine is unlikely to be due to changes in the release of the acid secretagogue histamine (Payne and Gerber, 1997), or the activity of phosphodiesterase (Gerber et al., 1984, 1985). Phosphodiesterase metabolizes cAMP, and inhibitors of this enzyme, such as high concentrations of theophylline and caffeine, may stimulate gastric acid secretion (Barnette et al., 1995). Furthermore, adenosine does not mediate its effect by altering the synthesis of prostaglandin E_2, an inhibitor of gastric acid secretion (Gerber et al., 1984; Geiger and Glavin, 1985). These findings suggest that, in the dog, adenosine may act directly on A_1 receptors of the parietal cells to inhibit gastric acid secretion.

In dogs, adenosine has also been shown to modulate the release of gastrin (Schepp et al., 1990), a potent gastric acid secretagogue (Hersey and Sachs, 1995). In isolated canine antral cells, R-PIA was shown to significantly inhibit forskolin-stimulated gastrin release, while CV 1808 had no effect on this action (Schepp et al., 1990). CV1808, however, slightly stimulated basal gastrin release and potentiated bombesin-stimulated gastrin release (Schepp

et al., 1990). Since R-PIA and CV 1808 preferentially bind to the A_1 and A_2 receptors, respectively, these results suggest that A_1 receptors may be involved in inhibiting gastrin release, while the A_2 receptors are involved in stimulating gastrin release.

In guinea pigs, adenosine was shown to inhibit histamine and gastrin-stimulated acid secretion from oxyntic cells (Heldsinger et al., 1986). Adenosine and its analogs inhibited histamine-stimulated hydroxyl ion production with the following rank order of potency: R-PIA > NECA > adenosine. The preferential action of R-PIA on A_1 receptors suggests that this subtype is involved in the inhibitory action of adenosine on gastric acid secretion (Heldsinger et al., 1986). This adenosine A_1 receptor-mediated inhibitory action is similar to that in the dog (Gerber et al., 1985).

The gastric effects of adenosine have been studied extensively in the rat. Various studies have shown that adenosine inhibits, stimulates, or does not alter gastric acid secretion (see Table 2), depending on the route of drug administration, the state of anesthesia, and whether or not the vagus is intact. Intraperitoneal (i.p.), subcutaneous (s.c.), intraduodenal (i.d.), oral, intragastric, and intracerebroventricular (i.c.v.) administration of adenosine and its analogs inhibited gastric acid secretion (Puurunen et al., 1986; Scarpignato et al., 1987; Puurunen and Huttunen, 1988; Westerberg and Geiger, 1989; Cho and Ogle, 1990), while intravenous (i.v.) administration of these drugs stimulated gastric acid secretion (Puurunen et al., 1986). The localization of the adenosine receptors and their preferential exposure to adenosine through different routes of administration may account for these functional differences. The various effects of adenosine suggest that this nucleoside may regulate gastric acid secretion through more than one site of action. Possible sites of action are addressed in the following discussion.

Table 2: Adenosine and adenosine agonists on gastric acid secretion in the rat

Administration	Drug	State	Effect	Reference
i.p.	R-PIA, S-PIA, 2-CA, NECA	conscious	↓ basal acid secretion	[1]
i.d	adenosine	anaesthetized	no effect	[2]
i.d.	R-PIA	anaesthetized	↓ basal acid secretion	[2]
oral	R-PIA	anaesthetized	↓ basal gastric secretion	[2]
intragastric	R-PIA	anaesthetized	↓ basal gastric secretion	[2]
s.c.	R-PIA	anaesthetized	↓ basal acid secretion	[2]
s.c.	adenosine	anaesthetized	↓ basal gastric secretion	[2-5]
s.c.	R-PIA	conscious	↓ basal gastric secretion	[6]
s.c.	S-PIA	conscious	no effect	[6]
s.c.	adenosine	conscious	↓ indomethacin-induced acid secretion	[7]
i.v.	R-PIA	vagotomized & anaesthetized	no effect	[8]
	RPIA & NECA	isolated parietal cells	no effect	[8]
i.v.	adenosine, NECA, R-PIA, CHA, S-PIA, 2-CA	anaesthetized	↑ basal acid secretion	[8]
i.v.	R-PIA	vagotomized & anaesthetized	no effect	[9]
i.c.v.	R-PIA	anaesthetized	no effect	[9]
i.c.v.	R-PIA	conscious	↓ basal acid secretion	[9]
i.c.v.	adenosine, NECA, R-PIA, S-PIA,	conscious	↓ basal acid secretion	[10]
i.c.v.	NECA	vagotomized & conscious	no effect	[10]
i.c.v.	NECA	anaesthetized	no effect	[10]

[1] Westerberg and Geiger, 1989; [2] Scarpignato et al., 1987; [3-5] Cho and Ogle, 1990; Cho et al., 1991; Cho et al., 1995; [6] Glavin et al., 1987; [7] Bozkurt et al., 1998; [8] Puurunen et al., 1987; [9] Puurunen et al., 1986; [10] Puurunen and Huttunen, 1988.

In conscious rats, the i.c.v. administration of adenosine has been shown to inhibit gastric acid secretion. This effect may be mediated by the action of adenosine on the central nervous system. The regulation of gastric acid secretion by the central nervous system involves the dorsomotor nucleus of the vagus (DMNV), the hypothalamus and the nucleus tractus solitarius (NTS) (Hersey and Sachs, 1995). The hypothalamus and NTS receive sensory input from the periphery and regulate the activity of the DMNV. Activation of the DMNV can stimulate gastric acid secretion. Central stimuli from the hypothalamus and the NTS are integrated at the DMNV, and efferent fibers originating from the DMNV travel to the stomach via the vagus nerve. The vagus also contains fibers originating from the nucleus ambiguus, but these nerves are mainly involved in controlling gastric motility (Hersey and Sachs, 1995). The i.c.v. administration of adenosine analogs to conscious rats with intact vagi inhibited gastric acid secretion with the following rank order of potency: NECA > R-PIA > S-PIA (Puurunen and Huttunen, 1988). The higher potency of NECA suggests that the A_2 receptor is involved in this action. The i.c.v. administration of NECA, however, did not alter gastric acid secretion in vagotomized rats (Puurunen and Huttunen, 1988) or anaesthetized rats with intact vagi (Puurunen and Huttunen, 1988). Anesthetics can suppress vagal tone (Murthy et al., 1982; Yamamura et al., 1983). Thus, the central A_2 receptors may inhibit gastric acid secretion by suppressing central stimulatory vagal impulses to the stomach. Furthermore, the inhibitory effect of NECA was attenuated by the removal of brain monoamines such as noradrenaline, dopamine and serotonin, but was not affected by opioid or prostaglandin inhibitors (Puurunen and Huttunen, 1988). Thus, in the brain, the A_2 receptors may mediate the inhibitory effect on acid secretion by altering the release of biogenic monoamines.

Gastric acid secretion was also inhibited by s.c., i.p., i.d., orally and intragastrically administered adenosine agonists (see Table 2). However, it is unclear whether this action is mediated centrally or peripherally. In one study, the s.c. administration of R-PIA was able to inhibit gastric acid secretion, while the s.c. administration of S-PIA had no effect (Glavin et al., 1987). In another study, the potency of NECA in inhibiting acid secretion was equal to that of R-PIA when administered i.p. (Westerberg and Geiger, 1989). Thus, both the A_1 and A_2 receptors may be involved in the inhibition of gastric acid secretion.

Gastric acid secretion is regulated peripherally through several mechanisms. Acetylcholine, gastrin, and histamine stimulate gastric acid secretion by acting on the M_3 receptor, CCK_B receptor, and H_2 receptor, respectively, which are expressed on parietal cells (Hersey and Sachs, 1995). Somatostatin and prostaglandins can also act directly on parietal cells to inhibit acid secretion (Schubert and Shamburek, 1990). Thus, adenosine may modulate gastric acid secretion by regulating the release of these substances. Unlike in other species, adenosine does not act directly on the parietal cells to alter gastric acid secretion in the rat. NECA and R-PIA were shown to have no effect on basal, histamine-stimulated, or carbachol-stimulated aminopyrine accumulation in the rat isolated enriched parietal cell preparation (Puurunen et al., 1987). Histamine and carbachol were shown to stimulate acid secretion by increasing cAMP and inositol phosphate levels, respectively, but neither NECA nor R-PIA elicited this effect (Puurunen et al., 1987).

Histamine is released from enterochromaffin-like (ECL) cells of the stomach (Sachs et al., 1997). Its release is stimulated by gastrin, Ach, and β-adrenergic agonists, and is inhibited by somatostatin (Hersey and Sachs, 1995). Although the effect of adenosine on histamine has not been examined in the rat, adenosine was shown not to alter histamine

release in both the dog and rabbit (Ainz et al., 1993; Payne and Gerber, 1997). Acetylcholine is released from the enteric nervous system and can stimulate gastric acid secretion either by acting directly on the parietal cells or indirectly by stimulating gastrin secretion or inhibiting somatostatin secretion (Hersey and Sachs, 1995). Studies on gastrointestinal motility have demonstrated that adenosine inhibits acetylcholine release from the myenteric plexus of several species. Adenosine was found to inhibit nicotinic neurotransmission in the antrum (Christofi et al., 1992) and ileum (Gustafsson, 1981). In the submucosal plexus, adenosine inhibits the presynaptic release of acetylcholine through activation of the A_1 receptor (Barajas-Lopez et al., 1991). Although adenosine can regulate acetylcholine release, this action has only been implicated in the modulation of gastric motility. It is unclear whether alterations in acetylcholine release are involved in the adenosine-induced inhibition of gastric acid secretion.

Adenosine may inhibit gastric acid secretion by stimulating the release of the gastric acid inhibitor, somatostatin. Somatostatin is released from D-cells of the gastric mucosa and from enteric nerves (McIntosh, 1985). The stomach contains two distinct populations of D-cells, one residing in the corpus and the other in the antrum. In the present study, the term "corpus" refers to the acid-secreting portion of the stomach (see Fig. 36). However, a number of studies also refer to this region as the "fundus". Thus, in this chapter, both "corpus" and "fundus" are used to describe the acid-secreting region of the stomach. In Chapter 3 (Results), the term "fundus" will be used exclusively to describe the non-glandular region of the stomach.

Somatostatin-containing D-cells of the fundus are localized at the basal mucosa and are characterized as small rounded or pyramid-shaped cells with long processes (Keast et al.,

1985). In the antrum, D-cell density is higher than in the fundus (Keast et al., 1985). Antral D-cells are also localized at the base of the gastric gland (Buchan et al., 1985a) and are characterized as flask-shaped cells with long processes (Keast et al., 1985). The fundic and antral D-cells of the rat are distinct and respond differently to gastric acidity and the presence of food (Schwarting et al., 1986). Antral D-cells are open to the luminal environment (Hersey and Sachs, 1995). These cells detect changes in the intraluminal pH, and respond to decreases in gastric pH by releasing somatostatin (Schusdziarra et al., 1983). Increases in gastric pH, such as those induced by omeprazole treatment, have been shown to decrease somatostatin gene expression (Brand and Stone, 1988; Wu et al., 1990a; Wu et al., 1990b; Sandvik et al., 1995), plasma somatostatin concentration, antral somatostatin concentration, and antral D-cell density (Allen et al., 1986; Lee et al., 1992; Pawlikowski et al., 1992). Omeprazole is a drug that inhibits gastric acid secretion by binding irreversibly to the proton pump, H^+K^+ATPase, on parietal cells (Hirschowitz et al., 1995). The prandial state of the stomach also alters somatostatin synthesis and release. After prolonged fasting (96 h), antral D-cell density has also been shown to decrease (Schwarting et al., 1986). Fundic D-cells are closed cells that do not interact with the luminal environment. Unlike antral D-cells, changes to the intraluminal pH and prolonged fasting do not alter fundic somatostatin release (Schusdziarra et al., 1983) and D-cell density (Schwarting et al., 1986), respectively. Instead, somatostatin release from fundic D-cells may be stimulated by intrinsic (Schubert et al., 1991) and extrinsic (Sandvik et al., 1993) nerve stimulation.

The distribution of gastric somatostatin-containing nerve fibers differs among species. In the rat stomach, these fibers are not present in the mucosa, external muscle or myenteric plexus, but a few single somatostatin-containing fibers have been observed in the muscularis

mucosae (Keast et al., 1985). In another study, somatostatin-containing fibers were absent from the mucosa and submucosal plexus; however, a few somatostatin-containing fibers were demonstrated in the smooth muscle and in the myenteric ganglia (Ekblad et al., 1985). Somatostatin is released from the long processes extending out from the D-cells, and has been shown to act in a paracrine manner to inhibit gastrin release from G-cells, acid secretion from parietal cells (Park et al., 1987; Kusumoto et al., 1995) and histamine release from ECL cells (Prinz et al., 1993). Fundic D-cells have also been shown to be in close proximity to the vasculature (Kusumoto et al., 1995), and antral D-cell processes have been shown to project to the blood capillaries (Kusumoto and Grube, 1987). Thus, somatostatin may be released into the blood and act in an endocrine manner. Somatostatin release is inhibited by cholinergic agents (Koop et al., 1982), enkephalins (McIntosh et al., 1983), substance P (Kwok et al., 1985; McIntosh et al., 1987a), neurokinins and tachykinins (Kwok et al., 1988) and is stimulated by β-adrenergic agents (Koop et al., 1982; Koop et al., 1983), histamine (McIntosh et al., 1987a), cholecystokinin (CCK) (DelValle et al., 1996), secretin/VIP (Chiba et al., 1980; Saffouri et al., 1984), calcitonin gene related peptide (CGRP) (Manela et al., 1995; Rasmussen et al., 2001), glucagon (Chiba et al., 1980) and bombesin/gastrin releasing peptide (GRP) (Martindale et al., 1982).

Our laboratory has demonstrated that basal somatostatin release can also be stimulated by adenosine in a concentration-dependent manner (Kwok et al., 1990). The stimulatory effect of adenosine on SLI was not altered by atropine and hexamethonium, cholinergic antagonists, or by propranolol, a β-adrenergic antagonist. Therefore, adenosine-induced attenuation of gastric SLI release does not involve changes in cholinergic or adrenergic neurotransmission (Kwok et al., 1990). Adenosine was also shown not to mediate its

stimulatory action on SLI through its vasodilatory effects since nitroprusside, a potent vasodilator, did not alter gastric SLI release (Yip and Kwok, 2004).

It is possible that adenosine acts directly on A_{2A} receptors on D-cells to stimulate somatostatin release. Activation of A_{2A} receptors has been shown to stimulate adenylate cyclase activity, cAMP production, PKC activity, and to activate L-type Ca^{2+} channels (see Table 1). The same second messengers were shown to be involved in mediating somatostatin release from D-cells. In human cultured D-cells, somatostatin release was stimulated by activation of PKC, production of cAMP, and increases in intracellular Ca^{2+} concentration (Buchan et al., 1990). In rat fundic D-cells, bombesin-induced release of somatostatin was also found to be mediated through a calcium-phospholipid-dependent mechanism (Schaffer et al., 1997). Furthermore, in canine fundic D-cells, CCK-stimulated somatostatin release was mediated by an increase in intracellular Ca^{2+} induced by activation of L-type Ca^{2+} channels (DelValle et al., 1996). Since the A_{2A} receptors are linked to second messengers which are involved in stimulating somatostatin release, it is possible that activation of A_{2A} receptors on D-cells stimulates somatostatin release.

Experiments have shown that somatostatin gene transcription is regulated by cAMP (Montminy et al., 1996a, 1996b). A cAMP response element (CRE), which confers cAMP inducibility, has been identified in the somatostatin gene (Comb et al., 1986) and can mediate the transcriptional induction of somatostatin. Activation of the A_{2A} receptors has been shown to increase the expression of genes that contain a cAMP response element (CRE), such as the tyrosine hydroxylase gene (Chae and Kim, 1997). Adenosine may therefore regulate the synthesis and release of somatostatin by activating A_{2A} receptors on D-cells. The cellular localization of these receptors, however, has not been determined in the stomach.

Gastrin is a major hormonal stimulant of gastric acid secretion (Edkins, 1905; Kovacs et al., 1989). In the stomach, it is synthesized in G-cells of the antral mucosa. These cells are open to the luminal environment, and their activity is partially regulated by the gastric contents. Gastrin secretion is enhanced by a number of chemical and physical factors such as acetylcholine (Matsuno et al., 1997), β-adrenergic agonists (DeSchryver-Kecskemeti et al., 1981; Koop et al., 1983), bombesin/GRP (Martindale et al., 1982; Guo et al., 1988; Campos et al., 1990), vagotomy (Stening and Grossman, 1970; Pederson et al., 1984), proteins and amino acids (Guo et al., 1988; DelValle and Yamada, 1990), gastric distension (Lloyd et al., 1992), and intraluminal pH (Holst et al., 1983). Gastric pH serves as a negative feedback mechanism since luminal acidification ($pH \leq 3.5$) has been shown to inhibit the further release of gastrin (Magee, 1996). Increased gastric pH induced by omeprezole treatment, conversely, has been shown to increase gastrin mRNA, antral gastrin and plasma gastrin levels (Brand and Stone, 1988; Inauen et al., 1988; Larsson et al., 1988; Dockray et al., 1991).

Gastrin release is inhibited by the antral somatostatin-secreting D-cells (Saffouri et al., 1980). D-cells may act as chemoreceptors for intraluminal pH and modulate somatostatin secretion to control gastrin secretion (Hersey and Sachs, 1995). An inhibition of basal somatostatin release may stimulate gastrin secretion since basal gastrin secretion was shown to be tonically restrained by somatostatin (Saffouri et al., 1980). Somatostatin acts on somatostatin 2 receptors located on the G-cells (Zaki et al., 1996), and inhibits gastrin secretion by inhibiting adenylate cyclase activity (DelValle et al., 1990).

Our laboratory has shown that adenosine can inhibit immunoreactive gastrin (IRG) release from the isolated vascularly perfused rat stomach in a concentration-dependent

manner (Kwok et al., 1990). Adenosine was also found to inhibit carbachol- and norepinephrine-stimulated gastrin secretion in rat antral mucosa cells (DeSchryver-Kecskemeti et al., 1981), and to inhibit basal and carbachol-stimulated gastrin secretion in the rat antral mucosal fragments (Harty and Franklin, 1984). The effect of adenosine on gastrin secretion is probably not centrally mediated since the administration of NECA (i.c.v.) did not alter serum gastrin concentrations (Puurunen and Huttunen, 1988). The adenosine receptor subtype involved in inhibiting gastrin release in the rat has not been examined. However, in the dog, adenosine was suggested to inhibit gastrin secretion by activating A_1 receptors localized on G-cells (Schepp et al., 1990).

Activation of A_1 receptors inhibits adenylate cyclase activity, cAMP production, and Ca^{2+} channel activity, and has also been shown to alter PKC activity (See Table 1) (Ralevic and Burnstock, 1998). The same second messengers are involved in regulating gastrin secretion. In isolated rat and pig parietal cells, cAMP is required to elicit aminopyrine accumulation (Cabero et al., 1993; Li et al., 1995). Cyclic AMP has also been shown to potentiate bombesin-evoked gastrin release from isolated perfused rat stomachs (Guo et al., 1988) and to stimulate gastrin release from isolated human G-cells in culture (Campos et al., 1990). Activation of PKC (Campos et al., 1990) and an elevation in intracellular Ca^{2+} concentration have also been shown to stimulate gastrin release (Campos et al., 1990; Buchan and Meloche, 1994; Seensalu et al., 1997). The release of gastrin from isolated G-cells was also shown to be mediated by the cytosolic Ca^{2+} concentration (Campos et al., 1990; Buchan and Meloche, 1994; Seensalu et al., 1997). Thus, it is possible that activation of A_1 receptors on G-cells leads to an inhibition of gastrin release through an inhibition of cAMP production and Ca^{2+} channel activity. The A_1 receptor may also be involved in

regulating gastrin gene expression since the expression of this gene has been shown to be cAMP-dependent (Shiotani and Merchant, 1995).

II. Hypothesis

I hypothesize that activation of gastric adenosine receptors plays a role in the regulation of gastric exocrine and endocrine secretion. In particular, adenosine may inhibit gastric acid secretion by inhibiting gastrin release and stimulating somatostatin release. I propose that adenosine acts on adenosine A_1 receptors on G-cells and A_{2A} receptors on D-cells to inhibit gastrin and stimulate somatostatin release, respectively. I also hypothesize that changes to the gastric state, such as those induced by fasting and omeprazole treatment, may alter adenosine receptor gene and protein expression to regulate gastrin and somatostatin release.

III. Rationale

Gastric acid secretion may be inhibited by adenosine through the regulation of gastrin and somatostatin release since adenosine has previously been shown to alter IRG and SLI release in the isolated vascularly perfused stomach. Adenosine was shown to inhibit basal and carbachol-stimulated gastrin release from isolated G-cell preparations (Schepp et al., 1990). Thus, this compound may act directly on A_1 receptors localized on G-cells to inhibit gastrin release. Adenosine may also stimulate somatostatin release by acting on A_{2A} receptors localized on the gastric D-cells since previous studies performed in our laboratory have shown that the effect of adenosine was not altered by cholinergic and adrenergic agonists. In addition, gastric adenosine receptor expression may be altered under different physiological conditions since changes to the prandial and acid secretory state of the stomach

have been shown to regulate the synthesis and release of gastrin and somatostatin (Brand and Stone, 1988; Wu et al., 1991; Sandvik et al., 1993).

IV. Objectives and specific aims

The objectives of this thesis were to determine the adenosine receptor subtypes that are involved in regulating SLI and IRG release, to provide information about the distribution, localization, and structure of these receptors, and to study how adenosine receptors are regulated under different physiological states of the stomach. The specific aims of this study are outlined below:

1. Determine the adenosine receptor subtypes involved in regulating IRG and SLI release in the isolated vascularly perfused rat stomach, using selective adenosine receptor agonists and antagonists.
2. Determine the distribution of adenosine receptor gene expression in morphologically and functionally distinct regions of the rat stomach using RT-PCR, and determine the structure of the gastric A_1, A_{2A}, and A_3 receptor by cloning and sequencing.
3. Develop a sensitive (competitive RT-PCR and Real-Time RT-PCR) assay to measure adenosine A_1 and A_{2A} receptor gene expression in various regions of the rat stomach.
4. Examine whether adenosine receptor gene expression and function are regulated by different physiological states of the stomach, in particular, fasting and omeprazole treatment.
5. Investigate the cellular localization and distribution of gastric A_1 and A_{2A} receptors in the stomach by immunohistochemistry.

Chapter 2: Methods

Animals were treated in accordance with the guidelines of the University of British Columbia Committee on Animal Care.

I. Effect of adenosine analogs on gastric SLI and IRG release

1. Isolated vascularly perfused rat stomach preparation

Male Wistar rats (250 to 325 g) were housed in light and temperature controlled rooms with free access to food and water. Animals were deprived of food, but had free access to water for at least 14 h prior to experiments. Rats were anesthetized with an i.p. injection (60 mg/kg) of sodium pentobarbital (Somnotol®, MTC Pharmaceuticals, Cambridge, ON, Canada). The surgical procedures used to isolate the stomach for perfusion have previously been described (Kwok et al., 1990; Kwok and McIntosh, 1990).

The stomach was exposed by an abdominal midline incision. The superior mesenteric artery and vasculature supplying the left and right adrenal glands and kidneys were tied or cut between double ligatures. The pancreas and spleen were carefully dissected out from the stomach without damaging the gastroepiploic artery. To empty the content of the stomach, a drainage cannula was inserted into the stomach through the gastroduodenal junction. The pancreas, spleen, small and large intestines were then removed. Arterial perfusion was achieved by inserting a cannula into the aorta with the tip lying adjacent to the celiac artery. Perfusate was introduced into the stomach via this arterial cannula. Six hundred units of heparin (Sigma-Aldrich, St. Louis, MO) in 2 ml saline solution were immediately injected. The portion of the body above the diaphragm was removed. The vena

cava was tied, and the portal vein was cannulated to allow for the collection of venous effluent.

The stomach was perfused at a rate of 3 ml/min using a peristaltic pump (Cole-Parmer Instrument Co. Chicago, IL). The perfusate was composed of Krebs' solution (120 mM NaCl, 4.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, 25 mM $NaHCO_3$, and 5.1 mM dextrose) containing 0.2% bovine serum albumin (BSA, RIA grade; Sigma-Aldrich) and 3% dextran (Clinical grade; Sigma-Aldrich). The perfusate was continuously gassed with a mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7.4. Both the perfusate and the stomach preparation were kept at 37°C by thermostatically-controlled heating units throughout the experiment.

2. Drug administration and sample collection

Venous effluent was collected via the portal cannula. After a 30 min equilibration period, samples were collected at 5 min intervals. Three basal samples were obtained before drugs were introduced. Drugs were administered into the perfusate via side-arm infusion at a rate calculated to give the final perfusion concentrations. The following drugs were purchased from Sigma-Aldrich: adenosine hemisulphate salt, N^6-cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl) phenethylamino-5'N-ethylcarboxamidoadenosine HCl (CGS 21680), 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-ß-D-ribofuranuronamide (IB-MECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and 3,7-dimethyl-1-propargylxanthine (DMPX). 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) was procured from Tocris Cookson Inc. (Ellisville, MO). Adenosine analogs were first dissolved in a small volume of

dimethylsulfoxide (DMSO) (BDH, Toronto, ON) and subsequently diluted with saline or perfusate to 0.03 or 0.5% before perfusing into the stomach. The perfusion of DMSO alone, at these concentrations, did not alter basal IRG or SLI release. Five min samples were collected into ice-cold polyethylene scintillation vials containing 0.3 ml of Trasylol® (aprotinin, 10,000 KIU/ml; Miles Labs., Etobicoke, ON). For the measurement of SLI release, 0.5 ml aliquots of each sample were transferred into ice-cold test-tubes containing 0.05 ml Trasylol. For the measurement of IRG release, 1 ml aliquots of each sample were transferred into ice-cold test tubes. Aliquots of samples were frozen and stored at –20°C until assayed.

3. Radioimmunoassay (RIA) for the measurement of SLI release

The RIA used to measure SLI has previously been described (McIntosh et al., 1978; McIntosh et al., 1987a; McIntosh et al., 1987b), and has been used routinely in our laboratory (Kwok et al., 1988; Kwok et al., 1990).

Iodination of somatostatin: Somatostatin was iodinated using chloramine-T. In neutral buffer, chloramine-T oxidizes iodide and allows it to bind, by electrophilic substitution, to residues containing an aromatic group, such as tyrosine. Five μg of ^{1}Tyr-somatostatin (Penninsula Laboratories Inc., Belmont, CA) was dissolved in 10 μl of distilled water, followed by the addition of 10 μl 0.5 M phosphate buffer (pH 7.5). Then 10 μl of chloramine-T (2 μg/μl 0.05 M phosphate buffer, pH 7.5, EM Sciences, Darmstadt, Germany) and 1 mCi of Na[^{125}I] (in 10 μl of NaOH) were added to the peptide solution. The mixture was incubated for 30 sec, and the reaction was terminated by the addition of 10 μl sodium metabisulphite (5 μg/μl 0.05 M phosphate buffer, pH 7.5, EM Sciences). One milliliter of

hormone free plasma and 20 mg of QUSO G32 (Philadelphia Quartz Co., Valley Forge, PA) was then added. The mixture was vortexed, and centrifuged for 2-3 min. The supernatant (fraction A) was removed and kept. The pellet was resuspended in 1 ml of water. The mixture was again vortexed and centrifuged. The supernatant (fraction B) was removed, and the pellet was resuspended in 1 ml distilled water. The procedure was repeated. The supernatant (fraction C) was removed, and the pellet was resuspended in 1 ml of solution containing glacial acetic acid/acetone/water (1:39:40). This solution is used to elute the labeled peptide. The mixture was centrifuged for 20 min, and the supernatant (fraction D) was removed. The pellet was re-suspended in 1 ml of water (fraction E). Fractions A-E were counted, and fraction D, containing the labeled protein, was diluted to 1×10^6 cpm/10 µl in 0.1 M acetic acid containing 0.5% BSA (Miles, Kankake, IL). Aliquots of 100 µl were lyophilized, and stored at -20°C.

The somatostatin label was purified on the day of the assay. The lyophilized label was re-dissolved in 2 mM ammonium acetate buffer (pH 4.6) and then applied to a column (12 cm × 1 cm) containing CM-cellulose CM 52 (Whatman Biosystem Ltd., Kent, England) which was previously equilibrated with the same buffer. The column was washed with approximately 50 ml of this buffer, before the label was eluted with 200 mM ammonium acetate buffer (pH 4.6). Buffer was introduced into the column at a rate of ~1 ml/min using a peristaltic pump. Two min fractions were collected during elution. Aliquots (10 µl) from each fraction were counted. The peak fraction was neutralized with 5 M NaOH, diluted to 3,500 ± 500 cpm/100 µl, and used for the assays.

Standards: To prepare the standards, somatostatin-14 (Bachem California, Torrance, CA) was dissolved in 0.1 M acetic acid containing 0.05% BSA (Miles, Kankake, IL) to a

final concentration of 5 μg/50 μl. These 50 μl aliquots were lyophilized and stored at -20°C. On the day of the assay, the lyophilized standard was re-dissolved in 100 μl of distilled water and 400 μl of assay buffer to yield a stock of 10 μg/ml. This stock was then serially diluted with assay buffer to obtain standard concentrations of: 500, 250, 125, 62.5, 31.25, 15.625, 7.8, and 3.9 pg/ml.

Assay: Each assay tube contained 100 μl of standard or sample, 100 μl of label, and 200 μl of somatostatin antibody (ascites SOMA 03 monoclonal antibody, final dilution of 1:4,000,000). The specificity of this antibody has previously been described (McIntosh et al., 1987b). The somatostatin antibody and label were diluted in assay buffer [23.77 mM Sodium barbital, 3.9 mM sodium acetate, 43.6 mM sodium chloride, and 0.247 mM ethylmercurithiosalicylic acid sodium salt (Eastman Kodak Co., Rochester, NY)]. The concentration of the drugs used in the present experiments did not cross-react with the antibody. Non-specific binding (NSB) tubes for standards and samples were prepared and contained buffer in place of the antibody, while zero standard tubes contained buffer in place of the standard. Total count (TC) tubes contained only 100 μl of labeled somatostatin. Triplicates were prepared for total count, standard, NSB standard, and zero tubes, while duplicates were prepared for the NSB sample and each unknown sample. Assay tubes were incubated for 72 h at 7°C. Free and bound labeled somatostatin were separated by adding 1 ml of dextran-coated activated charcoal [1.25% activated charcoal (Fischer Scientific, Fairlawn, NJ), 0.25% dextran T-70 (Pharmacia, Uppsala, Sweden), and 0.1% hormone free plasma in 0.05 M phosphate buffer, pH 7.5]. Tubes were incubated for 10 min, and centrifuged at 3,000 rpm for 30 min at 10°C. The supernatant was discarded and the charcoal pellet was air dried for >6 h, before the radioactivity was counted. The percentage bound of

sample was calculated using the following equation: % Bound = [(TC-standard)/TC - (TC-NSB)/TC)]cpm × 100. The standard curve of the % bound vs. Log(standard concentration) was plotted using GraphPad Prism (v. 3.0, GraphPad software, San Diego, CA), and unknown somatostatin concentrations were determined by interpolation using the standard curve. Inter- and intra-assay variations were found to be <3% and <6.5%, respectively.

4. Radioimmunoassay for the measurement of IRG release

IRG was measured by specific RIA as previously described (Jaffe and Walsh, 1978; Fujimiya and Kwok, 1997).

Iodination of Gastrin: Gastrin was iodinated using chloramine-T. Five μg of synthetic human gastrin-1 (Penninsula Laboratories, Merseyside, England) dissolved in 10 μl 0.4 M phosphate buffer (pH 7.4) was combined with 10 μl choloramine-T, (5 μg/μl in 0.04 M phosphate buffer, pH 7.4) and 0.3 mCi Na [^{125}I]. After a 30 sec incubation, the reaction was terminated with the addition of 10 μl sodium metabisulphite (5 μg/μl in 0.04 M phosphate buffer, pH 7.4). The iodinated gastrin was purified using a DEAE-Sephadex A25 (Amersham Biosciences, Uppsala, Sweden) column (12 cm × 0.5 cm) equilibrated with 0.05 M imidazole buffer (pH 7.5). The label was eluted with 1 M NaCl in a 0.05 M imidazole buffer (pH 7.5) gradient at a flow rate of 1.0 ml/min. A typical elution profile for the labeled gastrin is shown in Figure 6. The four fractions, collected after the second peak, were screened by RIA. The fraction of labeled gastrin that provided the best standard curve, as indicated by measuring control samples of 50 and 100 pg/ml gastrin was further diluted and used.

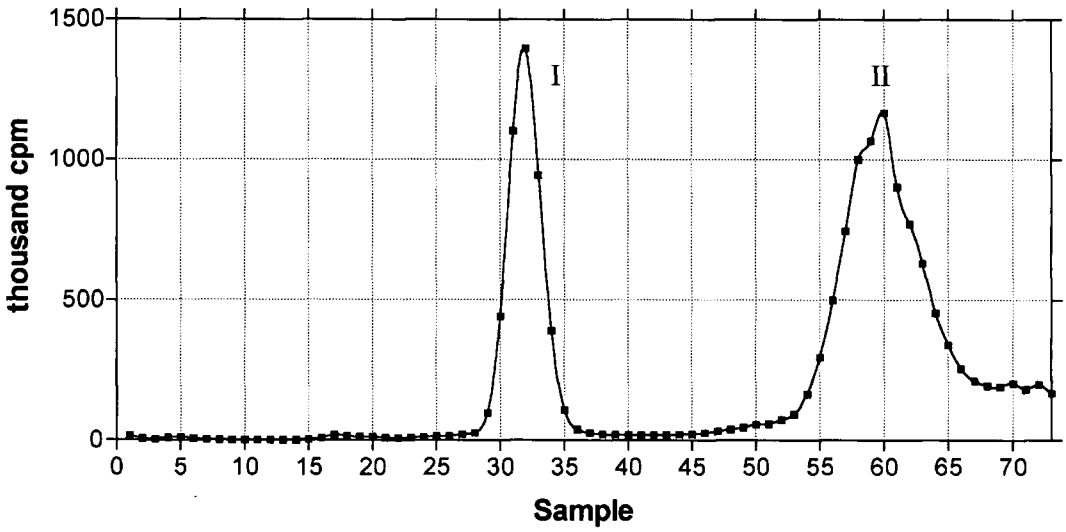


Fig. 6. Typical elution profile for the separation of labeled gastrin. ^{125}I-conjugated synthetic human gastrin-I was purified on a DEAE-Sephadex A25 column. The label was eluted with 1 M NaCl in a 0.05 M imidazole buffer gradient (pH 7.5) at a flow rate of 1.0 ml/min. Fractions in the second peak have been shown to exhibit higher specific activity (Jaffe and Walsh, 1978). Thus, the four fractions collected after peak II (# 61-64) were selected for screening by radioimmunoassay.

Standards: Human synthetic gastrin-1(2000 pg/ml; Penninsula Laboratories, San Carlos, CA) was serially diluted with assay buffer to obtain 1,000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8, and 3.9 pg/ml standard samples.

Assay: The assay buffer was composed of 0.02 M sodium barbital (pH 8.4) containing 0.5% BSA (RIA grade, Sigma-Aldrich). Each assay tube contained 700 µl assay buffer, 100 µl standard (0 to 1000 pg/ml) or sample, 100 µl gastrin antibody (PM1, rabbit polyclonal, final dilution of 1:90,000) and 100 µl labeled gastrin (~2000 counts/tube). The gastrin antibody (PM-1) was kindly provided by Dr. R. Pederson (Physiology, UBC). NSB samples contained buffer in place of the antibody, while zero standard tubes contained buffer in place of the sample, and total count tubes contained only labeled somatostatin. Triplicates were prepared for total count, standard, NSB standard, and zero tubes, while duplicates were prepared for the NSB sample, and each unknown sample. Assay tubes were incubated for 48 h at 7°C. Free and bound labeled gastrin were separated by the addition of 200 µl dextran-coated activated charcoal (1.25% activated charcoal, 0.25% dextran T-70, and 6.5% charcoal extracted plasma in 0.04 M phosphate buffer, pH 6.5). Samples were incubated for 10 min, and centrifuged at 3,000 rpm for 30 min at 10°C. The supernatant was discarded and the charcoal pellet was dried for >6 h, before the radioactivity was counted. The % bound was calculated as described for the SLI RIA. The IRG of unknown samples were determined by interpolation using the standard curve. The inter- and intra-assay variations of this assay were found to be <5.9% and <3.2%, respectively.

5. Data analysis

SLI measurements: Although the basal release rate of SLI varied among animals, previous experiments have demonstrated that basal SLI release is well maintained during a 50 min perfusion period (Saffouri et al., 1980; Kwok et al., 1988, 1990). Therefore, results are expressed as mean ± SEM of SLI release (%), and calculated as follows: [SLI release (pg/min) during a 5 min period ÷ SLI release (pg/min) during period 1] × 100. To compare the effect of various analogs, results are also expressed as percent change (SLI release), which is calculated as follows: [mean SLI release in the presence of drug – mean basal SLI release (periods 1-3)] pg/min ÷ [mean basal SLI release (periods 1-3)] pg/min × 100.

IRG measurements: Basal IRG release is also maintained in the perfused stomach preparation (Saffouri et al., 1980; Pederson et al., 1984; Kwok et al., 1990). The mean ± SEM of IRG release (%) was also calculated as described above. The percentage inhibition of IRG release by various analogs was calculated as follows: [mean basal IRG release (periods 1-3) - mean IRG release in the presence of drug] pg/min ÷ [mean basal IRG release (periods 1-3)] pg/min × 100.

Statistics: Statistical significance ($P < 0.05$) was determined using one-way ANOVA followed by Dunnett's multiple comparison test and the paired or unpaired Student's t-test when appropriate. Statistics were performed using GraphPad Prism, and the EC_{50} for different drugs were also estimated using the same software.

II. Adenosine receptor mRNA: distribution, structure, and quantification

1. Distribution of gastric adenosine receptor mRNA

RT-PCR was performed to determine whether the mRNA of all four adenosine receptor subtypes are expressed in various regions of the rat stomach, including the fundus, corpus, antrum, and the mucosa.

1.1 Primer design and synthesis

Adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor PCR primers were designed based on previously published cDNA sequences of these receptors in the rat brain (See Table 3). Primers were designed with the assistance of Mr. Jeff Hewitt (Department of Biochemistry and Molecular Biology, UBC) using the software program PCGene. Upper and lower primer regions were selected based on several criteria. Regions with similar melting temperature (Tm) were selected. Primers had a GC content of 40-60%. Complementarity between the upper and lower primers, runs of $\geq$3 Gs or Cs at the 3' end, and the occurrence of a T at the 3' end were avoided. Primer sets, designated as short length (SL) primers, were used to amplify only a portion of the adenosine receptor cDNA. These were designed for all four adenosine receptors. Full length (FL) primers, which amplify the entire coding region of the receptors, were designed for the A_1 and A_{2A} receptors. The sequences of these primers are listed in Table 3. The primers were synthesized by the Nucleic Acids Protein Services (NAPS) Unit at UBC and were diluted in autoclaved distilled water. Since single stranded DNA at a concentration of 33

Table 3: Adenosine receptor RT-PCR primers and PCR conditions

Primer Set[1]	Accession No.[2]	Position (brain sequences)	Primer Sequence	Amplicon Length	Annealing Temp. (°C)[3]	[$MgCl_2$] mM[4]
A_1SL	M64299	373-392 bp 636-655 bp	Upper: 5'TTC CAG GCT GCC TAC ATT GG3' Lower: 5'CAA TGG CGA GCA GAG CCA GA3'	283 bp	64	1.5
A_1FL	M64299	322-341 bp 1388-1407 bp	Upper: 5' GTC TGC TGA TGT GCC CAG CT3' Lower: 5' ACA GGG TGG GAC AGG GAG AA 3'	1086 bp	58	1.5
A_{2A}SL	S47609	99-118 bp 520-539 bp	Upper: 5' GTG GAG CTG GCC ATC GCT GT 3' Lower: 5' GCC GCA GGT CTT CGT GGA GT 3'	441 bp	60	1.5
A_{2A}FL	S47609	41-61 bp 1355-1377	Upper: 5'CTG CTG AGC CTG CCC AAG TGT3' Lower: 5'CCC TTC TCT TTG GGT TAC CCG3'	1335 bp	58	4.5
A_{2B}SL	M91466	223-242 bp 583-602 bp	Upper: 5' AGA CCC CCA CCA ACT ACT TT 3' Lower: 5' GCT CTT ATT CGT GAT GCC AT 3'	380 bp	53	1.5
A_3SL	M94152	319-338 bp 788-807 bp	Upper: 5' AAA GCC AAC AAT ACC ACG AC 3' Lower: 5' GGA GCT GTT TTG AGA GAG CT 3'	489 bp	58	3.0

[1] Primer sets designated as "SL" (short length) span only part of the coding region of the receptor. Those designated as "FL" (full-length) span the entire coding region of the receptor.

[2] Accession number of adenosine receptor sequences in the rat brain

[3] Optimal annealing temperatures used for PCR

[4] Optimal $MgCl_2$ concentration used for PCR

μg/ml has an A_{260} reading of 1, concentrations were determined spectrophotometrically using the following equation: Concentration (μg/ml) = A_{260} × 33 μg/ml × 100 (dilution factor).

1.2 Optimization of RT-PCR conditions

Several variables were examined to determine the optimal conditions for the A_1, A_{2A}, A_{2B}, and A_3 PCR reactions, including different $MgCl_2$ concentration (0.5 mM–7 mM) and annealing temperature during PCR (50-70°C). The optimum conditions for all RT-PCR reactions performed in this study are listed in Table 3.

1.3 Tissue extraction

Male Wistar rats (200-250g) were housed in light and temperature controlled rooms, with free access to food and water. Rats were anaesthetized with Somnotol® (60 mg/kg, i.p.). The fundus, corpus, and antrum were dissected out, rinsed in sterile ice-cold saline, flash frozen in liquid nitrogen, and stored at -80°C until total RNA was extracted. The total RNA from the gastric mucosa (containing the gastrin secreting G-cells and somatostatin secreting D-cells) was obtained as follows: The whole stomach was rinsed in sterile ice-cold saline and pinned onto a cork board with the luminal surface facing up. The mucosa was gently scraped off using a sterile glass slide, and total RNA was extracted immediately, as described in the next section. The striatum was also dissected from the brain and used as a positive control since this tissue has been shown to express the mRNA of all four adenosine receptors (Dixon et al., 1996).

1.4 Total RNA extraction and quantification

Total RNA was initially extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method, previously described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Since this method is fairly time consuming, total RNA extractions were subsequently performed using Trizol® reagent (Invitrogen, Carlsbad, CA). This reagent produced higher yields of total RNA at greater speed. The choice of extraction method did not affect RT-PCR results, and both methods are described in the next section. All materials and reagents used in the preparation of RNA and first strand cDNA were DNase- and RNase-free. Solutions were prepared using filtered distilled water which has been treated with diethylpyrocarbonate (DEPC) (Sigma-Aldrich), as previously described (Sambrook et al., 1989a).

For the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), tissues were ground to fine particles in a sterile mortar containing liquid nitrogen. Samples were transferred to sterile tubes, and homogenized on ice in Solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol] (1 ml/100 mg tissue) using the Tissumizer® tissue homogenizer (Tekmar Co. Cincinnati, OH). The following reagents were then added, 2 M sodium acetate (0.1 ml/100 mg), water-saturated phenol (1 ml/100 mg), and chloroform-isoamyl alcohol [49:1] (0.2 ml/100 mg). The mixture was vortexed for 10 sec and incubated on ice for 15 min. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The aqueous phase was removed and RNA was precipitated from this phase by the addition of isopropanol (1 ml/100 mg). Samples were incubated for 1 h at -20°C, and then centrifuged at 10,000 g for 20 min at 4°C. The resulting pellet was dissolved in Solution D (0.3 ml/100 mg) at 37°C. An equal volume

of isopropanol was added and the mixture was allowed to reprecipitate at -20°C for 1 h. The mixture was centrifuged at 10,000 g for 10 min at 4°C. Isopropanol was then removed and the RNA pellet was resuspended in 75% ethanol (1 ml/100 mg). After removal of the ethanol, the RNA pellet was vacuum-dried for 15 min, dissolved in DEPC-treated water (100 µl/100 mg), aliquoted, and stored at -80°C.

To extract total RNA using Trizol® reagent, tissues were homogenized on ice in Trizol® (1 ml/100 mg tissue) using the Tissumizer® tissue homogenizer. Samples were incubated at room temperature for 5 min, followed by the addition of chloroform (0.2 ml/ml Trizol®). Samples were shaken vigorously by hand for 15 sec, incubated in room temperature for 3 min and then centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was removed, and the total RNA was precipitated from this phase by the addition of isopropanol (0.5 ml/ml Trizol®). Samples were incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. The resulting RNA pellet was washed in 75% ethanol (1ml/ml Trizol®), and centrifuged at 7,500 g for 5 min at 4°C. The ethanol was removed and the RNA pellet was air-dried for 10 min at room temperature. The total RNA was finally rediluted in DEPC-treated water (100 µl/ml Trizol®), aliquoted, and stored at -80°C.

RNA purity and concentration: The total RNA purity was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), while the concentration was estimated by measuring the A_{260}. The spectrophotometer was calibrated with DEPC-treated water, and total RNA samples were diluted 100-fold in DEPC-treated water prior to measurement. Pure RNA gives an A_{260}/A_{280} ratio of 2 (Chomczynski and Sacchi, 1987). The A_{260}/A_{280} ratio for all our samples exceeded 1.8. Since total RNA at a concentration of 40

μg/ml has an A_{260} of 1, total RNA concentrations were determined using the following equation: concentration (μg/ml) = A_{260} × 40 μg/ml × 100 (dilution factor).

1.5 Formaldehyde gel electrophoresis

The integrity of the RNA samples was examined by formaldehyde gel electrophoresis to ensure that the samples were not degraded during total RNA extraction. Formaldehyde gel electrophoresis was performed using a method modified from the Molecular Cloning Laboratory Manual (Sambrook et al., 1989a). RNA samples were prepared on ice by mixing 10 μg of total RNA (in a volume of 4.5 μl) with 10 μl deionized formamide and 2 μl 5× formaldehyde gel running buffer [0.1 M MOPS, 40 mM sodium acetate, and 5 mM EDTA]. The mixture was incubated for 15 min at 55°C. This was followed by the addition of 1 μl ethidium bromide (1 mg/ml) and 2 μl formaldehyde gel loading buffer [50% glycerol, 1 mM EDTA (pH 8.0), 0.25 % bromphenol blue, 0.25% xylene cyanol]. Samples were electrophoresed through a 1% (w/v) agarose gel containing 5× formaldehyde gel running buffer and formaldehyde in a final concentration of 1× and 2.2 M, respectively. The gel was submerged in 1× gel loading buffer. The samples were loaded along with a 0.24-9.5 kb RNA ladder (Invitrogen), and run at 100 V for 1 h. Gels were viewed under UV light using the Stratagene Eagle Eye II system (La Jolla, CA). Images were acquired and saved using the Eagle Sight image analysis software (version 3.1, 1997, Stratagene).

1.6 DNase I treatment

RNA samples were treated with DNase I (Amersham Pharmacia, Piscataway, NJ) to remove any residual DNA contamination that may remain in samples after total RNA

extraction. DNase I treatment was performed at room temperature in 1× first strand buffer [50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$] containing 1 U DNase I/µg total RNA. The reaction was allowed to proceed for 15 min, before 1 µl of 25 mM EDTA was added to stop the reaction (Sanyal et al., 1997). Samples were then incubated for an additional 10 min at 65°C.

1.7 First strand cDNA synthesis

First strand cDNA was synthesized from 5 µg of DNase I treated total RNA using Superscript II RNase H- Reverse Transcriptase (Invitrogen). Total RNA (5 µg) was reverse transcribed in 1× first strand buffer [50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$] containing 200 ng random hexamers, 10 mM DTT, 0.5 mM dNTP mix, and 200 U Superscript II RNase H- Reverse Transcriptase, in a total volume of 20 µl. As a negative control for PCR, a sample was prepared using autoclaved distilled water in place of total RNA.

1.8 PCR

The PCR reaction mixture for all PCR reactions consisted of 2 µl cDNA in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], containing 0.2 mM dNTP mix, 1.5-4.5 mM $MgCl_2$ (see Table 3 for $MgCl_2$ concentrations), 100 ng forward primer, 100 ng reverse primer (see Table 3 for primer sequences), and 1 U Platinum Taq DNA polymerase (Invitrogen), in a total volume of 50 µl. For each experiment, a positive control sample containing striatal cDNA, and a negative control sample were included. The PCR was performed as follows using the Robocycler Temperature cycler (Stratagene, La Jolla, CA): cDNA was completely

denatured (2 min at 94°C) during the first step of PCR. Samples were then subjected to 30 cycles of amplification. Each cycle consisted of a 45 sec denaturation period at 94°C, a 1 min annealing period at 53°C - 60°C (See Table 3), and a 1 min extension period at 72°C. The sample was then subjected to a final extension period for 5 min at 72°C. PCR products were visualized by gel electrophoresis as described below.

1.9 Agarose gel electrophoresis

To determine the size of the amplicons produced by PCR, agarose gel electrophoresis was performed as previously described (Sambrook et al., 1989b). The PCR products (10 μl) were mixed with 3 μl gel loading dye [0.25% bromphenol blue in 50% glycerol, 10 mM Tris, 1 mM EDTA, pH 8], and loaded on a 2.5% agarose gel (w/v) containing 1× Tris acetate EDTA (TAE) buffer [40 mM Tris acetate and 1 mM EDTA (pH 8.5)] and ethidium bromide (0.5 μg/ml). The gel was placed in 1× TAE buffer and run at 100 V for 45 min. Bands were visualized and photographed under UV light using the Stratagene Eagle Eye II System and the Eagle Sight Image Analysis software program.

1.10 Restriction enzyme digestion

To confirm the identities of the PCR amplicons, restriction enzyme digestion was performed. PCR products generated by the A_1, A_{2A}, A_{2B} and A_3 RT-PCR reactions were concentrated, and cleaved with restrictive enzymes that were selected based on their cleavage sites. Cleavage sites within the PCR amplicons were determined using PCGENE. The following restriction enzymes were used to cleave the following PCR amplicons: Nar I and Sac I for the A_1 receptor; EcoRI, Mbo I, Pst I, and Rsa I for the A_{2A} receptor; Ava II and Hpa

II for the A_{2B} receptor; Ava II and Hinf I for the A_3 receptor. All restriction enzymes were purchased from Amersham Pharmacia (Uppsala, Sweden). To concentrate the DNA, PCR products (300 µl) were mixed with 150 µl of 7.5 M ammonium acetate and 600 µl 95% ethanol. Samples were incubated for 1 h at -20°C and then centrifuged at 10,000 g for 20 min at 4°C. The resulting pellet was washed in 1 ml 70% ethanol, and centrifuged at 10,000 g for 5 min at 4°C. The ethanol was removed and the pellet was re-dissolved in 50 µl of autoclaved distilled water. The restriction enzyme digestion was performed using the buffer provided with the enzyme, according to the manufacturer's instructions. For these reactions, 5 µl of the concentrated PCR product was digested in a final volume of 10 µl. Samples were electrophoresed through a 1.5-2 % agarose gel along with a 1 kb ladder (Invitrogen), as described earlier. The Eagle Sight Image analysis software was used to determine the size of the fragments. This measurement is based on the rate of DNA migration through the agarose gel. The software produces a standard curve of migration distance vs. Log[fragment size (bp)] using the fragments of the 1 kb ladder. The size of the fragments generated by restriction enzyme digestion was interpolated by comparing the migration distance of the fragment against the standard curve.

2. Structure of the adenosine receptors

To determine if the gastric A_1 and A_{2A} receptors undergo alternative splicing and if the structure of these receptors are the same as those in the brain, the entire coding region of the gastric A_1 and A_{2A} receptors were amplified by RT-PCR, cloned, and sequenced. In addition, part of the gastric A_{2B} and A_3 receptor gene sequence was also determined. These A_{2B} and A_3 gene sequences are located between the short length upper and lower primers

listed in Table 3. This portion of the A_3 receptor gene is of particular interest since its sequence can reveal whether the A_3i variant of the A_3 receptor is expressed in the stomach.

2.1 Detection of A_1 and A_{2A} receptor coding regions by RT-PCR

Primers: The primers used for this experiment are listed in Table 3. The A_1FL and A_{2A}FL primers span the entire coding region of the A_1 and A_{2A} receptor, respectively.

RT-PCR: To examine whether alternative splicing occurs for the A_1 and A_{2A} receptors, RT-PCR was performed in all gastric tissues, including the fundus, corpus, antrum and mucosa using the A_1FL and A_{2A}FL primers. RT-PCR was performed as previously described using the PCR conditions listed in Table 3. Fundus, corpus, antrum and mucosa RNA was used as the template for these reactions. A sample containing striatal RNA and a sample containing no template was also included as positive and negative controls, respectively.

2.2 Cloning and DNA sequence analysis of the adenosine receptors

PCR products and purification: RT-PCR was performed for all four receptors using rat gastric mucosal RNA as the template and the A_1FL, A_{2A}FL, A_{2B}SL, and A_3SL primers. The PCR products were concentrated using the QIAquick PCR purification kit (Qiagen, Mississauga, ON). According to manufacturer's instructions, 1.5 ml Buffer PB was mixed with 300 μl of the PCR product. The DNA was bound to a QIAquick column by centrifugation and washed with PE buffer. The DNA was finally eluted with 50 μl of autoclaved distilled water. The purified DNA was electrophoresed through a 2% agarose gel, as described earlier, and extracted from the gel using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. The fragment was excised from the

gel using a sterile razor blade and weighed. Buffer QX1 (300 µl/100 mg gel) was added, and the mixture was vortexed for 30 sec. QIAEX II buffer (5 µl) was then added and the sample was incubated at 50°C for 10 min, and vortexed every 2 min, and the sample was then centrifuged at 10,000 g for 30 sec. The pellet was washed with Buffer QX1 (500 µl), followed by two washes in Buffer PE (500 µl). The pellet was air dried for 10-15 min, and rediluted in autoclaved distilled water.

Ligation and transformation: The purified adenosine receptor transcripts were ligated into the pGEM-T vector (Promega, Madison, WI) by combining 5 µl of the purified A_1FL, A_{2A}FL, A_{2B}SL or A_3SL PCR product with 1 µl 10× T4 DNA Ligase Buffer, 1 µl pGEM-T vector (50 ng), 1 µl T4 DNA ligase and 2 µl autoclaved distilled water. The mixture was incubated overnight at 4°C, and then heated to 70°C for 10 min, and cooled to room temperature. DHα-competent E. coli cells (Invitrogen) were transformed with the ligated PCR product/pGEM-T vector by combining 2 µl of this sample with 50 µl of cells. The mixture was incubated on ice for 20 min, heat shocked at 37°C for 50 sec, and placed on ice. The volume of Luria-Bertani (LB) broth [0.1% NaCl containing tryptone (10g/L) and yeast extract (5g/L)] added to cells depended on the size of the PCR products, which were transformed into the cells. Cells containing the A_1FL, A_{2A}FL, A_{2B}SL, and A_3SL transcript were combined with 900 µl, 1 ml, 1.5 ml, and 1.5 ml of LB, respectively. Cells were incubated for 1 h at 37°C, and then plated on LB plates containing ampicillin (100 µg/ml), isopropyl β-D-thiogalactopyranoside (0.5 mM) and 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (80 µg/ml). Cells were grown overnight at 37°C. Colonies containing inserts

appear white since the inserts interrupts the lacZ gene in the pGEM-T vector. White colonies were screened by RT-PCR.

Colonies containing the correct insert were grown in 5 ml of LB broth containing ampicillin (100 μg/ml). Plasmid DNA was purified from these cells using the QIAprep Miniprep kit (Qiagen). The sample was centrifuged at 3,000 rpm for 5 min at 4°C to pellet the cells. Cells were resuspended in 250 μl Buffer P1, followed by the addition of 250 μl of Buffer P2. Cells were allowed to lyse for 5 min prior to the addition of 350 μl N3 Buffer. The sample was then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was applied to a QIAprep column and centrifuged for 30 sec. The column was washed with 750 μl PE buffer, and plasmid DNA was eluted from the column with 50 μl of Buffer EB [10 mM Tris-Cl (pH 8.5)]

DNA sequence analysis: The DNA sequences were determined at the NAPS Unit (UBC) using both the T7 and SP6 primers. This procedure was successful in obtaining the A_1Fl, A_{2B}SL and A_3SL sequences. However, due to the length of the A_{2A}FL sequence, 2 additional primers located within the A_{2A}FL transcript were required to complete the sequence analysis. These primers were based on the rat brain A_{2A} receptor gene (accession #S47609). Their sequences are as follows: 5' TTG TCC TGG TCC TCA CGC 3' (position 313-330 bp) and 5' AGG GCC GGG TGA CCT GTC 3' (position 541-558 bp). The sequence of the rat brain A_1 and A_{2A} receptor coding regions, and the partial cDNA sequence of the A_{2B} and A_3 receptors were aligned with the published sequences of these receptors in the rat brain using the "University of Southern California (USC) Sequence Alignment Server" (www-hto.usc.edu/software). These sequences were submitted to GenBank, and assigned accession numbers (see Results).

3. Quantification of gastric A_1 and A_{2A} receptor gene expression

3.1 Quantification by competitive RT-PCR

Competitive RT-PCR was performed to quantify the level of adenosine A_1 and A_{2A} receptor gene expression in various regions of the stomach, including the fundus, corpus, antrum, and mucosa. The general steps for measuring A_1 and A_{2A} receptor gene expression using competitive RT-PCR are as follows: 1) synthesize, purify, and quantify the A_1 and A_{2A} receptor RNA competitors; 2) perform the competitive RT-PCR assay by preparing samples containing a fixed amount of tissue total RNA and various amounts of competitor RNA as the template; 3) separate and visualize the PCR products by agarose gel electrophoresis; 4) determine the relative intensities of the tissue template and competitor bands; 5) calculate the tissue template level based on the relative intensity of the competitor vs. the template band. The intensities of both bands are approximately equal when the concentration of the template is equal to the concentration of the competitor. Each step is described in detail below.

3.1.1 RNA competitor design and synthesis

Previous experiments have shown that the reverse transcription step can vary considerably between assays (Ferre, 1992). Thus, to control for the efficiency of the reverse transcription step, RNA competitors were synthesized for this experiment instead of DNA competitors. The RNA competitors were synthesized by deleting a small portion (< 15%) of the adenosine receptor transcripts. The deletion allows for differentiation between the template and competitor bands when PCR products are visualized by electrophoresis. The small deletion also controls for differences in PCR amplification efficiency that may occur

due to differences in RNA length and secondary structure. The adenosine receptor RNA competitors were synthesized as described in the following section and in Figure 7. The general method has previously been described by Tang et al. (1996).

Vectors containing the A_1 and A_{2A} competitor: To synthesize vectors containing the A_1 receptor competitor, RT-PCR was performed using the A_1FL primer set (see Table 3). The RT-PCR products were concentrated using the QIAquick PCR purification kit, and gel purified using the QIAquick gel extraction kit, as described earlier. The A_1 competitor was synthesized by deleting a 136 bp fragment from the A_1 receptor template to produce a competitor containing 950 bp. To produce this deletion, the A_1 template was digested with Bpm I (New England BioLabs, Beverly, MA), as directed by the manufacturer. Three fragments (515 bp, 435 bp and 136 bp) were formed. The sample was electrophoresed through a 2% agarose gel as previously described. The two fragments containing the 5' and 3' primer sites (515 bp and 435 bp) were excised from the gel, purified using the QIAquick gel extraction kit, and religated with the pGEM-T vector using T4 ligase. The A_{2A} competitor was synthesized in a similar manner. RT-PCR was performed using the A_{2A}SL primers (see Table 3). The A_{2A} competitor was synthesized by deleting a 57 bp fragment from the A_{2A} template to produce a 384 bp competitor. The A_{2A} template was digested with Tsp509 I (New England BioLabs), according to manufacturer's instructions, to produce 3 fragments (286 bp, 98 bp, and 57 bp). The two fragments containing the primer sites (286 bp and 98 bp) were gel-purified and religated with the pGEM-T vector.

Transformation and in vitro Transcription: Vectors containing the modified A_1, and A_{2A} inserts were transformed into DHα competent cells, as described earlier. Bacterial colonies were screened by PCR to ensure that the colonies that were chosen contain the

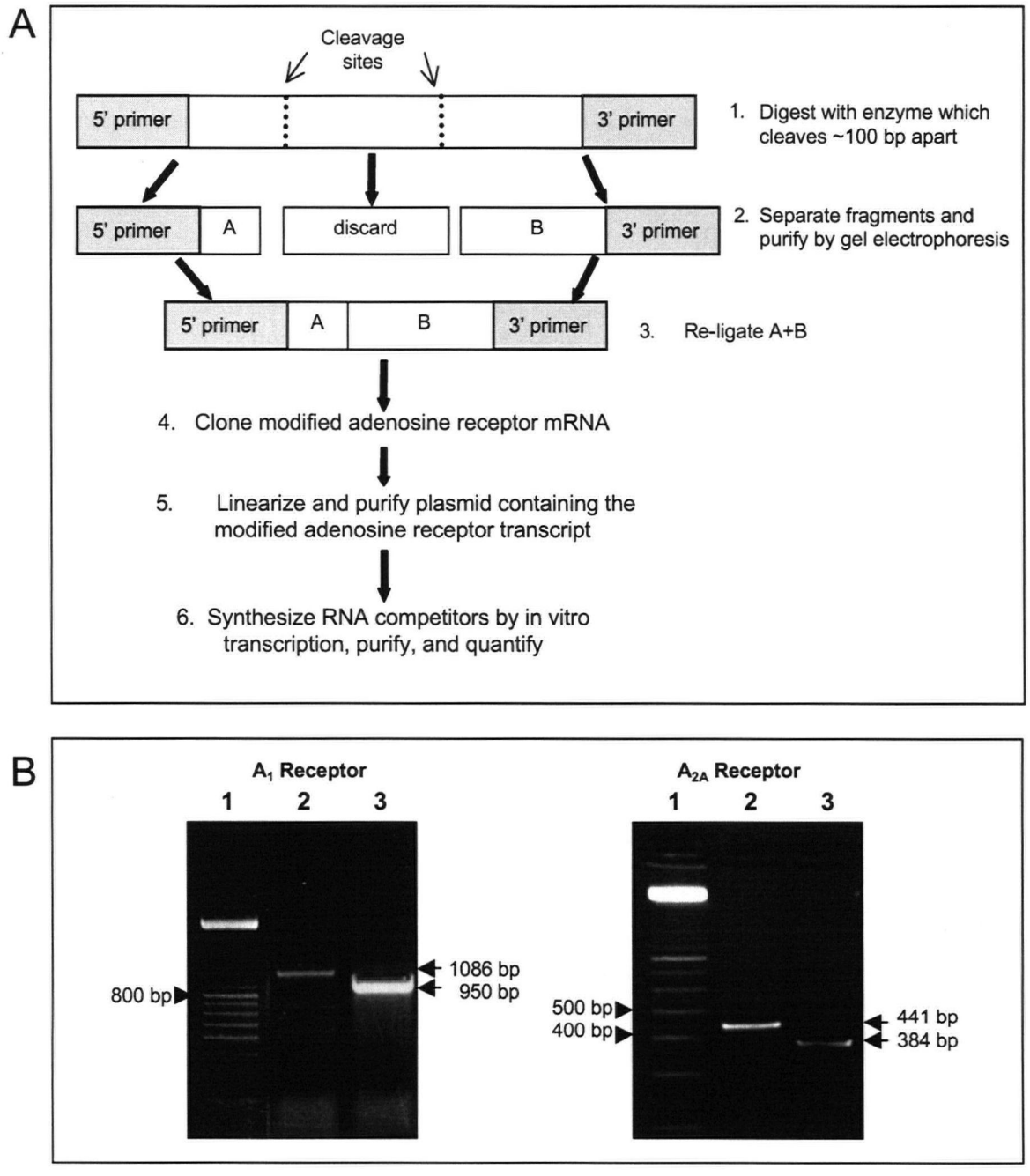


Fig. 7. Design and synthesis of the adenosine A_1 and A_{2A} receptor RNA competitors. A) The general protocol used for the design and synthesis of A_1 and A_{2A} competitors. B) Agarose gels showing PCR amplicons generated using corpus tissue total RNA as the template (lanes 2; A_1, 1086 bp; A_{2A}, 441 bp) or A_1 and A_{2A} RNA competitor as the template (lanes 3; A_1, 950 bp; A_{2A}, 384 bp). A 100 kb ladder is shown on lanes 1. The competitor and tissue template bands can be differentiated easily by their size.

modified adenosine receptor transcripts. The modified A_1 and A_{2A} receptor transcripts generated PCR amplicons which were 950 and 382 bp in length, respectively (see Fig. 7B). Plasmids from the chosen colonies were isolated using the QIAprep Miniprep kit and linearized by Not I (Amersham Pharmacia) digestion, according to manufacturer's instructions. Complete digestion was confirmed by agarose gel electrophoresis. The linearized plasmids were then purified and concentrated as described earlier.

In vitro transcription was performed using the Riboprobe in vitro Transcription System - T7 (Promega). To synthesize the competitor RNA, a reaction mixture (20 μl) containing 1× Transcription Optimized buffer [40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl], 10 mM DTT, 20 U RNasin ribonuclease inhibitor, 0.5 mM rNTPs, 0.2 μg linearized template, and 20 U T7 RNA polymerase, was incubated at 37°C for 1 h. The template DNA was digested with 0.2 U of RQ1 RNase-free DNase. The RNA competitors were purified using the RNeasy mini kit (Qiagen). The volume of the sample was increased to 100 μl with DEPC-treated water. Buffer RLT (350 μl) and ethanol (250 μl) was then added. The sample was applied to a RNeasy mini spin column and centrifuged for 15 sec at 8,000 g. The column was washed twice with 500 μl of Buffer RPE, and the RNA competitors were eluted from the spin column with 30 μl of DEPC-treated water. The purity and concentration of the RNA competitors were determined spectrophotometrically, as described earlier. The integrity of the competitors was examined by gel electrophoresis through a formaldehyde gel. RNA competitors were then serially diluted from 0.1 pmol to 0.01 amol, aliquoted, and stored at -80°C. RNA competitors were thawed only once prior to use to avoid damage caused by multiple freeze-thaw cycles.

3.1.2 Competitive RT-PCR assay

Total RNA extraction: Tissues were dissected from various regions of the stomach, including the fundus, corpus, antrum and mucosa. As a positive control, the level of A_1 and A_{2A} receptor gene expression was also examined in the striatum. This tissue has been shown to express extremely high levels of A_{2A} receptor mRNA and relatively high levels of A_1 receptor mRNA (Dixon et al., 1996). Total RNA was extracted using Trizol® Reagent, and the purity and concentration of the RNA were determined by measuring the absorbance of A_{260}/A_{280}, and A_{260}, as described earlier. The A_{260}/A_{280} ratio for all samples exceeded 1.8.

First strand cDNA synthesis: First strand cDNA was synthesized using Superscript II as described earlier with the following modification. Various amounts of A_1 or A_{2A} receptor competitor RNA (from 0.01 amol to 1 pmol) and a fixed amount of total RNA (2.5 μg) were used as the template for the reaction. The cDNA that was synthesized was then used as the template for PCR.

PCR: PCR was performed using the A_1FL and A_{2A}SL primer sets listed in Table 3. The A_1 and A_{2A} receptor competitive PCR reactions mixture (50 μl total) contained 0.5 μl and 2.0 μl of cDNA, respectively, in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl] with 0.2 mM dNTP mix, 1.5-4.5 mM $MgCl_2$ (see Table 3), 100 ng forward primer, 100 ng reverse primer, and 1 U Platinum Taq DNA Polymerase. PCR was performed using the Robocycler Temperature cycler as described previously. However, the cycles of amplification were limited to 30 cycles to avoid reaching the plateau phase. Reaching this phase of the reaction has previously been shown to introduce errors into the RNA measurements (Wang et al., 1989; Freeman et al., 1999). The PCR products, containing the competitor and template amplicons, were separated by electrophoresis through a 2.5%

agarose gel containing ethidium bromide, as described earlier. Bands were visualized and photographed under UV light using the Eagle Eye II System.

To determine whether altering the initial amount of cDNA in the PCR mixture affects the final A_1 and A_{2A} mRNA measurements, an equal volume of undiluted, 2× , 4×, or 8× diluted cDNA was used as the template for these PCR reactions.

3.1.3 Competitive RT-PCR data analysis

During the acquisition of gel images, special care was taken not to allow any bands to reach saturation density. The saturation of the bands can be detected by the Eagle Sight Image analysis software program. The images were saved, and band densities were determined by densiometric analysis using the Eagle Sight image analysis software (see Figure 8A).

Analysis of competitive RT-PCR images has previously been described (Piatak et al., 1993; Auboeuf and Vidal, 1997). The competitor band densities were corrected for inherent variation in band density resulting from the difference between template and competitor PCR product length. The correction factor is determined by dividing the size of the tissue template amplicon by the size of the competitor amplicon. Competitor band densities, therefore, were multiplied by the following correction factors: 1.14 (1086 bp/950 bp) for the A_1 competitor and 1.15 (441 bp/383 bp) for the A_{2A} receptor competitor. Levels of A_1 and A_{2A} receptor mRNA in each tissue sample were determined by plotting the Log [density ratio (corrected competitor band/template band)] against the Log (initial competitor concentration). Since the amount of A_1 and A_{2A} template mRNA is equal to the initial

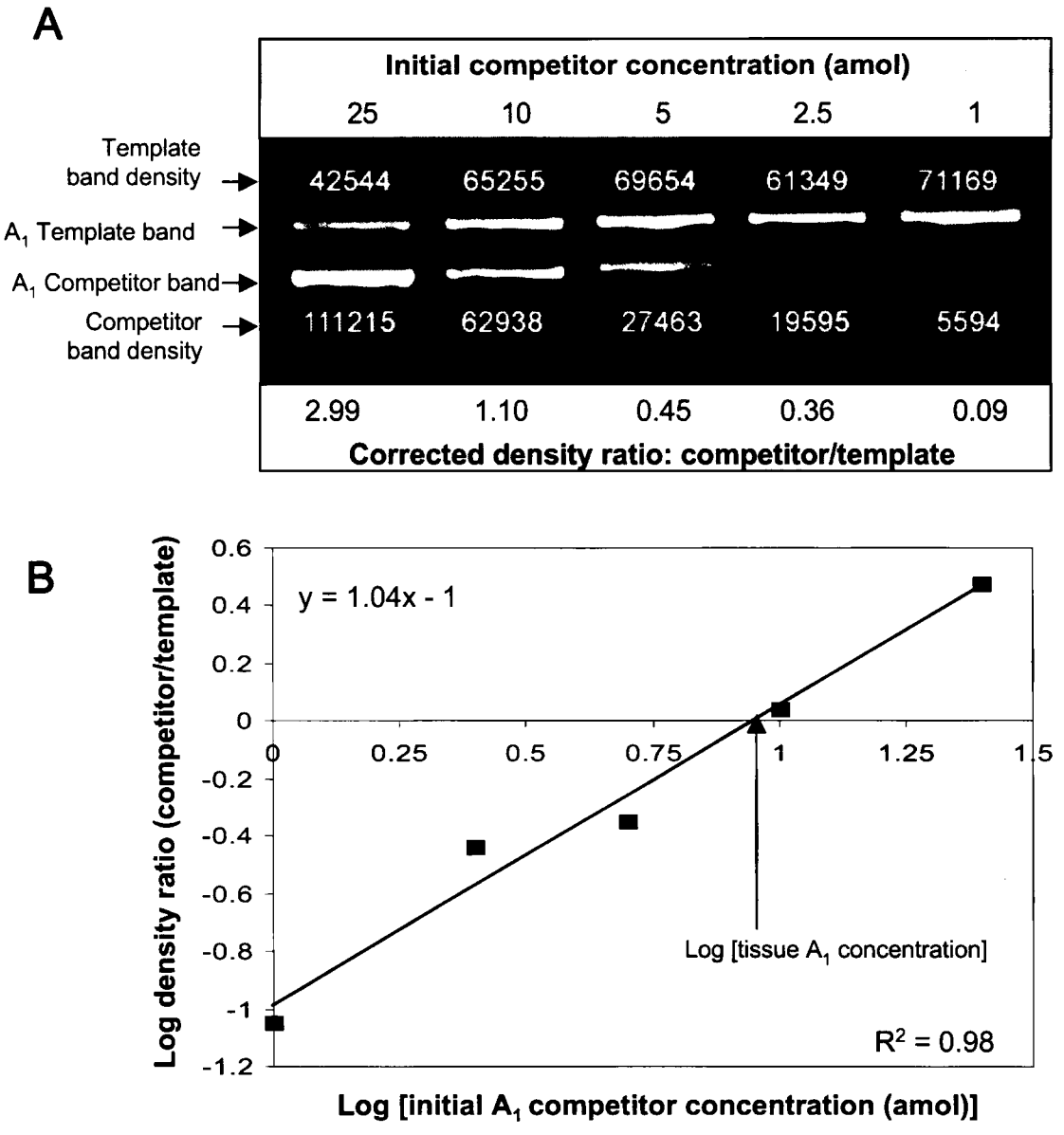


Fig. 8. Analysis of competitive RT-PCR data. A) Results of an A_1 receptor competitive RT-PCR assay performed using various amounts of A_1 competitor and a fixed amount (2.5 μg) of corpus tissue total RNA. Band densities were acquired using the Eagle Sight software. The competitor band density was multiplied by a correction factor to account for the difference in band density resulting from the smaller size of the competitor (see Methods). B) The Log density ratio (competitor band/ template band) was plotted against the Log (initial competitor concentration). The Log density ratio of the (competitor band/template band) is equal to zero when the template and corrected competitor band densities are equivalent. Thus, the A_1 receptor mRNA concentration in the tissue is determined using the x-intercept of this graph.

amount of competitor RNA when the template and corrected competitor band are of equal density, the initial amount of tissue A_1 and A_{2A} template was determined at the x-intercept (See Figure 8B). Data were expressed in units of amol of A_1 or A_{2A} receptor mRNA/μg total RNA. These values were then converted to the number of copies of A_1 or A_{2A} receptor mRNA transcript/μg total RNA. Statistical significance was determined using Student's unpaired t-test, and performed using GraphPad Prism; $P < 0.05$ was considered significant.

3.2 Quantification by Real-Time RT-PCR

The gene expression of the A_1 and A_{2A} receptor were also quantified using Real-Time RT-PCR when the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) became available. Absolute quantification of gastric A_1 and A_{2A} receptor gene expression was measured using a standard curve generated by known amounts of A_1 and A_{2A} receptor mRNA. Standard curve samples were prepared using *in vitro* transcribed A_1 or A_{2A} receptor RNA.

A two-step Real-Time PCR assay was performed. This technique is described in Fig. 9, and is based on the 5' nuclease activity of Taq DNA polymerase (Heid et al., 1996; Lockey et al., 1998). A double-labeled fluorogenic probe containing a reporter dye such as 6-carboxyfluorescein (FAM) and a quencher dye such as 6-carboxytetramethylrhodamine (TAMRA) is included in each PCR reaction. The probe hybridizes to the template at a position flanked by the forward and reverse primers. During PCR amplification, the hybridized probe is cleaved by the 5' nuclease activity of Taq DNA polymerase. When the

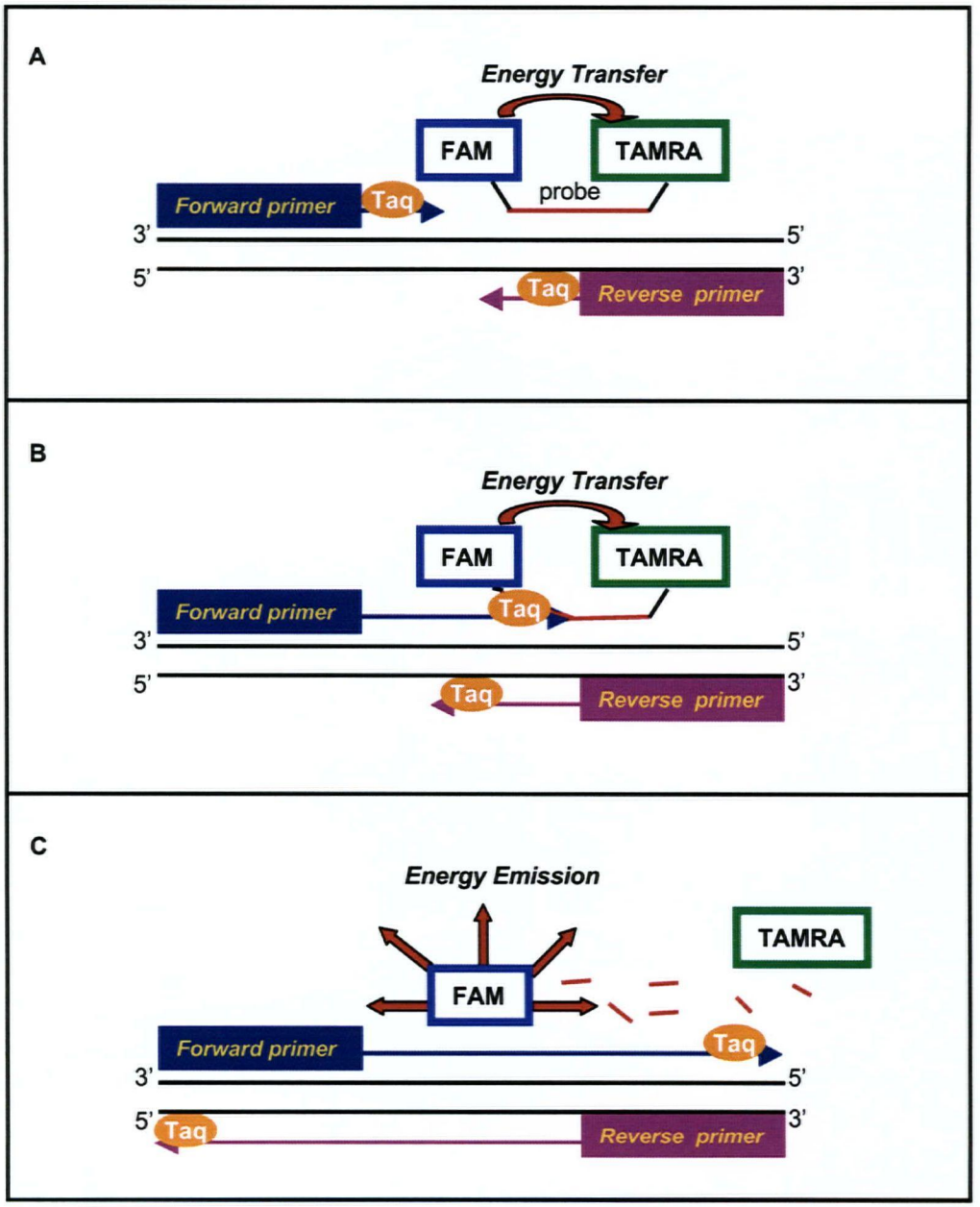


Fig. 9. Cleavage of the fluorogenic probe during Real-Time RT-PCR. During the extension phase of the Real-Time RT-PCR reaction, the primers and probes are bound to the template DNA. Taq polymerase synthesizes the new DNA strand (A), and encounters the probe (B). As Taq polymerase continues to synthesize the new strand, the 5' to 3' exonuclease activity of this enzyme degrades the probe. When the probe is intact, the energy emitted by the reporter dye, FAM is absorbed by the quencher dye, TAMRA, due to their close proximity. After the probe is degraded, FAM and TAMRA separate and the energy emitted by FAM is no longer quenched by TAMRA (C) and is detected by the ABI prism 7700 Sequence Detection System.

probe is intact, the quencher dye will suppress reporter fluorescence due to Forster type energy transfer. Upon cleavage of the probe, reporter emissions are no longer quenched and are detected by the ABI Prism Sequence Detector 7700. The methods used to synthesize the A_1 and A_{2A} receptor primers and probes are described in detail below.

3.2.1 Design and synthesis of primers and probes

Primers and probes were designed using the software program Primer Express (version 1.0, Applied Biosystems) according to the following guidelines. The guanine (G) and cytosine (C) content of the primers and probes were kept between 30-80% and ≥ 4 runs of an identical nucleotide were avoided. The melting temperatures (Tm) of the primers were kept between 58-60°C, and each primer contained ≤ 2 G/C bases at the 3' end. The Tm of the probes was 10°C higher than that of the primers. Probe sequences contained more C than G, and no G at the 5' end.

For both probes, the reporter dye, FAM was linked to the 5' end and the quencher dye, TAMRA was linked to the 3' end. The fluorogenic probes were synthesized by Synthegen, LLC (Houston, Texas) and the primers were synthesized by the NAPS Unit at UBC. The sequences of the A_1 and A_{2A} primers and probes are listed in Table 4.

3.2.2 Synthesis of A_1 and A_{2A} receptor RNA standards

RNA transcripts expressing the entire coding region of the adenosine A_1 and A_{2A} receptors were used as the standards for quantification during Real-Time RT-PCR. To synthesize these standards, plasmids containing the A_1FL and A_{2A}FL transcripts were used.

Table 4: A_1 and A_{2A} receptor Real-Time RT-PCR assay: primers and probes

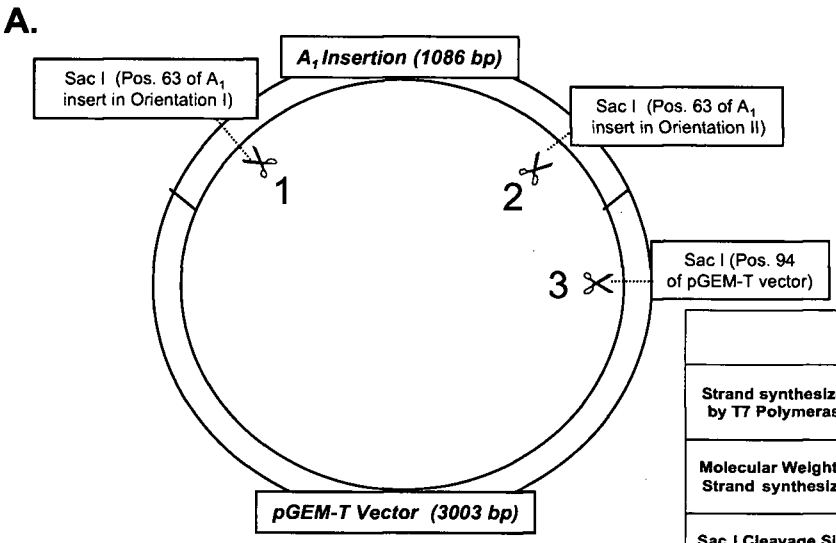
Rat A_1 Adenosine Receptor[1]					
	Position	**Length**	**Tm**	**%GC**	**Sequence**
Forward	963	22	59	59	5' CGGTGACCCCCAGAAGTACTAC 3'
Probe	989	26	70	58	5' CAGCGACTTGGCGATCTTCAGCTCCT 3'
Reverse	1039	21	59	52	5' GGGCAAAGAGGAAGAGGATGA 3'
Amplicon	962-1039	77	84	60	
Rat A_{2A} Adenosine Receptor[2]					
	Position	**Length**	**Tm**	**%GC**	**Sequence**
Forward	910	22	58	55	5' ACCCCTTCATCTACGCCTACAG 3'
Probe	936	22	69	68	5' CGGGAGTTCCGCCAGACCTTCC 3'
Reverse	978	20	59	55	5' CGTGGGTTCGGATGATCTTC 3'
Amplicon	910-978	69	84	59	

[1] Based on A_1 receptor sequence in the rat brain; Accession number: M64299

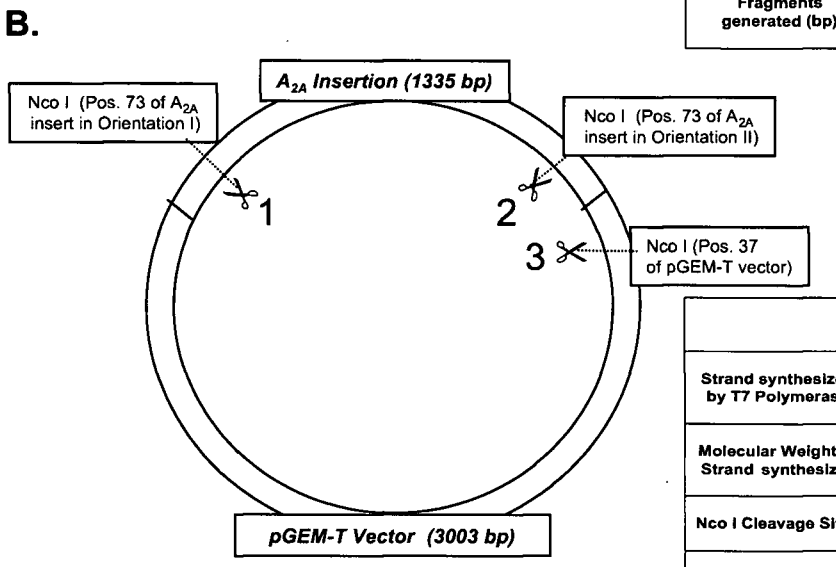
[2] Based on A_{2A} receptor sequence in the rat brain; Accession number: S47609

Plasmids were generated by our previous cloning experiments, described in section 2.2. The orientation of the adenosine A_1 and A_{2A} receptor insertions in the plasmid was determined to examine whether the 5' or 3' strand of the insertion would be synthesized by T7 polymerase during *in vitro* transcription. To determine the orientation of the A_1 and A_{2A} insertions, a Sac I digestion and Nco I digestion were performed, respectively. These restriction enzymes were chosen for the position of their cleavage sites, which allows the differentiation between the two orientations. Only the plasmids with insertions in Orientation I (see Fig. 10) were selected. In this orientation, T7 RNA polymerase synthesizes the 5' strand of the cloned inserts during *in vitro* transcription. The selected plasmids were linearized by Not I digestion. This enzyme does not cleave the A_1 or A_{2A} insert, but cleaves at a site located after the cloned insert. The linearized plasmids were purified using the Qiagen miniprep kit, as described earlier. Run-off transcripts were synthesized using the Riboprobe *in vitro* transcription kit and T7 RNA polymerase, as described previously in section III. RNA standards were treated with DNase I, and then purified using the Qiagen RNeasy mini Kit. The quality of the RNA standards was examined by gel electrophoresis, as described earlier.

The concentrations of the RNA standards were estimated spectrophotometrically, and then accurately determined using the RiboGreen Reagent Quantitation kit (Molecular Probes, Eugene, OR). Serial dilutions of the A_1 and A_{2A} RNA standards were made. RNA concentrations were determined in 1× TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.5) in DEPC-treated water] containing RiboGreen RNA quantitation reagent (0.025 µl /ml), a sensitive fluorescent nucleic acid stain. When bound to RNA, RiboGreen reagent has an



	Orientation I	Orientation II
Strand synthesized by T7 Polymerase	5' strand	3' strand
Molecular Weight of Strand synthesized	368.11 kD	373.44 kD
Sac I Cleavage Sites	1 & 3	2 & 3
Fragments generated (bp)	3270 & 818	3737 & 351



	Orientation I	Orientation II
Strand synthesized by T7 Polymerase	5' strand	3' strand
Molecular Weight of Strand synthesized	450.16 kD	453.10 kD
Nco I Cleavage Sites	1 & 3	2 & 3
Fragments generated (bp)	4291 & 47	3019 & 1319

Fig. 10. Orientation of A_1 and A_{2A} receptor transcript insertion into pGEM-T vector. Plasmids containing the A_1 (A) and A_{2A} (B) transcripts were cleaved with restriction enzymes, Sac I and Nco I, respectively. When the transcript is inserted in one orientation (orientation I), plasmids were cleaved at sites 1 and 3, and when inserted in the other orientation (orientation II), plasmids were cleaved at sites 2 and 3. The size of the fragments generated by restriction enzyme digestion are shown in the tables.

excitation wavelength of 480 nm and emission wavelength of 520 nm. The A_1 and A_{2A} RNA standards were quantified by measuring the fluorescence against a standard curve composed of samples containing known amounts of ribosomal RNA standard (16S and 23S rRNA from E. coli cells; provided by the manufacturer). The fluorescence measurements were performed using the FL600 Microplate Fluorescence reader (Biotek Inc., Winooski, VT), and RNA concentrations (ng/ml) were determined using the KC4 Kineticalc Software (version 2.6, Biotek Inc.). RNA concentrations were converted from ng/ml to copies/ml using the molecular weight of the standards: A_1 RNA standard: 368.11 kD; A_{2A} RNA standard: 450.16 kD. RNA standards were serially diluted to 10^3 - 10^{12} copies/µl in RNase-free water, aliquoted, and stored at -80°C. Standards were thawed only once prior to use.

3.2.3 Two step Real-Time RT-PCR assay

Real-Time RT-PCR was performed to measure the level of adenosine A_1 and A_{2A} receptor mRNA in the fundus, corpus, antrum, mucosa and striatum. Total RNA was extracted using Trizol® reagent, as described in section I, and quantified using the RiboGreen RNA Quantitation Kit. Total RNA was treated with DNase I. Two-step Real-Time PCR was performed using Superscript II RNase H-Reverse transcriptase, and the TaqMan PCR Core Reagents Kit (Applied Biosystems, Foster City, CA) as described below.

Step 1 - Reverse transcription: According to the manufacturer's instructions, 1 µg of DNase I-treated tissue RNA was reverse transcribed in a total volume of 10 µl containing 200 ng random hexamers, 20 U RNAguard RNase inhibitor, 1× first strand buffer, 10 mM DTT, 0.5 mM dNTP mix, and 100 U Superscript II RNase H- Reverse Transcriptase. Various amounts of RNA standard and a negative control sample containing DNase I-treated

RNase-free water in place of template, were reverse transcribed simultaneously. The latter was subsequently used as the template for the negative control during Real-Time PCR.

Step 2 - PCR: The standard curve for each Real-Time PCR assay was composed of at least 6 concentrations of either the A_1 or A_{2A} receptor standard. Each assay also contained a negative control sample containing no template. All reactions were performed in triplicate. The optimization of the A_1 and A_{2A} Real-Time RT-PCR assay conditions are described in the following section (3.2.5.). The PCR reaction consisted of a final volume of 25 µl reaction mixture containing 1× TaqMan Buffer A, 200 µM of each dATP, dCTP, and dGTP, 400 µM dUTP, 0.01 U/µl AmpErase uracil-N-glycosylase (UNG) and 0.025 U/µl AmpliTaq Gold DNA Polymerase from the TaqMan PCR Core Kit. This reaction mixture also contained 0.5 µl of tissue or standard cDNA, or 0.5 µl of the control sample (which does not contain template), 100 nM probe, various concentrations of forward and reverse primer (A_1 receptor assay contains 300 nM of each, A_{2A} receptor assay contains 100 nM of each) and $MgCl_2$ (A_1 receptor assay contains 7.5 mM, A_{2A} receptor assay contains 4.5 mM). The reaction was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems) with the following cycling parameters: a 2 min hold at 50°C for UNG incubation, a 10 min hold at 95°C for AmpliTaq Gold activation, followed by 40 cycles of amplification consisting of a 15 s denaturation step at 95°C and a 1 min anneal/extend period at 60°C.

3.2.4 Real-Time RT-PCR data collection and analysis

The data was collected by the Sequence Detection Software (version 1.6.3, Applied Biosystems) during each cycle of PCR amplification. At the end of the PCR, the data was analyzed by the software program, and an amplification plot was generated. The

amplification plot shows the normalized reporter emissions (Rn) vs. cycle number. Reporter emissions were normalized with the passive reference dye, ROX, which was present in the TaqMan Buffer A. The threshold cycle (C_T), where the software detects an increase in fluorescence associated with exponential growth, was determined using the first 15 cycles to represent the baseline. The threshold cycle (C_T) of each reaction occurred where the Rn exceeded the baseline Rn by the value of the threshold. The average Rn and standard deviation of Rn in the first 15 cycles were determined. To ensure that the change in Rn is associated with exponential growth of the amplicon during quantification, the threshold was determined by multiplying the standard deviation of the first 15 cycles by a factor of 10.

To quantify the levels of adenosine A_1 and A_{2A} receptor mRNA, standard curves of C_T vs. Log (initial A_1 or A_{2A} receptor RNA standard concentrations) were generated. The initial concentration of each unknown sample was determined by interpolation using the C_T value determined by the assay. The correlation coefficient of each standard curve was >0.95. The threshold cycle of the control samples containing no template exceeded 40 cycles in every assay, indicating the absence of DNA contamination. For the absolute quantification of adenosine A_1 and A_{2A} receptor mRNA levels, measurements were not normalized using the housekeeping genes, and the results were expressed as copies of mRNA per μg total RNA. Statistical significance was determined using GraphPad Prism and the two-tailed unpaired Student's t-test; $P \leq 0.05$ was considered significant.

3.2.5 Optimization of Real-Time RT-PCR

Real-Time RT-PCR assays were optimized by adjusting the primer, probe, and $MgCl_2$ concentrations. RT-PCR samples containing 100, 200, or 300 nM of probe and either 100,

200, or 300 nM of primer were prepared. A_1 or A_{2A} RNA (1×10^5 copies) was used as the template for these reactions. The conditions resulting in a fairly low C_T were selected for Real-Time RT-PCR. Due to the expense of the probe, the lowest acceptable concentration of this component was selected (see Table 5). Various $MgCl_2$ concentrations (3.5, 4.5, 5.5, 6.5, and 7.5 nM) were then subsequently tested using these primer and probe concentrations (see Table 6). The following optimized A_1 and A_{2A} Real-Time RT-PCR assay conditions were established. The A_1 receptor assay contained 100 nM probe, 300 nM primers, and 7.5 nM $MgCl_2$, while the A_{2A} receptor assay contained 100 nM probe, 100 nM primers, and 4.5 nM $MgCl_2$.

III. Regulation of adenosine receptor gene expression and function in altered physiological states of the stomach

The effect of fasting and omeprazole treatment on A_1 and A_{2A} receptor mRNA levels was examined to determine if changes in the physiological state of the stomach alter adenosine receptor gene expression. The gene expression of gastrin and somatostatin was also measured to determine if changes in adenosine receptor gene expression occurred with changes in gastric peptide expression. The effect of omeprazole on adenosine agonist-induced changes in IRG and SLI release was also examined to further determine if changes in adenosine receptor gene expression may result in changes in adenosine receptor function.

Table 5: Optimization of primer and probe concentrations for Real-Time RT-PCR

Probe Concentration (nM)	Primer Concentration (nM)	A_1 Assay: C_T[1]	A_{2A} Assay: C_T[1]
100	100	40.0	29.1
	200	40.0	29.4
	300	35.5	30.1
200	100	40.0	28.9
	200	35.1	29.1
	300	40.0	29.9
300	100	40.0	29.5
	200	40.0	29.2
	300	40.0	29.1

[1] The threshold cycle (C_T) was obtained using 1×10^5 copies of A_1 or A_{2A} RNA standard as the template for Real Time RT-PCR. Real-Time RT-PCR was performed as described in the methods section.

Table 6: Optimization of $MgCl_2$ concentrations for Real-Time RT-PCR

$MgCl_2$ Concentration (nM)	A_1 Assay: C_T[1]	A_{2A} Assay: C_T[1]
3.5	40.0	28.6
4.5	40.0	27.6
5.5	40.0	28.2
6.5	40.0	27.6
7.5	30.3	27.6

[1] The threshold cycle (C_T) was obtained using 1×10^5 copies of A_1 or A_{2A} RNA standard as the template for Real Time RT-PCR. Real-Time RT-PCR was performed as described in the methods section.

1. Quantification of A_1 and A_{2A} receptor, somatostatin and gastrin gene expression

1.1 Animal treatment

Fasting: Male Wistar rats (250-300g) housed in light and temperature-controled rooms with free access to water were deprived of food for 24 or 36 h. Control animals were allowed free access to both food and water. Rats were anaesthetized with Somnotol® (60 mg/kg i.p.), and tissues were extracted between 11 a.m. to 12 p.m. for all groups.

Omeprazole treatment: Male Wistar rats (250-270g) housed in light and temperature-controlled rooms with free access to food and water were used for this experiment. Omeprazole (AstraZeneca, Molndal, Sweden) was prepared for administration according to the manufacturer's instructions. Omeprazole (50 μmol/ml) was thoroughly dispersed in vehicle using the Tissumizer® tissue homogenizer. The vehicle consisted of 0.25% Methocel® (Dow, Midland, Michigan) containing 2mg/ml $NaHCO_3$ (pH 9.0). The drug was aliquoted, frozen, and stored at -20°C. The drug was thawed overnight at 4°C and brought to room temperature prior to administration. A new aliquot of drug was used daily to prevent degradation due to repeated freezing and thawing. Test groups were treated by gavage with omeprazole at a dose of 400 μmol/kg once daily between 9 and 10 a.m. for either 1 or 3 days. This omeprazole concentration and length of treatment were selected since gastric acid secretion is decreased by up to 80% after 1 day of treatment at 400 μmol/kg (Larsson et al., 1988; Lee et al., 1992), and steady state gastric acid inhibition is achieved after 3 days of treatment with the same dose (Carlsson et al., 1986). Control groups were treated similarly with vehicle. Animals were sacrificed 24 h after the last treatment of

omeprazole. Rats were anaesthetized with Somnotol® (60 mg/kg, i.p.), and tissue extraction occurred between 10 a.m. to 11 a.m.

1.2 Tissue extraction

The corporeal mucosa, corporeal muscle, and antrum, were dissected. These regions were selected because the corporeal mucosa contains somatostatin-secreting D-cells, the corporeal muscle contains somatostatin-releasing nerve fibers, and the antrum contains D-cells, gastrin-secreting G-cells, and somatostatin-releasing nerve fibers. Care was taken to avoid inclusion of tissue at the antro-corpus border. The corpus tissue was rinsed in ice-cold saline, and the mucosa was removed from the corpus with gentle scraping using a sterile glass slide. The mucosa was then rinsed off the slide with Trizol® reagent. Total RNA was extracted immediately from the corporeal mucosa as described in previous section. The corporeal muscle was flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Since the antrum is a relatively small tissue, total RNA was extracted from the whole antrum to ensure consistency between samples. The antral tissue was rinsed in sterile ice-cold saline, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

1.3 Sample preparation

Total RNA was extracted using Trizol Reagent®, and concentrations were determined using the RiboGreen Quantitation Kit, as described earlier. Total RNA samples were then treated with DNase I and reverse transcribed as described in section II (3.2).

1.4 Real-Time RT-PCR

The Real-Time RT-PCR procedure established in the present study was employed to measure adenosine A_1 and A_{2A} receptor mRNA levels. For these experiments, glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (rRNA), somatostatin and gastrin gene expression levels were also measured.

GAPDH and 18S rRNA gene expression: GAPDH mRNA and 18S rRNA levels were used as endogenous controls, and were measured using the Rodent GAPDH Control Reagents Kit (Applied Biosystems) and the TaqMan ribosomal RNA control reagents kit (Applied Biosystems), respectively. GAPDH mRNA and 18S rRNA levels were determined using the protocol described in the PE Applied Biosystems User Bulletin #2. A relative standard curve was constructed using the Rodent Control RNA Standard (50 ng/μl) provided with the Rodent GAPDH Control Reagents Kit. Rodent Control RNA (100 ng) was reverse transcribed into 10 μl of cDNA using Superscript II RNase H- Reverse Transcriptase. This sample was designated as the 10 ng/μl sample since 10 ng of Rodent control total RNA was used to produce 1 μl of cDNA. Reverse transcription was performed as described in section II (3.2). The 10 ng/μl sample was diluted to 7.5, 5, 2.5, 1, 0.5, and 0.1 ng/μl, and these samples were used to construct the relative standard curve for the Real-Time RT-PCR assay. The 18S and GAPDH Real-Time RT-PCR assays were performed according to the manufacturer's instruction. Data were collected and analyzed as described in section II (3.2.4). Since fasting did not affect 18S rRNA levels, this value was subsequently used to normalize A_1 receptor, A_{2A} receptor, somatostatin and gastrin gene expression for possible differences arising from variations in total RNA extraction efficiency, first strand cDNA synthesis efficiency, and total RNA and cDNA degradation.

Gastrin and somatostatin gene expression: Relative gastrin and somatostatin gene expression levels were measured using a sample of rat antrum total RNA to produce the standard curve. Antral total RNA (1 μg) was reverse transcribed into 10 μl of cDNA using Superscript II RNase H- Reverse Transcriptase. This sample was designated as the 100 ng/μl sample since 100 ng total RNA was reverse transcribed to produce 1 μl of cDNA. This sample was diluted to various concentrations to produce a relative standard curve. Standard sample concentrations ranged from 0.1 ng/μl to 100 ng/μl

The primers and probes used to measure somatostatin and gastrin gene expression were designed using Primer Express and are listed in Table 7. The same guidelines which were used to design the A_1 and A_{2A} receptor primers and probes were used here (see Section 3.2.1). The primers and probes were synthesized by the NAPS Unit at UBC, and by Synthegen, respectively. Gastrin and somatostatin mRNA levels were measured by performing a two-step Real-Time RT-PCR assay as described in section II (3.2.3). The primer, probe and $MgCl_2$ concentrations used for these assays were optimized according to the method described in section II (3.2.5). For both assays, the primer and probe concentrations were found to be 100 nM, and the $MgCl_2$ concentration was found to be 7.5 mM.

1.5 Data collection and analysis

Data were collected as described in section 3.2.4. To compare gene expression levels between the test and control animals, measurements were first normalized using the endogenous housekeeping-gene (18S rRNA) expression levels. This was performed according to the procedures described in the Applied Biosystems User bulletin #2: The

Table 7: Gastrin and somatostatin Real-Time RT-PCR assay: primers and probes

Rat Gastrin[1]					
	Position	**Length**	**Tm**	**%GC**	**Sequence**
Forward	6	22	61	50	5'TGCCACAACAGTTAACCGTTCC3'
Probe	80	28	69	57	5'TGCTGGCTCTAGCTACCTTCTCGGAAGC3'
Reverse	129	20	61	60	5'CTGGGAGCGAGGTTTCCAAG3'
Amplicon	6-129	124	82	54	
Rat Somatostatin[2]					
	Position	**Length**	**Tm**	**%GC**	**Sequence**
Forward	234	21	59	52	5'TGAGCCCAACCAGACAGAGAA3'
Probe	260	23	69	65	5'CCCTGGAGCCTGAGGATTTGCCC3'
Reverse	308	21	58	57	5'CTCATCTCGTCCTGCTCAGCT3'
Amplicon	234-308	75	84	60	

[1] Based on rat gastrin mRNA sequence; Accession number: M38653

[2] Based on rat somatostatin mRNA sequence; Accession number: M25890

adenosine A_1 or A_{2A} receptor, gastrin or somatostatin gene expression levels for each individual sample were divided by the housekeeping-gene expression level to obtain a normalized value of measurement (M_N). The mean ± S.E.M. of M_N for the fed control and fasted test group were calculated. The mean of each control group was then expressed as 100%, while the gene expression levels of the test groups were expressed as a percentage of controls. Statistical significance was determined using the two-tailed unpaired Student's t-test, where $P \leq 0.05$ is considered significant.

2. Effect of omeprazole on A_1 and A_{2A} receptor-induced changes in SLI and IRG release

Male Wistar rats (250-275g) were treated with omeprazole as described in the previous section. Rats were deprived of food for 12 h but had free access to water prior to stomach perfusion. Stomach perfusion was performed 24 h after the last drug treatment. Animals were anaesthetized with Somnotol (60mg/kg), and stomachs were prepared for perfusion as decribed in Section I (1).

The A_1 receptor agonist CPA and A_{2A} receptor agonist CGS 21680 were administered by side arm infusion at a rate to give a final concentration of 0.1 or 1 μM. These concentrations were selected because CPA has been shown to significantly inhibit and stimulate gastric SLI release when it is administered at a concentration of 0.1 and 1 μM, respectively. Both concentrations of CPA have also been shown to inhibit IRG release. The administration of CGS 21680 has been shown to significantly stimulate gastric SLI release at a concentration of 0.1 μM and to maximally stimulate SLI release at a concentration of 1 μM (see results). The effect of omeprazole treatment on CPA induced changes in SLI and IRG

release and CGS-induced changes in SLI release was examined. Samples were collected and stored as described in section I (2). Radioimmunoassay was performed to measure SLI and IRG release as described on section I (3 & 4).

The release of SLI and IRG were both expressed as the mean ± SEM for each 5 min sample. SLI and IRG release (%), and the % change in release was performed as described in Section I, 5. Statistical significance was determined using the paired Student's t-test, where $P \leq 0.05$ is considered significant. Single-factor ANOVA for repeated measures followed by Dunnett's t-test was also performed using GraphPad Prism.

IV. Cellular localization of the gastric A_1 and A_{2A} receptors

1. Tissue preparation for immunohistochemistry (IHC)

The methods used for IHC have previously been described (Yip et al., 2003). Four male Wistar rats (225-250 g) were anaesthetized with Somnotol (60 mg/kg i.p.). Stomachs were dissected out, cut along the greater curvature, and thoroughly rinsed with ice cold saline. Tissue specimens were immersion fixed in freshly prepared 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 30 min (A_{2A} receptor IHC) or overnight (A_1 receptor IHC) at 4°C. This was followed by cryoprotection in 0.1 M phosphate buffer containing 20% sucrose for 12 h. The corpus (excluding the region adjacent to the fundus and antrum) and the antrum were mounted on corks, embedded in O.C.T. compound (Miles, Etobicoke, ON) and frozen in isopentane (-60°C) for 1 min. Cryostat sections of 30 μm thickness were cut at -25°C and placed in 0.1M phosphate buffered saline (PBS) containing 0.1% sodium azide.

Random tissue sections were placed in 0.1 M PBS (pH 7.4) and stained as floating sections to enhance the penetration of antisera into these sections. Prior to the application of primary antibody, sections were sequentially incubated in 0.1 M PBS containing 50 mM NH_4Cl for 30 min, 0.1 M PBS containing 100 mM glycine for 10 min, and 0.1 M PBS containing 1% BSA and 0.3% Triton X-100 for 1 h.

Primary and secondary antibodies were diluted in 0.1 M PBS containing 1% BSA, 0.3% Triton X-100, and 0.1% sodium azide. Sections were incubated in adenosine A_1 or A_{2A} receptor primary antibody for 72 h at 4°C, washed in 0.1 M PBS (3 × 15 min), and incubated with cyanine Cy3-conjugated secondary antibody overnight at 4°C. Sections were again washed in 0.1 M PBS (3 × 15 min) and either double stained or mounted onto glass slides.

For double staining, tissue sections were incubated in one of the other primary antibodies for 72 h at 4°C, washed in 0.1 M PBS (3 × 15 min) and incubated with Alexa Fluor® 488-conjugated secondary antibody overnight at 4°C. Sections were again washed in 0.1 M PBS (3 × 15 min) and mounted onto glass slides. Coverslips were placed on sections using a mixture of 0.1 M PBS in glycerin (1:9) and sealed with nail polish.

2. Adenosine receptor antibodies and control peptides

The rabbit anti-A_1 adenosine receptor antibody was purchased from Sigma-Aldrich and was developed using amino acids 309-326 of the rat A_1 adenosine receptor C-domain as the immunogen. This antibody has been shown to cross-react strongly with the rat A_1 receptor (Nakata, 1993; Ciruela et al., 1995; Rivkees et al., 1995). The specificity of this antibody in rat tissues has been examined by Western blotting (Carruthers et al., 2001). When samples were deglycosylated, this antibody detected only one band of the expected

size (38-40k Da). In addition, pre-absorption of this antibody with the A_1 receptor peptide resulted in a lack of immunoreactivity (Caruthers, 2001). The A_1 receptor antibody was used at a dilution of 1:500 and immunostaining was detected using Donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoReasearch, West Grove, PA) at a concentration of 1:2000.

Since an anti-rat A_{2A} receptor antibody was unavailable, a rabbit anti-canine A_{2A} receptor antibody was used. This antibody was purchased from Alpha Diagnostic International (San Antonio, TX) and was generated using an immunogen corresponding to a 30 amino acid sequence in the 4^{th} intracellular domain of the canine A_{2A} receptor. This region has 43% homology with the rat A_{2A} receptor and has been shown to cross-react with the rat adenosine A_{2A} receptors (Diniz et al., 2003). Its specificity has previously been established (Nie et al., 1999; Christofi et al., 2001). This antibody was used at a dilution of 1:200 and immunostaining was detected using donkey anti-rabbit IgG conjugated to Cy3 at a concentration of 1:2000.

Since the blocking peptide for the A_1 receptor antibody was not available from Sigma-Aldrich, one was custom made by the NAPS Unit at UBC. This synthetic peptide was based on the following immunogen sequence provided by Sigma-Aldrich: Cys-Gln-Pro-Lys-Pro-Pro-Ile-Asp-Glu-Asp-Leu-Pro-Glu-Glu-Lys-Ala-Glu-Asp. The blocking peptide was re-dissolved in 0.1 M PBS (pH 7.4), and the concentration was determined using the BCA assay (Pierce, Rockford, IL). The blocking peptide for the A_{2A} receptor antibody was obtained from Alpha Diagnostic International. Preabsorption of the A_{2A} receptor primary antibody with this control peptide has been shown to abolish A_{2A} receptor labeling (Christofi et al., 2001).

3. Antibodies used for double-staining

To examine whether the adenosine A_1 and A_{2A} receptors are localized on specific cell types, double staining was performed to identify somatostatin, gastrin, H^+K^+-ATPase β, von Willebrand's factor (VWF), and protein gene product (PGP) 9.5. The secondary antibody, donkey anti-mouse IgG conjugated to Alexa Fluor® 488 (1:2000, Molecular Probes, Eugene, OR) was used to detect this staining. Somatostatin-IR was used to identify D-cells of the corporeal and antral mucosa and somatostatin-IR nerves within the muscle layers and enteric plexi. Somatostatin was stained using the somatostatin antibody (SOMA 8; 1:500) provided by the MRC regulatory peptide group (UBC). G-cells of the antral mucosa were identified by gastrin staining using the gastrin antibody (109-21; 1: 30,000) provided by the late Dr. John Walsh. Both these antibodies have been used to identify G-cells (Pederson et al., 1984) and D-cells (Buchan et al., 1985b) in the rat stomach. The parietal cells were identified by staining the β-subunit of the proton pump (H^+K^+-ATPase). The H^+K^+-ATPase β antibody was purchased from Affinity Bioreagents Inc. (Golden, CO) and used at a concentration of 1:2000, as suggested by the manufacturer. To identify vascular endothelial cells lining blood vessels in the stomach, VWF was stained. This protein is synthesized by endothelial cells and is stored in the Weibel-Palade bodies of the endothelial cells (Schmugge et al., 2003). The VWF antibody was used at a dilution of 1:100 and was purchased from Serotec (Oxford, England). PGP 9.5 staining was used as a neuronal marker. This protein is expressed in the cytoplasm of central and peripheral neurons (Schofield et al., 1995), and has previously been used to study the innervation of the gastrointestinal tract (Yip et al., 2003). The PGP 9.5 antibody was used at a dilution of 1:200 and was purchased from Abcam Limited (Cambridge, UK). The specificity of these antibodies have previously been characterized

(Wall et al., 1980; Buchan et al., 1985b; Vincent et al., 1985; Wilson et al., 1988; Chow and Forte, 1993).

4. Confocal microscopy

A_1 receptor immunoreactivity (A_1R-IR) and A_{2A} receptor immunoreactivity (A_{2A}R-IR) were viewed using the Biorad Radiance 2000 confocal scanning laser system mounted on a Nikon Eclipse TE300 inverted microscope. The system utilizes a krypton gas laser with an excitation wavelength of 568 nm and emission filter 575-625 nm (for visualization of Cy3), and an excitation wavelength of 488 and emission filter 500-530 nm (for visualization of Alexa 488). Lens magnification of ×40 was used with a zoom factor of 1.0 and z-step of 0.5 to 1.0 μm, while lens magnification of ×60 was used with a zoom factor of 1.0-1.6 and a z-step of 0.3 to 0.5 μm. The software program Lasersharp 2000 (version 4.1, Biorad) was used to scan tissues sequentially using the red and green collection channels and the Kalman collection filter (n=2). The Kalman collection filter averages the images collected to increase the signal to noise ratio. Images of 512×512 pixels were obtained and analyzed using NIH image (National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop (version 7.0, Adobe Systems Inc.). To determine whether co-localization occurs, a single optical image collected from the red channel (A_1R-IR or A_{2A}R-IR) was overlaid on the corresponding single optical image collected from the green channel (somatostatin, gastrin, VWF, PGP, H^+K^+-ATPase, VIP or CGRP-IR) using Adobe Photoshop.

5. Quantification of co-localization

Co-localization of A_1R-IR and A_{2A}R-IR with somatostatin-IR and gastrin-IR was quantified in the antral and corporeal mucosa by examining at least 3 tissue sections from 4 different animals. For quantification of A_1R-IR with gastrin-IR, at least 12 fields of view were analyzed. A_1R-IR was significantly more abundant than A_{2A}R-IR. The corporeal and antral mucosa contained only 1.1±0.2 and 1.3±0.2 A_{2A}R-IR cells per view, respectively. Thus, a total of at least 55 fields of view were analyzed for quantification of A_{2A}R-IR with somatostatin-IR.

6. Control studies

Immunoneutralization experiments were performed to confirm antibody specificity. The A_1R antibody was neutralized by incubating 1µl of the stock antibody (Sigma-Aldrich) with 0.1 µg of the blocking peptide in 0.5 ml 0.1 M PBS containing 1% BSA, 0.3% Triton X-100, and 0.1% sodium azide for 2 nights at 4°C. As suggested by the manufacturer, the A_{2A}R antibody was neutralized by incubating 1 µg of antibody with 40 µg of control peptide in 0.2 ml of 0.1 M PBS containing 1% BSA, 0.3% Triton X-100, and 0.1% sodium azide for 2 nights at 4°C. A positive control lacking the control peptide was also prepared for both antibodies. Tissues stained with the neutralized antibody did not demonstrate A_1R-IR or A_{2A}R-IR, while tissues stained with the positive control demonstrated extensive A_1R-IR and A_{2A}R-IR.

Additional control experiments were also performed to ensure that non-specific binding did not occur. These included sections which were processed as follows: 1) incubation in 1% BSA in place of the primary antibody, 2) incubation in adenosine A_1 or A_{2A}

receptor primary antibody without secondary antibody, and 3) incubation in adenosine A_1 or A_{2A} primary antibody followed by Alexa 488-conjugated anti-mouse IgG secondary antibody. No immunostaining was observed following these procedures. To ensure that bleed-through did not occur, single-stained sections were examined under the microscope using both filters. Bleed-through was not detected for any of the antibodies used.

Chapter 3: Results

I. Effect of adenosine on gastric IRG and SLI release

1. Effect of adenosine analogs on gastric IRG release

The effect of CPA (A_1-selective agonist), CGS 21680 (A_{2A}-selective agonist), and IB-MECA (A_3-selective agonist) on the inhibition of IRG release from the isolated vascularly perfused stomach was examined. The basal release of IRG during periods 1-3 remained relatively constant for all experiments. The basal IRG release (periods 1 to 3) ranged from 114±19 to 117±19pg/min (Fig. 11). When 1 μM CPA was administered, gastric IRG release was inhibited immediately upon drug perfusion (Fig. 11). The inhibition was apparent throughout the drug perfusion period, and the release of IRG returned to basal levels within 5 min after drug removal. The effect of various concentrations of CPA on IRG release was also examined. Results are expressed in % inhibition and are summarized in Fig. 12. CPA caused a concentration-dependent inhibition of IRG release. Maximal inhibition was achieved at a concentration of 1 μM. The administration of 10 μM CPA did not further suppress IRG release. The empirical EC_{50} of CPA in inhibiting IRG release was estimated to be 0.067 μM, with a confidence interval between 0.014 and 0.325 μM. The administration of CGS 21680 (0.1 μM) and IB-MECA (0.1 μM) did not alter basal IRG release.

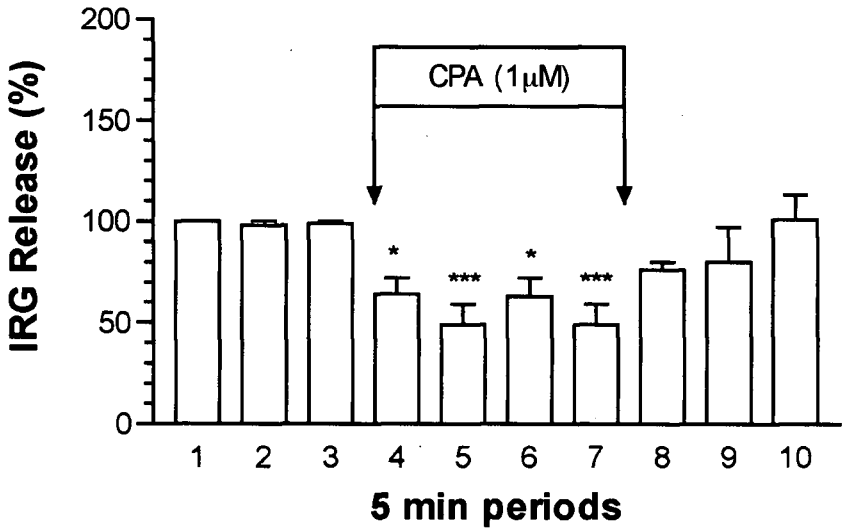


Fig. 11. Effect of CPA on gastric IRG release. Results are expressed as IRG release (%) as described in the Methods section. Each column represents the mean ± SEM of 5 experiments. *P < 0.05 and ***P < 0.001 when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test.

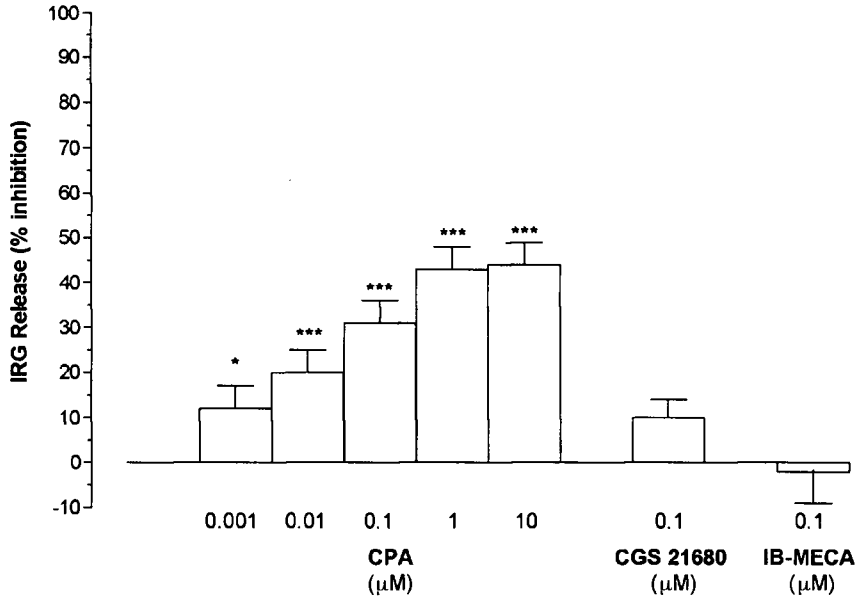


Fig. 12. Effect of various concentrations of CPA, and 0.1 μM of CGS 21680 and IB-MECA on IRG release. Results are expressed as the % inhibition of IRG release, and calculated as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments; $^{*}P < 0.05$ and $^{***}P < 0.001$ when the mean IRG release (pg/min) during drug perfusion (periods 4-7) is compared to the mean basal IRG release (periods 1-3).

2. Effect of adenosine receptor antagonists on gastric IRG release

Adenosine has been shown to inhibit gastric IRG release (Kwok et al., 1990). To determine the receptor subtype involved in this adenosine-mediated action, the effect of the A_1 receptor-selective antagonist, DPCPX, and the A_2 receptor-selective antagonists, DMPX, on adenosine-induced inhibition of IRG release were examined. Neither antagonists altered basal IRG release (Fig. 13). Fig. 14 shows the effect of DPCPX and DMPX on adenosine-induced changes in IRG release. After a 15 min basal period, 1 μM of the antagonist was administered alone for 5 min prior to the concomitant perfusion with adenosine (10 μM) for 15 min. In the presence of 1 μM DPCPX, the adenosine-induced inhibition of IRG release was completely abolished. DMPX (1 μM), however, had no effect on adenosine-induced changes in IRG release.

3. Effect of adenosine receptor agonists on gastric SLI release

To determine which adenosine receptor subtype is involved in regulating gastric SLI release, the effect of CPA (A_1-selective agonist), CGS 21680 (A_{2A}-selective agonist), and IB-MECA (A_3-selective agonist) were examined. The basal release of SLI during periods 1-3 remained relatively constant for all experiments. In experiments examining the effect of agonists (Fig. 15 and 16), the rates of basal SLI release (period 1-3) were 174±24 to178±27 pg/min (CPA 0.1 μM), 173±19 to 180±21 pg/min (CPA 1 μM), 92±18 to 95±18 pg/min (CGS 21680) and 191±39 to 195±41 pg/min (IB-MECA). When administered at a concentration of 0.1 μM, CPA immediately inhibited gastric SLI release (Fig. 15A). The inhibition continued throughout the drug perfusion period, and SLI release returned to basal

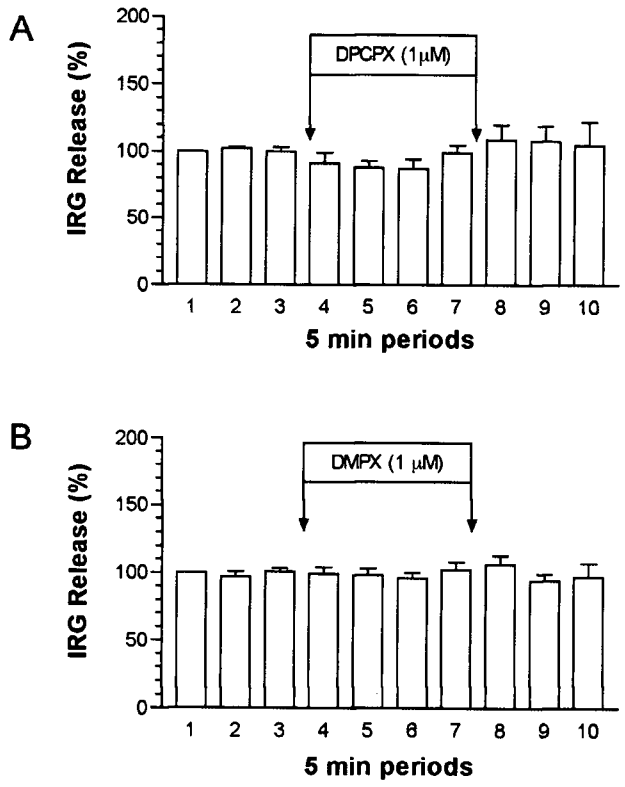


Fig. 13. Effect of DPCPX and DMPX on gastric IRG release. Each column represents the mean ± SEM of at least 5 experiments. Statistics were performed using repeated measures ANOVA followed by Dunnett's multiple comparison test. Neither drug altered basal IRG release.

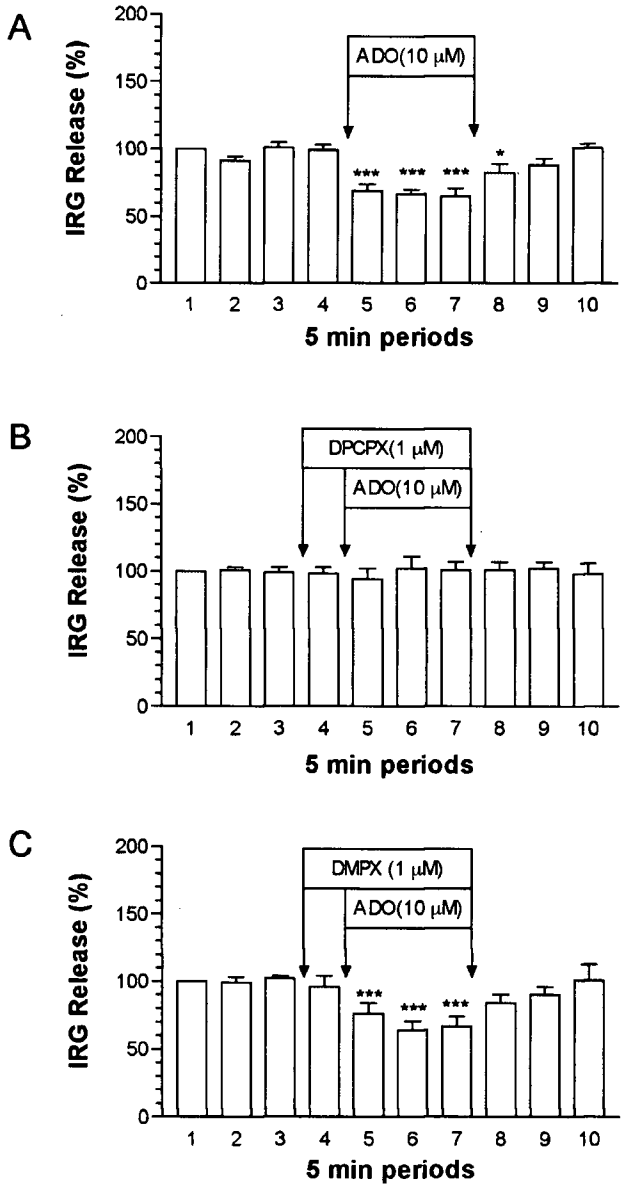


Fig. 14. Effect of DPCPX and DMPX on adenosine-induced changes in IRG release. A) Effect of adenosine on IRG release; B) Effect of DPCPX on adenosine-induced changes in IRG release; C) Effect of DMPX on adenosine-induced changes in IRG release. Results are expressed as IRG release (%) as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments. *$P < 0.05$ and ***$P < 0.001$ when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test.

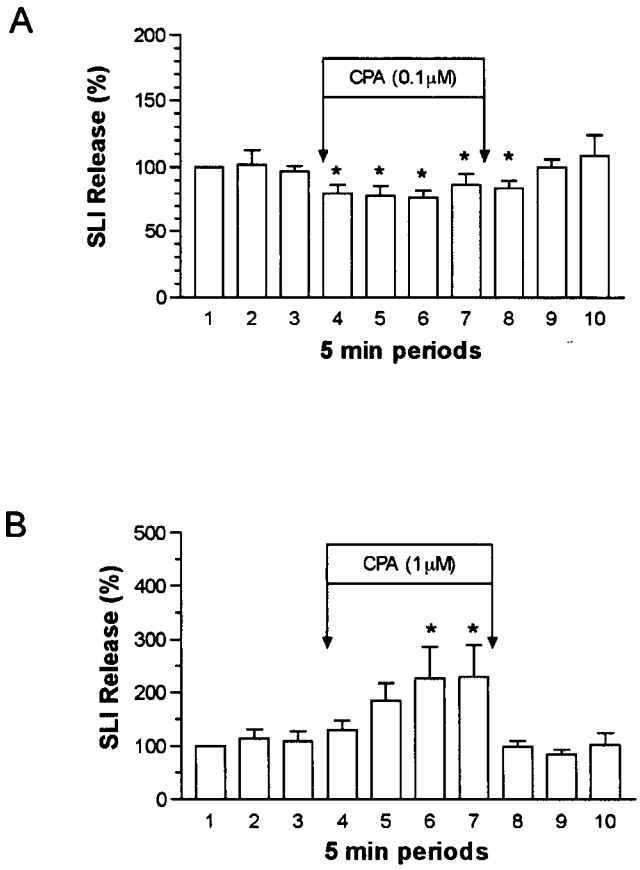


Fig. 15. Effect of 0.1 μM (A) and 1 μM (B) CPA on gastric SLI release. Results are expressed as SLI release (%) as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments. *P < 0.05 when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test.

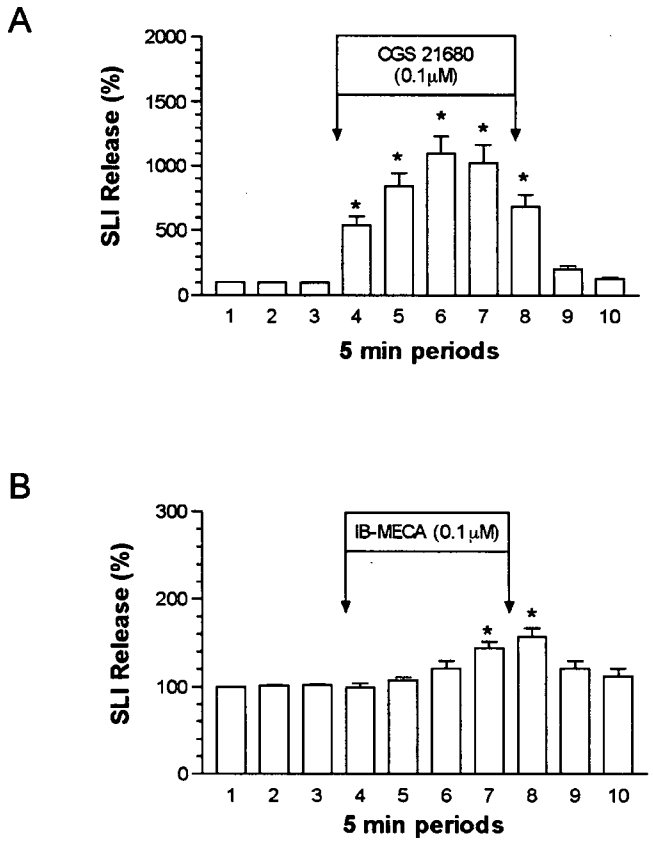


Fig. 16. Effect of 0.1 μM CGS 21680 (A) and IB-MECA (B) on gastric SLI release. Results are expressed as SLI release (%) as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments. *P < 0.05 when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test. Note the difference in the scale of the ordinate.

levels 10 min after the drug was removed. Conversely, when CPA was administered at a concentration of 1 μM, an increase in SLI release was observed 15 min after the start of drug perfusion. SLI release returned to basal levels within 5 min after the cessation of CPA perfusion (Fig. 15B). The administration of 0.1 μM CGS 21680 caused a significant increase in SLI release immediately upon its administration (Fig. 16A). The release of SLI continued to gradually increase throughout the drug perfusion period, and reached a plateau at period 6 (1100±135 % of basal SLI release). SLI release returned to basal levels 10 min after the drug was removed. IB-MECA (0.1 μM) did not significantly affect SLI release during the first 15 min of drug administration (Fig. 16B), but did significantly increase gastric SLI release to 144 ±8% after 15 min of perfusion. SLI release returned to basal levels 10 min after the withdrawal of drug administration.

To examine the concentration dependent effect of CPA, CGS, and IB-MECA in stimulating SLI release, stomachs were perfused with 1 nM to 10 μM of these drugs. Results are expressed as percentage changes of SLI release and shown in Fig. 17. CGS 21680 was the most potent adenosine agonist in stimulating SLI release. CGS 21680 significantly increased SLI release starting at the lowest concentration examined (10 nM; 238±38%). Maximal augmentation was observed at 1 μM (1237±120%). The effect elicited by 1 μM and 10 μM of CGS 21680 were not significantly different. Both IB-MECA and CPA were less potent than CGS 21680. Although higher concentrations (1 and 10 μM) of CPA and IB-MECA enhanced SLI release, lower concentrations of CPA (10 nM and 0.1 μM) significantly inhibited SLI release ($P < 0.05$). The % changes of SLI release in the presence of 10 nM and 0.1 μM CPA were -12±3 and -19±5%, respectively. Although IB-MECA may alter SLI release (%) in individual samples (4-7) during drug perfusion (Fig. 16B), it did not

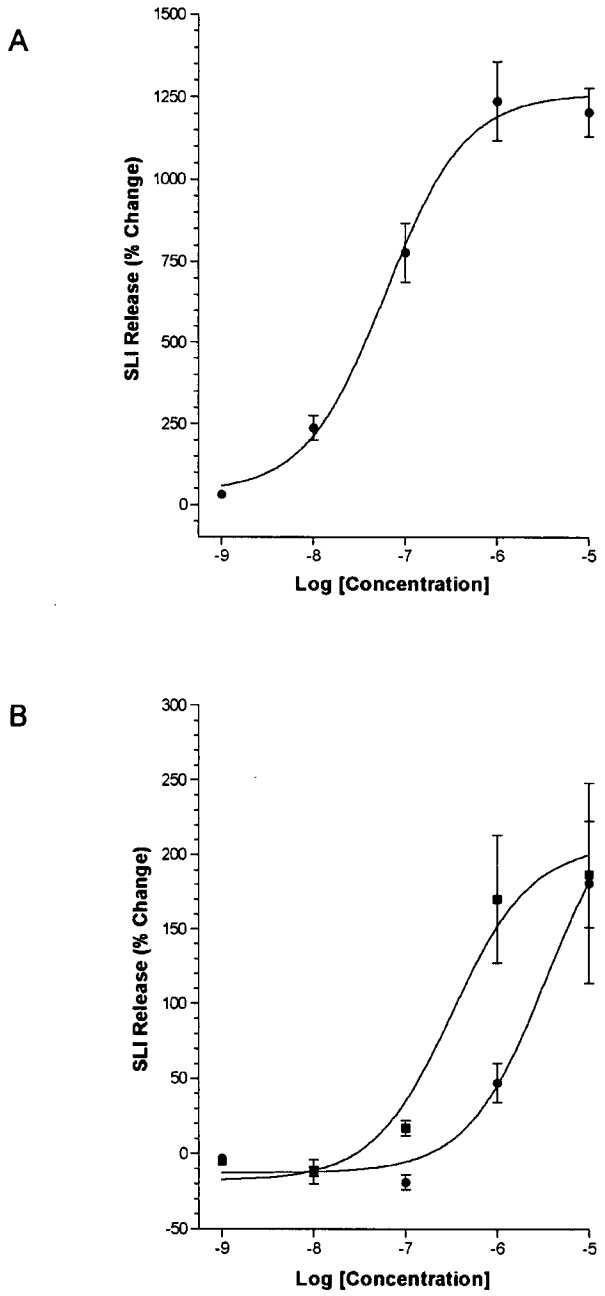


Fig. 17. Effect of various concentrations of adenosine agonists on gastric SLI release. A: The effect of CGS 21680 on SLI release. B: The effect of CPA (●) and IB-MECA (■) on SLI release. Results are expressed as percentage (%) changes and calculated as described in the Methods section. Each point represents the mean ± SEM of at least 5 experiments. Note the difference in the scale of the ordinate.

significantly alter SLI release (% change) when perfused at concentrations from 1 nM to 0.1 μM.

The EC_{50} of CGS 21680 in stimulating SLI release was estimated to be 0.06 μM, with a 95% confidence interval between 0.02 and 0.17 μM. The empirical EC_{50} of CPA and IB-MECA were estimated to be 3.5 μM (95% confidence interval: 0.5 μM to 23 μM) and 0.3 μM (95% confidence interval: 0.02 μM and 4 μM), respectively. The rank order of potency of these analogs in augmenting SLI release, therefore, was CGS 21680 > IB-MECA > CPA.

4. Effect of adenosine receptor antagonists on gastric SLI release

The effect of DMPX, an A_2 receptor-selective antagonist was studied to examine the role of A_2 receptors in adenosine and adenosine agonist-induced gastric SLI release. When perfused through the gastric vasculature for 20 min, 1 μM DMPX did not alter basal SLI release, while 10 μM DMPX significantly suppressed SLI release. Experiments similar to those described for Fig. 14 were then performed to test the effect of DMPX on adenosine and adenosine receptor agonist-induced release of SLI; 1 or 10 μM DMPX was administered 5 min prior to the concomitant perfusion with the agonist for 15 min. Results are summarized in Fig. 18. In the presence of 1 μM DMPX, adenosine induced-SLI release was attenuated. DMPX (1 μM) also inhibited the response elicited by CGS 21680 (0.01 and 0.1 μM) and IB-MECA (1μM), and completely abolished the effects of CPA (1 μM) on gastric SLI release. CGS 21680-induced release of SLI was significantly suppressed by DMPX in a concentration-dependent manner. The % change of SLI release induced by 0.01 μM CGS 21680 in the presence of 10 μM DMPX was not significantly different from the basal SLI release.

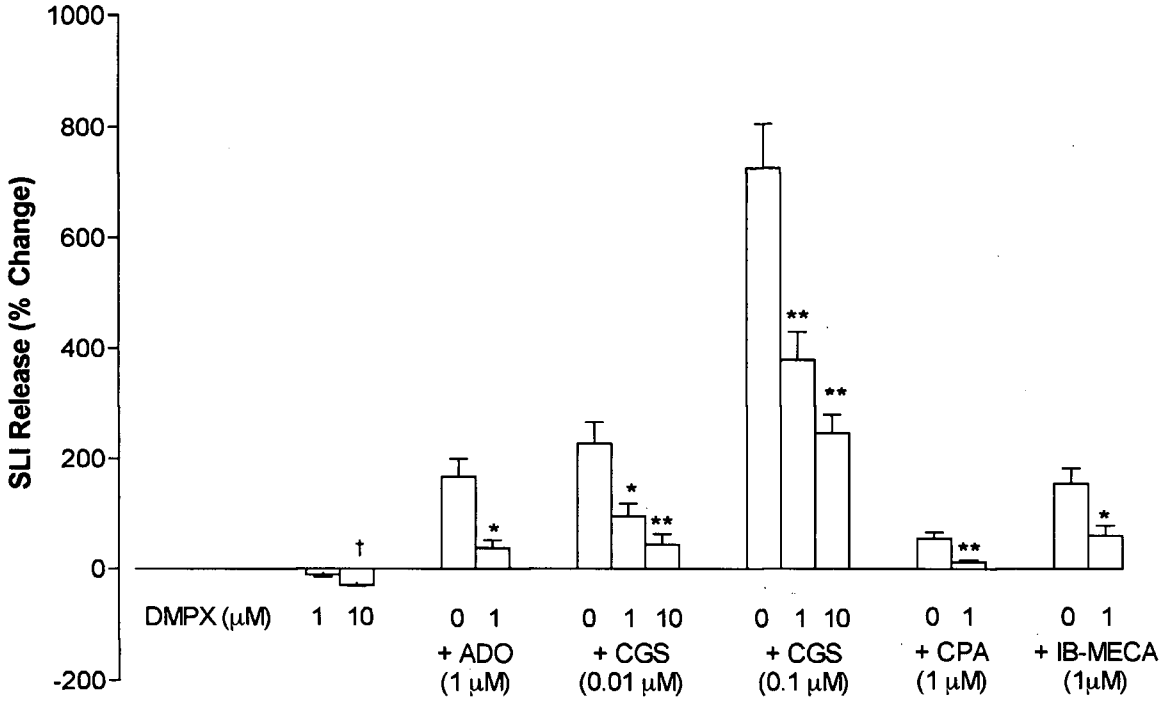


Fig. 18. Effect of DMPX on basal, adenosine, and adenosine agonist-stimulated SLI release. The effect of DMPX on CPA-, CGS 21680- (CGS), and IB-MECA-induced SLI release was examined. Results are expressed as % changes. Each column represents the mean ± SEM of at least 5 experiments; $^{\dagger}P < 0.05$ when the mean SLI release (pg/min) in the presence of DMPX (periods 4-7) is compared to the mean basal SLI release (periods 1-3), $*P < 0.05$ and $**P < 0.01$ when the mean release of SLI (periods 4-7) in the presence of agonists was compared with the mean release of SLI in the presence of both DMPX and agonists.

To test if the stimulatory effect on SLI release is mediated by the A_{2A} receptor, the effect of the potent selective A_{2A} receptor antagonist, ZM 241385, was examined. When perfused alone, ZM 241385 (1 μM) significantly inhibited basal SLI release during period 5 and 7 (Fig. 19A). However, when the % change of SLI release was calculated, SLI release in the presence of 1 μM ZM 241385 did not differ significantly from basal SLI release (Fig. 20). ZM 241385 abolished the stimulatory effect of adenosine; in the presence of ZM 241385 and adenosine, the release of SLI was inhibited (Fig. 20). When perfused concomitantly with CGS 21680 (0.1 μM), ZM 241385 (1 μM) also abolished the stimulatory effect of CGS 21680, and a significant inhibition of SLI release was apparent (Fig. 19). The effect of 10 μM ZM 241385 on CGS 21680 (0.1 μM) were similar to that of 1 μM ZM 241385. The stimulatory effect of CPA (1μM) and IB-MECA (1 μM) on SLI release was also completely abolished by ZM 241385. In the presence of both the agonist and antagonist, an inhibition of SLI was apparent (Fig. 20A). The effect of the A_1 selective antagonist DPCPX was also examined. DPCPX (1 μM) significantly inhibited basal SLI release, but had no effect on CGS 21680- (0.1 μM) stimulated SLI release (Fig. 20).

5. Involvement of endogenous adenosine on SLI release

To examine the role of endogenous adenosine in modulating SLI release, experiments were also performed to test the effect of EHNA, an ADA inhibitor, on gastric SLI release using the same experimental protocol described in Fig. 13. EHNA (1-10 μM) caused a concentration-dependent increase in SLI release, while 0.1 μM EHNA had no effect (Fig. 21). The stimulatory effect of adenosine on SLI release was potentiated in the presence of EHNA.

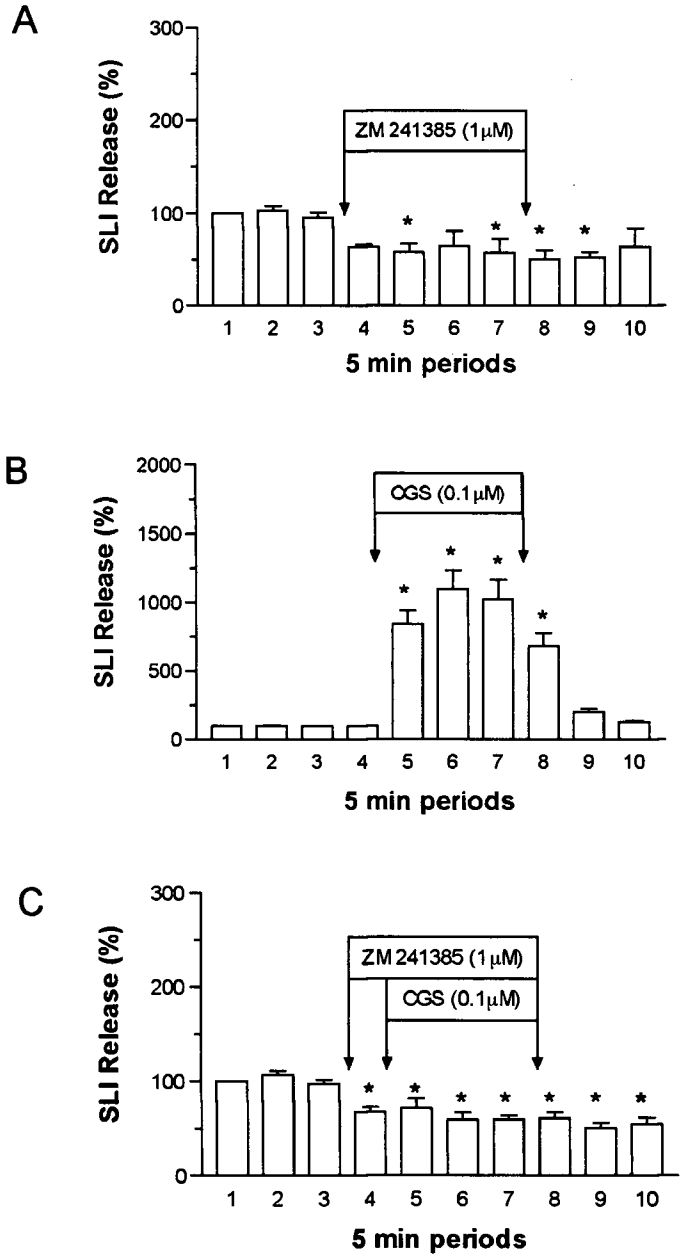


Fig. 19. Effect of ZM 241385 on basal and stimulated SLI release. A: The effect of ZM 241385 on basal SLI release; B: The effect of CGS 21680 (CGS) on basal SLI release; C: The effect of ZM 241385 on CGS-stimulated SLI release. Each column represents the mean ± SEM of at least 4 experiments; *$P < 0.05$ when compared with period 3 of respective experiments using repeated measures ANOVA followed by Dunnett's multiple comparison test.

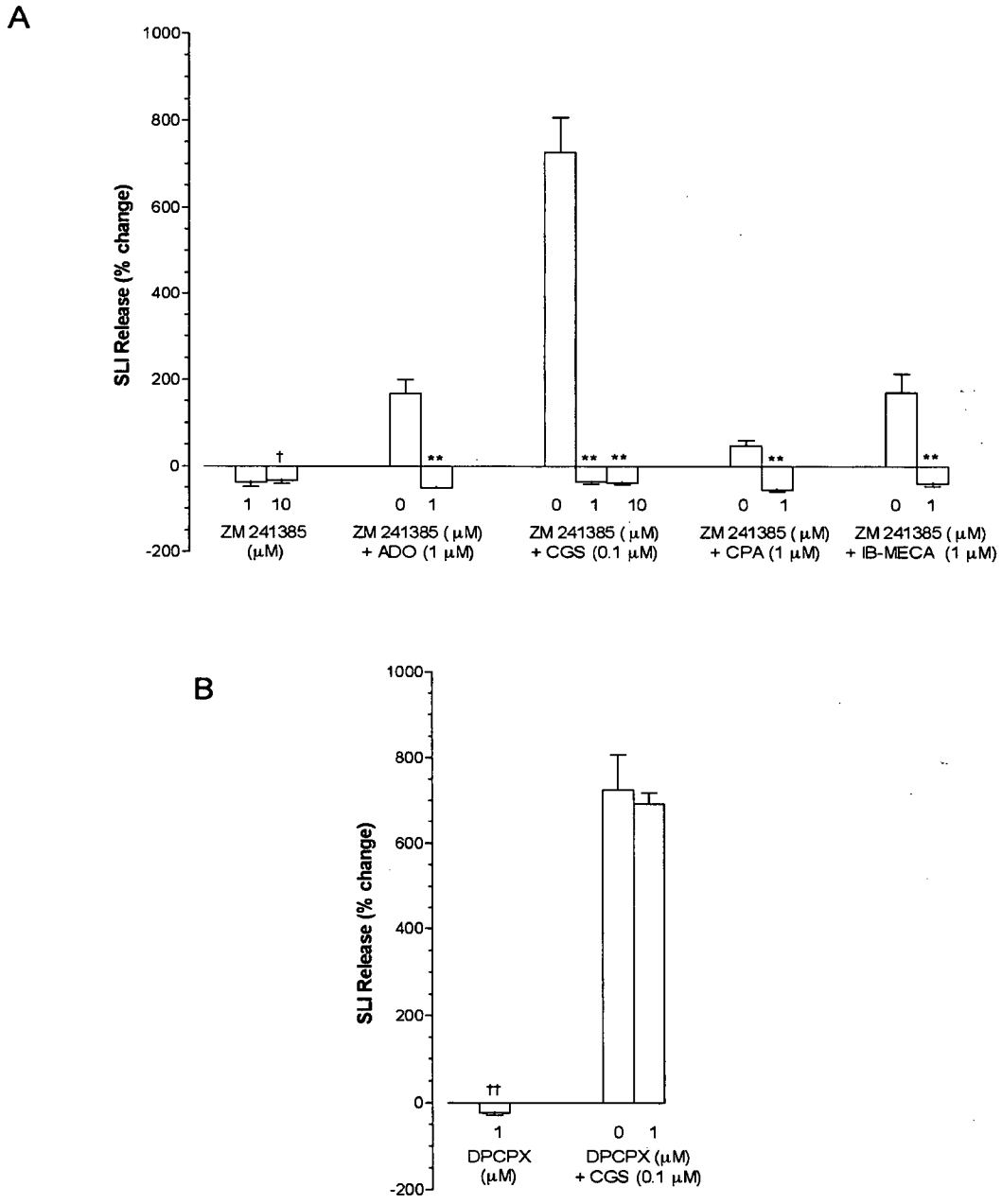


Fig. 20. Effect of ZM 241385 (A) and DPCPX (B) on basal and adenosine (ADO) and agonist-stimulated SLI release. The effect of ZM 241385 on CPA-, CGS 21680- (CGS), and IB-MECA-induced SLI release was examined. Results are expressed as % changes. Each column represents the mean ± SEM of at least 4 experiments; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ when the mean SLI release (pg/min) in the presence of ZM 241385 or DPCPX (periods 4-7) was compared to the mean basal SLI release (periods 1-3), $^{**}P < 0.01$ when the mean release of SLI (periods 4-7) in the presence of agonists was compared with the mean release of SLI in the presence of both ZM 241385 and agonists.

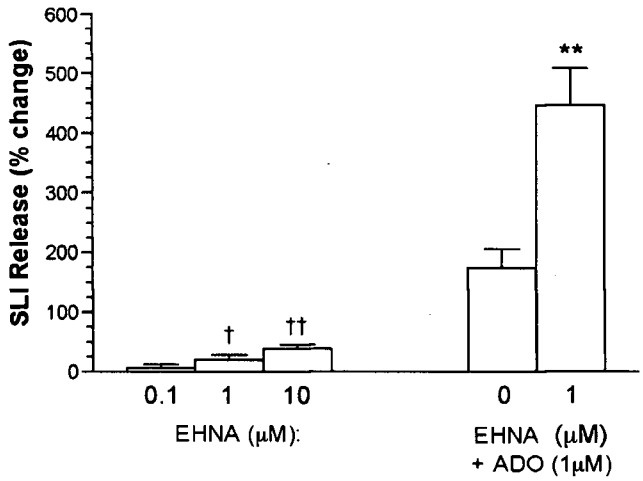


Fig. 21. Effect of EHNA on basal and adenosine (ADO)-induced SLI release. Results are expressed as % changes of SLI release, and each column represents the mean ± SEM of at least 6 experiments; †$P < 0.05$ and ††$P < 0.01$ when comparing the mean SLI release (pg/min) in the presence of EHNA during periods 4-7 with that of periods 1-3 (basal release), **$P < 0.01$ when compared with the ADO-induced release of SLI.

II. Distribution and sequence analysis of adenosine receptor mRNA

1. Distribution of adenosine receptor mRNA in various regions of the stomach

The regional distribution of all four gastric adenosine receptors was examined using RT-PCR. Results obtained from these experiments demonstrate that the mRNAs of all four adenosine receptors are present in every gastric region examined, including the fundus, corpus, antrum, and mucosa (Fig. 22). The mRNA of these receptors was also detected in the rat striatum, which was used as a positive control. Each PCR amplicon was of the expected length (A_1: 1086 bp; A_{2A}: 441 bp; A_{2B} 380 bp; A_3; 489 bp). Since adenosine A_1 and A_{2A} receptors were shown to play a role in the control of gastric regulatory peptide release, these receptors were examined in more detail. RT-PCR was therefore performed using primers which spanned the entire coding region of the rat A_1 and A_{2A} receptor gene. Results demonstrate that alternative splicing does not occur in any region of the stomach since only one specific A_1 and A_{2A} receptor transcript was detected in all gastric regions studied (Fig. 23). The specificity of the RT-PCR reactions was confirmed by restriction enzyme digestion of the RT-PCR amplicons (Fig. 24-28). The observed fragment sizes shown on these figures are estimates based on the migration distance of the fragment. Small fragments of less than 150 bp were often not visible by gel electrophoresis due to the low intensities of their fluorescence under UV light.

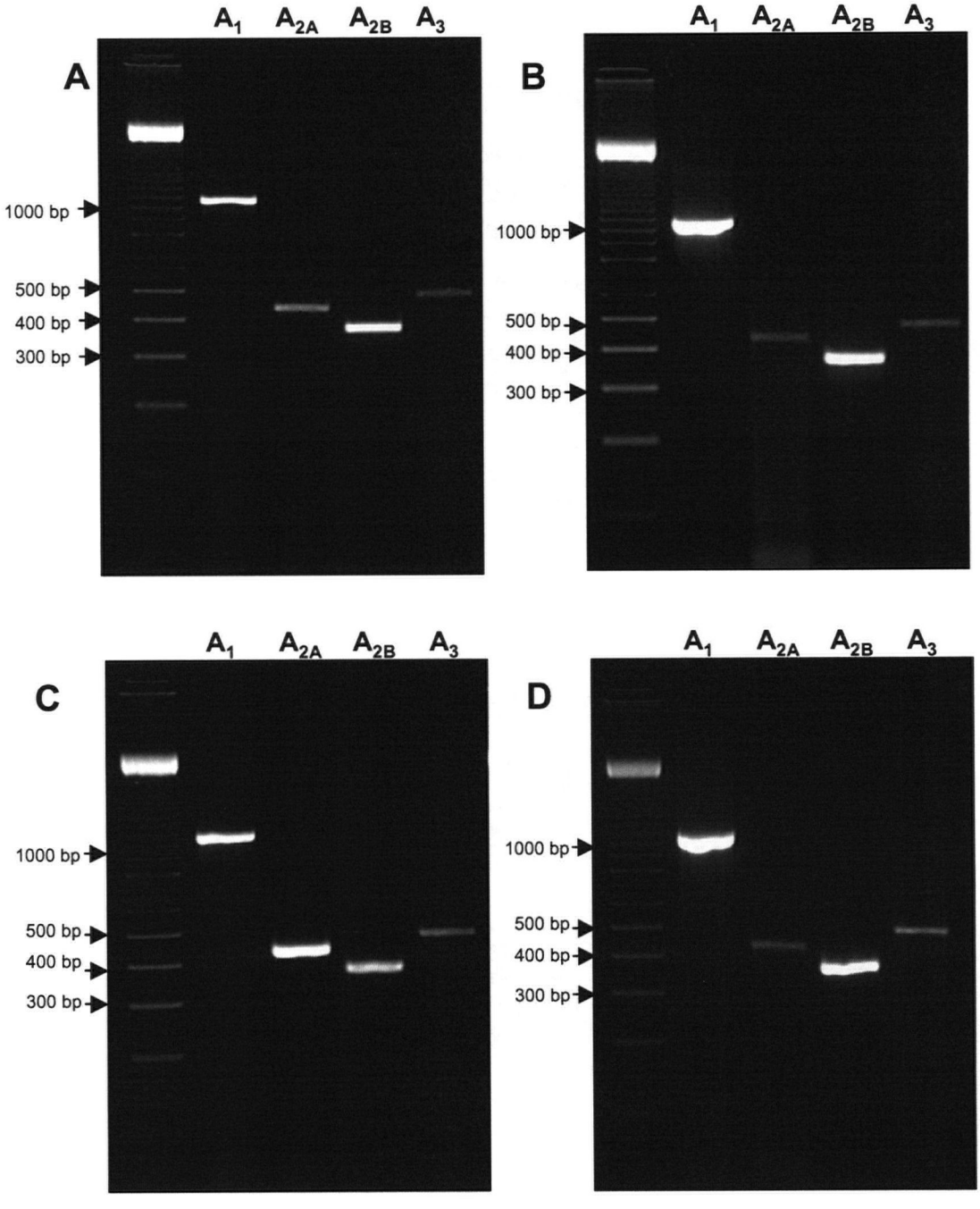


Fig. 22. Results of A_1, A_{2A}, A_{2B} and A_3 RT-PCR performed using rat gastric tissues. RT-PCR was performed using tissues from the rat fundus (A), corpus (B), antrum (C) and mucosa (D) and primers designed to detect the A_1 (1086 bp), A_{2A} (441 bp), A_{2B} (380 bp), and A_3 (489 bp) receptor mRNA (see Table 3). All four adenosine receptor subtypes are present in the gastric tissues examined.

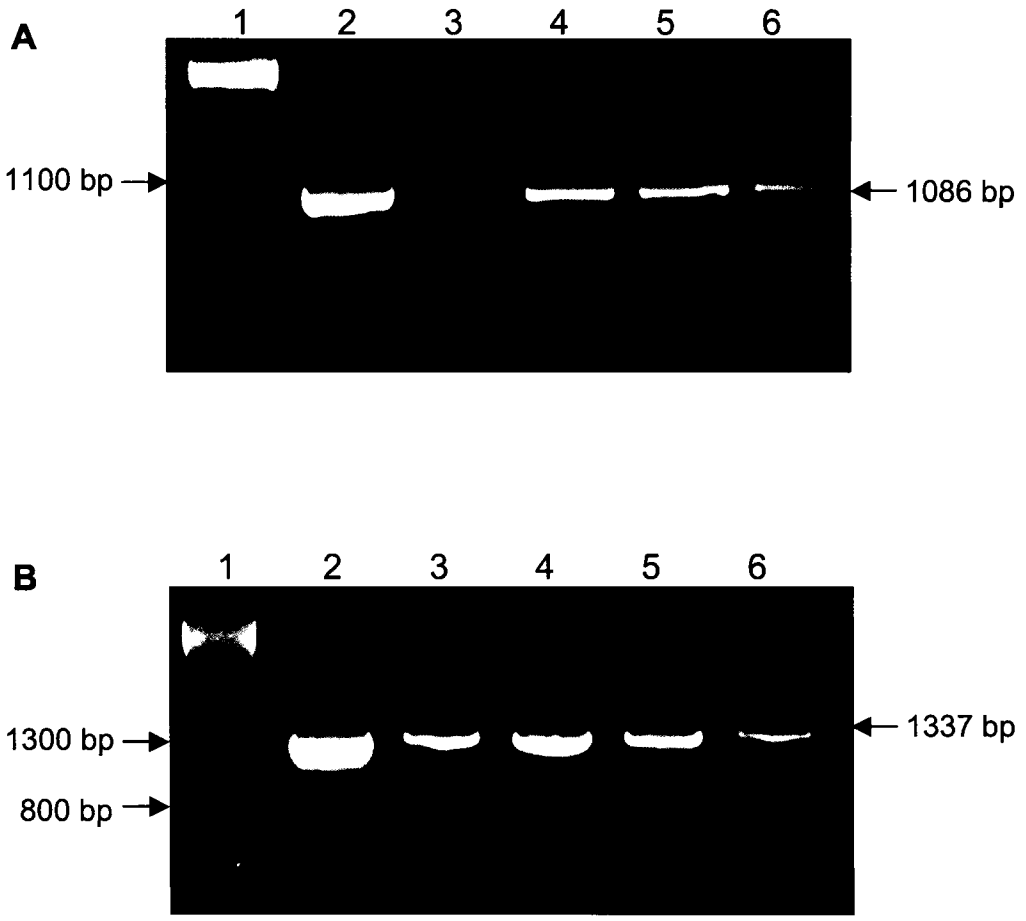
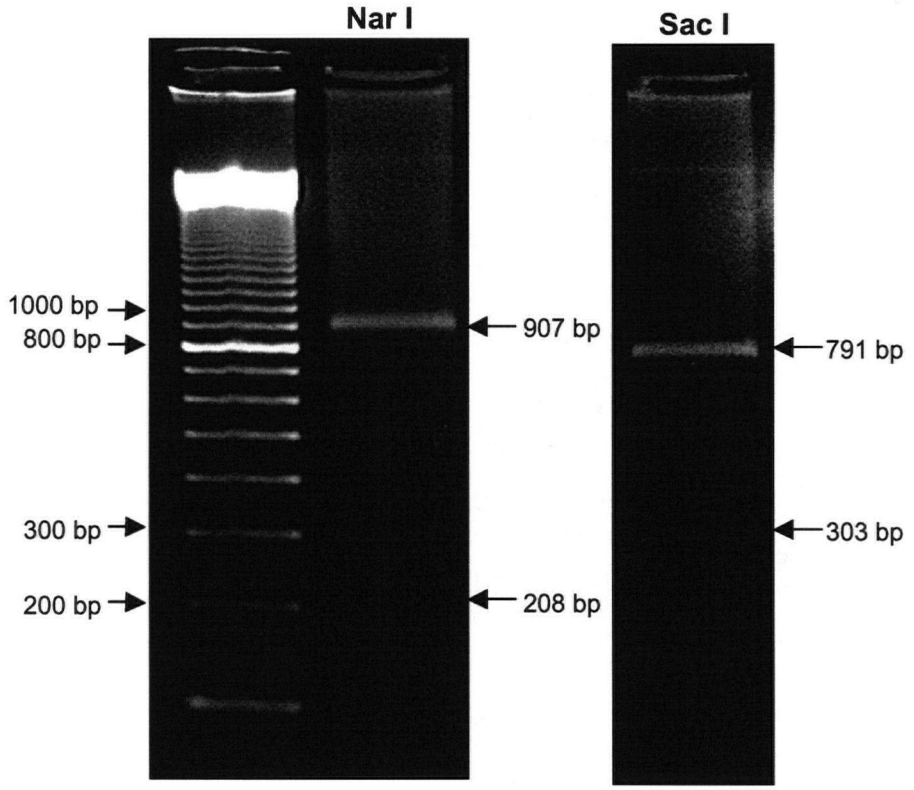


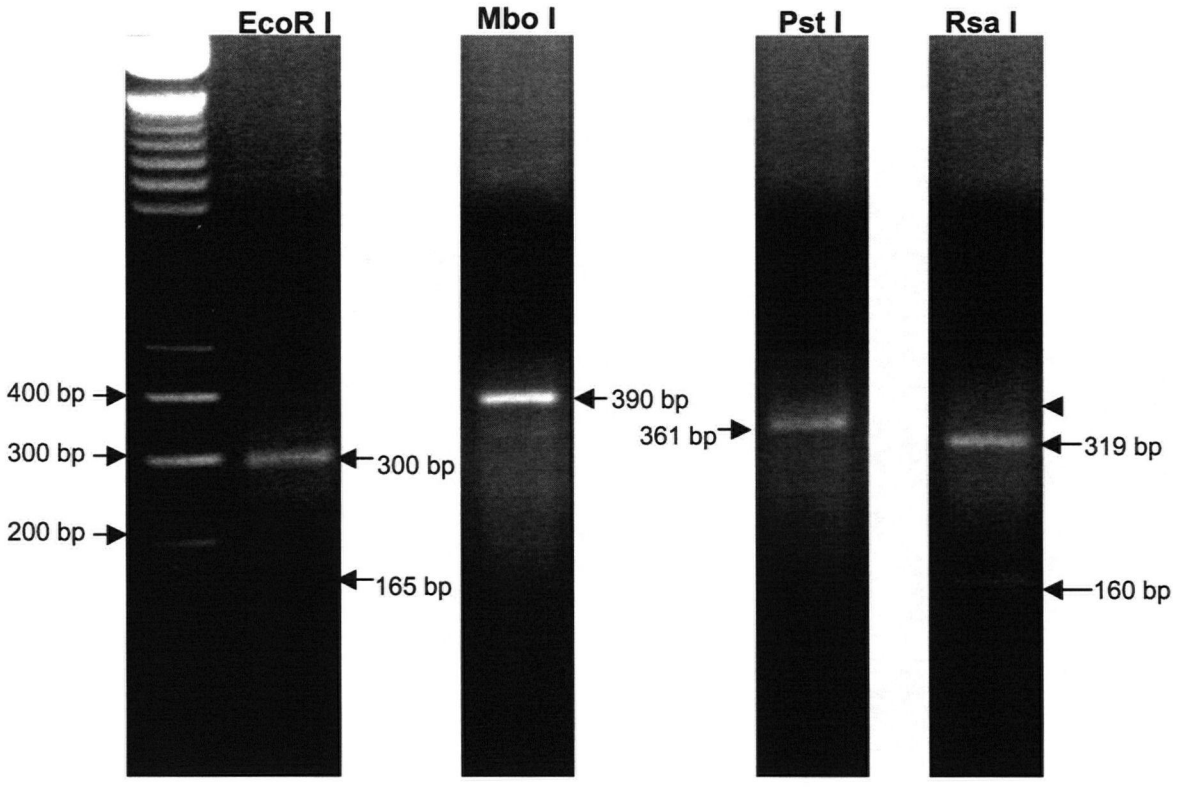
Fig. 23. Amplification of the A_1 (A) and A_{2A} (B) receptor coding region by RT-PCR. RT-PCR was performed using tissues obtained from the striatum (positive control; lane 2), fundus (lane 3), corpus (lane 4), antrum (lane 5) and mucosa (lane 6). The A_1FL and A_{2A}FL primer sets (see Table 3) used span the entire coding region of the rat adenosine A_1 and A_{2A} receptor and generated only one amplicon, of the expected length, for each tissue examined.



Uncleaved size & region amplified*	Enzyme	Recognition site	Cleavage site	Expected fragments (bp)	Observed fragments (bp)
1086 bp (322-1407 bp)	Nar I	GG/CGCC	527	880; 206	907; 208
	Sac I	GAGCT/C	631	776; 310	791; 303

*Positions based on A_1 receptor sequence in the rat brain (Accession No. M64299)

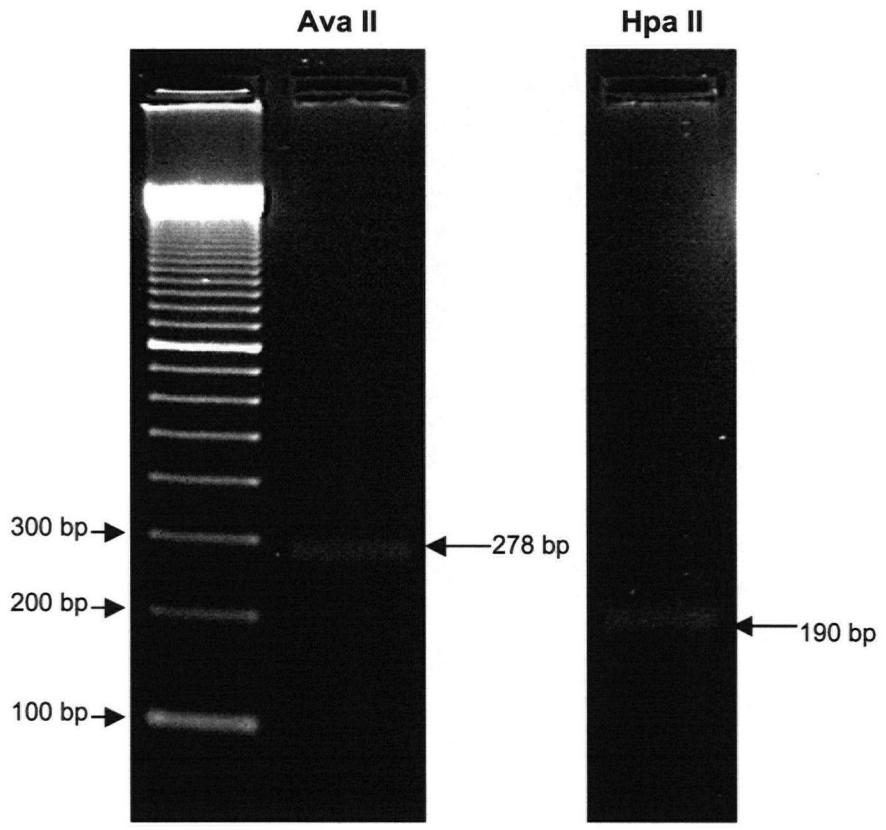
Fig. 24. Restriction enzyme digestion of the adenosine A_1 receptor PCR amplicon. RT-PCR products synthesized using the A_1FL primer set were concentrated, purified, and cleaved with restriction enzymes, Nar I and Sac I, and separated on a 2% agarose gel. Expected fragment sizes are listed in the table. The size of the observed fragments were estimated using the Stratagene Eagle Sight Image Analysis Software, as described in the Methods.



Uncleaved size & region amplified*	Enzyme	Recognition site	Cleavage site	Expected fragments (bp)	Observed fragments (bp)
441bp from 99-449 bp	EcoR I	G/AATTC	385	286; 155	300; 165
	Mbo I	/GATC	160	380; 61	390
	Pst I	CTGCA/G	178	362; 79	361
	Rsa I	GT/AC	399	300; 141	319; 160

*Positions based on A_{2A} receptor sequence in the rat brain (Accession No. S47609)

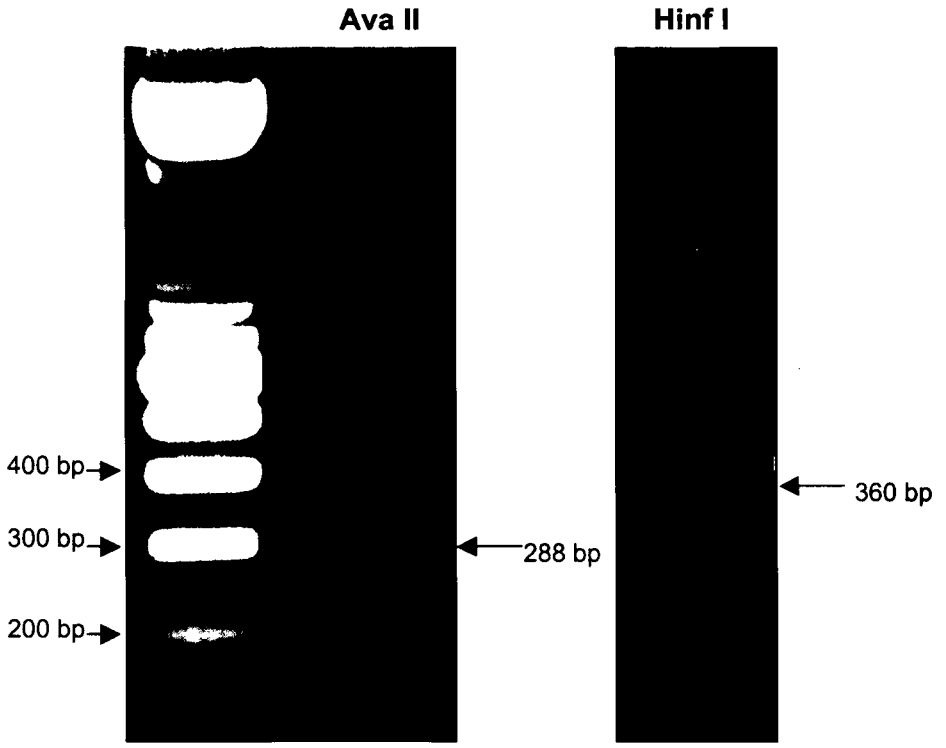
Fig. 25. Restriction enzyme digestion of the adenosine A_{2A} receptor PCR amplicon. RT-PCR products synthesized using the A_{2A}SL primer set were concentrated, purified, and cleaved with restriction enzymes, EcoR I, Mbo, I, Pst I, and Rsa I. Fragments were separated on a 2% agarose gel. Expected fragment sizes are listed in the table. The sizes of the observed fragments were estimated using the Stratagene Eagle Sight Image Analysis Software, as described in the Methods.



Uncleaved size & region amplified*	Enzyme	Recognition site	Cleavage site	Expected fragments (bp)	Observed fragments (bp)
380 bp from 223-603 bp	Ava II	G/G(A or T)CC	479	256; 124	278
	Hpa II	C/CGG	413	190; 190	190

*Positions based on A_{2B} receptor sequence in the rat brain (Accession No. M91466)

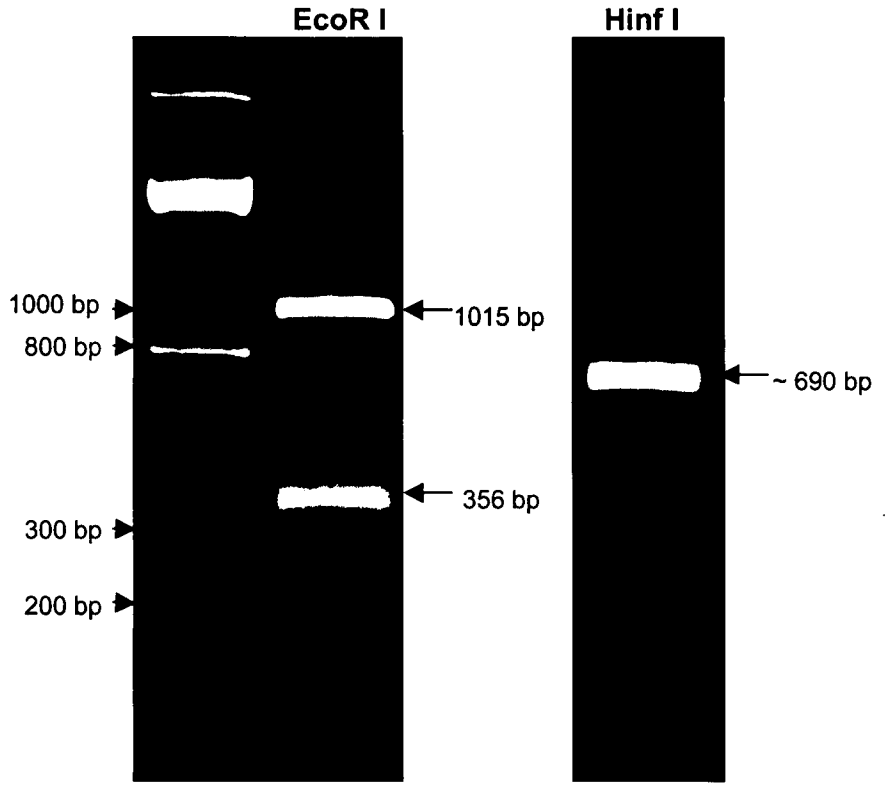
Fig. 26. Restriction enzyme digestion of the adenosine A_{2B} receptor PCR amplicon. RT-PCR products synthesized using the A_{2B}SL primer set were concentrated, purified, and cleaved with restriction enzymes, Ava II and Hpa II. Fragments were separated on a 2% agarose gel. Expected fragment sizes are listed in the table. The sizes of the observed fragments were estimated using the Stratagene Eagle Sight Image Analysis Software, as described in the Methods.



Uncleaved size & region amplified*	Enzyme	Recognition site	Cleavage site	Expected fragments (bp)	Observed fragments (bp)
489 bp from 319-788 bp	Ava II	G/G(A or T)CC	452; 546	133; 262; 94	288
	Hinf I	G/A(ACG or T)TC	653	334; 155	360

*Positions based on A_3 receptor sequence in the rat brain (Accession No. M94152)

Fig. 27. Restriction enzyme digestion of the adenosine A_3 receptor PCR amplicon. RT-PCR products synthesized using the A_3SL primer set were concentrated, purified, and cleaved with restriction enzymes, Ava II and Hinf I. Fragments were separated on a 1% agarose gel. Expected fragment sizes are listed in the table. The sizes of the observed fragments were estimated using the Stratagene Eagle Sight Image Analysis Software, as described in the Methods.



Uncleaved size & region amplified*	Enzyme	Recognition site	Cleavage site	Expected fragments (bp)	Observed fragments (bp)
1337 bp from 41-1377 bp	EcoR I	G/AATTC	385	992; 345	1015; 356
	Hinf I	G/A(ACG or T)TC	719	658; 679	~690

*Positions based on A_{2A} receptor sequence in the rat brain (Accession No. S47609)

Fig. 28. Restriction enzyme digestion of the full length adenosine A_{2A} receptor amplicon. RT-PCR products synthesized using the A_{2A}FL primer set were concentrated, purified, and cleaved with restriction enzymes, EcoR I and Hinf I. Fragments were separated on a 2% agarose gel. Expected fragment sizes are listed in the table. The sizes of the observed fragments were estimated using the Stratagene Eagle Sight Image Analysis Software, as described in the Methods.

2. A_1 and A_{2A} receptor coding region and A_{2B} and A_3 receptor partial cDNA sequence

Experiments were performed to determine the sequences of the A_1 and A_{2A} receptor coding regions, and the sequences of the partial A_{2B} and A_3 receptor cDNAs. The mucosal A_1 and A_{2A} receptor sequences cloned using the full length A_1 and A_{2A} receptor primers (Table 3) were submitted to Genbank and assigned accession numbers AF042079 and AF228684, respectively. The A_{2B} and A_3 receptor sequences cloned using the short length A_{2B} and A_3 receptor primers (Table 3) were assigned accession numbers AF084241 and AF102804, respectively. Results of the sequencing experiments demonstrate that the coding region of the gastric mucosal A_1 receptor (accession # AF042079) was identical to the published sequence in the rat brain (Mahan et al., 1991). The sequence of the gastric mucosal A_{2A} receptor coding region (accession # AF228684) was also found to be identical to that in the brain (Fink et al., 1992). Fig. 29 and 30 show the alignments of the rat mucosal and rat brain A_1 and A_{2A} receptor coding regions.

DNA sequence analysis was also performed on the PCR amplicons generated using the A_{2B}SL and A_3SL primer sets listed in Table 3. An alignment of these regions with corresponding regions of the rat brain receptors is shown in Fig. 31. The sequence of the mucosal A_{2B} receptor PCR amplicon is identical to that of the rat brain. However, analysis of the A_3 receptor PCR amplicon reveals a silent mutation at position 492. At the third position of the codon for alanine, adenine (A) is replaced by thymine (T). The position of this mutation corresponds to amino acid #59 of the A_3 receptor protein, which is situated in the second transmembrane domain (Salvatore et al., 1993).

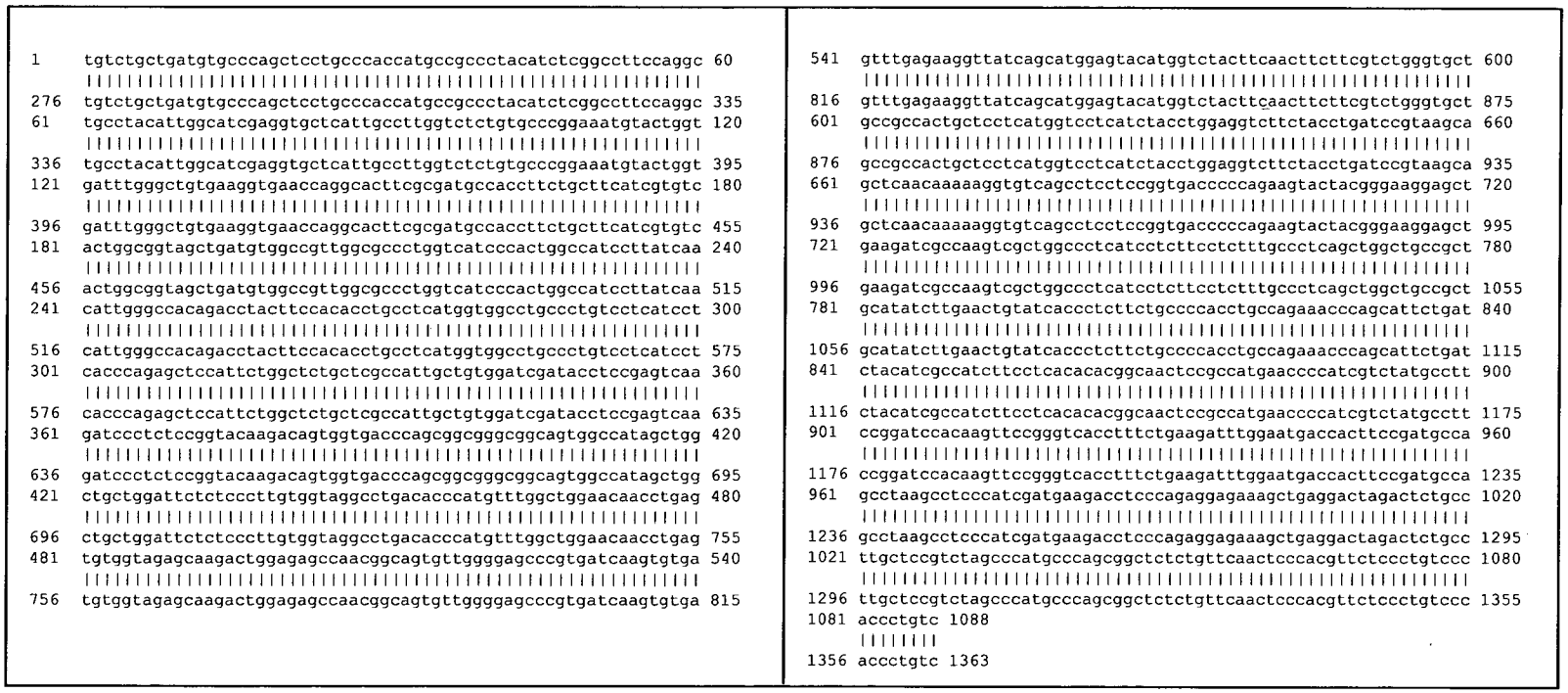


Fig. 29. Alignment of the rat mucosal and brain A_1 receptor coding regions. The rat mucosal A_1 receptor cDNA sequence (top strand; accession number: AF042079) is aligned with the corresponding region the rat brain receptor (bottom strand; accession number: M64299)

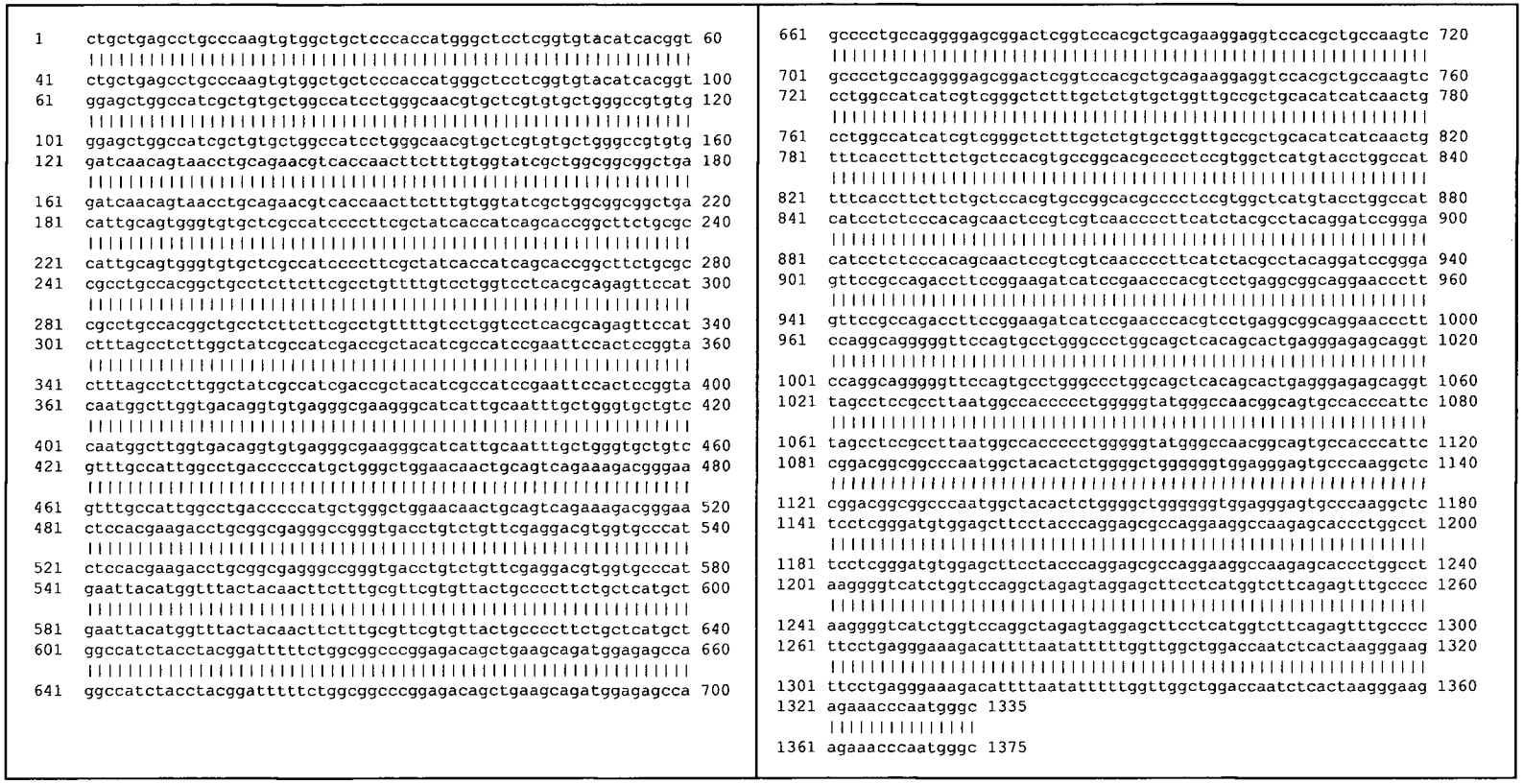


Fig. 30. Alignment of the rat mucosal and brain A_{2A} receptor coding regions. The rat mucosal A_{2A} receptor cDNA sequence (top strand; accession number: AF228684) is aligned with the corresponding region the rat brain receptor (bottom strand; accession number: S47609)

A

```
1   actactttctggtgtccctggcgacggcggacgtggctgtgggactcttcgccatcccct 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
235 actactttctggtgtccctggcgacggcggacgtggctgtgggactcttcgccatcccct 294
61  ttgccatcaccatcagcctgggcttctgcacggactttcacagctgcctcttcctcgcct 120
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
295 ttgccatcaccatcagcctgggcttctgcacggactttcacagctgcctcttcctcgcct 354
121 gcttcgtgctggtgctcacacagagctccatctttagcctcttggcggtggctgtcgacc 180
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
355 gcttcgtgctggtgctcacacagagctccatctttagcctcttggcggtggctgtcgacc 414
181 ggtatctggccattcgcgtcccgctcaggtataaaggtttggtcactggaacacgagcaa 240
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
415 ggtatctggccattcgcgtcccgctcaggtataaaggtttggtcactggaacacgagcaa 474
241 gagggatcatcgctgtcctctgggtccttgcctttggcattggactgactcctttcctgg 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
475 gagggatcatcgctgtcctctgggtccttgcctttggcattggactgactcctttcctgg 534
301 gttggaacagtaaagaccgtgccaccagcaactgcacagaacctggggatggcatc 356
    |||||||||||||||||||||||||||||||||||||||||||||||||||||
535 gttggaacagtaaagaccgtgccaccagcaactgcacagaacctggggatggcatc 590
```

B

```
1   aaagccaacaataccacgacgagtgccttgtggttgcaaatcacctacgtcaccatggag 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
319 aaagccaacaataccacgacgagtgccttgtggttgcaaatcacctacgtcaccatggag 378
61  gctgccattggtctctgtgctgtagtgggcaacatgctggtcatctgggtggtcaagctg 120
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
379 gctgccattggtctctgtgctgtagtgggcaacatgctggtcatctgggtggtcaagctg 438
121 aaccgcactctgaggaccaccaccttctatttcatcgtctccctagcactggctgacatt 180
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
439 aaccgcactctgaggaccaccaccttctatttcatcgtctccctagcactggcagacatt 498
181 gctgttggggtgctggtcataccttggccattgccgtcagcctggaggtccagatgcac 240
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
499 gctgttggggtgctggtcataccttggccattgccgtcagcctggaggtccagatgcac 558
241 ttctatgcctgccttttcatgtcctgtgtgcttctggtcttcacccatgcttccatcatg 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
559 ttctatgcctgccttttcatgtcctgtgtgcttctggtcttcacccatgcttccatcatg 618
301 tccttgctggccattgctgtagaccgatacctgcgagtcaagctgacagtcagatataga 360
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
619 tccttgctggccattgctgtagaccgatacctgcgagtcaagctgacagtcagatataga 678
361 acggttaccactcaaagaagaatatggctattcctgggcctctgctggctagtgtccttt 420
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
679 acggttaccactcaaagaagaatatggctattcctgggcctctgctggctagtgtccttt 738
421 ctggtgggactgacccccatgtttggctggaatagaaaagtgaccttagagctctctcaa 480
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
739 ctggtgggactgacccccatgtttggctggaatagaaaagtgaccttagagctctctcaa 798
481 aacagctcc 489
    |||||||||
799 aacagctcc 807
```

Fig. 31. Alignment of the rat mucosal and brain A_{2B} and A_3 cDNA sequences. The partial cDNA sequence of the rat mucosal A_{2B} (A) and A_3 (B) receptor was aligned with corresponding regions of the rat brain receptors. A: Top strand: gastric mucosal A_{2B} receptor sequence (accession number: AF084241); Bottom strand: brain A_{2B} receptor sequence (accession number: M91466). B: Top strand: gastric mucosal A_3 receptor sequence (accession number: AF102804) ; Bottom strand: brain A_3 receptor sequence (accession number: M94152). A silent mutation at position 174 bp of the mucosal A_3 receptor cDNA sequence is detected.

III. Quantification of gastric adenosine A_1 and A_{2A} receptor mRNA expression

To study the changes in adenosine A_1 and A_{2A} receptor gene expression induced by various states of the stomach, competitive RT-PCR and Real-Time RT-PCR assays were developed.

1. Competitive RT-PCR

Fig. 32 shows the results of a representative A_1 and A_{2A} receptor competitive RT-PCR assay which was performed to quantify A_1 and A_{2A} receptor mRNA levels in the rat striatum. This tissue has been shown to contain a moderate level of A_1 receptor mRNA and an extremely high level of A_{2A} receptor mRNA. In agreement with previous studies, the level of striatal A_{2A} receptor mRNA (6.6×10^7 copies/μg total RNA) was significantly greater than the level of A_1 receptor mRNA (3.9×10^6 copies/μg total RNA). See Fig. 32C. When the competitive RT-PCR samples are electrophoresed through an agarose gel, the density of the template band was shown to increase as the initial amount of competitor added was decreased. The Log [density ratio (corrected competitor band/template band)] plotted against the Log (initial competitor concentration) displayed a linear relationship with a R^2 value of > 0.95, and a slope of approximately 1.0. The graphs generated by the A_1 and A_{2A} competitive RT-PCR assays were shown to have slopes of 1.05±0.04, and 1.07±0.05, respectively, for all assays performed. An ideal slope of 1.0 indicates that the amplification efficiency of the template is equal to that of the competitor (Freeman et al., 1999). The R^2 values for all assays performed in these studies were > 0.95.

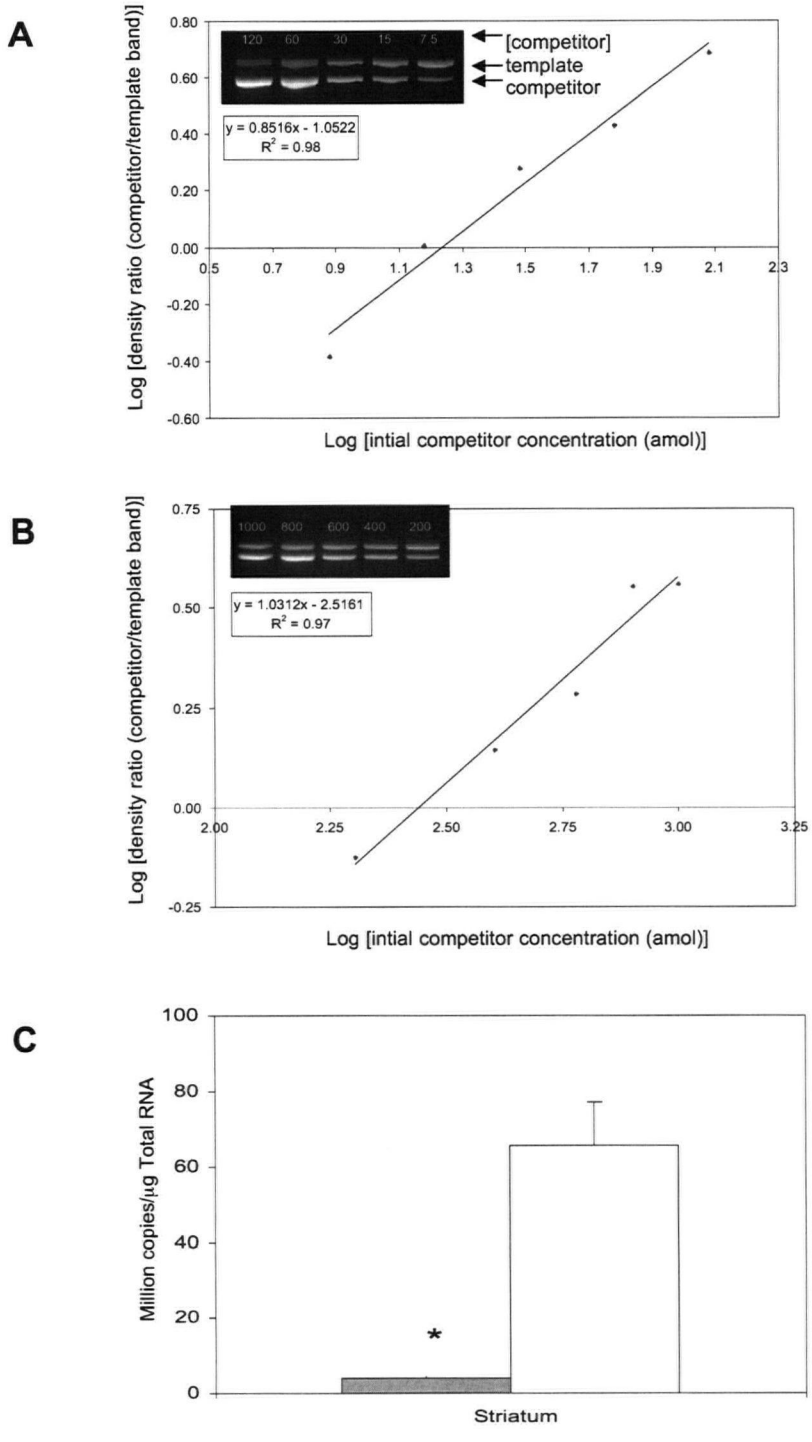


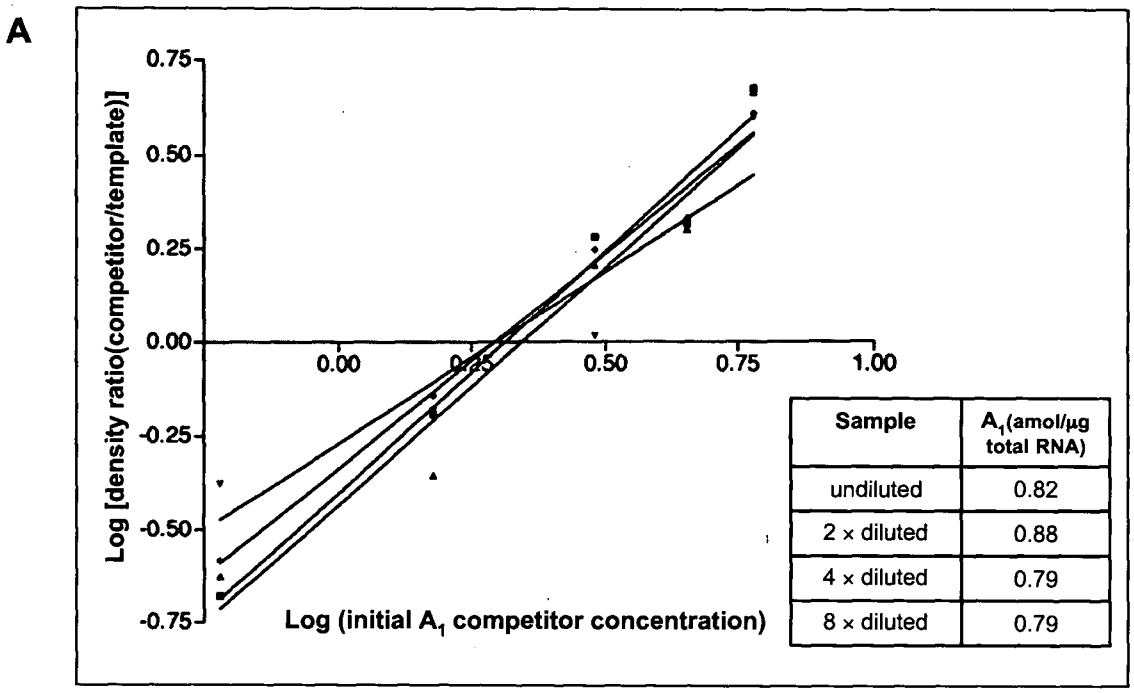
Fig. 32. Striatal A_1 and A_{2A} receptor gene expression measured by competitive RT-PCR. Results of a representative A_1 (A) and A_{2A} (B) receptor competitive RT-PCR assay performed using 2.5 µg of striatum total RNA and various amounts of A_1 and A_{2A} RNA competitor as the template. See inset for competitor concentrations (amol) used. C: A_1 (filled bar) and A_{2A} (empty bar) receptor mRNA expression in the striatum (* $P < 0.05$, $n = 4$)

Similar mRNA measurements were also obtained regardless of the amount of PCR template used. A_1 and A_{2A} receptor mRNA measurements were not significantly altered by using equal volumes of undiluted, 2×, 4×, or 8× diluted cDNA as the template for PCR (Fig. 33).

The results of representative A_1 and A_{2A} competitive RT-PCR assays performed using fundus, corpus, antrum and mucosa total RNA are shown in Fig. 34 and 35. A_1 and A_{2A} receptor mRNA levels in all gastric regions were lower than those measured in the striatum (Fig. 32C). The level of A_1 receptor mRNA found in the corpus, antrum and mucosa were approximately 3-fold higher than in the fundus (Fig. 36). Among the gastric tissues, the highest level of A_{2A} receptor mRNA was found in the corpus. The fundus, antrum and mucosa contained similar levels of A_{2A} receptor mRNA, which were approximately 2 fold less than in the corpus. In the antrum and mucosa, the level of A_1 receptor mRNA was significantly higher than the level of A_{2A} receptor mRNA, but in the fundus and corpus these levels were not significantly different.

2. Real-Time RT-PCR

Fig. 37A shows a representative amplification plot generated by a Real-Time RT-PCR assay for measuring adenosine A_1 receptor mRNA. The corresponding standard curve is also shown in Fig. 37B. Results show that this assay was able to amplify and measure adenosine A_1 receptor mRNA within a 7 log range of A_1 receptor RNA standard concentrations. When the highest concentration of standard was used as the template (1×10^9 copies/μl), the reaction was affected by limiting reagents at cycle 22, and the plateau phase of the reaction was reached by cycle 26. However, this does not affect quantification since measurements were made during the exponential phase at the threshold cycle. The standard



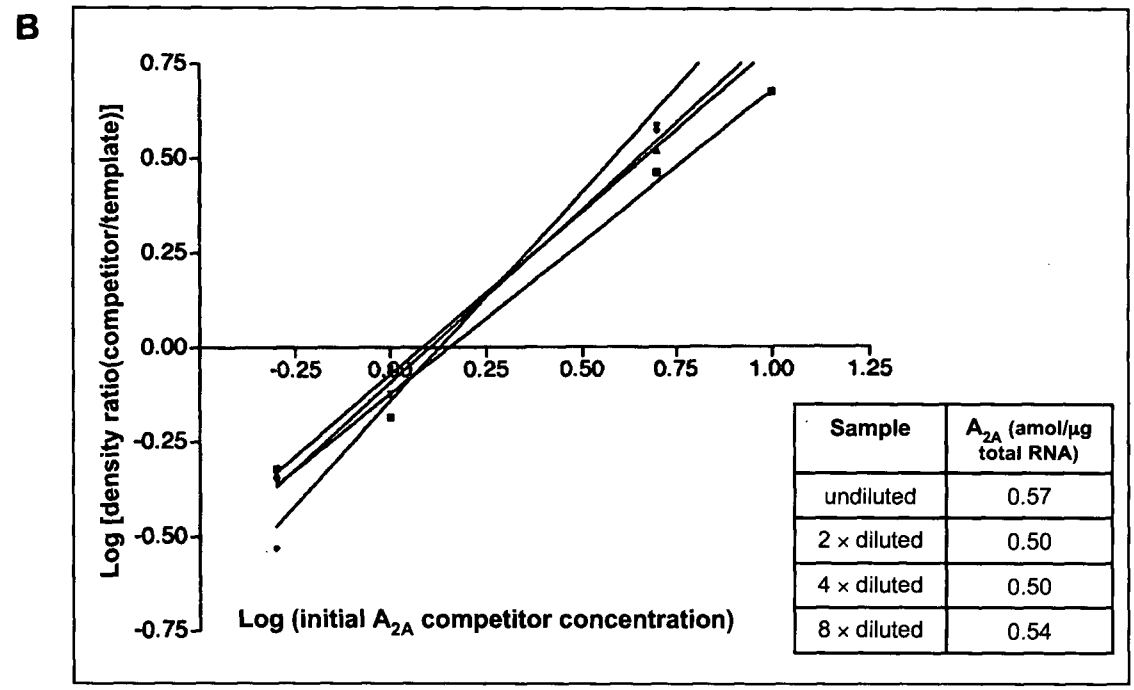


Fig. 33. Effect of template concentrations on quantification of A_1 and A_{2A} receptor mRNA by competitive RT-PCR. A_1 (A) and A_{2A} (B) receptor competitive RT-PCR experiments were performed using undiluted (■), 2× (▲), 4× (▼), or 8× (◆) diluted cDNA as the template. The cDNA samples were synthesized using a fixed amount (2.5 µg) of corpus total RNA and various amounts of A_1 or A_{2A} receptor RNA competitor.

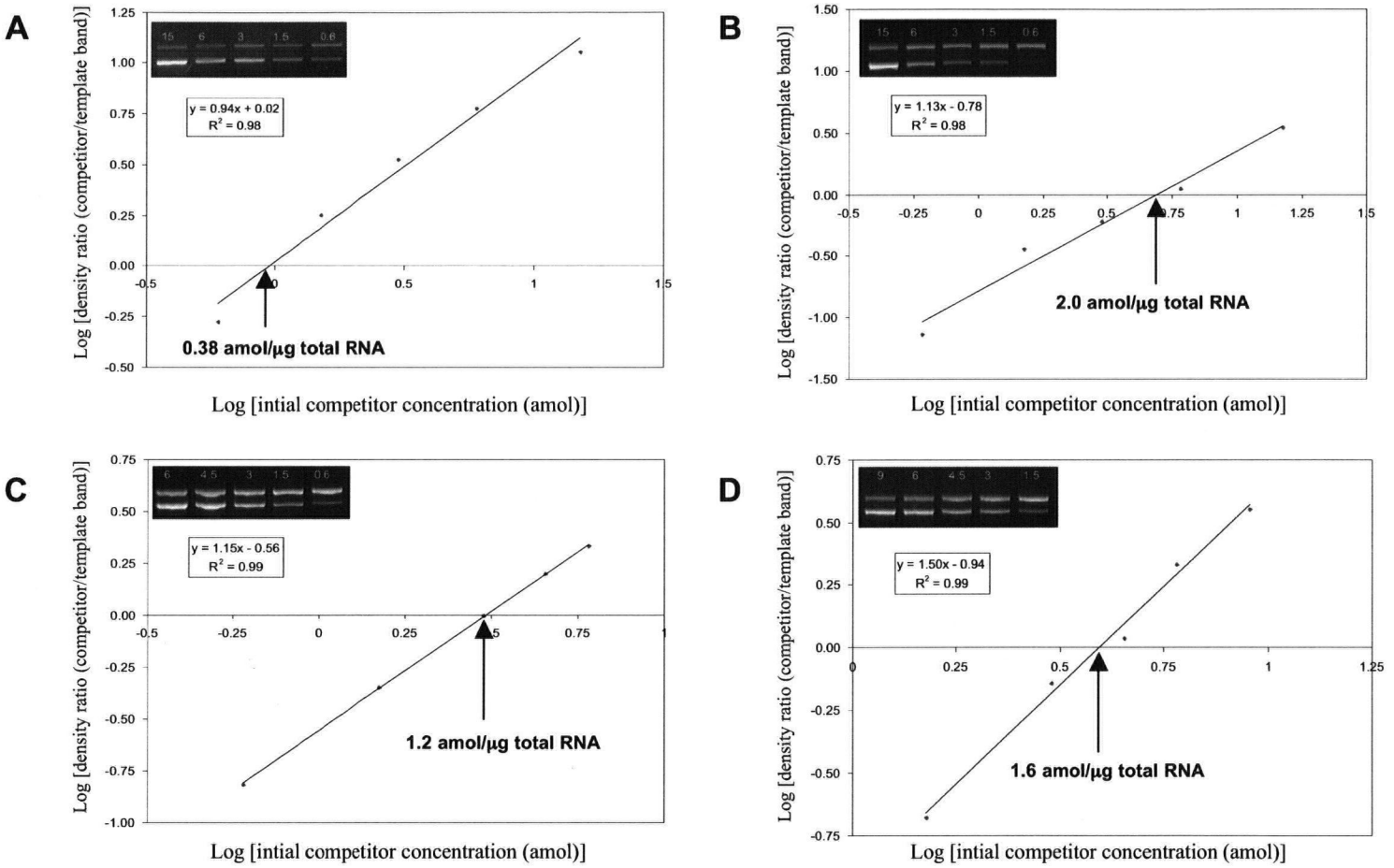


Fig. 34. Quantification of gastric A_1 receptor gene expression by competitive RT-PCR. Results of representative competitive RT-PCR assays for the fundus (A), corpus (B), antrum (C) and mucosa (D) are presented. The A_1 receptor gene expression levels measured in these tissues were 0.38, 2.0, 1.2, and 1.6 amol/µg total RNA, respectively.

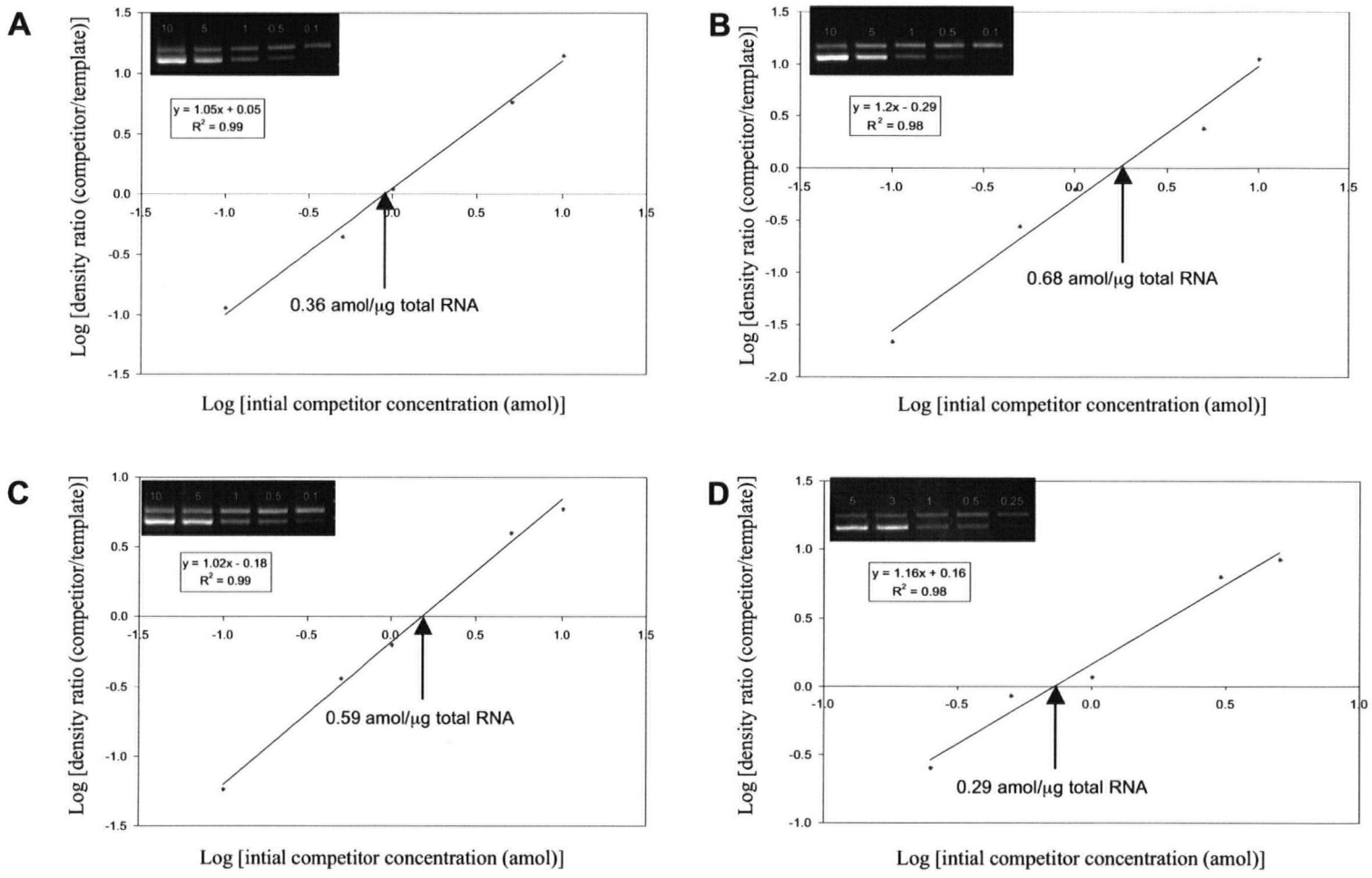


Fig. 35. Quantification of gastric A_{2A} receptor gene expression by competitive RT-PCR. Results of representative competitive RT-PCR assays for the fundus (A), corpus (B), antrum (C) and mucosa (D) are presented. The A_{2A} gene expression levels measured in these tissues were 0.36, 0.68, 0.59 and 0.29 amol/μg total RNA, respectively.

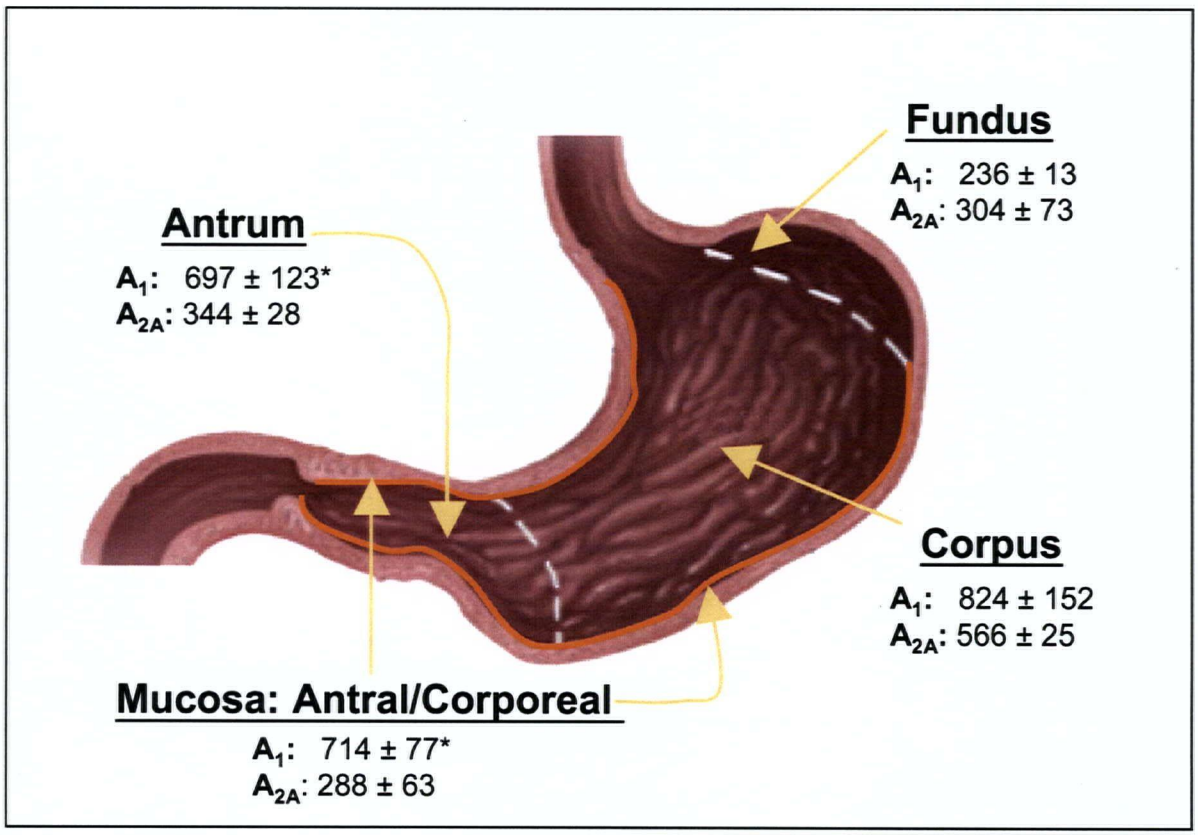


Fig. 36. Competitive RT-PCR quantification of A_1 and A_{2A} receptor gene expression in the rat stomach. Adenosine receptor mRNA levels (in thousand copies/μg total RNA) are shown for each region. *$P < 0.05$ when A_1 receptor mRNA levels are compared to A_{2A} receptor mRNA levels, $n \geq 4$.

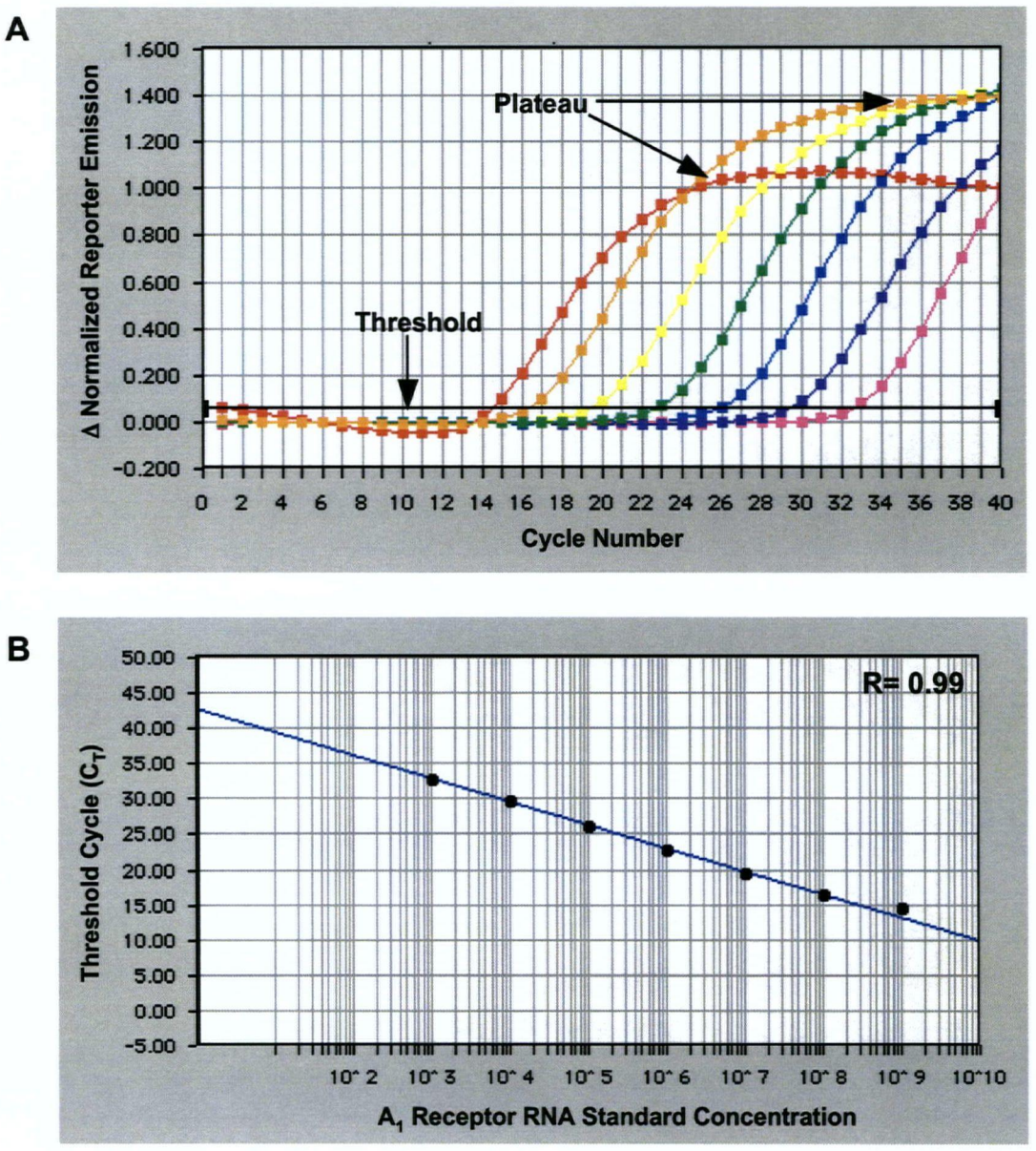


Fig. 37. Results of a representative A_1 receptor Real-Time RT-PCR assay. A: An amplification plot showing changes in normalized reporter emission after each cycle of PCR. Samples contained 1×10^9 (■), 1×10^8 (■), 1×10^7 (■), 1×10^6 (■), 1×10^5 (■), 1×10^4 (■), 1×10^3 (■) initial copies of A_1 receptor RNA. B: The corresponding standard curve generated by the amplification plot. The threshold cycle (C_T) represents the cycle where the change in normalized reporter emission exceed the threshold.

curve generated by plotting the threshold cycle against the Log(A_1 RNA standard concentration) was found to be linear with a R value of 0.99. Fig. 38 shows the amplification plot and standard curve obtained for a representative A_{2A} receptor Real-Time PCR assay. The standard curve was also linear within a 7 log range of A_{2A} RNA standard concentrations, with a R value of 0.98.

Fig. 39 shows the absolute levels of adenosine A_1 and A_{2A} receptor mRNA measured in the striatum and in various regions of the stomach, including the fundus, corpus, antrum, mucosa, corporeal muscle and corporeal mucosa. In agreement with previous studies, adenosine A_1 and A_{2A} receptor levels were highest in the striatum (Dixon et al., 1996). Striatal A_1 and A_{2A} receptor mRNA levels were approximately 2-fold and 70-fold higher than the highest gastric A_1 and A_{2A} mRNA levels in the corporeal muscle region, respectively. In all gastric tissues, A_1 receptor mRNA levels were significantly higher than A_{2A} receptor mRNA levels. In the fundus, corpus, antrum, mucosa, corporeal muscle and corporeal mucosa, the A_1 receptor mRNA levels were 3.4, 7.8, 8.3, 28.4, 14.4, and 14.6 times higher than A_{2A} receptor mRNA levels, respectively. The highest and lowest levels of A_1 receptor mRNA were found in the corporeal muscle and corporeal mucosa, respectively. No significant difference in the A_1 receptor mRNA levels was observed among the corpus, antrum and the whole stomach mucosa. A_{2A} receptor mRNA levels were not significantly different among the fundus, corpus and antrum, but were lowest in the whole gastric mucosa and in the corporeal mucosa.

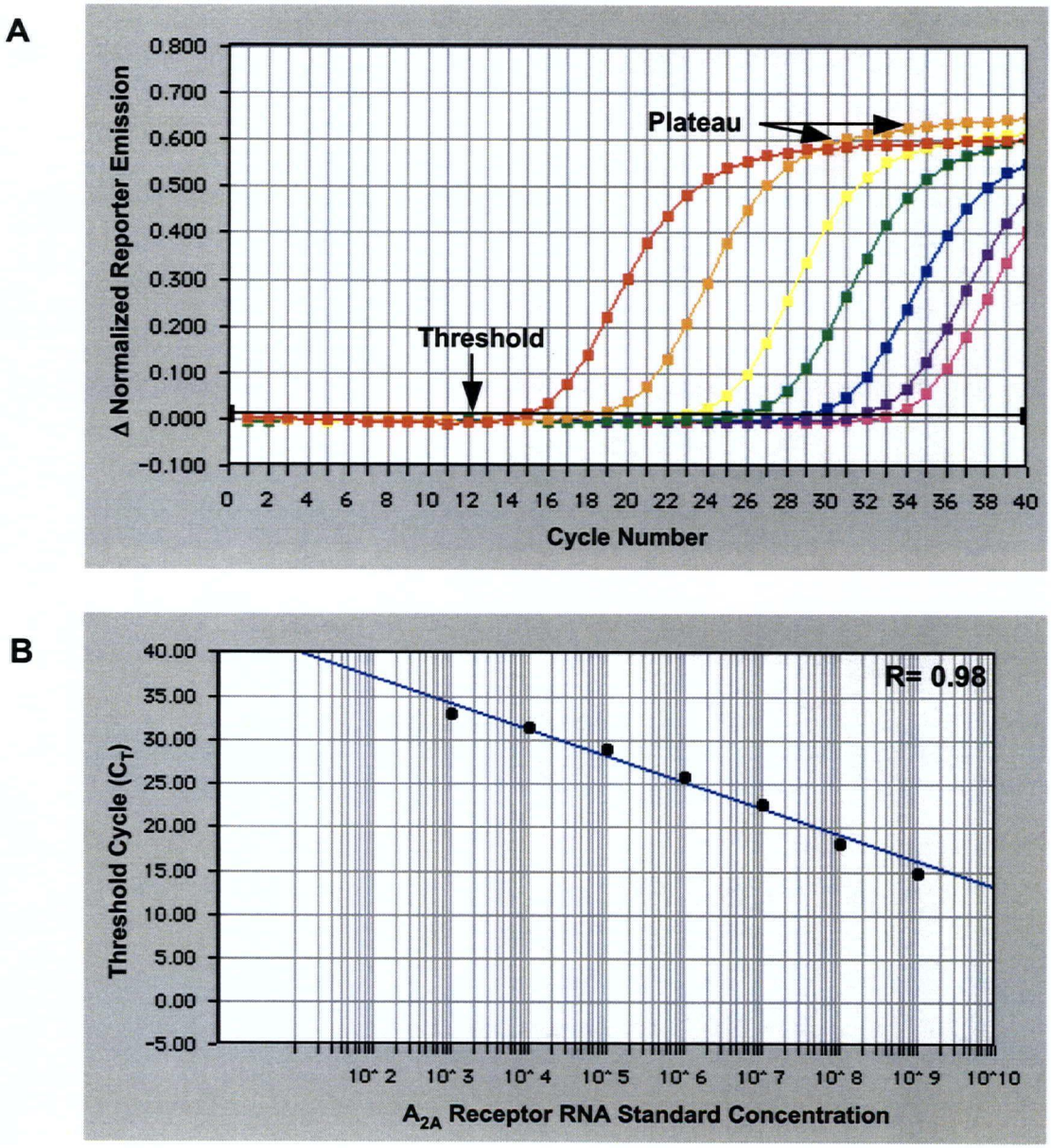


Fig. 38. Results of a representative A_{2A} Real-Time RT-PCR assay. A: An amplification plot showing changes in normalized reporter emission after each cycle of PCR. Samples contained 1×10^9 (■), 1×10^8 (■), 1×10^7 (■), 1×10^6 (■), 1×10^5 (■), 1×10^4 (■), 1×10^3 (■) initial copies of A_{2A} receptor RNA. B: The corresponding standard curve generated by the amplification plot. The threshold cycle (C_T) represents the cycle where the change in normalized reporter emission exceed the threshold.

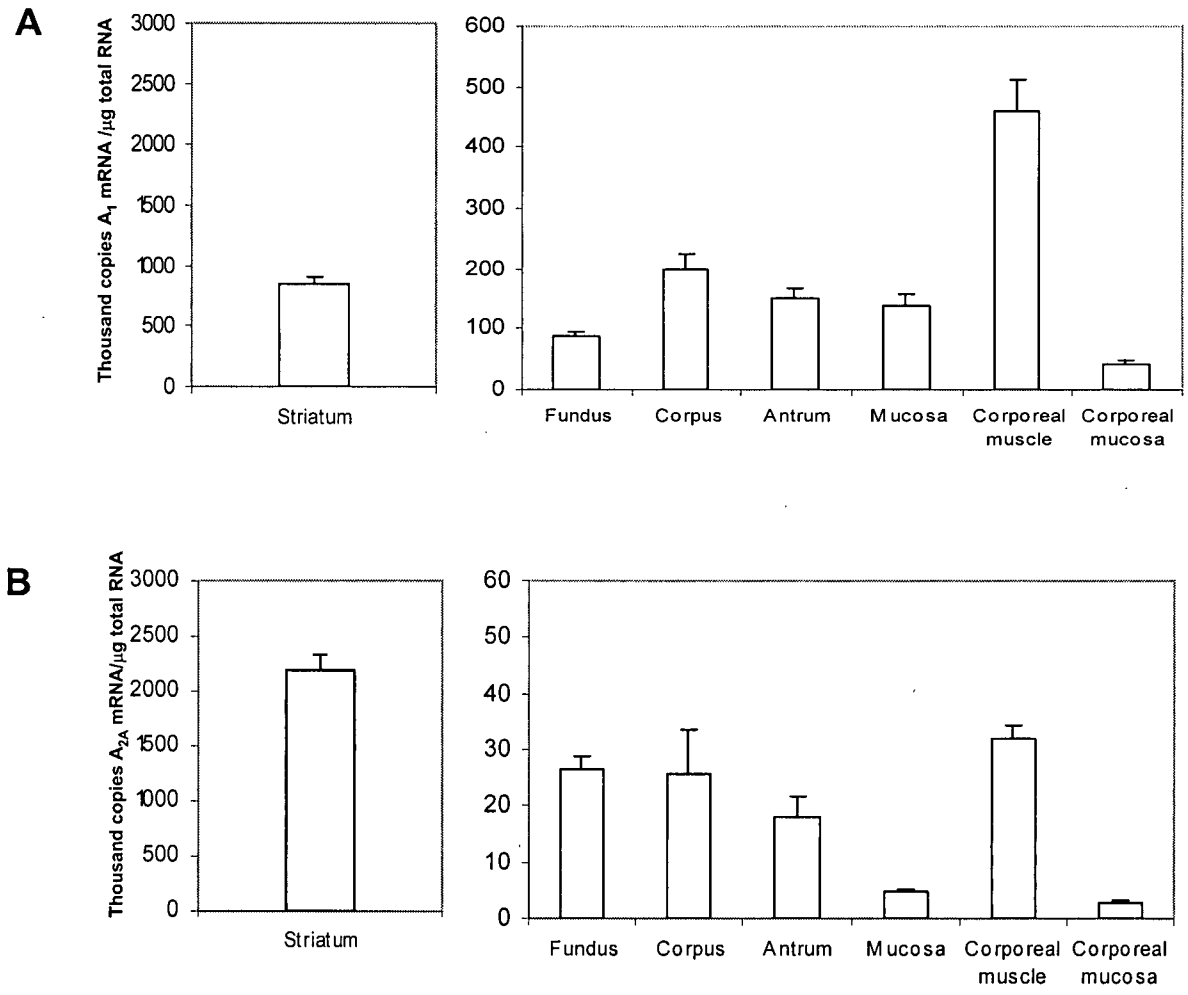


Fig. 39. Real-Time RT-PCR quantification of gastric and striatal adenosine A_1 (A) and A_{2A} (B) receptor gene expression. The level of A_1 and A_{2A} receptor gene expression was highest in the striatum. A_1 receptor gene expression was also significantly higher than A_{2A} receptor gene expression in all gastric tissues examined ($n > 4$). Note the difference in the scale of the ordinate.

IV. Effect of fasting on adenosine receptor, somatostatin and gastrin gene expression

To examine whether the prandial state of the stomach affects adenosine receptor gene expression, rats were fasted for either 24 or 36 h, and gene expression was quantified using Real-Time RT-PCR. Real-Time RT-PCR was utilized for this study since this technique is more sensitive and specific than competitive RT-PCR (Heid et al., 1996). In these experiments, gastrin and somatostatin gene expression were also measured by Real-Time RT-PCR to determine if changes in adenosine receptor gene expression occur with changes in gastric peptide gene expression.

1. Effect of fasting on body weight and Total RNA concentration

Rats subjected to 24 and 36 h of fasting were shown to exhibit significant weight loss compared to control animals (see Table 8). Fasting for 36 h, but not 24 h, was also shown to significantly decrease the mass of the antral tissue. Corporeal mucosa and corporeal muscle mass were not affected by either 24 or 36 h of fasting. The total RNA concentration (μg total RNA/mg tissue) was also altered by fasting in some regions (see Table 8). Fasting for 24 and 36 h significantly decreased the total RNA concentration in the antrum, while fasting for 24 h significantly decreased total RNA concentration in the corporeal muscle.

2. Effect of fasting on 18S rRNA and GAPDH gene expression

The expression levels of housekeeping genes were measured and used as the endogenous control to standardize the amount of sample added to each reaction. Two

Table 8: Effect of fasting and omeprazole treatment on body weight, tissue weight and total RNA concentration

		Fasting		Omeprazole	
		24 h	**36 h**	**1 day**	**3 day**
Change in weight (g)	Test	-28±2*	-35±1*	5±2	17±3
	Control	6±1	13±1	6±2	18±2
Tissue weight (mg)	**Antrum**				
	Test	155±10	117±10*	121±10	163±17
	Control	163±16	182±20	115±11	153±26
	Corporeal Mucosa				
	Test	55±4	69±11	63±4	74±6
	Control	63±6	65±10	65±8	83±9
	Corporeal Muscle				
	Test	792±23	815±41	689±68	658±53
	Control	804±41	804±40	634±17	665±56
Total RNA (μg/mg tissue)	**Antrum**				
	Test	1.1±0.1*	1.0±0.1*	2.2±0.2	1.7±0.3
	Control	1.4±0.1	1.7±0.2	2.1±0.2	2.0±0.3
	Corporeal Mucosa				
	Test	2.7±0.3	2.4±0.5	2.3±0.6	2.7±0.3
	Control	2.8±0.3	3.9±0.6	2.2±0.2	2.1±0.2
	Corporeal Muscle				
	Test	4.7±0.4*	4.7±0.2	5.9±0.3	4.1±0.3
	Control	5.9±0.3	4.6±0.2	6.6±0.6	4.5±0.4

*$P < 0.05$ when test animals were compared to controls using the unpaired Student's t-test, $n \geq 8$ per group.

commonly used housekeeping genes were measured, GAPDH and 18S rRNA. Results show that GAPDH mRNA levels were not significantly altered after 24 h of fasting in all gastric tissues examined. However, after 36 h of fasting, significant decreases in GAPDH mRNA levels were observed in the antrum (53% of control), corporeal mucosa (56% of control) and corporeal muscle (70% of control) (Fig. 40). The levels of 18S rRNA were not altered by 24 or 36 h of fasting in all tissues studied (See Fig. 40B). Therefore, 18S rRNA levels were used as the endogenous standard to control for differences in total RNA concentration and RNA integrity in the following studies.

3. Effect of fasting on adenosine A_1 and A_{2A} receptor expression

Following a 24 h fast, A_1 receptor mRNA levels in the corporeal mucosa and antrum were significantly increased to 134 ± 11% and 157 ± 19% of control levels, respectively (Fig. 41A). No significant change in A_1 receptor gene expression was observed in these tissues when the fasting period was extended to 36 h. In addition, fasting for 24 and 36 h did not alter A_1 receptor mRNA expression in the corporeal muscle.

The effect of fasting on A_{2A} receptor mRNA expression differed between the corporeal mucosa and the antrum. In the corporeal mucosa, adenosine A_{2A} receptor expression was not altered by 24 h of fasting. However, following 36 h of fasting, A_{2A} receptor expression was significantly reduced to 65 ± 6 % of the control levels (Fig. 41B). In the antrum, the gene expression of adenosine A_{2A} receptor was significantly increased to 173 ± 21 % of control levels after a 24 h fast, but unchanged following a 36 h fast. Similar to A_1 receptor gene expression, fasting did not alter A_{2A} receptor gene expression in the corporeal muscle.

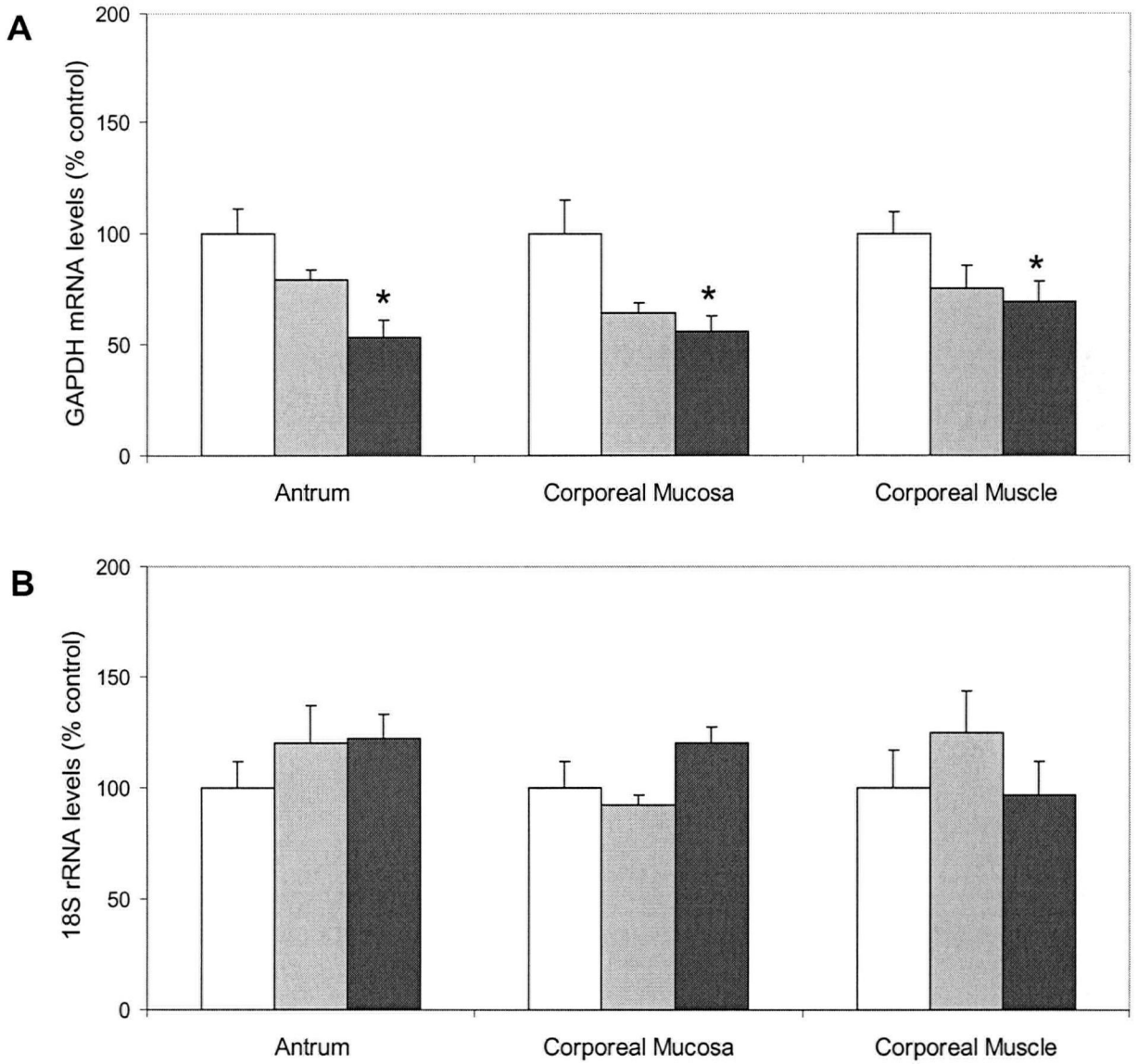


Fig. 40. Effect of fasting on gastric housekeeping gene expression. GAPDH mRNA (A) and 18S rRNA (B) levels were measured in rats fasted for 24 h (grey) or 36 h (black). Results are expressed as a percentage of the control (white) as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$; $n \geq 7$.

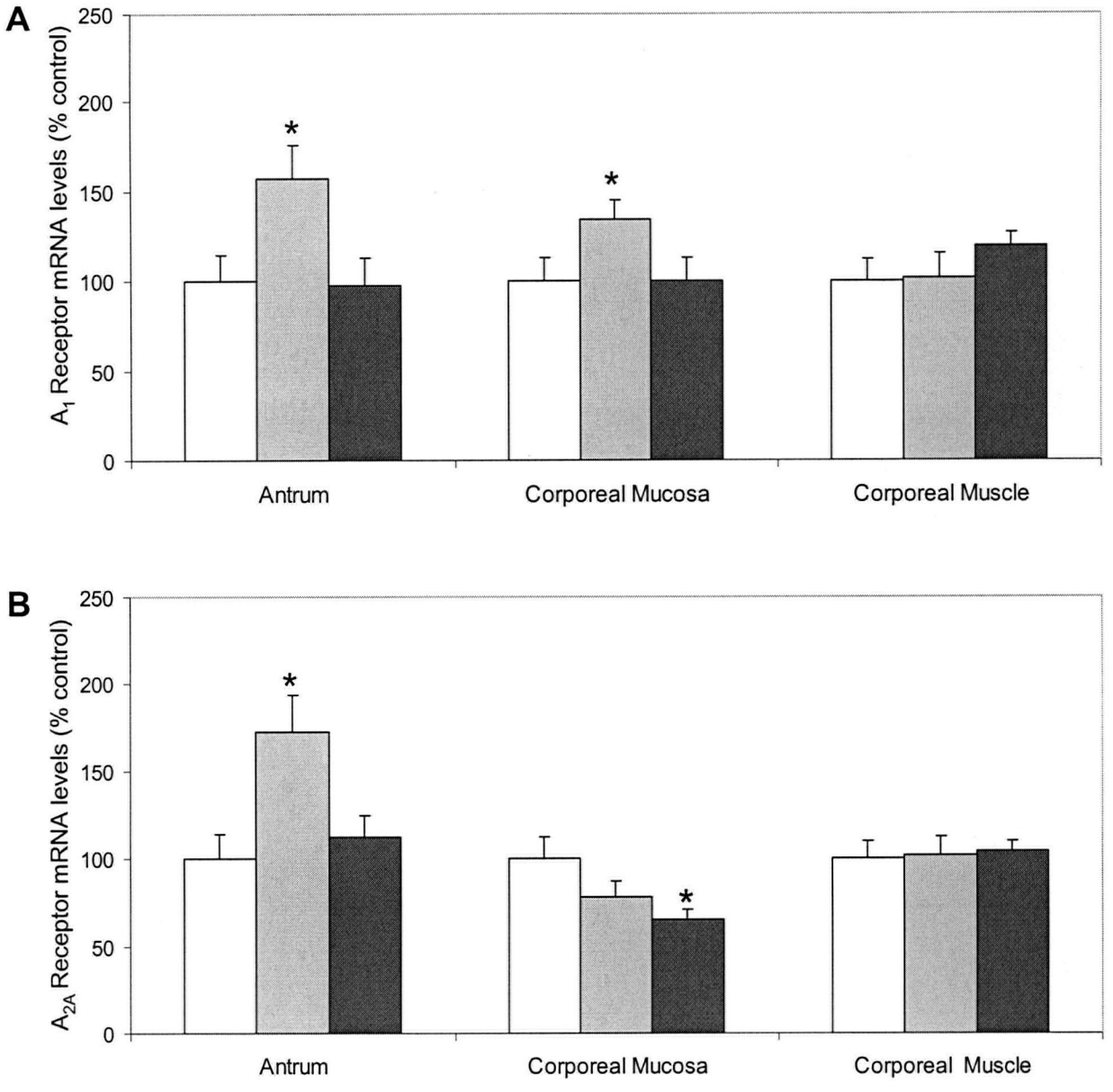


Fig. 41. Effect of fasting on adenosine receptor gene expression. A_1 receptor mRNA (A) and A_{2A} receptor mRNA (B) levels were measured in rats fasted for 24 h (grey) or 36 h (black). Results are expressed as a percentage of the control (white) as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$; $n \geq 7$.

4. Effect of fasting on gastrin and somatostatin gene expression

Antral gastrin gene expression was significantly reduced to 50 ± 9% and 54 ± 10% of control levels after 24 and 36 h of fasting, respectively (Fig. 42A). Following fasting, changes in somatostatin gene expression (Fig. 42B) resembled changes in A_{2A} receptor gene expression (Fig. 41B). In the corporeal mucosa, somatostatin mRNA levels were not affected by 24 h of fasting, but significantly decreased (53 ± 5 % of controls) after 36 h of fasting. In the antrum, somatostatin gene expression was significantly augmented (196 ± 27% of controls) after 24 h of fasting, but unaltered after 36 h of fasting. Similar to A_{2A} receptor gene expression, fasting did not affect somatostatin gene expression in the corporeal muscle. When the changes in somatostatin gene expression were compared to changes in adenosine A_{2A} receptor gene expression, a linear relationship with a correlation coefficient of 0.96 was observed (Fig. 43).

V. Omeprazole treatment on adenosine receptor and gastric peptide gene expression

To examine if the inhibition of gastric acid secretion alters adenosine receptor gene expression, rats were treated with 400 μmol/kg omeprazole daily for 1 or 3 days. Omeprazole inhibits gastric acid secretion by binding irreversibly to the proton pump, H^+K^+ATPase, on parietal cells (Hirschowitz et al., 1995). Gastrin and somatostatin gene expression were also measured to examine if their expression is also altered with adenosine receptor gene expression.

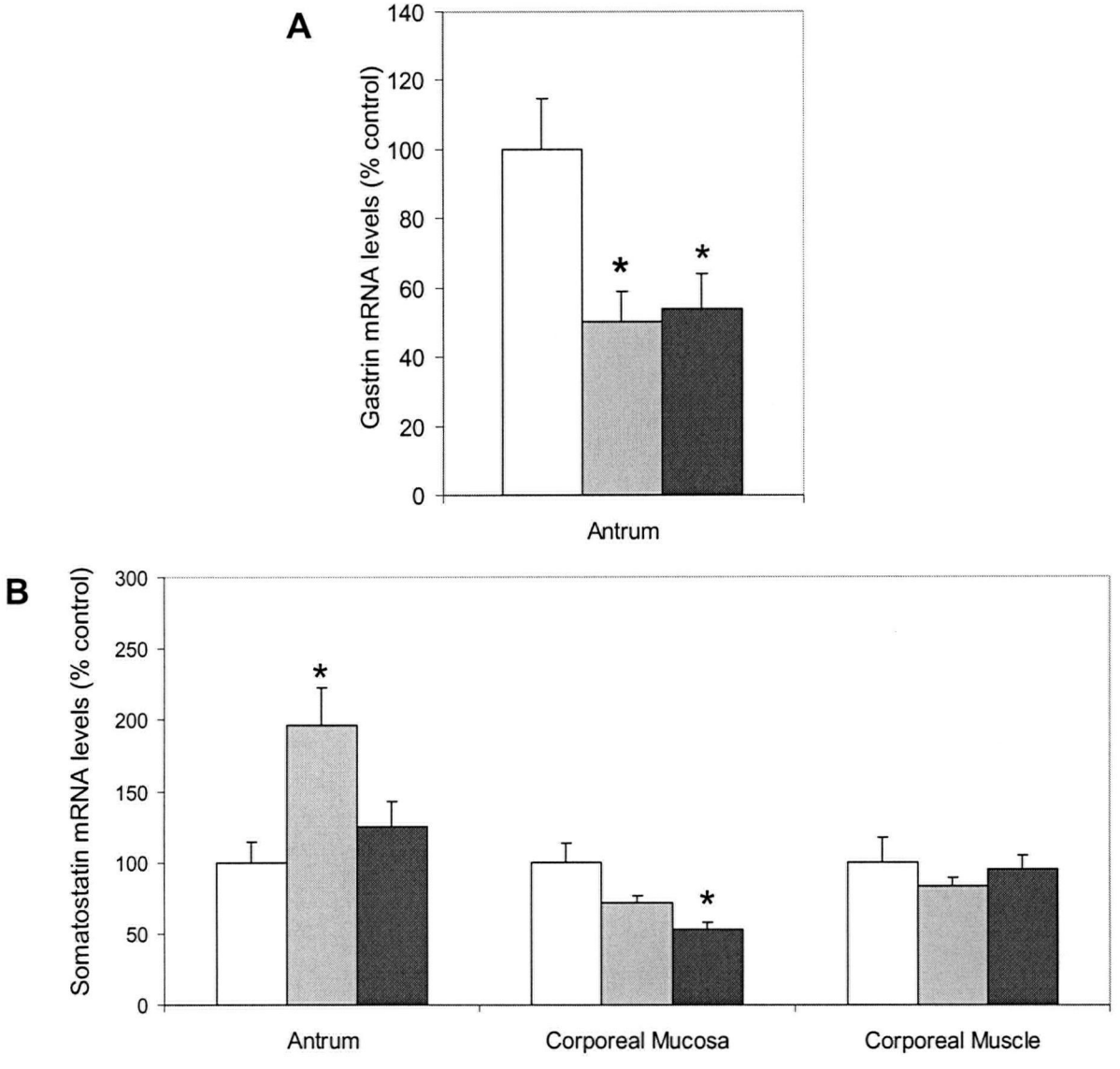


Fig. 42. Effect of fasting on gastric peptide gene expression. Gastrin mRNA (A) and somatostatin mRNA (B) levels were measured in rats fasted for 24 h (grey) or 36 h (black). Results are expressed as a percentage of the control (white) as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$; $n \geq 7$.

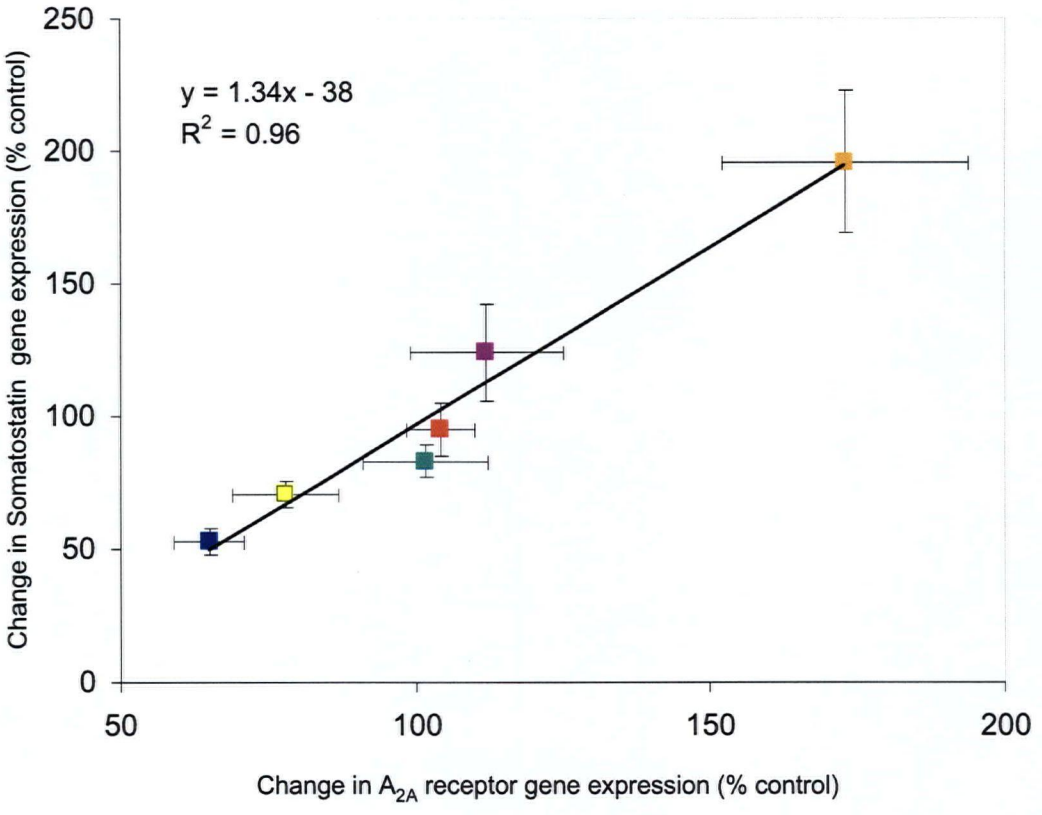


Fig. 43. Comparison between fasting-induced changes in somatostatin and adenosine A_{2A} receptor gene expression. Changes in gene expression were measured after 24 or 36 h of fasting in various gastric tissues: corporeal mucosa (36 h; blue), corporeal mucosa (24 h; yellow), corporeal muscle (24 h, green); corporeal muscle (36 h; red), antrum (36 h; purple), antrum (24 h; orange). Each point represents the mean ± SEM of at least 7 animals.

1. Effect of omeprazole treatment on 18S rRNA and GAPDH gene expression

Omeprazole treatment for 1 or 3 days did not alter the weight of the rats, the mass of the gastric tissues, or the total RNA concentration of these tissues (see Table 8). Housekeeping gene expression levels were measured and used as the endogenous control. Similar to the effects of fasting, omeprazole treatment also altered GAPDH mRNA levels in some regions. GAPDH gene expression was significantly decreased to 65±5% of control levels in the corporeal mucosa after 3 days of treatment, and significantly increased to 130±9% in the corporeal muscle after 1 day of treatment (Fig. 44A). However, levels of 18S rRNA were not altered by 1 or 3 days of omeprazole treatment (Fig. 44B). Therefore, this housekeeping gene was used as the endogenous control for the Real-Time RT-PCR quantification of A_1 and A_{2A} receptor, somatostatin and gastrin mRNA levels.

2. Effect of omeprazole treatment on adenosine A_1 and A_{2A} receptor expression

Omeprazole significantly decreased A_1 receptor gene expression in the antrum to 66±12% and 47±8% of control levels after 1 day and 3 days of treatment, respectively (see Fig. 45A). In the corporeal mucosa, A_1 receptor gene expression was significantly decreased to 62±6% of control levels after 1 day of omeprazole treatment, but changes were not apparent after 3 days of treatment.

A_{2A} receptor gene expression was significantly decreased to 57±7% of control levels in the antrum after 3 days of treatment (Fig. 45B). In the corporeal mucosa, A_{2A} receptor gene expression was also decreased to 67±14% of control levels after 1 day of omeprazole treatment, but changes were not apparent 3 days after treatment. Both A_1 and A_{2A} receptor

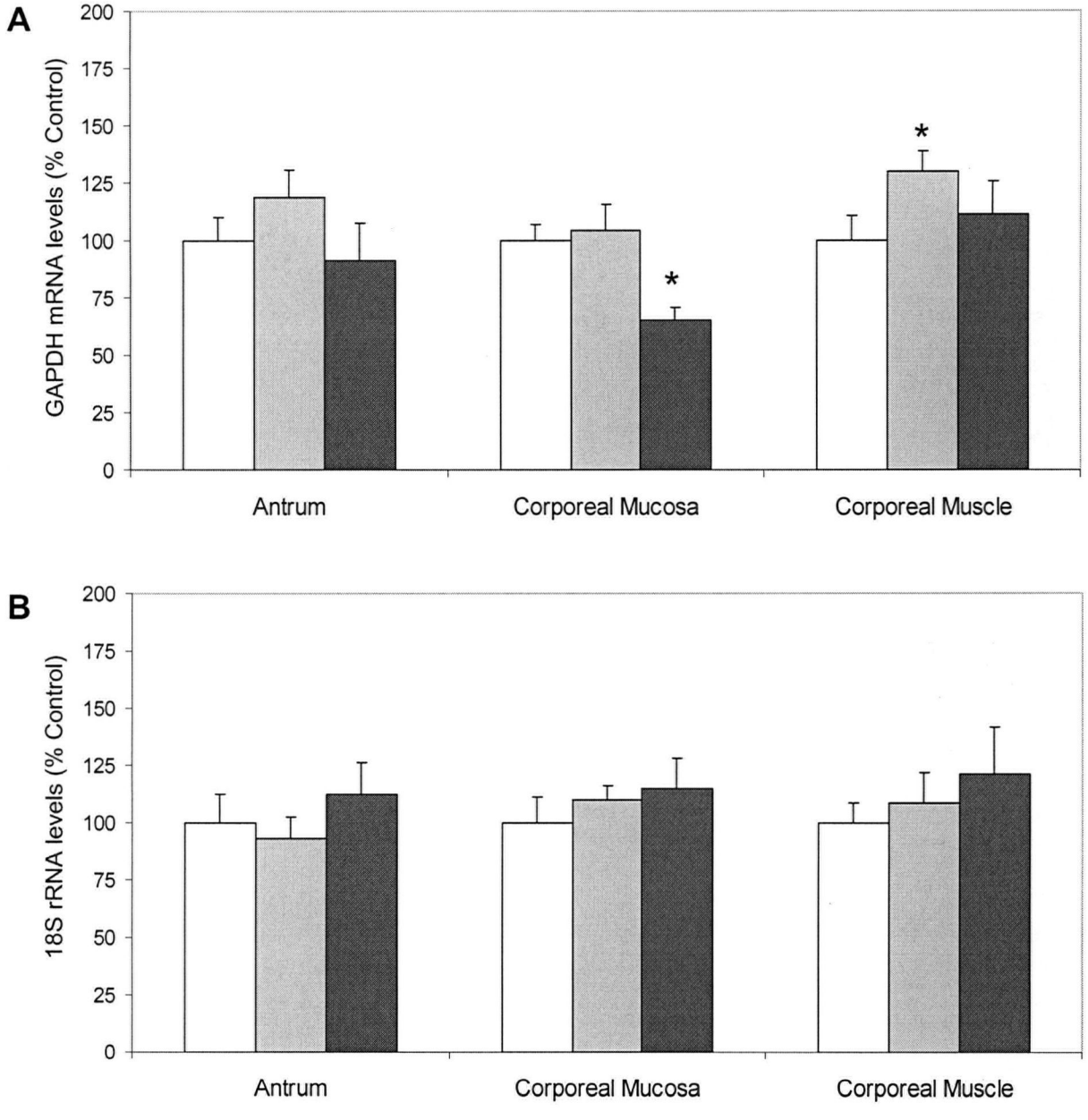


Fig. 44.: Effect of omeprazole treatment on gastric housekeeping gene expression. GAPDH mRNA (A) and 18S rRNA (B) levels were measured in rats treated with omeprazole for 1 (grey) or 3 days (black). Results are expressed as a percentage of the control (white), as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$; $n \geq 7$.

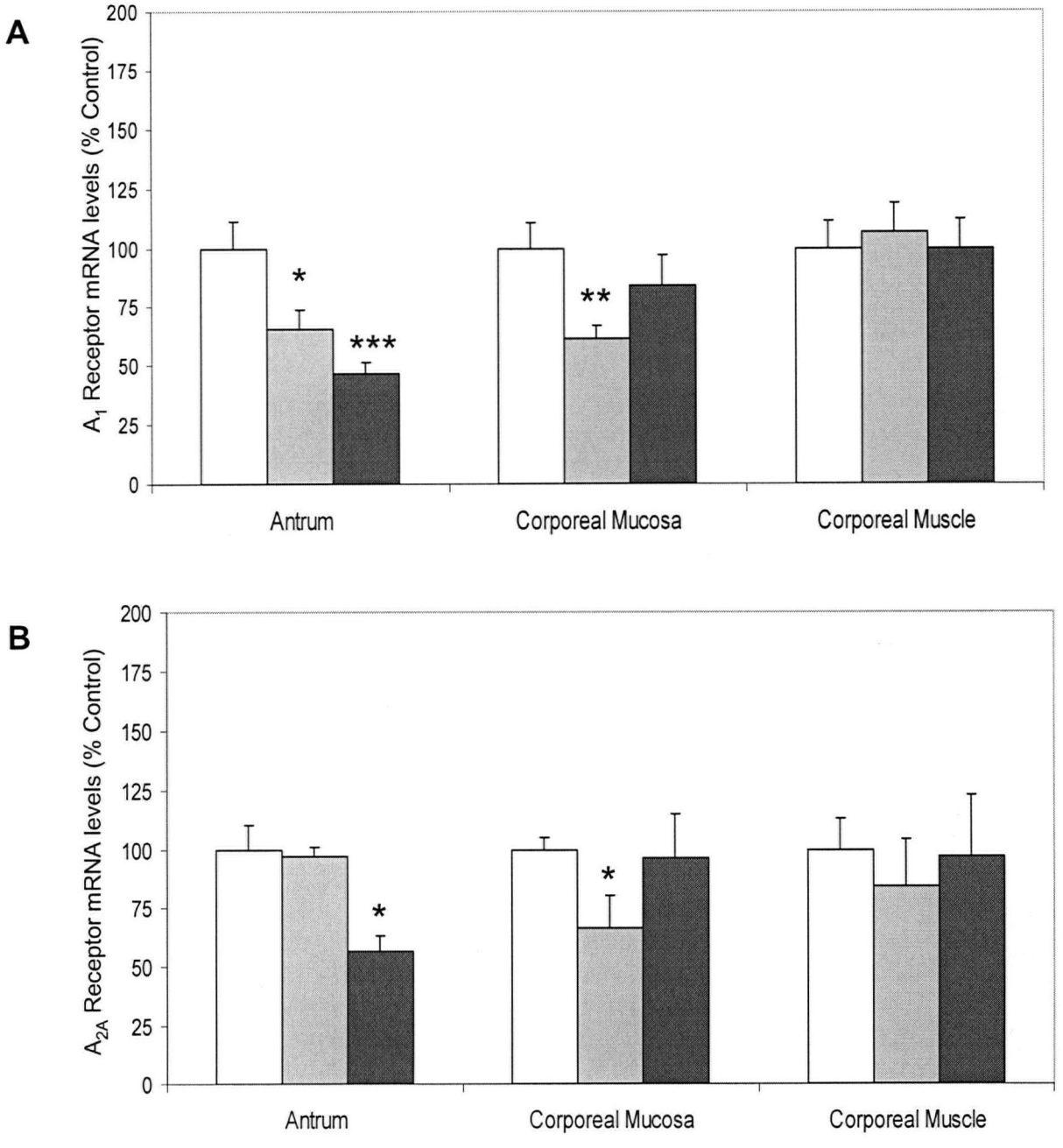


Fig. 45. Effect of omeprazole treatment on adenosine A_1 and A_{2A} receptor gene expression. A_1 receptor (A) and A_{2A} (B) receptor mRNA levels were measured in rats treated with omeprazole for 1 (grey) or 3 (black) days. Results are expressed as a percentage of the control (white) as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n \geq 7$.

gene expressions were not altered by either 1 or 3 days of omeprazole treatment in the corporeal muscle.

3. Effect of omeprazole treatment on gastrin and somatostatin gene expression

Antral gastrin gene expression was significantly increased to 201 ± 18% and 268 ±42% of control levels after 1 and 3 days of omeprazole treatment, respectively (Fig. 46A). In contrast, antral somatostatin gene expression was significantly reduced to 71±6% and 58±4% of controls levels, and corporeal mucosal somatostatin gene expression was reduced to 54±13% and 54±10% of control levels after 1 day and 3 days of treatment, respectively (Fig. 46B). In the corporeal muscle, somatostatin gene expression was not altered by 1 day of treatment. However, after 3 days of treatment, somatostatin gene expression was significantly reduced to 45±6% of control levels.

VI. Omeprazole on adenosine agonist-induced changes in IRG and SLI release

The effect of omeprazole treatment on adenosine-agonist induced changes in IRG and SLI release was examined. After 1 or 3 days of omeprazole treatment, stomachs were perfused with either CPA (A_1-selective agonist) or CGS 21680 (A_{2A}-selective agonist) at a concentration of 0.1 μM or 1 μM. The lower concentration of 0.1 μM was chosen based on the estimated EC_{50} value of CPA (0.067 μM) and CGS 21680 (0.06 μM) in inhibiting IRG and stimulating SLI release, respectively. The effect of 1.0 μM concentration was examined since this concentration of CPA and CGS 21680 elicited

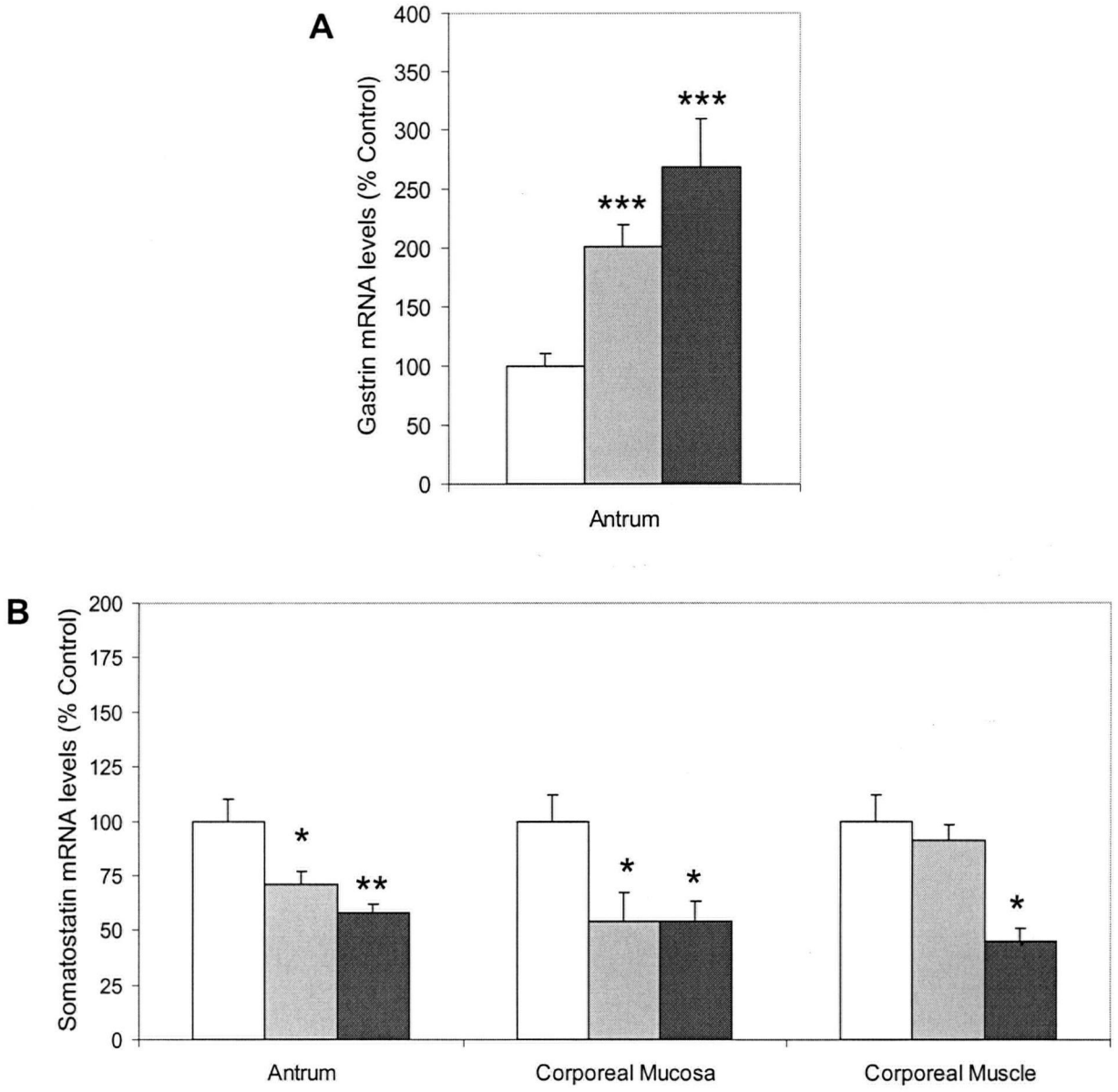


Fig. 46. Effect of omeprazole treatment on gastric peptide gene expression. Gastrin (A) and somatostatin (B) mRNA levels were measured in rats treated with omeprazole for 1 (grey) or 3 (black) days. Results are expressed as a percentage of the control (white), as described in the Methods section. Statistics were performed using the Student's unpaired t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n \geq 7$.

maximal inhibition and stimulation of IRG and SLI release, respectively (see Section I of this chapter). The effect of CGS 21680 on IRG release was not examined since this compound did not alter IRG release (see section 1.1).

1. Effect of omeprazole on CPA-induced changes in IRG release

Fig. 47 shows the effect of 1 μM CPA on IRG release in animals treated with omeprazole for 3 days. The administration of CPA resulted in a significant inhibition of IRG release in both control and treated animals. However, there was no difference in the release of IRG between these two groups. Results of CPA-induced IRG release in omeprazole-treated and control animals are summarized in Fig. 48. There were no changes in CPA-induced IRG release between treated and control animals.

2. Effect of omeprazole on agonist-induced changes in SLI release

Fig. 49 shows the effect of 1 μM CGS 21680 on SLI release in control and 3 day omeprazole-treated animals. In these experiments, SLI release was enhanced by CGS 21680 in both treated and control animals. However, the CGS 21680-induced SLI release was significantly attenuated by 3 day omeprazole treatment. Results of CGS 21680-induced SLI release in control and 1 or 3 day omeprazole-treated animals are summarized in Fig. 50.

As described earlier, the administration of 0.1 μM CPA significantly inhibited gastric SLI release, while the administration of 1 μM CPA significantly stimulated SLI release (see Section I). These observations were also apparent in the current experiments (Fig. 51). The inhibition of SLI release induced by 0.1 μM CPA was not altered by either 1 or 3 days of

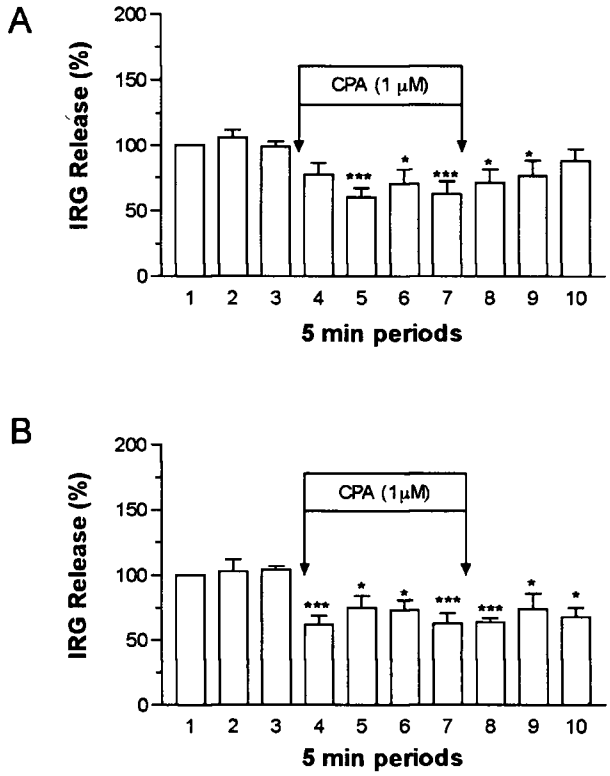


Fig. 47. Effect of CPA (1 μM) on gastric IRG release from the perfused stomachs of 3 day omeprazole-treated (A) and control (B) animals. Results are expressed as IRG release (%) as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments. *P < 0.05 and ***P < 0.001 when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test. The IRG release during each 5 min period was not significantly different between omeprazole-treated and control animals.

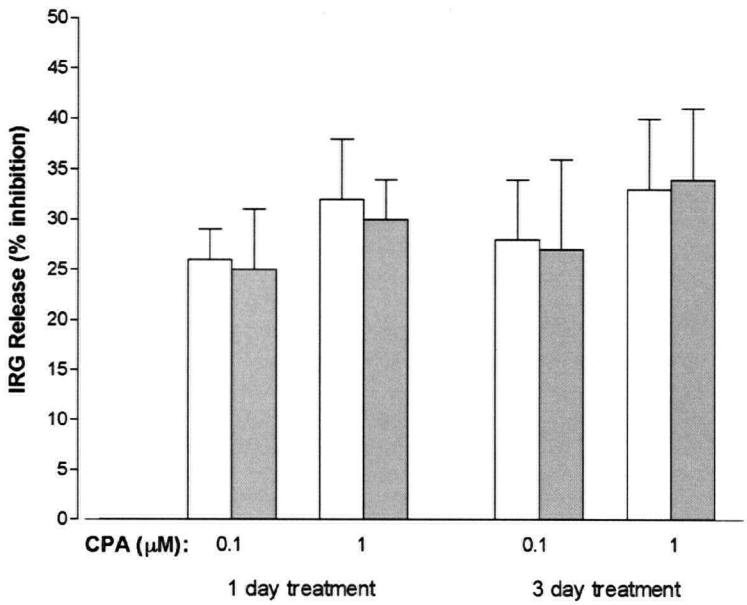


Fig. 48. Effect of omeprazole treatment on CPA-induced changes in IRG release. The percentage inhibition of IRG release between control (empty bar) and omeprazole-treated (filled bar) animals was compared using the Student's unpaired t-test, and were found not to differ. Each column represents the mean ± SEM of at least 5 experiments.

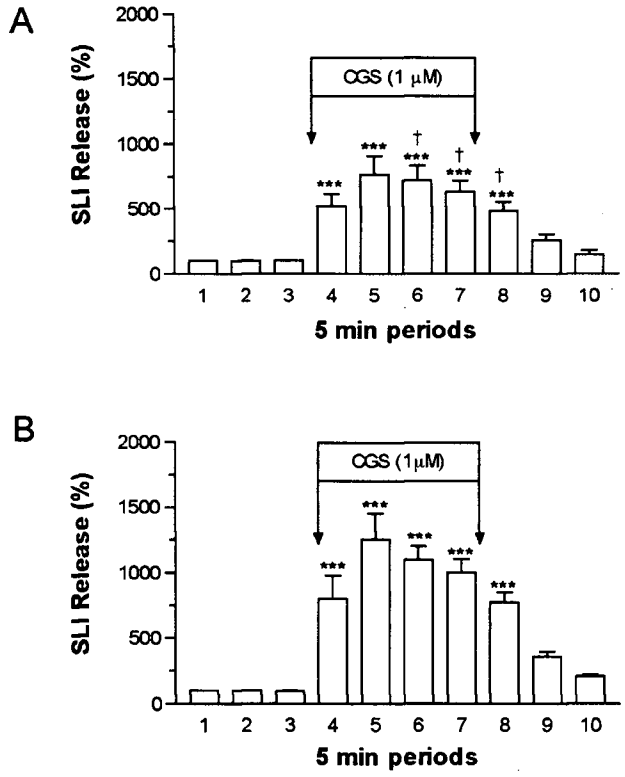


Fig. 49. Effect of CGS 21680 (CGS; 1 μM) on gastric SLI release in 3 day omeprazole-treated (A) and control (B) animals. Results are expressed as SLI release (%) as described in the Methods section. Each column represents the mean ± SEM of at least 5 experiments. ***$P < 0.001$ when compared with period 3 using repeated measures ANOVA followed by Dunnett's multiple comparison test. The SLI release during each 5 min period was compared between omeprazole-treated and control rats using the Student's unpaired t-test and found to differ significantly in periods 6, 7, and 8; $^{\dagger}P < 0.05$.

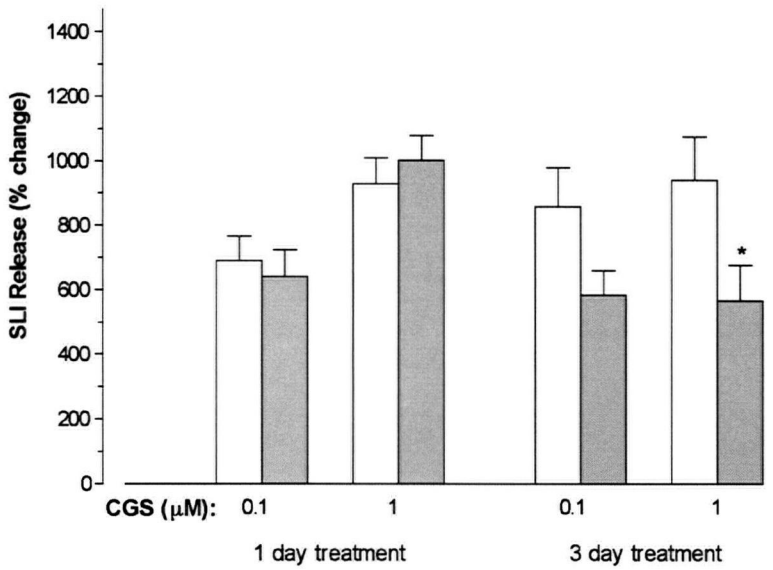


Fig. 50. Effect of omeprazole treatment on CGS 21680-induced changes in SLI release. The percentage change in SLI release between the control (empty bar) and omeprazole-treated (filled bar) animals was compared using the Student's unpaired t-test. Each column represents the mean ± SEM of at least 5 experiments. *P < 0.05.

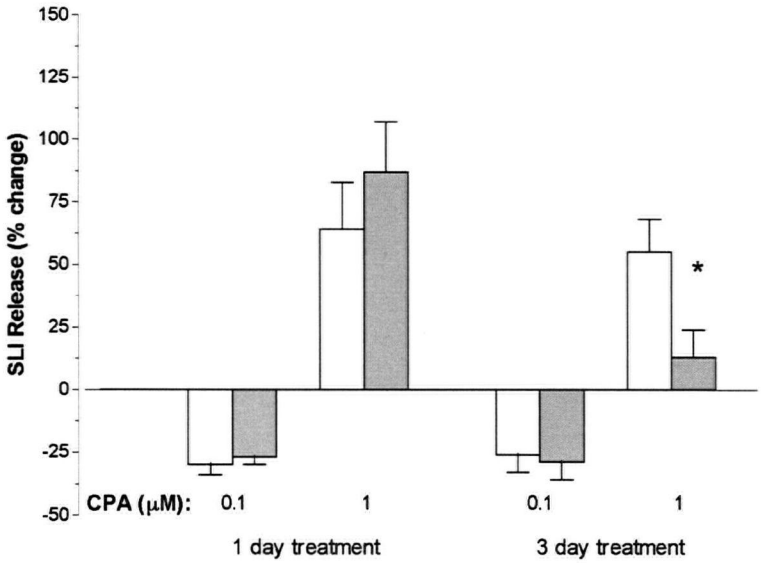


Fig. 51. Effect of omeprazole treatment on CPA-induced changes in SLI release. The percentage change in SLI release between the control (empty bar) and omeprazole-treated (filled bar) animals was compared using the Student's unpaired t-test. Each column represents the mean ± SEM of at least 5 experiments. *P < 0.05.

omeprazole treatment. However, 3 days of omeprazole treatment was shown to significantly attenuate the stimulatory effect of 1 μM CPA on SLI release (Fig. 51).

VII. Cellular localization of the gastric adenosine A_1 and A_{2A} receptors

Immunohistochemistry experiments were performed to examine the cellular localization of the A_1 and A_{2A} receptors in the rat stomach, and confocal images of various layers of the corpus and antrum were acquired. Fig. 52 shows representative confocal images obtained from the mucosa, muscularis mucosa, submucosal plexus, circular muscle, myenteric plexus, and longitudinal muscle of the rat corpus and antrum.

1. Cellular localization and distribution of A_1R-IR

Fig. 53 and 54 show that A_1R-IR is distributed throughout the corpus and antrum of the stomach. Abundant A_1R-IR cells were dispersed throughout the corporeal mucosa, except at the tips of the mucosa (Fig. 53A and B). In the antrum, A_1R-IR cells were observed along the basal region of the antral mucosa (Fig. 54A and B). Distinct A_1R-IR was also observed on cell bodies and nerve fibers of the myenteric plexus, and on nerve fibers of the submucosal plexus, circular muscle, and longitudinal muscle, on blood vessels localized throughout the stomach (Fig. 53 and 54: C & D) and on nerve fibers of the muscularis mucosae. Tissues stained with the immunoneutralized A_1 receptor antibody did not exhibit any distinct A_1R-IR (Fig. 53 and 54: E and F). Tissue sections stained with the secondary antibody, but without the prior incubation with A_1 receptor primary antibody also demonstrated a lack of immunoreactivity (Fig. 53G and 54G)

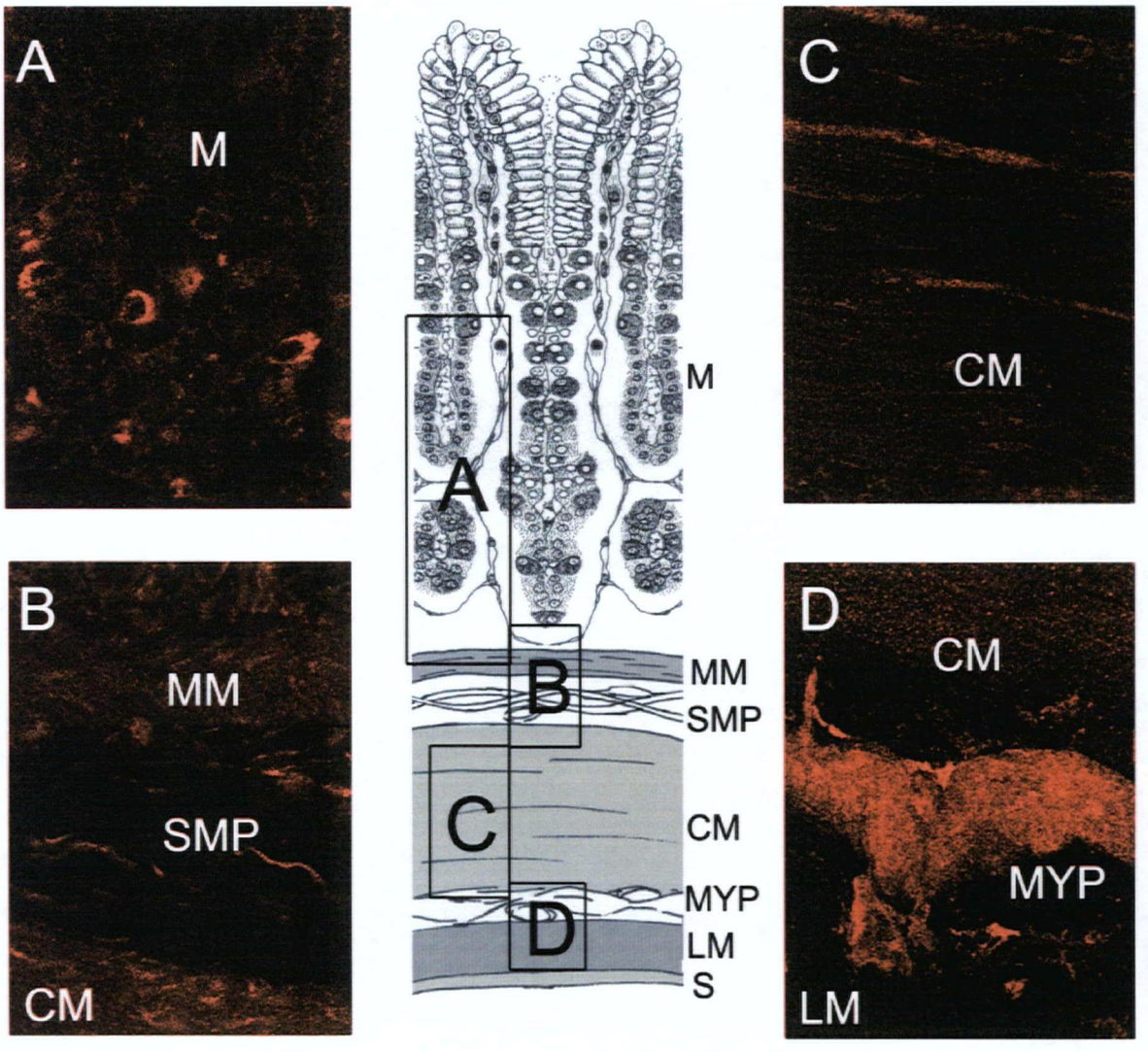


Fig. 52. Representative A_1R-IR and A_{2A}R-IR in various regions of the rat stomach. A diagram representing a cross-sectional view of the stomach wall is shown with the following layers identified: mucosa (M), muscularis mucosae (MM), submucosal plexus (SMP), circular muscle (CM), myenteric plexus (MYP), longitudinal muscle (LM), and serosa (S). Typical confocal images taken from the regions highlighted by the boxes are shown. A: A_1R-IR in the basal region of the antral mucosa; B: A_1R-IR in the submucosal plexus of the antrum; C: A_{2A}R-IR in the circular muscle of the corpus; D: A_{2A}R-IR in the myenteric region of the antrum.

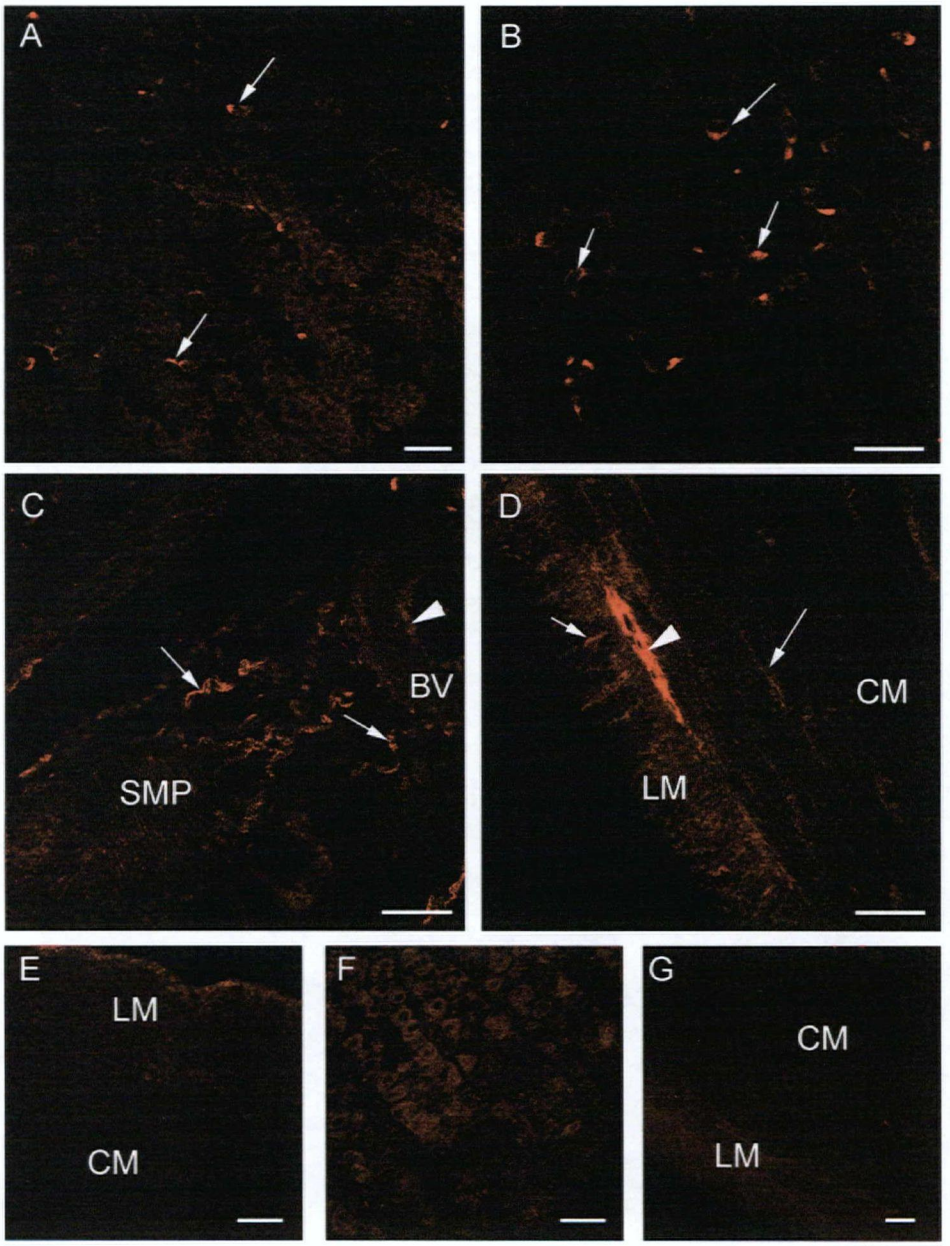


Fig. 53. Confocal images of A_1R-IR in the corpus of the rat stomach. A: A_1R-IR cells (arrows) were shown to be distributed throughout the corporeal mucosa. B: Intense A_1R-IR was observed on mucosal cells viewed under high magnification (arrows). C: A_1R-IR on endothelial cells lining a blood vessel (BV; arrowhead) and on nerve fibers (arrows) of the submucosal plexus (SMP). D: A_1R-IR on nerves fibers and cell bodies of the myenteric plexus (arrowhead), and on nerve fibers of the circular (CM) and longitudinal (LM) muscle layers (arrows) of the corpus. Tissue sections stained with the immunoneutralized A_1 receptor primary antibody in muscle (E) and mucosal (F) layers. Tissue section stained with the secondary antibody, donkey anti-rabbit IgG conjugated to Cy3, but without prior incubation with A_1 receptor primary antibody (G). (z-step = 1.0 μm, scale bars = 25 μm for all images).

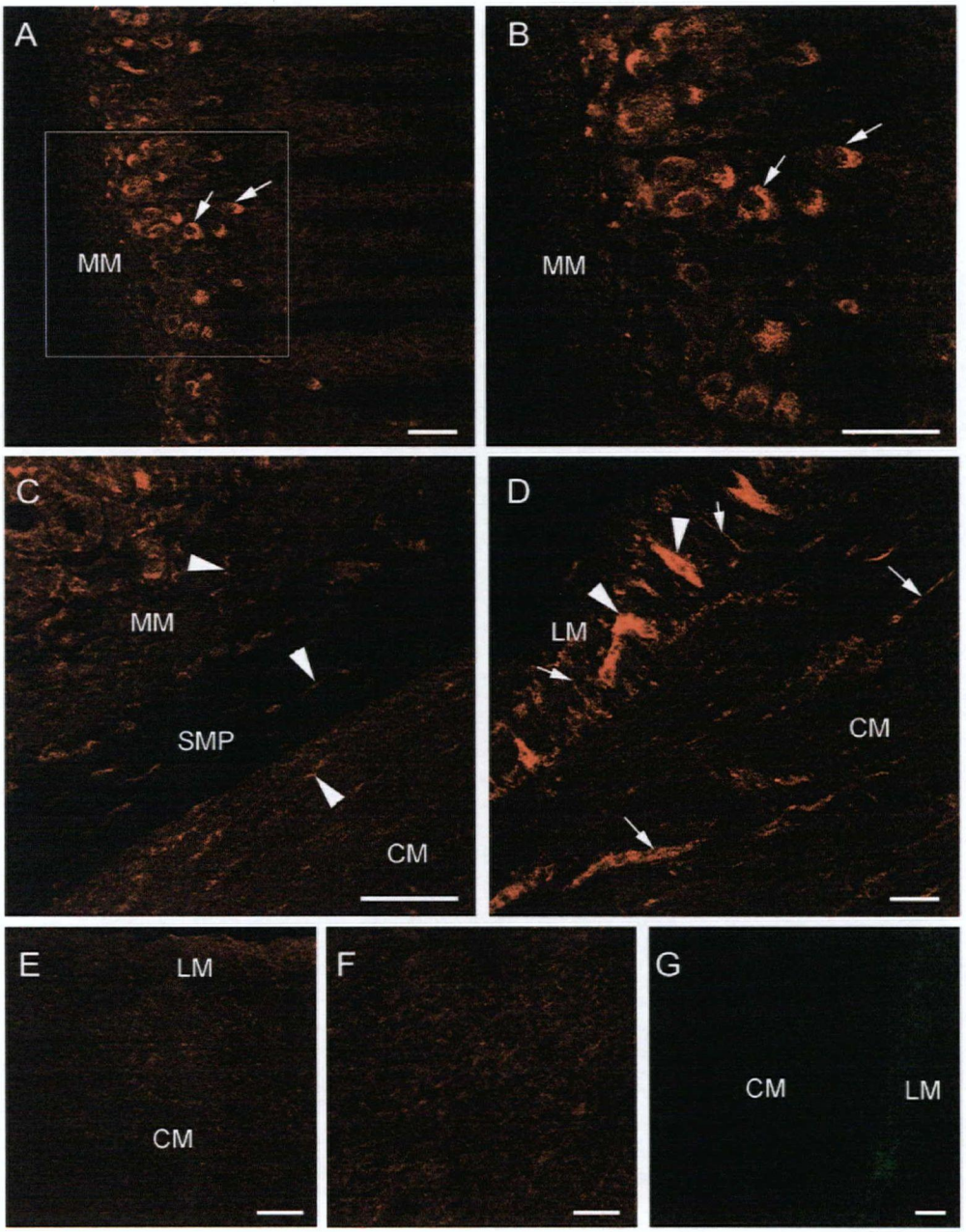


Fig. 54. Confocal images of A_1R-IR in the antrum of the rat stomach. A: A_1R-IR cells (arrows) were shown to be concentrated along the base of the antral mucosa (MM = muscularis mucosae). A high magnification image of the boxed area is shown in B. Note the punctate pattern of staining. C: A_1R-IR on nerve fibers (arrowheads) of the submucosal plexus (SMP), circular muscle (CM) and muscularis mucosae. D: A_1R-IR on nerves fibers and cell bodies of the myenteric plexus (arrowheads), and on nerve fibers of the circular and longitudinal (LM) muscle layers (arrows). Tissue sections stained with the immunoneutralized A_1 receptor primary antibody in muscle (E) and mucosal (F) layers. Tissue section stained with the secondary antibody, donkey anti-mouse IgG conjugated to Alexa Fluor 488, but without the prior incubation with A_1 receptor primary antibody (G). (z-step = 1.0 μm, scale bars = 25 μm for all images).

2. Double staining with the A_1 receptor antibody

Double staining experiments were performed to examine if the A_1 receptors are localized on gastrin-secreting G-cells. Results show that gastrin-IR was abundant throughout the base of the antral mucosa. Cells were flask-shaped and some were shown to project short processes. Double staining revealed that all gastrin-IR cells examined expressed A_1R-IR (Fig. 55). A_1R-IR was most intense in antral mucosal cells that express gastrin-IR. However, not all A_1R-IR cells contained gastrin-IR. Quantification revealed that approximately 28±2% of A_1R-IR cells in the antral mucosa also expressed gastrin-IR.

Double staining of A_1R-IR with somatostatin-IR was performed to examine whether A_1 receptors are localized on D-cells of the corpus and antrum or somatostatin-containing nerves throughout the stomach. Intense and abundant somatostatin-IR was observed in the antral and corporeal mucosa, while sparse somatostatin-IR was observed throughout the rest of the stomach. Double staining revealed that all somatostatin-IR cells of the corporeal mucosa expressed A_1R-IR (Fig. 56A-C). A_1R-IR staining was especially intense at the processes of the D-cells (Fig. 56D-F). In the antrum, only 24±2% of the somatostatin-IR cells contained A_1R-IR (Fig. 56G-I). Although a relatively large proportion of the A_1R-IR of the antral mucosa is co-localized with gastrin-IR, only 8.5±0.6 % of the A_1R-IR cells in this region was co-localized with somatostatin-IR. A_1 receptor-IR was not co-localized with somatostatin-IR in any other region of the stomach, including the myenteric plexus region, where sparse somatostatin staining was observed (see Fig. 57).

To determine whether the A_1 receptors are localized on the parietal cells, double staining was performed to detect H^+K^+ATPase β, a marker for parietal cells. Dense and

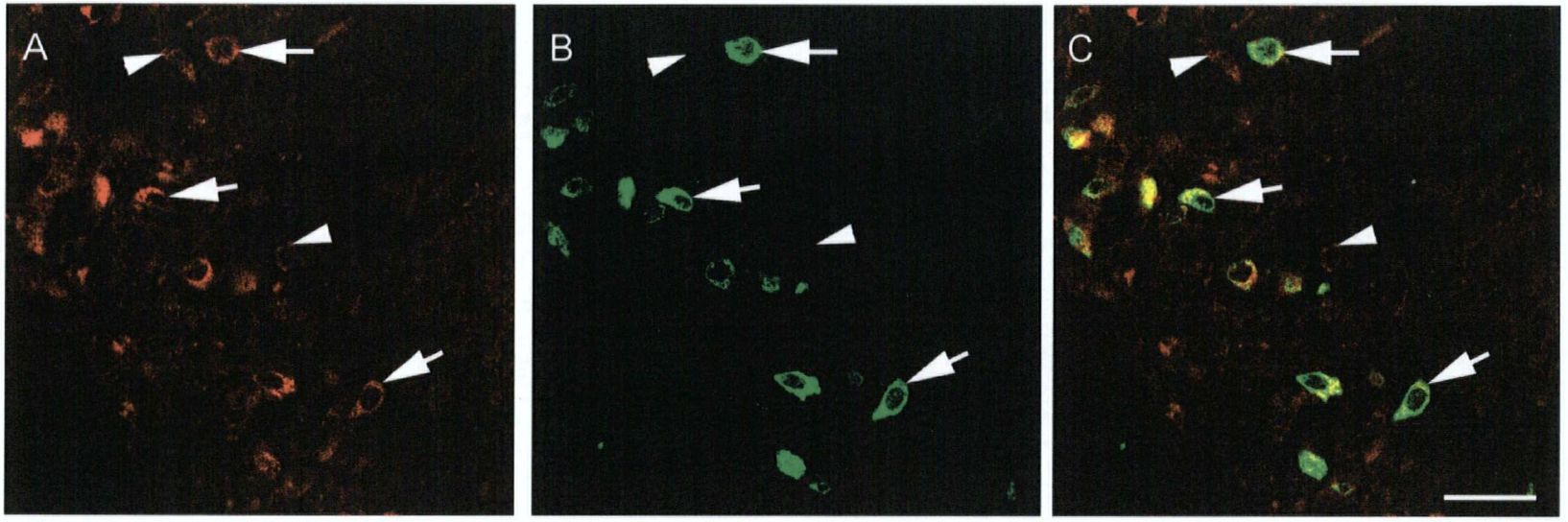


Fig. 55. Double staining of A_1R-IR with gastrin-IR. Confocal images show co-localization of A_1R-IR (A) with gastrin-IR (B) in the antral mucosa. Cells containing both immunoreactivities appear yellow in the merged image (C). All gastrin-IR cells contain A_1R-IR (arrows). A_1R-IR cells not expressing gastrin-IR are identified by arrowheads (z-step = 0.5 μm; scale bar = 25 μm for all images).

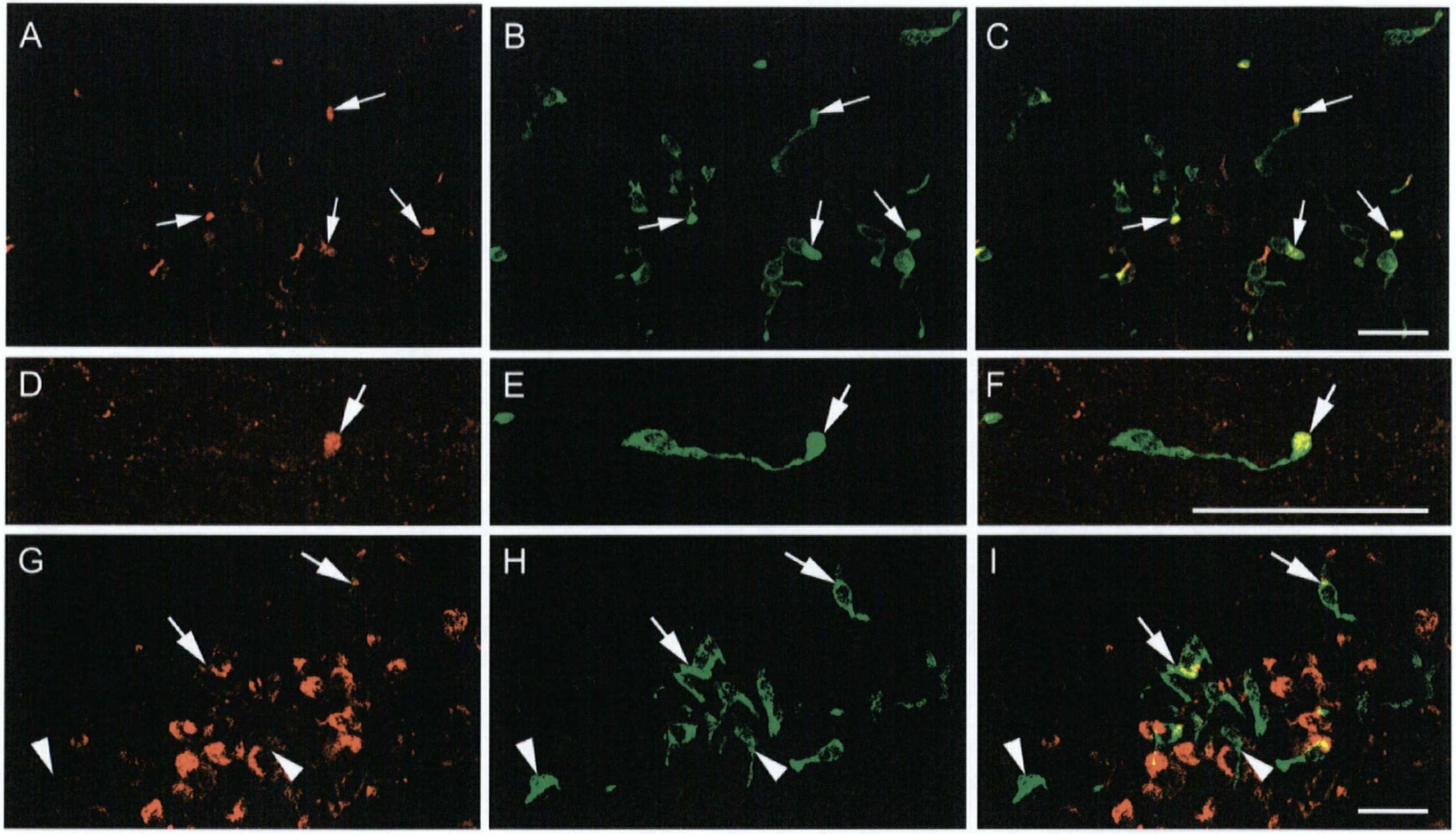


Fig. 56. Double staining of A_1R-IR with somatostatin-IR in the gastric mucosa. Confocal images show co-localization of A_1R-IR (red) with somatostatin-IR (green) in the rat gastric corporeal (A-F) and antral (G-I) mucosa. Cells expressing both A_1R-IR and somatostatin-IR are indicated by arrows and appear yellow (C, F, & I). A_1R-IR is expressed on all somatostatin-IR cells of the corpus (A-C), and appears concentrated at the processes of the D-cell (D-F). A_1R-IR is also co-localized with somatostatin-IR in some cells in the antral mucosa (G-I). Somatostatin-IR cells not expressing A_1R-IR are indicated by the arrowheads. (z-step = 0.5 μm; scale bars = 25 μm for all images).

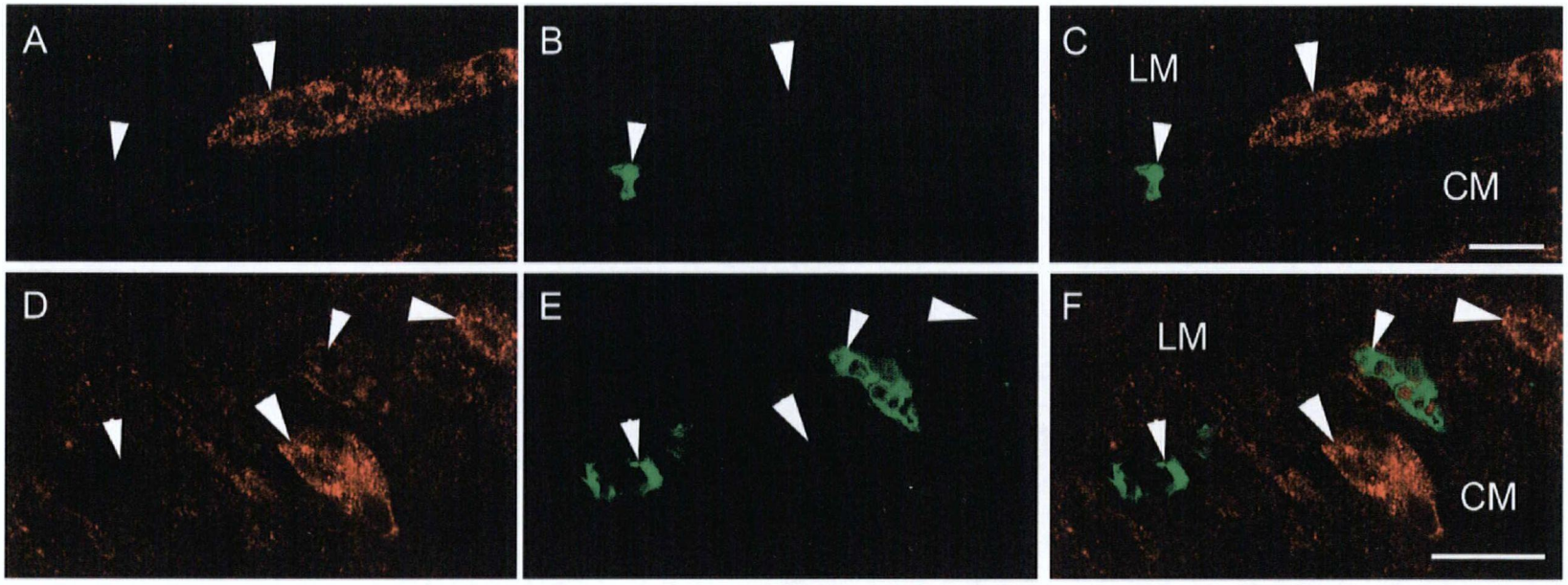


Fig. 57. Double staining of A_1R-IR and somatostatin-IR in the gastric muscle. Confocal images show the lack of co-localization of A_1R-IR (red; large arrowheads) with somatostatin-IR (green; small arrowheads) in the myenteric plexus of the rat corpus (A-C) and antrum (D-F). LM = longitudinal muscle, CM = circular muscle. (z-step of confocal images = 0.5 μm; scale bars = 25 μm).

intense H^+K^+ATPase β staining was observed throughout the corporeal mucosa. Double-staining of H^+K^+ATPase β-IR with A_1R-IR, however, demonstrated a lack of co-localization (Fig. 58).

To determine with certainty whether A_1R-IR are localized on blood vessels, double staining was performed to detect VWF, a marker for the vascular endothelial cells. A_1R-IR was found to be co-localized with VWF in both the corpus (Fig. 59A-C) and the antrum (Fig. 59D-F). However, A_1R-IR was not co-localized with VWF-IR in the mucosa, except in arterioles at the base of the mucosa (Fig. 59G-I). Careful analysis of single confocal images (z-step 1.0 μm) revealed that some A_1R-IR was also observed in close proximity to VWF-IR but was not co-localized with VWF-IR (Fig. 60). This A_1R-IR likely represents A_1 receptors on vascular smooth muscle cells.

Double staining for PGP 9.5 was performed to determine whether A_1R-IR is expressed on nerve fibers. PGP 9.5-IR was found to be co-localized with A_1R-IR on cell bodies and nerve fibers of the myenteric plexus of the corpus (Fig. 61A-C) and antrum (Fig. 61D-F). PGP-9.5-IR was also co-localized with A_1R-IR on nerve fibers throughout the muscle layers of the corpus (Fig. 61G-I) and antrum (Fig. 61J-L).

3. Cellular localization and distribution of A_{2A}R-IR

Results show that the distribution of A_{2A}R-IR was similar in the corpus and antrum (Fig. 62 and 63). In both regions, intense A_{2A} receptor staining was observed on mucosal cells (Fig. 62A and 63A), on nerve fibers of the submucosal plexus (Fig. 62B and 63B), on cell bodies and nerve fibers of the myenteric plexus, and on nerve fibers of the circular muscle layer, longitudinal muscle layer (Fig. 62C and 63C) and muscularis mucosae.

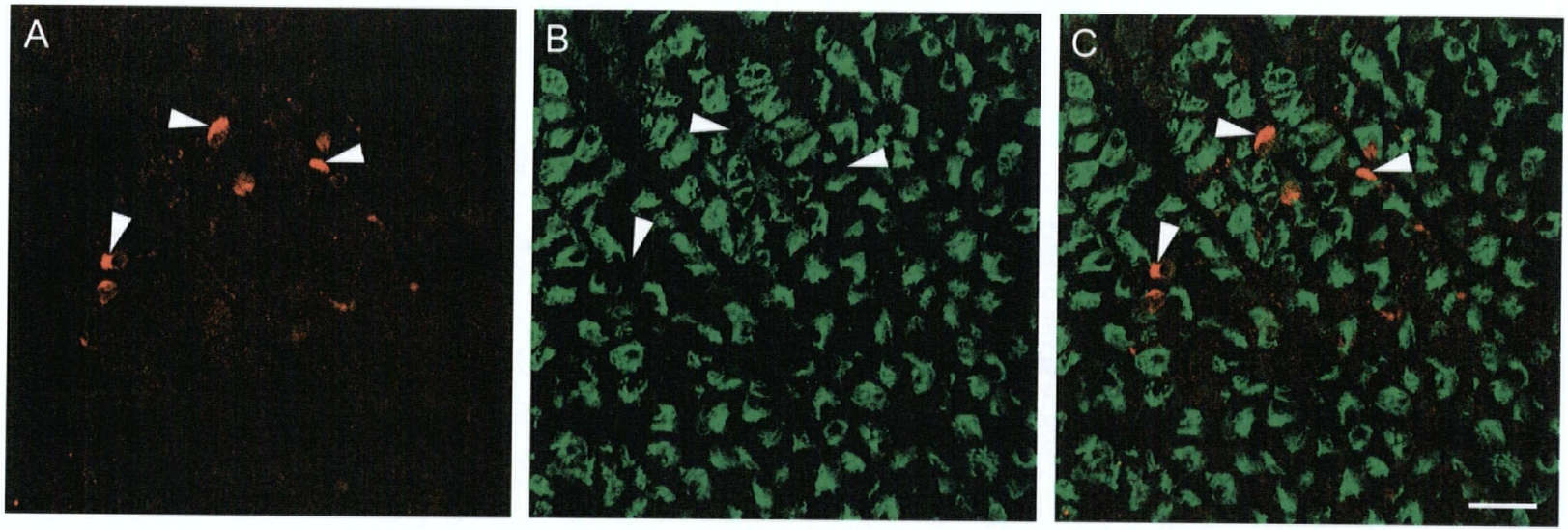


Fig. 58. Double-staining of A_1R-IR with H^+K^+-ATPase β-IR. A_1R-IR (A) is shown not to co-localize with H^+K^+-ATPase β-IR (B) in the corporeal mucosa. (z-step of confocal images = 0.5 μm; scale bar = 25 μm).

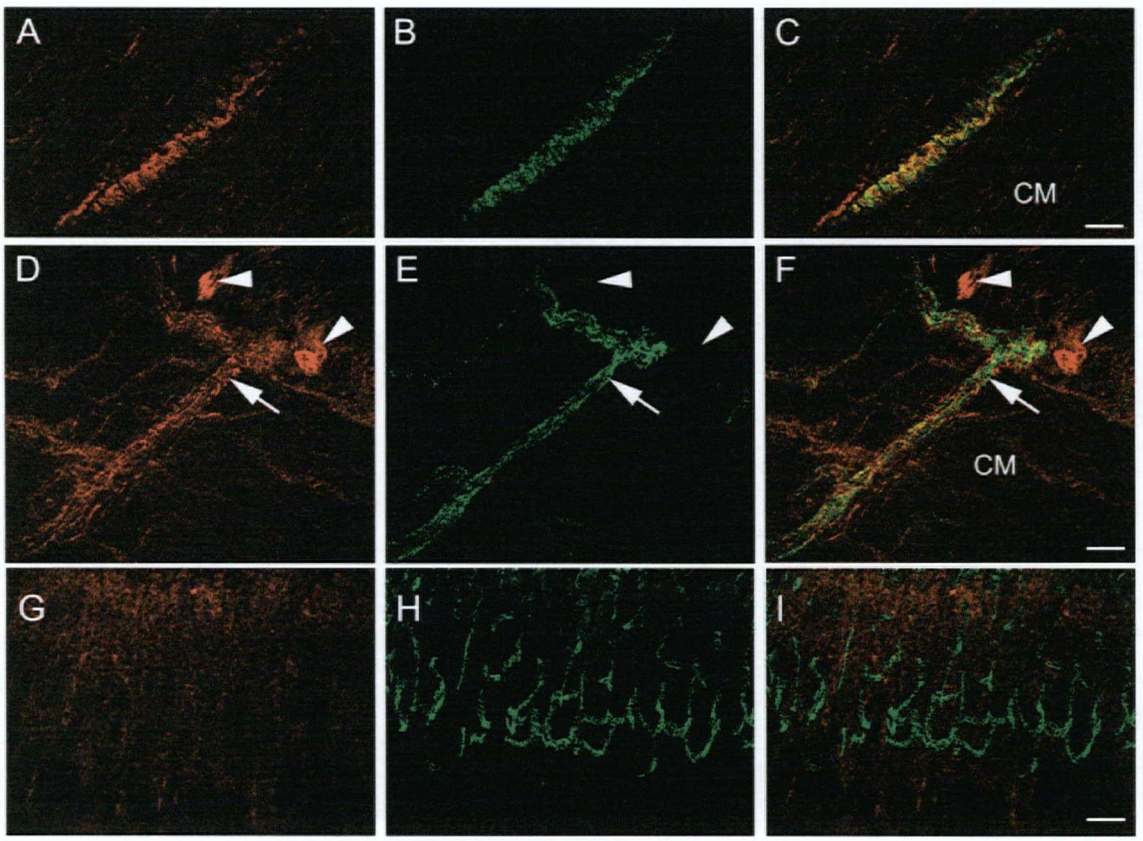


Fig. 59. Double staining of A_1R-IR (red) with VWF-IR (green). Images show the co-localization of A_1R-IR with VWF-IR in the circular muscle (CM) of the corpus (A-C) and antrum (D-F). The arrowheads (D-F) identify A_1R-IR in the myenteric plexus. Regions expressing both immunoreactivities appear yellow. A_1R-IR is not co-localized with VWF-IR in the fine capillaries of the corporeal mucosa (G-I). Images shown in A-C, D-F and G-I represent the immunoreactivity of 1, 20, and 36 stacked images (z-step = 1.0 μm), respectively. Images were stacked to adequately illustrate the immunoreactivity within the entire blood vessel or capillary bed. Scale bars = 25 μm.

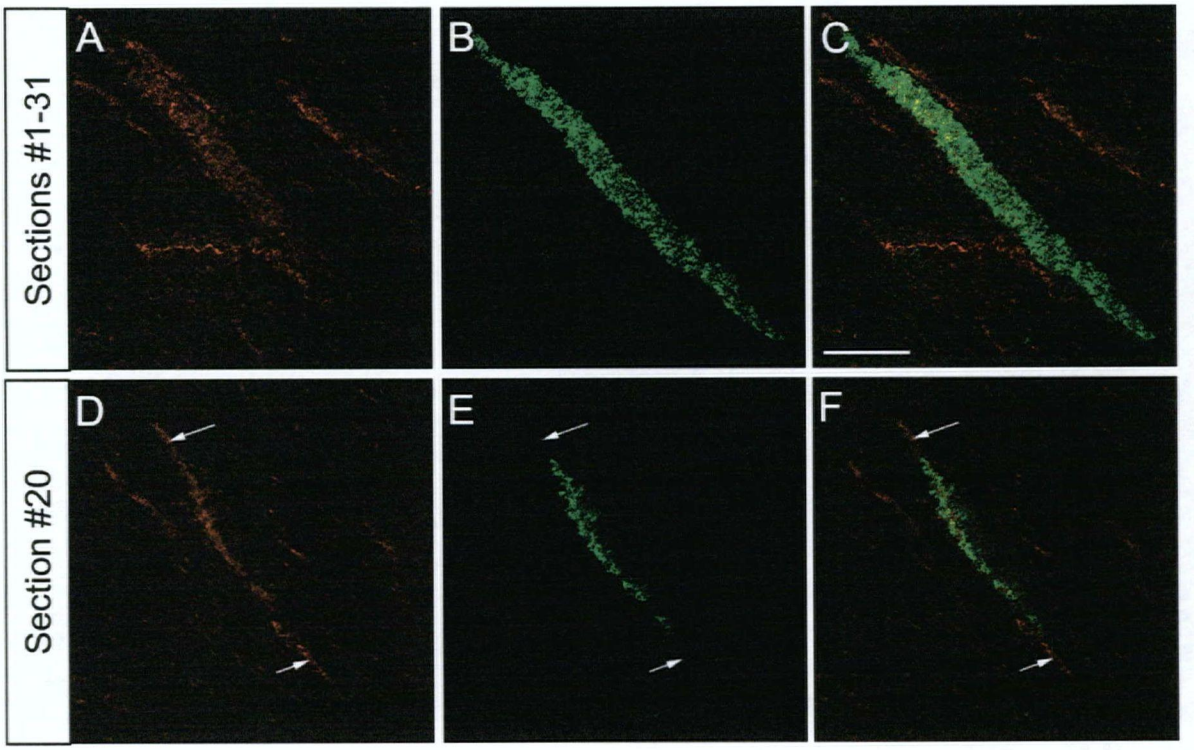


Fig. 60. Localization of A_1R-IR (red) relative to VWF-IR (green) in the corporeal muscle. Images shown in A-C and D-F represent the immunoreactivity of 31 stacked sections, and a single section (z-step = 1.0 μm), respectively. A_1R-IR is co-localized with VWF-IR, and is also found adjacent to VWF-IR. The A_1R-IR indicated by the arrows on panel D may represent A_1R localized on on vascular smooth muscle cells. (Scale bars = 50 μm).

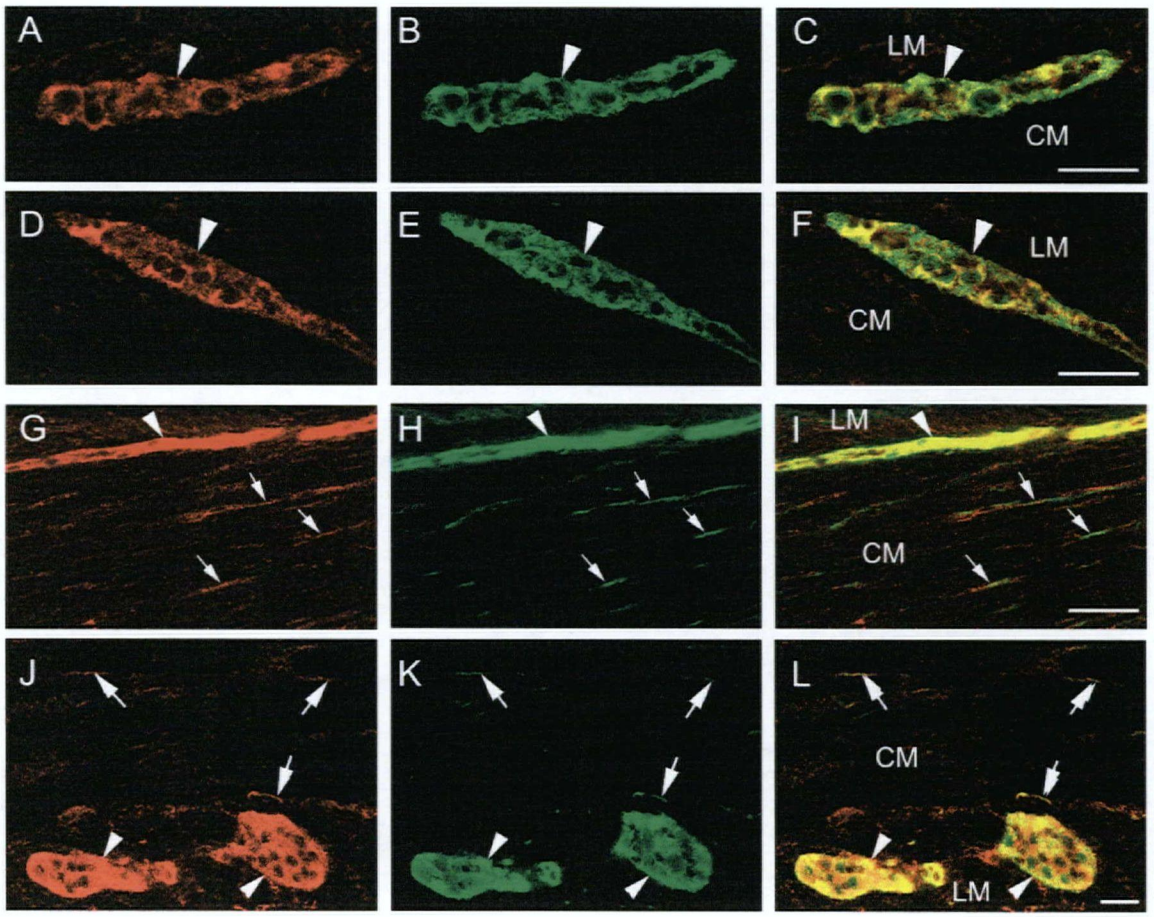


Fig. 61. Double staining of A_1R-IR (red) with PGP 9.5-IR (green). Confocal images show the co-localization of both immunoreactivities in the myenteric plexus of the corpus (C and I, arrowheads) and antrum (F and L, arrowheads), and in the circular muscle of the corpus (I) and antrum (L). Cells expressing both immunoreactivities appear yellow in the merged images. (z-step = 0.5 μm for all images, scale bars = 25 μm).

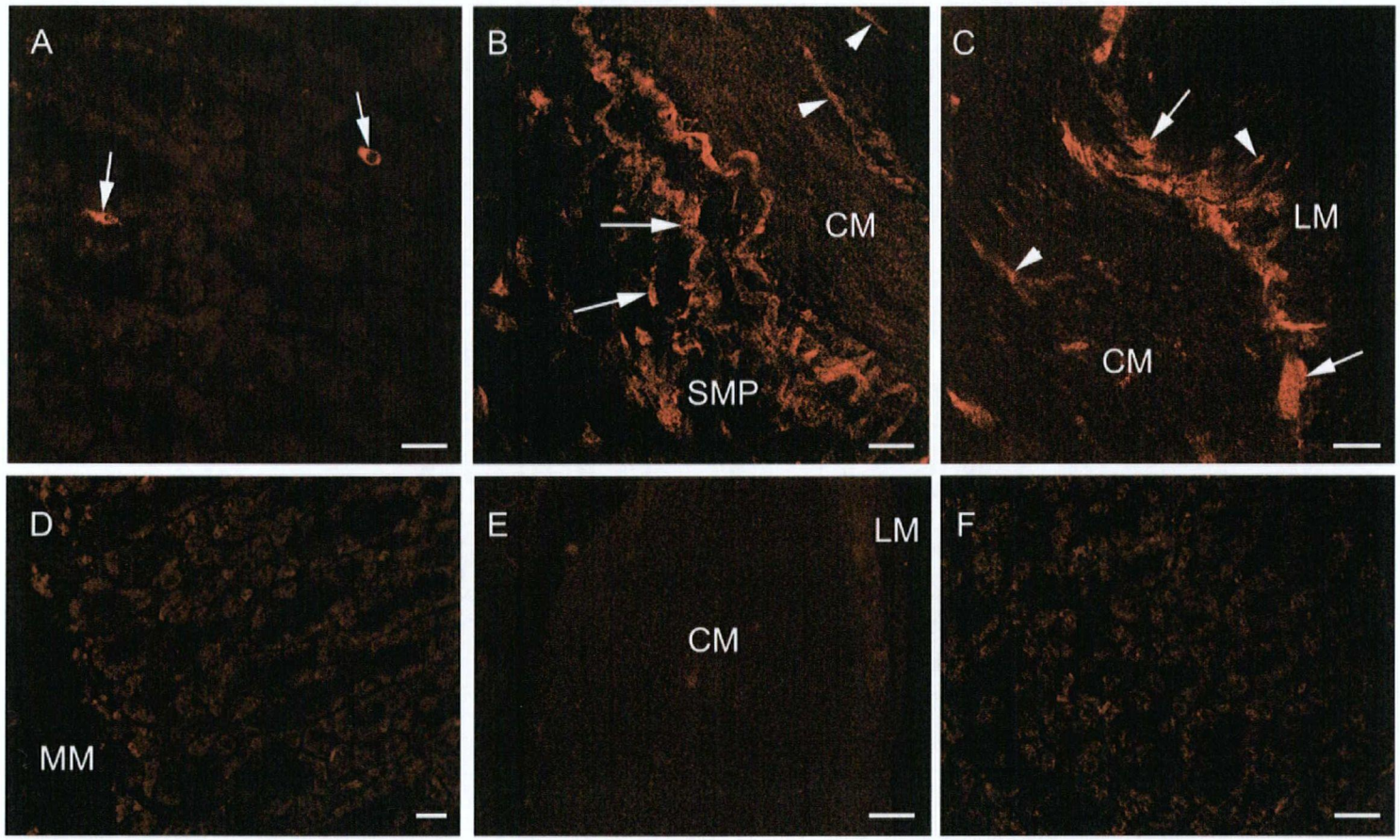


Fig. 62. Confocal images of A_{2A}R-IR in the corpus of the rat stomach. A: A_{2A}R-IR cells (arrows) were distributed throughout the corporeal mucosa. B: A_{2A}R-IR on nerve fibers in the submucosal plexus (SMP, arrows) and circular muscle (CM, arrowheads). C: A_{2A}R-IR on nerves fibers and cell bodies of the myenteric plexus (arrows), and on nerve fibers of the circular and longitudinal (LM) muscle layers (arrowheads). Tissue sections stained with the immunoneutralized A_{2A} receptor primary antibody in the mucosa (D) and muscle (E) layers. Tissue section stained with the secondary antibody, donkey anti-rabbit IgG conjugated to Cy3, but without prior incubation with A_{2A} receptor primary antibody (F). (z-step = 1.0 μm, scale bars = 25 μm for all images).

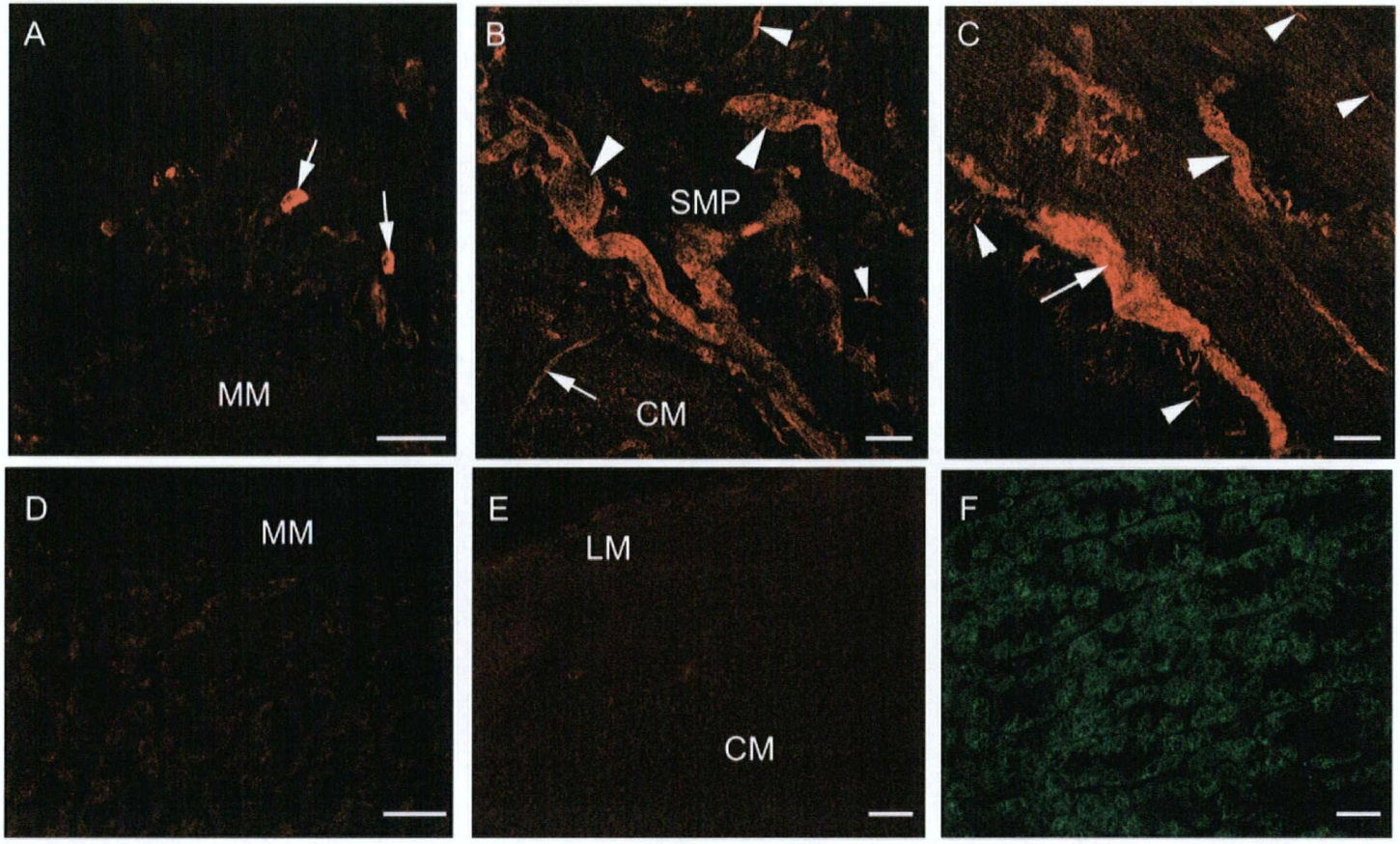


Fig. 63. Confocal images of A_{2A}R-IR in the antrum of the rat stomach. A: A_{2A}R-IR cells (arrows) were distributed throughout the antral mucosa, MM = muscularis mucosae. B: A_{2A}R-IR on blood vessels (large arrowheads) and nerve fibers (small arrowheads) of the submucosal plexus (SMP), and on nerve fibers of the circular muscle (CM; arrows). C: A_{2A}R-IR on nerves fibers and cell bodies of the myenteric plexus (arrows), on nerve fibers of the circular (CM) and longitudinal (LM) muscle layers (small arrowheads) and on blood vessels of the circular muscle (large arrowheads). Tissue sections stained with the immunoneutralized A_{2A} receptor primary antibody in the mucosal (D) and muscle (E) layers. Tissue section stained with the secondary antibody, donkey anti-mouse IgG conjugated to Alexa Fluor 488, but without prior incubation with A_{2A} receptor primary antibody (F). (z-step = 1.0 μm, scale bars = 25 μm for all images).

A_{2A}R-IR was also observed on blood vessels of both the corpus and antrum. The specificity of the A_{2A} receptor antibody was confirmed by immunoneutralization. Tissues stained with the immunoneutralized A_{2A} receptor antibody were shown not to exhibit A_{2A}R-IR (Fig. 62 and 63: D and E). Tissue sections stained with the secondary antibody, but without the prior incubation with A_{2A} receptor primary antibody also demonstrated a lack of A_{2A}R-IR immunoreactivity (Fig. 62F and 63F).

4. Double staining with the A_{2A} receptor antibody

Double staining was performed to examine if the A_{2A} receptors are localized on the D-cells, G-cells, parietal cells, neuronal cells and vascular endothelial cells. Somatostatin-IR was abundant in the mucosa, but sparse in other layers of the stomach. Co-localization of A_{2A}R-IR with somatostatin-IR was frequently observed on both corporeal (Fig. 64A-C) and antral mucosal cells (Fig. 64D-F). Quantification of this co-localization demonstrated that 33±4% and 32±8% of A_{2A}R-IR cells expressed somatostatin-IR in the corporeal and antral mucosa, respectively. On rare occasions, A_{2A}R-IR was also shown to co-localize with somatostatin-IR in the myenteric plexus and muscle layers of the corpus (Fig. 65) and antrum (Fig. 66). The co-localization of A_{2A}R-IR with gastrin-IR was not observed in any region of the antral mucosa (Fig. 67). A_{2A}R-IR was also not co-localized with H^+K^+ATPase β-IR in the corporeal mucosa (Fig. 68). However, extensive co-localization of A_{2A}R-IR with VWF-IR was observed throughout the corporeal and antral muscle layers, and the myenteric and submucosal plexi (Fig. 69). A_{2A}R-IR was not co-localized with VWF-IR in the gastric mucosa. Analysis of single confocal images (z-step 1.0 μm) also reveals that A_{2A}R-IR is localized adjacent to VWF-IR with a pattern similar to that of VWF-IR (Fig. 70). This

staining may represent A_{2A}R-IR on vascular smooth muscle cells. Double staining of A_{2A}R-IR with PGP 9.5-IR was also observed in cell bodies and nerve fibers of the myenteric plexus, nerve fibers of the submucosal plexus, circular and longitudinal muscle layers, and muscularis mucosae (Fig. 71).

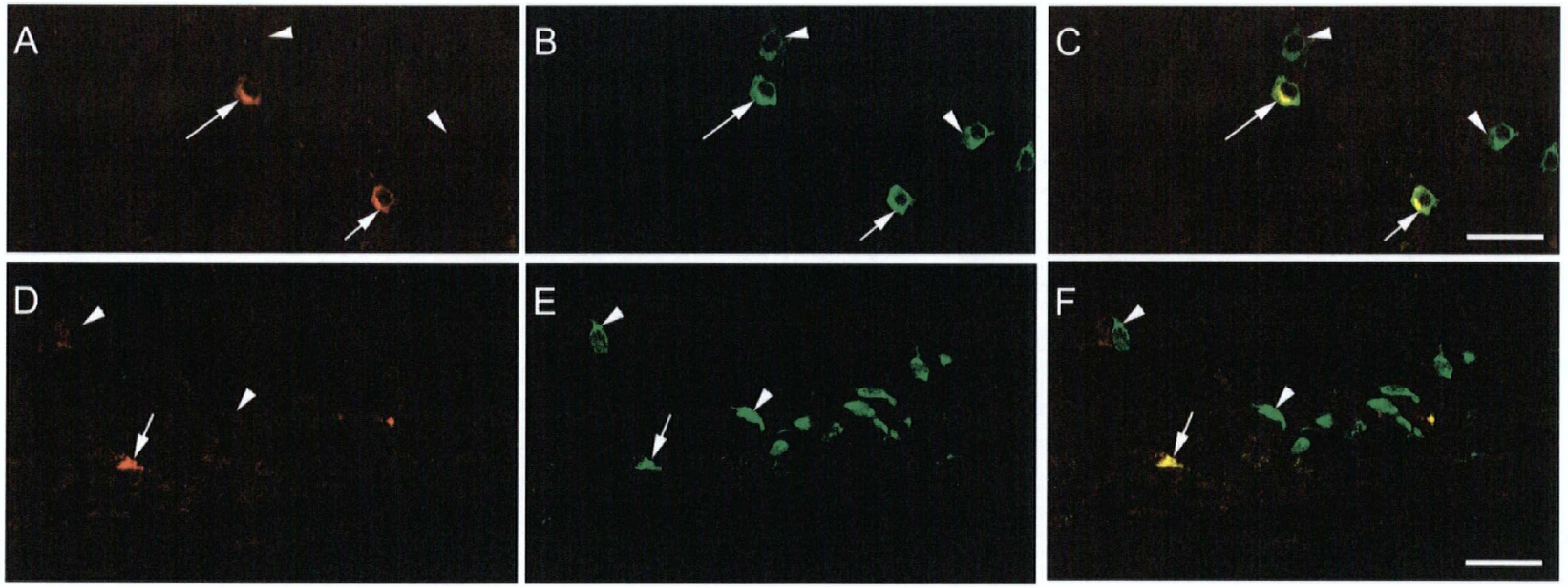


Fig. 64. Double staining of A_{2A}R-IR with somatostatin-IR in the gastric mucosa. Confocal images showing the co-localization of A_{2A}R-IR (red) with somatostatin-IR (green) in the rat corporeal (A-C) and antral (D-F) mucosa. Cells expressing both A_{2A}R-IR and somatostatin-IR are indicated by arrows and appear yellow (C & F). Somatostatin-IR cells not expressing A_{2A}R-IR are indicated by the arrowheads. (z-step = 0.5 μm, scale bars = 25 μm for all images).

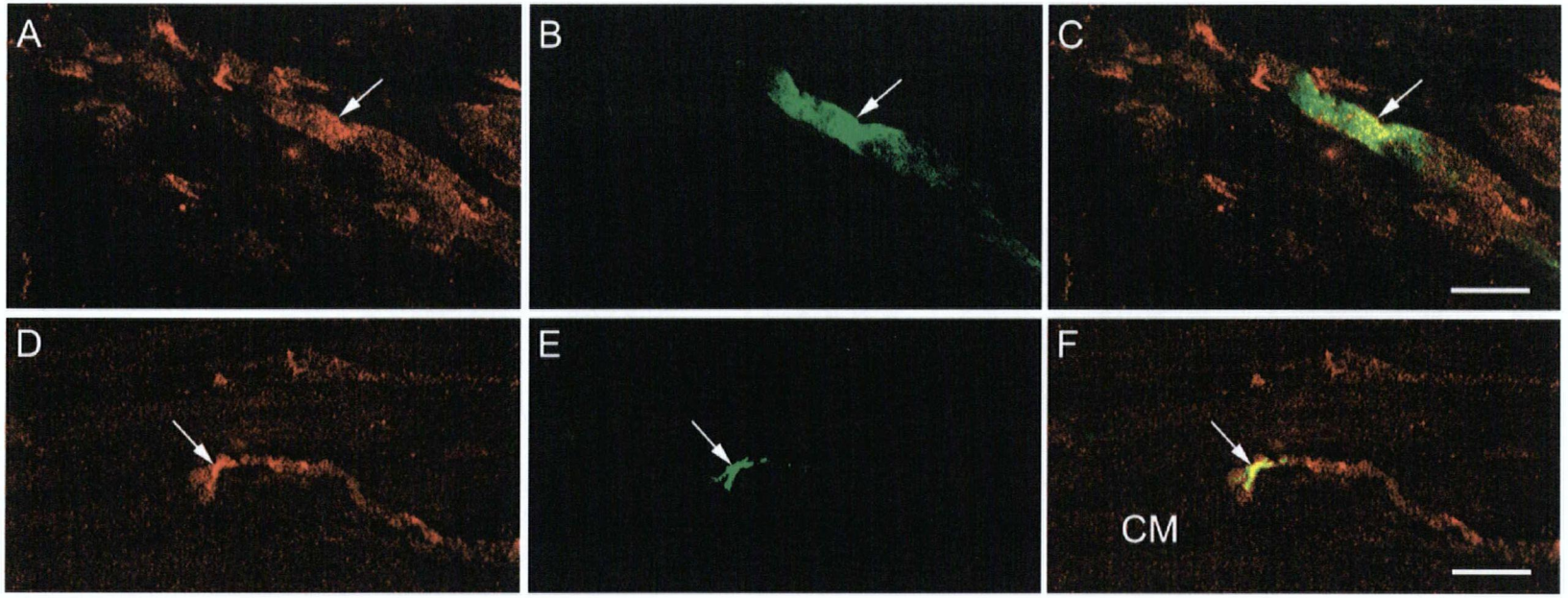


Fig. 65. Double staining of A_{2A}R-IR with somatostatin-IR in the gastric muscle of the corpus. Confocal images show co-localization of A_{2A}R-IR (red) with somatostatin-IR (green) in the myenteric plexus (A-C) and circular muscle (D-F) of the rat corpus. Co-localization does occur (yellow; arrows); however, this was only observed occasionally. CM = circular muscle. (z-step = 0.5 μm, scale bars = 25 μm).

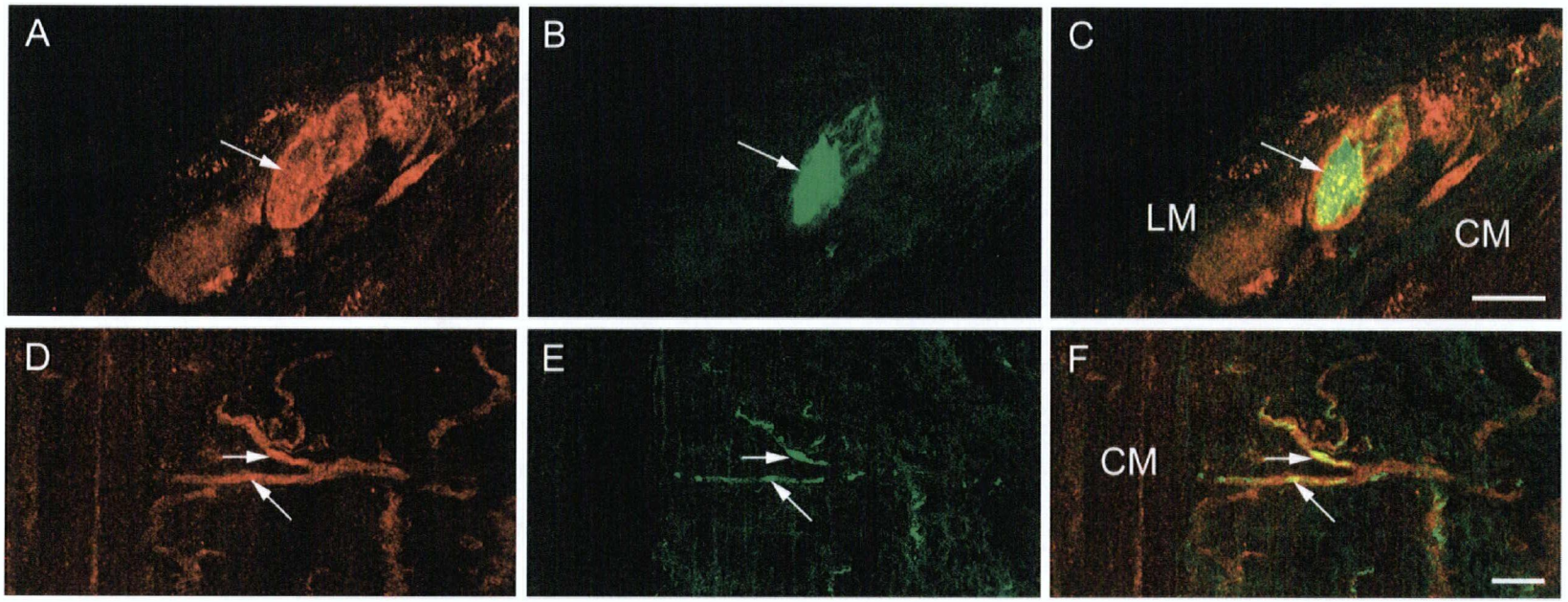


Fig. 66. Double staining of A_{2A}R-IR with somatostatin-IR in the gastric muscle of the antrum. Confocal images show co-localization of A_{2A}R-IR (red) with somatostatin-IR (green) in a cell body of the myenteric plexus (A-C) and nerve fibers in the circular muscle (D-F) of the rat antrum. Co-localization was shown to occur (yellow), however this was not frequent. LM = longitudinal muscle, CM = circular muscle. (z-step = 0.5 μm, scale bars = 25 μm).

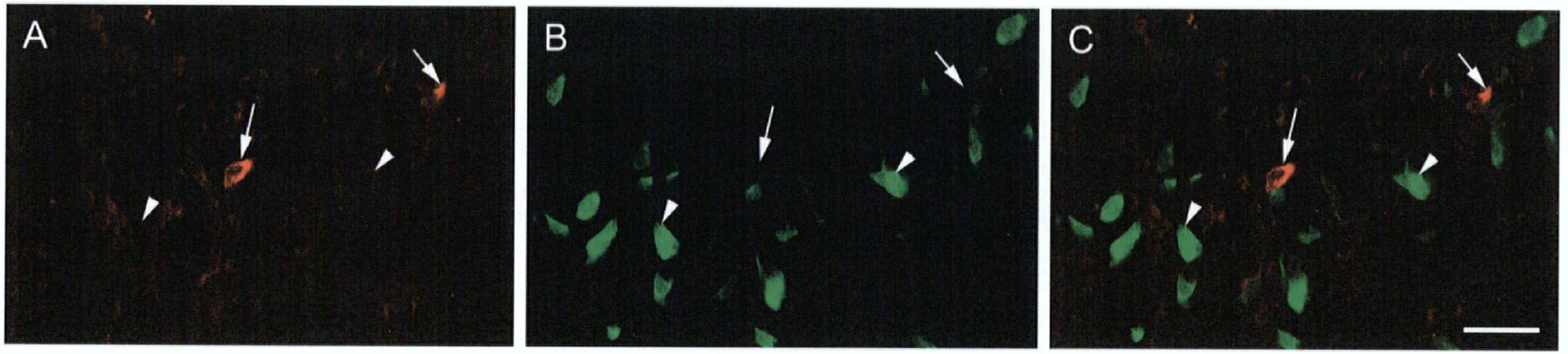


Fig. 67. Double staining of A_{2A}R-IR with gastrin-IR. Confocal images show the lack of co-localization of A_{2A}R-IR (A) with gastrin-IR (B) in the antrum. Cells containing A_{2A}R-IR (arrows) do not express gastrin-IR (arrowheads). (z-step = 0.5 μm; scale bars = 25 μm).

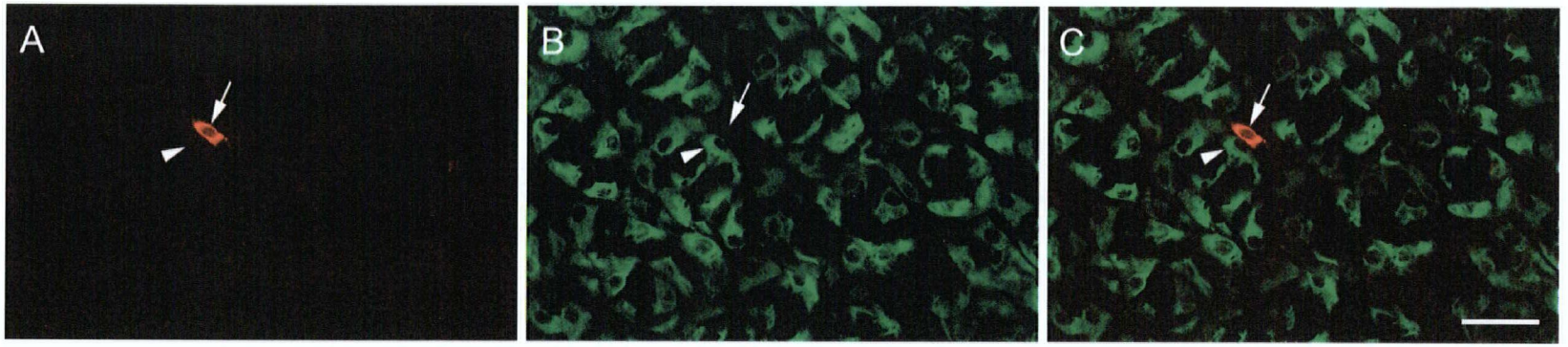


Fig. 68. Double-staining of A_{2A}R-IR (red) with H^+K^+-ATPase β-IR (green). A_{2A}R-IR (A; arrow) is shown not to co-localize with H^+K^+-ATPase β-IR (B; arrowhead) in the corporeal mucosa. (z-step = 0.5 μm; scale bar = 25 μm).

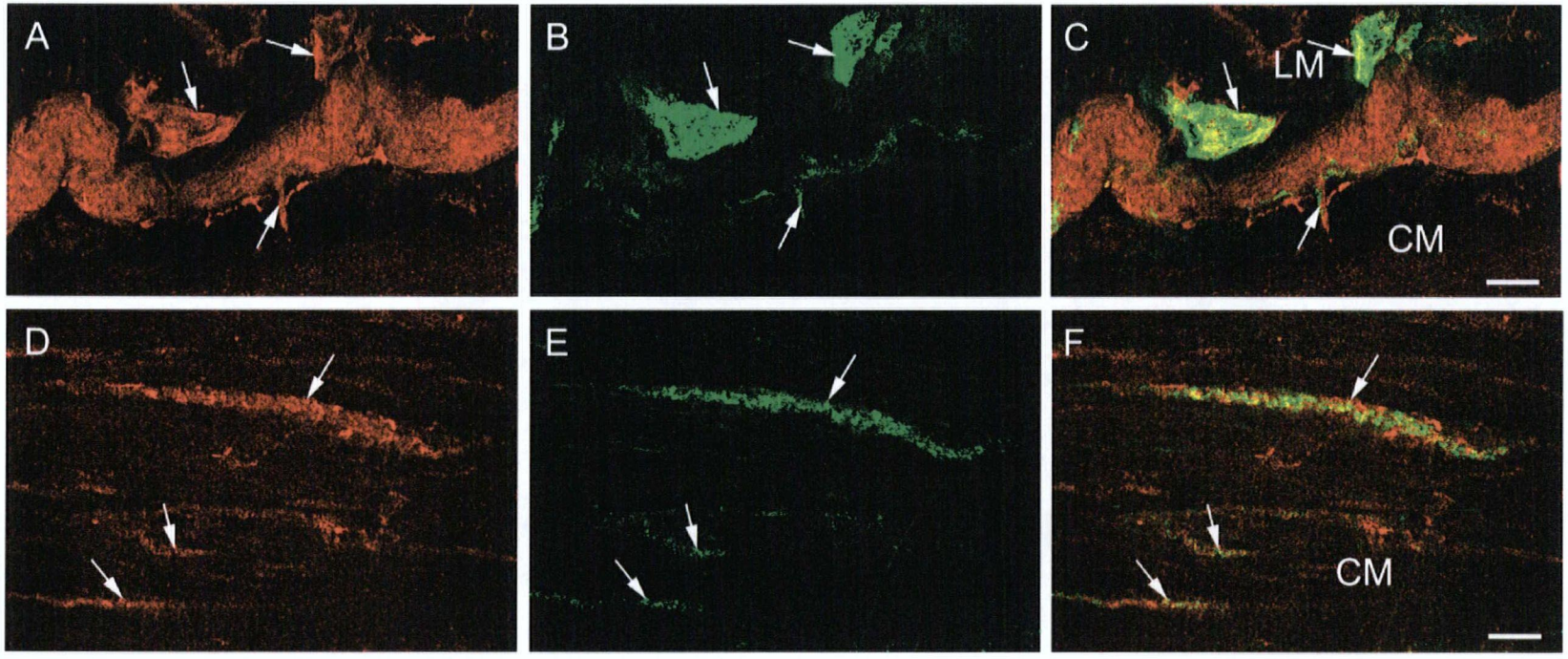


Fig. 69. Double staining of A_{2A}R-IR (red) with VWF-IR (green). Confocal images show the co-localization of A_{2A}R-IR with VWF-IR in the myenteric plexus region of the corpus (A-C; arrows) and in the circular muscle of the antrum (D-F; arrows). Regions expressing both immunoreactivities appear yellow. (z-step = 1 μm; scale bars = 25 μm).

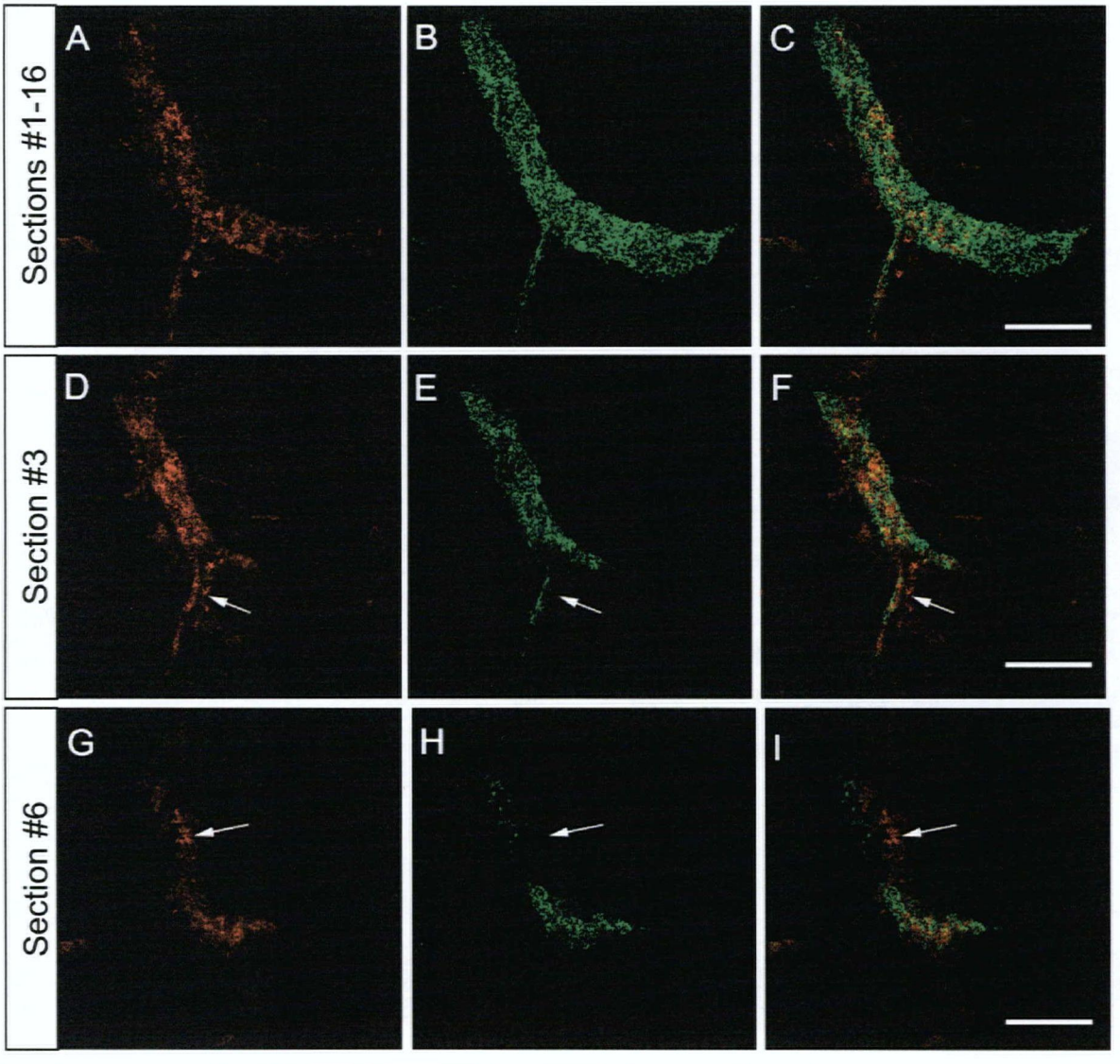


Fig. 70. Localization of A_{2A}R-IR relative to VWF-IR in the corporeal muscle. Images shown in A-C represent the immunoreactivity of 16 stacked sections. Images shown on D-F and G-I represent the immunoreactivity taken from a single section (z-step = 1.0 μm). A_{2A}R-IR (red) is localized with VWF-IR (green), and is also found to be expressed in close proximity to VWF-IR. The A_{2A}R-IR indicated by the arrows on panel D and G may represent A_{2A} receptors localized on on vascular smooth muscle cells. (scale bar s= 50 μm).

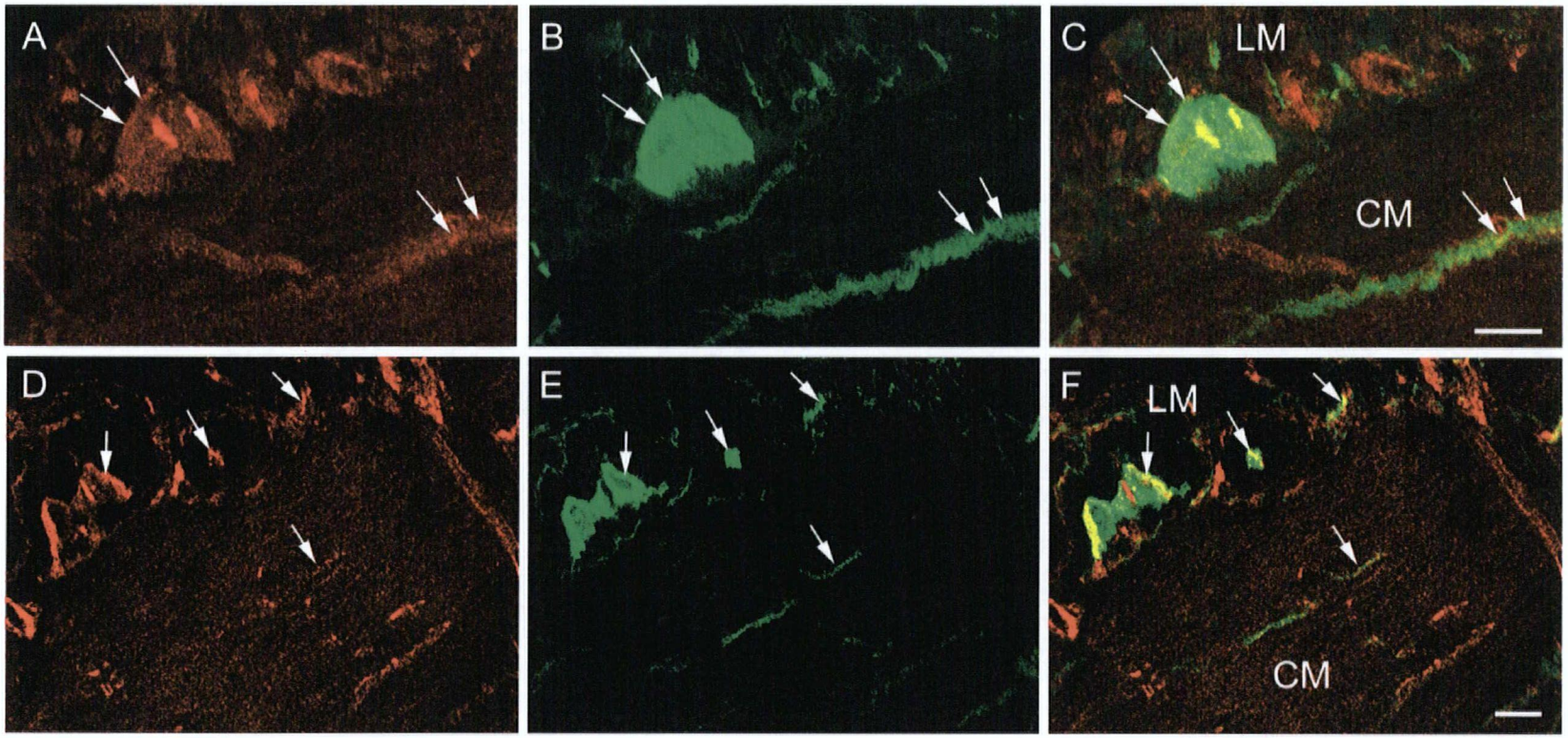


Fig. 71. Double staining of A_{2A}R-IR (red) with PGP 9.5-IR (green). Confocal images show the co-localization of A_{2A}R-IR with PGP 9.5-IR in nerve fibers of the myenteric plexus and circular muscle of the corpus (A-C) and antrum (D-F) (arrows). Cells expressing both immunoreactivities appear yellow (C and F). (CM = circular muscle; LM= longitudinal muscle; z-step = 0.5 μm; scale bars = 25 μm.).

Chapter 4: Discussion

This study is the first to establish the significant roles of adenosine A_1 and A_{2A} receptors in modulating gastric somatostatin and gastrin release. Biological and immunohistochemical results suggest that adenosine may inhibit IRG release, at least in part, by activating A_1 receptors on the G-cells and may alter SLI release by activating both A_1 and A_{2A} receptors on the D-cells. Results of the molecular biological experiments further demonstrate that the adenosine A_1 and A_{2A} receptors are structurally identical to those in the brain, and are expressed in all functionally and morphologically distinct regions of the stomach. The expression and function of these receptors can be altered by different physiological states of the stomach, suggesting that adenosine receptor expression may be changed under different gastric secretory states. This discussion is organized into the following sections: Section I, the adenosine receptor subtypes involved in regulating gastric IRG and SLI release; Section II, the distribution of adenosine receptor mRNA in functionally distinct regions of the stomach and the structure of the gastric adenosine receptors; Section III, the quantitative RT-PCR techniques developed to measure gastric adenosine receptor gene expression; Section IV, the changes in A_1 and A_{2A} receptor gene expression and function by alterations in the physiological states of the stomach; Section V, the significant implications of the cellular localization and distribution of gastric A_1 and A_{2A} receptor, as illustrated by immunohistochemistry experiments.

I. Alteration of IRG and SLI release by selective adenosine analogs

While the effect of adenosine on inhibiting IRG release (DeSchryver-Kecskemeti et al., 1981; Harty and Franklin, 1984; Kwok et al., 1990) and stimulating SLI release (Kwok et al., 1990) has been described, the adenosine receptor subtypes involved have not been identified with certainty. The present study, therefore, was performed to elucidate the adenosine receptor(s) involved in these actions. Using adenosine receptor subtype-selective analogs, the present study demonstrates that the inhibition of IRG release was mediated by the activation of adenosine A_1 receptors, while the release of SLI was inhibited and stimulated by the activation of the A_1 and A_{2A} receptors, respectively.

1. Effect of adenosine receptor-selective analogs on IRG release

To determine the adenosine receptor subtype involved in the inhibition of IRG release, the effect of various adenosine analogs was examined in the isolated vascularly perfused rat stomach preparation. This preparation has been used in our earlier investigation of the effect of adenosine on IRG release (Kwok et al., 1990). Adenosine was found to suppress IRG release concentration-dependently. In the present study, only the administration of CPA significantly inhibited IRG release. CPA is a potent A_1 receptor agonist that exhibits a greater binding affinity for the A_1 receptor than the A_2 and A_3 receptors (Klotz et al., 1998; Ji and Jacobson, 1999). The administration of CGS 21680 and IB-MECA did not significantly alter basal IRG release. CGS21680 is a potent A_{2A} receptor agonist, which exhibits a 140-fold selectivity for A_{2A} receptors over A_1 receptors (Hutchison et al., 1989), and IB-MECA is a potent A_3 receptor agonist (Gallo-Rodriguez et al., 1994) that exhibits a greater affinity for A_3 receptors than A_1 and A_2 receptors. CPA significantly

inhibits basal IRG release starting at a concentration of 1 nM. However, CGS 21680 and IB-MECA were unable to elicit an effect even at a concentration of 100 nM. At this concentration, both analogs have been shown to potently elicit various A_{2A} and A_3 receptor-mediated actions in the rat (Hutchison et al., 1989; Zucchi et al., 2001). The potent inhibitory effect of CPA and the lack of effect of CGS 21680 and IB-MECA suggest that the A_1 receptor is likely the subtype involved in modulating IRG release.

To further test this hypothesis, the effect of selective A_1 and A_2 receptor antagonists on adenosine-induced inhibition of IRG release was examined. DPCPX, a selective A_1 receptor antagonist that exhibits a 700-fold preference for the A_1 receptor over the A_2 receptor (Bruns et al., 1987), completely abolished the inhibition of IRG release induced by adenosine. DMPX, an A_2 receptor-selective antagonist (Seale et al., 1988), however, did not alter either basal or adenosine-induced IRG release. The complete blockade of the inhibitory effect of adenosine by DPCPX and the lack of an effect by DMPX further support the suggestion that the A_1 receptor subtype is responsible for the inhibition of IRG release.

Results of these experiments do not identify the site at which adenosine acts to produce its inhibitory effect, but several sites are suggested. Using immunohistochemistry, the present study demonstrates that A_1 receptors were localized on nerve fibers throughout the enteric plexi and on gastrin-containing G-cells. In the enteric plexi, Christofi and co-workers have suggested that these receptors reside in presynaptic nerve terminals and function to modulate neurotransmitter release (Christofi et al., 1992). In addition, adenosine has been shown to inhibit the release of acetylcholine and noradrenaline in the guinea pig enteric plexi (Barajas-Lopez et al., 1991; Christofi et al., 1992), most likely through activation of A_1 receptors at the presynaptic nerve terminal (Nitahara et al., 1995). In the

isolated vascularly perfused rat stomach, IRG release is regulated by cholinergic and adrenergic agents (Koop et al., 1982, 1983). Thus, adenosine may regulate IRG release indirectly by modulating neurotransmitter release in the enteric plexi.

Evidence also suggests that adenosine may regulate IRG release by acting directly on the G-cells. In rat antral mucosal fragments, adenosine inhibits basal and carbachol-stimulated gastrin release (Harty and Franklin, 1984). Adenosine has also been shown to inhibit forskolin-stimulated gastrin release in isolated canine G-cells (Schepp et al., 1990). Furthermore, the use of subtype-selective analogs specifically implicated the A_1 receptors in mediating this inhibitory effect (Schepp et al., 1990). Additional support for a direct action on G-cells is provided by the present immunohistochemistry experiments which demonstrate intense A_1 receptor staining on all antral G-cells of the rat stomach. The presence of adenosine A_{2A} receptors on G-cells was not detected. This observation lends support to the biological experiments, which demonstrated the lack of effect of CGS 21680 on IRG release.

In summary, these results clearly show that the adenosine A_1 receptor subtype is involved in suppressing IRG release in the rat. In addition, I propose that adenosine may regulate IRG release directly by acting on the G-cells, and/or indirectly by modulating neurotransmitter release in the enteric plexi.

2. Effect of adenosine receptor-selective analogs on gastric SLI release

The adenosine receptor subtype(s) involved in regulating SLI release was also examined by studying the effect of various adenosine analogs. In the isolated vascularly perfused rat stomach, our laboratory has demonstrated that adenosine may inhibit or augment gastric SLI release depending on the concentration used (Kwok et al., 1990). Results of the present study suggest that the stimulatory effect of adenosine is most likely mediated by

activation of the A_{2A} receptor. The rank order of potency of adenosine analogs in augmenting SLI release was shown to be: CGS 21680 > IB-MECA > CPA. At 1 μM, CGS 21680, an A_{2A} receptor-selective agonist, was shown to elicit an increase in SLI release (% change) that was approximately 26-fold and 7-fold greater than the increase elicited by the same concentration of CPA and IB-MECA, respectively. CGS 21680 potently stimulated gastric SLI release with an EC_{50} of 0.06 μM. Although CGS 21680 is selective for the A_{2A} receptor, it can act on both A_{2A} and A_{2B} receptors. The stimulatory effect of CGS 21680 on SLI release, however, is not likely due to the activation of A_{2B} receptors since this drug has a much lower affinity for the A_{2B} receptors (Brackett and Daly, 1994). In the present study, the EC_{50} of CGS 21680 in augmenting SLI release was found to be in the sub-micromolar range (0.06 μM), as suggested for A_{2A} receptor-mediated actions (Brackett and Daly, 1994). In PC12 membranes expressing A_{2A} receptors, the stimulation of adenylate cyclase activity by CGS 21680 was characterized by an EC_{50} of 0.07 μM (Brackett and Daly, 1994). In NIH 3T3 membranes expressing A_{2B} receptors, 100 μM and 3 μM CGS 21680 was shown have little effect on adenylate cyclase activity and cAMP production, respectively (Brackett and Daly, 1994; Klotz et al., 1998). Thus, actions mediated by the A_{2A} and A_{2B} receptors can be differentiated by the potency of CGS 21680 in eliciting those effects. The sub-micromolar EC_{50} value of CGS 21680 in augmenting SLI release suggests the involvement of A_{2A} receptors, but not A_{2B} receptors.

The involvement of A_{2A} receptors is also supported by the effect of various selective adenosine antagonists on adenosine and adenosine agonist-induced SLI release. When administered at 1 μM, DMPX, an A_2 receptor antagonist, completely abolished the stimulatory effect elicited by CPA (1 μM), and significantly attenuated IB-MECA- (1μM)

and CGS 21680- (0.01 and 0.1 μM) stimulated SLI release. The inhibitory effect of DMPX on CGS 21680- (0.01 and 0.1 μM) induced SLI release was concentration-dependent. To ascertain whether the A_{2A} receptors are involved in the augmentation of SLI release, the effect of the potent non-xanthine A_{2A} receptor antagonist ZM 241385 (Poucher et al., 1995) on SLI release was tested. This A_{2A} receptor-selective antagonist was able to completely abolish the stimulatory effect of CGS 21680 and adenosine on SLI release when administered at a concentration of 1 μM. This compound is 6,700-fold more selective for A_{2A} receptors than A_1 receptors (Poucher et al., 1995), while DMPX is only 4-fold more selective for A_2 receptors than A_1 receptors (Seale et al., 1988). These results support the proposal that A_{2A} receptors are involved in stimulating SLI release.

The A_1 receptors are not likely involved in the augmentation of SLI release since DMPX and ZM 241385 completely abolished CPA-induced SLI release. Furthermore, the A_1 receptor-selective antagonist, DPCPX, did not alter the effect of CGS 21680 on SLI release. The involvement of the A_3 receptor in augmenting SLI release is also unlikely since the stimulatory effect induced by IB-MECA was sensitive to blockade by DMPX and was completely abolished by ZM 241385. In addition, studies have shown that adenosine-induced SLI release can be abolished by the administration of the non-selective antagonist 8-PT (Kwok et al., 1990). Since the rat A_3 receptors were shown to be resistant to blockade by xanthine derivatives (Zhou et al., 1992) and A_3 receptor-mediated effects in the rat were resistant to blockade by 8-PT (van Galen et al., 1994; Peachey et al., 1996), the involvement of A_3 receptors in augmenting SLI release is unlikely. The stimulatory action of CPA and IB-MECA on SLI release, thus, may be due to their non-specific effect on A_{2A} receptors when administered at high concentrations. Potent A_{2B} receptor-selective antagonists were

not available, so it is not possible to thoroughly examine the role of the A_{2B} receptor in augmenting SLI release. However, the EC_{50} of CGS 21680 in eliciting this effect strongly suggests that the A_{2B} receptors are not involved. Thus, the augmentation of gastric SLI release by adenosine is most likely mediated solely by the A_{2A} receptor subtype.

In addition to the stimulatory effect of adenosine on SLI release, studies have shown that at low concentrations (10 nM), adenosine can also significantly inhibit SLI release (Kwok et al., 1990). Similar results were obtained in the present experiments, in which a low concentration of CPA (0.1 μM) was shown to significantly inhibit SLI release. It is possible that CPA acts specifically on A_1 receptors to inhibit SLI release at low concentrations, while this compound acts non-specifically on A_{2A} receptors to stimulate SLI release at higher concentrations. This proposal agrees well with studies demonstrating that, in tissues containing several adenosine receptor subtypes, A_1 receptors are preferentially activated by lower levels of adenosine. In the rat, studies have shown that adenosine elicits A_1-, A_{2A}-, A_{2B}-, and A_3- mediated effects with EC_{50} values of 73, 150, 5100, and 6500 nM, respectively (Daly and Padgett, 1992; Zhou et al., 1992; Peakman and Hill, 1994). The present proposal that the inhibition of SLI release is mediated by A_1 receptors also explains the observation that basal SLI release was lower in the presence of ZM 241385 and adenosine than in the presence of ZM 241385 alone. When administered alone, ZM 241385 may inhibit SLI release by blocking the effect of endogenous adenosine on the A_{2A} receptors, thereby unmasking the A_1 receptor-mediated inhibition of SLI release. When ZM 241385 is administered together with adenosine, adenosine will only act on A_1 receptors to inhibit SLI release. Consequently, the inhibitory effect of adenosine is enhanced in the presence of ZM 241385. These results demonstrate that adenosine may play a dual role in the regulation of

SLI release in the rat. Activation of A_1 and A_{2A} receptors may result in inhibition and stimulation of SLI release, respectively.

Results of the perfusion experiments do not clearly identify where adenosine acts to regulate SLI release, but results from the immunohistochemistry experiments suggest several possibilities. These studies demonstrate that A_1 and A_{2A} receptors were expressed in the gastric vasculature, the enteric plexi, and D-cells. Previous studies performed in our laboratory have shown that the stimulatory effect of adenosine on SLI release is not likely due to its vasodilatory action in the stomach. Like CGS 21680, nitroprusside, a vasodilator, was found to decrease perfusion pressure in the isolated vascularly perfused rat stomach. However, nitroprusside was shown not to alter SLI release (Yip and Kwok, 2004). The presence of A_{2A} receptors on enteric nerves suggests that adenosine may alter SLI release by regulating neurotransmitter release since cholinergic and β-adrenergic agents are capable of modulating gastric SLI release (Saffouri et al., 1980; Koop et al., 1983). Earlier work performed in our laboratory has demonstrated that the cholinergic blockers, atropine and hexamethonium, and the β-adrenergic blocker, propranolol, did not alter adenosine-induced changes in SLI release (Kwok et al., 1990). Thus, it is unlikely that adenosine stimulates SLI release through A_{2A} receptor-mediated changes in acetylcholine and noradrenaline release. The possibility that adenosine may alter gastric SLI release through non-cholinergic and non-adrenergic neurotransmission cannot be eliminated since both A_1 and A_{2A} receptors were localized on nerve fibers throughout the enteric plexi and muscle layers. The enteric peptides CGRP (Ren et al., 1993; Manela et al., 1995) and VIP (Chiba et al., 1980) can stimulate the release of gastric SLI. Thus, the possibility that adenosine modulates the release of these peptides to subsequently enhance SLI release remains to be examined. However, the

localization of both A_1 and A_{2A} receptors on the corporeal and antral somatostatin-secreting D-cells strongly suggests that adenosine can regulate SLI release, at least in part, by acting directly on the gastric D-cells. The presence of both receptors on the D-cells further lends support to our proposal that adenosine may inhibit and stimulate SLI release by acting on the A_1 and A_{2A} receptors, respectively.

3. Regulation of gastric SLI and IRG release by adenosine

Results from the present study suggest that endogenous adenosine may be involved in regulating basal SLI release. The administration of DMPX and ZM 241385 significantly inhibited basal SLI release, suggesting that basal SLI release may be maintained by activation of A_{2A} receptors. When EHNA, an ADA inhibitor (Mendelson et al., 1983), was administered, both basal and adenosine-stimulated SLI release were enhanced. ADA is expressed in the gastric epithelia (Witte et al., 1991) and is responsible for metabolizing adenosine to inosine (Geiger et al., 1997). The enhancement of basal and adenosine-stimulated SLI release by EHNA suggests that ADA may play a role in regulating endogenous adenosine levels in the rat stomach. The significant regulatory role of ADA in maintaining adenosine concentrations has been demonstrated in ADA-deficient mice, in which a lack of ADA activity has been shown to significantly increase the endogenous adenosine level in the stomach (Xu and Kellems, 2000). Previous studies performed in our laboratory have also demonstrated that the adenosine uptake inhibitor dipyridamole can enhance basal and adenosine-induced SLI release (Kwok et al., 1990), indicating that endogenous adenosine is also regulated by the cellular reuptake of this nucleoside through equilibrative adenosine transporters. The extracellular adenosine level in the isolated vascularly perfused rat stomach may, therefore, be regulated by the cellular reuptake and

metabolic degradation of adenosine. Since EHNA and dipyridamole can alter basal and adenosine-induced SLI release, endogenously released adenosine may be involved in maintaining the release of SLI and IRG under normal physiological states of the stomach.

Although the origin of endogenous adenosine in the present study has not been determined, extracellular adenosine in the stomach may originate from several sources. In the perfused stomach preparation, endogenous adenosine may be derived from the metabolism of ATP released into the stomach (see Chapter 1, Section I, 1.3). ATP may be released by enteric nerves or smooth muscle cells of the gastrointestinal tract (Burnstock, 1975; Nitahara et al., 1995). In the stomach, purinergic nerves of the myenteric plexus project to the circular and longitudinal muscles (Burnstock, 1975). ATP was demonstrated to be co-released with other neurotransmitters such as nitric oxide (Selemidis et al., 1997), VIP (Furness et al., 1995) and pituitary adenylate cyclase-activating polypeptide (Furness et al., 1995; Imoto et al., 1998), and may be released by nerve depolarization (Su et al., 1971). In guinea pigs, ATP was also been shown to be released from smooth muscle cells of the ileum (Nitahara et al., 1995). Thus, ATP released from gastric neurons and smooth muscle cells may be an important source of endogenous adenosine in the rat stomach. Adenosine derived from the metabolism of ATP, thus, may depend on neural activity of the enteric plexi.

The endogenous adenosine level can change under various conditions. During normoxic conditions, endogenous adenosine concentrations are in the nanomolar range. In the whole blood and plasma of rats, adenosine concentrations have been found to be 79 nM (Phillis et al., 1992) and 320 nM (Conlay et al., 1997), respectively, while in the jejunum, the venous adenosine concentration was found to be 62 nM (Sawmiller and Chou, 1990). Significant elevations in adenosine concentrations can occur during periods of stress, such as

during hypoxia, ischemia, and hypoglycemia. The production of ATP is reduced when low glucose or oxygen levels produce an elevated level of AMP (Milusheva et al., 1996), which stimulates 5' nucleotidase activity to promote adenosine formation (Worku and Newby, 1983). The level of adenosine, thus, may increase to micromolar concentrations during ischemia (Latini et al., 1999). For example, ischemia has been shown to increase the adenosine concentration in the striatum from a basal level of 41- 210 nM (Ballarin et al., 1991; Pazzagli et al., 1995; Melani et al., 1999) to an ischemic level of 3 μM (Melani et al., 1999). The physiological state of the stomach may also alter the endogenous level of adenosine. In humans, the acidity of the stomach may alter adenosine concentrations by altering ADA and 5' nucleotidase activity. In particular, in the fundic mucosa, ADA activity and gastric acid output have been observed to change in parallel (Namiot et al., 1990). Hypersecretors of acid exhibited increased ADA activity, while patients suffering from achlorhydria exhibited lowered ADA activity (Namiot et al., 1990). The activity of 5' nucleotidase was found to be reduced in the gastric juice of patients suffering from gastric cancer, gastric ulcers, and gastritis (Durak et al., 1994). The activity of ADA and 5' nucleotidase, therefore, may be regulated by the acidity of the stomach. This observation is consistent with studies, in the rat skeletal muscle, showing that increased acidity can stimulate 5' nucleotidase activity (Cheng et al., 2000). The prandial state of the stomach may also alter the adenosine level. In the canine jejunum, the venous adenosine level was shown to be increased by the presence of food in the intestinal lumen (Sawmiller and Chou, 1990). Changes to the prandial state or acidity of the stomach may, therefore, alter gastric adenosine concentrations and subsequently influence gastric actions mediated by adenosine, such as the regulation of SLI and IRG release. As discussed earlier, activation of A_1 receptors has been

shown to inhibit IRG and SLI release, while activation of A_{2A} receptors has been shown to stimulate SLI release. A_1 receptors are preferentially activated by lower concentrations of adenosine, while A_{2A} receptors are preferentially activated by higher concentrations of adenosine. Therefore, during conditions that lead to an increase in the endogenous adenosine concentration, A_{2A} receptors may be preferentially activated to stimulate SLI release, while during conditions that would lead to a decrease in the endogenous adenosine concentration, A_1 receptors may be preferentially activated to inhibit SLI and IRG release.

II. Distribution and structure of gastric adenosine receptor mRNA

The mRNAs of all four adenosine receptors are expressed in extremely low levels within the whole rat stomach (Dixon et al., 1996). The localization of adenosine receptors on functionally and morphologically distinct regions of the stomach is unknown. However, functional studies suggest that these receptors may be localized on specific regions of the stomach. For example, studies demonstrating the protective effect of adenosine on gastric mucosal damage (Geiger and Glavin, 1985; Westerberg and Geiger, 1987; Cho and Ogle, 1990; Cho et al., 1991; Bozkurt et al., 1998) and studies showing the inhibitory and stimulatory effect of adenosine on gastrin and somatostatin release, respectively (Kwok et al., 1990), suggest that the adenosine receptors may be expressed on the gastric mucosa.

The current study is the first to demonstrate the localization of all four adenosine receptors in the gastric corpus, antrum, and mucosa of the rat using RT-PCR. The specificity of each RT-PCR reaction was confirmed by restriction enzyme digestion using multiple restriction enzymes. Small fragments generated by restriction enzyme digestion (< 150 bp) were often not detected by agarose gel electrophoresis due to their low intensity fluorescence. The larger fragments (≥ 150 bp) were easily detected, and their sizes compared well with the

expected sizes, differing by < 9%. The A_1, A_{2A}, A_{2B} and A_3 receptor RT-PCR reactions established for this study, therefore, are highly specific.

The presence of adenosine receptors in the corpus, antrum and mucosa may have important functional implications since these regions contain cells which secrete gastric acid, somatostatin and/or gastrin. The parietal cells, G-cells and D-cells reside in the gastric mucosa of the stomach (Dockray et al., 1996; Sachs et al., 1997). The corpus contains parietal cells and D-cells, while the antrum contains G-cells and a population of D-cells that are distinct from those in the corpus. In addition to their presence in these tissues, all four adenosine receptors were also expressed in the gastric fundus, the non-glandular region of the stomach that is responsible for the accommodation of food. The presence of A_1 and A_{2A} receptor mRNA throughout the stomach suggests the possibility that adenosine receptors may be expressed on various sites of the stomach, including the parietal cells, G-cells and D-cells, nerve fibers and vasculature. Indeed, the localization and distribution of adenosine receptors were confirmed on some of these structures by the present immunohistochemistry experiments.

The structure of the A_1 and A_{2A} receptors was also examined in the present study by RT-PCR and by cloning and sequencing of the RT-PCR amplicons. Only one A_1 and A_{2A} receptor amplicon is produced when RT-PCR was performed using primers that span the entire coding region of the receptor. Northern blot analysis has previously demonstrated the existence of two A_1 receptor transcripts in various tissues of the rat, including the brain and stomach (Mahan et al., 1991; Reppert et al., 1991). Two distinct A_{2A} receptor transcripts have also been demonstrated in rat PC12 cells (Chu et al., 1996). The A_1 and A_{2A} receptor genes both contain 2 exons, which are separated by one intron (Ralevic and Burnstock,

1998). The synthesis of only one RT-PCR amplicon in the present study demonstrates that the intron is not alternatively spliced to generate variant forms of the A_1 and A_{2A} receptor in the rat stomach. Cloning and sequencing of the mucosal A_1 and A_{2A} receptor amplicons further demonstrate that the coding region of these receptors is identical to published sequences in the rat brain (Mahan et al., 1991; Fink et al., 1992). Thus, only one form of the gastric A_1 and A_{2A} receptors exists in the stomach.

The multiple A_1 and A_{2A} receptor mRNA transcripts, detected by previous Northern blot analysis studies (Mahan et al., 1991; Reppert et al., 1991; Chu et al., 1996) may arise from differences in the non-coding regions. In rat PC12 cells, the A_{2A} receptor gene was shown to express two different promoters (Chu et al., 1996). These two promoters drive the synthesis of two distinct A_{2A} receptor transcripts that differ in the 5'UTR. Similarly, in humans, two separate promoters were found in the A_1 receptor gene (Ren and Stiles, 1995). These promoters drive the synthesis of two distinct A_1 receptor transcripts which were also shown to differ in the 5'UTR. It is unclear whether this also occurs in the rat stomach since the promoter regions of the rat A_1 receptor gene have not yet been identified.

Unlike the A_1 and A_{2A} receptors, two forms of the A_3 receptor exist in various rat tissues (Zhou et al., 1992; Sajjadi et al., 1996). The predominant form was cloned by Zhou and coworkers (1992), and was shown to contain 320 amino acids. The alternate form, the A_3i receptor, was cloned by Sajjadi and coworkers (1996) and was found to be expressed in the rat brain and spleen (Zhou et al., 1992; Sajjadi et al., 1996). This form contains a 17 amino acid insertion which is produced by alternative splicing of the intron. The RT-PCR primers used in the present study were able to detect both the A_3 and A_3i receptor transcripts. Results of our study demonstrate that alternative splicing was not shown to occur in any

region of the rat stomach since only one A_3 receptor transcript was detected. Cloning and sequencing of the PCR amplicon revealed that the A_3 receptor, but not the A_3i receptor, is expressed in the rat stomach. A silent mutation was also shown to occur at position 492 (second transmembrane domain). This mutation has previously been observed in the A_3i receptor transcript of the rat brain (Sajjadi et al., 1996). Other point mutations reported in the A_3i receptor, however, were not observed in this region of the rat mucosal A_3 receptor gene.

Results of these studies demonstrate that all four adenosine receptors are expressed in functionally distinct regions of the rat stomach, and that each subtype exists in only one structural form. Furthermore, the gastric A_1 and A_{2A} receptors are structurally identical to the A_1 and A_{2A} receptors of the brain in the rat.

III. Quantification of A_1 and A_{2A} receptor gene expression

1. Competitive RT-PCR and Real-Time RT-PCR assay development

In the rat stomach, the level of adenosine A_1 and A_{2A} receptor mRNA is extremely low (Dixon et al., 1996). Quantification of gastric adenosine receptor expression using conventional techniques such as Northern blot and slot blot is not possible since moderate levels of mRNA expression and relatively large sample sizes are required. In the present study, competitive RT-PCR and Real-Time RT-PCR techniques were developed to measure absolute A_1 and A_{2A} receptor gene expression in different regions of the rat stomach. Both methods are widely accepted techniques for quantifying low levels of gene expression in small tissue samples, such as the gastric mucosa (Raeymaekers, 1995; Heid et al., 1996). Quantitative RT-PCR has been used to measure A_1 and A_{2A} receptor gene expression in the rat brain (Rose'Meyer et al., 2003). The relative changes in A_1 receptor mRNA expression

have been determined in the rat brain using competitive RT-PCR (Othman et al., 2002) and in the rat heart using Real-Time PCR (Ashton et al., 2003). The present study is the first to quantify the absolute level of A_1 and A_{2A} receptor gene expression in the rat stomach, where the expression of these receptors are extremely low (Dixon et al., 1996). Both competitive RT-PCR and Real-Time RT-PCR assays were developed to measure this gene expression.

Competitive RT-PCR is based on the competitive co-amplification of a fixed quantity of target template with known amounts of internal standard or RNA competitor. The A_1 and A_{2A} receptor competitive RT-PCR assays developed in this study were able to measure gene expression ranging from 10^5 to10^7 copies/μg total RNA. Each reaction tube contained a fixed amount of tissue RNA; 0.125 μg and 0.5 μg were used in the A_1 and A_{2A} receptor assays, respectively. The amount of template used was optimized so that the tissue template and competitor band were both easily visualized, and so that both the tissue template and competitor cDNA amplified with equal efficiency during PCR. Using the competitive RT-PCR assay, striatal A_1 and A_{2A} receptor mRNA levels were found to be at least 4.7 times and 116 times greater than in any region of the stomach, respectively. This is consistent with studies demonstrating moderate A_1 and abundant A_{2A} receptor gene expression in the striatum and extremely low A_1 and A_{2A} receptor gene expression in the stomach (Dixon et al., 1996). A_1 receptor gene expression was shown to be significantly higher than A_{2A} receptor gene expression in the antrum and mucosa, but not in the fundus and the corpus. These results do not agree well with results from the Real-Time RT-PCR experiments. Real-Time RT-PCR results demonstrate that A_1 receptor gene expression is significantly higher than A_{2A} receptor gene expression in all gastric regions examined. The discrepancy between the competitive RT-PCR and Real-Time RT-PCR results is addressed later in this section.

Real-Time RT-PCR is based on the cleavage of a fluorogenic probe that hybridizes to the template at a position located between the forward and reverse primers (See Fig. 9). The Real-Time RT-PCR assays used to measure A_1 and A_{2A} receptor gene expression were shown to be considerably more sensitive than the corresponding competitive RT-PCR assay. The Real-Time RT-PCR assays were able to measure a range of concentrations exceeding a 7 log scale, and as few as 500 copies of A_1 or A_{2A} receptor RNA/μg total tissue RNA. The latter is equivalent to 25 copies of A_1 or A_{2A} receptor transcript per reaction since each reaction mixture only contained 0.05 μg of total RNA as the template. Using Real-Time RT-PCR, A_1 receptor gene expression was lowest in the corporeal mucosa, while A_{2A} receptor gene expression was lowest in the whole stomach and corporeal mucosa. These results correspond well with the results of the immunohistochemistry studies. A_1R-IR and A_{2A}R-IR were found on some mucosal cells, but the majority of A_1R-IR and A_{2A}R-IR was localized on the nerve fibers and vasculature of the muscle and myenteric plexus of the corpus and antrum. In the antral mucosa, A_1R-IR was localized on all G-cells and some D-cells, while A_{2A}R-IR was only localized on some D-cells. Thus, the higher level of A_1 compared to A_{2A} receptor mRNA in the antrum and whole stomach mucosa may reflect the higher number of G-cells compared to D-cells in these regions (Lehy et al., 1979).

Striatal A_1 and A_{2A} receptor mRNA levels measured by Real-Time RT-PCR were shown to be at least 2- and 70-fold higher, respectively, than levels measured in any region of the stomach. The mRNA levels of the A_1 and A_{2A} receptor measured using this technique were consistently lower than those measured using competitive RT-PCR; A_1 and A_{2A} receptor mRNA levels were found to be 2.4±0.2 and 21±3 times lower, respectively. The absolute level of A_1 receptor gene expression has not previously been quantified in the rat

stomach or striatum. Only the relative level of A_1 receptor gene expression in various tissues have been determined using RT-PCR (Dixon et al., 1996). The striatal A_{2A} receptor mRNA levels measured by Real-Time RT-PCR (2.2×10^6 copies/μg total RNA), however, compared well with levels measured in a previous study (Lee et al., 1999). The striatal A_{2A} receptor gene expression measured by Lee and co-workers (1999) was approximately 1.4×10^6 copies/μg total RNA, using a similar experimental protocol. The sequence of the primers and fluorogenic probe, and the total RNA extraction protocol used, however, were different, and may account for the small difference between their striatal A_{2A} mRNA level and that found in the present study. The striatal A_{2A} receptor level determined by competitive RT-PCR (6.6×10^7 copies/μg total RNA) was not in the same range as that measured by Real-Time RT-PCR.

Real-Time RT-PCR is likely the more accurate and reliable technique. The added component of the fluorogenic probe significantly increases the specificity of the Real-Time RT-PCR assay. This technique is more sensitive since the fluorogenic signal emitted by the reporter dye is detected by a highly sensitive CCD camera. Studies have shown that Real-Time RT-PCR can reliably detect a single specific DNA molecule in a high background of non-specific DNA (Lockey et al., 1998). Our assays were able to consistently measure as few as 25 copies of A_1 and A_{2A} receptor RNA per reaction. Changes in reporter fluorescence are also monitored in a Real-Time manner. Thus, quantification is made at the start of the exponential phase and the errors that may be introduced by reaching the plateau phase are avoided (Raeymaekers, 1995; Schmittgen, 2001).

The difference between the A_1 and A_{2A} receptor mRNA levels measured by competitive RT-PCR and Real-Time RT-PCR is unclear. Several factors can introduce

errors to competitive RT-PCR measurements, but in the present study, care was taken to avoid these. For example, during competitive RT-PCR, the number of cycles of amplification was limited to 30 to avoid reaching the plateau phase. As the reaction approaches the plateau phase, there is an increased likelihood of heteroduplex formation. These complexes are formed when the competitor and tissue template PCR products anneal. These formations can significantly decrease the sensitivity and accuracy of competitive RT-PCR quantification (Henley et al., 1996). These complexes would appear as an additional band above the competitor and tissue template bands. However, in the present study, heteroduplex formations were not observed for either the A_1 or A_{2A} receptor competitive RT-PCR assays. Accurate quantification with competitive RT-PCR also requires that the template and the competitor behave in a similar manner during the reverse transcription and PCR step (Schmittgen, 2001). In this study, RNA competitors were used instead of DNA competitors to control for variations in the reverse transcription step (Orlando et al., 1998). In addition, homologous competitors, which have a sequence similar to that of the tissue template, were also used to minimize differences in the amplification efficiency during PCR. An equal amplification efficiency of the template and competitor is required for reproducible and accurate quantification (Connolly et al., 1995). The amplification efficiencies were determined in this study by plotting the log [density ratio (competitor/template) band] against the log (initial competitor concentration) for each assay. When the slope of the competitive RT-PCR relationship is equal to 1, both the template and competitor cDNA should amplify with equal efficiency (Connolly et al., 1995). The slopes obtained for the A_1 and A_{2A} receptor competitive RT-PCR assays were found to be 1.05 ± 0.04 and 1.07 ± 0.05, respectively. Thus, a significant difference in amplification efficiency between the tissue

template and competitor cDNA was not apparent. Some studies have shown that, even when these slopes are ideal (≅ 1.0), competitive RT-PCR can yield results that differ significantly from actual levels. For example, Hayward et al. have shown that when a known quantity of Na^+K^+-ATPase mRNA was measured by competitive RT-PCR, the value reported was almost 4-fold higher although the slope of the competitive RT-PCR relationship remained at 1.01 (Hayward et al., 1998). These investigators further demonstrated, using mathematical modeling, that small differences in the amplification efficiency between the competitor and unknown template can drastically alter competitive RT-PCR measurements, while still producing a relationship with a slope of 1.0. A 20% difference in amplification efficiency between the competitor and unknown template was found to result in a 9 to 20-fold difference between the actual mRNA level and the value estimated by competitive RT-PCR (Hayward et al., 1998). In our studies, A_1 and A_{2A} receptor mRNA levels measured by competitive RT-PCR were found to be 2.4 and 21-fold higher, respectively, than levels measured by Real-Time RT-PCR. Thus, a difference in the amplification efficiency between the competitor and unknown template may exist in our assays, despite the precautions taken. The A_1 and A_{2A} competitors contain a 136 and 57 bp deletion. Decreasing the size of the deletion has been shown to increase the accuracy of competitive RT-PCR (Hayward et al., 1998). However, the resulting similarity between the size of the competitor and unknown template make it difficult to distinguish between the two bands during gel electrophoresis.

In view of the limitations of competitive RT-PCR, Real-Time RT-PCR was used in our subsequent experiments to measure changes in A_1 and A_{2A} receptor gene expression after various treatments, including food deprivation and omeprazole treatment. This technique is highly specific and is not subject to the limitations of competitive RT-PCR. In addition, this

technique was chosen over competitive RT-PCR since striatal A_{2A} receptor mRNA levels measured by Real-Time RT-PCR compared well with previously published results (Lee et al., 1999). The extremely high sensitivity of this method also allows for quantification of A_1 and A_{2A} receptor gene expression in small gastric tissue samples such as the mucosa, where the expression levels of these genes are extremely low.

2. Changes of gastric A_1 and A_{2A} receptor gene expression and function by altered states of the stomach

The present study shows that the A_1 and A_{2A} receptors participate in the regulation of IRG and SLI release in the rat stomach. However, it is unclear if the expression of these receptors is altered by different secretory states of the stomach. Changes to adenosine receptor gene expression may subsequently alter the modulatory effect of adenosine on IRG and SLI release. Previous studies have shown that oxidative stress (Nie et al., 1998; Kobayashi and Millhorn, 1999) or stress induced by acute renal failure (Gould et al., 1997) and sleep deprivation (Basheer et al., 2000) can alter adenosine receptor gene and/or protein expression. The present study demonstrates that stress induced by fasting and omeprazole treatment may also alter adenosine receptor gene expression.

Real-Time RT-PCR was performed to measure changes in A_1 and A_{2A} receptor, gastrin and somatostatin gene expression. All measurements were standardized with an endogenous control to account for differences resulting from variations in total RNA extraction efficiency, RNA integrity and RNA concentration. Two common house-keeping genes, GAPDH and 18S rRNA, were considered for use as the endogenous control. These genes can only be used as the endogenous control if their expression is not altered by the experimental treatment. In the current study, GAPDH gene expression was significantly

decreased in the corpus mucosa after 24 and 36 h of fasting, and in the antrum after 36 h of fasting. Similar results have been reported in animals which were subjected to 48 h of fasting (Yamada et al., 1997a). GAPDH mRNA levels were also significantly altered in the corporeal muscle and mucosa after 1 and 3 days of omeprazole treatment, respectively. These findings are consistent with another study, which demonstrated that a single dose of omeprazole was capable of increasing GAPDH mRNA levels in the corpus (Sandvik et al., 1995). Furthermore, several studies have cautioned against the use of GAPDH mRNA as an endogenous control since its expression can be altered by a variety of conditions and agents such as hypoxia, oxidative stress, heat shock, glucose and insulin (Schmittgen and Zakrajsek, 2000; Suzuki et al., 2000). In the current study, 18S rRNA levels were not affected by fasting or omeprazole treatment. Thus, this gene was used as the endogenous control for the measurement of all gene expression. Previous experiments have also demonstrated that 18S rRNA expression was not altered by 48 h of fasting (Yamada et al., 1997a). Using 18S rRNA expression as the endogenous control, both fasting and omeprazole treatment were shown to alter adenosine A_1 and A_{2A} receptor gene expression in the corporeal mucosa and antrum, but not in the corporeal muscle layer.

In the fasting experiments, an increase in A_1 receptor gene expression was observed in both the corporeal mucosa and antrum after 24 h, but not after 36 h, of fasting. Similarly, fasting was shown to increase A_{2A} receptor gene expression in the antrum after 24, but not after 36 h, of fasting. Although adenosine receptor protein expression may change in parallel with its mRNA level, the return of adenosine receptor gene expression to control levels after 36 h of fasting does not necessarily imply that adenosine receptor protein expression also returns to control levels. It is possible that the elevated adenosine receptor mRNA levels

observed after 24 h of fasting resulted in increased adenosine receptor expression, which is maintained even after 36 h of fasting. The effect of fasting on A_{2A} receptor gene expression differed in the antrum and corporeal mucosa. In the antrum, A_{2A} receptor gene expression was shown to increase after 24 h of fasting. In the corporeal mucosa, its gene expression was shown to gradually decrease and become significant after 36 h of fasting. The different effect of fasting on A_{2A} receptor gene expression in the antrum and corporeal mucosa suggests that the gene expression of this receptor may be differentially regulated in these two gastric regions. The lack of change in A_1 and A_{2A} receptor gene expression in the corporeal muscle suggests that neither gene is altered by fasting in this tissue.

It is currently unclear how adenosine receptor gene expression is altered by fasting. The absence of food in the stomach has been shown to increase gastric acidity (Matsumoto et al., 1989; Jiang et al., 2002). In the rat, Jiang and colleagues (2002) demonstrated that fasting for 24 h significantly decreased the gastric intraluminal pH to 2.0. Similarly, Matsumoto and co-workers (1989) demonstrated that fasting significantly decreased gastric intraluminal pH from a non-fasting level of 3.5 to 2.5, 2.0, and 1.6 after 1, 2, and 3 days of fasting, respectively, and that the intraluminal pH did not return to control levels until after 8 days of fasting. These studies suggest that changes in gastric acidity during fasting may alter gastric adenosine receptor gene expression. In agreement with this proposal, omeprazole-induced achlorhydria was also shown to alter A_1 and A_{2A} receptor gene expression. Omeprazole (400 μmol/kg) was found to completely inhibit gastric acid secretion within 2 h of administration (Lee et al., 1992). Gastric acid secretion was decreased by up to 80% 24 h after the first treatment (Larsson et al., 1988; Lee et al., 1992), and continuous inhibition of acid secretion was achieved after 3 days of treatment (Carlsson et al., 1986). Results show

that omeprazole treatment inhibited both A_1 and A_{2A} receptor gene expression in the antrum, which is contrary to what occurs during fasting. Since fasting increases gastric acidity and omeprazole decreases gastric acidity, intraluminal acidity may play a role in altering adenosine receptor gene expression. In support of this proposal, the gene expression of gastric A_1 receptors was observed to be increased after fasting but decreased after omeprazole treatment. Results of our immunohistochemistry experiments demonstrate that A_1 receptors were expressed on D-cells and G-cells and A_{2A} receptors were expressed on D-cells of the gastric mucosa. In the antrum, both of these cell types are open to the luminal environment and, thus, can respond to changes in gastric acidity, while the D-cells of the corpus are closed and do not readily respond to changes in gastric acidity (Sachs et al., 1997). Therefore, it is possible that gene expression of adenosine receptors in the antrum is readily altered by changes in gastric acidity, while adenosine receptor gene expression in D-cells of the corpus mucosa may not be directly affected by gastric acidity. This suggestion may explain why A_{2A} receptor gene expression in the corporeal mucosa did not alter according to the changes in gastric acidity. In particular, A_{2A} receptor gene expression was decreased by both fasting and omeprazole treatment.

The possible mechanisms involved in the regulation of adenosine receptor gene expression by gastric acidity have not been examined in the present study. However, there is evidence to suggest that alterations in the activities of various mitogen-activated protein kinases (MAPK) may be involved. Malek and colleagues (1999) have shown that activation of ERK1, ERK2 and JNK can decrease adenosine receptor A_{2A} mRNA levels in rat PC12 cells. The activities of various MAPK respond to changes in both intracellular and extracellular pH (Feifel et al., 2002). Exposure of human epidermoid carcinoma cell line

A431 to low extracellular pH was shown to increase the activity of MAPK (ERK2, JNK, and p38 MAP kinase), and exposure of mouse Swiss 3T3 cells to low extracellular pH was shown to stimulate the activity of MAPK (p42, p44 MAP kinase, ERKs, p38 MAP kinase and JNK) (Xue and Lucocq, 1997). In the Barrett's esophagus adenocarcinoma cell line (SEG-1), increased exposure to acidic media increased ERK, p38 MAP kinase, and JNK activities (Souza et al., 2002). The activity of p38 MAP kinase was also shown to be increased in esophageal mucosa cells of patients who suffer from Barrett's esophagus (Souza et al., 2002). Barrett's esophagus is associated with gastroesophageal reflux disease. Thus, the increased p38 MAP kinase activity in these cells is likely due to the increased exposure to acid. The studies mentioned above have demonstrated that changes in acidity may alter MAPK activity. Increases in MAPK activity may subsequently alter adenosine receptor gene expression. The effect of specific MAPK on A_1 receptor gene expression has not been determined. However, in the rat pituitary-derived cell line, A_1 receptor transcription is stimulated by epidermal growth factor (EGF) (Navarro et al., 1999). EGF has also been shown to activate MAPK pathways (Yamada et al., 1997b). Although MAPK activity may alter adenosine receptor gene expression in some cell lines (Malek et al., 1999), this has not been examined in the stomach.

Adenosine receptor gene expression may also be regulated by changes in intracellular pH. During periods of oxidative stress, such as hypoxia and ischemia, intracellular pH is decreased (Kintner et al., 2000). Previous studies have shown that oxidative stress can induce A_1 receptor gene expression in DDT1-MF2 cell line (Nie et al., 1998), and that hypoxia can stimulate A_{2A} receptor gene expression (Kobayashi and Millhorn, 1999). During fasting, it is possible that increased intraluminal acidity leads to subsequent increases

in intracellular acidity in the gastric epithelium. In the guinea pig gastric gland, decreasing the luminal pH from 3.0 to 1.9 was shown to decrease intracellular pH in cells of the gastric gland (Schreiber et al., 2000). Similar results have also been found in the isolated Necturus antral mucosa, where acidification of the luminal pH to 2.0 was shown to significantly decrease intracellular pH of the antral mucosal cells (Kivilaakso and Kiviluoto, 1988; Kiviluoto et al., 1990). Since the intraluminal pH level has been shown to decrease from 3.5 to 2.0 after 24 h and 48 h of fasting (Matsumoto et al., 1989; Jiang et al., 2002), it is possible that the increase in A_1 and A_{2A} receptor mRNA levels observed in the antrum after 24 h of fasting result from intracellular acidification.

Although fasting and omeprazole treatment may regulate adenosine A_1 and A_{2A} receptor gene expression by altering the intraluminal acidity, it is unclear whether adenosine receptor gene expression may also respond to other factors. For example, during fasting, adenosine receptor gene expression may be altered by the absence of specific nutritional components of food in the stomach. The presence of peptones, phenylalanine and olive oil, in the gastric lumen has been shown to regulate the gene expression of gastrin and somatostatin (Wu et al., 1990b; Wu et al., 1991). Omeprazole treatment has also been shown to increase plasma levels of GRP (Takehara et al., 1996), which has been shown to regulate the activity of D-cells and G-cells. D-cells of the antrum and corpus can be regulated directly (Schaffer et al., 1997) and indirectly (Schubert et al., 1991) by GRP, respectively, while G-cells can be directly modulated by GRP (Campos et al., 1990). The G-cells and D-cells were shown to express adenosine receptors. Thus, fasting and omeprazole-treatment may also induce changes in adenosine receptor gene expression by preventing the exposure

of the gastric lumen to nutritional components of food and by enhanced GRP release, respectively.

Since activation of A_1 and A_{2A} receptors inhibited gastrin and stimulated somatostatin release, respectively, changes in A_1 and A_{2A} receptor expression may also regulate gastrin and somatostatin synthesis and release. Therefore, the effect of fasting and omeprazole treatment on gastrin and somatostatin gene expression was also examined. Results show that gastrin gene expression was significantly inhibited by 24 and 36 h of fasting. These findings are consistent with previous studies demonstrating a significant reduction in gastrin gene expression after 12, 24, 48 and 72 h of fasting (Dimaline et al., 1991; Wu et al., 1991; Larsson and Hougaard, 1993; Sandvik et al., 1993; Yamada et al., 1997a). These results are also consistent with the decrease in antral gastrin concentration, G-cell number and serum gastrin content observed after fasting (Lichtenberger et al., 1975; Track et al., 1978; Shulkes et al., 1983; Dimaline et al., 1991; Larsson and Hougaard, 1993).

Omeprazole treatment, on the other hand, was shown to increase gastrin mRNA levels after 1 and 3 days of treatment. Increased gastrin gene expression has also been demonstrated after 4 and 14 days of omeprazole treatment (Wu et al., 1990a; Dockray et al., 1991). The increased gastrin mRNA levels are most likely responsible for the enhanced levels of antral gastrin content and plasma gastrin concentrations (Brand and Stone, 1988; Inauen et al., 1988; Larsson et al., 1988; Dockray et al., 1991), which have been observed following short term (5 days or less) omeprazole treatment.

Both fasting and omeprazole treatment were shown to induce reciprocal changes in A_1 receptor and gastrin gene expression. Our immunohistochemistry results demonstrate that A_1 receptors are expressed on G-cells, which suggests that increased expression and

activation of A_1 receptors may directly affect gastrin gene expression. Adenosine has been shown to modulate the expression of various genes in the murine heart (Asakura et al., 2002), and may modulate gene expression by activating A_1 receptors to inhibit cAMP production. The transcription of certain genes is cAMP-dependent (Dragon et al., 2002). In particular, gastrin gene expression was regulated by cAMP levels (Shiotani and Merchant, 1995). Since activation of A_1 receptors inhibits adenylate cyclase activity and cAMP formation (Ralevic and Burnstock, 1998), it is possible that activation of A_1 receptor on G-cells may inhibit gastrin gene expression by inhibiting cAMP accumulation. In addition, somatostatin has been shown to inhibit gastrin synthesis by suppressing adenylate cyclase activity and subsequent cAMP production (DelValle and Yamada, 1990).

Somatostatin gene expression was also altered by fasting and omeprazole treatment. The antral somatostatin mRNA level was shown to be increased following 24 h of fasting, but returned to control levels after 36 h of fasting. Previous experiments have demonstrated a significant increase in antral somatostatin gene expression following both 24 h (Wu et al., 1991) and 36 h of starvation (Sandvik et al., 1995). The cause of this discrepancy is unclear. However, it may be due to the use of a different housekeeping gene as the endogenous control. Sandvik and co-workers utilized GAPDH mRNA as the endogenous control. Sandvik reported no significant change in GAPDH mRNA expression after 36 h of fasting (Sandvik et al., 1995), contrary to this study and another report (Yamada et al., 1997a). In the corporeal mucosa, the somatostatin mRNA level was reduced following 36 h of fasting. This decrease is consistent with previous studies demonstrating a significant decrease in the somatostatin mRNA level in the oxyntic region following a 48 h fasting period (Sandvik et al., 1993).

Results of the present experiments show that antral somatostatin gene expression was significantly decreased after 1 and 3 days of omeprazole treatment (400μmol/kg). These findings are similar to those reported in previous studies (Brand and Stone, 1988; Wu et al., 1990a; Wu et al., 1990b; Sandvik et al., 1995). The lowered antral somatostatin mRNA levels are consistent with the decreased plasma concentration of somatostatin, antral somatostatin concentration, and antral D-cell density that have been shown to occur in the rat stomach during achlorhydria (Allen et al., 1986; Lee et al., 1992; Pawlikowski et al., 1992). In the corpus, the present study shows that somatostatin mRNA levels were significantly decreased after omeprazole treatment. These observations are in agreement with several studies demonstrating reduced corporeal somatostatin mRNA expression during achlorhydria (Tari et al., 1991; Sandvik et al., 1995). Similar to our results, Tari and co-workers reported that the somatostatin mRNA level in the corpus gradually decreased over a period of 3 days during drug treatment (Tari et al., 1991). Other studies have shown that omeprazole can also cause an increase, rather than a decrease, in corporeal somatostatin gene expression (Kapuscinski and Shulkes, 1995; Bolkent et al., 2001). However, these observations were made 8 h after a single treatment of omeprazole (400 μmol/kg) (Kapuscinski and Shulkes, 1995) or after long-term (2 months) omeprazole treatment (Bolkent et al., 2001).

The present study demonstrates that somatostatin gene expression was inhibited by achlorhydria in the corporeal muscle. Our immunohistochemistry experiments demonstrate that, in this region, somatostatin was expressed on very few nerve fibers of the circular muscle and myenteric plexus. Achlorhydria has previously been shown to alter neuropeptide mRNA expression in the enteric plexi (Dimaline et al., 1992). In particular, Dimaline and colleagues have shown that omeprazole treatment can increase the antral GRP mRNA level.

The increase in somatostatin gene expression in this tissue may be secondary to changes in GRP levels, since GRP has been shown to stimulate somatostatin release (Martindale et al., 1982).

Both fasting and omeprazole treatment induced similar changes in A_{2A} receptor and somatostatin gene expression. Results of perfusion experiments performed in the present study demonstrate that activation of A_{2A} receptors stimulated somatostatin release. Furthermore, results of the immunohistochemistry study demonstrate the expression of A_{2A} receptors on D-cells. Thus, it is possible that changes in A_{2A} receptor expression regulate somatostatin synthesis and release. Previous studies have shown that the rat somatostatin gene contains a cAMP response element (Montminy et al., 1986) and that its expression is regulated by cAMP levels (Montminy et al., 1996b). The A_{2A} receptor couples to the G_s protein and can stimulate adenylate cyclase activity to increase cAMP accumulation (Dalziel and Westfall, 1994; Fredholm et al., 2001). A number of studies have demonstrated that activation of A_{2A} receptors induces the expression of genes regulated by the cAMP response element (CRE). Asher et al. have demonstrated that activation of A_2 receptors can induce cAMP-dependent gene expression through the phosphorylation of CRE binding protein (CREB) (Asher et al., 2002). In vascular smooth muscle cells, activation of the A_2 receptor was suggested to upregulate lysyl oxidase gene expression (Ravid et al., 1999). In addition, CGS 21680 (A_{2A}-selective agonist) has been shown to induce tyrosine hydroxylase gene expression in rat PC12 cells (Chae and Kim, 1997). Like the somatostatin gene, both the lysyl oxidase and tyrosine hydroxylase genes also contain a CRE in the 5' upstream promoter region. Therefore, it is possible that activation of adenosine A_{2A} receptors also increases somatostatin gene expression through activation of the CRE. Although the direct modulation

of the gastrin and somatostatin gene expression by activation of the A_1 and A_{2A} receptors remains a possibility, specific experiments have not been performed in the present study to confirm this proposal. The regulation of gastrin and somatostatin mRNA expression is complex and may also depend on a number of other factors.

To examine whether changes in adenosine receptor mRNA levels may result in actual changes in adenosine receptor function, the effect of omeprazole treatment on adenosine agonist-induced changes in IRG and SLI release was also examined. The level of gene expression often changes in parallel with protein synthesis and receptor function. However, the results of the present study suggest that changes in gastric adenosine receptor gene expression do not always result in changes in receptor function.

CPA, an A_1 receptor agonist, was demonstrated to be a potent inhibitor of IRG release in the isolated vascularly perfused rat stomach. When administered at low ($\leq$0.1 μM) and high ($\geq$1 μM) concentrations, CPA suppressed and stimulated SLI release, respectively. A similar concentration-dependent effect has been demonstrated using adenosine (Kwok et al., 1990). After treatment with omeprazole, A_1 receptor gene expression was reduced in the corporeal mucosa and antrum. Following 1 or 3 days of omeprazole treatment, CPA-induced inhibition of IRG and SLI release was not altered. However, the stimulatory effect on SLI release induced by the higher concentration (1 μM) of CPA was significantly decreased following 3 day treatment with omeprazole. As discussed earlier, the inhibition of IRG and SLI release induced by CPA is likely mediated by activation of A_1 receptors, while the augmentation of SLI release induced by high concentrations of CPA is likely mediated by the non-selective activation of A_{2A} receptors. The attenuation of CPA-stimulated SLI release after omeprazole treatment, therefore suggests that A_{2A} receptor expression is reduced after 3

days of omeprazole treatment. In agreement with this proposal, the stimulatory action of the A_{2A} receptor agonist CGS 21680 on SLI release was also reduced after 3 days of omeprazole treatment.

Both 0.1 and 1 μM CGS 21680 potently stimulated the release of SLI in earlier experiments. Although the stimulatory effect of 0.1 μM CGS 21680 on SLI release was suppressed after 3 days of treatment, this effect was not statistically significant. The release of SLI induced by 1 μM CGS 21680, however, was significantly attenuated after 3 day omeprazole treatment. This attenuation of A_{2A} receptor function is consistent with the reduced level of antral A_{2A} receptor gene expression observed after 3 days of omeprazole treatment. Taken together, results from the gene expression and perfusion experiments suggest that omeprazole treatment decreases the number of functional A_{2A} receptors, but does not always produce parallel changes in adenosine receptor expression. In the present study, A_{2A} receptor gene expression was also reduced in the corporeal mucosa after 1 day of omeprazole treatment, but significant changes in CGS 21680-induced SLI release were not observed at this time. Thus, alterations in A_{2A} receptor mRNA levels may not necessarily produce changes in A_{2A} receptor function. It is possible that, although A_{2A} receptor mRNA levels may be altered after 24 h of achlorhydria, actual changes in A_{2A} receptor expression may require more than 24 h to occur.

The protein synthesis and expression of the adenosine receptors may also be controlled by other factors. The synthesis of both the A_1 and A_{2A} receptors can be regulated at the translational level (Ren and Stiles, 1994b; Chu et al., 1996; Lee et al., 1999). The translation of the human A_1 receptor was shown to be inhibited by two upstream AUG codons in exon 4 (Ren and Stiles, 1994b), while translation of the human A_{2A} receptor is

inhibited by the presence of several upstream open-reading frames and regions of high GC content (Chu et al., 1996). The expression of the rat A_{2A} receptor is suppressed by the presence of an upstream out-of-frame AUG codon in the 5' untranslated region (Lee et al., 1999). Thus, in addition to the regulation of A_1 and A_{2A} receptor expression by its mRNA levels, receptor expression can also be controlled by translational mechanisms. It is uncertain whether fasting and omeprazole treatment also alter the translation of the A_1 and A_{2A} receptor genes.

Since A_1 and A_{2A} receptor gene expression was examined in whole tissue extracts, it is difficult to assess whether changes in A_1 and A_{2A} receptor mRNA expression only occur in anatomical structures that are involved in the direct control of IRG and SLI release, such as the G-cells and D-cells. Our immunohistochemistry experiments have demonstrated that, in the stomach, the adenosine A_1 and A_{2A} receptors are not expressed solely on these cells. The significant functional implications of the widespread localization of A_1 and A_{2A} receptors are discussed in the following section.

IV. Cellular localization of the adenosine A_1 and A_{2A} receptor

From the discussion above, it is clear that both adenosine A_1 and A_{2A} receptors play a role in regulating gastric secretions. However, studies examining the cellular localization of gastric adenosine receptors are lacking. Autoradiography studies have not been performed in this tissue and *in situ* hybridization experiments are not sensitive enough to detect the low levels of adenosine receptor mRNA in the stomach (Dixon et al., 1996). The present study uses the technique of immunohistochemistry to determine the cellular localization of

adenosine A_1 and A_{2A} receptors in the rat stomach. This study is the first to provide the anatomical basis for various gastric actions of adenosine.

In the present study, A_1R-IR and A_{2A}R-IR were found to be widely distributed throughout the stomach. Both were present on cells of the mucosa, cell bodies of the myenteric plexus, nerve fibers of the muscle layers and enteric plexi, and the gastric vasculature. The regional localization and relative density of A_1R-IR and A_{2A}R-IR in the gastric tissues agree well with results of the Real-Time RT-PCR experiments. Overall, A_1R-IR appeared to be more abundant than A_{2A}R-IR. Although some mucosal cells were shown to contain intense adenosine receptor staining, the majority of A_1R-IR and A_{2A}R-IR was localized in the enteric plexi. The localization of the gastric A_1 and A_{2A} receptors in various regions of the stomach also agrees well with their distribution in other tissues of the gastrointestinal tract. Although the anatomical distribution of adenosine receptors in the rat intestines has not been determined, immunohistochemical localization of the A_1 and A_{2A} receptors has been demonstrated in the human intestines (Christofi et al., 2001). Some anatomical structures, which were labeled in the present study, were also labeled in the human intestines. A_1R-IR was demonstrated in both the myenteric and submucosal plexi of the human jejunum and colon, respectively. Neurons of the circular muscle of these tissues contained A_1R-IR. Using the same A_{2A} receptor antibody as the present study, Christofi and co-workers (2001) also demonstrated the expression of A_{2A}R-IR in myenteric and submucosal neurons of the human jejunum and colon. In the jejunum, A_{2A}R-IR was found to be localized in epithelial cells, while, in the colon, staining of this receptor was observed in myenteric neurons. A_{2A}R-IR was also detected on some nerve fibers in the intestines, in particular, in the longitudinal muscle of the jejunum and circular muscle of the colon. The

neuronal localization of these receptors in the stomach and intestine agrees with the role of adenosine in the regulation of motility. The cellular localization of these receptors in the mucosa and vasculature provide the anatomical basis for the known effect of adenosine on somatostatin and gastrin release and vascular actions in the stomach, respectively.

1. Adenosine A_1 and A_{2A} receptors of the gastric mucosa

Results show that cells containing distinct A_1R-IR and A_{2A}R-IR were distributed throughout the corporeal and antral mucosa. These findings support the role of adenosine in regulating various gastric secretions. Tissue sections were double stained to determine whether these receptors are localized on specific cells of the mucosa, in particular, the acid-secreting parietal cells, gastrin-secreting G-cells, and somatostatin-secreting D-cells. Although ECL cells are known to be present in the mucosa, the localization of adenosine receptors on these cells was not examined. ECL cells are difficult to identify by immunohistochemistry since the preservation of histamine and histidine decarboxylase, ECL cell markers, require special fixation techniques (Bordi et al., 2000). ECL cells are commonly identified by chromogranin A immunostaining. However, this product is not exclusive to the ECL cells, and is also found in other endocrine cells such as G-cells. Results of our studies suggest that the A_1R and A_{2A}R are unlikely to be expressed on ECL cells of the rat. ECL cells make up a large proportion of the endocrine cell population, and reside at the base of the oxyntic mucosa (Dockray et al., 1996; Bordi et al., 2000). In the corporeal mucosa, the majority of A_1R-IR was localized on D-cells. The A_1 receptors that were not on D-cells were scattered throughout the mucosa and were not confined to the basal region. A_{2A}R-IR was not abundant in the corporeal mucosa, and was also scattered throughout the oxyntic mucosa. Since the ECL cells make up a large proportion of the endocrine cell

population and are localized only at the base of the oxyntic mucosa, any appreciable A_1 or A_{2A} receptor expression on these cells should be apparent. The effect of adenosine on histamine release has not been studied in rats. However, previous studies have shown that adenosine does not alter histamine release in either dog or rabbit (Ainz et al., 1993; Payne and Gerber, 1997). These studies, in combination with our immunohistochemistry results, suggest that adenosine is unlikely to alter histamine release in the rat.

Adenosine has been shown to stimulate or inhibit gastric acid secretion in the rat stomach, depending on the route of drug administration. When administered orally or intragastrically, adenosine and its analogs have been shown to decrease basal gastric acid secretion (see Table 2). These routes of administration expose adenosine to the gastric mucosa. Thus, adenosine may mediate its gastric acid inhibitory effect by acting on adenosine receptors in mucosal cells. Although radioligand binding studies have shown extremely low specific binding of ^{3}H-PIA, a selective A_1 receptor agonist, on isolated rat parietal cell preparations (Puurunen et al., 1987), results of the present experiments rule out the possible existence of A_1 receptors on parietal cells. Results suggest that adenosine does not act directly on the parietal cells since neither A_1 nor A_{2A} receptors were found on these cells. In the present study, parietal cells were identified by the staining of the H^+K^+-ATPase β subunit of the proton pump. Abundant H^+K^+-ATPase β-IR was localized throughout the isthmus, neck, and base of the oxyntic glands, as previously shown (Jiang et al., 2002). Double-staining experiments demonstrate that H^+K^+-ATPase β-IR was not co-localized with either A_1R-IR or A_{2A}R-IR. The absence of adenosine receptors on parietal cells also agrees with previous studies showing that R-PIA (A_1 agonist) and NECA (A_2 agonist) do not alter carbachol- and histamine-stimulated ^{14}C-aminopyrine accumulation in isolated rat parietal

cells (Puurunen et al., 1987). Adenosine analogs also did not alter basal or histamine-stimulated adenylate cyclase activity or carbachol-stimulated inositol phosphate formation in these cells (Puurunen et al., 1987).

1.1 Adenosine A_1 receptors on gastrin-secreting G-cells

Adenosine has been shown to inhibit gastrin release from the rat stomach (Kwok et al., 1990). The present experiments further demonstrate that this action involves A_1 receptors, but not A_2 or A_3 receptors. In agreement with these observations, our immunohistochemistry experiments demonstrate that A_1R-IR was present on all mucosal cells expressing gastrin-IR, while A_{2A}R-IR was not observed on any of these cells. The presence of A_1 receptors on G-cells suggests that adenosine may act directly on the G-cells to regulate gastrin release. This direct action may also occur in dogs since adenosine has been shown to inhibit forskolin-stimulated gastrin release through the activation of A_1 receptors in primary cultures of canine G-cells (Schepp et al., 1990).

Several possible mechanisms may be involved in the A_1 receptor-mediated inhibition of gastrin release. The release of gastrin is stimulated by the activation of multiple second messenger pathways. In isolated G-cells, activation of adenylyl cyclase, PLC and PKC are involved in stimulating gastrin release (Campos et al., 1990; Buchan and Meloche, 1994; Seensalu et al., 1997). Activation of A_1 receptors has been shown to inhibit adenylate cyclase activity, the most common signaling pathway recognized for the A_1 receptor (Fredholm et al., 2001). In G-cells, this second messenger pathway may be activated to inhibit basal gastrin release. Activation of the A_1 receptor may also oppose the actions of agents, such as forskolin, that act by stimulating adenylate cyclase. Activation of A_1 receptors has also been shown to alter PLC and PKC activity in some tissues (see Table 1).

In the stomach, these pathways may also be involved in the inhibitory action of adenosine on gastrin release. The release of gastrin is induced by the influx of extracellular calcium (Buchan and Meloche, 1994). High local concentrations of calcium are required for the exocytosis of gastrin (Oomori et al., 1997), and previous studies showed that gastrin secretion from G-cells requires the activation of L-type Ca^{2+} channels (Ray et al., 1997). Activation of A_1 receptors inhibits L-type channel activity in atrial myocytes (Belardinelli et al., 1995). Thus, these receptors may also inhibit gastrin secretion through the inactivation of these channels.

1.2 Adenosine A_1 and A_{2A} receptors on somatostatin secreting D-cells

The present study demonstrates that both A_1 and A_{2A} receptors were involved in modulating somatostatin release. Results of double staining immunohistochemistry experiments further demonstrates that both receptor subtypes were expressed on somatostatin-secreting D-cells of the corpus and antrum, suggesting that adenosine may act directly on D-cells to regulate somatostatin release.

In isolated rat oxyntic D-cells, somatostatin release has been shown to involve both phospholipid-dependent and cAMP-dependent signaling pathways (Schaffer et al., 1997). In human oxyntic D-cells, somatostatin release was also stimulated by the activation of adenylate cyclase and PKC pathways (Buchan et al., 1990, 1993). The adenosine A_{2A} receptor is coupled to the G_s protein and has been shown to stimulate adenylate cyclase activity (see Table 1). In striatal neurons, stimulation of this receptor has also been shown to activate the PKC pathway. Thus, A_{2A} receptors may stimulate somatostatin release by activating adenylate cyclase and PKC pathways. Conversely, A_1 receptors are coupled to the pertussis toxin-sensitive G protein, G_i, and activation of this receptor may inhibit

somatostatin release through the inhibition of adenylate cyclase activity. The activation of a pertussis-toxin sensitive G protein has previously been implicated in mediating the inhibitory effect of carbachol on pentagastrin-stimulated somatostatin release in canine oxyntic D-cells (Chiba et al., 1989).

Somatostatin is released from D-cell processes by an exocytotic process requiring high local concentrations of Ca^{2+}. In isolated oxyntic D-cells, CCK-stimulated somatostatin release was shown to involve the influx of extracellular Ca^{2+} through the L-type Ca^{2+} channels (DelValle et al., 1996). The release of somatostatin from D-cells is also stimulated by membrane depolarization (Patel, 1999). In the present study, confocal microscopy performed at high magnification reveals that most A_1R-IR appeared to be concentrated at the end of the D-cell processes. These receptors have previously been localized on presynaptic nerve terminals, and have been shown to inhibit the exocytosis of synaptic vesicles through the inactivation of N-type and L-type Ca^{2+} channels (Ralevic and Burnstock, 1998). Activation of A_1 receptors has also been shown to hyperpolarize neurons by increasing K^+ conductance (Belardinelli et al., 1995). Thus, in D-cells, A_1 receptors may inhibit somatostatin release by regulating exocytosis or by modulating the membrane potential.

2. Adenosine A_1 and A_{2A} receptors of the enteric plexi

The localization of gastric A_1 and A_{2A} receptors in nerve fibers of the myenteric and submucosal plexi is not surprising since studies have demonstrated adenosine-mediated actions in the intestinal enteric plexi (Burnstock, 2001; Christofi, 2001). Adenosine receptors in this region are likely involved in regulating gastric motility by modulating neurotransmitter release. Numerous studies examining the purinergic control of gastrointestinal motility have been performed in guinea pigs (Christofi, 2001). In general,

activation of A_1 and A_{2A} receptors has been shown to inhibit and stimulate gastrointestinal motility, respectively. In the rat jejunum, activation of the A_1 receptors has been shown to inhibit peristalsis (Hancock and Coupar, 1995), and in the ileum, these receptor have been shown to enhance descending relaxation (Storr et al., 2002). In the rat stomach, activation of A_1 receptors in the enteric plexi has been shown to mediate gastric relaxation (Matharu and Hollingsworth, 1992). Conversely, activation of A_2 receptor has been suggested to potentiate vagally-stimulated gastric contractions (Cho et al., 1993). Thus, adenosine A_1 and A_{2A} receptors are involved in the inhibitory (relaxation) and stimulatory (contraction) control of gastrointestinal motility, respectively. The effect of adenosine on gastrointestinal motility is likely mediated by its action on presynaptic and postsynaptic neurotransmission in the enteric plexi. Most studies that examine the neuromodulatory role of adenosine in the intestines have been performed in the guinea pig ileum, in particular, the guinea pig myenteric plexus-longitudinal muscle preparations. In the submucosal plexus of the guinea pig ileum, electrophysiological studies have shown that adenosine inhibits enteric neurotransmission by suppressing the excitability of myenteric neurons (Zafirov et al., 1985; Palmer et al., 1987). This inhibitory effect was mediated by presynaptic A_1 receptors on myenteric neurons (Christofi and Wood, 1993). Similarly, activation of presynaptic A_1 receptors were shown to inhibit neurotransmitter release from submucosal neurons (Barajas-Lopez et al., 1991). In the guinea pig ileum, adenosine inhibits acetylcholine release from enteric nerves (Sawynok and Jhamandas, 1976; Hayashi et al., 1978; Gustafsson et al., 1985). This inhibition was presynaptic in nature (Gustafsson et al., 1985; Christofi and Cook, 1986). A_1 receptor-mediated presynaptic inhibition of noradrenaline (Barajas-Lopez et al., 1991), neurokinin A (Broad et al., 1992) and substance P (Broad et al., 1993) release from enteric nerves endings

has also been demonstrated. In addition to its inhibitory effect on neurotransmitter release, adenosine may stimulate neurotransmitter release by acting directly on enteric neurons. This postsynaptic excitatory effect was shown to be mediated by A_{2A} receptors (Barajas-Lopez et al., 1991).

Although the effect of adenosine on the intestines has been studied extensively, the effect of this nucleoside is not well-studied in the stomach. In the gastric antrum of the guinea pig, adenosine and its analogs have been shown to inhibit nicotinic transmission in the myenteric plexus by acting on presynaptic adenosine receptors (Christofi et al., 1992). Furthermore, adenosine and its analogs have been shown to inhibit fast EPSPs in approximately 60% of gastric neurons (Christofi et al., 1992). Thus, the relatively high level of A_1R-IR and A_{2A}R-IR staining observed in the myenteric plexus of the rat stomach in the present study fits well with the known modulatory effects of adenosine on neurotransmission. In the rat stomach, adenosine has been demonstrated to protect against ethanol-induced gastric lesion formation (see Chapter 1, Section 3), but the mechanism involved has not been determined. Recent studies have shown that inhibition of gastric motility by anti-muscarinic drugs can protect the stomach against lesion formation induced by ethanol (Schicho et al., 2003). Thus, the A_1 receptor-mediated inhibition of acetylcholine release may be involved in protecting the stomach against ethanol injury.

Adenosine has also been shown to regulate gastric IRG and SLI release. Similar to its action on motility, the effect of adenosine on the release of these gastric peptides may be secondary to its effect on adrenergic and cholinergic neurotransmission. Adrenergic and cholinergic agents have been shown to alter the release of IRG and SLI (Saffouri et al., 1980; Koop et al., 1982, 1983; Schubert and Makhlouf, 1992). Previous experiments performed in

our laboratory have shown that the adenosine-induced release of SLI was not altered in the presence of the cholinergic blockers, atropine and hexamethonium, or the β-adrenergic blocker, propranolol (Kwok et al., 1990). Thus, adenosine is unlikely to modulate SLI release by acting on adrenergic or cholinergic nerves. The present study shows that adenosine receptors were expressed in the enteric plexi. Since various non-adrenergic, non-cholinergic nerve fibers are present in the plexi, it is possible that adenosine stimulates SLI release indirectly by modulating non-adrenergic, non-cholinergic neurotransmission. CGRP and VIP have been demonstrated to stimulate gastric somatostatin secretion (Chiba et al., 1980; Manela et al., 1995; Rasmussen et al., 2001). While the effect of adenosine on CGRP and VIP release has not been examined in the stomach, endogenous adenosine has been shown to tonically control CGRP and VIP release in the CNS (Sebastiao and Ribeiro, 2000).

The co-localization of A_{2A}R-IR with somatostatin-IR in some nerves of the myenteric plexus and circular muscle observed in the present study suggests that activation of A_{2A} receptors may elicit somatostatin release from nerve fibers. However, most of the somatostatin-IR was shown to reside in the D-cells, as previously reported (Vinik et al., 1981; Ekblad et al., 1985; Keast et al., 1985). Only sparse somatostatin staining was observed in the myenteric plexus and on nerve fibers. These somatostatin-IR fibers do not appear to project to the mucosa, and most of these fibers do not express A_{2A}R-IR. Thus, adenosine is more likely to act directly on mucosal D-cells to elicit SLI release.

The possibility that adenosine mediates its inhibitory effect on IRG release through adrenergic or cholinergic mechanisms has not been examined. It is also possible that adenosine mediates its inhibitory actions on IRG release by modulating the release of CGRP and VIP. These neurotransmitters have been shown to inhibit IRG release (Chiba et al.,

1980; Manela et al., 1995). However, the localization of A_1R on all gastrin-secreting G-cells of the mucosa demonstrated by the present study suggests that adenosine may inhibit gastrin secretion, at least in part, by acting on A_1 receptors on the G-cells. This proposal is supported further by the ability of adenosine to inhibit gastrin release from canine G-cells (Schepp et al., 1990) and rat antral mucosal cells in culture (DeSchryver-Kecskemeti et al., 1981).

3. Adenosine A_1 and A_{2A} receptors of the gastric vasculature

The vascular localization of the gastric adenosine receptors has been suggested by a number of studies demonstrating the action of adenosine on gastric mucosal blood flow (Cho and Ogle, 1990; Cho et al., 1991; Cho et al., 1995; Nagata et al., 1996). In the pulmonary artery (Biaggioni et al., 1989) and the renal vasculature (Holz and Steinhausen, 1987), activation of A_1 receptors has been shown to mediate vasoconstriction. Conversely, activation of A_{2A} receptors has been shown to mediate relaxation in coronary and pulmonary arteries, renal vasculature and the aorta (Tabrizchi and Bedi, 2001).

In the rat stomach, subcutaneous administration of adenosine has been shown to increase gastric mucosal blood flow (Cho et al., 1991; Cho et al., 1995). Adenosine has been shown to induce vasodilation in submucosal microvessels of the stomach by acting on A_2 receptors (Gerber and Guth, 1989; Nagata et al., 1996). This vasodilatory action may be involved in protecting the stomach against ethanol-induced gastric injury since the administration of ethanol has previously been shown to vasoconstrict submucosal blood vessels (Yonei and Guth, 1991). The perfusion of CGS 21680 into the isolated vascularly perfused rat stomach has also been demonstrated to decrease the gastric perfusion pressure

(Yip and Kwok, 2004), suggesting that the A_{2A} receptors are involved in mediating gastric vasodilation.

Adenosine has been proposed to act on A_{2A} receptors localized on vascular smooth muscle cells and endothelial cells (Tabrizchi and Bedi, 2001). In the rat mesenteric arterial bed, adenosine-induced vasodilation was shown to involve A_{2A} receptors localized on both of these cell types (Hiley et al., 1995). The immunohistochemical localization of the A_1 and A_{2A} receptors has previously been demonstrated in the vascular smooth muscle cells and endothelial cells of the human skeletal muscle (Lynge and Hellsten, 2000). Results of the current study clearly demonstrate that, in the stomach, both A_1 and A_{2A} receptors are localized on vascular endothelial cells and are also likely expressed on vascular smooth muscle cells.

The vasodilatory actions of adenosine in the stomach are mainly attributed to activation of A_{2A} receptors. An A_1 receptor-mediated action in the gastric vasculature has not yet been demonstrated. In the renal vasculature, the effect of adenosine was concentration-dependent. At low micromolar concentrations, adenosine elicited vasoconstriction via activation of A_1 receptors, while at higher concentrations (>10 μM), adenosine elicited vasodilation via activation of A_2 receptors (Tabrizchi and Bedi, 2001). In the rat gastric submucosal vessels, adenosine-induced vasodilation was demonstrated using >10 μM adenosine (Nagata et al., 1996). The effect of lower concentrations of adenosine has not been examined. The regulation of vascular tone by the A_1 receptor remains to be confirmed in the stomach.

In summary, the results of the immunohistochemistry study provide several new and significant insights into the distribution and localization of adenosine receptors. They

confirm the widespread distribution of A_1 and A_{2A} receptor expression in the enteric plexi and vasculature, and further support the suggestion that adenosine acts on these anatomical structures. The localization of A_1 and A_{2A} receptors on the G-cells and D-cells support the proposal that adenosine may act directly on these cells to modulate gastrin and somatostatin release, respectively. The lack of adenosine receptors on the parietal cells provides further evidence that adenosine does not regulate gastric acid secretion by acting directly on the parietal cells.

V. Conclusion

This study provides fundamental information on the functional role of A_1 and A_{2A} receptors in the rat stomach. Results suggest that adenosine may be an important regulator of gastric acid secretion. Unlike in other species, adenosine does not alter gastric acid secretion by acting directly on parietal cells in the rat. This lack of effect is confirmed in the present study by demonstrating the absence of A_1R-IR and A_{2A}R-IR on rat parietal cells. Instead, this study suggests that adenosine likely regulates gastric acid secretion by acting on A_1 receptors to inhibit gastrin and A_{2A} receptors to stimulate somatostatin release. The localization of A_1 and A_{2A} receptors on the G-cells and D-cells suggests that adenosine may act directly on these cells to modulate gastrin and somatostatin release. Since these receptors were also present on nerve fibers throughout the enteric plexi, the possibility that adenosine may modulate gastrin and somatostatin indirectly by altering neurotransmission cannot be ruled out. The localization of A_1 and A_{2A} receptors in the vasculature and enteric plexi also agrees with the known actions of adenosine in regulating gastric mucosal blood flow and motility.

The mucosal adenosine A_1 and A_{2A} receptors were shown to be structurally identical to those in the brain, and expressed in extremely low levels, using Real-Time RT-PCR and competitive RT-PCR. Real-Time RT-PCR was also used to examine the relationship between altered physiological states of the stomach and gastric A_1 and A_{2A} receptor gene expression. Like gastrin and somatostatin, the gene expression of A_1 and A_{2A} receptors is regulated by the gastric luminal environment. Fasting and omeprazole treatment were shown to alter adenosine receptor gene expression, most likely by changing the intraluminal acidity. Since these receptors are localized on G-cells and D-cells, changes in their expression may alter gastrin and somatostatin synthesis and secretion. Changes in adenosine receptor gene expression can be accompanied by corresponding changes in receptor function, suggesting that adenosine receptor expression may be determined, in part, by its gene expression.

The present experiments provide fundamental information regarding the function, distribution, and cellular localization of the gastric A_1 and A_{2A} receptors. Furthermore, this study suggests that under different physiological states of the stomach adenosine receptor expression may be altered to modulate gastrin and somatostatin synthesis and release, and, therefore, gastric acid secretion. Understanding the processes involved in the purinergic control of gastric functions is critical in understanding the role adenosine receptors may play in protecting the stomach in pathophysiological states. This understanding is vital to the future development of adenosine-based therapeutic agents.

References

Abbracchio MP, Brambilla R, Ceruti S, Kim HO, von Lubitz DK, Jacobson KA and Cattabeni F (1995) G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Mol Pharmacol* 48:1038-1045.

Ainz LF, Salgado C, Gandarias JM, Gomez R, Vallejo A and Gil-Rodrigo CE (1993) P1(A2/Ra)-purinoceptors may mediate the stimulatory effect of adenosine and adenosine analogs on acid formation in isolated rabbit parietal cells. *Pharmacol Res* 27:319-334.

Akatsuka Y, Egashira K, Katsuda Y, Narishige T, Ueno H, Shimokawa H and Takeshita A (1994) ATP sensitive potassium channels are involved in adenosine A2 receptor mediated coronary vasodilatation in the dog. *Cardiovasc Res* 28:906-911.

Allen JM, Bishop AE, Daly MJ, Larsson H, Carlsson E, Polak JM and Bloom SR (1986) Effect of inhibition of acid secretion on the regulatory peptides in the rat stomach. *Gastroenterology.* 90:970-977.

Anderson SM, Leu JR and Kant GJ (1988) Chronic stress increases the binding of the A1 adenosine receptor agonist, [3H]cyclohexyladenosine, to rat hypothalamus. *Pharmacol Biochem Behav* 30:169-175.

Andres CM and Fox IH (1979) Purification and properties of human placental adenosine kinase. *J Biol Chem* 254:11388-11393.

Asakura M, Kitakaze M, Sakata Y, Asanuma H, Sanada S, Kim J, Ogida H, Liao Y, Node K, Takashima S, Tada M and Hori M (2002) Adenosine-induced cardiac gene expression of ischemic murine hearts revealed by cDNA array hybridization. *Circ J* 66:93-96.

Asher O, Cunningham TD, Yao L, Gordon AS and Diamond I (2002) Ethanol stimulates cAMP-responsive element (CRE)-mediated transcription via CRE-binding protein and cAMP-dependent protein kinase. *J Pharmacol Exp Ther* 301:66-70.

Ashton KJ, Holmgren K, Peart J, Lankford AR, Paul Matherne G, Grimmond S and Headrick JP (2003) Effects of A1 adenosine receptor overexpression on normoxic and post-ischemic gene expression. *Cardiovasc Res* 57:715-726.

Atkinson MR, Townsend-Nicholson A, Nicholl JK, Sutherland GR and Schofield PR (1997) Cloning, characterisation and chromosomal assignment of the human adenosine A3 receptor (ADORA3) gene. *Neurosci Res* 29:73-79.

Auboeuf D and Vidal H (1997) The use of the reverse transcription-competitive polymerase chain reaction to investigate the in vivo regulation of gene expression in small tissue samples. *Anal Biochem* 245:141-148.

Bailey SJ, Hickman D and Hourani SM (1992) Characterization of the P1-purinoceptors mediating contraction of the rat colon muscularis mucosae. *Br J Pharmacol* 105:400-404.

Ballarin M, Fredholm BB, Ambrosio S and Mahy N (1991) Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta Physiol Scand* 142:97-103.

Barajas-Lopez C, Surprenant A and North RA (1991) Adenosine A1 and A2 receptors mediate presynaptic inhibition and postsynaptic excitation in guinea pig submucosal neurons. *J Pharmacol Exp Ther* 258:490-495.

Baraldi PG and Borea PA (2000) New potent and selective human adenosine A(3) receptor antagonists. *Trends Pharmacol Sci* 21:456-459.

Baraldi PG, Cacciari B, Romagnoli R, Merighi S, Varani K, Borea PA and Spalluto G (2000) A(3) adenosine receptor ligands: history and perspectives. *Med Res Rev* 20:103-128.

Barnette MS, Grous M, Cieslinski LB, Burman M, Christensen SB and Torphy TJ (1995) Inhibitors of phosphodiesterase IV (PDE IV) increase acid secretion in rabbit isolated gastric glands: correlation between function and interaction with a high-affinity rolipram binding site. *J Pharmacol Exp Ther* 273:1396-1402.

Basheer R, Porkka-Heiskanen T, Strecker RE, Thakkar MM and McCarley RW (2000) Adenosine as a biological signal mediating sleepiness following prolonged wakefulness. *Biol Signal Receptors* 9:319-327.

Basheer R, Rainnie DG, Porkka-Heiskanen T, Ramesh V and McCarley RW (2001) Adenosine, prolonged wakefulness, and A1-activated NF-kappaB DNA binding in the basal forebrain of the rat. *Neuroscience* 104:731-739.

Belardinelli L, Shryock JC, Song Y, Wang D and Srinivas M (1995) Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. *FASEB J* 9:359-365.

Berglindh T, Helander HF and Obrink KJ (1976) Effects of secretagogues on oxygen consumption, aminopyrine accumulation and morphology in isolated gastric glands. *Acta Physiol Scand* 97:401-414.

Biaggioni I, King LS, Enayat N, Robertson D and Newman JH (1989) Adenosine produces pulmonary vasoconstriction in sheep. Evidence for thromboxane A2/prostaglandin endoperoxide-receptor activation. *Circ Res* 65:1516-1525.

Blackburn MR (2003) Too much of a good thing: Adenosine overload in adenosine-deaminase-deficient mice. *Trends Pharmacol Sci* 24:66-70.

Bolkent S, Yilmazer S, Kaya F and Ozturk M (2001) Effects of acid inhibition on somatostatin-producing cells in the rat gastric fundus. *Acta Histochem* 103:413-422.

Bordi C, D'Adda T, Azzoni C and Ferraro G (2000) Classification of gastric endocrine cells at the light and electron microscopical levels. *Microsc Res Tech* 48:258-271.

Borkje B, Odegaard S, Skagen DW, Andersen KJ and Schrumpf E (1986) Enzyme activities in biopsy specimens from human gastric mucosa. *Scand J Gastroenterol* 21:1051-1057.

Bozkurt A, Yuksel M, Haklar G, Kurtel H, Yegen BC and Alican I (1998) Adenosine protects against indomethacin-induced gastric damage in rats. *Dig Dis Sci* 43:1258-1263.

Brackett LE and Daly JW (1994) Functional characterization of the A2b adenosine receptor in NIH 3T3 fibroblasts. *Biochem Pharmacol* 47:801-814.

Brand SJ and Stone D (1988) Reciprocal regulation of antral gastrin and somatostatin gene expression by omeprazole-induced achlorhydria. *J Clin Invest* 82:1059-1066.

Broad RM, McDonald TJ, Brodin E and Cook MA (1992) Adenosine A1 receptors mediate inhibition of tachykinin release from perifused enteric nerve endings. *Am J Physiol* 262:G525-531.

Broad RM, McDonald TJ and Cook MA (1993) Adenosine and 5-HT inhibit substance P release from nerve endings in myenteric ganglia by distinct mechanisms. *Am J Physiol* 264:G454-461.

Brownhill VR, Hourani SM and Kitchen I (1996) Differential ontogeny of adenosine receptors in the longitudinal muscle and muscularis mucosae of the rat isolated duodenum. *Eur J Pharmacol* 317:321-328.

Brundege JM, Diao L, Proctor WR and Dunwiddie TV (1997) The role of cyclic AMP as a precursor of extracellular adenosine in the rat hippocampus. *Neuropharmacology* 36:1201-1210.

Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD, Hays SJ and Huang CC (1987) Binding of the A1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn Schmiedebergs Arch Pharmacol* 335:59-63.

Bruns RF, Lu GH and Pugsley TA (1986) Characterization of the A2 adenosine receptor labeled by [3H]NECA in rat striatal membranes. *Mol Pharmacol* 29:331-346.

Buchan AM, Curtis SB and Meloche RM (1990) Release of somatostatin immunoreactivity from human antral D cells in culture. *Gastroenterology* 99:690-696.

Buchan AM and Meloche RM (1994) Signal transduction events involved in bombesin-stimulated gastrin release from human G cells in culture. *Can J Physiol Pharmacol* 72:1060-1065.

Buchan AM, Meloche RM, Kwok YN and Kofod H (1993) Effect of cholecystokinin and secretin on somatostatin release from cultured antral cells. *Gastroenterology* 104:1414-1419.

Buchan AM, Seal AM and Debas HT (1985a) Increased antral G-cell number and gastrin content in dogs after massive small bowel resection. *Dig Dis Sci* 30:236-239.

Buchan AM, Sikora LK, Levy JG, McIntosh CH, Dyck I and Brown JC (1985b) An immunocytochemical investigation with monoclonal antibodies to somatostatin. *Histochemistry.* 83:175-180.

Burnstock G (1975) Comparative studies of purinergic nerves. *J Exp Zool* 194:103-133.

Burnstock G (1978) A basis for distinguishing two types of purinergic receptor, in *Cell membrane receptors for drugs and hormones: a multidisiplinary approach* (Straub RW ed) pp 107-118, Raven press, New York.

Burnstock G (2001) Purinergic signalling in the gut, in *Purinergic and pyrimidinergic signalling I* (Abbracchio MP and Williams M eds) pp 141-238, Springer, Berlin.

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

Cabero JL, Li ZQ and Mardh S (1993) Gastrin action on aminopyrine accumulation in isolated pig parietal cells requires cAMP. *Biochim Biophys Acta* 1177:245-252.

Campos RV, Buchan AM, Meloche RM, Pederson RA, Kwok YN and Coy DH (1990) Gastrin secretion from human antral G cells in culture. *Gastroenterology* 99:36-44.

Carlsson E, Larsson H, Mattsson H, Ryberg B and Sundell G (1986) Pharmacology and toxicology of omeprazole--with special reference to the effects on the gastric mucosa. *Scand J Gastroenterol Suppl* 118:31-38.

Carruthers AM, Sellers LA, Jenkins DW, Jarvie EM, Feniuk W and Humphrey PP (2001) Adenosine A(1) receptor-mediated inhibition of protein kinase A-induced calcitonin gene-related peptide release from rat trigeminal neurons. *Mol Pharmacol* 59:1533-1541.

Centelles JJ, Franco R and Bozal J (1987) Distribution of adenosine deaminase in some rat tissues. Inhibition by ethanol and dimethyl sulfoxide. *Comp Biochem Physiol* 86:95-98.

Chae HD and Kim KT (1997) Stimulation of the A2A adenosine receptor increases expression of the tyrosine hydroxylase gene. *Brain Res Mol Brain Res* 44:31-38.

Cheng B, Essackjee HC and Ballard HJ (2000) Evidence for control of adenosine metabolism in rat oxidative skeletal muscle by changes in pH. *J Physiol* 522 Pt 3:467-477.

Chern Y, King K, Lai HL and Lai HT (1992) Molecular cloning of a novel adenosine receptor gene from rat brain. *Biochem Biophys Res Commun* 185:304-309.

Chiba T, Fujita T and Yamada T (1989) Carbachol inhibits stimulant-induced increases in fundic D-cell cytosolic Ca2+ concentration. *Am J Physiol* 257:G308-312.

Chiba T, Taminato T, Kadowaki S, Abe H, Chihara K, Seino Y, Matsukura S and Fujita T (1980) Effects of glucagon, secretin, and vasoactive intestinal polypeptide on gastric somatostatin and gastrin release from isolated perfused rat stomach. *Gastroenterology* 79:67-71.

Cho CH, Kaan SK and Wang HH (1995) Different pharmacological actions of adenosine on gastric function and mucosal damage in normotensive and spontaneously hypertensive rats. *Pharmacology* 50:261-265.

Cho CH, Liao NX, Liu XG, Hui WM, Luk CT and Lam SK (1991) Effects of adenosine on gastric and cardiovascular systems, and ethanol ulceration in male and female rats. *Eur J Pharmacol* 201:157-162.

Cho CH and Ogle CW (1990) Modulatory action of adenosine on gastric function and ethanol-induced mucosal damage in rats. *Dig Dis Sci* 35:1334-1339.

Cho CH, Qiu BS, Ballard HJ and Ogle CW (1993) Adenosine and cholinergic-induced gastric contraction in rats. *Digestion* 54:98-104.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.

Chow DC and Forte JG (1993) Characterization of the beta-subunit of the H(+)-K(+)-ATPase using an inhibitory monoclonal antibody. *Am J Physiol* 265:C1562-1570.

Christofi FL (2001) Unlocking mysteries of gut sensory transmission: is adenosine the key? *News Physiol Sci* 16:201-207.

Christofi FL and Cook MA (1986) Affinity of various purine nucleosides for adenosine receptors on purified myenteric varicosities compared to their efficacy as presynaptic inhibitors of acetylcholine release. *J Pharmacol Exp Ther* 237:305-311.

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

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

Christofi FL, Zhang H, Yu JG, Guzman J, Xue J, Kim M, Wang YZ and Cooke HJ (2001) Differential gene expression of adenosine A1, A2a, A2b, and A3 receptors in the human enteric nervous system. *J Comp Neurol* 439:46-64.

Chu YY, Tu KH, Lee YC, Kuo ZJ, Lai HL and Chern Y (1996) Characterization of the rat A2a adenosine receptor gene. *DNA Cell Biol* 15:329-337.

Ciruela F, Casado V, Mallol J, Canela EI, Lluis C and Franco R (1995) Immunological identification of A1 adenosine receptors in brain cortex. *J Neurosci Res* 42:818-828.

Cohen S and Booth GH, Jr. (1975) Gastric acid secretion and lower-esophageal-sphincter pressure in response to coffee and caffeine. *N Engl J Med* 293:897-899.

Comb M, Birnberg NC, Seasholtz A, Herbert E and Goodman HM (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* 323:353-356.

Conlay LA, Conant JA, deBros F and Wurtman R (1997) Caffeine alters plasma adenosine levels. *Nature* 389:136.

Connolly AR, Cleland LG and Kirkham BW (1995) Mathematical considerations of competitive polymerase chain reaction. *J Immunol Methods* 187:201-211.

Cooke AR (1976) Gastric damage by drugs and the role of the mucosal barrier. *Aust N Z J Med* 6 Suppl 1:Suppl 1:26-32.

Cornfield LJ, Hu S, Hurt SD and Sills MA (1992) [3H]2-phenylaminoadenosine ([3H]CV 1808) labels a novel adenosine receptor in rat brain. *J Pharmacol Exp Ther* 263:552-561.

Coupar IM and Hancock DL (1994) The adenosine agonist NECA inhibits intestinal secretion and peristalsis. *J Pharm Pharmacol* 46:801-804.

Cristalli G, Camaioni E, Costanzi S, Vittori S, Volpini R and Klotz KN (1998) Characterization of potent ligands at human recombinant adenosine receptors. *Drug Dev Res* 45:176-181.

Daly JW and Padgett WL (1992) Agonist activity of 2- and 5'-substituted adenosine analogs and their N6-cycloalkyl derivatives at A1- and A2-adenosine receptors coupled to adenylate cyclase. *Biochem Pharmacol* 43:1089-1093.

Dalziel HH and Westfall DP (1994) Receptors for adenine nucleotides and nucleosides: subclassification, distribution, and molecular characterization. *Pharmacol Rev* 46:449-466.

de Zwart M, Link R, von Frijtag Drabbe Kunzel JK, Cristalli G, Jacobson KA, Townsend-Nicholson A and AP IJ (1998) A functional screening of adenosine analogues at the adenosine A2B receptor: a search for potent agonists. *Nucleosides Nucleotides* 17:969-985.

Delahunty TM, Cronin MJ and Linden J (1988) Regulation of GH3-cell function via adenosine A1 receptors. Inhibition of prolactin release, cyclic AMP production and inositol phosphate generation. *Biochem J* 255:69-77.

DelValle J, Park J, Chiba T and Yamada T (1990) Cellular mechanisms of somatostatin action in the gut. *Metab Clin Exp* 39:134-137.

DelValle J, Wakasugi J, Takeda H and Yamada T (1996) Linkage of [Ca2+]i in single isolated D cells to somatostatin secretion induced by cholecystokinin. *Am J Physiol* 270:G897-901.

DelValle J and Yamada T (1990) Amino acids and amines stimulate gastrin release from canine antral G-cells via different pathways. *J Clin Invest* 85:139-143.

DeSchryver-Kecskemeti K, Greider MH, Rieders ER, Komyati SE and McGuigan JE (1981) In vitro gastrin secretion by rat antrum: effects of neurotransmitter agonists, antagonists, and modulators of secretion. *Lab Invest* 44:158-163.

Deussen A (2000) Metabolic flux rates of adenosine in the heart. *Naunyn Schmiedebergs Arch Pharmacol* 362:351-363.

Deussen A, Lloyd HG and Schrader J (1989) Contribution of S-adenosylhomocysteine to cardiac adenosine formation. *J Mol Cell Cardiol* 21:773-782.

Dickenson JM, Blank JL and Hill SJ (1998) Human adenosine A1 receptor and P2Y2-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. *Br J Pharmacol* 124:1491-1499.

Dimaline R, Evans D, Varro A and Dockray GJ (1991) Reversal by omeprazole of the depression of gastrin cell function by fasting in the rat. *J Physiol* 433:483-493.

Dimaline R, Sandvik AK, Barclay G and Dockray GJ (1992) Functional control of gastrin releasing peptide (GRP) mRNA in rat stomach. *FEBS Lett* 301:291-293.

Diniz C, Leal S and Goncalves J (2003) Regional differences in the adenosine A(2) receptor-mediated modulation of contractions in rat vas deferens. *Eur J Pharmacol* 460:191-199.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ and Freeman TC (1996) Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol* 118:1461-1468.

Dockray GJ, Hamer C, Evans D, Varro A and Dimaline R (1991) The secretory kinetics of the G cell in omeprazole-treated rats. *Gastroenterology* 100:1187-1194.

Dockray GJ, Varro A and Dimaline R (1996) Gastric endocrine cells: gene expression, processing, and targeting of active products. *Physiol Rev* 76:767-798.

Dolphin AC, Forda SR and Scott RH (1986) Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. *J Physiol* 373:47-61.

Dragon S, Offenhauser N and Baumann R (2002) cAMP and in vivo hypoxia induce tob, ifr1, and fos expression in erythroid cells of the chick embryo. *Am J Physiol* 282.

Dunwiddie TV, Diao L and Proctor WR (1997) Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J Neurosci* 17:7673-7682.

Durak I, Ormeci N, Akyol O, Canbolat O, Kavutcu M and Bulbul M (1994) Adenosine deaminase, 5'-nucleotidase, xanthine oxidase, superoxide dismutase, and catalase activities in gastric juices from patients with gastric cancer, ulcer, and atrophic gastritis. *Dig Dis Sci* 39:721-728.

Edkins JS (1905) The chemical mechanism of gastric secretion. *J Physiol* 34:133.

Ekblad E, Ekelund M, Graffner H, Hakanson R and Sundler F (1985) Peptide-containing nerve fibers in the stomach wall of rat and mouse. *Gastroenterology* 89:73-85.

Feifel E, Obexer P, Andratsch M, Euler S, Taylor L, Tang A, Wei Y, Schramek H, Curthoys NP and Gstraunthaler G (2002) p38 MAPK mediates acid-induced transcription of PEPCK in LLC-PK(1)-FBPase(+) cells. *Am J Physiol Renal Physiol* 283:F678-688.

Feoktistov I and Biaggioni I (1995) Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* 96:1979-1986.

Feoktistov I, Goldstein AE and Biaggioni I (1999) Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol* 55:726-734.

Ferre F (1992) Quantitative or semi-quantitative PCR: reality versus myth. *PCR Meth Appl* 2:1-9.

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM and Reppert SM (1992) Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res Mol Brain Res* 14:186-195.

Fozard JR, Baur F and Wolber C (2003) Antagonist pharmacology of adenosine A2B receptors from rat, guinea pig and dog. *Eur J Pharmacol* 475:79-84.

Franco R, Casado V, Ciruela F, Saura C, Mallol J, Canela EI and Lluis C (1997) Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog Neurobiol* 52:283-294.

Fredholm BB (1982) Adenosine actions and adenosine receptors after 1 week treatment with caffeine. *Acta Physiol Scand* 115:283-286.

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

Freeman WM, Walker SJ and Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 26:112-122, 124-115.

Freissmuth M, Selzer E and Schutz W (1991) Interactions of purified bovine brain A1-adenosine receptors with G-proteins. Reciprocal modulation of agonist and antagonist binding. *Biochem J* 275 (Pt 3):651-656.

Fujimiya M and Kwok YN (1997) Effect of carbachol on vascular and luminal release of immunoreactive gastrin from isolated perfused rat duodenum. *Dig Dis Sci* 42:634-639.

Furlong TJ, Pierce KD, Selbie LA and Shine J (1992) Molecular characterization of a human brain adenosine A2 receptor. *Brain Res Mol Brain Res* 15:62-66.

Furness JB, Young HM, Pompolo S, Bornstein JC, Kunze WA and McConalogue K (1995) Plurichemical transmission and chemical coding of neurons in the digestive tract. *Gastroenterology* 108:554-563.

Gallo-Rodriguez C, Ji XD, Melman N, Siegman BD, Sanders LH, Orlina J, Fischer B, Pu Q, Olah ME, van Galen PJ and et al. (1994) Structure-activity relationships of N6-benzyladenosine-5'-uronamides as A3-selective adenosine agonists. *J Med Chem* 37:636-646.

Gao ZG, Chen A, Barak D, Kim SK, Muller CE and Jacobson KA (2002) Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A3 adenosine receptor. *J Biol Chem* 277:19056-19063.

Geiger JD and Glavin GB (1985) Adenosine receptor activation in brain reduces stress-induced ulcer formation. *Eur J Pharmacol* 115:185-190.

Geiger JD, Parkinson PE and Kowaluk EA (1997) Regulators of endogenous adenosine levels as therapeutic agents, in *Purinertgic Approaches in Experimental Therapeutics* (Jacobson KA and Jarvis MF eds) pp 55-84, Wiley-Liss, Inc., New York.

Gerber JG, Fadul S, Payne NA and Nies AS (1984) Adenosine: a modulator of gastric acid secretion in vivo. *J Pharmacol Exp Ther* 231:109-113.

Gerber JG and Guth PH (1989) Role of adenosine in the gastric blood flow response to pentagastrin in the rat. *J Pharmacol Exp Ther* 251:550-556.

Gerber JG, Nies AS and Payne NA (1985) Adenosine receptors on canine parietal cells modulate gastric acid secretion to histamine. *J Pharmacol Exp Ther* 233:623-627.

Gerwins P and Fredholm BB (1991) Glucocorticoid receptor activation leads to up-regulation of adenosine A1 receptors and down-regulation of adenosine A2 responses in DDT1 MF-2 smooth muscle cells. *Mol Pharmacol* 40:149-155.

Gerwins P and Fredholm BB (1995) Activation of phospholipase C and phospholipase D by stimulation of adenosine A1, bradykinin or P2U receptors does not correlate well with protein kinase C activation. *Naunyn Schmiedebergs Arch Pharmacol* 351:194-201.

Gil-Rodrigo CE, Bergaretxe I, Carou M, Galdiz B, Salgado C and Ainz LF (1996) Inhibitory action of extracellular adenosine 5'-triphosphate on parietal cells isolated from rabbit gastric mucosa. *Gen Physiol Biophys* 15:251-264.

Gil-Rodrigo CE, Galdiz B, Gandarias JM, Gomez R and Ainz LF (1990) Characterization of the effects of adenosine, adenosine 5'-triphosphate and related purines on acid secretion in isolated rabbit gastric glands. *Pharmacol Res* 22:103-113.

Glavin GB, Westerberg VS and Geiger JD (1987) Modulation of gastric acid secretion by adenosine in conscious rats. *Can J Physiol Pharmacol* 65:1182-1185.

Gould J, Morton MJ, Sivaprasadarao A, Bowmer CJ and Yates MS (1997) Renal adenosine A1 receptor binding characteristics and mRNA levels during the development of acute renal failure in the rat. *Br J Pharmacol* 120:947-953.

Green A, Milligan G and Dobias SB (1992) Gi down-regulation as a mechanism for heterologous desensitization in adipocytes. *J Biol Chem* 267:3223-3229.

Grover AK, Agrawal DK, Ahmad S, Daniel EE, Kwan CY, Oakes PJ, Sipos SN, Berenski C and Jung C (1985) Target size of 5'-nucleotidase in smooth muscle. *J Biochem (Tokyo)* 98:573-575.

Grover AK, Oakes P, Sipos SN, Kwan CY and Garfield RE (1983) Studies on canine gastric antrum smooth muscle: preparation and characterization of a plasma membrane enriched fraction. *Can J Physiol Pharmacol* 61:927-940.

Gubitz AK, Widdowson L, Kurokawa M, Kirkpatrick KA and Richardson PJ (1996) Dual signalling by the adenosine A2a receptor involves activation of both N- and P-type calcium channels by different G proteins and protein kinases in the same striatal nerve terminals. *J Neurochem* 67:374-381.

Guo YS, Bhalla VK, Thompson JC and Singh P (1988) Effect of cyclic nucleotides on bombesin-evoked gastrin release from isolated perfused rat stomach. *Regul Pept* 22:361-369.

Gustafsson L (1981) Influence of adenosine on responses to vagal nerve stimulation in the anesthetized rabbit. *Acta Physiol Scand* 111:263-268.

Gustafsson LE, Wiklund NP, Lundin J and Hedqvist P (1985) Characterization of pre- and post-junctional adenosine receptors in guinea-pig ileum. *Acta Physiol Scand* 123:195-203.

Hancock DL and Coupar IM (1995) Functional characterization of the adenosine receptor mediating inhibition of peristalsis in the rat jejunum. *Br J Pharmacol* 115:739-744.

Harty RF and Franklin PA (1984) Effects of exogenous and endogenous adenosine on gastrin release from rat antral mucosa. *Gastroenterology* 86:1107.

Hayashi E, Mori M, Yamada S and Kumitomo M (1978) Effects of purine compounds on cholinergic nerves. Specificity of adenosine and related compounds on acetylcholine release in electircally stimulated guinea pig ileum. *Eur J Pharmacol* 48:297-307.

Hayward AL, Oefner PJ, Sabatini S, Kainer DB, Hinojos CA and Doris PA (1998) Modeling and analysis of competitive RT-PCR. *Nucleic Acids Res* 26:2511-2518.

Heid CA, Stevens J, Livak KJ and Williams PM (1996) Real time quantitative PCR. *Genome Res* 6:986-994.

Heldsinger AA, Vinik AI and Fox IH (1986) Inhibition of guinea-pig oxyntic cell function by adenosine and prostaglandins. *J Pharmacol Exp Ther* 237:351-356.

Henley WN, Schuebel KE and Nielsen DA (1996) Limitations imposed by heteroduplex formation on quantitative RT-PCR. *Biochem Biophys Res Commun* 226:113-117.

Hersey SJ and Sachs G (1995) Gastric acid secretion. *Physiol Rev* 75:155-189.

Hiley CR, Bottrill FE, Warnock J and Richardson PJ (1995) Effects of pH on responses to adenosine, CGS 21680, carbachol and nitroprusside in the isolated perfused superior mesenteric arterial bed of the rat. *Br J Pharmacol* 116:2641-2646.

Hirschowitz BI, Keeling D, Lewin M, Okabe S, Parsons M, Sewing K, Wallmark B and Sachs G (1995) Pharmacological aspects of acid secretion. *Dig Dis Sci* 40:3S-23S.

Holst JJ, Jensen SL, Knuhtsen S, Nielsen OV and Rehfeld JF (1983) Effect of vagus, gastric inhibitory polypeptide, and HCl on gastrin and somatostatin release from perfused pig antrum. *Am J Physiol* 244:G515-522.

Holz FG and Steinhausen M (1987) Renovascular effects of adenosine receptor agonists. *Ren Physiol* 10:272-282.

Hutchison AJ, Webb RL, Oei HH, Ghai GR, Zimmerman MB and Williams M (1989) CGS 21680C, an A2 selective adenosine receptor agonist with preferential hypotensive activity. *J Pharmacol Exp Ther* 251:47-55.

IJzerman A, Van Galen PJ and Jacobson KA (1992) Molecular modeling of adenosine receptors. I. The ligand binding site on the A1 receptor. *Drug Des Discov* 9:49-67.

IJzerman AP, van der Wenden EM, van Galen PJ and Jacobson KA (1994) Molecular modeling of adenosine receptors. The ligand binding site on the rat adenosine A2A receptor. *Eur J Pharmacol* 268:95-104.

Imoto A, Inoue R, Tanaka M and Ito Y (1998) Inhibitory NANC neurotransmission in choledocho-duodenal junction of rabbits--a possible role of PACAP. *J Auton Nerv Syst* 70:189-199.

Inauen W, Wyss PA, Kayser S, Baumgartner A, Schurer-Maly CC, Koelz HR and Halter F (1988) Influence of prostaglandins, omeprazole, and indomethacin on healing of experimental gastric ulcers in the rat. *Gastroenterology.* 95:636-641.

Jacobson KA (1996) Specific Ligands for the Adenosine Receptor Family. *Neurotransmissions* 12:1-6.

Jacobson KA, Ravi RG, Nandanan E, Kim HS, Moro S, Kim YC, Lee K, Barak D, Marquez VE and Ji XD (2001) Ribose modified nucleosides and nucleotides as ligands for purine receptors. *Nucleosides, Nucleotides & Nucleic Acids* 20:333-341.

Jacobson MA, Johnson RG, Luneau CJ and Salvatore CA (1995) Cloning and chromosomal localization of the human A2b adenosine receptor gene (ADORA2B) and its pseudogene. *Genomics* 27:374-376.

Jaffe B and Walsh JH (1978) Gastrin and related peptides, in *Methods of Hormone Radioimmunoassay* (Jaffe bM and Behrman HR eds) pp 455-477, Academic Press, New York.

Ji XD and Jacobson KA (1999) Use of the triazolotriazine [3H]ZM 241385 as a radioligand at recombinant human A2B adenosine receptors. *Drug Des Discov* 16:217-226.

Jiang X, Suzaki E and Kataoka K (2002) Immunofluorescence detection of gastric H(+)/K(+)-ATPase and its alterations as related to acid secretion. *Histochem Cell Biol* 117:21-27.

Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S and Freissmuth M (1994) Species difference in the G protein selectivity of the human and bovine A1-adenosine receptor. *J Biol Chem* 269:32077-32084.

Johansson B, Ahlberg S, van der Ploeg I, Brene S, Lindefors N, Persson H and Fredholm BB (1993) Effect of long term caffeine treatment on A1 and A2 adenosine receptor binding and on mRNA levels in rat brain. *Naunyn Schmiedebergs Arch Pharmacol* 347:407-414.

Kadowaki M, Takeda M, Tokita K, Hanaoka K and Tomoi M (2000) Molecular identification and pharmacological characterization of adenosine receptors in the guinea-pig colon. *Br J Pharmacol* 129:871-876.

Kapuscinski M and Shulkes A (1995) Secretory and biosynthetic responses of gastrin and somatostatin to acute changes in gastric acidity. *J Gastroenterol Hepatol* 10:405-412.

Keast JR, Furness JB and Costa M (1985) Distribution of certain peptide-containing nerve fibres and endocrine cells in the gastrointestinal mucosa in five mammalian species. *J Comp Neurol* 236:403-422.

Kim J, Wess J, van Rhee AM, Schoneberg T and Jacobson KA (1995) Site-directed mutagenesis identifies residues involved in ligand recognition in the human A2a adenosine receptor. *J Biol Chem* 270:13987-13997.

Kim YC, Ji X, Melman N, Linden J and Jacobson KA (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *J Med Chem* 43:1165-1172.

Kintner DB, Anderson MK, Fitzpatrick JH, Jr., Sailor KA and Gilboe DD (2000) 31P-MRS-based determination of brain intracellular and interstitial pH: its application to in vivo H+ compartmentation and cellular regulation during hypoxic/ischemic conditions. *Neurochem Res* 25:1385-1396.

Kivilaakso E and Kiviluoto T (1988) Intracellular pH in isolated Necturus antral mucosa in simulated ulcerogenic conditions. *Gastroenterology* 95:1198-1205.

Kiviluoto T, Paimela H, Mustonen H and Kivilaakso E (1990) Intracellular pH in isolated Necturus antral mucosa exposed to luminal acid. *Gastroenterology* 98:901-908.

Klotz KN (2000) Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch Pharmacol* 362:382-391.

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB and Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes - characterization of

stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* 357:1-9.

Kobayashi S and Millhorn DE (1999) Stimulation of expression for the adenosine A2A receptor gene by hypoxia in PC12 cells. A potential role in cell protection. *J Biol Chem* 274:20358-20365.

Konturek SJ, Brzozowski T, Pajdo R, Konturek PC, Kwiecien S, Sliwowski Z, Pawlik M, Ptak A, Drozdowicz D and Hahn EG (2001) Gastric preconditioning induced by short ischemia: the role of prostaglandins, nitric oxide and adenosine. *Med Sci Monit* 7:610-621.

Koop H, Behrens I, Bothe E, Koschwitz H, McIntosh CH, Pederson RA, Arnold R and Creutzfeldt W (1983) Adrenergic control of rat gastric somatostatin and gastrin release. *Scand J Gastroenterol* 18:65-71.

Koop H, Behrens I, Bothe E, McIntosh CH, Pederson RA, Arnold R and Creutzfeldt W (1982) Adrenergic and cholinergic interactions in rat gastric somatostatin and gastrin release. *Digestion* 25:96-102.

Kovacs TO, Walsh JH, Maxwell V, Wong HC, Azuma T and Katt E (1989) Gastrin is a major mediator of the gastric phase of acid secretion in dogs: proof by monoclonal antibody neutralization. *Gastroenterology* 97:1406-1413.

Krasnow S and Grossman MI (1949) Stimulation of gastric secretion in man by theophylline ethylenediamine. *Proc Soc Exp Biol Med* 71:335-336.

Kull B, Svenningsson P and Fredholm BB (2000) Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Mol Pharmacol* 58:771-777.

Kusumoto Y and Grube D (1987) Somatotostatin (D-) cells in the rat pyloric antrum, with special reference to the destination of their cytoplasmic processes. *Biomed Res* 8:145-151.

Kusumoto Y, Sato AG, Kaneda K and Nakamae E (1995) Cytology and microenvironment of somatostatin (D) cells in the gastric fundic mucosa of rodents. *Hiroshima J Med Sci* 44:105-112.

Kwok YN, McIntosh C and Brown J (1990) Augmentation of release of gastric somatostatin-like immunoreactivity by adenosine, adenosine triphosphate and their analogs. *J Pharmacol Exp Ther* 255:781-788.

Kwok YN and McIntosh CH (1990) Release of substance P-like immunoreactivity from the vascularly perfused rat stomach. *Eur J Pharmacol* 180:201-207.

Kwok YN, McIntosh CH, Pederson RA and Brown JC (1985) Effect of substance P on somatostatin release from the isolated perfused rat stomach. *Gastroenterology* 88:90-95.

Kwok YN, McIntosh CH, Sy H and Brown JC (1988) Inhibitory actions of tachykinins and neurokinins on release of somatostatin-like immunoreactivity from the isolated perfused rat stomach. *J Pharmacol Exp Ther* 246:726-731.

Larsson H, Carlsson E, Ryberg B, Fryklund J and Wallmark B (1988) Rat parietal cell function after prolonged inhibition of gastric acid secretion. *Am J Physiol* 254:G33-39.

Larsson LI and Hougaard DM (1993) Sensitive detection of rat gastrin mRNA by in situ hybridization with chemically biotinylated oligodeoxynucleotides: validation, quantitation, and double-staining studies. *J Histochem Cytochem* 41:157-163.

Latini S, Bordoni F, Pedata F and Corradetti R (1999) Extracellular adenosine concentrations during in vitro ischaemia in rat hippocampal slices. *Br J Pharmacol* 127:729-739.

Le F, Townsend-Nicholson A, Baker E, Sutherland GR and Schofield PR (1996) Characterization and chromosomal localization of the human A2a adenosine receptor gene: ADORA2A. *Biochem Biophys Res Commun* 223:461-467.

Le Poole IC, Yang F, Brown TL, Cornelius J, Babcock GF, Das PK and Boissy RE (1999) Altered gene expression in melanocytes exposed to 4-tertiary butyl phenol (4-TBP): upregulation of the A2b adenosine receptor 1. *J Invest Dermatol* 113:725-731.

Lee CW and Jarvis SM (1988) Nucleoside transport in rat cerebral-cortical synaptosomes. Evidence for two types of nucleoside transporters. *Biochem J* 249:557-564.

Lee H, Hakanson R, Karlsson A, Mattsson H and Sundler F (1992) Lansoprazole and omeprazole have similar effects on plasma gastrin levels, enterochromaffin-like cells, gastrin cells and somatostatin cells in the rat stomach. *Digestion* 51:125-132.

Lee YC, Chang CW, Su CW, Lin TN, Sun SH, Lai HL and Chern Y (1999) The 5' untranslated regions of the rat A2A adenosine receptor gene function as negative translational regulators. *J Neurochem* 73:1790-1798.

Lee YC, Lai HL, Sun CN, Chien CL and Chern Y (2003) Identification of nuclear factor 1 (NF1) as a transcriptional modulator of rat A(2A) adenosine receptor. *Brain Res Mol Brain Res* 111:61-73.

Lehy T, Gres L and Ferreira de Castro E (1979) Quantitation of gastrin and somatostatin cell populations in the antral mucosa of the rat. Comparative distribution and evolution through different life stages. *Cell Tissue Res* 198:325-333.

Li ZQ, Cabero JL and Mardh S (1995) Gastrin and carbachol require cAMP to elicit aminopyrine accumulation in isolated pig and rat parietal cells. *Am J Physiol* 268:G82-89.

Libert F, Parmentier M, Lefort A, Dinsart C, Van Sande J, Maenhaut C, Simons MJ, Dumont JE and Vassart G (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* 244:569-572.

Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, Vassart G, Ensinger HA and Mendla KD (1992) Cloning and functional characterization of a human A1 adenosine receptor. *Biochem Biophys Res Commun* 187:919-926.

Lichtenberger LM, Lechago J and Johnson LR (1975) Depression of antral and serum gastrin concentration by food deprivation in the rat. *Gastroenterology* 68:1473-1479.

Lloyd HG, Deussen A, Wuppermann H and Schrader J (1988) The transmethylation pathway as a source for adenosine in the isolated guinea-pig heart. *Biochem J* 252:489-494.

Lloyd KC, Raybould HE, Tache Y and Walsh JH (1992) Role of gastrin, histamine, and acetylcholine in the gastric phase of acid secretion in anesthetized rats. *Am J Physiol* 262:G747-755.

Lockey C, Otto E and Long Z (1998) Real-time fluorescence detection of a single DNA molecule. *Biotechniques* 24:744-746.

Lohse MJ, Klotz KN, Lindenborn-Fotinos J, Reddington M, Schwabe U and Olsson RA (1987) 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)--a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 336:204-210.

Londos C, Cooper DM and Wolff J (1980) Subclasses of external adenosine receptors. *Proc Natl Acad Sci U S A* 77:2551-2554.

Lorbar M, Fenton RA, Duffy AJ, Graybill CA and Dobson JG, Jr. (1999) Effect of aging on myocardial adenosine production, adenosine uptake and adenosine kinase activity in rats. *J Mol Cell Cardiol* 31:401-412.

Lupica CR, Cass WA, Zahniser NR and Dunwiddie TV (1990) Effects of the selective adenosine A2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J Pharmacol Exp Ther* 252:1134-1141.

Lynge J and Hellsten Y (2000) Distribution of adenosine A1, A2A and A2B receptors in human skeletal muscle. *Acta Physiol Scand* 169:283-290.

Mabley J, Soriano F, Pacher P, Hasko G, Marton A, Wallace R, Salzman A and Szabo C (2003) The adenosine A3 receptor agonist, N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide, is protective in two murine models of colitis. *Eur J Pharmacol* 466:323-329.

Magee DF (1996) Pyloric antral inhibition of gastrin release. *J Gastroenterol* 31:758-763.

Mahan LC, McVittie LD, Smyk-Randall EM, Nakata H, Monsma FJ, Jr., Gerfen CR and Sibley DR (1991) Cloning and expression of an A1 adenosine receptor from rat brain. *Mol Pharmacol* 40:1-7.

Malek RL, Nie Z, Ramkumar V and Lee NH (1999) Adenosine A(2A) receptor mRNA regulation by nerve growth factor is TrkA-, Src-, and Ras-dependent via extracellular regulated kinase and stress-activated protein kinase/c-Jun NH(2)-terminal kinase. *J Biol Chem* 274:35499-35504.

Manela FD, Ren J, Gao J, McGuigan JE and Harty RF (1995) Calcitonin gene-related peptide modulates acid-mediated regulation of somatostatin and gastrin release from rat antrum. *Gastroenterology* 109:701-706.

Martindale R, Kauffman GL, Levin S, Walsh JH and Yamada T (1982) Differential regulation of gastrin and somatostatin secretion from isolated perfused rat stomachs. *Gastroenterology* 83:240-244.

Matharu MS and Hollingsworth M (1992) Purinoceptors mediating relaxation and spasm in the rat gastric fundus. *Br J Pharmacol* 106:395-403.

Matsumoto A, Asada S, Saitoh O, Tei H, Okumura Y, Hirata I and Ohshiba S (1989) A study on gastric ulcers induced by long-term fasting in rats. *Scand J Gastroenterol Suppl* 162:75-78.

Matsuno M, Matsui T, Iwasaki A and Arakawa Y (1997) Role of acetylcholine and gastrin-releasing peptide (GRP) in gastrin secretion. *J Gastroenterol* 32:579-586.

McIntosh C, Arnold R, Bothe E, Becker H, Kobberling J and Creutzfeldt W (1978) Gastrointestinal somatostatin: extraction and radioimmunoassay in different species. *Gut.* 19:655-663.

McIntosh CH (1985) Gastrointestinal somatostatin: distribution, secretion and physiological significance. *Life Sci* 37:2043-2058.

McIntosh CH, Bakich V, Kwok YN, Wong J and Brown JC (1987a) The effects of substance P, histamine and histamine antagonists on somatostatin and gastrin release from the isolated perfused rat stomach. *Regul Pept* 19:253-263.

McIntosh CH, Kwok YN, Mordhorst T, Nishimura E, Pederson RA and Brown JC (1983) Enkephalinergic control of somatostatin secretion from the perfused rat stomach. *Can J Physiol Pharmacol* 61:657-663.

McIntosh CHS, Kwok YN, Tang C and Brown JC (1987b) The use of monoclonal antibodies in RIA for gastrointestinal hormones. *J Clin Immunoassay* 10:79-84.

Meghji P, Middleton KM and Newby AC (1988) Absolute rates of adenosine formation during ischaemia in rat and pigeon hearts. *Biochem J* 249:695-703.

Melani A, Pantoni L, Corsi C, Bianchi L, Monopoli A, Bertorelli R, Pepeu G and Pedata F (1999) Striatal outflow of adenosine, excitatory amino acids, gamma-aminobutyric acid, and taurine in awake freely moving rats after middle cerebral artery occlusion: correlations with neurological deficit and histopathological damage. *Stroke* 30:2448-2454; discussion 2455.

Mendelson WB, Kuruvilla A, Watlington T, Goehl K, Paul SM and Skolnick P (1983) Sedative and electroencephalographic actions of erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA): relationship to inhibition of brain adenosine deaminase. *Psychopharmacology* 79:126-129.

Meno JR, Ngai AC, Ibayashi S and Winn HR (1991) Adenosine release and changes in pial arteriolar diameter during transient cerebral ischemia and reperfusion. *J Cereb Blood Flow Metab* 11:986-993.

Milusheva EA, Doda M, Baranyi M and Vizi ES (1996) Effect of hypoxia and glucose deprivation on ATP level, adenylate energy charge and [Ca2+]o-dependent and independent release of [3H]dopamine in rat striatal slices. *Neurochem Int* 28:501-507.

Montminy M, Brindle P, Arias J, Ferreri K and Armstrong R (1996a) Regulation of somatostatin gene transcription by cAMP. *Adv Pharmacol* 36:1-13.

Montminy M, Brindle P, Arias J, Ferreri K and Armstrong R (1996b) Regulation of somatostatin gene transcription by cyclic adenosine monophosphate. *Metab Clin Exp* 45:4-7.

Montminy MR, Sevarino KA, Wagner JA, Mandel G and Goodman RH (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc Natl Acad Sci U S A* 83:6682-6686.

Moriwaki Y, Yamamoto T and Higashino K (1999) Enzymes involved in purine metabolism--a review of histochemical localization and functional implications. *Histol Histopathol* 14:1321-1340.

Moriwaki Y, Yamamoto T, Yamaguchi K, Takahashi S and Higashino K (1996) Immunohistochemical localization of aldehyde and xanthine oxidase in rat tissues using polyclonal antibodies. *Histochem Cell Biol* 105:71-79.

Muller CE (2001) A1 adenosine receptors and their ligands: overview and recent developments. *Farmaco* 56:77-80.

Muller CE, Geis U, Hipp J, Schobert U, Frobenius W, Pawlowski M, Suzuki F and Sandoval-Ramirez J (1997) Synthesis and structure-activity relationships of 3,7-dimethyl-1-propargylxanthine derivatives, A2A-selective adenosine receptor antagonists. *J Med Chem* 40:4396-4405.

Murrison EM, Goodson SJ, Edbrooke MR and Harris CA (1996) Cloning and characterisation of the human adenosine A3 receptor gene. *FEBS Lett* 384:243-246.

Murthy VS, Zagar ME, Vollmer RR and Schmidt DH (1982) Pentobarbital-induced changes in vagal tone and reflex vagal activity in rabbits. *Eur J Pharmacol* 84:41-50.

Nagata H, Sekizuka E, Morishita T, Tatemichi M, Kurokawa T, Mizuki A and Ishii H (1996) Adenosine A2-receptor mediates ethanol-induced arteriolar dilation in rat stomach. *Am J Physiol* 271:G1028-1033.

Nakata H (1993) Development of an antiserum to rat-brain A1 adenosine receptor: application for immunological and structural comparison of A1 adenosine receptors from various tissues and species. *Biochim Biophys Acta* 1177:93-98.

Namiot Z, Marcinkiewicz M, Jaroszewicz W, Stasiewicz J and Gorski J (1993) Mucosal adenosine deaminase activity and gastric ulcer healing. *Eur J Pharmacol* 243:301-303.

Namiot Z, Rutkiewicz J, Stasiewicz J, Baranczuk E and Marcinkiewicz M (1991) Adenosine deaminase activity in the gastric mucosa in patients with gastric ulcer. Effects of ranitidine and sucralfate. *Eur J Pharmacol* 205:101-103.

Namiot Z, Rutkiewicz J, Stasiewicz J and Gorski J (1990) Adenosine deaminase activity in the human gastric mucosa in relation to acid secretion. *Digestion* 45:172-175.

Navarro A, Zapata R, Canela EI, Mallol J, Lluis C and Franco R (1999) Epidermal growth factor (EGF)-induced up-regulation and agonist- and antagonist-induced desensitization and internalization of A1 adenosine receptors in a pituitary-derived cell line. *Brain Res* 816:47-57.

Nicholls J, Brownhill VR and Hourani SM (1996) Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *Br J Pharmacol* 117:170-174.

Nicholls J, Hourani SM and Kitchen I (1992) Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *Br J Pharmacol* 105:639-642.

Nie Z, Mei Y, Ford M, Rybak L, Marcuzzi A, Ren H, Stiles GL and Ramkumar V (1998) Oxidative stress increases A1 adenosine receptor expression by activating nuclear factor kappa B. *Mol Pharmacol* 53:663-669.

Nie Z, Mei Y, Malek RL, Marcuzzi A, Lee NH and Ramkumar V (1999) A role of p75 in NGF-mediated down-regulation of the A(2A) adenosine receptors in PC12 cells. *Mol Pharmacol* 56:947-954.

Nitahara K, Kittel A, Liang SD and Vizi ES (1995) A1-receptor-mediated effect of adenosine on the release of acetylcholine from the myenteric plexus: role and localization of ecto-ATPase and 5'-nucleotidase. *Neuroscience* 67:159-168.

Olah ME (1997) Identification of A2a adenosine receptor domains involved in selective coupling to Gs. Analysis of chimeric A1/A2a adenosine receptors. *J Biol Chem* 272:337-344.

Olah ME and Stiles GL (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. *Ann Rev Pharmacol Toxicol* 35:581-606.

Olah ME and Stiles GL (2000) The role of receptor structure in determining adenosine receptor activity. *Pharmacol Ther* 85:55-75.

Oomori Y, Satoh Y, Ishikawa K and Gesase AP (1997) Exocytosis in the antral gastrin cells of mouse, rat, and guinea pig after stimulation by carbamylcholine. *Cell Tissue Res* 289:463-472.

Orlando C, Pinzani P and Pazzagli M (1998) Developments in quantitative PCR. *Clin Chem Lab Med* 36:255-269.

Ota S, Hata Y, Hiraishi H, Mutoh H, Terano A and Sugimoto T (1992) The effects of acid secretagogues on protective agents of gastric cells from adult rabbits in vitro. *J Clin Gastroenterol* 14:S156-161.

Ota S, Hiraishi H, Terano A, Mutoh H, Kurachi Y, Shimada T, Ivey KJ and Sugimoto T (1989) Effect of adenosine and adenosine analogs on [14C]aminopyrine accumulation by rabbit parietal cells. *Dig Dis Sci* 34:1882-1889.

Othman T, Legare D, Sadri P, Lautt WW and Parkinson FE (2002) A preliminary investigation of the effects of maternal ethanol intake during gestation and lactation on brain adenosine A(1) receptor expression in rat offspring. *Neurotoxicol Teratol* 24:275-279.

Pajdo R, Brzozowski T, Konturek PC, Kwiecien S, Konturek SJ, Sliwowski Z, Pawlik M, Ptak A, Drozdowicz D and Hahn EG (2001) Ischemic preconditioning, the most effective gastroprotective intervention: involvement of prostaglandins, nitric oxide, adenosine and sensory nerves. *Eur J Pharmacol* 427:263-276.

Pak MA, Haas HL, Decking UK and Schrader J (1994) Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology* 33:1049-1053.

Palmer JM, Wood JD and Zafirov DH (1987) Purinergic inhibition in the small intestinal myenteric plexus of the guinea-pig. *J Physiol* 387:357-369.

Palmer TM, Gettys TW and Stiles GL (1995a) Differential interaction with and regulation of multiple G-proteins by the rat A3 adenosine receptor. *J Biol Chem* 270:16895-16902.

Palmer TM, Poucher SM, Jacobson KA and Stiles GL (1995b) 125I-4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol, a high affinity

antagonist radioligand selective for the A2a adenosine receptor. *Mol Pharmacol* 48:970-974.

Park J, Chiba T and Yamada T (1987) Mechanisms for direct inhibition of canine gastric parietal cells by somatostatin. *J Biol Chem* 262:14190-14196.

Patel YC (1999) Somatostatin and its receptor family. *Front Neuroendocrinol* 20:157-198.

Pawlikowski M, Karbownik M, Lewinski A, Pisarek H, Wajs E and Szkudlinski M (1992) Effects of omeprazole on the number of immunoreactive gastrin- and somatostatin-cells in the rat gastric mucosa. *Histol Histopathol* 7:153-156.

Payne NA and Gerber JG (1997) Effect of adenosine and histamine receptor stimulation on canine histamine release to pentagastrin. *Digestion* 58:352-360.

Pazzagli M, Corsi C, Fratti S, Pedata F and Pepeu G (1995) Regulation of extracellular adenosine levels in the striatum of aging rats. *Brain Res* 684:103-106.

Peachey JA, Hourani SM and Kitchen I (1996) Differential development of adenosine A1 and A2b receptors in the rat duodenum. *Br J Pharmacol* 119:949-958.

Peachey JA, Hourani SM and Kitchen I (1999) Ontogeny of adenosine receptors in the longitudinal muscle and muscularis mucosae of the rat distal colon. *Naunyn Schmiedebergs Arch Pharmacol* 359:140-146.

Peakman MC and Hill SJ (1994) Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *Br J Pharmacol* 111:191-198.

Pederson RA, Kwok YN, Buchan AM, McIntosh CH and Brown JC (1984) Gastrin release from isolated perfused rat stomach after vagotomy. *Am J Physiol* 247:G248-252.

Peterfreund RA, Gies EK and Fink JS (1997) Protein kinase C regulates adenosine A2a receptor mRNA expression in SH-SY5Y cells. *Eur J Pharmacol* 336:71-80.

Peterfreund RA, MacCollin M, Gusella J and Fink JS (1996) Characterization and expression of the human A2a adenosine receptor gene. *J Neurochem* 66:362-368.

Phillis JW, O'Regan MH and Perkins LM (1992) Measurement of rat plasma adenosine levels during normoxia and hypoxia. *Life Sci* 51:PL149-152.

Phillis JW, Walter GA, O'Regan MH and Stair RE (1987) Increases in cerebral cortical perfusate adenosine and inosine concentrations during hypoxia and ischemia. *J Cereb Blood Flow Metab* 7:679-686.

Piatak M, Jr., Luk KC, Williams B and Lifson JD (1993) Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *Biotechniques* 14:70-81.

Pierce KD, Furlong TJ, Selbie LA and Shine J (1992) Molecular cloning and expression of an adenosine A2b receptor from human brain. *Biochem Biophys Res Commun* 187:86-93.

Poucher SM, Keddie JR, Brooks R, Shaw GR and McKillop D (1996) Pharmacodynamics of ZM 241385, a potent A2a adenosine receptor antagonist, after enteric administration in rat, cat and dog. *J Pharm Pharmacol* 48:601-606.

Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PW, Jones G and Coll MG (1995) The in vitro pharmacology of ZM 241385, a potent, non-xanthine A2a selective adenosine receptor antagonist. *Br J Pharmacol* 115:1096-1102.

Prentice DJ and Hourani SM (1997) Adenosine analogues relax guinea-pig taenia caeci via an adenosine A2B receptor and a xanthine-resistant site. *Eur J Pharmacol* 323:103-106.

Prinz C, Kajimura M, Scott DR, Mercier F, Helander HF and Sachs G (1993) Histamine secretion from rat enterochromaffinlike cells. *Gastroenterology* 105:449-461.

Puurunen J, Aittakumpu R and Tanskanen T (1986) Vagally mediated stimulation of gastric acid secretion by intravenously administered adenosine derivatives in anaesthetized rats. *Acta Pharmacol Toxicol* 58:265-271.

Puurunen J and Huttunen P (1988) Central gastric antisecretory action of adenosine in the rat. *Eur J Pharmacol* 147:59-66.

Puurunen J, Ruoff HJ and Schwabe U (1987) Lack of direct effect of adenosine on the parietal cell function in the rat. *Pharmacol Toxicol* 60:315-317.

Raeymaekers L (1995) A commentary on the practical applications of competitive PCR. *Genome Res* 5:91-94.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492.

Ramkumar V, Olah ME, Jacobson KA and Stiles GL (1991) Distinct pathways of desensitization of A1- and A2-adenosine receptors in DDT1 MF-2 cells. *Mol Pharmacol* 40:639-647.

Ramkumar V, Wilson M, Dhanraj DN, Gettys TW and Ali H (1995) Dexamethasone up-regulates A3 adenosine receptors in rat basophilic leukemia (RBL-2H3) cells. *J Immunol* 154:5436-5443.

Rasmussen TN, Schmidt P, Poulsen SS and Holst JJ (2001) Effect of calcitonin gene-related peptide (CGRP) on motility and on the release of substance P, neurokinin A, somatostatin and gastrin in the isolated perfused porcine antrum. *Neurogastroenterol Motil* 13:353-359.

Ravid K, Smith-Mungo LI, Zhao Z, Thomas KM and Kagan HM (1999) Upregulation of lysyl oxidase in vascular smooth muscle cells by cAMP: role for adenosine receptor activation. *J Cell Biochem* 75:177-185.

Ray JM, Squires PE, Meloche RM, Nelson DW, Snutch TP and Buchan AM (1997) L-type calcium channels regulate gastrin release from human antral G cells. *Am J Physiol* 273:G281-288.

Reeves JJ, Coates J, Jarvis JE, Sheehan MJ and Strong P (1993) Characterization of the adenosine receptor mediating contraction in rat colonic muscularis mucosae. *Br J Pharmacol* 110:1255-1259.

Ren H and Stiles GL (1994a) Characterization of the human A1 adenosine receptor gene. Evidence for alternative splicing. *J Biol Chem* 269:3104-3110.

Ren H and Stiles GL (1994b) Posttranscriptional mRNA processing as a mechanism for regulation of human A1 adenosine receptor expression. *Proc Natl Acad Sci U S A* 91:4864-4866.

Ren H and Stiles GL (1995) Separate promoters in the human A1 adenosine receptor gene direct the synthesis of distinct messenger RNAs that regulate receptor abundance. *Mol Pharmacol* 48:975-980.

Ren H and Stiles GL (1998) A single-stranded DNA binding site in the human A1 adenosine receptor gene promoter. *Mol Pharmacol* 53:43-51.

Ren H and Stiles GL (1999) Dexamethasone stimulates human A1 adenosine receptor (A1AR) gene expression through multiple regulatory sites in promoter B. *Mol Pharmacol* 55:309-316.

Ren J, Young RL, Lassiter DC and Harty RF (1993) Calcitonin gene-related peptide mediates capsaicin-induced neuroendocrine responses in rat antrum. *Gastroenterology* 104:485-491.

Reppert SM, Weaver DR, Stehle JH and Rivkees SA (1991) Molecular cloning and characterization of a rat A1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol Endocrinol* 5:1037-1048.

Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Hyde RJ, Karpinski E, Cass CE, Baldwin SA and Young JD (2001) Recent molecular advances in studies of the concentrative Na+-dependent nucleoside transporter (CNT) family: identification and characterization of novel human and mouse proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *Mol Membr Biol* 18:65-72.

Rivkees SA, Chen M, Kulkarni J, Browne J and Zhao Z (1999) Characterization of the murine A1 adenosine receptor promoter, potent regulation by GATA-4 and Nkx2.5. *J Biol Chem* 274:14204-14209.

Rivkees SA, Price SL and Zhou FC (1995) Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain Res* 677:193-203.

Rivkees SA and Reppert SM (1992) RFL9 encodes an A2b-adenosine receptor. *Mol Endocrinol* 6:1598-1604.

Robinson AJ and Dickenson JM (2001) Regulation of p42/p44 MAPK and p38 MAPK by the adenosine A1 receptor in DDT1MF-2 cells. *Eur J Pharmacol* 413:151-161.

Rose'Meyer RB, Mellick AS, Garnham BG, Harrison GJ, Massa HM and Griffiths LR (2003) The measurement of adenosine and estrogen receptor expression in rat brains following ovariectomy using quantitative PCR analysis. *Brain Res Brain Res Protoc* 11:9-18.

Sachs G, Zeng N and Prinz C (1997) Physiology of isolated gastric endocrine cells. *Ann Rev Physiol* 59:243-256.

Saffouri B, DuVal JW, Arimura A and Makhlouf GM (1984) Effects of vasoactive intestinal peptide and secretin on gastrin and somatostatin secretion in the perfused rat stomach. *Gastroenterology* 86:839-842.

Saffouri B, Weir GC, Bitar KN and Makhlouf GM (1980) Gastrin and somatostatin secretion by perfused rat stomach: functional linkage of antral peptides. *Am J Physiol* 238:G495-501.

Saitoh O, Saitoh Y and Nakata H (1994) Regulation of A2a adenosine receptor mRNA expression by agonists and forskolin in PC12 cells. *Neuroreport* 5:1317-1320.

Sajjadi FG, Boyle DL, Domingo RC and Firestein GS (1996) cDNA cloning and characterization of A3i, an alternatively spliced rat A3 adenosine receptor variant. *FEBS Lett* 382:125-129.

Sakai Y, McLean J, Grover AK, Garfield RE, Fox JE and Daniel EE (1981) Isolation and characterization of subcellular membranes from canine stomach smooth muscle. *Can J Physiol Pharmacol* 59:1260-1267.

Sala-Newby GB, Skladanowski AC and Newby AC (1999) The mechanism of adenosine formation in cells. Cloning of cytosolic 5'-nucleotidase-I. *J Biol Chem* 274:17789-17793.

Salvatore CA, Jacobson MA, Taylor HE, Linden J and Johnson RG (1993) Molecular cloning and characterization of the human A3 adenosine receptor. *Proc Natl Acad Sci U S A* 90:10365-10369.

Sambrook J, Fritsch EF and Maniatis T (1989a) Extraction, purification, and analysis of messenger of RNA from eukaryotic cells, in *Molecular cloning: a laboratory manual* (Nolan C ed) pp 7.1-7.87, Cold Spring Harbor Laboratory Press, Plainview.

Sambrook J, Fritsch EF and Maniatis T (1989b) Gel electrophoresis of DNA, in *Molecular cloning: a laboratory manual* (Nolan C ed) pp 6.1-6.62, Cold Spring Harbor Laboratory Press, Plainview.

Sandvik AK, Dimaline R, Brenna E and Waldum HL (1995) Differential expression and regulation of SSTR2 messenger RNA in rat gastric antrum and corpus. *Am J Physiol* 269:G542-547.

Sandvik AK, Dimaline R, Forster ER, Evans D and Dockray GJ (1993) Differential control of somatostatin messenger RNA in rat gastric corpus and antrum. Role of acid, food, and capsaicin-sensitive afferent neurons. *J Clin Invest* 91:244-250.

Sanyal A, O'Driscoll SW, Bolander ME and Sarkar G (1997) An effective method of completely removing contaminating genomic DNA from an RNA sample to be used for PCR. *Mol Biotechnol* 8:135-137.

Sawmiller DR and Chou CC (1990) Jejunal adenosine increases during food-induced jejunal hyperemia. *Am J Physiol* 258:G370-376.

Sawynok J and Jhamandas KH (1976) Inhibition of acetylcholine release from cholinergic nerves by adenosine, adenine nucleotides and morphine: antagonism by theophylline. *J Pharmacol Exp Ther* 197:379-390.

Scarpignato C, Tramacere R, Zappia L and Del Soldato P (1987) Inhibition of gastric acid secretion by adenosine receptor stimulation in the rat. *Pharmacology* 34:264-268.

Schaffer K, Herrmuth H, Mueller J, Coy DH, Wong HC, Walsh JH, Classen M, Schusdziarra V and Schepp W (1997) Bombesin-like peptides stimulate somatostatin release from rat fundic D cells in primary culture. *Am J Physiol* 273:G686-695.

Schepp W, Soll AH and Walsh JH (1990) Dual modulation by adenosine of gastrin release from canine G-cells in primary culture. *Am J Physiol* 259:G556-563.

Schicho R, Schemann M, Pabst MA, Holzer P and Lippe IT (2003) Capsaicin-sensitive extrinsic afferents are involved in acid-induced activation of distinct myenteric neurons in the rat stomach. *Neurogastroenterol Motil* 15:33-44.

Schmittgen TD (2001) Real-time quantitative PCR. *Methods* 25:383-385.

Schmittgen TD and Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* 46:69-81.

Schmugge M, Rand ML and Freedman J (2003) Platelets and von Willebrand factor. *Transfus Apheresis Sci* 28:269-277.

Schofield JN, Day IN, Thompson RJ and Edwards YH (1995) PGP9.5, a ubiquitin C-terminal hydrolase; pattern of mRNA and protein expression during neural development in the mouse. *Brain Res Dev Brain Res* 85:229-238.

Schreiber S, Nguyen TH, Stuben M and Scheid P (2000) Demonstration of a pH gradient in the gastric gland of the acid-secreting guinea pig mucosa. *Am J Physiol Gastrointest Liver Physiol* 279:G597-604.

Schubert ML, Jong MJ and Makhlouf GM (1991) Bombesin/GRP-stimulated somatostatin secretion is mediated by gastrin in the antrum and intrinsic neurons in the fundus. *Am J Physiol* 261:G885-889.

Schubert ML and Makhlouf GM (1992) Neural, hormonal, and paracrine regulation of gastrin and acid secretion. *Yale J Biol Med* 65:553-560.

Schubert ML and Shamburek RD (1990) Control of acid secretion. *Gastroenterol Clin North Am* 19:1-25.

Schulte G and Fredholm BB (2000) Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol Pharmacol* 58:477-482.

Schusdziarra V, de la Fuente A, Swobodnik W, Fussganger R and Pfeiffer EF (1983) Somatostatin release from perifused rat and human gastric mucosa. *Clin Physiol Biochem* 1:12-16.

Schwarting H, Koop H, Gellert G and Arnold R (1986) Effect of starvation on endocrine cells in the rat stomach. *Regul Pept* 14:33-39.

Seale TW, Abla KA, Shamim MT, Carney JM and Daly JW (1988) 3,7-Dimethyl-1-propargylxanthine: a potent and selective in vivo antagonist of adenosine analogs. *Life Sci* 43:1671-1684.

Sebastiao AM and Ribeiro JA (2000) Fine-tuning neuromodulation by adenosine. *Trends Pharmacol Sci* 21:341-346.

Seensalu R, Avedian D, Barbuti R, Song M, Slice L and Walsh JH (1997) Bombesin-induced gastrin release from canine G cells is stimulated by Ca2+ but not by protein kinase C, and is enhanced by disruption of rho/cytoskeletal pathways. *J Clin Invest* 100:1037-1046.

Selemidis S, Satchell DG and Cocks TM (1997) Evidence that NO acts as a redundant NANC inhibitory neurotransmitter in the guinea-pig isolated taenia coli. *Br J Pharmacol* 121:604-611.

Sexl V, Mancusi G, Holler C, Gloria-Maercker E, Schutz W and Freissmuth M (1997) Stimulation of the mitogen-activated protein kinase via the A2A-adenosine receptor in primary human endothelial cells. *J Biol Chem* 272:5792-5799.

Shinozuka K, Maeda T and Hayashi E (1985) Possibilities for adenosine modulation of peristaltic reflex in guinea pig isolated ileum. *J Pharmacobiodyn* 8:877-884.

Shiotani A and Merchant JL (1995) cAMP regulates gastrin gene expression. *Am J Physiol* 269:G458-464.

Shulkes A, Caussignac Y, Lamers CB, Solomon TE, Yamada T and Walsh JH (1983) Starvation in the rat: effect on peptides of the gut and brain. *Aust J Exp Biol Med Sci* 61 (Pt 5):581-587.

Sinclair CJ, LaRiviere CG, Young JD, Cass CE, Baldwin SA and Parkinson FE (2000) Purine uptake and release in rat C6 glioma cells: nucleoside transport and purine metabolism under ATP-depleting conditions. *J Neurochem* 75:1528-1538.

Souza RF, Shewmake K, Terada LS and Spechler SJ (2002) Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. *Gastroenterology.* 122:299-307.

Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD and Reppert SM (1992) Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. *Mol Endocrinol* 6:384-393.

Stening GF and Grossman MI (1970) Gastric acid response to pentagastrin and histamine after extragastric vagotomy in dogs. *Gastroenterology* 59:364-371.

Stone TW and Simmonds HA (1991) *Purines: basic and clinical aspects*. Kluwer academic publishers, Dordrecht.

Storr M, Thammer J, Dunkel R, Schusdziarra V and Allescher HD (2002) Modulatory effect of adenosine receptors on the ascending and descending neural reflex responses of rat ileum. *BMC Neurosci* 3:21.

Su C, Bevan JA and Burnstock G (1971) [3H]adenosine triphosphate: release during stimulation of enteric nerves. *Science* 173:336-338.

Sundaram U, Hassanain H, Suntres Z, Yu JG, Cooke HJ, Guzman J and Christofi FL (2003) Rabbit chronic ileitis leads to up-regulation of adenosine A1/A3 gene products, oxidative stress, and immune modulation. *Biochem Pharmacol* 65:1529-1538.

Suzuki M, Tomaru A, Kishibayashi N and Karasawa A (1995) Effects of the adenosine A1-receptor antagonist on defecation, small intstinal propulsion and gastric emptying in rats. *Jpn J Pharmacol* 68:119-123.

Suzuki T, Higgins PJ and Crawford DR (2000) Control selection for RNA quantitation. *Biotechniques* 29:332-337.

Svenningsson P and Fredholm BB (1997) Glucocorticoids regulate the expression of adenosine A1 but not A(2A) receptors in rat brain. *J Pharmacol Exp Ther* 280:1094-1101.

Tabrizchi R and Bedi S (2001) Pharmacology of adenosine receptors in the vasculature. *Pharmacol Ther* 91:133-147.

Takehara Y, Sumii K, Tari A, Yoshihara M, Sumii M, Haruma K, Kajiyama G, Wu SV and Walsh JH (1996) Evidence that endogenous GRP in rat stomach mediates omeprazole-induced hypergastrinemia. *Am J Physiol* 271:G799-G804.

Tally KJ, Hrnjez BJ, Smith JA, Mun EC and Matthews JB (1996) Adenosine scavenging: a novel mechanism of chloride secretory control in intestinal epithelial cells. *Surgery* 120:248-254.

Tang L, Parker M, Fei Q and Loutzenhiser R (1999) Afferent arteriolar adenosine A2a receptors are coupled to KATP in in vitro perfused hydronephrotic rat kidney. *Am J Physiol* 277:F926-933.

Tari A, Wu V, Sumii M, Sachs G and Walsh JH (1991) Regulation of rat gastric H+/K(+)-ATPase alpha-subunit mRNA by omeprazole. *Biochim Biophys Acta* 1129:49-56.

Thorn JA and Jarvis SM (1996) Adenosine transporters. *Gen Pharmacol* 27:613-620.

Townsend-Nicholson A, Baker E, Schofield PR and Sutherland GR (1995) Localization of the adenosine A1 receptor subtype gene (ADORA1) to chromosome 1q32.1. *Genomics* 26:423-425.

Track NS, Creutzfeldt C, Arnold R and Creutzfeldt W (1978) The antral gastrin-producing G-cell: biochemical and ultrastructural responses to feeding. *Cell Tiss Res* 194:131-139.

Traversa U, Rosati AM, Florio C and Vertua R (1994) Effects of chronic administration of adenosine antagonists on adenosine A1 and A2a receptors in mouse brain. *In Vivo* 8:1073-1078.

Ushijima I, Mizuki Y, Hara T, Obara N, Minematsu N and Yamada M (1992) Effects of lithium and purinergic compounds on the behavioral and physiological aspects of restraint stress in rats. *Pharmacol Biochem Behav* 42:431-435.

Ushijima I, Mizuki Y and Yamada M (1985) Development of stress-induced gastric lesions involves central adenosine A1-receptor stimulation. *Brain Res* 339:351-355.

Vallejo AI, Bo X and Burnstock G (1996) P2Y purinoceptors in gastric gland plasma membranes. *Eur J Pharmacol* 312:209-214.

van Calker D, Muller M and Hamprecht B (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* 33:999-1005.

van Galen PJ, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, AP IJ, Stiles GL and Jacobson KA (1994) A binding site model and structure-activity relationships for the rat A3 adenosine receptor. *Mol Pharmacol* 45:1101-1111.

Vincent SR, McIntosh CH, Buchan AM and Brown JC (1985) Central somatostatin systems revealed with monoclonal antibodies. *J Comp Neurol* 238:169-186.

Vinik AI, Gaginella TS, O'Dorisio TM, Shapiro B and Wagner L (1981) The distribution and characterization of somatostatin-like immunoreactivity in epithelial cells, submucosa, and muscle of the rat stomach and intestine. *Endocrinology* 109:1921-1926.

Vizi ES, Sperlagh B and Baranyi M (1992) Evidence that ATP released from the postsynaptic site by noradrenaline, is involved in mechanical responses of guinea-pig vas deferens: cascade transmission. *Neuroscience* 50:455-465.

Wall RT, Counts RB, Harker LA and Striker GE (1980) Binding and release of factor VIII/von Willebrand's factor by human endothelial cells. *Br J Haematol* 46:287-298.

Wang AM, Doyle MV and Mark DF (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A* 86:9717-9721.

Westerberg VS and Geiger JD (1987) Central effects of adenosine analogs on stress-induced gastric ulcer formation. *Life Sci* 41:2201-2205.

Westerberg VS and Geiger JD (1989) Adenosine analogs inhibit gastric acid secretion. *Eur J Pharmacol* 160:275-281.

Wiklund NP and Gustafsson LE (1987) On the nature of endogenous purines modulating cholinergic neurotransmission in the guinea-pig ileum. *Acta Physiol Scand* 131:11-18.

Wilson PO, Barber PC, Hamid QA, Power BF, Dhillon AP, Rode J, Day IN, Thompson RJ and Polak JM (1988) The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br J Exp Pathol* 69:91-104.

Witte DP, Wiginton DA, Hutton JJ and Aronow BJ (1991) Coordinate developmental regulation of purine catabolic enzyme expression in gastrointestinal and postimplantation reproductive tracts. *J Cell Biol* 115:179-190.

Worku Y and Newby AC (1983) The mechanism of adenosine production in rat polymorphonuclear leucocytes. *Biochem J* 214:325-330.

Worpenberg S, Burk O and Klempnauer KH (1997) The chicken adenosine receptor 2B gene is regulated by v-myb. *Oncogene* 15:213-221.

Wu SV, Giraud A, Mogard M, Sumii K and Walsh JH (1990a) Effects of inhibition of gastric secretion on antral gastrin and somatostatin gene expression in rats. *Am J Physiol* 258:G788-793.

Wu SV, Sumii K, Tari A, Mogard M and Walsh JH (1990b) Regulation of gastric somatostatin gene expression. *Metab Clin Exp* 39:125-130.

Wu V, Sumii K, Tari A, Sumii M and Walsh JH (1991) Regulation of rat antral gastrin and somatostatin gene expression during starvation and after refeeding. *Gastroenterology* 101:1552-1558.

Xaus J, Mirabet M, Lloberas J, Soler C, Lluis C, Franco R and Celada A (1999) IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J Immunol* 162:3607-3614.

Xu PA and Kellems RE (2000) Function of murine adenosine deaminase in the gastrointestinal tract. *Biochem Biophys Res Commun* 269:749-757.

Xue L and Lucocq JM (1997) Low extracellular pH induces activation of ERK 2, JNK, and p38 in A431 and Swiss 3T3 cells. *Biochem Biophys Res Commun* 241:236-242.

Yaar R, Cataldo LM, Tzatsos A, Francis CE, Zhao Z and Ravid K (2002) Regulation of the A3 adenosine receptor gene in vascular smooth muscle cells: role of a cAMP and GATA element. *Mol Pharmacol* 62:1167-1176.

Yakel JL, Warren RA, Reppert SM and North RA (1993) Functional expression of adenosine A2b receptor in Xenopus oocytes. *Mol Pharmacol* 43:277-280.

Yamada H, Chen D, Monstein HJ and Hakanson R (1997a) Effects of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. *Biochem Biophys Res Commun* 231:835-838.

Yamada M, Ikeuchi T and Hatanaka H (1997b) The neurotrophic action and signalling of epidermal growth factor. *Prog Neurobiol* 51:19-37.

Yamamura T, Kimura T and Furukawa K (1983) Effects of halothane, thiamylal, and ketamine on central sympathetic and vagal tone. *Anesth Analg* 62:129-134.

Yip L and Kwok YN (2004) Role of adenosine A2A receptor in the regulation of gastric somatostatin release. *J Pharmacol Exp Ther* (in press).

Yip L, Kwok YN and Buchan AM (2003) Cellular localization and distribution of neurokinin-1 receptors in the rat stomach. *Auton Neurosci* 104:95-108.

Yonei Y and Guth PH (1991) Ethanol-induced gastric injury. Role of submucosal venoconstriction and leukotrienes. *Dig Dis Sci* 36:601-608.

Zafirov DH, Palmer JM and Wood JD (1985) Adenosine inhibits forskolin-induced excitation in myenteric neurons. *Eur J Pharmacol* 113:143-144.

Zaki M, Harrington L, McCuen R, Coy DH, Arimura A and Schubert ML (1996) Somatostatin receptor subtype 2 mediates inhibition of gastrin and histamine secretion from human, dog, and rat antrum. *Gastroenterology* 111:919-924.

Zhao Z, Francis C and Ravid K (1999) Characterization of the mouse A3 adenosine receptor gene: exon/intron organization and promoter activity. *Genomics* 57:152-155.

Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL and Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc Natl Acad Sci U S A* 89:7432-7436.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* 362:299-309.

Zucchi R, Yu G, Ghelardoni S, Ronca F and Ronca-Testoni S (2001) A3 adenosine receptor stimulation modulates sarcoplasmic reticulum Ca(2+) release in rat heart. *Cardiovasc Res* 50:56-64.

Appendix 1: Table of abbreviations

2-CA	2-chloroadenosine
8-PT	8-phenyltheophylline
8-SPT	8-(p-sulfophenyl)theophyline
A_1R-IR	A_1 receptor immunoreactivity
A_{2A}R-IR	A_{2A} receptor immunoreactivity
ADA	adenosine deaminase
ADP	adenosine diphosphate
AK	adenosine kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic AMP
CCK	cholecystokinin
CGRP	calcitonin gene related peptide
CGS 15943	9-chloro-2(2-furyl)[1,2, 4]triazolo [1,5-c]quinazolin-5-amine
CGS 21680	2-p-(2-carboxyethyl)phenethylamino-5'N-ethylcarboxamidoadenosine
CHA	5'-N^6-cyclohexyladenosine
CPA	N^6-cyclopentyladenosine
CRE	cAMP response element
CREB	CRE binding protein
C_T	threshold cycle
CV1808	2-phenylaminoadenosine
DEPC	diethlypyrocarbonate
DMNV	dorsomotor nucleus of the vagus
DMPX	3,7-dimethyl-1-propargylxanthine
DMSO	dimethylsulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
ECL	enterochromaffin-like
EGF	epidermal growth factor
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride
E-NPP	ecto-nucleotide pyrophosphatase
E-NTPase	ecto-nucleoside triphosphate diphoshohydrolase
EPSP	excitatory postsynaptic potential
FAM	6-carboxyfluorescein
FL	full length
GAPDH	glyceraldehyde-3'-phosphate dehydrogenase
GRP	gatrin releasing peptide
i.c.v.	intracerebroventricular
i.d.	intraduodenal
i.p.	intraperitoneal
i.v.	intravenous

IB-MECA	1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-ß-D-ribofuranuronamide
IHC	immunohistochemistry
IR	immunoreactivity
IRG	immunoreactive gastrin
IUPHAR	International Union of Pharmacology
LB	Luria-Bertani
MAPK	mitogen activated protein kinases
NAD^+	nicotinamide adenine dinucleotide
NECA	N-ethylcarboxamidoadenosine
NFκB	nuclear factor κB
NSB	non-specific binding
NTS	nucleus tractus solitarius
PCR	polymerase chain reaction
PGP	protein gene product
PIA	N^6-(2-phenylisopropyl)adenosine
PKC	protein kinase C
PLC	phospholipase C
RIA	radioimmunoassay
Rn	reporter emissions
R-PIA	R(-)-N^6-(2-phenylisopropyl)adenosine
RT-PCR	reverse-transcriptase PCR
s.c.	subcutaneous
SAH	S-adenosyl homocysteine
SAHH	S-adenosyl homocysteine hydrolase
	S-adenosyl methionine
SL	short length
SLI	somatostatin-like immunoreactivity
S-PIA	(S)-N^6-(2-phenylisopropyl) adenosine
TAMRA	6-carboxytetramethylrhodamine
TC	total count
Tm	melting temperature
UTR	untranslated region
VIP	vasoactive intestinal peptide
VWF	von Willebrand's factor
ZM 241385	4-(2-[7-Amino-2 (2furyl)[1,2,4]triazolo [2,3-a][1,3,5]triazin-5ylamino]ethyl)phenol