Effect of Omeprazole on Gastric Adenosine $A_1$ and $A_{2A}$ Receptor

Gene Expression and Function

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Non-standard abbreviations used in paper: N6-cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl)phenethylamino-5’N-ethylcarboxamidoadenosine (CGS 21680), immunoreactive gastrin (IRG), somatostatin-like immunoreactivity (SLI), reverse transcription-polymerase chain reaction (RT-PCR), bovine serum albumin (BSA), radioimmunoassay (RIA), 6-carboxyfluorescein (FAM), 6-carboxytetramethylrhodamine (TAMRA), normalized reporter emissions (Rn), threshold cycle (C_T), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

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Abstract

Adenosine has been shown to inhibit immunoreactive gastrin (IRG) release and to stimulate somatostatin-like immunoreactivity (SLI) release by activating adenosine A1 and A2A receptors, respectively. Since the synthesis and release of gastrin and somatostatin are regulated by the acid secretory state of the stomach, the effect of achlorhydria on A1 and A2A receptor gene expression and function were examined. Omeprazole-induced achlorhydria was shown to suppress A1 and A2A receptor gene expression in the antrum and corporeal mucosa, but not in the corporeal muscle. Omeprazole treatment produced reciprocal changes in A1 receptor and gastrin gene expression, and parallel changes in A2A receptor and somatostatin gene expression. The localization of A1 and A2A receptors on gastrin-secreting G-cells and somatostatin-secreting D-cells, respectively, suggests that changes in adenosine receptor expression may modulate the synthesis and release of gastrin and somatostatin. Thus, the effect of omeprazole on adenosine receptor-mediated changes in IRG and SLI release was also examined in the vascularly perfused rat stomach. Following omeprazole treatment, the A1 receptor-mediated inhibition of IRG and SLI release induced by N6-cyclopentyladenosine (A1 receptor-selective agonist) was not altered, but the A2A receptor-mediated augmentation of SLI release induced by 2-p-(2-carboxyethyl)phenethylamino-5’N-ethylcarboxamidoadenosine (A2A-selective agonist) was significantly attenuated. These findings agree well with the corresponding omeprazole-induced decrease in antral A2A receptor mRNA expression. Overall, the present study suggests that adenosine receptor gene expression and function may be altered by omeprazole treatment. Acid-dependent changes in adenosine receptor expression may represent a novel purinergic regulatory feedback mechanism in controlling gastric acid secretion.
Adenosine has been demonstrated to protect the stomach against stress-induced ulcer formation (Geiger and Glavin, 1985; Westerberg and Geiger, 1987), to inhibit gastric acid secretion (Gerber et al., 1985; Heldsinger et al., 1986; Scarpignato et al., 1987) and to modulate the release of gastric regulatory peptides (Kwok et al., 1990). Unlike in other species (Gerber et al., 1985; Heldsinger et al., 1986), adenosine does not alter gastric acid secretion in the rat by acting directly on the acid-secreting parietal cells (Puurunen et al., 1987). Instead, our laboratory has shown that in the isolated vascularly perfused rat stomach, the exogenous administration of adenosine suppressed and augmented the release of immunoreactive gastrin (IRG) and somatostatin-like immunoreactivity (SLI), respectively (Kwok et al., 1990). Thus, in these animals, adenosine may exert its inhibitory action on acid secretion indirectly by regulating the release of the gastric acid secretagogue, gastrin, and the acid inhibitor, somatostatin. In addition, adenosine deaminase, a metabolic enzyme of adenosine, has been shown to enhance basal and carbachol-stimulated IRG release in rat antral mucosal fragments (Harty and Franklin, 1984), and to inhibit basal gastric SLI release (Yip and Kwok, 2004), while dipyridamole, an adenosine uptake blocker, and erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride, an inhibitor of adenosine deaminase, were shown to enhance basal SLI release in the perfused rat stomach (Kwok et al., 1990; Yip and Kwok, 2004). These findings suggest that endogenous adenosine may play a role in controlling the release of gastrin and somatostatin, and consequently, gastric acid.

Adenosine elicits its actions by activating specific G-protein-coupled receptors that are classified into the A1, A2A, A2B and A3 receptor subtypes (Fredholm et al., 2001). The inhibitory and stimulatory effects of adenosine on IRG and SLI release are likely mediated by the activation of A1 and A2A receptors, respectively (Schepp et al., 1990; Yip and Kwok, 2004; Yip
et al., 2004). Furthermore, the basal release of SLI is likely to be under the control of the A2A receptors since ZM 241385 (A2A receptor-selective antagonist) was shown to suppress basal SLI release (Yip and Kwok, 2004). The A1 and A2A receptors were shown to be expressed throughout the antral and corporeal mucosa, and in the enteric plexi (Yip and Kwok, 2004; Yip et al., 2004). The cellular localization of A1 receptors on gastrin-containing G-cells and A2A receptors on somatostatin-containing D-cells further suggests that adenosine may act directly to alter the release of IRG and SLI. Although adenosine A1 and A2A receptors may regulate gastric acid secretion by modulating gastrin and somatostatin release, it has not been determined whether the gene expression of these receptors could be altered by changes in the acid secretory state of the stomach. Our laboratory has demonstrated that fasting, a condition that increases gastric acidity (Matsumoto et al., 1989), upregulated gastric A1 and A2A receptor gene expression and altered gastrin and somatostatin gene expression (Yip and Kwok, 2002). The proton pump inhibitor, omeprazole, has also been demonstrated to alter gastrin and somatostatin gene expression (Brand and Stone, 1988; Wu et al., 1990; Sandvik et al., 1995). However, it is unclear if omeprazole-induced achlorhydria also alters adenosine receptor gene expression. The objective of the present study was to examine whether short-term omeprazole treatment alters adenosine A1 and A2A receptor gene expression and function. The gene expression of gastrin and somatostatin was also measured since changes in adenosine receptor expression may be associated with changes in gastrin and somatostatin gene expression and release. To examine if omeprazole-induced changes in adenosine receptor gene expression also results in corresponding changes in adenosine receptor-mediated SLI and IRG release, the effect of selective adenosine agonists on SLI and IRG release were also studied following omeprazole treatment.
Materials and Methods

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

**Omeprazole treatment:** Male Wistar rats (250-270g) housed in light- and temperature-controlled rooms with free access to food and water were used. As suggested by the manufacturer, omeprazole (50 µmol/ml; AstraZeneca, Molndal, Sweden) was thoroughly dispersed in 0.25% Methocel® (Dow, Midland, Michigan) containing 2 mg/ml NaHCO₃ (pH 9.0) using the Tissumizer® tissue homogenizer (Tekmar Co. Cincinnati, OH). The drug was aliquoted, frozen, and stored at -20°C. To prevent drug degradation due to repeated freezing and thawing, a new aliquot of drug was thawed overnight at 4°C and brought to room temperature prior to administration. Test groups were treated by gavage with a dose of 400 µmole/kg omeprazole once daily between 9 and 10 a.m. for either 1 or 3 days. These regimens were selected since gastric acid secretion is decreased by up to 80% after 1 day of treatment with 400 µmole/kg omeprazole (Larsson et al., 1988; Lee et al., 1992), and steady state inhibition is achieved after 3 days of treatment at this dose (Carlsson et al., 1986). Rats were not treated for more than 3 days since G-cell and/or D-cell density are altered after 4 days (Bolkent and Yilmazer, 1997) and 5 days (Pawlikowski et al., 1992) of treatment. Control groups were treated similarly with the vehicle. Animals were anaesthetized with an i.p. injection of 60 mg/kg sodium pentobarbital (Somnotol®, MTC Pharmaceuticals, Cambridge, ON, Canada) 24 h after the last treatment, and tissue extraction occurred between 10 a.m. to 11 a.m.
Quantification of adenosine $A_1$ and $A_{2A}$ receptor and gastric peptide gene expression

The quantitative Real-Time RT-PCR developed to measure $A_1$ and $A_{2A}$ receptor gene expression have previously been described (Yip and Kwok, 2004; Yip et al., 2004). Two-step Real-Time RT-PCR assays were used to quantify the gene expression of adenosine $A_1$ and $A_{2A}$ receptors, gastrin and somatostatin in various gastric regions following omeprazole treatment. Primers and probes were designed using the Primer Express Sequence Design software program (v. 1.0, Applied Biosystems, Foster City, CA). The reporter dye, 6-carboxyfluorescein (FAM) and the quencher dye, 6-carboxytetramethylrhodamine (TAMRA) were linked to the 5’ and 3’ ends of the probes, respectively. The sequences of the forward primers, reverse primers, and probes used are listed in Table 1. The primers and probes were synthesized by the Nucleic Acid Protein Services (NAPS) Unit (University of British Columbia) and Synthegen, LLC (Houston, TX), respectively.

$A_1$ and $A_{2A}$ gene expression: RNA transcripts expressing the entire coding region of the $A_1$ and $A_{2A}$ receptors were used as the standards for Real-Time RT-PCR. The standards were synthesized from plasmids containing the $A_1$ or $A_{2A}$ receptor transcript by in vitro transcription using the Riboprobe in vitro transcription kit and T7 RNA polymerase (Promega, Madison, WI), as previously described (Yip and Kwok, 2004; Yip et al., 2004). Adenosine receptor RNA standards were DNase I (Amersham Pharmacia, Piscataway, NJ)-treated and purified. Standard concentrations were determined using the Ribogreen Reagent Quantitation kit (Molecular Probes, Eugene, OR), the FL600 Microplate Fluorescence reader (Biotek Inc., Winooski, VT), and the KC4 Kineticalc Software (version 2.6, Biotek Inc.), according to manufacturer’s
instructions. RNA standards were serially diluted to $1 \times 10^3$ to $1 \times 10^{12}$ copies/µl in RNase-free water, aliquoted, stored at -80°C, and thawed only once prior to use.

**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 standards for 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 then serially diluted to various concentrations (0.1 ng/µl to 100 ng/µl) to produce a relative standard curve.

**Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA gene expression:** The expression of GAPDH mRNA and 18S rRNA was measured using the Rodent GAPDH Control Reagents Kit (Applied Biosystems) and the TaqMan ribosomal RNA Control Reagents Kit (Applied Biosystems), respectively, according to 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 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 RNA was used to produce 1 µl of cDNA. 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 rRNA and GAPDH Real-Time RT-PCR assays were performed according to the manufacturer’s instruction.
Tissue and total RNA extraction

The corporeal mucosa, corporeal muscle, and antrum were dissected out. These regions were examined since 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. Since the antrum is a relatively small tissue, total RNA was extracted from the whole antrum to ensure consistency between samples. The corpus was separated into the corporeal mucosa and muscle. The whole 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 using Trizol® reagent (Invitrogen Corp. Carlsbad, CA), and total RNA was extracted immediately using the same reagent. The corporeal muscle and antrum were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted from all tissues using Trizol® reagent, according to manufacturer’s instructions and concentrations were determined using the RiboGreen Quantitation Kit (Molecular Probes). Total RNA samples were treated with DNase I 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 MgCl₂] 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. Samples were then incubated for an additional 10 min at 65°C.
Two step Real-Time RT-PCR

**Reverse transcription:** One 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 (Pharmacia), 1× first strand buffer, 10 mM DTT, 0.5 mM dNTP mix, and 100 U Superscript II RNase H-Reverse Transcriptase. At least 6 concentrations of each standard and a sample containing DNase I-treated RNase-free water in place of the template (negative control) were reverse transcribed simultaneously. The reverse transcribed RNA standards were used to construct the standard curve for the Real-Time RT-PCR assay.

**PCR:** Each assay consisted of at least 6 standard curve samples, a negative control sample, and unknown samples. All reactions were performed in triplicates. The PCR reaction mixture (25 µl) consisted of 1× TaqMan buffer A, 200 µM of each dATP, dCTP, and dGTP, 400 µM dUTP, 0.01 U/µl AmpErase uracil-N-glycosylase, and 0.025 U/µl AmpliTaq Gold DNA polymerase from the TaqMan PCR Core Kit (Applied Biosystems). Reaction mixtures also contained 0.5 µl tissue cDNA, standard cDNA or negative control, 100 nM probe, 100 nM (A2A receptor, gastrin and somatostatin assay) or 300 nM (A1 assay) each of the forward and reverse primers, and 7.5 mM MgCl2 (A1 receptor, gastrin and somatostatin assay) or 4.5 mM MgCl2 (A2A receptor assay). The reaction was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems) with the following cycling parameters: 2-min hold at 50°C for uracil-N-glycosylase incubation, 10-min hold at 95°C for AmpliTaq Gold activation, followed by 40 cycles of amplification consisting of a 15-sec denaturation step at 95°C and 1-min anneal/extend period at 60°C.

**Data collection and analysis:** Data were collected during each PCR cycle, and analyzed using the Sequence Detection Software (v. 1.6.3, Applied Biosystems). An amplification plot showing normalized reporter emissions (Rn) vs. cycle number was generated. The threshold cycle (Cₜ),
the cycle where an increase in fluorescence is associated with exponential growth, was
determined by the software using the fluorescence emitted during the first 15 cycles. A standard
curve of $C_T$ vs. Log (initial standard concentrations) was then 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 $> 0.95$, and the
$C_T$ of the negative control exceeded 40 cycles in every assay, indicating the absence of DNA
contamination. To compare gene expression levels between the test and control animals,
measurements were first normalized with the gene expression of the endogenous control (18S
rRNA). This was performed according to the procedures described in the Applied Biosystems
User bulletin #2. The adenosine $A_1$ receptor, $A_2A$ receptor, gastrin or somatostatin gene
expression level of each individual sample was divided by the 18S rRNA level to obtain a
normalized value of measurement. The mean ± S.E.M. of this normalized value was calculated
for control and omeprazole-treated groups. The mean of each control group was expressed as
100%, and gene expression levels of the omeprazole-treated groups were expressed as a
percentage of the control. Statistical significance was determined using the two-tailed unpaired
Student’s t-test, and was performed using GraphPad Prism (v. 3.0, GraphPad software, San
Diego, CA); $P \leq 0.05$ was considered significant.

**Stomach perfusion**

Rats were housed in light- and temperature-controlled rooms with free access to food and water.
Omeprazole-treated animals were deprived of food for 12-14 h, but had free access to water prior
to stomach perfusion. Rats were anesthetized with Somnotol® (60 mg/kg). The procedures used
to prepare the stomach for perfusion have previously been described (Kwok et al., 1990).
Following the exposure of the stomach through an abdominal midline incision, the superior mesenteric artery and vasculatures supplying the left and right adrenal glands and kidneys were occluded or cut between double ligatures. The pancreas and spleen were then dissected along the greater curvature of the stomach, while preserving the right gastroepiploic artery. A cannula was secured into the gastroduodenal junction for drainage of any gastric contents. The spleen, pancreas, small and large intestines were then dissected out. Arterial perfusion was achieved through a cannula inserted into the aorta with the tip lying adjacent to the celiac artery. An injection of 2 ml saline containing 600 U of heparin (Sigma, St. Louis, MO) was introduced into the gastric circulation through this arterial cannula followed by perfusate. Venous effluent was collected via the portal vein cannula. The preparation was equilibrated for 30 min before 5-min samples were collected into ice-cold scintillation vials containing 0.3 ml of Trasylol® (aprotinin, 10,000 KIU/ml; Miles Labs., Etobicoke, ON). Aliquots (0.5 ml) of samples were immediately transferred into ice-cold test-tubes containing 0.05 ml aprotinin and stored at -20°C until assayed.

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 (in mM: NaCl, 120; KCl, 4.4; CaCl₂, 2.5; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.5; NaHCO₃, 25 and dextrose, 5.1) containing 0.2% BSA (RIA grade; Sigma) and 3% dextran (Clinical grade; Sigma). The perfusate was continuously gassed with a mixture of 95% O₂ and 5% CO₂ to maintain a pH of 7.4. Both the perfusate and the preparation were kept at 37°C by thermostatically-controlled heating units throughout the experiment. Drugs were introduced into the perfusate via side-arm infusion at a rate calculated to give the final perfusion concentrations. N⁶-cyclopentyladenosine (CPA) and 2-p-(2-carboxyethyl)phenethylamino-5’N-ethylcarboxamidoadenosine HCl (CGS 21680) were purchased from Sigma-Aldrich. Drugs were first dissolved in a small volume of
DMSO (BDH, Toronto, ON) and subsequently diluted with saline or perfusate to 0.03 or 0.5% DMSO before perfusing into the stomach. At these concentrations, DMSO did not alter basal IRG or SLI release. The perfusion concentrations of the A1 receptor agonist, CPA, and A2A receptor agonist, CGS 21680, were 0.1 or 1 µM. The lower concentration (0.1 µM) was selected to approximate the EC50 of CPA (0.067 µM with a 95% confidence interval between 0.014 and 0.325 µM) (Yip et al., 2004) and CGS 21680 (0.06 µM with a 95% confidence interval between 0.02 and 0.17 µM) (Yip and Kwok, 2004) in inhibiting and stimulating IRG and SLI release, respectively. The higher concentration (1 µM) was also examined since CPA and CGS 21680 elicited their maximal effects at this concentration.

**Measurement of gastric peptides and data analysis**

The specific RIA employed for the measurement of IRG (Jaffe and Walsh, 1978; Fujimiya and Kwok, 1997) and SLI (McIntosh et al., 1987; Kwok et al., 1990) have previously been described. The gastrin antibody (PM1) used to measure IRG was kindly provided by Dr. R. Pederson (Physiology, UBC). The inter- and intra-assay variation was less than 6 and 4%, respectively. The monoclonal antibody, SOMA-3 (MRC-RPG), was used to measure SLI. The inter- and intra-assay variation of the RIA was less than 12 and 8%, respectively. The drugs used in the present study did not cross-react with these antibodies.

Although the basal release rate of IRG and SLI release varied among animals, previous experiments have demonstrated that basal IRG (Pederson et al., 1984; Kwok et al., 1990) and SLI release (Kwok et al., 1990) were maintained in the perfused stomach. Therefore, results were expressed as mean ± SEM of IRG or SLI release (%), which was calculated as follows: 

\[
\frac{\text{release (pg/min) during a 5 min period}}{\text{release (pg/min) during period 1}} \times 100
\]

Results were
also expressed as percentage (%) inhibition of IRG release, which was calculated as follows:

\[
\frac{[\text{mean basal IRG release (periods 1-3)} - \text{mean IRG release in the presence of drug (periods 4-7)}]}{\text{mean basal IRG release (periods 1-3)}} \times 100.
\]

The percent (%) change of SLI release was calculated as follows: (mean SLI release in the presence of drug – mean basal SLI release) pg/min ÷ (mean basal SLI release) pg/min × 100. 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.
Results

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

Omeprazole treatment was found to alter GAPDH mRNA levels in various gastric regions. The gene expression of GAPDH in the corporeal mucosa was significantly decreased to 65±5% of control levels after 3 days of omeprazole treatment. In the corporeal muscle, the GAPDH mRNA level was significantly increased to 130±9% after 1 day of treatment (Fig. 1A). In all gastric regions examined, 18S rRNA levels were not altered by 1 or 3 days of omeprazole treatment (Fig. 1B). Since omeprazole treatment did not alter 18S rRNA levels, this gene was used as the endogenous control for the subsequent quantification of gastric A₁ receptor, A₂A receptor, somatostatin, and gastrin gene expression using Real-Time RT-PCR.

**Effect of omeprazole treatment on adenosine A₁ and A₂A receptor gene expression**

Fig. 2A shows that antral adenosine A₁ receptor gene expression was significantly reduced to 66±12% and 47±8% of control levels after 1 and 3 days of omeprazole treatment, respectively. In the corporeal mucosa, A₁ receptor gene expression was significantly decreased to 62±6% of control levels after 1 day of treatment, but changes were not apparent after 3 days of treatment. The gene expression of the A₂A receptor was significantly reduced in the antrum to 57±7% of control levels after 3 days of treatment (Fig. 2B). A₂A receptor gene expression was also decreased in the corporeal mucosa, to 67±14% of control levels, after 1 day of treatment. However, changes were not detected in this tissue after 3 days of treatment. In the corporeal muscle, neither A₁ receptor nor A₂A receptor gene expression was altered by omeprazole treatment.
Effect of omeprazole treatment on gastrin and somatostatin gene expression

Antral gastrin gene expression was significantly increased to 201±18% and 268±42% of controls after 1 and 3 days of omeprazole treatment, respectively (Fig. 3A). In contrast, after 1 and 3 days of omeprazole treatment, antral somatostatin gene expression was significantly reduced to 71±6% and 58±4% of controls, respectively. The somatostatin gene expression in the corporeal mucosa was also reduced to 54±13% and 54±10% of control levels after 1 and 3 days of treatment, respectively (Fig. 3B). In the corporeal muscle, a significant decrease (45±6%) in somatostatin gene expression was observed following 1 day of omeprazole treatment.

Effect of omeprazole on CPA-induced changes in IRG release

Previous studies have shown that adenosine A1 receptors are involved in the inhibition of IRG release. Thus, the effect of omeprazole treatment on CPA (A1-selective agonist)-induced changes in IRG release was examined. Fig. 4A shows the effect of 0.1 µM CPA on IRG release in animals after 1-day treatment with omeprazole. Basal IRG release during periods 1 to 3 in control (222±46 to 238±52 pg/min) and treated (175±19 to 198±23 pg/min) animals were comparable. The administration of CPA (0.1 µM) resulted in a significant inhibition of basal IRG release in both control and treated animals. However, no significant difference in CPA-induced IRG release was observed between these two groups. Fig. 4B shows the effect of 1 µM CPA on IRG release in controls and animals treated with omeprazole for 3 days. The basal release of IRG during periods 1 to 3 in control (203±24 to 212±29 pg/min) and treated (231±36 to 251±46 pg/min) animals were also comparable. The perfusion of CPA (1 µM) significantly inhibited IRG release. However, this response also did not differ between 3-day omeprazole treated and control animals. For comparison, results are also expressed as percentage inhibitions.
and summarized in Fig. 5. No significant changes in the inhibitory effect of CPA (0.1 µM and 1 µM) on IRG release occurred between 1-day and 3-day omeprazole treated and vehicle-treated control animals.

Effect of omeprazole on CPA- and CGS 21680-induced changes of SLI release

Previous studies have demonstrated that adenosine A₁ and A₂A receptors are involved in the inhibition and augmentation of SLI release, respectively. Thus, the effect of omeprazole treatment on CPA and CGS 21680 (A₂A selective agonist)-induced changes in SLI release were examined. Fig. 6 shows the effect of 1 µM CGS 21680 on SLI release in control and 3 day omeprazole-treated animals. The basal release of SLI during periods 1 to 3 in controls (152±48 to 157±51 pg/min) was not significantly different from that of treated animals (194±45 to 197±47 pg/min). In these experiments, SLI release was enhanced by CGS 21680 in both groups of animals. However, CGS 21680-induced SLI release was significantly attenuated by 3 days of omeprazole treatment. The percentage change in SLI release induced by CGS 21680 (0.1 and 1 µM) in control and 1- and 3-day omeprazole-treated animals are summarized in Fig. 7. After 3 days of omeprazole treatment, CGS 21680-induced augmentation of SLI released was shown to be significantly reduced.

Previous studies have demonstrated that the administration of 0.1 µM CPA significantly inhibited gastric SLI release, while the administration of 1 µM CPA significantly stimulated SLI release (Yip and Kwok, 2004). These observations were also observed in the current study (Fig. 8 and 9). Fig. 8A shows the effect of 0.1 µM CPA on SLI release after 1 day of omeprazole treatment. Basal SLI release did not differ significantly between control (227±33 to 234±30 pg/min) and treated (194 ±27 to 197±21 pg/min) animals. The administration of 0.1 µM CPA
significantly reduced SLI release. However, this inhibitory response did not differ between control and 1-day omeprazole-treated animals. Fig. 8B shows the effect of 1 μM CPA on SLI release after 3 days of omeprazole treatment. Basal SLI release was comparable between control (158±33 to 165±37 pg/min) and treated (134±26 and 138±30 pg/min) animals. The administration of CPA (1 μM) significantly increased SLI release. This stimulatory response was shown to be attenuated after 3 days of omeprazole treatment. The effect of omeprazole treatment on CPA-induced changes in SLI release is summarized on Fig. 9. The inhibition of SLI release induced by 0.1 μM CPA was not altered by either 1 or 3 days of omeprazole treatment. However, 3 days of treatment did significantly attenuate the stimulatory effect of 1 μM CPA on SLI release (Fig. 9).
Discussion

In the present study, the gene expression of GAPDH, a commonly used housekeeping gene, was shown to be significantly increased in the corporeal muscle and decreased in the corporeal mucosa after 1 and 3 days of omeprazole treatment, respectively. These findings are consistent with another study demonstrating increased GAPDH mRNA levels in the rat corpus after omeprazole treatment (Sandvik et al., 1995). In contrast, 18S rRNA gene expression was not altered by omeprazole treatment. Therefore, 18S rRNA was used as the endogenous control for the measurement of A₁ receptor, A₂A receptor, somatostatin, and gastrin gene expression. Results demonstrate that both gastrin and somatostatin mRNA levels were altered by omeprazole treatment. The observation that 1 and 3 days of omeprazole treatment led to increased gastrin mRNA levels agrees well with other studies showing increased expression following 4 and 14 days of omeprazole treatment (Wu et al., 1990; Dockray et al., 1991). The present study also demonstrates decreased antral somatostatin gene expression after 1 and 3 days of treatment, which also agrees well with the findings of other investigators (Brand and Stone, 1988; Wu et al., 1990; Sandvik et al., 1995). Similar to previous studies (Tari et al., 1991; Sandvik et al., 1995), the present study also demonstrated reduced somatostatin mRNA expression in the corporeal mucosa and muscle following omeprazole treatment.

We have previously demonstrated that adenosine may regulate gastric acid secretion by suppressing IRG and stimulating SLI release via activation of A₁ and A₂A receptors, respectively (Yip and Kwok, 2004; Yip et al., 2004). The present study suggests that the gene expression and function of these receptors may be regulated by changes in the acid secretory state of the stomach. Omeprazole treatment inhibited A₁ receptor gene expression in the antrum (1 and 3 day treatment) and corporeal mucosa (1 day treatment), and A₂A receptor gene expression in the
antrum (3 day treatment) and corporeal mucosa (1 day treatment). The precise cellular localization of both receptors have been examined in these tissues (Yip and Kwok, 2004; Yip et al., 2004). A1 receptors were shown to be expressed on D-cells and G-cells, while A2A receptors are expressed on D-cells. Thus, it is plausible that omeprazole-induced changes in adenosine receptor gene expression may occur in the mucosal G-cells and/or D-cells of the corpus and antrum. Both of these adenosine receptors have also been localized in the enteric plexi. However, changes in A1 and A2A receptor gene expression were not observed in the corporeal muscle after omeprazole treatment, suggesting that adenosine receptor gene expression in the corporeal enteric plexi was unlikely altered by omeprazole treatment.

Conditions that increase intracellular acidity, such as hypoxia or ischemia, have previously been shown to upregulate A1 and A2A receptor gene expression in DDT1-MF2 cells (Nie et al., 1998) and PC12 cells (Kobayashi and Millhorn, 1999), respectively. In addition, fasting, which increases gastric acidity (Matsumoto et al., 1989), has been shown to upregulate both gastric A1 and A2A receptor mRNA levels (Yip and Kwok, 2002). In the present study, adenosine receptor gene expression was reduced following omeprazole-induced achlorhydria. This observation fits the proposal that adenosine receptor gene expression may respond to changes in acidity.

When omeprazole-induced changes in adenosine receptor and gastric regulatory peptide gene expression were compared, a reciprocal relationship was observed between changes in A1 receptor and gastrin gene expression. Although a clear relationship between A2A receptor and somatostatin gene expression was not apparent, the expression of both genes were reduced in gastric tissues after various lengths of omeprazole treatment. Omeprazole-induced achlorhydria may decrease adenosine receptor expression, and subsequently alter the synthesis and release of
gastrin and somatostatin. Both gastrin and somatostatin gene expression are enhanced by cAMP formation (Shiotani and Merchant, 1995; Montminy et al., 1996). Activation of A₁ and A₂A receptors inhibits and stimulates cAMP formation, respectively (Ralevic and Burnstock, 1998). Thus, activation of adenosine receptors may alter cAMP levels to regulate gastrin and somatostatin gene expression. Studies have shown that A₂A receptor stimulation can induce the expression of genes regulated by cAMP (Chae and Kim, 1997; Ravid et al., 1999). Therefore, a reduction in A₂A receptor gene expression may lead to a subsequent decrease in somatostatin gene expression.

Although the possible regulatory mechanism(s) involved in the modulation of adenosine receptor gene expression by omeprazole was not examined in the present study, the expression of the A₁ and A₂A receptor gene may be regulated by changes in gastric acidity, as suggested for gastrin and somatostatin gene expression. Omeprazole, however, has also been shown to induce the expression of various genes, through the activation of specific intracellular signaling pathways (Backlund et al., 1997; Kikuchi and Hossain, 1999). Thus, the direct regulation of A₁ and A₂A receptor gene expression by omeprazole, therefore, cannot be ruled out.

Omeprazole-induced changes in adenosine receptor mRNA expression may result in similar changes in receptor density, and thus receptor function. The present study demonstrates that changes in gastric adenosine receptor gene expression can occur with corresponding changes in adenosine receptor function. We have previously suggested that high concentrations of CPA (≥1 µM) may stimulate SLI release through the non-specific activation of A₂A receptors (Yip and Kwok, 2004). Following 3 days of omeprazole treatment, the stimulatory effect of 1 µM CPA on SLI release was decreased, suggesting a reduction in A₂A receptor expression. This proposal is supported by the observation that the stimulatory effect of the A₂A receptor agonist, CGS
21680, on SLI release was also significantly attenuated after 3 days of omeprazole treatment. The reduced $A_{2A}$ receptor-mediated function is consistent with the reduced level of $A_{2A}$ receptor gene expression observed in the antrum after 3 days of omeprazole treatment. Together, these results suggest that omeprazole may decrease the number of $A_{2A}$ receptors in the antrum by reducing $A_{2A}$ receptor gene expression. Our previous studies have implicated the involvement of the $A_{2A}$ receptor in the tonic control of basal SLI release by endogenously released adenosine (Yip and Kwok, 2004). Furthermore, in comparison to the $A_{1}$ receptors, the relative abundance of the gastric mucosal $A_{2A}$ receptors was found to be relatively low (Yip and Kwok, 2004; Yip et al., 2004), suggesting the existence of a fairly small $A_{2A}$ receptor reserve in this region. Thus, changes in $A_{2A}$ receptor expression may readily alter somatostatin secretion.

Contrary to the $A_{2A}$ receptor, the $A_{1}$ receptor is highly expressed in the gastric mucosa (Yip et al., 2004). $A_{1}$ receptors are expressed on all G-cells, all corporeal D-cells and some antral D-cells (Yip et al., 2004). Thus, a large $A_{1}$ receptor population may be present in the rat gastric mucosa. In the present study, omeprazole treatment reduced $A_{1}$ receptor gene expression in the antrum and corporeal mucosa, but did not alter $A_{1}$ receptor-mediated inhibition of IRG and SLI release. The large reserve of $A_{1}$ receptors may prevent decreased $A_{1}$ receptor expression from altering gastrin and somatostatin release, thus, resulting in a discrepancy between changes in $A_{1}$ receptor mRNA levels and $A_{1}$ receptor function. However, this discrepancy may also be due to other factors. The present study examined the effect of short-term (1 and 3 day) omeprazole treatment. Although changes in actual receptor expression may require more time to occur, the effect of a longer treatment regimen was not examined. In rats treated with omeprazole for more than 3 days, both G-cell and D-cell density have been shown to be altered (Pawlikowski et al., 1992; Bolkent and Yilmazer, 1997). Any changes in adenosine receptor
mRNA expression may be complicated by the alterations in G-cell and D-cell numbers since adenosine receptors are present on both these endocrine cell types. The adenosine receptors are also expressed in different regions of the stomach including the enteric plexi and vasculature. Thus, it is also possible that changes in adenosine receptor mRNA expression may occur in anatomical structures that are not involved in the control of IRG and SLI release. The regulation of adenosine receptor gene expression by post-transcriptional mechanism, such as changes in the translational rate (Ren and Stiles, 1994; Chu et al., 1996; Lee et al., 1999), also cannot be ruled out. Thus, the disparity between adenosine receptor mRNA levels and receptor function may result from various factors.

The results of this study suggest that adenosine A1 and A2A receptor gene expression may respond to changes in the acid secretory state of the stomach. Omeprazole-treatment inhibited both A1 and A2A receptor gene expression in gastric tissues. Although A1 receptor-mediated inhibition of IRG and SLI release was not altered by omeprazole treatment, A2A receptor-mediated augmentation of SLI release was attenuated. The omeprazole-induced inhibition of A2A receptor function corresponds with the reduced level of antral A2A receptor mRNA. These findings suggest that gastric acidity may modulate the purinergic control of IRG and SLI release through the alteration of adenosine receptor expression. The possible modification of adenosine receptor expression by changes in intraluminal acidity may represent a novel purinergic regulatory feedback mechanism to control gastric acid secretion.
References


Footnotes:

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A portion of this work was included in Linda Yip’s Ph.D. thesis entitled: Adenosine A1 and A2A Receptors in the Rat Stomach: Biological Actions, Cellular Localization, Structure, and Gene Expression.

Part of this work will be presented at the Purines 2004 Meeting, and a portion of this work has previously been published in abstract form:


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Figure Legends

Fig. 1. Effect of omeprazole treatment on gastric housekeeping gene expression. GAPDH mRNA (A) and 18S rRNA (B) levels were measured in rats treated with vehicle or omeprazole (OM) for 1 or 3 days; n ≥ 7. Results are expressed as a percentage of the control as described in the Methods section; * P < 0.05.

Fig. 2. Effect of omeprazole treatment on adenosine A1 and A2A receptor gene expression. A1 receptor (A) and A2A receptor (B) mRNA levels were measured in rats treated with omeprazole (OM) for 1 or 3 days; n ≥ 7. Results are expressed as a percentage of the control as described in the Methods section; * P < 0.05, ** P <0.01, ***P < 0.001.

Fig. 3. Effect of omeprazole treatment on gastric peptide gene expression. Gastrin (A) and somatostatin (B) mRNA levels were measured in rats treated with omeprazole (OM) for 1 or 3 days; n ≥ 7. Results are expressed as a percentage of the control, as described in the Methods section; * P < 0.05, ** P <0.01, ***P < 0.001.

Fig. 4. Effect of CPA on gastric IRG release in 1-day (A) and 3-day (B) omeprazole (OM)-treated animals. Results are expressed as IRG release (%) as described in the Methods section. Each column represents the mean ± SEM. *P < 0.05 and ***P < 0.001 when compared with period 3 using repeated measures ANOVA followed by Dunnett’s multiple comparison test (n ≥ 5). The IRG release during each 5-min period was not significantly different between omeprazole-treated and control animals when compared using the Student’s unpaired t-test.
Fig. 5. Effect of omeprazole (OM) treatment on CPA-induced changes in IRG release. The percentage inhibition of IRG release between omeprazole-treated animals and their respective controls was compared using the Student’s unpaired t-test, and were found not to differ. Each column represents the mean ± SEM (n ≥ 5).

Fig. 6. Effect of CGS 21680 (CGS; 1µM) on gastric SLI release in control (A) and 3-day omeprazole (OM)-treated (B) animals. Results are expressed as SLI release (%) as described in the Methods section. Each column represents the mean ± SEM (n ≥ 5); ***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 during periods 6, 7, and 8; †P < 0.05.

Fig. 7. Effect of omeprazole (OM) treatment on CGS 21680-induced changes in SLI release. The percentage change in SLI release between omeprazole-treated animals and their respective controls was compared using the Student’s unpaired t-test. Each column represents the mean ± SEM (n ≥ 5); *P < 0.05.

Fig. 8. Effect of CPA on gastric SLI release in 1-day (A) and 3-day (B) omeprazole (OM)-treated animals. Results are expressed as SLI release (%) as described in the Methods section. Each column represents the mean ± SEM. *P < 0.05 and ***P < 0.001 when compared with period 3 using repeated measures ANOVA followed by Dunnett’s multiple comparison test (n ≥
5). The SLI release during each 5-min period was compared between omeprazole-treated and control rats using the Student’s unpaired t-test. In 1-day treated rats perfused with 0.1 μM CPA (A), SLI release did not differ from controls. In 3-day treated rats perfused with 1 μM CPA (B), SLI release was found to differ significantly during periods 4 and 5; †P < 0.05 and ††P < 0.01.

Fig. 9. Effect of omeprazole (OM) treatment on CPA-induced changes in SLI release. The percentage change in SLI release between omeprazole-treated animals and their respective controls was compared using the Student’s unpaired t-test. Each column represents the mean ± SEM (n ≥ 5); *P < 0.05.
Table 1: Primers and probe sequences for Real-Time RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>Position</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt; Receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>963</td>
<td>5’ CGGTGACCCCCCAGAAGTACTAC 3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1039</td>
<td>5’ GGGCAAAGAGGAAGAGGATGA 3’</td>
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<tr>
<td>Probe</td>
<td>989</td>
<td>5’ CAGCGACTTGCGATCTTCAGCTCCT 3’</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt; Receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>910</td>
<td>5’ ACCCCTTCATCTACGCCTACAG 3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>978</td>
<td>5’ CGTGAGTTCGGATGATCTTC 3’</td>
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<tr>
<td>Probe</td>
<td>936</td>
<td>5’ CGGGAGTTCCGCCAGACCTTCC 3’</td>
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<tr>
<td>Gastrin</td>
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<td>Somatostatin</td>
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<td>5’CTCATCTCGTCACGTCAGCT3’</td>
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<tr>
<td>Probe</td>
<td>260</td>
<td>5’CCCTGGAGCCTTGAGGATTTGCC3’</td>
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Sequences are based on the following genes: ¹Rat brain A<sub>1</sub> receptor (Accession number: M64299); ²Rat brain A<sub>2A</sub> receptor (Accession number: S47609), ³Rat gastrin (Accession number: M38653), ⁴Rat somatostatin (Accession number: M25890)
Fig. 1

A

GAPDH mRNA levels (% Control)

- control
- OM-treated (1 day)
- OM-treated (3 day)

Antrum
Corporeal Mucosa
Corporeal Muscle

B

18S rRNA levels (% Control)

Antrum
Corporeal Mucosa
Corporeal Muscle

* Denotes significant difference from control.
Fig. 2

**Fig. 2**: Bar graphs showing the relative mRNA levels of A1 receptors in the antrum, corporeal mucosa, and corporeal muscle. The graphs compare control conditions with OM-treated (1 day) and OM-treated (3 days) conditions.

- **A.** A1 Receptor mRNA levels (% Control)
  - Control
  - OM-treated (1 day)
  - OM-treated (3 days)

- **B.** A2A Receptor mRNA levels (% Control)
  - Control
  - OM-treated (1 day)
  - OM-treated (3 days)
Fig. 3
Fig. 4
Fig. 5

IRG Release (% inhibition)

Control
OM-treated

CPA (µM):

0.1
1 day treatment

1

0.1
3 day treatment

1
**Fig. 7**

The bar chart illustrates the SLI release (% change) in response to different concentrations of CGS (μM) and treatment durations (1 day vs. 3 day treatment). The chart compares control and OM-treated conditions.

- **Control** (white bars):
  - CGS (μM): 0.1
  - CGS (μM): 1
  - CGS (μM): 0.1
  - CGS (μM): 1

- **OM-treated** (black bars):
  - CGS (μM): 0.1
  - CGS (μM): 1
  - CGS (μM): 0.1
  - CGS (μM): 1

Significance indicated by asterisks (*) for specific comparisons.
Fig. 8
Fig. 9

SLI Release (% change)

CPA (μM): 0.1 1 0.1 1

1 day treatment 3 day treatment

control OM-treated

*