Role of Adenosine A$_2$A Receptor in the Regulation of Gastric Somatostatin Release

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Received October 22, 2003; accepted January 16, 2004

ABSTRACT

Adenosine has been demonstrated to inhibit gastric acid secretion. In the rat stomach, this inhibitory effect may be mediated indirectly by increasing the release of somatostatin-like immunoreactivity (SLI). Results show that adenosine analogs augmented SLI release in the isolated vascularity perfused rat stomach. The rank order of potency of the analogs in stimulating SLI release was 2-p-(2-carboxyethyl)-phenethylamino-5’-N-ethylycarboxamidoadenosine (CGS 21680) ≈ 5’-N-ethylcarboxamidoadenosine > 2-chloroadenosine > R-(−)-N’-(2-phenylisopropyl)adenosine > 1-deoxy-1-[6-[[3-iodophenyl]methylamino]-9H-purin-9-yl]-N-methyl-β-d-ribofuranuronamide > N’-cyclohexyladenosine ≈ N’-cyclohexyladenosine > S-(+)-N’-(2-phenylisopropyl) adenosine, suggesting the involvement of the A$_2$A receptor. In agreement, 4-(2-[7-amino-2-(2-furyl)]-1,3,5-triazol-2,3-a-[1,3,5]triazin-5-ylamino)ethyl)phenol (ZM 241385), an A$_2$A receptor antagonist, was shown to abolish the adenosine- and CGS 21680-stimulated SLI release. Immunohistochemical studies reveal the presence of A$_2$A receptor immunoreactivity on the gastric plexi and mucosal D-cells, but not on parietal cells and G-cells, suggesting that adenosine may act directly on D-cells or indirectly on the gastric plexi to augment SLI release. The present study also demonstrates that the structure of the mucosal A$_2$A receptor is identical to that in the rat brain, and that alternative splicing of this gene does not occur. A real-time reverse transcription-polymerase chain reaction assay has also been established to quantify the levels of A$_2$A receptor mRNA. Results show that gastric tissues contained significantly lower levels of A$_2$A receptor mRNA compared with the striatum. The lowest level was detected in the mucosa. In conclusion, adenosine may act on A$_2$A receptors to augment SLI release and consequently control gastric acid secretion.

This work was supported by the Canadian Apatosens Research Foundation, Canada Foundation for Innovation, W. Sheng Fund, and the former British Columbia Health Research Foundation. L.Y. was supported by the Cordula and Gunter Paetzold Fellowship and the University of British Columbia Graduate Fellowship. A portion of this work was included in L.Y.’s Ph.D. dissertation entitled, Adenosine A$_1$ and A$_2$A Receptors in the Rat Stomach: Biological Actions, Cellular Localization, Structure, and Gene Expression. Citation of abstracts where part of this work was previously presented: Kwok YN and Hui J (1992) Effect of selective adenosine A1 and A2 analogs on the release of gastric somatostatin-like immunoreactivity. Regul Pept 46:189; Kwok YN (1994) Purinergic control of release of somatostatin in the rat stomach. Pathophysiol Suppl 12:18; Yip L and Kwok Y (2002) Gastric A1 and A2A receptors: cellular localization, gene sequence and gene expression levels. Drug Dev Res 56:551; and Yip L, Leung CH, and Kwok YN (2003) Cellular localization of adenosine A1 and A2A receptors in the rat stomach. FASEB J 17:12A40. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.103.061986.

ABBREVIATIONS: ADA, adenosine deaminase; SLI, somatostatin-like immunoreactivity; IR, immunoreactivity; RT-PCR, reverse transcription-polymerase chain reaction; BSA, bovine serum albumin; RIA, radioimmunoassay; 2-CA, 2-chloroadenosine; CPA, N’-cyclopyrlyladenosine; CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5’-N-ethylycarboxamidoadenosine; CHA, 5’-N-ethylcarboxamidoadenosine; NECA, N’-ethylcarboxamidoadenosine; R-PIA, R-(−)-N’-(2-phenylisopropyl)adenosine; S-PIA, S-(+)-N’-(2-phenylisopropyl)adenosine; IB-MECA, 1-deoxy-1-[6-[[3-iodophenyl]methylamino]-9H-purin-9-yl]-N-methyl-β-d-ribofuranuronamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ZM 241385, 4-(2-[7-amino-2-(2-furyl)]-1,3,5-triazol-2,3-a-[1,3,5]triazin-5-ylamino)ethyl)phenol; ENHA, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride; DMSEO, dimethyl sulfoxide; PBS, phosphate-buffered saline; A$_2$A-R-IR, A$_2$A receptor-immunoreactivity; PGP 9.5, protein gene product 9.5; WWF, von Willebrand’s factor; bp, base pair(s); UNG, uracil-N-glycosylase; C, threshold cycle.

A61986/1141112

Printed in U.S.A.
In animal studies, adenosine and its analogs have been shown to protect against gastric ulcers induced by stress (Geiger and Glavin, 1985; Westerberg and Geiger, 1987). This protective effect has been attributed to the inhibitory action of adenosine on acid secretion. Adenosine was shown to suppress gastric acid secretion in several species, including dogs (Gerber et al., 1985; Gerber and Payne, 1988), guinea pigs (Heldsinger et al., 1986), and rats (Glavin et al., 1987; Scarpignato et al., 1987; Westerberg and Geiger, 1989). However, the site of action of adenosine in eliciting this inhibitory response differs among species. Adenosine has been shown to inhibit gastric acid secretion by acting on the acid-secreting parietal cells of guinea pigs (Heldsinger et al., 1986) and dogs (Gerber et al., 1985; Gerber and Payne, 1988). In addition to this direct action, adenosine has also been shown to inhibit the secretion of the acid secretagogue gastrin from canine antral G-cells (Schepp et al., 1990). In rats, adenosine analogs did not alter basal or histamine-stimulated aminopyrine uptake in isolated enriched parietal cell preparations (Puurunen et al., 1987). Thus, in this species, adenosine most likely inhibits gastric acid secretion indirectly.

Our laboratory has previously demonstrated that the administration of adenosine to the isolated, vascularly perfused rat stomach inhibited immunoreactive gastrin and stimulated somatostatin-like immunoreactivity (SLI) release (Kwok et al., 1990), suggesting that adenosine may regulate gastric acid secretion by modulating gastrin and somatostatin release. Somatostatin, a potent inhibitor of gastrin secretion, is released from gastric mucosal D-cells (Hersey and Sachs, 1995). Although the stimulatory effect of adenosine on SLI release was shown to be mediated by extracellular adenosine receptors (Kwok et al., 1990), the receptor subtype(s) involved has not been determined. Therefore, the first objective of the present study was to determine the adenosine receptor subtype involved in the stimulatory action of adenosine on SLI release using the isolated vascularly perfused rat stomach and selective adenosine analogs. Preliminary studies suggest that the A<sub>2A</sub> receptor may be involved. However, the presence of this receptor in functionally distinct regions of the stomach is unknown. The localization of the A<sub>2A</sub> receptor on specific cells of the stomach, such as the somatostatin-secreting D-cells, is also undetermined. Currently, only the presence of extremely low levels of A<sub>2A</sub> receptor mRNA has been demonstrated in the whole rat stomach using RT-PCR (Dixon et al., 1996). Therefore, the second objective of this study was to determine the cellular localization, distribution, and gene expression levels of the A<sub>2A</sub> receptor in the rat stomach, using immunohistochemistry, RT-PCR, and real-time RT-PCR, respectively. In addition, structural information regarding the gastric A<sub>2A</sub> receptor is lacking. The differential expression of multiple A<sub>1</sub> and A<sub>2</sub> receptor transcripts has been demonstrated in different tissues (Fredholm et al., 2001). Because the presence of multiple receptor forms could have important functional implications, the final objective of this study was to determine the cDNA sequence of the rat gastric A<sub>2A</sub> receptor by cloning and sequencing the entire coding region of the mucosal A<sub>2A</sub> receptor.

Materials and Methods

Stomach Perfusion

Animals were treated in accordance with the guidelines of the University of British Columbia Committee on Animal Care. Male Wistar rats (250–325 g) were housed in light- and temperature-controlled rooms with free access to food and water. Animals were deprived of food for at least 14 h, but they had free access to water, before stomach perfusion. Rats were anesthetized with 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 been described previously (Kwok et al., 1988, 1990). Two milliliters of saline solution containing 600 U of heparin (Sigma-Aldrich, St. Louis, MO) was introduced into the stomach via the arterial cannula, followed by perfusate. Venous effluent was collected via a portal vein cannula. After a 30-min equilibration period, 5-min samples were collected into ice-cold scintillation vials containing 0.3 ml of Trasylol (aprotinin, 10,000 KIU/ml; Miles Laboratories, Etobicoke, ON, Canada). Aliquots (0.5 ml) were immediately transferred into ice-cold test tubes containing 0.05 ml of Trasylol 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 (120 mM NaCl, 4.4 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 1.5 mM KH₂PO₄, 25 mM NaHCO₃, and 5.1 mM dextrose) containing 0.2% BSA (RIA grade; Sigma-Aldrich) and 3% dextran (clinical grade; Sigma-Aldrich). 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. In some experiments, the perfusion pressure was recorded using a Statham blood pressure transducer (P52 Db) connected at the level of the aortic cannula, a SE 905 converter, and a Gould chart recorder. The perfusion pressure during basal condition was between 52 and 89 mm Hg. The percent change in perfusion pressure was calculated as follows: [recorded pressure during drug perfusion – pressure during basal periods (mm Hg)/2.5 min] × pressure during basal periods (mm Hg/2.5 min) × 100.

Drugs were introduced into the perfusate via side-arm infusions at a rate calculated to give the final perfusion concentrations. The following drugs were purchased from Sigma-Aldrich: adenosine hemisulfate salt, 2-chloroadenosine (2-CA), N<sup>6</sup>-cyclopentyladenosine (CPA), 2-p-[2-carboxethyl-ethyl]adenosine-5'-N-ethylcarboxamidoadenosine HCl (CGS 21680), N<sup>6</sup>-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA), R(-)-N<sup>6</sup>-2-(phenylisopropyl)adenosine (R-PIA), S(-)-N<sup>6</sup>-2-(phenylisopropyl)adenosine (S-PIA), 1-deoxy-1-[(3R,4S)-6-(2-carboxyethyl)aminomethyl-9H-purin-9-yl]-N-methyl-β,β-dibofuranuronomide (IB-MECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), adenosine deaminase (type VII; ADA), and sodium nitroprusside. 4-[(2-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 DMSO (BDH, Toronto, ON, Canada) and subsequently diluted with saline or perfusate to 0.03 or 0.5% before perfusing into the stomach. ADA (100 U/ml) was dialyzed in saline containing 0.2% BSA, and the concentration was adjusted according to the final volume before it was diluted with perfusate for perfusion. The perfusion of DMSO alone, at these concentrations, did not alter basal SLI release; the percent change of SLI release in the presence of 0.03 and 0.5% DMSO was −1 ± 2 and −3 ± 3%, respectively.

RIA and Data Analysis

The specific RIA used for the measurement of SLI content in samples has been described previously (Kwok et al., 1988, 1990). The monoclonal antibody SOMA-3 was used. The drugs used in the present study did not cross-react with this antibody. The inter- and intra-assay variation of the RIA was less than 12 and 8%, respectively.
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; Yip & Kwok, 1998, 1999). Therefore, results were expressed as mean ± S.E.M. of SLI release (percentage), which was calculated as follows: [SLI release (picograms per minute) during a 5-min period] ÷ SLI release (picograms per minute) during period 1] × 100. To compare the effect of analogs, results were 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)] picograms per minute ÷ [mean basal SLI release (periods 1–3)] picograms per minute × 100. Statistical significance (P < 0.05) was determined using one-way ANOVA followed by Dunnett’s multiple comparison test and paired or unpaired Student’s t test when appropriate. Statistics and estimation of the EC50 values were performed using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, CA).

**Immunohistochemistry**

Gastric corpus and antrum tissues from male Wistar rats were fixed overnight, cryoprotected, and sectioned as described previously (Yip et al., 2003). Free-floating sections (30 µm) were sequentially incubated in 0.1 M PBS containing 50 mM NH4Cl (30 min), 0.1 M PBS containing 100 mM glycine (10 min), and blocking buffer (0.1 M PBS containing 1% BSA and 0.3% Triton X-100, 1 h). Antibodies were diluted in blocking buffer containing 0.1% sodium azide. Sections were incubated with A2A receptor primary antibody (1:200) for 72 h at 4°C, washed (0.1 M PBS; 3 × 15 min), and incubated with cyanine Cy3-conjugated secondary antibody overnight at 4°C. Sections were again washed and either double stained or mounted onto glass slides. For double staining, tissue sections were incubated with another primary antibody for 72 h at 4°C, washed, and incubated with Alexa Fluor 488-conjugated secondary antibody overnight at 4°C. Sections were then washed, mounted onto glass slides, and coverslipped using a mixture of 0.1 M PBS in glycerin (1:9) and sealed with nail polish.

This rabbit anti-canine A2A receptor antibody (Alpha Diagnostic International, San Antonio, TX) cross-reacts with rat tissues and has been used in this species for immunohistochemistry (Diniz et al., 2003). Its specificity has been established in rat tissues (Nie et al., 1999). In the present study, the specificity of this antibody was confirmed by neutralizing the primary antibody with the control peptide, as suggested by the supplier. Tissues stained with the neutralized antibody did not demonstrate A2A receptor immunoreactivity (A2A R-IR). Additional control experiments were also performed to ensure that nonspecific binding did not occur; these included incubating sections with 1% BSA in place of the primary antibody, without the secondary antibody, or with Alexa 488-conjugated anti-mouse IgG secondary antibody alone. No immunostaining was observed after these procedures. For double-staining experiments, monoclonal primary antibodies against somatostatin (1:500; Soma 8, MRC Regulatory peptide group, University of British Columbia, Vancouver, BC, Canada), gastrin (1:30,000; 109-21 provided by the late Dr. John Walsh), PGP 9.5 (1:200; ab8189; Abcam Limited, Cambridge, UK), human von Willebrand’s factor (1:50; Serotec, Oxford, UK), and H’K’-ATPase β (1:200; Affinity Bioreagents, Golden, CO) were used. A2A R-IR was visualized using the secondary antibody, donkey anti-rabbit IgG conjugated to Cy3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). All other immunoreactivities were visualized using donkey anti-mouse IgG conjugated to Alexa Fluor 488 (1:2000; Molecular Probes, Eugene, OR).

**Confocal Microscopy**

Tissues were viewed using the Radiance 2000 confocal scanning laser system (Bio-Rad, Hercules, CA) mounted on a Nikon Eclipse TE300 inverted microscope. The system uses 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 to 530 nm (for visualization of Alexa Fluor 488). Bleed-through was not detected for any of the antibodies used. Magnification of 40× was used with a zoom factor of 1.0 and 2× objective of 0.5 to 1.0 µm, whereas lens magnification of 60× was used with a zoom factor of 1.0 to 1.6 and a 2× objective of 0.3 to 0.5 µm. The software program Lasersharp 2000 (version 4.1; Bio-Rad) was used to scan tissues sequentially using the red and green color channels, and the Kalman collection filter (n = 2). Images with a resolution of at least 512 × 512 pixels were obtained and then analyzed using NIH Image (National Institutes of Health, Bethesda, MD) and Adobe Photoshop (version 7.0; Adobe Systems, San Jose, CA). To determine whether co-localization occurs, the image collected from the red channel (A2A R-IR) was overlaid on the image collected from the green channel (somatostatin, gastrin, VWF, PGP 9.5, or H’K’-ATPase β-IR) using Adobe Photoshop.

**Quantification of A2AR-IR with Somatostatin-IR**

Colocalization of A2A R-IR with somatostatin-IR was quantified in the antral and corporeal mucosa by examining at least three tissue sections from four different animals. For each tissue section, four or more fields of view, chosen at random, were examined at a magnification of 40×. In total, 55 fields of view were analyzed for colocalization in the corpus and antrum. The corporeal and antral mucosa contained 1.1 ± 0.2 and 1.3 ± 0.2 A2A R-IR cells per view, respectively.

**RT-PCR**

**Primer Design and Synthesis.** PCR primers were designed based on previously published rat brain A2AR cDNA sequences (accession no. S47609) (Fink et al., 1992), using the software program PCGene (IntelliGenetics, Mountain View, CA). The forward and reverse primer sequences are 5’-CTG CTG AGC CTG CCC AAG TGT3’ (corresponding to position 41–61 bp) and 5’-CCC TTC TCT TTG GGT TAC CCG3’ (corresponding to position 1355–1377 bp), respectively. The amplicon generated (1337 bp) spans the entire coding region of the A2A receptor gene. The primers were synthesized by the Nucleic Acid Protein Services Unit at University of British Columbia.

**Tissue and Total RNA Extraction.** Male Wistar rats (200–250 g) were anesthetized, and the fundus, corpus, and antrum were dissected out, rinsed in sterile ice-cold saline, and flash frozen in liquid nitrogen. The gastric mucosa was obtained by gently scraping the luminal surface of the stomach using a sterile glass slide. Total RNA was extracted immediately from the mucosa and striatum. The latter tissue has been shown to express high levels of A2A receptor mRNA (Dixon et al., 1996) and was used as a positive control. Total RNA was extracted from tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The total RNA concentration was determined by the following calculation: concentration (micrograms per milliliter) = A260 × 40 µg/ml × 100 (dilution factor).

**DNase I Treatment, First Strand cDNA Synthesis, and PCR.** 

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 of DNase I (Invitrogen), according to manufacturer’s instructions. First strand cDNA was synthesized from 5 µg of DNase I-treated total RNA using Superscript II RTase H-Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. As a negative control for RT-PCR, a sample was also prepared using autoclaved distilled water in place of total RNA.

The PCR reaction mixture (50 µl) consisted of 2 µl of cDNA in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), and 50 mM KCl] containing 0.2 mM dNTP mix, 4.5 mM MgCl2, 100 ng each of forward and reverse primer, and 1 U of Platinum TaqDNA Polymerase (Invitrogen). A positive control sample containing stratial cDNA and a negative control from the first strand synthesis step were included.
for all experiments. The PCR was performed using the Robocycler temperature cycle (Stratagene, La Jolla, CA). Thirty cycles of amplification were performed. Each cycle consisted of a 45-s denaturation period at 94°C, a 1-min annealing period at 58°C, and a 1-min extension period at 72°C. PCR products were separated by gel electrophoresis, visualized, and photographed under UV light using the Stratagene Eagle Eye II system.

Cloning and Sequencing of the Mucosal A2A Receptor Gene. The PCR product generated using mucosa tissue as the template was ligated into the pGEM-T vector (Promega, Madison, WI). DH5α-competent Escherichia coli (Invitrogen) were transformed with this vector and grown in LB plates containing ampicillin (100 μg/ml), isopropyl β-d-thiogalactoside (0.5 mM), and X-Gal (80 μg/ml). Plasmid DNA was purified using the QiAprep Miniprep kit (QIAGEN, Valencia, CA). Samples were sequenced at the Nucleic Acid Protein Services Unit at University of British Columbia and by Synthegen, LLC (Houston, TX), respectively.

RNA transcripts expressing the entire coding region of the A2A receptors were used as the standards for quantification during real-time RT-PCR. The standards were synthesized using plasmids generated by the previous cloning experiments by in vitro transcription using the Riboprobe in vitro transcription kit and T7 RNA polymerase (Promega). A2a receptor RNA standards were DNase I-treated, and purified. A2a RNA standard concentrations were determined using the Ribogreen reagent quantitation kit (Molecular Probes), the FL600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT), and the KC4 Kineticale software (version 2.6; Bio-Tek Instruments). A2a RNA standards were serially diluted to 1 x 10^11 to 1 x 10^14 copies/μl in RNase-free water, aliquoted, stored at -80°C, and thawed only once before use.

Two-Step Real-Time RT-PCR. A2a receptor gene expression was measured in extracts from the whole fundus, corpus, antrum, the corporeal mucosa, and corporeal muscle layers, and the whole stomach mucosa (n = 4). The methods for tissue extraction were described above. To obtain the corporeal mucosa and muscle tissue, the corporeal mucosa was gently scraped off the luminal surface of the corpus using a sterile glass slide. The remaining corporeal muscle tissue was flash frozen in liquid nitrogen. Total RNA was extracted immediately from corporeal and whole stomach mucosa samples. The striatum was also used as a positive control in this experiment. Total RNA was isolated, quantified, and DNase I-treated as described previously.

Step 1: Reverse transcription. One microgram of DNase I-treated tissue RNA was reverse transcribed in a total volume of 10 μl of containing 200 ng of random hexamers, 20 U of RNaseguard RNase inhibitor, 1X first strand buffer, 10 mM dithiothreitol, 0.5 mM dNTP mix, and 100 U of Superscript II RNase H-Reverse Transcriptase. At least six concentrations of the A2a receptor RNA standard, ranging from 1 x 10^3 to 1 x 10^7 copies/μl, and a sample containing DNase I-treated RNase-free water in place of the template, were reverse transcribed simultaneously. The reverse transcribed RNA standards were used to construct the standard curve for the real-time PCR assay, and the sample containing the sterile water was used as the template for the negative control.

Step 2: PCR. Each assay consisted of six standard curve samples, a negative control sample, and unknown samples. All reactions were performed in triplicates. The PCR reaction mixture (25 μl) consisted of 1X TaqMan buffer A, 200 μM of each dATP, dCTP, and dGTP, 400 μM dUTP, 0.01 μM AmpErase uracil-N-glycosylase and 0.025 μM AmpliTaq Gold DNA polymerase from the TaqMan PCR core kit (Applied Biosystems) and also contained 0.5 μl of tissue cDNA, standard cDNA or negative control, 100 nM probe, 100 nM each of the forward and reverse primers, and 4.5 mM MgCl2. 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-s 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 (version 1.6.3; Applied Biosystems). An amplification plot showing normalized reporter emissions (Rn) versus 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 Ct versus Log (initial A2a receptor standard concentrations) was generated (Fig. 10C). The initial concentration of each unknown sample was determined by interpolation using the Ct value determined by the assay. The correlation coefficient of each standard curve was >0.95, and the Ct of the no template controls exceeded 40 cycles in every assay, indicating the absence of DNA contamination. Results were expressed as copies of mRNA per microgram of total RNA. Statistical significance was determined using GraphPad Prism and the two-tailed unpaired Student's t test, where P < 0.05 was considered significant.

Results

Effect of Adenosine Agonists on Gastric SLI Release.Various adenosine receptor-selective and -nonselective agonists were used in the present study to examine the adenosine receptor(s) involved in the regulation of SLI release, because specific agonists for individual receptor subtypes are unavailable. The effect of the A1- (CHA, CPA, R-PIA, and S-PIA), A2a- (CGS 21680), A2b (NECA), and A3 (IB-MECA)-selective, and the nonselective agonists (2-CA) on SLI release was tested and compared. The basal release rate of SLI during periods 1 to 3 was shown to remain relatively constant in experiments examining the effect of 0.1 μM CPA (174 ± 24 to 178 ± 27 pg/min), 1 μM CPA (146 ± 20 to 162 ± 19 pg/min), 0.1 μM CGS 21680 (92 ± 18 to 95 ± 18 pg/min), and 0.1 μM IB-MECA (191 ± 39 to 199 ± 47 pg/min) on SLI release (Fig. 1). Figure 1A shows that the administration of 0.1 μM CPA
caused a significant inhibition of SLI release. SLI release returned to basal levels 5 min after the cessation of CPA perfusion. However, when 1 μM CPA was perfused into the stomach, the release of SLI was enhanced starting at period 6. The increased release returned to basal levels upon withdrawal of the drug (Fig. 1B). When 0.1 μM CGS 21680 was perfused into the stomach, the increase in SLI release was immediately apparent (Fig. 1C). Upon the cessation of CGS 21680 perfusion, SLI release returned to basal levels within 10 min. The perfusion of IB-MECA also increased SLI release significantly at periods 7 and 8. SLI release returned to basal levels 10 min after the withdrawal of the drug perfusion (Fig. 1D).

To test the concentration dependence of adenosine analogs in stimulating the release of SLI, similar experiments were performed using different concentrations of adenosine analogs. Results are expressed as percent changes in SLI release and are summarized in Fig. 2. These analogs caused concentration-dependent increases in SLI release. CGS 21680 and NECA caused significant augmentation of SLI release starting at 0.01 μM, and a maximal response was achieved at 1 μM. The EC50 of both CGS 21680 and NECA in stimulating SLI release was estimated to be 0.06 μM, with a 95% confidence interval between 0.02 and 0.17 μM and 0.03 and 0.14 μM, respectively. At higher concentrations (1 and 10 μM), CHA and CPA enhanced SLI release. Although it is not apparent in Fig. 2, lower concentrations of CHA (0.1 μM) and CPA (0.01 and 0.1 μM) significantly (P < 0.05) inhibited SLI release (Fig. 1A). The percent changes of SLI release in the presence of 0.1 μM CHA, and 0.01 and 0.1 μM CPA were –16 ± 6, and –12 ± 3 and –19 ± 5%, respectively. The rank order of potency of the analogs in augmenting SLI release was CGS 21680 > NECA > 2-CA > R-PIA > IB-MECA > CPA = CHA > S-PIA.

The effect of CGS 21680 on perfusion pressure was also examined. The administration of CGS 21680 significantly decreased perfusion pressure in a concentration-dependent manner (Fig. 3). To test whether the stimulated SLI release is due to a vasodilatory action, the effect of nitroprusside, a potent vasodilator, on perfusion pressure and SLI release was examined. Nitroprusside (1 μM) also significantly decreased perfusion pressure (Fig. 3), but did not alter gastric SLI release; the percent change in SLI release was shown to be 0 ± 2%.

Effect of ZM 241385 and DPCPX on SLI Release. The effect of the antagonists ZM 241385 (A2A-selective) and
DPCPX (A1-selective) on basal and agonist-stimulated SLI release were examined. To test the effect of ZM 241385 on CGS 21680-stimulated SLI release, the antagonist was perfused 5 min before the concomitant perfusion of both agonist and antagonist for 15 min. Figure 4A shows that the release of SLI was augmented when 0.1 μM CGS 21680 was introduced into the stomach alone for 15 min. The perfusion of ZM 241385 decreased basal SLI release (Fig. 4B). When ZM 241385 was perfused concomitantly with CGS 21680, the stimulated release of SLI was abolished (Fig. 4C). For comparison, results are expressed in percent change of SLI release and summarized in Fig. 5. Both 1 and 10 μM ZM 241385 suppressed basal SLI release and blocked the effect of 0.1 μM CGS 21680 on SLI release. The percent changes of SLI release during the perfusion of both 1 and 10 μM ZM 241385 with 0.1 μM CGS 21680 were similar to the percent change in SLI release when the antagonist (1 or 10 μM) was perfused alone. ZM 241385 was also shown to block adenosine (1 μM)-induced SLI release (Fig. 5). The percent change of SLI release during the perfusion of ZM 241385 together with adenosine (–51 ± 1%) is lower than that of the antagonist alone (–38 ± 10%).

The effect of 1 μM DPCPX on basal and 0.1 μM CGS 21680-induced SLI release was also tested. DPCPX inhibited basal SLI release (Fig. 5). However, the CGS 21680-induced SLI release was not altered in the presence of DPCPX.

Involvement of Endogenous Adenosine in SLI Release. The effect of ADA and EHNA, an ADA inhibitor, on SLI release was examined. Drugs were perfused into the stomach for 20 min after a 15-min basal period. Results are summarized in Fig. 6. EHNA and ADA caused a concentration-dependent increase and decrease in basal SLI release, respectively (Fig. 6A). Adenosine-induced SLI release was also enhanced and inhibited by the presence of EHNA and ADA, respectively (Fig. 6B).

Cellular Localization and Distribution of A2A-R-IR. The perfusion studies suggest that the A2A receptor is involved in the augmentation of SLI release. Therefore, experiments were performed to examine the cellular localization and distribution of A2A receptors in the rat stomach. Results show that the distribution of A2A-R-IR was similar in the corpus and antrum. In both regions, intense A2A receptor staining was observed on mucosal cells, cell bodies and nerve fibers of the myenteric plexus, and nerve fibers of the circular muscle layer, longitudinal muscle layer, muscularis mucosae, and submucosal plexus (Fig. 7). A2A-R-IR was also observed on blood vessels of both the corpus and antrum.

Double-staining for A2A-R-IR and somatostatin-IR was performed to examine whether A2A receptors are expressed on somatostatin-secreting D-cells of the mucosa. Somatostatin-IR was abundant in the mucosa, but sparse in other layers of the stomach. Colocalization of A2A-R-IR with somatostatin-IR was frequently observed on cells of both the corporeal (Fig. 8, A–C) and antral mucosa (Fig. 8, D–F). Quantification of this colocalization demonstrated that 33 ± 4 and 32 ± 8% of A2A-R-IR cells also expressed somatostatin-IR in the corporeal and antral mucosa, respectively. In rare occa-
sessions, A2AR-IR was also shown to colocalize with somatostatin-IR in the myenteric plexus of the corpus (Fig. 8, G–I) and antrum (Fig. 8, J–L).

Double staining experiments were also performed to localize A2AR-IR in relation to the IR of gastrin, H⁺K⁺-ATPase β (parietal cell marker), VWF (endothelial cell marker), and PGP 9.5 (neuronal marker). Results show that A2AR-IR was not colocalized with H⁺K⁺-ATPase β-IR (Fig. 9, A–C) or gastrin-IR (Fig. 9, D–F). However, extensive colocalization of VWF-IR with A2AR-IR was observed in the muscle layers, myenteric and submucosal plexi (Fig. 9, G–I). In both the corpus and antrum, A2AR-IR was shown to be colocalized with PGP 9.5-IR 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. 9, J–L).
Regional Distribution, Structure, and Abundance of Adenosine A2A Receptor mRNA. RT-PCR demonstrated the presence of A2A receptor mRNA in all gastric regions examined, including the fundus, corpus, antrum, and mucosa. Abundant A2A receptor mRNA was also detected in the rat striatum, which was used as a positive control (Fig. 10A). For each tissue, only one RT-PCR amplicon was generated. In addition, cloning and sequencing results demonstrated that the coding region of the gastric mucosal A2A receptor (submitted to GenBank; accession no. AF228684) was identical to the published sequence in the rat brain (Fink et al., 1992) (accession no. S47609).

Results also show that A2A receptor gene expression levels did not differ significantly among the fundus, corpus, and antrum. The mRNA level of the A2A receptor was lower in the whole stomach mucosa and corporeal mucosa than in the whole fundus, corpus, and antrum (Fig. 10B). The A2A receptor mRNA levels in gastric tissues were significantly lower in comparison with the striatum. The striatum contained 2.2 × 10^6 copies of A2A receptor mRNA/μg total RNA, which was at least 70× higher than in any region of the stomach. The standard curve generated by the A2A receptor real-time PCR assay was able to measure a 7 log range of concentrations (Fig. 10C). Thus, striatal and gastric A2A receptor gene expression levels were quantified simultaneously in the same assay.

Discussion

Studies have shown that adenosine may play a role in regulating gastric acid secretion and protecting the stomach against ulceration. In the rat, adenosine has not been shown to act directly on parietal cells, and our laboratory has suggested that adenosine may exert its inhibitory effect on acid secretion by releasing somatostatin (Kwok et al., 1990). Results of the present experiments demonstrate that adenosine-induced SLI release is likely mediated by activation of the adenosine A2A receptors. This was suggested by the following rank order of potency of adenosine analogs in augmenting SLI release: CGS 21680 ≈ NECA > 2-CA > R-PIA >
IB-MECA > CPA > CHA > S-PIA. CGS 21680 is a potent A2A receptor agonist that exhibits a 140-fold selectivity for A2A receptors over A1 receptors (Hutchison et al., 1989). Results shows that the stimulatory effect of CGS 21680 (1 and 10 μM) on SLI release (percent increase) was at least 6-fold greater than that elicited by similar concentrations of CPA or CHA. Although CPA and CHA were able to stimulate basal SLI release, it is conceivable that at these high concentrations they were acting nonspecifically on A2A rather than A1 receptors. The lack of an effect of DPCPX (1 μM) on CGS 21680-induced SLI release also suggests that A1 receptors are not involved. This concentration of DPCPX has been shown to completely abolish A1 receptor-mediated responses (Lohse et al., 1987). The stimulatory effect of CGS 21680 is unlikely mediated by A2B receptors because this compound has a much lower affinity for the A2B receptor (Brackett and Daly, 1994). Although CGS 21680 and NECA stimulated gastric SLI release equipotently and with similar efficacy, the calculated EC50 values for both compounds were in the submicromolar range (0.06 μM), rather than the micromolar range, as suggested for A2B receptors (Brackett and Daly, 1994; Klotz et al., 1998). In addition, the stimulatory effect of CGS 21680 and adenosine was completely blocked by the A2A-selective antagonist ZM 241385 (Poucher et al., 1995). Although the A3-selective agonist IB-MECA (Gallo-Rodriguez et al., 1994) also enhanced SLI release, the effect was about 7-fold less than CGS 21680. In rats, A3 receptor mediated effects were shown to be resistant to 8-phenyltheophylline blockade (van Galen et al., 1994; Peachey et al., 1996). The observation that 8-phenyltheophylline can abolish adenosine-induced SLI release (Kwok et al., 1990) supports the lack of A3 receptor involvement. The possibility that the adenosine A2A receptor-mediated augmentation of SLI release is secondary to a vasodilatory action may also be ruled out because the vasodilator nitroprusside did not alter SLI release.

Fig. 9. Double-staining of A2AR-IR with H+ K+ -ATPase β, gastrin, VWF, and PGP 9.5-IR in the rat stomach. Cells expressing both immunoreactivities look yellow (I and L). A to C, A2AR-IR (A) was shown not to colocalize with H+ K+ -ATPase β-IR (B) in the corpus mucosa (C). D to F, A2AR-IR (D) was also shown not to colocalize with gastrin-IR (E) in the antral mucosa (F). G–I, A2AR-IR (G) is colocalized with VWF-IR (H) on blood vessels in the myenteric region of the antrum (I). LM, longitudinal muscle; CM, circular muscle. J to L, A2AR-IR (G) is colocalized with PGP 9.5 (H) in nerve fibers and cell bodies of the myenteric plexus (arrowhead), and nerve fibers of the circular and longitudinal muscle (arrows) of the corpus. Scale bars, 25 μm (z-step is 1.0 μM for all images).
Results show that the A₁-selective analogs CPA (0.01 and 0.1 μM) and CHA (0.1 μM) caused a small but significant inhibition of SLI release, suggesting that A₁ receptor activation may exert an inhibitory effect on SLI release. A similar result was obtained with 0.01 μM adenosine (Kwok et al., 1990). This A₁ receptor-mediated inhibitory effect may be a result of the preferential activation of A₁ receptors by low concentrations of adenosine, as suggested previously (Ralevic and Burnstock, 1998). Results also show that blockade of A₂A receptors by ZM 241385 resulted in significant suppression of basal SLI release. This observation may be due to the unmasking of the inhibitory A₁ receptors. Under this condition, endogenous adenosine may act only on the A₁ receptors. This proposal was also supported by the observation that the inhibition of SLI release caused by the perfusion of both ZM 241385 and adenosine was greater than that caused by the antagonist alone. The stimulatory effect of adenosine, CPA, and CHA on SLI release may then be attributed to their abilities to activate A₂A receptor at higher concentrations. The effect of DPCPX on A₁ receptor-mediated inhibition of SLI release was not tested because the administration of DPCPX alone also inhibited SLI release. The explanation for the inhibition is, at present, unclear.

Results also show that EHNA, an ADA inhibitor (Mendelson et al., 1983), increased basal and potentiated adenosine-stimulated SLI release, suggesting that endogenous adenosine is involved in the regulation of SLI release. Similar results were obtained when the adenosine uptake inhibitor...
dipyridamole was used (Kwok et al., 1990). In the canine parietal cell preparation, the addition of ADA enhanced histamine-stimulated aminopyrine uptake (Gerber and Payne, 1988). In the present study, the administration of ADA was shown to decrease basal SLI release. Although the present study did not examine the effectiveness of the various doses of ADA on endogenous adenosine metabolism, ADA was shown to suppress the effect of exogenously administered adenosine. Therefore, an increase or decrease in the availability of adenosine may alter SLI release leading to changes in gastric acid secretion.

Results of the present experiments also demonstrate that the sequence of the coding region of the gastric mucosal A2A receptor was identical to that in the rat brain (Fink et al., 1992). Because multiple transcripts of the A2A receptor were not detected in the PCR experiments, alternative splicing of this receptor is unlikely to occur in the rat stomach. The present experiments demonstrate that this A2A receptor gene was expressed in several functionally and morphologically distinct regions of the stomach, including the fundus, corpus, antrum, and mucosa. The A2A receptor mRNA levels in these tissues were quantified using real-time RT-PCR. This assay was able to measure as little as 5 x 10^5 copies of A2A receptor RNA/µg total tissue RNA and over a 7 log range of concentrations. Results demonstrate that the lowest level of A2A receptor mRNA level was present in the gastric mucosa and the level in the striatum was at least 70-fold higher than any gastric regions. These results correspond well with results obtained from the immunohistochemistry studies. Although intense and distinct A2A-IR was present on some mucosal cells, the majority of A2A-IR was observed on nerve fibers and vasculature of the muscle layers and the myenteric plexus.

The present study shows that somatostatin-IR resided in the D-cells, as reported previously (Ekbлад et al., 1985; Keast et al., 1985). Sparse staining for this peptide was observed in the myenteric plexus and on nerve fibers of the circular muscle. These fibers seem not to project to the mucosa. The present study shows that A2AR-IR was present on mucosal D-cells. Although A2AR-IR also localized with somatostatin-IR in the myenteric plexus, this occurred at rare. Thus, it is likely that adenosine and its analogs act directly on mucosal D-cells to elicit SLI release. Increases in gastric and gastric acid secretion have been shown to stimulate SLI release (Hersey and Sachs, 1995). The lack of colocalization of A2A-R-IR with gastrin-IR or H^+K^-ATPase β suggests that the effect of adenosine on SLI release is not mediated indirectly by its action on G-cells or parietal cells.

In addition to somatostatin-IR, A2AR-IR was also colocalized with VWF-IR throughout the corpus and antrum. This observation agrees well with the known vascular action of adenosine (Tabrizchi and Bedi, 2001) and with the results of the present perfusion pressure study. The localization of A2A receptors in the nerve fibers of the myenteric and submucosal plexus is not surprising because previous studies have demonstrated their presence in the enteric plexi. A2A receptors have been shown to be expressed on myenteric and submucosal neurons of the jejunum and colon (Christofi et al., 2001) and are involved in the modulation of enteric neural transmission (Barajas-Lopez et al., 1991). Activation of neuronal A2A receptors has also been shown to modulate noradrenaline and acetylcholine-mediated neural transmission (Sebastiao and Ribeiro, 1996). Although the gastric release of SLI is regulated by these neurotransmitters (Saffouri et al., 1980; Koop et al., 1983), it is unlikely that adenosine modulates SLI release through A2A receptor-mediated changes in acetylcholine and noradrenaline release. We have previously shown 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). However, the possibility that adenosine stimulates SLI release indirectly by modulating noncholinergic and non-adrenergic neural transmission cannot be ruled out.

In conclusion, the present study demonstrates that the brain and gastric mucosal A2A receptor are structurally identical. A sensitive real-time RT-PCR method has been established to quantify adenosine A2A receptor gene expression. Stimulation of gastric A2A receptors enhances the release of SLI and may subsequently inhibit gastric acid secretion. Adenosine is likely to exert this effect, at least in part, by acting directly on A2A receptors of gastric D-cells. This suggestion is supported by immunohistochemistry studies showing the colocalization A2A-R-IR with somatostatin-IR on D-cells.

Acknowledgments

We thank Henry Chi Hang Leung for excellent technical assistance.

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