Stimulation of gastric acid secretion by rabbit parietal cell A$_{2B}$ adenosine receptor activation

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Arin RM, Vallejo AI, Rueda Y, Fresnedo O, Ochoa B. Stimulation of gastric acid secretion by rabbit parietal cell A$_{2B}$ adenosine receptor activation. Am J Physiol Cell Physiol 309: C823–C834, 2015. First published October 14, 2015; doi:10.1152/ajpcell.00224.2015.—Adenosine modulates different functional activities in many cells of the gastrointestinal tract; some of them are believed to be mediated by interaction with its four G protein-coupled receptors. The renewed interest in the adenosine A$_{2B}$ receptor (A2BR) subtype can be traced by studies in which the introduction of new genetic and chemical tools has widened the pharmacological and structural knowledge of this receptor as well as its potential therapeutic use in cancer and inflammation- or hypoxia-related pathologies. In the acid-secreting parietal cells of the gastric mucosa, the use of various radioligands for adenosine receptors suggested the presence of the A$_2$ adenosine receptor subtype(s) on the cell surface. Recently, we confirmed A2BR expression in native, nontransformed parietal cells at rest by using flow cytometry and confocal microscopy. In this study, we show that A2BR is functional in primary rabbit gastric parietal cells, as indicated by the fact that agonist binding to A2BR increased adenylate cyclase activity and acid production. In addition, both acid production and radioligand binding of adenosine analogs to isolated cell membranes were potently blocked by selective A2BR antagonists, whereas ligands for A$_1$, A$_{2A}$, and A$_3$ adenosine receptors failed to abolish adenosine A$_{2B}$ receptor activation. We conclude that rabbit gastric parietal cells possess functional A2BR proteins that are coupled to $G_s$ and stimulate HCl production upon activation. Whether adenosine- and A2BR-mediated functional responses play a role in human gastric pathophysiology is yet to be elucidated.

$G_s$ protein-coupled A$_{2B}$ adenosine receptor; parietal cell; gastric acid secretion

The parietal cell of the gastric mucosa is a highly specialized cell that is responsible for the production and secretion of concentrated hydrochloric acid (HCl) into the gastric lumen and, in humans, also of the Castle’s intrinsic factor. To fulfill these tasks in a regulated manner, the parietal cell is equipped with an exclusive collection of apical and basolateral ion transporter proteins that are exposed on the membrane whenever their functions are required (see e.g., review Ref. 45). In fact, the parietal cell undergoes dramatic morphological transformations in the transition from the resting to the secreting state. Upon stimulation by secretagogues in particular, the acid-secreting pump (H$^+$/K$^+$-ATPase) transits from the intracellular tubulovesicles, where it is stored, towards the apical surface of the cell, and remains there throughout the active acid secretory phase, allowing for the secretion of a proton in exchange for a potassium cation consuming the energy equivalent to one ATP molecule breakdown (53). Two other apical mechanisms are also needed to secrete HCl into the gastric lumen: secretion of Cl$^-$, the other atomic component of HCl, and K$^+$ recycling, which is necessary for maintaining constant luminal potassium concentrations for proper H$^+$-K$^+$-ATPase functioning (29).

Gastric acid secretion is a tightly regulated process. It is closely connected with the function of the other specialized cells that are also homed by the gastric glands: the pepsinogen-producing chief cells and the histamine-secreting enterochromaffin-like (ECL) cells and gastrin-secreting G cells that promote acid secretion (29;45). However, it is also connected with the release of inhibitory substances, such as somatostatin (8). Globally, a collection of neural stimuli, efferent and afferent, endocrine and paracrine agents, acting directly at apical and basolateral membrane receptors/transporters of the parietal cell or acting indirectly through the other cells of the gastric mucosa, as well as mechanical and chemical stimuli participate in acid secretion physiology (e.g., see recent reviews in Refs. 13, 29). The primary stimulatory processes are considered to be of histaminergic nature via $G_s$-coupled H$_2$ receptor activation and of cholinergic nature via activation of $G_q$-coupled muscarinic receptors or receptors for gastrin (69), but other actors are also on the stage.

Adenosine is an endogenous purine nucleoside, a metabolite produced continuously in intracellular and extracellular locations that modulates different physiological functions (25). The effects of extracellular adenosine are mediated by four subtypes of adenosine receptors that belong to the family of seven transmembrane G protein-coupled proteins known as A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$ (26). Among the adenosine receptor subtypes, the A$_{2B}$ receptor (A2BR) is the less known due to the relatively low affinity of adenosine at this receptor [with a micromolar $K_i$ value (24 $\mu$M) at the human receptor after heterologous expression] (26, 27) and the relatively limited progress in the discovery of selective agonists and antagonists. The surge of interest in A2BR can be traced to studies in which ectopic expression of A2BR, combined with genetic approaches and the generation of mice deficient in A2BR, has widened the pharmacological, structural, and functional knowledge of this receptor, as well as its potential therapeutic use in cancer and inflammation- or hypoxia-related pathologies (1, 2, 19, 22, 39, 60). It is especially relevant that during inflammatory ischemia extracellular adenosine is elevated to levels sufficient for A2BR activation (1) and that limited oxygen availability alters adenosine signaling at the receptor level, with A2BR being
specifically overexpressed by the binding of hypoxia-inducible factor (HIF)-1 to its gene promoter (20, 44). This suggests that the adenosine-A2B tandem can be engaged to control inflammation during tissue hypoxia or to fine tune other tissue responses when and where the concentration of the nucleoside and the density of the A2BRs is abundant.

High A2BR levels of expression have been reported in different parts of the intestinal tract (1, 21, 42) where it appears to be involved in modulation of the vascular tone and inflammation and intestinal secretion and motility (16, 17, 20, 28, 56, 65). However, little is known about the functional relevance of A2BR in gastric physiology. Ota et al. (54) were the first to report that there might be adenosine A2 receptors on parietal cells modulating gastric acid secretion in rabbits. Other pharmacological studies by Ainz and colleagues (3, 4, 31) demonstrated that adenosine and some of its analogs stimulated gastric acid secretion in glands and parietal cells from rabbit gastric mucosa and suggested the involvement of the A2 adenosine receptor subtype(s) based on the potencies elicited by the agonists tested. Recently, we demonstrated that the ADORA2B gene is expressed at the mRNA and protein levels in rabbit gastric parietal cells and we also confirmed the presence of A2BR on the cell surface by using flow cytometry and confocal microscopy (6). Interestingly, A2BR was found to be in close apposition to surface adenosine deaminase (ADA), the enzyme that degrades extracellular adenosine by deaminating it to inosine. The two proteins presented a high degree of colocalization, particularly in the contact areas of adjacent cells (6). With this background, the hypothesis emerged that adenosine and its analogs might have a role in gastric acid secretion via A2BR binding in the parietal cell. Using nontransformed primary cells, we show here that A2BR is functional in the parietal cell of the rabbit gastric mucosa and mediates acid secretion modulation, as indicated by the fact that agonist binding to A2BR increased adenylate cyclase activity and H⁺ secretion, and that both acid production and A2BR activation were blocked by A2BR-selective antagonists but not by the A₁, A₂A, and A₃ adenosine receptor blockade.

MATERIALS AND METHODS

Materials. ·[¹⁴C]laminopyrine (AP), ·[³H]5'-N-ethyl-carboxamidoadenosine (NECA), ·[³H]1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), and ·[³H]cAMP were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). 2-Chloro-adenosine (2-CADO), methyl-1-deoxy-1-[6-[(3-iodophenyl)methyl]-amino]-9-[1,5-c]pyrimidine (SCH58261) were supplied by Tocris (Bristol, UK). 6-cyclopentyl-adenosine (CPA), ·N-(4-nitrobenzyl)-6-thioï-[¹³C]adenosine (ADORA2B), and ·[¹³C]adenosine, adenosine, carbachol (CCh), R-Nº-phenylisopropyl-adenosine (R-PIA), Nº-cyclopentyl-adenosine (CPA), S-(4-nitrobenzy)-6-thioïnosine (NBTI), IBMX, and forskolin were from Sigma-Aldrich (St Louis, MO). 4-(-2-[7-Amino-2-[2-furyl]-1,2,4]triazolo [2,3-a] [1,3,5]triazin-5-ylamino)ethyl)phenol (ZM241385), DPCPX, and 5-amin-7-[phenethyl]-2-([1 furyl]pyrazol4,3-e) [1,2,4]triazolo [1,5-c]pyrimidine (SCH58261) were supplied by Tocris (Bristol, UK). 1-Deoxy-1-[-[(3-iodophenyl)ethyl]amino]-9H-purin-9-yl-N-methyl-β-D-ribofuranuronamide (IB-MECA) was obtained from Research Biochemicals (San Diego, CA), histamine chlorhydrate from MERCK (Darmstadt, Germany) and ADA from ICN Biomedical (Irvine, CA).

Parietal cell isolation and enrichment. Male and female New Zealand rabbits (2.5–4 kg body wt) were used in accordance with the Spanish (RD 1201/2005) and European (2003/65/CE Directive and 2007/526/CE recommendation) guidelines for the use of laboratory animals. The isolation of gastric mucosa cells by pronase and collagenase digestion of the mucosa and the isolation and enrichment of parietal cells by centrifugal elutriation were carried out following the procedures described in earlier works (4, 5). Briefly, after perfusion with PBS, the stomach was removed and the cardia and antral regions were discarded. The corpus was rinsed in PBS and the mucosa was separated and minced and the mucosa fragments were digested. Average cell viability was 90% and purity 75%. All parietal cell preparations used in this study were monitored for functionality by measuring the dose response of hydrochloric acid secretion to 10⁻⁷ to 10⁻³ M histamine and were considered to represent a normal population of resting parietal cells.

Determination of acid production. Acid formation was evaluated in isolated parietal cells by the [¹³C]AP accumulation method as previously described (54) with minor modifications. Briefly, cells (1 × 10⁶ cells/ml) were incubated (110 cycles/min, 37°C for 45 min.) with the label AP (0.01 μCi) in the presence or the absence (100% or basal AP accumulation) of the investigated drug in 1 ml of buffer A (132.4 mM NaCl, 1 mM NaH₂PO₄, 5 mM NaH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 1 mg/ml BSA, 2 mg/ml glucose, and 2.38 mg/ml HEPES, pH 7.4). The reaction was stopped by the addition of 1 ml ice-cold buffer A; the cells were sedimented (600 g for 3 min), and the supernatant was aspirated and the cells were digested with 100 μl of 4 M perchloric acid. After incubation at 50°C for 15 min, aliquots were placed in vials containing 5 ml of a suitable scintillation cocktail and radioactivity counted. Incubates were performed in triplicate. The radioactivity accumulated in the presence of 0.1 mM dinitrophenol was subtracted from all basal and test drug data.

Isolation of parietal cell membranes. Plasma membranes from parietal cells were prepared as described earlier (5). Parietal cells (60 × 10⁶ cells/ml) were homogenized with a Potter-Elvehjem homogenizer in ice-cold buffer B (20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 0.5 mM EDTA, 0.54 mM dithiothreitol, 5 μg/ml leupeptin, and 15.7 μg/ml benzamidine). The 700-g pellet was discarded and the supernatant was collected and placed on top of a 47% sucrose solution and centrifuged (100,000 g, 4°C for 45 min) in a swinging rotor. Plasma membranes were then collected from the interface and resuspended in 50 mM Tris-HCl buffer, pH 7.4 (buffer C), washed once (120,000 g, 4°C for 20 min), incubated with ADA (2 U/ml, room temperature for 1 h) to metabolize endogenous adenosine, and resolated. Membranes were finally resuspended in buffer C and stored at −80°C until used. Protein concentration was determined by the Bradford method (10) using BSA as standard.

Radioligand binding experiments. Binding assays were carried out as described by Casadó et al. (15) using 0.5 mg membrane protein/ml. In saturation experiments, membranes were incubated with 20 to 2560 nM of the agonist [³H]NECA at room temperature for 1 h in buffer C. Free and membrane-bound radioligands were quickly separated by vacuum filtration through Whatman GF/F filters previously soaked in 0.3% polyethyleneimine, pH 10, for 2 h. The filters were then bathed in 6 ml of buffer C and placed afterwards in 10 ml of a scintillation cocktail for radioactivity measurement. All binding assays were performed in triplicate. Nonspecific binding, measured in the presence of saturating 100 μM NECA, was subtracted from the total binding data to calculate the specific binding. For competition binding experiments, membranes were incubated with 200 nM [³H]NECA for 1 h and 10⁻12 to 10⁻³ M of unlabelled NECA were added as competitor. Incubations were terminated and the radioactivity measured as described above. [³H]NECA association-dissociation kinetics experiments were carried out by incubating 50 nM [³H]NECA with membranes. For association measurements, 500-μl aliquots were taken at the indicated times and processed. Dissociation was induced by addition of the appropriate unlabeled ligand after an association period of 30 min; 500-μl aliquots were taken at different intervals for filtration and subsequent analysis, as described above. Further association-dissociation experiments were performed using 50 nM of the A₁ receptor agonist [³H]DPCPX, 50 nM of the A₂A agonist [³H]NECA, and 5 nM of the A₃ receptor agonist [³H]NECA for the
association and 50 μM DPCPX, 50 μM ZM241385, and 5 μM IB-MECA as their respective displacing agents.

**Determination of adenylyl cyclase activity.** Adenylyl cyclase activity was measured in cell membranes by quantification of the cAMP generated from ATP substrate using a competitive protein binding procedure (52). After a 5-min preincubation at 30°C of membrane suspensions (0.8 mg protein/ml) in buffer C supplemented with 5 mM Mg-Cl and 1 mM DTT, the reaction was initiated by adding 200 μM ATP and stopped 10 min later. The samples were then centrifuged at 12,000 g for 2 min, and supernatants (50 μl) were taken for cAMP determination in 96-well microplates. To each well, 50 μl of [3H]cAMP (54 Ci/mmol) and 100 μl of 0.3 mg/ml bovine adrenalin protein kinase A (PKA) were added in a total volume of 250 μl of buffer C. A standard curve of cAMP (from 0.2 to 6 pmol of cAMP/well) was included in each plate. Plates were incubated at 4°C for 150 min and then free and PKA-bound [3H]cAMP were separated by vacuum filtration through Whatman GF/B filters using a Skatron Micro 96 Harvester. Filters were washed twice with 3 ml of buffer C and placed afterwards in 3 ml of a scintillation cocktail and radioactivity counted.

**Assessment of calcium mobilization in individual cells by microfluorimetry.** The cytosolic Ca2+ concentration was recorded in single parietal cells using a multiple excitation microfluorescence system (Cairn Research, Kent, UK), essentially as described in Ref. 38. Following isolation, cells were washed three times in DMEM:F12 (1:1, vol/vol), seeded (1–2 × 106 cells) on Matrigel precoated coverslips, and cultured at 37°C in 5% CO2 atmosphere in DMEM:F12 (1:1, vol/vol) supplemented with 15 mM HEPES, pH 7.4, 2 mM L-glutamine, 10 nM hydrocortisone, 1 mg/ml insulin, 0.1 mg/ml transferrin, 5 g/ml geniticin, and 2 mg/ml BSA. After 1 h, cells were washed in the above medium supplemented with 1 mg/ml BSA and subsequently loaded with 5 μM fura-2/AM for 60 min at 37°C. The coverslip was placed in a superfusion chamber of a Nikon Diaphot microscope thermostatted at 30°C and the cells were excited alternately at 340, 360, and 380 nm to monitor the fluorescence emitted at 510 nm. Control and calibration procedures as well as the algorithms used to calculate the cytosolic Ca2+ concentration are described previously (38).

**Calculations and statistical analysis.** We used GraphPad Prism version 5.02 (GraphPad Software) to compare dose-response curves with models of one or two binding sites, to draw graphs, and to calculate the parameters that define the binding: maximum binding capacity (Bmax), radioligand-receptor dissociation constant in equilibrium (Ka) in saturation assays, the dissociation (koff) constant in kinetics assays, and the IC50 value and inhibition constant (Ki) in competitive binding assays, IC50, and for statistics. Parameters were calculated directly from nonlinear regression curves. All data are presented as means ± SE of at least three independent experiments, each performed in triplicate. Quantitative variables were analyzed by one-way or two-way ANOVA with Bonferroni posttest for multiple comparisons or unpaired Student’s two-tailed t-test to determine differences between two groups. Differences were considered significant at a value of P ≤ 0.05.

**RESULTS**

**Pharmacological identification of A2BR in membranes isolated from gastric parietal cells.** Confocal microscopy and flow cytometry analyses demonstrated that the A2BR protein is expressed at the surface of the acid-secreting parietal cell of rabbit gastric mucosa (6). To investigate the functional significance of this receptor, we analyzed first the kinetic behavior of membranes purified by ultracentrifugation from parietal cells towards A2BR agonist and antagonist binding in classical pharmacological assays performed at room temperature. Equilibrium binding of increasing concentrations (from 20 to 2,560 nM) of the radiolabeled adenosine derivative [3H]NECA to membranes showed a monophasic saturation curve (Fig. 1A) with a binding site density, Bmax, of 228 ± 28 nmol/mg of protein and an affinity of the receptor to the radioligand, Ki, of 1.8 ± 0.4 μM. As observed in Fig. 1B, the time dependence of 50 nM [3H]NECA association to membranes revealed that equilibrium was rapidly reached with a kobs of 0.67 ± 0.8 min−1. Competition assays were performed with unlabeled NECA at concentrations ranging from 10−12 to 10−3 M after membrane saturation with 200 nM [3H]NECA (Fig. 1C), and the resulting data were compared with models of one or two binding sites using GraphPad software. When the competition curve was fitted to a unique binding site model (R² = 0.9231), the binding constant for the competitor ligand, Kd, was 2 μM and the concentration at which the inhibition is 50% of maximum, IC50, was 2.38 μM. These affinity values of NECA are in the range of those described for A2BR in a variety of models (Table 1) (14, 21, 26). Fitting the experimental data to a biphasic competition curve (R² = 0.9297) showed that there was a population of binding sites with affinity values (IC50: 3.24 μM) that were very close to those calculated from the monophasic analysis and a second population of binding sites with affinity characteristics in the nanomolar range (IC50: 10.23 nM). This IC50 value corresponds well with those described for NECA binding to glucose-regulated protein of 94 kDa (GRP94) in rabbit gastric parietal cell membranes at 4°C (5) and to purified human (36) or bovine (57) GRP94. This chaperone is normally confined to the endoplasmic reticulum but escapes the KDEL-mediated retention system and anchors to the plasma membrane in some cell types and circumstances, but the significance of surface GRP94 expression and of its ability to bind adenosine analogs with high affinity (57) remains unclear.

NECA is a nonspecific agonist for adenosine receptors. To investigate the contribution of [3H]NECA binding to other adenosine receptors potentially expressed on the parietal cell surface, we used specific displacers at the concentrations that are reported to allow us to distinguish between A1, A2A, A2B, and A3 adenosine receptors (Table 1 and references therein). Graphs in Fig. 1, D–H, show the time course of the displacement of the bound radioligand by 50 μM of A2BR agonists NECA (D) and 2-CADO (E), by 50 μM of the A1 receptor ligands DPCPX (F) and R-PIA (not shown), and by 50 μM of the selective A2A receptor antagonist ZM241385 (H) after receptor saturation with 50 nM [3H]NECA. Figure 1I shows the displacement of 5 nM [3H]NECA binding by 5 μM of the A3 receptor agonist IB-MECA. Results indicate that ligand binding is antagonized only by the A2BR agonists NECA (with a koff of 1.3 ± 0.3 min−1) and 2-CADO (with a koff of 2.3 ± 3.1 min−1). On the contrary, all other tested compounds failed to displace [3H]NECA binding, suggesting that it does not correspond to adenosine A1, A2A, and A3 receptor binding. R-PIA at 200 μM, the concentration used in other cell types to displace binding to A2BR (14), also did not have any inhibitory effect (Fig. 1G). In another set of experiments, a time-course analysis of the dissociation of 50 nM [3H]DPCPX binding to the A1 receptor using 50 μM DPCPX as displacer and of 5 nM [3H]NECA binding to the A3 receptor using 5 μM IB-MECA as displacer was conducted. Findings (not shown) revealed that the specific binding of either radiolabel agent was
negligible, thus corroborating that the gastric parietal cell surface is unlikely to contain a physiologically relevant density of A$_2$$_2$ and A$_3$ receptors.

Overall, these results strongly support the concept that A2BR is the only adenosine receptor subtype able to bind NECA, adenosine, and other adenosine analogs in the parietal cell membrane of the rabbit gastric mucosa at rest. Should other adenosine receptors be expressed simultaneously in this target cell, which a priori might be entirely possible, their concentrations would be too low for conducting pharmacological studies.

Acid production is stimulated by A2BR activation in primary gastric parietal cells. Adenosine and adenosine analogs have been shown to stimulate acid formation in isolated rabbit parietal cells (4, 54), but the underlying mechanism is unknown. To analyze the involvement of the A2BR in the process of gastric acid secretion, we first determined AP accumulation, as a surrogate index of the produced H$^+$, in isolated parietal cells stimulated with increasing concentrations of 2-CADO and compared it with the rise produced by histamine, the essential activating agent for the parietal cell in vivo (Fig. 2A).

As shown in Fig. 2B, 2-CADO increased AP accumulation in a dose-dependent manner, with an EC$_{50}$ value of 280 $\mu$M. A2BR activation, in a more potent (EC$_{50}$ of $\sim$37 $\mu$M) but a far less effective inducer of acid production (up to 100% increase at 10$^{-4}$ M) than 2-CADO, whereas adenosine, the natural agonist, was somewhat less potent and effective than NECA, stimulating AP accumulation by around 75–100% at 10$^{-3}$ M. In confirmation of previous studies (4, 54), NECA behaved as a more potent (EC$_{50}$ of 280 $\pm$ 6 $\mu$M) than 2-CADO, whereas adenosine, the natural agonist, was somewhat less potent and effective than NECA, stimulating AP accumulation by around 75–100% at 10$^{-4}$ M concentration (data not shown). Conceivably, although at a higher concentration range than histamine, A2BR activation, in
2-CADO, such as 10 nM, potentiated the AP accumulation response to concentrations of 10^{-7} M (Fig. 3A) and the A2A receptor antagonist SCH58261 at 10^{-9} M had no effect (Fig. 3B). The two compounds were used at the concentrations reported to exhibit their selective antagonistic action (Table 1 and references therein). Moreover, binding of the selective A1 receptor agonist CPA (from 10^{-12} to 10^{-7}) or inhibition of 2-CADO response by DPCPX at 10^{-9} M, the concentration at which it antagonizes A1 receptor binding, had no effect on acid production (Fig. 3, C and D).

Adenosine is the natural ligand for adenosine receptors but its efficacy and potency on acid secretion at the parietal cell is weak (4, 54). However, in view of the fact that surface ADA can degrade endogenous adenosine and bind extracellularly adenosine receptors affecting their functionality (32, 33) and that ADA and ARD were found to establish contact sites at the plasma membrane of the rabbit parietal cell (6), we wondered whether endogenous adenosine could have an impact on the functional acid secretory unit. To this end, acid production was determined in cells treated with ADA at concentrations between 0.025 and 0.3 U/ml to inactivate the endogenous agonist, and in cells left untreated. As Fig. 4A shows, ADA-treated cells exhibited an AP uptake rate that was significantly lower (20–45%) than untreated cells when tested at rest. In contrast, ADA treatment (0.1 U/ml) did not affect the dose-dependent response of acid secretion to histamine (Fig. 4B) or 2-CADO (not shown), suggesting that the acid secreted by the parietal cell in basal, resting conditions is sustained in part by the adenosine molecules that are physiologically associated to the cell membrane or released from cells during incubation whilst this effect is irrelevant when acid secretagogues enter the scene.

Collectively, these findings indicate that adenosine and adenosine analogs stimulate acid formation in isolated rabbit parietal cells through A2BR interaction.

**A2BR signaling is transduced by adenylyl cyclase activation in the parietal cell.** A2BR is known to be coupled to G_s and G_{i} proteins and this mediates its effects (21, 47). Most A2BRs are coupled to G_{i} proteins and activate adenylyl cyclase resulting in the intracellular production of cAMP and subsequent activation of PKA. In some cell types, activation of calcium-dependent mechanisms involving the G_{q} family of proteins to activate PLC and increase intracellular [Ca^{2+}] has been reported (23, 47). The two signaling pathways play a key role in the recruitment of the H^{+}-K^{+}-ATPase to the apical membrane. To elucidate the operating signal transduction systems, we estimated adenylyl cyclase activity and mobilization of intracellular Ca^{2+} in the parietal cell in response to A2BR activation.

Confirmation that A2BR activation is coupled to G_{i} stimulation was achieved by direct measurement of adenylyl cyclase activity in membranes obtained from isolated parietal cells. Control experiments in which membranes were treated with particular by 2-CADO, can lead to stimulation levels of acid formation that are not very far from those achieved by histamine. In fact, routine 2-CADO stimulation reached 80–90% of the maximal histamine response, while NECA and adenosine had lesser effects, averaging 30–40% of maximal response (results not shown). Thus 2-CADO was selected to perform the studies described below.

To elucidate whether the 2-CADO-promoted stimulation of acid secretion could be mediated by increases in intracellular cAMP levels, a dose-response study to 2-CADO was analyzed in the presence of 10^{-4} M IBMX. IBMX is an inhibitor of the cAMP phosphodiesterase, so that A2BR activation should be followed by additive effects on the response of parietal cells. As seen with histamine (Fig. 2C), IBMX substantially potentiated the AP accumulation response to concentrations of 2-CADO, such as 10^{-6} and 10^{-5} M (Fig. 2D), that were barely effective alone. These data point to the involvement of G_{s}-coupled adenosine A2A receptor or A2BR in the 2-CADO action.

Equilibrative nucleoside transporters (ENTs) allow adenosine to cross the cell membrane following its concentration-dependent gradient. 2-CADO can also be transported in both directions across the plasma membrane via ENTs (61). Hence, an increase in extracellular adenosine or 2-CADO could be expected to be compensated by directing the flow through ENTs towards the intracellular space. Comparison of the acid secretion rates in response to 2-CADO in normal and ENT-blocked parietal cells by 5 × 10^{-4} M NBNTI (61) allows us to estimate the contribution of this compensatory flux to the effect of extracellular 2-CADO mediated by its membrane receptor. Results in Fig. 2E show that the 2-CADO-mediated stimulation of acid production is similar in the two conditions, indicating that an interaction of the agonist with the plasma membrane is responsible for such a stimulated production of hydrochloric acid and that its ENT-mediated uptake is not influencing acid secretion.

Subsequent experiments were designed to identify the adenosine receptor subtype mediating acid secretion stimulation by agonistic and antagonistic binding assays. Findings in Fig. 3 demonstrate that A2BR is the only receptor engaged in the 2-CADO-mediated acid production activation, as the 2-CADO response was abolished by antagonizing A2BR using DPCPX at 10^{-7} M (Fig. 3A) and the A2A receptor antagonist SCH58261 at 10^{-9} M had no effect (Fig. 3B). The two compounds were used at the concentrations reported to exhibit their selective antagonistic action (Table 1 and references therein). Moreover, binding of the selective A1 receptor agonist CPA (from 10^{-12} to 10^{-7}) or inhibition of 2-CADO response by DPCPX at 10^{-9} M, the concentration at which it antagonizes A1 receptor binding, had no effect on acid production (Fig. 3, C and D).

### Table 1. Adenosine receptor affinities of agonists and antagonists

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<tr>
<th>Agonists</th>
<th>A1</th>
<th>A2A</th>
<th>A2B</th>
<th>A3</th>
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<tr>
<td>Adenosine</td>
<td>100 (h) (67)</td>
<td>310 (h) (67)</td>
<td>15,000 (h) (67)</td>
<td>290 (h) (67)</td>
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<tr>
<td>NECA</td>
<td>14 (h) (67)</td>
<td>20 (h) (67)</td>
<td>140 (h) (67)</td>
<td>25 (h) (67)</td>
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<tr>
<td>2-CADO</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1,890 (h) (26)</td>
<td>N.D.</td>
</tr>
<tr>
<td>R-PIA</td>
<td>2.04 (h) (41)</td>
<td>N.D.</td>
<td>150,000 (h) (11)</td>
<td>33 (h) (58)</td>
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<tr>
<td>IB-MECA</td>
<td>51 (h) (37)</td>
<td>2,900 (h) (37)</td>
<td>11,000 (h) (37)</td>
<td>1.8 (h) (37)</td>
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<tr>
<td>CPA</td>
<td>2.3 (h) (37)</td>
<td>794 (h) (37)</td>
<td>18,600 (h) (37)</td>
<td>72 (h) (37)</td>
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<table>
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<tr>
<th>Antagonists</th>
<th>3.0 (h) (62)</th>
<th>129 (h) (37)</th>
<th>51 (h) (62)</th>
<th>795 (h) (64)</th>
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<tr>
<td>DPCPX</td>
<td>0.21 (rb) (24)</td>
<td>60 (h) (62)</td>
<td>63.8 (h) (11)</td>
<td>243 (h) (62)</td>
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<td>ZM241385</td>
<td>774 (h) (37)</td>
<td>1.6 (h) (37)</td>
<td>75 (h) (37)</td>
<td>743 (h) (37)</td>
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<td>SCH58261</td>
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<td>5.0 (h) (37)</td>
<td>1,110 (h) (37)</td>
<td>1,200 (h) (37)</td>
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Numbers in parentheses are reference numbers. N.D., no data available in human or rabbit; h, human; rb, rabbit; 2-CADO, 2-chloro-adenosine; CPA, N\textsuperscript{\textregistered}-cyclopentyl-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; IB-MECA, 1-deoxy-1-[[3-(iodophenyl)methyl]-amino]-9H-purin-9-yl-N-methyl-β-N-ribonuronamide; NECA, 5'-N-ethyl-carboxamidoadenosine; CPA, N\textsuperscript{\textregistered}-cyclopentyl-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; IB-MECA, 1-deoxy-1-[[3-(iodophenyl)methyl]-amino]-9H-purin-9-yl-N-methyl-β-N-ribonuronamide; NECA, 5'-N-ethyl-carboxamidoadenosine; CPA, N\textsuperscript{\textregistered}-cyclopentyl-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; IB-MECA, 1-deoxy-1-[[3-(iodophenyl)methyl]-amino]-9H-purin-9-yl-N-methyl-β-N-ribonuronamide; NECA, 5'-N-ethyl-carboxamidoadenosine.
with $10^{-4}$ M histamine or $10^{-4}$ M forskolin, a direct activator of adenylate cyclase activity, resulted in high increases of cAMP production from ATP substrate. In the same way, $10^{-4}$ M NECA (Fig. 5A) and $10^{-4}$ M 2-CADO (Fig. 5B) significantly enhanced adenylate cyclase activity to an extent of $\approx 80\%$ in the two cases. As expected, this increase was strongly potentiated by costimulation with $10^{-4}$ M histamine or forskolin, whereas NECA and 2-CADO were not able to potentiate the histamine or forskolin stimulatory effects. The $EC_{50}$ value at the human receptor after overexpression in CHO cells is high (24 $\mu$M) and the intracellular cAMP accumulation levels were used as the readout (27). We examined the response of membrane adenylate cyclase to increasing NECA concentrations and found that the stimulatory effect was moderate, although significant, until a threshold concentration was reached (Fig. 5C). When the data were fitted to a dose-response model with variable slope (Fig. 5C), an $EC_{50}$ value of 18 $\mu$M was obtained, indicating that our functional studies in native parietal cells yielded affinity values of A2BR for adenosine analogs similar to that reported in heterologous A2BR-expressing cells.

To determine whether transduction by $G_{\alpha}$ was also involved in A2BR activation, cytosolic calcium waves were recorded by...
We observed the elevation of intracellular Ca\(^{2+}\)/H^11001 produced by CCh (51), an activator of the muscarinic M3 receptor, manifested as an initial spike, representing Ca\(^{2+}\)/H^11001 release from intracellular stores, followed by a lower level plateau caused by an inward current of Ca\(^{2+}\)/H^11001. Compared with positive CCh, ATP, or histamine controls, no calcium mobilization occurred after exposure to NECA or 2-CADO in parietal cells. This lack of calcium mobilization via A2BR was recorded whatever the order of addition of agents. Simultaneous costimulation with histamine was required to see an effect, which, in any case, was not more potent than of histamine alone, indicating that calcium signaling is not involved in the A2BR-mediated response of acid secretion in our cell model.

DISCUSSION

Adenosine is the endogenous agonist at all adenosine receptors. Under resting physiological conditions, adenosine levels in the interstitial fluid are between 30 and 300 nM (7). This is sufficient to activate A_1, A_2A, and A_3 adenosine receptors whenever these proteins are abundantly expressed on the cell surface. The local adenosine level increases 10-fold during hypoxia and 100- to 1,000-fold in ischemia (7, 18, 34, 72), allowing cell responses mediated via A2BR in these settings. The pharmacological and functional studies presented here have been conducted using parietal cells obtained from normal rabbit gastric mucosa, which are representative populations of native, nontransformed primary cells at rest. They provide evidence that A2B is the adenosine receptor that is preferentially, if not exclusively, expressed on the parietal cell membrane and mediates acid production via Gs activation. Moreover, activation kinetics indicate that the A2BR might act at full power to activate acid production in extreme pathophysiological conditions of the local microenvironment; i.e., exhibiting high concentrations of agonist.

Functional A2BR expression has been identified in a variety of organs and tissues of various species, including humans (reviewed in Refs. 1, 21, 26). Furthermore, pharmacological and structural characterization of the human A2BR has been investigated both after its heterologous expression in cell lines such as HEK 293 cells (47), HMC-1 mast cells (47), CHO cells (27, 43, 63), or the colonic epithelial line T84 (43) and also in primary lymphocytes (48), macrophages (66), or endothelial cells (23). However, there has not been any reported attempt to characterize A2BR expression in the parietal cell biochemi-
cally, apart from our recent report in rabbits (6). Our study shows that in rabbits, the gastric parietal cell is endowed with a density of A2BRs that allows its activation to associate with acid secretion stimulation even though the affinity constants of agonists are similar to those reported in other tissues and cell models.

Our conclusion that A2BR is the only adenosine receptor subtype expressed on the parietal cell surface of relevance for agonist binding and acid secretion stimulation is supported by the following observations. First, the binding saturation curve for the nonspecific agonist NECA that turned out to be monophasic with a $B_{max}$ of 228 nmol/mg of protein and a $K_d$ of 1.8 $\mu$M (Fig. 1A). This binding site density value is markedly higher in the parietal cells than that displayed in other primary cells; it is commonly around the picomolar range or less, for instance, in chromaffin cells from bovine adrenal medulla (14) or HEK 293 cells (47). Second, we have observed a low affinity value of radiolabel NECA, which is similar to that usually estimated for A2BR (14, 26), and the absence of displacement of NECA binding by ligands selective for the $G_\text{i}$-coupled $A_1$ or $A_3$ adenosine receptors or for the $G_\text{s}$-coupled

Fig. 4. Endogenous adenosine helps sustain basal acid production in parietal cells. Acid production was determined in parietal cells isolated from rabbit gastric mucosa exposed to 0.025 to 0.3 U/ml of adenosine deaminase (ADA; A) or stimulated with concentrations between $10^{-7}$ and $10^{-3}$ M of histamine in the presence or absence of 0.1 U/ml ADA (B). Acid production was measured as aminopyrine accumulated in cells. Curves were fitted to % of aminopyrine uptake in basal, unstimulated conditions (100%) vs. ADA or histamine concentration using a sigmoid dose-response model with variable slope. Values correspond to the means ± SE of 5 independent experiments performed in triplicate. Where absent, error bars were smaller than the symbol. One-way ANOVA was performed to assess the effect of ADA at rest and two-way ANOVA to assess the effect of histamine and the difference in the response due to ADA. Significantly different from the corresponding value without ADA by the Bonferroni posttest: *$P < 0.05$, ***$P < 0.001$.

Fig. 5. Activation of cell membrane adenylate cyclase by A2BR agonists. Adenylate cyclase activity was determined in membranes isolated from parietal cells in the absence (basal) or presence of a $10^{-4}$ M concentration of NECA (A), 2-CADO (B), histamine or forskolin, alone or in combination, or increasing concentrations of NECA (C) as described in MATERIALS AND METHODS. Data were normalized with respect to the basal value (100%) for each experiment. In C, the curve was fitted to % of adenylate cyclase activity in basal, unstimulated conditions (100%), vs. NECA concentration using a sigmoid dose-response model with variable slope. Basal activities averaged 10.3 ± 4.0 pmol of cAMP produced per mg of protein. Values are the means ± SE for at least 3 independent experiments performed in triplicate. Significantly different by the Student’s $t$-test: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, from the corresponding basal value and #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$, from the group in which NECA or 2-CADO were tested alone.
It is interesting to note that when \(^{3}H\)NECA was employed as the displaceable radioligand and NECA as the displacing ligand, the binding inhibition curve fitted with almost the same statistical quality one-site \((R^2 = 0.9231)\) and two-site \((R^2 = 0.9297)\) equation models (Fig. 1C). This suggests that, besides recognizing A2BR, NECA might recognize one other different receptor or binding protein or, alternatively, two distinct affinity states of A2BR. This second population of binding sites had affinities for NECA in the nanomolar range \((10.23 \text{ nM at } 25^\circ \text{C})\). We documented in previous work that GRP94 was expressed at the surface of rabbit gastric parietal cells anchored to the basolateral domain, with active regions exposed at the plasma membrane. Membranes were able to bind radiolabeled NECA much better at 4°C than at room temperature, with high affinity \((K_d = 466.6 \text{ nM})\), and in a radioligand-displaced manner \((K_i = 18.9 \text{ nM})\) (5), all key features of GRP94 binding (57). Thus this second population of binding sites might well be GRP94. However, although unlikely, the presence of two distinct affinity states of A2BR on the rabbit parietal cell cannot be excluded. In view of the fact that A2BR is thought to exhibit conformational changes promoted by allosteric modulators (63), surface ADA modulates allosterically ligand binding to adenosine receptors (32, 33), and A2BR and ADA establish contact sites at the plasma membrane of the parietal cell (6), it is therefore possible that the occurrence of this type of regulation induced by ADA vicinity might happen.

A link between adenosine and gastric acid secretion was suggested more than two decades ago. From the published work, it seems evident that adenosine has species-dependent actions on regulating acid secretion, which may be inhibitory or stimulatory, and also that it has direct and indirect actions. However, findings are still controversial and inconclusive due to the fact that, in most cases, a glandular model with its inherent elevated cellular complexity was used, whereas in other investigations, studies were done in isolated parietal cells (for a recent review, see Ref. 13). Apparent discrepancies can be exemplified by reports in murine models, in which adenosine stimulates the gastric somatostatin release via activation of A2A (70) and inhibits gastric production via activation of A1 (71), suggesting that adenosine may inhibit gastric acid secretion. However, adenosine and its analogs did not alter AP uptake in the rat parietal cell (55), whereby indirect actions might be inferred. Further studies using the perfused stomach of specific adenosine receptor knockout mice confirmed the extreme complexity of the glandular stomach system by showing that adenosine has dual actions on somatostatin release in the mouse: stimulatory at concentrations higher than 1 \(\mu\)M via A2A activation and inhibitory at low concentrations via A1 activation with no role for A2B and A3 (68). On the other hand, adenosine was found to inhibit acid secretion by acting directly on the parietal cell in guinea pigs (35) and dogs (30), while, in rabbits, adenosine stimulates acid secretion both in glands (3) and isolated parietal cells (4, 54). These differences are surprising and comparative studies would be necessary to reveal the common character or the specificity of the direct and indirect actions of adenosine on acid secretion. The fact that the aminoacidic sequence of the human A2BR protein is 93% identical to the rabbit and 88% to the mouse and rat A2BR protein intuitively suggests that these species could share the A2BR role in gastric acid secretion regulation.
To the best of our knowledge, the role of adenosine on the human parietal cell function or the gastric mucosa gland pathophysiologic has not been addressed in direct studies. Indirect studies assessing ADA activity in antral mucosal biopsies of helicobacter pylori infected patients (12) or in patients with chronic gastritis (49) showed no clear correlation between ADA activity and mucosal inflammation. In the fundic mucosa, a positive correlation between ADA activity and basal and maximal gastric acid output was found (50), suggesting a role for ADA in regulating acid secretion. In our primary cell model, using parietal cells isolated from the corpus, we show for the first time that degradation of endogenous adenosine by exposure to ADA led cells to decrease acid secretion (Fig. 4A). This suggests that endogenous adenosine can make a contribution to acid production in basal conditions. ATP is a well known cotransmitter of acetylcholine in synapsis of the peripheral nervous system (13) and also a major adenosine precursor. Hence, it is tempting to speculate that basal adenosine is part of the endogenous systems that help to control gastric secretion through ATP/acetylcholine release in vagal synapsis innervating the gastric parietal cell.

Involvement of A2BR in intestinal pathophysiology has been widely demonstrated. It is clear that there are paramount functional differences between the intestinal mucosa and the gastric mucosa. However, taking into consideration that A2BR-mediated functional responses were shown to participate in normal intestinal physiology as well as during inflammation (1, 20, 28) and that A2BR is upregulated in human and animal models of inflammatory diseases (42), it is worth underlining the similarities of A2BR activation that exist between the intestinal and the gastric mucosa. 1) Both, as mucosal tissues, are particularly prone to significant drops in PO2, resulting in inflammatory hypoxia (40). 2) Of the four adenosine receptor subtypes, functional A2BR are specifically expressed in intestinal epithelial cells (59) as well as in parietal cells (Fig. 1). 3) Downstream responses to intestinal epithelial and parietal cell A2BR activation include chloride secretion stimulation. Gastric acid secretion depends on the apical extrusion of Cl−ions, and it has been shown that the function of chloride carriers is regulated by A2BR in intestinal epithelial cells. In particular, during intestinal inflammation, adenosine acts as a paracrine mediator of chloride secretion in diarrheic processes resulting in activation of electrogenic Cl−secretion (17). Also in colonic epithelium cells, A2BR stimulates chloride secretion (65). 4) Adenosine and its analogs do not activate Gs (Fig. 6) and cAMP/PKA is the only identified signaling pathway triggered by A2BR in the parietal cell (Fig. 5), as in the intestinal epithelium (59). Taken together, these observations suggest that A2BR activation may share common upstream effectors and some downstream responses in the gastrointestinal mucosa as a whole.

Adenosine has been defined as an endogenous distress, anti-inflammatory agent released by cells in conditions that are metabolically unfavorable. Our studies suggest that A2BR agonism might be more relevant than anticipated in acid secretion pathophysiology. A scheme summarizing our findings is shown in Fig. 7. Because of the lack of a precise localization for A2BR in the polarized parietal cell, for simplicity, the model locates the receptor in the apical membrane. Adenosine may originate extracellularly or be released to and taken up from the extracellular space of the parietal cell by several transport systems. Extracellular adenosine has a very short half-life on the extracellular surface due to a compensation of concentrations between the extra and the intracellular compartments, mainly achieved via ENTs. In the rabbit parietal cell, ENTs do not seem to play a significant role in acid production, as the 2-CADO-mediated acid secretion stimulation was unaffected by ENTs inhibition. By binding to Gs-coupled A2BR, adenosine and adenosine analogs stimulate gastric acid secretion. The potency for 2-CADO was two orders of magnitude inferior to histamine, while their efficiencies were almost similar. In previous experiments, we identified expression of GRP94 only in the basolateral membrane of the parietal cell (5) as well as the specific binding of GRP94 to adenosine analogs with parameters of a true binding protein; the parietal cell (5) as well as the specific binding of GRP94 to adenosine analogs with parameters of a true binding protein.
ated functional responses play a role in human gastric pathophysiology deserve elucidation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


