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

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Parietal cell A$_{2B}$ adenosine receptor characterization

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Authors' contributions

RMA, AIV and BO conceived the study, and participated in its design and coordination. RMA carried out the radioligand binding studies and acid production measurements. OF and YR participated in data interpretation and statistical analysis, as well as the figures' conception and drawing. All authors wrote the manuscript and approved the final manuscript.
Abstract

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 A2B 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 A2 adenosine receptor subtype(s) on the cell surface. Recently, we confirmed A2BR expression in native, non-transformed 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 analogues to isolated cell membranes were potently blocked by selective A2BR antagonists, whereas ligands for A1, A2A and A3 adenosine receptors failed to abolish activation. We conclude that rabbit gastric parietal cells possess functional A2BR proteins that are coupled to Gs 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.

Keywords

Gs-protein-coupled A2B adenosine receptor, parietal cell, gastric acid secretion.
1. Introduction

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 (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 1 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 (13; 29)]. The primary stimulatory processes are considered to be of histaminergic nature via Gs-coupled H₂ receptor activation and of cholinergic nature via activation of Gq-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 A₁, A₂A, A₂B and A₃ (26). Among the adenosine receptor subtypes, the A₂B receptor (A₂BR) is the less known due to the relatively low affinity of adenosine at this receptor (with a micromolar EC₅₀ value (24 µM) 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-A2BR 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 $A_{2B}$ adenosine receptors are 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 and co-workers were the first to report that there might be adenosine A$_2$ receptors on parietal cells modulating gastric acid secretion in rabbits (54). Other pharmacological studies by Ainz, Gil-Rodrigo and co-workers demonstrated that adenosine and some of its analogues stimulated gastric acid secretion in glands and parietal cells from rabbit gastric mucosa (3; 4; 31), and suggested the involvement of the A$_2$ 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 co-localization, particularly in the contact areas of adjacent cells (6). With this background, the hypothesis emerged that adenosine and its analogues might have a role in gastric acid secretion via A2BR binding in the
parietal cell. Using non-transformed 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 $A_1$, $A_{2A}$ and $A_3$ adenosine receptor blockade.

2. Materials and Methods

2.1. Materials. $[^{14}C]$Aminopyrine (AP), $[^3H]5'$-N-ethyl-carboxamido-adenosine (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), NECA, adenosine, carbachol (CCh), R-N$^6$-phenylisopropyl-adenosine (R-PIA), N$^6$-cyclopentyl-adenosine (CPA), S-(4-nitrobenzyl)-6-thioinosine (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-amino-7-(phenylethyl)-2-(1-furyl)pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (SCH58261) were supplied by Tocris (Bristol, UK). 1-deoxy-1-[[3-iodophenyl)methyl]-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 adenosine deaminase (ADA) from ICN Biomedical (Irvine, CA).

2.2. Parietal cell isolation and enrichment. Male and female New Zealand rabbits (2.5-4 kg bw) 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 cardial 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 hydrochloride acid secretion to $10^{-7}$ to $10^{-3}$ M histamine, and were considered to represent a normal population of resting parietal cells.

2.3. Determination of acid production. Acid formation was evaluated in isolated parietal cells by the $[^{14}C]$AP accumulation method as previously described (54) with minor modifications. Briefly, cells (1x$10^6$ cells/ml) were incubated (110 cycles/min, 37ºC 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 NaH$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 5.4 mM KCl, 1.2 mM MgSO$_4$, 1 mM CaCl$_2$, 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 (600g for 3 min), and the supernatant aspirated and the cells digested with 100 µl of 4 M perchloric acid. After incubation at 50ºC 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 tested drug data.

2.4. Isolation of parietal cell membranes. Plasma membrane from parietal cells were prepared as described earlier (5). Parietal cells (60x$10^6$ 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 interphase 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 reisolated. 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.

2.5. Radioligand binding experiments. Binding assays were carried out as described by Casadó and co-workers (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/B filters previously soaked in 0.3% polyethylenimine, 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. Non-specific 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 unlabeled NECA was 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 \[^3\text{H}\]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 A1R agonist \[^3\text{H}\]DPCPX, 50 nM of 
the A2A agonist \[^3\text{H}\]NECA and 5 nM of the A3R agonist \[^3\text{H}\]NECA for the 
association and 50 µM DPCPX, 50 µM ZM241385 and 5 µM IB-MECA as their 
respective displacing agents.

2.6. Determination of adenylate cyclase activity. Adenylate 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 30ºC of membrane suspensions (0.8 mg protein/ml) in buffer C 
supplemented with 5 mM Mg2Cl and 1 mM DTT, the reaction was initiated by 
adding 200 µM ATP and stopped 10 min later. The samples were then centrifuged 
at 12,000g for 2 min, and supernatants (50 µl) were taken for cAMP determination 
in 96-well microplates. To each well, 50 µl of \[^3\text{H}\]cAMP (54 Ci/mmol) and 100 µl of 
0.3 mg/ml bovine adrenal 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 4ºC for 150 min and then free and 
PKA-bound \[^3\text{H}\]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.
2.7. Assessment of calcium mobilization in individual cells by microfluorimetry. The cytosolic Ca\(^{2+}\) concentration was recorded in single parietal cells using a multiple excitation microfluorescence system (Cairn Research Ltd, Kent, UK), essentially as described in ref. (38). Following isolation, cells were washed three times in DMEM:F12 (1:1, v/v), seeded (1-2 x 10\(^6\) cells) on Matrigel-precoated coverslips and cultured at 37°C in 5% CO\(_2\) atmosphere in DMEM:F12 (1:1, v/v) supplemented with 15 mM HEPES, pH 7.4, 2 mM L-glutamina, 10 nM hídrocortisona, 1 mg/ml insulin, 0.1 mg/ml gentamycin, 0.5 μg/ml transferrin, 5 μg/ml sodium selenite, 10 mM glucose, 5 μg/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 superperfusion chamber of a Nikon Diaphot microscope thermostatized at 30°C and the cells were excited alternatively 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 Ca\(^{2+}\) concentration are described previously (38).

2.8. Calculations and Statistical analysis. We used GraphPad Prism version 5.02 (GraphPad software, California) 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 (\(B_{\text{max}}\)), radioligand-receptor dissociation constant in equilibrium (\(K_D\)) in saturation assays, the dissociation (\(k_{\text{off}}\)) constants in kinetics assays and the IC\(_{50}\) value and inhibition constant (\(K_i\)) in competitive binding assays, EC\(_{50}\), and for statistics. Parameters were calculated directly from non-linear regression curves. All data are presented as mean ± SEM of at least three independent experiments, each performed in
triplicate. Quantitative variables were analyzed with one-way or two-way ANOVA with Bonferroni post-test 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 \( P \leq 0.05 \).

3. Results

3.1. Pharmacological identification of A2B adenosine receptor in membranes isolated from gastric parietal cells

Confocal microscopy and flow cytometry analyses demonstrated that the A2B adenosine receptor 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 2560 nM) of the radiolabeled adenosine derivative [\(^3\)H]NECA to membranes showed a monophasic saturation curve (Fig. 1A) with a binding site density, \( B_{\text{max}} \), of 228 ± 28 nmol/mg of protein and an affinity of the receptor to the radioligand, \( K_D \), of 1.8 ± 0.4 µM. As observed in Fig. 1B, the time-dependence of 50 nM [\(^3\)H]NECA association to membranes revealed that equilibrium was rapidly reached with a \( k_{\text{obs}} \) 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 [\(^3\)H]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 (\( R^2 = 0.9231 \)), the binding constant for the competitor ligand, \( K_i \), was 2 µM and
the concentration at which the inhibition is 50% of maximum, IC$_{50}$, 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 (R$^2$=0.9297) showed that there was a population of binding sites with affinity values (IC$_{50}$, 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 (IC$_{50}$, 10.23 nM). This IC$_{50}$ value corresponds well with those described for NECA binding to glucose-regulated protein of 94 kDa (GRP94) in rabbit gastric parietal cell membranes at 4ºC (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 analogues with high affinity (57) remains unclear.

NECA is a non-specific agonist for adenosine receptors. To investigate the contribution of [$^3$H]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 A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$ adenosine receptors (Table 1 and references therein). Graphs in Fig. 1D-1H 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 A$_1$ receptor ligands DPCPX (F) and R-PIA (not shown), and by 50 µM of the selective A$_{2A}$ receptor antagonist ZM241385 (H) after receptor saturation with 50 nM [$^3$H]NECA. Fig. 1I shows the displacement of 5 nM [$^3$H]NECA binding by 5 µM of the A$_3$ receptor agonist IB-MECA. Results
indicate that ligand binding is antagonized only by the A2BR agonists NECA (with a $k_{off}$ of $1.3 \pm 0.3 \text{ min}^{-1}$) and 2-CADO (with a $k_{off}$ of $2.3 \pm 3.1 \text{ min}^{-1}$). On the contrary, all other tested compounds failed to displace $[3\text{H}]$NECA binding, suggesting that it does not correspond to adenosine A$_1$, A$_2$A and A$_3$ receptor binding. Neither did R-PIA at 200 µM, the concentration used in other cell types to displace binding to A2BR (14), have any inhibitory effect (Fig. 1G). In another set of experiments, a time-course analysis of the dissociation of 50 nM $[3\text{H}]$DPCPX binding to A$_1$ receptor using 50 µM DPCPX as displacer and of 5 nM $[3\text{H}]$NECA binding to A$_3$ receptor using 5 µM IB-MECA as displacer were 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$_1$ 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 analogues 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.

3.2. Acid production is stimulated by A$_{2B}$ adenosine receptor activation in primary gastric parietal cells

Adenosine and adenosine analogues 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 A$_{2B}$ adenosine receptor 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 EC50 value of 280 ± 6 µM and a maximal accumulation of 10-fold above basal at 10⁻³ M. In confirmation of previous studies (4; 54), NECA behaved as a more potent (EC50 of ~37 µM) 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 10⁻⁴-10⁻³ M concentration (data not shown). Conceivably, although at a higher concentration range than histamine, A2BR activation, in 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 M and 10⁻⁵ M (Fig. 2D), that were barely effective alone. These
data point to the involvement of Gs-coupled adenosine A<sub>2A</sub> or A<sub>2B</sub> receptors 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 ENTs-blocked parietal cells by 5x10<sup>-6</sup> M NBTI (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 ENTs-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<sup>-7</sup> M (Fig. 3A) and the A<sub>2A</sub> receptor antagonist SCH58261 at 10<sup>-9</sup> 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 A<sub>1</sub> receptor agonist CPA (from 10<sup>-12</sup> to 10<sup>-7</sup>) or inhibition of 2-CADO response by DPCPX at 10<sup>-9</sup> M, the concentration at which it antagonizes A<sub>1</sub> 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 A2BR and ADA 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 which 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 analogues stimulate acid formation in isolated rabbit parietal cells through A<sub>2B</sub> adenosine receptor interaction.
3.3. \(A_2B\) adenosine receptor signaling is transduced by adenylate cyclase activation in the parietal cell

\(A_2B\) receptors are known to be coupled to \(G_s\) and \(G_q\) proteins and this mediates its effects (21; 47). Most \(A_2B\) adenosine receptors are coupled to \(G_s\) proteins and activate adenylate 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 \([Ca^{2+}]_i\) 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 adenylate cyclase activity and mobilization of intracellular \(Ca^{2+}\) in the parietal cell in response to \(A_2B\) activation.

Confirmation that \(A_2B\) activation is coupled to \(G_s\) stimulation was achieved by direct measurement of adenylate cyclase activity in membranes obtained from isolated parietal cells. Control experiments in which membranes were treated 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 about 80% in the two cases. As expected, this increase was strongly potentiated by co-stimulation 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, though 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 EC50 value of 18 µM was obtained, indicating that our
functional studies in native parietal cells yielded affinity values of A2BR for
adenosine analogues similar to that reported in heterologous A2BR-expressing
cells.

To determine whether transduction by Gq was also involved in A2BR activation,
cytosolic calcium waves were recorded by microfluorimetry in individual parietal
cells in response to various effectors (Fig. 6). We observed the elevation of
intracellular Ca2+ produced by CCh (51), an activator of the muscarinic M3 receptor,
manifested as an initial spike, representing Ca2+ release from intracellular stores,
followed by a lower level plateau caused by an inward current of Ca2+. Compared to
positive CCh, ATP or histamine (HA) 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 co-stimulation 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.

4. 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 A1, A2A and A3 adenosine receptors
whenever these proteins are abundantly expressed on the cell surface. The local adenosine level increases 10-fold during hypoxia and 100- to 1000-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, non-transformed 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 A2B adenosine receptor 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 (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 biochemically, apart from our recent report in rabbits (6). Our study shows that in rabbits, the gastric parietal cell is endowed with a density of A2B adenosine receptors 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, which turned out to be monophasic with a $B_{\text{max}}$ of 228 nmol/mg of protein and a $K_D$ of 1.8 µM (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 pmol 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 Gα-coupled A₁ or A₃ adenosine receptors or for the Gs-coupled A₂A adenosine receptor (Fig. 1, F-I). Third, we found that the specific binding of radiolabel NECA at the concentrations expected to bind A₃ or of radiolabel DPCPX to A₁ adenosine receptor was negligible (not shown). Fourth, it is clear that NECA and 2-CADO stimulated membrane adenylate cyclase activity (Fig. 5A), and that the 2-CADO mediated stimulation of acid secretion was both up-regulated by inhibiting degradation of cAMP through phosphodiesterase activity in intact cells (Fig. 2D) and abolished by A₂B but not by A₂A adenosine receptor invalidation (Fig. 3, A and B). These results are compatible with a functional expression of the A₂B adenosine receptor subtype in the parietal cell, mediating via Gs a stimulatory role in gastric acid secretion. Presence of the A₂ receptor subtype in gastric parietal cells was suggested years ago (3; 54) and recently, A2BR has been proven to exist at the cell surface by immunocytochemical analysis of isolated parietal cells (6). However, this is the first report demonstrating A2BR engagement in the parietal cell’s acid secretory function in any species.
It is interesting to note that when [³H]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 nM at 25°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 ($K_D$ was 466.6 nM), and in a radicicol-displaced manner ($K_i$ 18.9 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. But 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 (13)]. Apparent discrepancies can be exemplified by reports in murine models, in which adenosine stimulates the gastric somatostatin release via activation of A$_{2A}$ (70) and inhibits gastrin production via activation of A$_1$ (71), suggesting that adenosine may inhibit gastric acid secretion. However, adenosine and its analogues 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 µM via A$_{2A}$ activation and inhibitory at low concentrations via A$_1$ activation with no role for A$_{2B}$ and A$_3$ (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, direct studies on the role of adenosine on the human parietal cell function or the gastric mucosa gland pathophysiology has not been addressed. 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 co-transmitter 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 A₂B receptors 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 analogues do not activate Gᵣ (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 which are metabolically unfavourable. Our studies suggest that A₂B adenosine receptor 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 analogues stimulate gastric acid secretion. The
potency for 2-CADO was two orders of magnitude inferior to histamine’s, 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 analogues with parameters of a true
binding protein; however, its relevance in the parietal cell function was not explored.

In summary, our studies show that rabbit gastric parietal cells possess functional
A2BR proteins that are coupled to Gs and stimulate HCL production upon activation.
Given that the functional role of A2BR in gastric acid secretion is in its infancy, a
discussion on potential therapeutical opportunities is necessarily speculative.
Whether adenosine- and A2BR-mediated functional responses play a role in human
gastric pathophysiology deserve elucidation.

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Table 1. Adenosine receptor affinities of agonists and antagonists.

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<tr>
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<th>$K_i$ (nM)</th>
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<tr>
<td></td>
<td>$A_1$</td>
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<tr>
<td><strong>Agonists</strong></td>
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</tr>
<tr>
<td>Adenosine</td>
<td>100 (h) (67)</td>
</tr>
<tr>
<td>NECA</td>
<td>14 (h) (67)</td>
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<tr>
<td>2-CADO</td>
<td>N.D.</td>
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<tr>
<td>R-PIA</td>
<td>2.04 (h) (41)</td>
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<tr>
<td>IB-MECA</td>
<td>51 (h) (37)</td>
</tr>
<tr>
<td>CPA</td>
<td>2.3 (h) (37)</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>3.0 (h) (62)</td>
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<tr>
<td></td>
<td>0.21 (rb) (24)</td>
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<tr>
<td>ZM241385</td>
<td>774 (h) (37)</td>
</tr>
<tr>
<td>SCH58261</td>
<td>725 (h) (37)</td>
</tr>
</tbody>
</table>

N.D., no data available in human or rabbit: h, human; rb, rabbit.

2-CADO, 2-chloro-adenosine; CPA, $N^6$-cyclopentyl-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; GRP94, glucose-regulated protein of 94 kDa; IB-MECA, 1-deoxy-1-[[3-iodophenyl][methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-
ribofuranuronamide; NECA, 5'-N-ethyl-carboxamidoadenosine; R-PIA, (R)-N^6-phenylisopropyl-adenosine; SCH58261, 5-amino.7-(phenylethyl)-2-(1-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; ZM241385, [4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol].
Figure Captions

Figure 1. Saturation curves of agonist binding to parietal cell plasma membranes and displacement of \([{}^3\text{H}]\text{NECA}\) specific binding by adenosine receptor ligands.

Membranes (0.5 mg protein/ml) from rabbit parietal cells were incubated at room temperature (~25°C) with 20 to 2560 nM \([{}^3\text{H}]\text{NECA}\) for 1 h (A) or 50 nM \([{}^3\text{H}]\text{NECA}\) for up to 60 min (B) as described in Material and Methods. Specific binding was defined as the difference between total binding and non-specific binding, where non-specific binding was that determined in the presence of 100 µM NECA. The $B_{\text{max}}$ value was 228 ± 28 nmol/mg of protein and $K_D$ was 1.8 ± 0.4 µM. The curve in A was fitted to specific bound versus equilibrium free concentration of \([{}^3\text{H}]\text{NECA}\) using a one-site binding model. (C) In competitive binding assays, NECA was used as competitor at concentrations between $10^{-12}$ and $10^{-3}$ M with 200 nM \([{}^3\text{H}]\text{NECA}\) for 1 h. The curve was fitted to % of the maximum specific binding of \([{}^3\text{H}]\text{NECA}\) using a sigmoid dose-response model with variable slope. Time-course dissociation of the \([{}^3\text{H}]\text{NECA}\) (50 nM) bound to membranes promoted by 50 µM NECA (D), 50 µM 2-CADO (E), 50 µM DPCPX (F), 200 µM R-PIA (G) and 50 µM ZM241385 (H). In (I), dissociation of 5 nM \([{}^3\text{H}]\text{NECA}\) binding by 5 µM IB-MECA was analyzed. Values are means ± SEM of at least three independent experiments performed in triplicate. Where absent, error bars were smaller than the symbol.

Figure 2. Stimulation of acid production by histamine, 2-CADO and IBMX.

Parietal cells isolated from rabbit gastric mucosa were stimulated with concentrations between $10^{-7}$ and $10^{-3}$ M of histamine or 2-CADO in the absence (A and B) or presence of $10^{-4}$M IBMX (C and D) and acid production was measured as aminopyrine accumulated in cells. In E, the effect of $10^{-6}$ M NBTI treatment on acid
production was estimated. Curves were fitted to % of aminopyrine uptake in basal, unstimulated conditions (100%) versus agonist concentration using a sigmoid dose-response model with variable slope. Values correspond to the mean ± SEM of at least three independent experiments performed in triplicate. Where absent, error bars were smaller than the symbol. Two-way ANOVA was performed to assess the effect of each histamine, 2-CADO or IBMX treatment. Significantly different from the corresponding value without IBMX by the Bonferroni post-test: *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Antagonists of A₂B but not other adenosine receptor subtypes abolish the 2-CADO-mediated activation of acid secretion. Parietal cells isolated from rabbit gastric mucosa were stimulated with increasing concentrations of the A₂BR agonist 2-CADO in the absence or presence of 10⁻⁷ M DPCPX (A), 10⁻⁹ M SCH58261 (B) or 10⁻⁹ M DPCPX (D). In C, cells were incubated with increasing concentrations of the A₁ agonist CPA and antagonized with 10⁻⁹ M DPCPX. Acid production was measured as aminopyrine accumulated in cells. Curves were fitted to % of aminopyrine uptake in basal, unstimulated conditions (100%) versus agonist concentration using a sigmoid dose-response model with variable slope. Values correspond to the mean ± SEM of at least three independent experiments performed in triplicate. Where absent, error bars were smaller than the symbol. Two-way ANOVA was performed to assess the effect of each agonist and the difference in the responses due to antagonists.

Figure 4. Endogenous adenosine helps sustain basal acid production in parietal cells.
Acid production was determined in parietal cells isolated from rabbit gastric mucosa (A) exposed to 0.025 to 0.3 U/ml of adenosine deaminase (ADA) or (B) stimulated with concentrations between $10^{-7}$ and $10^{-3}$ M of histamine in the presence or absence of 0.1 U/ml ADA. Acid production was measured as aminopyrine accumulated in cells. Curves were fitted to % of aminopyrine uptake in basal, unstimulated conditions (100%) versus ADA or histamine concentration using a sigmoid dose-response model with variable slope. Values correspond to the mean ± SEM of five 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 post-test: *$P<0.05$, **$P<0.001$. 

Figure 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 the Methods section. 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%), versus NECA concentration using a sigmoid dose-response model with variable slope. Basal activities averaged $10.3 \pm 4.0$ pmol of cAMP produced per mg of protein. Values are the means ± SEM for at least three independent experiments performed in triplicate. Significantly different by the Student’s $t$-test: *$P<0.05$, **$P<0.01$, and ***$P<0.001$. 

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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.

**Figure 6.** Lack of calcium responses of single parietal cells to A2BR agonists. (A) Cells were stimulated sequentially with 20 µM carbachol (CCh), 10 µM NECA, 10 µM NECA plus100 µM histamine (HA), 100 µM HA, 50 µM ATP, 50 µM UTP, 20 µM CCh. (B) Cells were stimulated with 20 µM CCh, 10 µM 2-CADO, 10 µM 2-CADO plus 100 µM HA, 100 µM HA and 20 µM CCh. Cytosolic calcium increases were measured by microfluorimetry in single cells and calculated as described in the Methods section. In each panel, data correspond to the intracellular calcium concentration (nM) of a representative cell of four different cultures.

**Figure 7.** Scheme summarizing the involvement of A2B adenosine receptor in acid secretion stimulation in the rabbit gastric parietal cell. During conditions of limited oxygen availability, extracellular adenosine signaling is enhanced and mainly derives from the phosphohydrolysis of its precursor nucleotides ATP, ADP and AMP by a coordinated two-step enzymatic process: CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5’-nucleotidase). Extracellular adenosine levels also depend on the activity of equilibrative nucleoside transporters (ENTs), that allow adenosine to cross the plasma membrane following its concentration-dependent gradient, and of ecto-adenosine deaminase (ADA) that degrades extracellular adenosine to inosine, inactive for acid production (RM Arin, unpublished observation). Agonist-binding at A2BR results in activation of adenylate cyclase and chloride acid production.
Pharmacological studies were performed with parietal cell membranes as a whole, so a basolateral or an apical localization to A2BR cannot be ascribed.


22. Feoktistov I and Biaggioni I. Role of adenosine A(2B) receptors in inflammation. 


Characterization of [3H]NECA binding

A

[3H]NECA dissociation assays

D

50 μM NECA

E

50 μM 2-CADO

F

50 μM DPCPX

G

200 μM R-PIA

H

50 μM ZM241385

I

5 μM IB-MECA
**A**

Aminopyrine accumulation (% of basal)

log [histamine] (M)

ANOVA
- Histamine: P < 0.001
- IBMX: P < 0.001

**B**

Aminopyrine accumulation (% of basal)

log [2-CADO] (M)

ANOVA
- 2-CADO: P < 0.001
- IBMX: P < 0.001

**C**

Aminopyrine accumulation (% of basal)

IBMX
- Control
- Histamine 10^-6 M
- Histamine 10^-3 M

ANOVA
- Histamine: P < 0.001
- IBMX: P < 0.001

**D**

Aminopyrine accumulation (% of basal)

IBMX
- Control
- 2-CADO 10^-5 M
- 2-CADO 10^-3 M

ANOVA
- 2-CADO: P < 0.001
- IBMX: P < 0.001

**E**

Aminopyrine accumulation (% of basal)

log [2-CADO] (M)

ANOVA
- 2-CADO: P < 0.001
- 2-CADO + NBTI: P = 0.1500
A. A2B antagonism

- 2-CADO
- 2-CADO + DPCPX 10^{-7} M

ANOVA
2-CADO: P < 0.001
DPCPX 10^{-7} M: P < 0.001

B. A2A antagonism

- 2-CADO
- 2-CADO + SCH 58261 10^{-9} M

ANOVA
2-CADO: P < 0.001
SCH 58261 = 0.0530

C. A1 agonist binding

- CPA
- CPA + DPCPX 10^{-9} M

D. A1 antagonism

- 2-CADO
- 2-CADO + DPCPX 10^{-9} M

ANOVA
2-CADO: P < 0.001
DPCPX 10^{-9} M: P = 0.2447
A

Histamine + ADA
ANOVA
Histamine: P < 0.001
ADA: P = 0.5621

Aminopyrine accumulation (% of basal)

ADA (U/mL)

B

Histamine
Histamine + ADA
ANOVA
Histamine: P < 0.001
ADA: P = 0.5621

Aminopyrine accumulation (% of basal)

log [histamine] (M)