Adenosine A1 receptor activation inhibits histamine release in gastric enterochromaffin-like cells

Rafael Martins de OLIVEIRA, Carolina Bernardi STEFANI, Angélica Aparecida Antoniellis SILVEIRA, Marcelo Lima RIBEIRO, José PEDRAZZOLI Jr. and Alessandra GAMBERO*.

Clinical Pharmacology and Gastroenterology Unit, São Francisco University Medical School, Bragança Paulista, SP, Brazil.

ABSTRACT: Adenosine acts as a gastroprotective factor decreasing inflammation and reducing gastric acid secretion. Quantitative RT-PCR was used to determine the expression of the adenosine receptor genes *(A1AR, A2AAR, A2BAR* and *A3AR)* and that of the gastrin receptor B gene *(CCKBR)* in isolated, short-term cultured enterochromaffin-like (ECL) cells. Both the *A1AR* and the *CCKBR* genes were expressed at a level higher than the other genes. Also, the effect of 2-chloroadenosine, a stable agonist of A1 and A2A receptors, was explored on ECL cells, with a resulting inhibition of both basal and gastrin-stimulated histamine release. Also, dipropylcyclopentylxanthine (DPCPX), a selective A1 antagonist, prevented the inhibitory effects of 2-chloroadenosine, suggesting the effect of 2-chloroadenosine is mediated by A1 receptors. It is concluded that isolated, short-term cultured ECL cells are a suitable model for studies relating gene expression and function, and that the gastroprotective actions of adenosine are at least partly mediated through A1 receptors.

Extracellular adenosine plays a role in cellular protection under physiological and pathological conditions acting through different adenosine receptors (AR), denominated A1AR, A2AAR, A2BAR and A3AR, and its signaling is terminated by the relatively short half-life of extracellular adenosine (Chen *et al.,* 2013, Sheth *et al.,* 2014).

Adenosine acts on A2AR decreasing production of proinflammatory cytokines such as IL-2, TNF-α and INF-γ by T lymphocytes, thus controlling inflammation, but may contribute to persistent *Helicobacter pylori* infection (Alam *et al.,* 2009, Odashima *et al.,* 2006). High gastric concentrations of adenosine increase somatostatin release via A2AAR (Yip and Kwok, 2004), which exerts in turn a tonic restraint on acid secretion by parietal cells, histamine secretion by ECL cells and gastrin secretion by G-cells (Rudholm *et al.,*

2009, Yip and Kwok, 2004). Adenosine directly inhibits gastrin release by G cells through A1AR (Yip *et al.,* 2004b). Gastrin controls histamine release by ECL cells through gastrin/CCK-2 receptors and it is a stimulus for gastric acid production through histamine H(2) receptors in parietal cells (von Rosenvinge and Raufman, 2010). Achlorhydria induced by omeprazol administration was able to suppress both A1AR and gastrin gene expression in G-cells and, A2AAR and somatostatin gene expression in D-cells, suggesting that changes in adenosine receptor expression may modulate the synthesis and release of gastrin and somatostatin (Yip *et al.,* 2004a).

We have explored here the expression of *A1AR, A2AAR, A2BAR* and *A3AR* genes by isolated, short-term cultured ECL cells and the modulation of histamine release by these cells by an A1/A2 agonist and a specific A1 receptor antagonist.

Stomachs from Sprague-Dawley rats (CEMIB; State University of Campinas, Campinas, SP, Brazil) were used. The study was performed in agreement with norms of the

Brazilian College for Animal Experimentation (COBEA), and was approved by the Ethics Committee of the São Francisco University, Bragança Paulista. Five to eight stomachs were excised out, everted to mucosal-side-out sacs and 8 mL of 0.9 mg/mL Pronase solution (Roche, Mannheim, Germany) was injected into the sacs, which were then incubated for 30 min, at 37 °C with oxygenation in medium A containing: 0.5 nM NaH_2PO_4; 1.0 nM Na_2HPO_4; 20 nM $NaHCO_3$; 70 nM NaCl; 5 nM KCl; 11 nM glucose; 1.0 nM EDTA; 50 nM HEPES (at pH 7.8) and BSA 5 mg/mL. The stomachs were then transferred for 10 min at 37 °C with oxygenation in medium B containing 1.0 mM $CaCl_2$ and 1.5 mM $MgCl_2$ (at pH 7.4) instead of EDTA. Another 30 min of incubation in Medium A was followed by 15 min of incubation in Medium B and the mucosal cells were collected by centrifugation (100 g, 10 min).

ECL cells were isolated as previously described (Lindstrom *et al.,* 2001, Stefani *et al.,* 2012). Briefly, mucosal cells were subjected to counter-flow elutriation (Beckman Instruments, Palo Alto, CA, USA) first using a standard chamber (20 mL/min; speed 2000 rpm) which was followed by a Sanderson chamber (18 mL/min, 2000 rpm). Cells obtained from elutriation were then layered on the two layers of a Nycodenz gradient (15% and 10.8%; NycoPrep – Universal; Axis-Shield, Norway) and centrifuged for 5 min at 1000 rpm. The cells above the 10.8% layer were collected (density 1.056 g/mL). Number and viability were evaluated by Trypan Blue exclusion. The purity of each ECL cell isolation was assessed by immunocytochemistry using an anti-histamine (1:1500) antibody as primary (Euro-diagnostica, Malmo, Sweden) and a biotinilated anti-rabbit (1:1000) antibody as secondary (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The final cell suspension contained 93.1% ± 1.1 ECL cells.

Total RNA was isolated from ECL cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The single-stranded cDNA was synthesized using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. Quantitative polymerase chain reaction (PCR) amplification was performed using Platinum SYBR Green (InvitrogenTM Life Technologies, Alameda, CA, USA) in a 7300 real-time PCR system (Applied Biosystems). The primers sequence were as follows, for sense/antisense:

A1AR - 5'-GGATCGATACCTCCGAGTCA-3'/5'-CAAGGGAGAGAATCCAGCAG-3';
A2AAR -5'-CAGATCCCCTGGAGAAGTCA-3'/5'-CAGCCCTTTCCTCACAAGAG-3';
A2BAR- 5'-CCTTTGGCATTGGACTGACT-3'/5'-CAGGGCAGCAGCTCTTATTC-3';
A3AR - 5'-TCTGTGCTGTAGTGGGCAAC-3'/5'-CAACAGCAATGTCAGCCAGT-3';
Cckbr 5'-GCTACAGCTTCACCACCACA-3'/5'-TCTCCAGGGAGGAAGAGGAT-3';

β-actin 5'-ACAAACCTGGTCCGTGCTAC-3'/5'-TAGGTAGAGTTCGCGGGAGA-3'.

The gene expressions were normalized to a constitutive gene (β-actin), and the relative fold induction was calculated according to the formula $2(-\Delta\Delta Ct)$ (Livak and Schmittgen, 2001). Biopsies of whole stomach were also assayed for comparison.

ECL cells (2500 cells/well) were cultured in 96-well plates pre-coated with Matrigel (1:10; BD Biosciences, San Jose, CA, USA) in DMEM/F12 Invitrogen) supplemented with 2% fetal bovine serum, 2mM glutamine, 100 IU/mL penicillin, 100 ug/mL streptomycin, 250 ng/mL amphotericin B, 10 μg/mL insulin, 5.5 μg/mL transferring, 5 ng/mL selenious acid, 10 nM hydrocortisone, 0.5 μg/mL BSA, 15 mM HEPES, 10 μM pyridoxal-5-phosphate and 1 pM gastrin-17 during 48 h, at 37 °C in a 95% O_2-5% CO_2 atmosphere.

For the histamine release experiments, the medium was aspirated and replaced with fresh serum-free medium (DMEM/F12 supplemented 2mM glutamine, 100 IU/mL
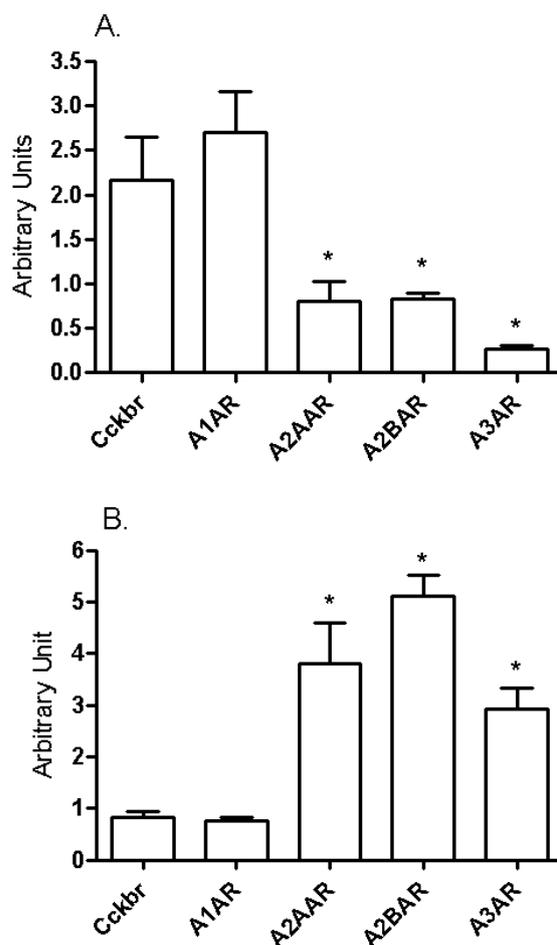

FIGURE 1. Gene expression in isolated ECL cells (A) and in whole stomach (B). Bars represent mean ± SEM, N=4; asterisks indicate statistically significant differences from CCKBR gene expression.

penicillin, 100 ug/mL streptomycin and 15 mM HEPES). Measurements of basal and gastrin (10^{-8} M, 30 min; Sigma-Aldrich, St Louis, MO, USA) stimulated histamine release were carried out in the supernatant of cells previously incubated with 2-chloroadenosine (0, 1 or 3 μM) alone or in combination with 1 μM dipropylcyclopentylxanthine (DPCPX) (Sigma-Aldrich) for 30 min using a commercial EIA kit (SPI Bio, Montigny le Bretonneux, France). Data are presented as means ± SEM (N=4).

Cell toxicity was estimated using the tetrazolium salt reduction test (MTT assay) in ECL cells after exposure to 2-chloroadenosine. Briefly, ECL cells were cultured with 2-chloroadenosine (30 min, 1 or 3 μM) or vehicle (0.1 % DMSO) at 37 °C and 5 % CO_2. The supernatant was discarded and MTT was added (5 mg/mL in PBS). Cells were incubated for 3 h at 37 °C and 5 % CO_2, after which 100 μL of 10 % sodium dodecyl sulfate in 0.01 M HCl were added to each well. Samples were then incubated for 18 h at 37 °C and absorbance was measured at 540 nm in a microplate reader (Multiscan MS; Labsystems, Joensuu, Finland). Data are presented as means ± SEM (N=3, each one in duplicate).

Multiple comparisons were made by analysis of variance followed by Dunnett's test (GraphPad Instat). A P value of less than 0.05 was considered significant.

Fig. 1A shows the quantitative expression of adenosine and gastrin receptor genes in isolated ECL cells. *A1AR* gene was expressed at the same level of gastrin receptor B gene *(CCKBR)*. *A2AAR, A2BAR* and *A3AR* genes were expressed at a significantly lower level. In whole stomach (Fig. 1B), the *A2AAR, A2BAR* and *A3AR* receptor genes were expressed at a higher level than *CCKBR* (p<0.05).

2-Chloroadenosine, a stable adenosine analogue, was able to inhibit both basal and gastrin-stimulated histamine release in ECL cells (Fig. 2A). This inhibitory action of 2-chloroadenosine was not due to a cytotoxic effect (MTT reduction levels, OD / 2.5 x 10^5 cell/mL, were 0.59, 0.57 and 0.53, for vehicle and 1 and 3 μM 2-chloradenosine, respectively). As expected, 2-chloroadenosine did not show an inhibitory effect on ECL cells previously exposed to DPCPX, a selective antagonist of adenosine A1 receptor (Fig. 2B).

The *A1AR* gene is expressed at a low level when whole stomach tissue was studied (Dixon *et al.,* 1996) However, our
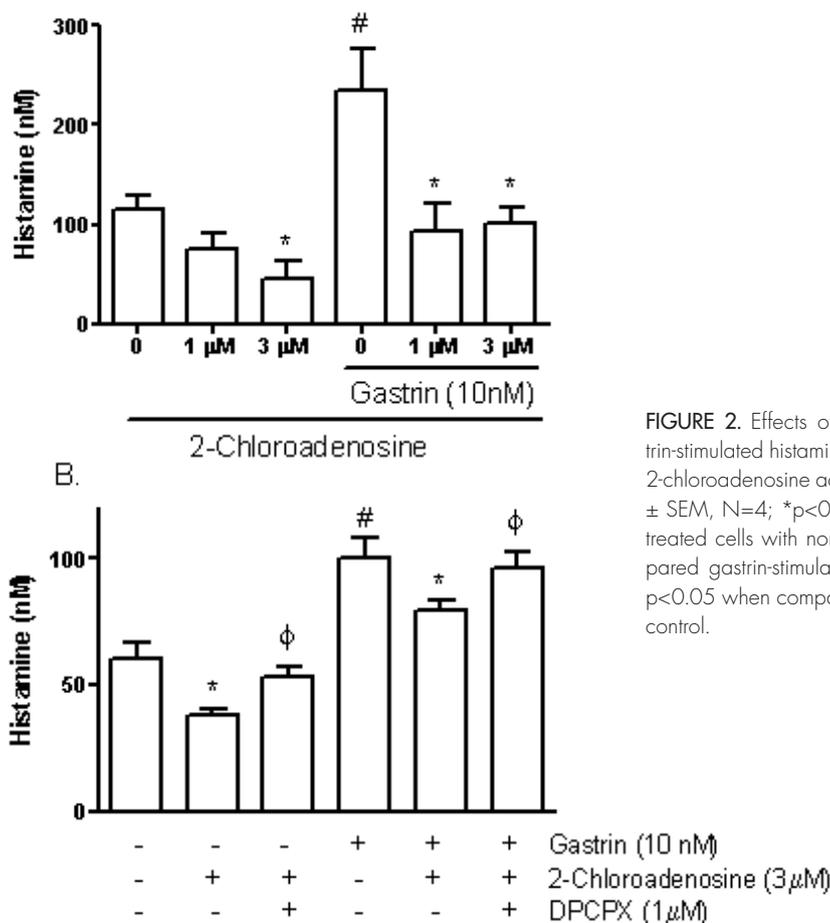

FIGURE 2. Effects of 2-chloroadenosine on basal and gastrin-stimulated histamine release (A) and effects of DPCPX upon 2-chloroadenosine action in ECL cells (B). Bars represent mean ± SEM, N=4; *p<0.05 when compared 2- chloroadenosine treated cells with non-treated controls. # p<0.05 when compared gastrin-stimulated cells with non-stimulated controls. φ p<0.05 when compared DPCPX treated cells with non-treated control.

current results showed that expression of the *A1AR* gene was greater in isolated ECL cells than in the whole stomach, and that this gene was expressed in isolated cells at a level comparable to that of *CCKBR*. Also, the higher expression of the *A1AR* gene seemed functionally related to inhibition of both basal and gastrin stimulated acid secretion by ECL cells.

Extracellular adenosine is increased during acute inflammation or limited oxygen availability (Eltzschig *et al.,* 2009) and its inhibitory action on gastric acid secretion (through a higher expression of the *A1AR* gene and the subsequent diminution of histamine release) may serve a gastroprotective function in those conditions. The inhibitory action of 2-chloroadenosine on basal and gastrin-stimulated histamine release in isolated ECL cells, suggests that the inhibitory action of adenosine on gastric acid secretion may be effected directly upon ECL cells. However, other adenosine actions may also be exerted on different cell types involved in gastric parietal cell control (Dixon, Gubitz, 1996, Yip and Kwok, 2004, Yip, Leung, 2004b).

Acknowledgements

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