Luminal adenosine stimulates chloride secretion through A₁ receptor in mouse jejunum

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ADENOSINE IS AN ENDOGENOUS nucleoside that can regulate a large number of physiological and pathophysiological processes (4, 9–16, 18, 24, 25, 31). Adenosine is generated by hydrolysis of intra- or extracellular adenine nucleotides (11). Extracellular adenosine can either be taken up by cells by processes (4, 9–16, 18, 24, 25, 31). Adenosine is generated by hydrolysis of intra- or extracellular adenine nucleotides (11).

Adenosine acts as a paracrine and/or autocrine mediator of chloride secretion in tracheal and intestinal epithelia (6, 37, 40). Intestinal secretion is driven by electrolytic chloride secretion by crypt cells; C1− ions then drive Na+ ions by electrical coupling, and water follows by osmotic coupling. The net amount of fluid secreted is substantially greater in the small intestine than in the colon, amounting to nearly 2 liters daily in humans because it has to buffer gastric, pancreatic, and biliary secretions within its lumen; yet it is balanced by the greater absorption capacity of the small intestine even in the absence of nutrients (15). In the colon, adenosine has been shown to regulate chloride secretion through occupancy of A2B receptors, which have been clearly demonstrated to occur on the basolateral membrane. This process may be relevant during inflammation, when extracellular adenosine production appears greatly increased (9, 24). However, much less is known about the receptors mediating chloride secretion when adenosine is applied apically (i.e., via the lumen), especially in the small intestine. We therefore decided to identify the adenosine receptor that is involved in the increase in secretion in response to luminal adenosine. To solve this problem, we elected to study jejunal chloride secretion in response to adenosine using mice genetically lacking a specific adenosine receptor subtype, i.e., either the A1 or the A2A receptor.

MATERIALS AND METHODS

Chemicals. Adenosine, phloridzin, indomethacin, forskolin, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Sigma (St. Louis, MO). The RNeasy kit was obtained from Qiagen (Hilden, Germany). The GeneAmp RNA PCR kit and the TaqMan Universal PCR master mix were purchased from Applied Biosystems (Applera, Stockholm, Sweden). Primers and probes were obtained from MWG-Biotech (Ebersberg, Germany). The rest of the chemicals were purchased from Sigma.

Mice. A1 and A2A knockout mice were generated as previously described (18, 25). Experiments were performed on adult male mice (2–3 mo old, 30–35 g body wt). Heterozygous A1 mice were of a mixed 129OlaHsd/C57Bl6 background. A2A heterozygous mice were backcrossed on a CD1 background for >20 generations before being intercrossed to generate the experimental wild-type and knockout mice. Mice were genotyped using PCR. Animals were maintained under controlled environmental conditions (12:12-h light-dark cycle, 21°C, 60% relative humidity, food and water ad libitum). The number of animals was kept to a minimum, and all efforts were made to avoid

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animal suffering. Experiments were carried out in strict accordance with both the Guide for the Care and Use of Laboratory Animals [DHENV Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DDR/NIH, Bethesda, MD 20205] and the European Community regulations for animal use in research (CEE no. 86/609). The Regional Ethics Committee for Animal Research approved all animal studies.

**Tissue isolation.** mRNA expression studies were performed using pooled tissue obtained by scraping from the jejunum of nine wild-type and nine A1 receptor knockout male mice. The mice were CO2 anesthetized before intestine removal.

**RNA extraction and cDNA synthesis.** RNA was isolated from the tissue using the reagents and protocols included in the Qiagen RNeasy kit. The tissue was placed in the Qiagen lysis buffer and homogenized by using a rotor-stator homogenizer at full speed for 2 min and then centrifuged for 3 min at 8,000 g. The supernatant was further homogenized by using a syringe and needle before RNA extraction with the Qiagen columns. RNA quantity and quality were determined spectrophotometrically at 260 and 280 nm with a Biophotometer (Eppendorf, Hamburg, Germany). The cDNA synthesis was performed with the GeneAmp RNA PCR kit using random hexamers and the MuLV reverse transcriptase.

**Real-time RT-PCR.** Semiquantitative real-time RT-PCR (17) was performed by using β-actin and subtype-specific (A1, A2A, A3B, and A3) adenosine receptor primers and probes synthesized according to sequences previously published (7) in an ABI Prism 7000 Sequence Detector System (Applied Biosystems). The real-time RT-PCR reactions were performed using the TaqMan Universal PCR master mix and probes labeled at the 5’-end with the reporter dye 6-carboxyfluorescein and at the 3’-end with the quencher dye 6-carboxytetramethyl-rhodamine. The passive reference dye ROX was included in the master mix to adjust automatically for background variability due to pipetting errors as well as differences in detection sensitivity across the plate. The four adenosine receptor (AR) subtypes and β-actin were analyzed in parallel using the same amount of cDNA (20 ng) for each reaction. The RT-PCR reactions were performed in triplicates. Values are expressed as the difference in the number of cycles to reach the detection threshold (Ct = cycle at threshold), using β-actin as reference (ΔΔCt = CΔT - CΔβ-actin).

**Measurements of short-circuit current.** Mice aged 2–5 mo were anesthetized with pentobarbital sodium, and the midportion of jejunum was dissected 2 cm after the ligament of Treitz. The jejunal mucosa was washed to remove all intestinal content using Krebs-bicarbonate-Ringer solution of the following composition (in mM) 140 Na+, 120 Cl−, 5.2 K+, 1.2 Mg2+, 1.2 Ca2+, 2.4 HPO4−2, 0.4 H2PO4−, 25 HCO3−, and 11.5 glucose. The jejunal sample was cut longitudinally along the mesenteric border, and under a dissecting microscope, the epithelium was stripped out of the subepithelial tissue, stretched, and sealed onto the basolateral side to a fixation ring with an opening diameter of 3 mm, using Histoacryl glue applied to the periphery of the plastic ring. The ring was mounted between the two halves of an Ussing chamber (exposed surface area: 0.8 mm2) the periphery of the plastic ring. The ring was mounted between the

**Impedance analysis.** The transepithelial resistance of jejunum is rather small (10–20 Ω·cm2), whereas the resistance of the bathing solution between the voltage electrodes in series with the epithelium is at least 40–50 Ω·cm2. Consequently, Isc will be underestimated and transepithelial resistances will be dominated by the solution of the bath. Therefore, we applied impedance analysis to discriminate between the resistance of the tissue and that of the bathing solution. These parameters were used to correct the Isc values. The electrical equivalent circuit of the cell membrane consists of a capacitor (lipid phase) in parallel with a resistor (ion conductance). The apical, as well as the basolateral, membranes can be represented by such a resistance-capacitance (RC) network. Figure 1A shows the equivalent circuit of the epithelium where Rb and Cb are the resistance and capacitance of the apical membrane, respectively. The basolateral membrane is represented by Rs and Cs. Both membranes, and thus the RC networks, are arranged in series and shunted by a conductive pathway residing in the paracellular pathway (41). Because the paracellular pathway of an epithelium-like small intestine has a very high conductance, the two-membrane model can be represented by a single lumped RC circuit, consisting of the transepithelial resistance (Rf) in parallel with the transepithelial capacitance (Cf) (Fig. 1B). The resistance of the solution between the voltage electrodes (Rs) resides in series with the epithelial RC model. Due to the heterogeneity of the preparation, the presence of the lateral inter-space and the dielectric dispersion in the lipid membranes the electrical impedance [Z(f)] is a complex function that can be approximated by the Cole-Cole equation

\[
Z(f) = R_s + \frac{R_f}{1 + (j2\pi f R_f C_f)^\alpha}
\]

where j represents the imaginary unit and f the frequency. The number α (<1) describes the nonideal RC behavior of the epithelium. The representation in a Nyquist plot of the impedance function of the ideal...
acin (100 mM) chloride secretion, an inhibitor of prostaglandin synthesis, indomethacin may induce a release of prostaglandins that could themselves increase Na-glucose cotransporter. Because the stripping of jejunal mucosa bathing solution to abolish the current related to the operation of the current component only, 1 mM phloridzin was added to the apical records were averaged.

The amplitudes of the current (I1) and voltage (V1) sine waves by regression analysis and calculated RT5 were as V1/I1. This procedure enabled us to update the RT5 values every 7 s. Simultaneously, with the second DSP board, the CT and Rs were determined by applying five high-frequency sine waves in the range of 2 to 16 kHz. From the current and voltage response to the high-frequency sine waves, we calculated the impedance of the epithelium and bathing solution arranged in series. At high frequencies, the impedance of the equivalent circuit in Fig. 1 equals the Rs. Therefore, Rs was calculated by extrapolation of the real part of impedance data to infinite frequency. RT was calculated by subtracting Rs from the RT5 values obtained with the 1-Hz sine wave. Low- and high-pass filters were used to avoid interference between the high- and low-frequency sine waves used for the Rs and RT5 measurements, respectively. Ise values were corrected by dividing the recorded values by Rf/RT5. Data obtained from four records were averaged.

Chloride secretory response to adenosine. To record the chloride current component only, 1 mM phloridzin was added to the apical bathing solution to abolish the current related to the operation of the Na-glucose cotransporter. Because the stripping of jejunal mucosa may induce a release of prostaglandins that could themselves increase chloride secretion, an inhibitor of prostaglandin synthesis, indomethacin (100 μM), was added to both bathing solutions. Thus before testing of the chloride secretory response to adenosine, all tissues were first exposed to phloridzin and to indomethacin. Adenosine and the A1 receptor antagonist DPCPX (100 μM), were added to the apical or basolateral solution, depending on the experimental protocol. Forskolin (10 μM) was always added to the basolateral bathing solution. The increase in SCC (expressed in μA/cm²) was calculated as the difference between the basal current and the peak current obtained within 15 min of adenosine or forskolin addition.

Statistics. Data are expressed as means ± SE. Statistical analysis was performed by using Student’s unpaired t-test.

RESULTS

Expression of adenosine receptors in mouse jejunum. The expression of adenosine receptors in the jejunum was assessed by semiquantitative real-time PCR analysis. In jejunum of wild-type mice, the abundance of adenosine receptor mRNA was characterized by the following rank order: A2B > A2A = A3 > A1. The A1 knockout jeuna expressed levels of A2A, A2B, and A3 similar to those found in wild-type tissue (Fig. 2), but, as expected, no A1 mRNA. The difference between A2B and A2A mRNA is approximately sevenfold. The difference between A2B and A1 mRNA is ~34-fold.

Role of adenosine A1 receptors. The addition of adenosine to the apical bathing solution increased Ise in jejun derived from control mice, although rather high concentrations were required (Fig. 3). That chloride secretion fully accounts for Ise in the presence of phloridzin was attested by its fall when both solutions were made chloride free by isosmotic replacement by gluconate (Fig. 4). The adenosine-mediated response was not seen in jeuna from mice lacking A1 receptors (Fig. 3, Table 1). This increase in Ise was therefore ascribed to occupancy of apical A1 receptors. This was further supported by the fact that the A1 receptor antagonist DPCPX abolished the stimulation of chloride secretion. Forskolin elicited a substantially larger response than adenosine, but the pattern was similar in jeuna from both groups of mice (Fig. 3). There was no statistically significant difference in adenosine-induced Ise between the two groups (Table 1). Furthermore, the response to forskolin was similar regardless of the previous addition of adenosine (data not shown), implying no synergistic effect. On the other hand, the addition of adenosine to the basolateral side of the jejunum increased the Ise response similarly in both groups of mice (control and A1 receptor knockout), thus eliminating a role for A1 receptor in this latter secretory response (Table 1).

Role of adenosine A2A receptors. Separate addition of adenosine to either the apical or basolateral side of jejuna from controls and from mice lacking the A2A receptor, induced identical increase in Ise (Table 2). Thus we find no role for the A2A receptor in this secretory response. The response to forskolin was always large and identical in both groups, in keeping with the important role of the cystic fibrosis transmembrane conductance regulator in jejunal crypts.

DISCUSSION

We have confirmed and extended previous reports on the distribution of adenosine receptors in the intestine (6). We show that mRNA for all four adenosine receptors is present in jejunum mucosa. The most abundant was A2B receptor mRNA, which is compatible with previous reports suggesting that A2B receptors mediate changes in gut epithelial chloride conductance (40). However, we have no direct proof that receptor protein of A2A, A2B, or A3 is present in mouse jejunum, but this is likely because A2B receptors are present on practically all cells, and A2A and A3 are present on vascular endothelium and/or cells of the immune system (12–16, 24), both of which are obviously present in jejunum.

Extracellular occurrence of adenosine (either by generation or release) is encountered in several ischemic or stressed conditions and mediates protective effects in heart, brain, kidney, skeletal muscle, and adipose tissue. Because of these
protective properties, adenosine is sometimes called a “retaliatory metabolite” (16, 31). Although this has not been extensively studied in the intestine, there is evidence that adenosine is biologically important there also. In ischemic and inflammatory conditions, polymorphonuclear leukocytes can migrate across the intestinal mucosa (32) and release 5’ adenosine monophosphate (26) that is degraded into adenosine at the apical pole by an ecto-5’-nucleotidase (often referred to as CD73), anchored within the luminal membrane by a glycosphosphoinositol linkage (39). This enzyme is also upregulated during hypoxia and ischemia (38). The secretory response to adenosine can also be considered beneficial because it will flush the mucosa and eliminate pathogens (hence preventing colonization). Strohmeier et al. (40) have characterized the chloride secretory response to adenosine added to either side in the mouse jejunum, the A1 receptor mRNA appeared to be least expressed of all subforms. If A1 receptors are predominately expressed in crypt cells, this would explain the low abundance. Indeed, our preliminary evidence is compatible with such a distribution (Lo¨vdahl C, Dare ´ E, and Beauwens R, unpublished data). As expected, the expression of A1 receptor mRNA was completely lost in the A1 receptor null mice. None of the other receptor mRNAs were affected, however. This tallies with previous data indicating that adenosine receptors do not alter the expression of each other to any appreciable extent (14). The luminal response to adenosine was absent in jejuna

Table 1. Effect of adenosine on chloride secretion by jejunal mucosa from A1 receptor knockout (A1−/−) or control (A1+/+) mice

<table>
<thead>
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<th>Series 1 experiments</th>
<th>A1+/+</th>
<th>A1−/−</th>
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<tr>
<td>Adenosine 100 µM ap</td>
<td>7±2</td>
<td>1±1*</td>
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<tr>
<td>Adenosine 1 mM ap</td>
<td>16±5</td>
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<tr>
<td>Forskolin</td>
<td>39±12</td>
<td>38±13</td>
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<th>Series 2 experiments</th>
<th>A1+/+</th>
<th>A1−/−</th>
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<tr>
<td>Adenosine 1 mM + DPCPX</td>
<td>1±1</td>
<td>1±1</td>
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<th>Series 3 experiments</th>
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<th>A1−/−</th>
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<tr>
<td>Adenosine 100 µM bl</td>
<td>16±4</td>
<td>14±3</td>
</tr>
<tr>
<td>Adenosine 1 mM bl</td>
<td>26±7</td>
<td>26±5</td>
</tr>
<tr>
<td>Forskolin</td>
<td>41±13</td>
<td>29±11</td>
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</table>

Three different series of experiments are shown. In the first set of experiments adenosine was added apically (ap) and sequentially at 100 µM and 1 mM followed by addition of forskolin on the basolateral side (10 µM), and the increase in short-circuit current (Isc) values (µA/cm²; means ± SE) are given for both groups of mice. In a second set of experiments, response to apical adenosine (1 mM) was completely abolished in the presence of the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 nM). Finally, in the third set of experiments, the response to basolateral (bl) adenosine (100 µM and 1 mM sequentially) was tested. For each group of experiments, n = 5. *P < 0.05.

T84 colonic epithelia. By RT-PCR, only adenosine A2B receptor was found to be significantly expressed, implying that it mediates all the effects of adenosine on chloride secretion at both sides of the epithelium (4). This conclusion may hold for human or mouse colon, but certainly cannot be extended to small intestine, because we definitely show that A1 receptors are important there.

In the mouse jejunum, the A1 receptor mRNA appeared to be least expressed of all subforms. If A1 receptors are predominately expressed in crypt cells, this would explain the low abundance. Indeed, our preliminary evidence is compatible with such a distribution (Lo¨vdahl C, Dare ´ E, and Beauwens R, unpublished data). As expected, the expression of A1 receptor mRNA was completely lost in the A1 receptor null mice. None of the other receptor mRNAs were affected, however. This tallies with previous data indicating that adenosine receptors do not alter the expression of each other to any appreciable extent (14). The luminal response to adenosine was absent in jejuna

Table 2. Effect of adenosine on chloride secretion by jejunal mucosa from A2A receptor knockout (A2A−/−) or control (A2A+/+) mice

<table>
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<th>Series 1 experiments</th>
<th>A2A+/+</th>
<th>A2A−/−</th>
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<tr>
<td>Adenosine 100 µM (ap)</td>
<td>11±2</td>
<td>12±2</td>
</tr>
<tr>
<td>Adenosine 1 mM (ap)</td>
<td>16±5</td>
<td>7±1</td>
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<tr>
<td>Forskolin</td>
<td>35±10</td>
<td>30±6</td>
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<th>Series 2 experiments</th>
<th>A2A+/+</th>
<th>A2A−/−</th>
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<td>Adenosine 100 µM (bl)</td>
<td>7±4</td>
<td>10±8</td>
</tr>
<tr>
<td>Adenosine 1 mM (bl)</td>
<td>21±7</td>
<td>19±4</td>
</tr>
<tr>
<td>Forskolin</td>
<td>30±5</td>
<td>29±6</td>
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Data are presented as in Table 1. In the first set of experiments, adenosine was added to the apical solution, whereas in the second set, it was added to the basolateral solution. There is no statistical difference in the chloride secretory response induced by adenosine in jejunal mucosa from A2A receptor knockout (A2A−/−) vs. that of control (A2A+/+) mice (for 1 mM adenosine, P = 0.123). For each group of experiments, n = 5.

Fig. 3. Effect of apical addition of adenosine (ado) on short-circuit current (Isc) across jejunal mucosa from either control (A1+/+) (A) or A1 receptor knockout (A1−/−; B) mice. Adenosine was sequentially increased from 10 to 1,000 µM. Representative experiment of n = 5. Phlor, phloridzin; indo, indomethacin; fsk, forskolin.

Fig. 4. Effect of removal of chloride on Isc across jejunal mucosa from control mice. Chloride was isosmotically replaced by gluconate (Cl−).
from A1 receptor knockout mice. Furthermore, the luminal response to adenosine observed in jejuna from control mice was also abolished by the specific A1 receptor antagonist DPCPX in agreement with previous findings (8, 27, 28, 34). The intracellular signaling pathway leading to increase in chloride secretion from A1 receptor occupancy is clearly unknown at the present time. In the jejuna, the CFTTR appears as the only chloride channel present in the apical membrane (21, 23), whereas in colonic cells, calcium-activated chloride channels also appear to play a role in chloride secretion (3). The CFTTR can be activated by mechanisms other than an increase in cAMP (21). In particular, it has been shown in several systems that increased intracellular calcium leads to increased trafficking and insertion of CFTTR molecules into the membrane (1, 5, 10). In the jejuna, such a mechanism may explain the stimulation of chloride secretion by apical ATP and UTP that occurs via activation of the Gq-coupled P2Y4 receptor (33) and that is abolished in CFTTR−/− mice (22). The A1-mediated chloride current induced by luminal adenosine might involve a similar mechanism because in airway epithelial cells, A1 agonists activate chloride secretion via mobilization of intracellular calcium (35). Other mechanisms also deserve consideration in light of the recent elegant studies of the group of Barrett and colleagues (2, 19), who established that transactivation of the EGF receptor (EGFR) is a new mechanism controlling intestinal chloride secretion with downstream effectors involving either phosphatidylinositol 3-kinase (PI3-kinase) or MAPK. For instance, the full chloride secretory response to VIP in T84 monolayers involves activation of PI3-kinase through EGF transactivation by a typical Gs-coupled receptor (2). On the other hand, transactivation of EGFR via Gq-coupled receptors would decrease chloride secretion in T84 epithelial cells as a result of activation of MAPK (19). However, the coupling to either downstream effector may not be so straightforward as the occupancy of Gq-coupled receptor (e.g., acetylcholine and adenosine A1 receptor) in rabbit hearts leads to PI3-kinase activation through EGFR transactivation (20). Finally, the coupling of adenosine receptors to MAPK has been established (36), but its potential role in modulating chloride secretion remains to be examined.

The second part of our study established that A2A adenosine receptors do not appear to play any significant role in chloride secretion, at least in mice, because Isc increased to a similar extent in jejuna from control and A2A receptor knockout mice. This does not rule out that A2A receptors could play a role in ion transport in vivo, because these receptors do influence blood flow.

The basolateral adenosine receptor controlling chloride secretion could not be identified in the present study but is likely of the A2B type (40). Coexistence of different subtypes of adenosine receptors within the same tissue is known to occur in several cells of the human jejuna, including mucosa, myenteric neuron, and muscle layers (6), as well as in guinea pig small intestine muscle cells (29, 30). Thus A2B adenosine receptor null mice are now required not only to establish the role of A2BR in the response to basolateral adenosine but also to completely rule out any participation of A2BR in the response to apical adenosine.

In conclusion, generation of adenosine into the lumen of the jejuna induces a chloride secretory response that is mediated by A1 adenosine receptor.

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