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 lacking the adenosine receptors of A1 (A1R) and A(2A) (A2AR) or control littermates. The jejunal epithelium was mounted in a Ussing chamber, and a new method on the basis of impedance analysis was used to calculate the short-circuit current (Isc) values. Chloride secretion was assessed by the Ios after inhibition of the sodium-glucose cotransporter by adding phlorizin to the apical bathing solution. The effect of apical adenosine on chloride secretion was lost in jejunum from mice lacking the A1R. There was no difference in the response to basolaterally applied adenosine or to apical forskolin. Furthermore, in jejunum from control mice, the effect of apical adenosine was also abolished in the presence of 8-cyclopentyl-1,3-dipropylxanthine, a specific A1R antagonist. Responses to adenosine were identical in jejuna from control and A1R knockout mice. This study demonstrates that A1R (and not A2AR) mediates the enhancement of chloride secretion induced by luminal adenosine in mice jejenum.

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 electrogenic chloride secretion by crypt cells; Cl− 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 A2AR 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 A2AR 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. A2AR 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
animal suffering. Experiments were carried out in strict accordance with both the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85–23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and the European Community regulations for animal use in research (CEC 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, A2B 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 by using the TaqMan Universal PCR master mix and probes labeled at the 5’-end with the reporter dye 6-carboxy-fluorescein and at the 3’-end with the quencher dye 6-carboxytetra-methyl-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) are expressed as the difference in the number of cycles to reach the reaction. The RT-PCR reactions were performed in triplicates. Values are analyzed in parallel using the same amount of cDNA (20 ng) for each 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) are expressed as the difference in the number of cycles to reach the reaction.

**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 Krebsbicarbonate-Ringer solution of the following composition (in mM) 140 Na+<sub>1</sub>, 120 Cl<sub>1</sub>, 5.2 K<sup>+</sup>·, 1.2 Mg<sup>2+</sup>, 1.2 Ca<sup>2+</sup>, 2.4 HPO<sub>4</sub><sup>2-</sup>, 0.4 H<sub>2</sub>PO<sub>4</sub>, 25 HCO<sub>3</sub>, and 11.5 glucose. The jejunal was opened longitudinally along the mesenteric border, and under a dissecting stereomicroscope, 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 mm<sup>2</sup>) with an opening diameter of 3 mm, using Histoacryl glue applied to longitudinally along the mesenteric border, and under a dissecting detection threshold (Ct)

The transepithelial resistance of jejunum is 86/609). The Regional Ethics Committee for Animal Research approved all animal studies. 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 $I_w$ 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 $R_b$ and $C_b$ are the resistance and capacitance of the apical membrane, respectively. The basolateral membrane is represented by $R_b$ and $C_b$. 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 ($R_T$) in parallel with the transepithelial capacitance ($C_T$) (Fig. 1B). The resistance of the solution between the voltage electrodes ($R_s$) resides in series with the epithelial RC model. Due to the heterogeneity of the preparation, the presence of the lateral interspace 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

$$Z(f) = R_s + \frac{R_T}{1 + (j2\pi f R_T C_T)^a}$$

where $j$ represents the imaginary unit and $f$ the frequency. The number $\alpha (<1)$ describes the nonideal RC behavior of the epithelium. The representation in a Nyquist plot of the impedance function of the ideal

Fig. 1. Equivalent circuit of the jejunum. A: two-membrane model of the epithelium. Electrical properties of the apical and basolateral membranes are represented by a resistance ($R$) in parallel with a capacitance ($C$). Apical membrane: $R_a$ and $C_a$, basolateral membrane: $R_b$ and $C_b$. B: lumped model of the transepithelium ($R_T$ and $C_T$) and series resistance ($R_s = R_{sol} + R_{sub}$), where $R_{sol}$ represents the resistance of the solution in between the voltage electrodes, and $R_{sub}$ is the subepithelial resistance. C: impedance function calculated $R_T = 20 \Omega$, $R_s = 40 \Omega$, and $C_T = 4 \mu F$ and $\alpha = 0.95$. The impedance is represented in a Nyquist plot. $R$ represents the real or resistive part of the impedance. $X$ is the imaginary part or reactance. Due to the distribution of the capacitance in the lateral interspace and dielectric dispersion, the center of the semicircle is depressed.
testing of the chloride secretory response to adenosine, all tissues were
acin (100
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
the amplitudes of the current (I_T) and voltage (V_T) sine waves by
regression analysis and calculated R_T as V_T/I_T. This procedure enabled us to update the R_T values every 7 s. Simultaneously, with
the second DSP board, the C_T and R_T 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 equiv-
ent circuit in Fig. 1 equals the R_T. Therefore, R_T was calculated by
extrapolation of the real part of impedance data to infinite frequency.
R_T was calculated by subtracting R_T from the R_T 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 R_T and R_T measurements, respectively. I_sc values were corrected by
dividing the recorded values by R_T/R_T. 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, indometh-
acin (100 \mu M), 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 nM), were added to the apical or
basolateral solution, depending on the experimental protocol. Forsko-
ilin (10 \mu M) was always added to the basolateral bathing solution. The
increase in SCC (expressed in \mu A/cm^2) was calculated as the differ-
ence between the basal current and the peak current obtained within
15 min of adenosine or forskolin addition.

Statistics. Data are expressed as means \pm 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 jejuna 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 \sim 34-fold.

Role of adenosine A1 receptors. The addition of adenosine to
the apical bathing solution increased I_sc in jejunum derived from
control mice, although rather high concentrations were
required (Fig. 3). That chloride secretion fully accounts for I_sc 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 jejunum from mice lacking A1 receptors (Fig. 3, Table 1).
This increase in I_sc 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 jejunum
from both groups of mice (Fig. 3). There was no statistically
significant difference in adenosine-induced I_sc 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 I_sc 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 jejunum from
controls and from mice lacking the A2A receptor, induced
identical increase in I_sc (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
jejunal mucosa. The most abundant was A2B receptor mRNA,
which is compatible with previous reports suggesting that A2B
receptors mediate changes in gut epithelial chloride conduc-
tance (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 various 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 glycosylphosphoinositol 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 more abundantly expressed in crypt cells, this would explain the low expression of each other to any appreciable extent (14). The luminal response to adenosine was absent in jejuna and colon (49). 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 less expressed of all subforms. If A1 receptors are predominantly expressed in crypt cells, this would explain the low abundance. Indeed, our preliminary evidence is compatible with such a distribution (Lövdahl C, Daré 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 and colon (49).

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

<table>
<thead>
<tr>
<th>Series</th>
<th>A1+/+ (μM)</th>
<th>A1−/− (μM)</th>
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<tbody>
<tr>
<td>Series 1 experiments</td>
<td>Adenosine 100</td>
<td>7 ± 2</td>
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<tr>
<td></td>
<td>Adenosine 1 mM</td>
<td>16 ± 5</td>
</tr>
<tr>
<td></td>
<td>Forskolin</td>
<td>39 ± 12</td>
</tr>
<tr>
<td>Series 2 experiments</td>
<td>Adenosine 1 mM + DPCPX</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Series 3 experiments</td>
<td>Adenosine 100 μM</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>Adenosine 1 mM</td>
<td>26 ± 7</td>
</tr>
<tr>
<td></td>
<td>Forskolin</td>
<td>41 ± 13</td>
</tr>
</tbody>
</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 (in 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.

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

<table>
<thead>
<tr>
<th>Series</th>
<th>A2A+/+</th>
<th>A2A−/−</th>
</tr>
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<tbody>
<tr>
<td>Series 1 experiments</td>
<td>Adenosine 100 μM (ap)</td>
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<tr>
<td></td>
<td>Adenosine 1 mM (ap)</td>
<td>16 ± 5</td>
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<td></td>
<td>Forskolin</td>
<td>35 ± 10</td>
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<tr>
<td>Series 2 experiments</td>
<td>Adenosine 100 μM (bl)</td>
<td>7 ± 4</td>
</tr>
<tr>
<td></td>
<td>Adenosine 1 mM (bl)</td>
<td>21 ± 7</td>
</tr>
<tr>
<td></td>
<td>Forskolin</td>
<td>30 ± 5</td>
</tr>
</tbody>
</table>

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.

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Fig. 3. Effect of apical addition of adenosine (ado) on short-circuit current (Isc) across jejunal mucosa from 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 jejunum 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 jejunum, the CFTR 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 CFTR 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 CFTR molecules into the membrane (1, 5, 10). In the jejunum, such a mechanism may explain the stimulation of chloride secretion by apical ATP and UTP that occurs via activation of the Gₛ-coupled P2Y₄ receptor (33) and that is abolished in CFTR⁻/⁻ 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 stream effectors involving either phosphatidylinositol 3-kinase that transactivation of the EGF receptor (EGFR) is a new deserve consideration in light of the recent elegant studies of 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 A₂α adenosine receptors do not appear to play any significant role in chloride secretion, at least in mice, because Iₑ increased to a similar extent in jejunum from control and A₂α receptor knockout mice. This does not rule out that A₂α 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 jejunum, 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 A₂B in the response to basolateral adenosine but also to completely rule out any participation of A₂B in the response to apical adenosine.

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

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