Activation of neuronal adenosine A_1 receptors suppresses secretory reflexes in the guinea pig colon

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Cooke, Helen J., Y.-Z. Wang, C. Y. Liu, H. Zhang, and F. L. Christofi. Activation of neuronal adenosine A_1 receptors suppresses secretory reflexes in the guinea pig colon. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G451–G462, 1999.—The role of adenosine A_1 receptors (A_1R) in reflex-evoked short-circuit current (I_sc) indicative of chloride secretion was studied in the guinea pig colon. The A_1R antagonist 8-cyclopentyltheophylline (CPT) enhanced reflex-evoked I_sc. Adenosine deaminase and the nucleoside transport inhibitor S-(4-nitrobenzyl)-6-thioinosine enhanced and reduced reflex-induced I_sc, respectively. The A_1R agonist 2-chloro-N\((\text{4-nitrobenzyl})\)cyclopentyladenosine (CCPA) inhibited reflex-evoked I_sc at nanomolar concentrations, and its action was antagonized by CPT. In the presence of either N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide to block the 5-hydroxytryptamine (5-HT)-mediated pathway or piroxicam to block the prostaglandin-mediated pathway, CCPA reduced the residual reflex-evoked I_sc. CCPA reduced the response to a 5-HT pulse without affecting the tetrodotoxin-insensitive I_sc responses to carbachol or forskolin. Immunoreactivity for A_1R was detected in the membrane (10% of neurons) and cytoplasm (90% of neurons) of neural protein gene product 9.5-immunoreactive submucosal neurons, in glia, and in the muscularis mucosa. A_1R immunoreactivity in a majority of neurons remained elevated in the cysteine despite preincubation with adenosine deaminase or CPT. A_1R immunoreactivity colocalized in synaptophysin-immunoreactive presynaptic varicosities of enteric ganglia, and, in these neurons, adenosine activates A_1R on submucosal neurons acts as a physiological brake to suppress reflex-evoked I_sc indicative of chloride secretion.

5-hydroxytryptamine; prostaglandin; submucous plexus; chloride secretion

ADENOSINE IS A UBQUITOUS molecule that has diverse actions in cardiopulmonary, renal, and gastrointestinal systems. Adenosine, a key metabolite of ATP, is distributed throughout the intracellular and extracellular compartments. Its concentration is dependent on processes related to production, release, reuptake, and metabolism (23). Extracellular levels of adenosine can rise as a consequence of increased intracellular hydrolysis of adenine nucleotides and its subsequent transport to the extracellular space or by increased ATP release into the extracellular compartment and its subsequent hydrolysis. Adenosine exerts its actions by binding to cell surface receptors belonging to the \(P_1\) purinoceptor family of receptors which includes A_1, A_2A, A_2B, and A_3 receptors cloned from human or animal sources (25). Highly specific affinity-purified anti-A_1, A_2A, A_2B, and A_3 receptor antibodies to peptide sequences of each receptor have been produced for immunohistochemistry (13, 30, 44). Because of substantial interspecies sequence homology among these receptors, the antibodies cross-react with receptors from several different species.

Adenosine A_1 receptors (A_1R) have been identified on isolated myenteric varicosities by ligand binding techniques (6, 7). Biochemical studies indicate that A_1R activation leads to inhibition of acetylcholine and tachykinin release from presynaptic varicoses of enteric ganglia (32). Furthermore, activation of this receptor on myenteric neurons leads to suppression of cholinergic and tachykinergic transmission to longitudinal muscle (6, 9). Electrophysiological studies provide evidence for both pre- and postsynaptic A_1R inhibition of slow synaptic transmission [slow excitatory postsynaptic potentials (EPSP)] (10, 11) and presynaptic inhibition of fast synaptic transmission (fast EPSP) (11, 12). In AH/type 2 neurons, the main postsynaptic action of adenosine is A_1R-mediated suppression of neuronal excitability, associated with a decrease in cell input resistance and a sustained membrane hyperpolarization lasting seconds to minutes (5, 12). In addition, adenosinergic suppression of the predominant slow excitatory synaptic inputs to myenteric neurons often reveals a robust slow inhibitory postsynaptic potential in AH/type 2 neurons (11). In contrast to findings in AH/type 2 neurons, A_1R are not present in a significant proportion of cell somas of S/type 1 neurons in enteric ganglia, and, in these neurons, adenosine elevates excitability and causes a slow EPSP-like effect by activating another receptor subtype (2). In submucosal neurons of the guinea pig small intestine, adenosine also acts at postsynaptic A_1 sites to inhibit voltage-activated calcium currents and release of acetylcholine, a transmitter involved in nicotinic fast excitatory transmission (1, 2).

A_1R belong to the family of G protein-coupled receptors. Thus far, two types of G protein-coupled receptors, namely, opioid and tachykinin receptors, have been shown to undergo receptor internalization into enteric neurons after being activated by agonists (24, 35, 40, 42). Within 30–60 min, these receptors are recycled to the membrane of enteric neurons. Because endogenous adenosine is continuously released and provides an ongoing inhibitory tone on neuronal excitability, neurotransmitter release, and synaptic transmission in the myenteric plexus (11, 32), it seems likely that a significant proportion of A_1R should be internalized at all times, if indeed A_1R undergo internalization and recycling.

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Because activation of A₁R on enteric neurons in some regions of the bowel is linked to inhibition of both neurotransmitter release and neuroeffector transmission, we investigated the role of neural A₁R in the submucous plexus in the regulation of 5-HT- and prostaglandin-mediated chloride secretory reflexes in the colon. Chloride secretion by colonic epithelial cells is modulated by submucosal secretomotor neurons that release the neurotransmitters vasoactive intestinal polypeptide (VIP) and acetylcholine (Fig. 1) (15, 38, 39). VIP and acetylcholine bind to VIP and muscarinic M₃ receptors on epithelial crypt cells to elevate intracellular cAMP and intracellular Ca²⁺ levels leading to chloride secretion. Synaptic input to these secretomotor neurons can be initiated by mechanical stimulation or mucosal stroking that releases 5-hydroxytryptamine (5-HT) from enterochromaffin cells and prostaglandins from an unknown cell type (26, 39). In one pathway (Fig. 1B), 5-HT activates 5-HT₁₃ receptors on submucosal primary afferents containing substance P, acetylcholine, and glutamate (15, 26, 29). The presence of NK₁ receptors on cholinergic secretomotor neurons in the ileum suggests that these neurons receive synaptic input directly from primary afferents via release of substance P (33). Activation of primary afferents also triggers VIP secretomotor neurons, but it is uncertain whether this occurs directly or indirectly via interneurons (38, 39). Prostaglandins also appear to activate submucous neurons independent of the 5-HT-activated pathway (Fig. 1A) (16, 17, 20). VIP secretomotor neurons in the ileum and cholinergic neurons in the colon are reported to be targets of prostaglandins (16, 17, 20). Understanding how these reflex circuits regulate chloride secretion is important because chloride secretion provides an essential driving force for sodium movement as well as fluid accumulation necessary for lubrication or for flushing the intestinal contents during host defense against microbial invasion.

The physiological role of adenosine in the modulation of these reflexes was explored with treatments that either limit or increase the availability of endogenous adenosine at A₁R or with exogenous application of agonists or antagonists. These treatments were also used to test whether activation of A₁R by endogenous adenosine undergoes internalization and recycling to the membrane like other G protein-coupled receptors. Laser confocal microscopy and immunofluorescent labeling for A₁R and neuronal protein gene product 9.5 (PGP 9.5), S-100 (glial), or synaptophysin (presynaptic varicosities which surround the cell soma) were used to identify the distribution of A₁R that may be involved in the reflexes. The results indicate that endogenous adenosine binding to A₁R on submucous neurons acts as a physiological brake to suppress short-circuit current (Iₛ𝑐) indicative of chloride secretion through the 5-HT- and prostaglandin-activated neural reflex pathways in the colon. The kinetics of internalization/recycling of A₁R appear to behave differently from other G protein-coupled receptors in enteric ganglia.

**METHODS**

Tissues for reflex studies. Male albino Hartley guinea pigs (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250–600 g were allowed food and water ad libitum. Animals were stunned and exsanguinated, a method approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. A 10- to 15-cm segment of the colon 5 cm proximal to the anus was removed, flushed with cold Krebs-Ringer solution, and cut along the mesenteric border. The longitudinal and circular muscle layers with the myenteric plexus were removed by blunt dissection to give sheets of submucosa-mucosa containing intact submucosal ganglia. All solutions were gassed with 95% O₂-5% CO₂ mixture and buffered at pH 7.2–7.4.

Experimental design. Conventional or modified flux chambers were used. The flux chambers were equipped with Krebs-Ringer-agar bridges connected to colalom half cells for measurement of transmural potential difference (PD) and aluminum foil electrodes for passing Iₛегодня from a voltage-clamp apparatus. Solution resistance between the PD-sensing bridges was compensated. The current necessary to change the transepithelial PD by 8 mV was used to monitor tissue conductance, a measure of tissue viability, and was calculated from Ohm’s law.

To determine whether drugs had effects on basal transport, or whether they affected calcium- or cAMP-mediated secretion, conventional flux chambers were used. Drugs were added to the mucosal or serosal compartment (10 ml), and Iₛегодня, a measure of active ion transport, was monitored by a voltage-clamp apparatus (VCC600, Physiologic Instruments, Houston, TX).

For studies of the effects of drugs on neural reflex-evoked secretion, muscle-stripped colonic segments were mounted in modified Ussing flux chambers with the mucosal side oriented upward (38). Both mucosal and serosal compartments (1.5 ml) were continuously perfused at a rate of 1.6 ml/min with Krebs-Ringer solution warmed to 37°C by a heat exchanger. This flow rate allowed a rapid washout of drugs from the mucosal compartment. For experiments designed to
evoked neural reflexes, a 2-mm-wide brush attached to a
micromanipulator was lowered to the mucosal surface. Stroked
was applied with a forward or backward motion for 1 s. After
two to three strokes at 5-min intervals, drugs were perfused
in the serosal or mucosal bath, and, 30–60 min later, a second stroke was applied to assess the effects of the drugs.

Mucosal 5-HT pulse. A pulse of 5-HT into the mucosal
surface was previously shown to activate 5-HT1B receptors on
submucosal primary afferent neurons and to activate the
reflex without the complication of using a physical stimulus
such as stroking that could release mediators from other
sources (16, 39). A 15-µl pulse of 100 µM 5-HT into the
mucosal bath (1.5 ml) was applied from a pipette held in a
micromanipulator. The pipette tip was positioned at a fixed
distance (2–3 mm) from the epithelial surface. An initial
5-HT pulse was initiated followed 30–60 min later by a second pulse.

Immunofluorescent labeling of A1R and imaging with laser
scan confocal microscopy. Guinea pigs were stunned and
exsanguinated as previously described (11). Segments of
colon were removed, placed in an ice-cold Krebs solution with
2 µM nicardipine, and bubbled with a mixture of 95% O2–5% CO2.
The tissue was opened and pinned flat with the luminal
side up on the Sylgard base of a culture dish. Fine microdissec-
tion was performed to remove mucosa, circular muscle, myenteric
plexus and longitudinal muscle layers, leaving the
submucous layer intact. In the initial experiments, the tissue
was fixed for 5 h at 4°C with either a modified Zamboni’s
fixative (2% paraformaldehyde plus 0.2% picric acid) or 0.5%
paraformaldehyde and then processed for immunofluorescent
labeling. Because 0.5% paraformaldehyde was not suitable
for colabeling studies with anti-PGP 9.5 or anti-S-100 protein
antibodies, the modified Zamboni’s fixative was used in most
experiments.

Colabeling experiments were conducted using antibodies
against PGP 9.5 that label neurons, synaptophysin that
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glandular cells. After fixation, the tissue was washed sequentially
with dimethyl sulfoxide and PBS, treated with goat
serum (1:10 dilution) for 30 min at room temperature, and then
incubated with antibodies overnight at 4°C. The primary
antibody was then incubated and then washed with PBS, the tissues were simulta-
nously incubated with anti-rabbit Texas red-conjugated
secondary antibody (1:100 dilution) and anti-mouse FITC-
conjugated secondary antibody for 3 h at room temperature,
washed with PBS, and mounted on slides.

A1R internalization. The role of internalization and recycling
to the membrane was investigated, as has been shown to
take place in other G protein-coupled receptors in the gut (35, 40, 41). Two protocols were used to assess the dynamics of A1R in
submucosal ganglia. 1) To reduce the fluidity of the cell
membrane and minimize any internalization of receptors,
immediately after the colon was removed from the animal, a
segment was microdissected in perfused ice-cold Krebs buffer
supplemented with 2 µM nicardipine and fixed for staining.
2) Immediately after the tissues were removed from the
animals, the colon segments were slit open and placed in a
warm (37°C) Krebs supplemented with 2 µM nicardipine, 1
µM tetrodotoxin, and either 10 µM 8-cyclopentyltheophylline
(CPT) to block the action of endogenous adenosine at A1R or 5
U/ml adenosine deaminase for 2 h to inactivate adenosine.
Denaturation of the enzyme was prevented by minimizing
bubbling. Under these conditions, any internalized
A1R may recycle back to the membrane and remain there
since adenosine can no longer activate A1R. Tetrodotoxin
blocks any neural contribution to released endogenous adeno-
sine. After the 2-h incubation, tissues were immediately
immersed in ice-cold Krebs supplemented with the same
agents. Tissues were then stretched and fixed with an approp-
rate fixative for designated time before being dissected.

Labeling was then viewed with the Zeiss LSM 410 laser
scanning confocal imaging system (Carl Zeiss). The argon-
krypton laser was used to excite tissues at 488 nm (FITC) and
568 nm (Texas red), respectively. The fluorescence emission
was first separated by a 560-nm dichromatic mirror. The FITC
fluorescence was further selected by a 515- to 540-nm band-
pass filter and that of Texas red was selected by a long-pass
filter of 590 nm. Under such conditions, the crossover fluores-
cence between the two channels was negligible (~2%). Speci-
mens were viewed through a ×40 oil immersion fluor-
objective (1.3 numerical aperture). The pinhole was set at 30,
which gave rise to a section thickness of ~1.0 µm. The averaged image of two to four consecutive scans was saved as
a 512 × 512 RGB tif image for later analysis. For simulta-
nous immunofluorescent labeling of A1R and S-100 protein
or other proteins, the images were acquired as single or dual fluoros-
cence channels. The final color of the RGB dual images depends
on the extent of colocalization of the two antigens (labeled by
green or red fluorochromes) in the same cells, i.e., yellow
denotes strong colocalization. These images provide qualita-
tive information about colocalization.

The colocalization of A1R immunoreactivity with other
proteins was further analyzed by colocalization software on
the Zeiss LSM 410 computer. This software computes a
scatter diagram from the two images that are acquired in
overlay mode and stored separately under image 1 (red) and
image 2 (green) in the video memory. The scatter diagram
treats all pixels that are in the same position in both images
as pairs and displays each pixel pair as x,y coordinates
(paired pixels) corresponding to the respective fluorescence intensities for images 1 and 2. The threshold fluorescent intensity value of
each individual image (red and green) was first determined
by a separate measure function of the software. Colocaliza-
tion was defined as follows. True colocalization occurred when
paired pixels had fluorescence intensities that were at least
40 units higher than the intensity of each negative control
image for each fluorochrome (i.e., secondary antibody conju-
gated to FITC or Texas red without primary antibody).
Colocalized pixels typically had intensities >110 units.
An area was then outlined on the scatter diagram that included
all pixels whose intensity values were >110 (which represent
colocalization). The regions that demonstrated positive label-
ing in both images according to our exclusion criteria (i.e., for
both molecules) were assigned a blue color, and this was
superimposed on the overlay RGB source image. A combined
RGB color scale with blue mask overlay was constructed and
included with each figure image displayed in this manuscript.
In appropriate figures, images are displayed as single channel (red or green), dual channel (red and green), and transformed
colocalization image of RGB overlay image together with
blue mask distribution of colocalization sites in the

Antisera. Anti-A1R affinity-purified rabbit polyclonal anti-
body (Alpha Diagnostic International, San Antonio, TX) to a
14-amino acid sequence corresponding to the third extracellu-
lar domain of the rat A1R was used. The peptide used for
making A1R antibodies shows 100% amino acid homology
with canine, rabbit, and human A1R sequences and 85% homology with the bovine sequence. The antibody to A1R
cross-reacts with human, pig, lamb, and rat. The glial-specific mouse monoclonal anti-S-100 antibody, done 15E2E2 (Biogenex, San Ramon, CA), was used as well as the mouse monoclonal antibody for neuron-specific PGP 9.5 (clone 13C4) against human brain PGP 9.5 (Biogenesis, Sandown, NH) which reacts with human, rat, and guinea pig. The mouse monoclonal anti-synaptophysin antibody (clone SVP-38) against the rat retinal synaptosomal-derived antigen (Sigma Immunochemicals, St. Louis, MO), which reacts with human, guinea pig, and pig synaptophysin, was used to stain neurons.

Bound antibodies were visualized by incubating tissues in Texas red (A1 receptor)-labeled or FITC-labeled (S-100 or PGP 9.5 or synaptophysin) secondary antibodies to rabbit IgG. In parallel control experiments, tissues were incubated with normal rabbit antiserum instead of the primary antibodies. The specificity of the labeling to A1R was tested in experiments in which anti-A1R antibody was first preabsorbed with a peptide corresponding to the antigenic site of the A1R (Alpha Diagnostic International, San Antonio, TX). Double label immunohistochemistry was used to identify neurons (absence of S-100 staining or positive PGP 9.5 staining), glial cells (positive S-100 staining), or varicosities (positive synaptophysin) that expressed the A1R receptor.

Statistics. Means ± SE are reported. The n values refer to the number of tissues, which approximates the number of animals unless otherwise stated. Student’s t-test was used to determine statistical significance at P < 0.05.

RESULTS

Basal and reflex-evoked ISc. Basal ISc in 64 control groups averaged 4 ± 3 µA/cm² and was not statistically different from zero. The ISc was previously shown to be due to the algebraic sum of small net fluxes of sodium, chloride, and a residual ion (27). During stroking, due to the algebraic sum of small net fluxes of sodium, chloride, and a residual ion (27). During stroking, ISc increased over baseline levels by 82 ± 5 µA/cm². The stroking-induced change in ISc was shown previously to be due to stimulation of electrogenic chloride secretion (38).

Role for endogenous adenosine in reflex-evoked ISc. To determine whether endogenous adenosine modulates ISc evoked by mucosal stroking, which elicits a neural reflex, the selective A1R antagonist CPT was added to the serosal bath. CPT (0.5 µM) had no effect on baseline ISc compared with vehicle controls (Table 1). However, CPT caused a concentration-dependent enhancement of the neurally evoked reflex response due to stroking (Fig. 2A). To rule out the possibility that any adenosine displaced from the A1R by CPT could affect excitatory A2A receptors on neurons to increase ISc and thereby also to contribute to the CPT response, 8-(3-chlorostyryl)-caffeine, a specific A2A receptor antagonist, was added. CPT (0.5 µM) enhanced stroking-evoked ISc to 153 ± 20% (n = 12; P < 0.05) of control response despite the presence of 1 µM 8-(3-chlorostyryl)caffeine; the enhancement is comparable to that obtained with CPT alone. The A2A receptor antagonist alone had little effect on baseline ISc (Table 1).

To determine whether altering endogenous levels of adenosine would modulate the neurally evoked response, two inhibitors were used. One of these, the enzyme adenosine deaminase, accelerates the conversion of adenosine to the inactive metabolite inosine and thereby reduces endogenous adenosine levels that could activate P1 purinoceptors (5). Adenosine deaminase (5 U/ml) enhanced the reflex-evoked response to 132 ± 9% of control response (n = 5; P < 0.05) without a significant effect on baseline ISc (Table 1). On the other hand, when endogenous adenosine levels were increased by adding submicromolar concentrations of S-(4-nitrobenzyl)-6-thioinosine (NBTI), ISc at 0 µM was 63 ± 8 µA/cm² (n = 3 or 4). *P < 0.05.

Table 1. Drug effects of adenosine analogs and related compounds on baseline ISc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Baseline ΔIsc µA/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 µM)</td>
<td>9</td>
<td>-5.6 ± 1.6</td>
</tr>
<tr>
<td>8-Chlorophenyltheophylline (0.5 µM)</td>
<td>9</td>
<td>-2.4 ± 1.7</td>
</tr>
<tr>
<td>Adenosine deaminase (5 U/ml)</td>
<td>9</td>
<td>1.9 ± 2.1</td>
</tr>
<tr>
<td>S-(4-nitrobenzyl)-6-thioinosine (0.5 µM)</td>
<td>4</td>
<td>-5.5 ± 1.5</td>
</tr>
<tr>
<td>8-(3-Chlorostyryl)caffeine (1 µM)</td>
<td>12</td>
<td>-2.5 ± 4.6</td>
</tr>
<tr>
<td>Tetrodotoxin (0.2 µM) + 2-chloro-N6-cyano-pentyladenosine (0.1 µM)</td>
<td>5</td>
<td>-2.2 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of tissues. ISc, short-circuit current.
with the degree of ongoing neural activity in the basal state (Fig. 3). When ongoing neural activity, defined by the reduction in $I_{sc}$ in response to 0.2 µM tetrodotoxin, was low, CCPA's effect on $I_{sc}$ was small; on the other hand, when the tetrodotoxin-induced reduction in $I_{sc}$ was large, so was the effect of CCPA.

CCPA caused a concentration-dependent inhibition of the mucosal stroking response but did not abolish it (Fig. 4A). The $EC_{50}$ was 6 nM. Reflex-evoked $I_{sc}$ was reduced to 29 ± 10 µA/cm² after 0.1 µM CCPA compared with control values of 155 ± 34 µA/cm² (n = 4). After a 30-min washout recovery period, reflex-evoked $I_{sc}$ rose to 101 ± 22 µA/cm². After reexposure to the same dose of CCPA, reflex-evoked $I_{sc}$ was reduced to 20 ± 5 µA/cm². Thus the A₁R did not appear to be desensitized, since they were still able to evoke maximum inhibition after a 30-min washout period. To determine whether the effect of 0.1 µM CCPA could be prevented by an A₁R antagonist, CPT was added in increasing concentrations (Fig. 4B). CPT attenuated CCPA's inhibition of the stroking response in a concentration-dependent manner.

Determination of sites of action of A₁R analogs on reflex-evoked $I_{sc}$. Another series of experiments was done to determine whether CCPA inhibited reflex-evoked $I_{sc}$ by acting on epithelial cells. Two secretagogues, carbachol and forskolin, known to increase $I_{sc}$ and cause chloride secretion by calcium- and cAMP-mediated pathways, respectively, were added to the serosal bath (4, 28). Because both carbachol and forskolin activate epithelial cells directly as well as indirectly via submucosal nerves, 0.2 µM tetrodotoxin was added to block the neurally mediated $I_{sc}$ response, and these responses were compared with responses in the absence of tetrodotoxin. In the absence of tetrodotoxin, 10 µM carbachol evoked a large increase in $I_{sc}$ that was significantly reduced by 0.1 µM CCPA (Fig. 5A, left). In the presence of tetrodotoxin, carbachol produced a smaller increase in $I_{sc}$ and this was unaffected by CCPA (Fig. 5A, right). The results are consistent with an effect of CCPA on the neural pathway to the epithelium and not directly on the epithelial cells.

The results with forskolin were similar to those with carbachol. In the absence of tetrodotoxin, 100 µM...
Con, control. * 5-HT1P antagonist when the 5-HT-mediated pathway was blocked with the tetrodotoxin. On the other hand, the prostaglandin-mediated pathway of the enterochromaffin cell was intact and not when it was blocked with tetrodotoxin.

In an attempt to determine whether the 5-HT-mediated limb of the neural reflex pathway was affected by CCPA, the prostaglandin-mediated pathway was blocked with piroxicam, a cyclooxygenase inhibitor (Fig. 1A). Piroxicam (10 µM) caused inhibition of the 5-HT-mediated pathway, which reflects 1_sc due to activation of the 5-HT pathway alone, was further reduced by 0.1 µM CCPA (Fig. 6A). Thus CCPA inhibited the prostaglandin-mediated pathway. On the other hand, when the 5-HT-mediated pathway was blocked with the 5-HT1P antagonist N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (HTP), at concentrations that were previously shown to be maximally effective, 1_sc was significantly reduced (Fig. 6B). The residual response, which reflects 1_sc due to activation of the prostaglandin-mediated pathway, was further attenuated by CCPA (Fig. 6B). This reduction indicated that CCPA affected the prostaglandin-mediated pathway, independently of the 5-HT pathway.

Another experimental protocol was used to verify that CCPA affected submucosal neurons within the 5-HT-mediated pathway (Fig. 1B). The mechanical stimulus of stroking releases both 5-HT and prostaglandins, whereas a mucosal pulse of 5-HT bypasses the enterochromaffin cell and activates 5-HT1P receptors directly on submucosal primary afferent neurons without releasing prostaglandins (16, 38, 39). Therefore, the 5-HT-mediated limb of the reflex was activated by a pulse of 5-HT. There was an increase in l_sc that was nearly abolished by 0.1 µM CCPA (Fig. 7). Thus CCPA still inhibited the l_sc response despite the fact that the enterochromaffin cell had been bypassed by a pulse of 5-HT. Consequently, one of CCPA’s main sites of action must be in the neural pathways innervating the epithelium that is dependent on the availability of 5-HT.

Immunofluorescent distribution of A1R immunoreactivity in submucosal ganglia. The distribution of A1R in submucosal ganglia of the guinea pig colon was further characterized in immunofluorescent colabeling studies with anti-A1R antibodies. Data analysis was from 7 guinea pigs and 35 separate microdissected tissues. Five additional animals were used in initial studies to optimize the staining procedures.

Colocalization of A1R and PGP 9.5 immunoreactivity. A1R immunoreactivity was prominent in many but not all PGP 9.5 immunoreactive cells as shown in Fig. 8B and C. Similar results were obtained in tissues fixed with modified Zamboni’s fixative (n = 3 tissues) or 0.5% paraformaldehyde (n = 3), although the staining for PGP 9.5 was stronger with the former fixation method. A mild 5-h fixation method was used to prevent destruction of the extracellular antigenic site for A1R. Preliminary studies (data not shown; n = 5 animals) showed that overnight fixations of tissues for A1R greatly increased background and labeled tissues indiscriminately. Overnight fixation for PGP 9.5 produced the strongest specific staining, and this finding explains the somewhat weaker immunofluorescence staining seen for PGP 9.5 in colabeling studies for A1R (Fig. 8C). A 5-h incubation was adopted for all subsequent dual labeling studies involving A1R. Single immunofluorescence labeling studies for A1R revealed a similar distribution profile of A1R immunoreactivity in large cells ranging in neuronal size from 20 to 50 µm (data not shown).

Colocalization of A1R and S-100 immunoreactivity. The distribution of A1R in S-100-immunoreactive glial cells is shown in Fig. 9. Nine tissues were colabeled with S-100 and A1R, six tissues were fixed in modified Zamboni’s fixative, and three were fixed in 0.5% paraformaldehyde. No significant differences in immunoreactive distribution/intensity were evident between the two fixation methods. Colocalization analysis revealed that A1R immunoreactivity was colocalized in glial cells (Fig. 9C and D). A1R immunoreactivity was unevenly distributed in glia, with some ganglia having more immunoreactive glia than others. All A1R-immunoreactive cells lacking S-100 immunoreactivity have larger cell diameters and represent submucosal neurons (Fig. 9B). Not all neurons had A1R immunoreactivity.

![Fig. 6. Effect of CCPA (0.1 µM) on 5-HT- and PG-mediated limb of reflex pathway. A: presence of 5-HT limb after blockade of prostaglandin synthesis with 10 µM piroxicam (PIR) (n = 5). B: presence of PG limb after blockade of 5-HT limb with 1–10 µM N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (HTP) (n = 6 or 7). Con, control. *P < 0.05 from control and PIR or HTP.](http://ajpgi.physiology.org/article-pdf/10.1152/ajpgi.00053.2017)
Cellular distribution of A1R immunoreactivity. Thin optical sectioning of tissues (i.e., 1 µm thick) with the laser confocal scanning microscope and viewing of the specimens with a high numerical aperture (1.3 numerical aperture) ×40 or ×100 objective revealed that A1R immunoreactivity was present in the cell soma of the neurons and distributed both in the cell membranes and cytoplasm of the neurons. Exclusive membrane localization of the A1R immunoreactivity was evident in ~10% of the neurons and occurred as a thin ring of fluorescence around the membrane (Fig. 10A). The size of the nonimmunoreactive area inside the ring of fluorescence is clearly bigger than the nucleus (i.e., twice the size of nucleus), indicating that the cytoplasm in addition to the nucleus is lacking A1R immunoreactivity. The edge/outline of the cell membrane was further identified by colabeling of the neurons for synaptophysin (Fig. 10, B and C). In the majority (~90%) of the neurons, A1R immunoreactivity was also present in the cytosol of the submucosal neurons (Figs. 8, B–D, 11D, and 12, A and B). A1R immunoreactivity codistributed with a significant proportion of synaptophysin immunoreactive varicosities (Fig. 8D). Similar data were obtained in six different tissues from three animals.

Selectivity of the anti-A1R antibody for adenosine A1R. In tissues in which the primary antibody was preabsorbed with a peptide (at 2–5 times the anti-A1R antibody concentration) corresponding to the immunogenic peptide recognized by the A1R antibody, all A1R immunoreactivity was abolished (Figs. 10D and 12, C and D), whereas immunoreactivity for either S-100 or synaptophysin was not affected. Similar results were obtained in six tissues from two different animals.

A1R immunoreactivity in other submucosal cell types. Prominent A1R immunoreactivity was present in the muscularis mucosae (Fig. 11D). A1R immunoreactivity was absent in the mucosal crypt cells of the colon (Fig. 11, A–C). This was the case in both whole-mount tissues and frozen sections. Less than 5% of frozen sections showed some nonselective labeling that is
Fig. 10. Immunolocalization of A1R immunoreactivity in cell membrane of a submucosal neuron of guinea pig colon. A: single-channel image of A1R immunoreactivity in membrane of a neuron; arrow indicates intense fluorescence labeling of cell membrane for A1R immunoreactivity. A small amount of A1R immunoreactivity is present in cytoplasm. B: single-channel image of synaptophysin (SYN)-immunoreactive varicose fibers that outline membrane of submucosal neuron depicted in A at arrow. C: dual-channel image for A1R and SYN. Yellow indicates colocalization. D: representative image showing that preabsorption of anti-A1R antibody with immunogenic peptide (CP) for A1R totally blocks A1R immunoreactivity without affecting SYN immunoreactivity in a submucous ganglion.

Fig. 11. Lack of A1R immunoreactivity in mucosal crypt glands of guinea pig colon. A: dual negative control image for FITC- and Texas red-conjugated secondary antibodies in microdissected submucous plexus with intact mucosa. Optical section is through mucosal crypt glands. B: lack of immunoreactivity for A1R and S-100 in mucosal crypt cells. Neurons and glial cells in submucous ganglia were labeled for A1R immunoreactivity (data not shown). C: transmitted light image of same area depicted in B to show crypts. D: frozen section cut in transverse direction showing immunoreactive labeling of glial cells, neurons, and smooth muscle cells of muscularis mucosae for A1R immunoreactivity. Crypt cells did not label for A1R immunoreactivity in these sections (data not shown). G, ganglion; arrowhead, likely to be a macrophage on surface of ganglion.

Fig. 12. Adenosine deaminase treatment of tissues to destroy endogenous adenosine does not alter cellular distribution of A1R immunoreactivity in submucosal neurons. A and B: in presence of adenosine deaminase (5 U/ml for 2 h). Significant A1R immunoreactivity remains in cytoplasm of neurons (Texas red-conjugated secondary antibody against anti-A1R antibody; secondary antibody against anti-S-100 antibody conjugated to FITC). Arrowheads represent A1R-immunoreactive nonneuronal cells, which likely represent immune cells, that are generally elevated after prolonged treatment with adenosine deaminase; prolonged exposure to adenosine deaminase may lead to metabolic disturbances in neurons due to adenosine depletion. C and D: both cytoplasmic and membrane labeling of A1R immunoreactivity is abolished by preabsorption of anti-A1R antibody with immunogenic peptide. This indicates that all labeling with anti-A1R antibody represents A1R.
sometimes seen with frozen sections, especially at the edge of tissues; no such immunoreactivity was present in whole-mount tissues (n = 4 tissues from 3 animals).

Influence of endogenous adenosine on A1R internalization. The percentage of submucosal neurons displaying cytosolic A1R immunoreactivity remained at near 90% after treatment of tissues with either 10 µM CPT (n = 4 tissues, 2 animals) or 5 U/ml adenosine deaminase (n = 6 tissues, 3 animals) for 1–2 h (Figs. 12, A and B) These treatments are sufficient to maximally block the interaction of endogenous adenosine with A1R.

DISCUSSION

Endogenous adenosine, through its action on A1R, appears to have little effect on baseline Isc but inhibits reflex-evoked Isc that was previously shown to be due to electrogenic chloride secretion (7, 15, 38, 39). This conclusion comes from the observations that the selective A1R antagonist enhanced the reflex-evoked change in Isc independent of the blockade of A2A receptors. Furthermore, lowering endogenous adenosine by addition of adenosine deaminase or increasing its concentration by inhibiting transporters that regulate transmembrane movements of nucleosides appropriately enhanced or inhibited reflex-evoked changes in Isc, respectively. The finding that the A1R antagonist had little effect on baseline Isc suggests that endogenous adenosine plays a small role, if any, in modulating basal electrogenic transport rates. We cannot rule out effects of adenosine A1R activation on electroneutral transport that is not reflected by Isc. It is only after stroking that the antagonist enhances reflex-evoked Isc. It is unclear whether stroking itself releases adenosine and its purine precursors. Although the source of endogenous adenosine is unknown, others have suggested that ATP and its major metabolites including adenosine are released from enteric neurons by electrical stimulation (31). Because adenosine is found in many different cells, we cannot rule out the possibility this nucleoside is released from nonneural cells as well (23).

The inhibitory effect of adenosine A1R activation on reflex-evoked Isc was verified by using a specific A1R agonist, CCPA. The effective concentration to achieve a half-maximal response was in the nanomolar range, consistent with an action of this agonist at A1R (25). Additional evidence for specificity of the agonist at A1R is provided by the ability of the selective A1 antagonist CPT to attenuate its inhibitory effect.

Functional investigation of the location of A1R suggests that they are unlikely to be found in abundance on epithelial cells, because CCPA was ineffective in altering carbachol- or forskolin-evoked Isc when the neurons were blocked with tetrodotoxin. Furthermore, A1R immunoreactivity was not detected in crypt cells that secrete chloride. Other studies suggest that excitatory A2B receptors are present on T84 colonic epithelial cells and their activation stimulates chloride secretion (43). Because both carbachol and forskolin are known to activate neural pathways to the epithelium as well as to trigger secretion by acting directly on epithelial cells, CCPA must be acting somewhere within the neural reflex circuit that innervates the epithelium (4, 28). Possible sites include enterochromaffin cells that release 5-HT and prostaglandin-producing cells or the neural pathways that they activate (15, 16, 38, 39). It is unknown whether enterochromaffin cells in the guinea pig colon express A1R, although A2 receptors have been reported for porcine intestine (36).

A pulse of 5-HT was used to bypass the enterochromaffin cells by directly activating intrinsic primary afferents that are synaptically coupled to secretomotor neurons (33, 39). Previous studies have clearly demonstrated that the mucosal pulse activates 5-HT, and not other 5-HT receptors (16, 38). Therefore, maximal blockade of this receptor is thought to block the 5-HT-activated limb of the reflex (16, 26). The inhibition of the 5-HT pulse by CCPA is consistent with an effect on neural circuits in the 5-HT-mediated pathway. Further support for the concept that adenosine’s effect on the reflex is to modulate neuronal activity comes from the observation that during neural blockade CCPA had no effect on baseline Isc nor on carbachol- or forskolin-stimulated secretion. However, when there was spontaneous neural activity, the CCPA-evoked reduction in Isc correlated with the degree of ongoing activity. Furthermore, CCPA was able to reduce the neurally mediated carbachol- and forskolin-stimulated secretory response (i.e., the response in the absence of neural blockade).

A1R immunoreactivity is evident in a significant population of submucous neurons that are immunoreactive for PGP 9.5 or lack immunoreactivity to the glial protein S-100. This provides further support for our conclusion that neural A1 purinoceptors on submucosal neurons inhibit the 5-HT- and prostaglandin-mediated limbs of the secretory reflex. Blockade of A1R immunoreactivity by preabsorption with the blocking peptide indicates that the anti-A1R antibody is binding specifically to A1R in submucosal neurons. A1R immunoreactivity was localized to both membranes and cytosolic regions of the ganglion cells, and only a minority of neurons show exclusively a membrane localization of the A1R immunoreactivity, seen as a thin ring around the cell soma that is well marked by a dense network of synaptophysin-immunoreactive varicosities. A1R antibody binding to sites on intracellular enzymes involved in intermediary metabolism found in all cells that have affinity for adenosine cannot explain the A1R immunoreactivity in the cytoplasm, because not all cell types had cytoplasmic staining for A1R. A1R were absent in many glia, numerous submucosal neurons and their varicosities, all crypt cells in this study, as well as many myenteric neurons in the human and guinea pig small intestine (Christofi, unpublished observations). Unlike NK1 receptors, A1R are expressed in glial cells in submucous ganglia and are usually located in close apposition to the edges of submucosal neurons, making it exceedingly more difficult to reveal membrane staining for A1R; this is even more so in the smaller neurons even with laser confocal imaging of thin optical sections. Although the ubiquitous distribution of A1R in both neurons and glia make it difficult to resolve nerve terminal sites, the strong colocalization of A1R and synaptophysin immunoreactivities in submu-
cous ganglia is consistent with the presence of A₁R on presynaptic varicose nerve terminals.

Internalization/recycling has been studied in enteric neurons only for two members of the G protein-coupled receptor family, namely, opioid and tachykinin receptors (24, 35, 40–42). After being activated by substance P, NK₁ receptors are rapidly internalized via clathrin-coated endosomes and within 30–60 min recycle back to the membrane (40, 42). Previous electrophysiological studies suggested that A₁R are expressed on the surface of the cell somas of enteric neurons (1, 2, 12). Consistent with these findings was the observation of a thin ring of A₁R immunoreactivity around some neurons. Our results also suggest that the A₁R also exists in the internalized form in our in vitro microdissected submucous plexus preparations. Our finding that endogenous adenosine provides an ongoing tonic suppression of reflexes indicates that the endogenous accumulation of adenosine is sufficient to activate A₁ sites on submucous neurons, which could lead to subsequent receptor internalization and the observed cytosolic immunoreactivity.

The source of adenosine is unknown, although it might be released from nerves or other surrounding cells under normal, damaged (microdissection), or ischemic situations in which metabolic demand exceeds metabolic availability. However, in experiments in which the receptor was blocked by the antagonist CPT or adenosine was degraded by the enzyme adenosine deaminase, recycling was not detected despite assay conditions that were shown previously to favor recycling of tachykinin receptors to the cell somal membranes of enteric neurons (40). Therefore, A₁R on submucous neurons behave differently from other G protein-coupled receptors shown previously to undergo receptor internalization/recycling in enteric neurons. A₁R are also different in that they represent the smallest of the G protein-coupled receptors cloned to date (i.e., 326 amino acids; Refs. 14, 30, 37). In contrast to NK₁ receptor activation by substance P, the inhibitory response to CCPA is reproducible without any appreciable desensitization following a prolonged occupancy of the A₁R by CCPA and a subsequent 30-min washout period. Therefore, sufficient numbers of functional high-affinity A₁R must be present in the membrane during this period of time, since their activation leads to suppression of secretory reflexes. One possibility is that the amount of A₁R recycled compared with the amount remaining in the cytosol may be too small to be detectable. The work of Ciruela et al. (14) in a vas deferens smooth muscle cell line, DDT₁MF-2, and of Ruiz et al. (37) in rat brain sheds some light on why blockade of the interaction of endogenous adenosine with A₁R did not result in detectable recycling to the membrane of a majority of submucous neurons. They report slow kinetics for agonist-induced internalization/recycling of the A₁R. Chronic treatment with agonist resulted in a time-dependent translocation of A₁R to intracellular vesicles that was evident at 5–12 h and maximal at 12–48 h (14). In the case of DDT₁MF-2, 30% of the receptors were internalized. The slow kinetics of the A₁R may be related to the lack of serine/threonine residues in the carboxy-terminal cytoplasmic tail, a finding that makes it unique in the family of G protein-coupled receptors (14).

Several neural sites of action of adenosinergic A₁R inhibition of secretory reflexes are possible. Experiments designed to evaluate which limb of the reflex is modulated by A₁R activation provide evidence that both pathways are affected. This conclusion is evidenced by CCPA’s inhibition of the residual reflex-evoked Iₛ in tissues treated with piroxicam, which blocks the prostaglandin-mediated limb, or with HTTP, which antagonizes 5-HT₃p receptors, a gateway to the 5-HT-mediated limb (16, 26). Concentrations used were those shown to be maximally effective in previous studies, and therefore, appropriate blockade should have been achieved (16, 38).

The mechanism of inhibition when neural A₁R are stimulated has not been investigated in submucosal neurons in the guinea pig colon. Current information is derived from electrophysiological studies on myenteric and submucosal neurons of the ileum (1, 2, 8, 11). Functional studies did not allow us to exclude the possibility that A₁ receptor activation on enterochromaffin cells contributes to the overall inhibitory response. The apparent lack of A₁R immunoreactivity in crypt glands would argue against the presence of A₁R on enterochromaffin cells. However, if receptor density is low on these sparsely distributed cells, A₁R may not have been readily detected. Another possibility is that the A₁R agonist CCPA was also acting at other inhibitory P₁ purinoceptors on enterochromaffin cells that are not recognized by the anti-A₁R antibody. Indeed, recent observations in a carcinoid tumor cell line indicate the presence of transcripts of other P₁ purinoceptors on these cells (Cooke, unpublished observations).

That neural A₁R play a significant role in attenuating reflex-evoked Iₛ is clear from pulse experiments in which the enterochromaffin cell was bypassed by a pulse of 5-HT. Another potential target of adenosine at A₁R is submucosal primary afferent neurons believed to be AH/type 2 neurons with Dogiel type II multipolar morphology (22). Suppression of the somal excitability of the primary afferent neuron would block or attenuate the neural reflex. Indeed, in both the myenteric and submucous plexuses, A₁R activation suppresses the excitability of most AH/type 2 neurons (12; Christofi, unpublished observations).

It is also possible that adenosine is acting presynaptically at nerve terminals to inhibit release of transmitters for slow excitatory synaptic transmission (i.e., slow EPSP) as has been reported for myenteric neurons (8, 10). Inhibitory A₁R on the primary afferent ending would prevent the relay of sensory information to the postsynaptic cell body when 5-HT activates 5-HT₃p receptors to initiate neural reflex activity (22). Adenosine would in essence short-circuit the reflex at the neural activation site.

The submucosal primary afferent neurons, which contain substance P, acetylcholine, and glutamate as putative neurotransmitters, are likely to synapse di-
rectly with cholinergic secretomotor neurons and either directly or via an interneuron to VIP secretomotor neurons (Figs. 1 and 10) (15, 26, 29). Exogenous application of each of these putative neurotransmitters evokes slow EPSP-like responses in submucosal neurons. Adenosine has been shown to suppress all slow EPSP in both S/type 1 myenteric neurons and AH/type 2 neurons by acting at pre- and postsynaptic sites in AH/type 2 and only presynaptic sites in S/type 1 neurons (8). Secretomotor neurons are S/type 1 neurons and therefore would not be expected to have A1R on cell somas. Nevertheless, we cannot rule out the possibility that transmitter release at neuroepithelial junctions could be inhibited by A1R on secretomotor nerve terminals.

The sites of action of CCPA in the submucous plexus to suppress the prostaglandin-mediated neural reflex are unknown. PGE2 is reported to activate directly VIP secretomotor neurons in the guinea pig ileum (17). The chloride secretory effects of PGE2, PGF2α, and PGI2 in the guinea pig colon are mediated in part by activation of submucosal neurons and release of transmitters that cause postsynaptic depolarization of the membrane potential associated with an enhanced spike discharge. Although part of the excitatory response is mediated by the activation of nicotinic cholinergic circuits that drive responsive neurons synaptically, nicotinic receptors are not involved in the stroking reflex (38). Therefore, attenuation of nicotinic cholinergic transmission as a mechanism for A1R-mediated inhibition of prostaglandin effects can be excluded from consideration (19–21). Neuronal effects of PGE2, PGD2, and PGI2 are, in part, mediated by muscarinic receptors (18). Adenosine could suppress the prostaglandin-mediated neural secretory reflex by acting at pre- or postsynaptic A1R on submucosal neurons that display prostaglandin-mediated excitatory responses.

The role of glial A1R in submucosal ganglia is unclear, but these receptors are unlikely to contribute to the inhibition of the secretory reflex responses studied here. There was strong labeling of the muscularis mucosae for A1R immunoreactivity, but the relevance of this receptors, if any, in secretory reflexes is unknown.

These results provide compelling evidence that endogenous adenosine suppresses secretory reflexes in the submucosal plexus of the guinea pig colon. The internalized A1R apparently behave differently from other G protein-coupled receptors in enteric neurons. Understanding the integrated response to elevated adenosine levels will have to take into account the balance between its role in intermediary metabolism and its extracellular actions at excitatory and inhibitory P1 (A1, A2A, A2B, A3) purinoceptors on submucosal neurons, as well as the contribution of adenosine from ATP release and breakdown to adenosine.

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