Activation of neuronal adenosine A₁ receptors suppresses secretory reflexes in the guinea pig colon

HELEN J. COOKE,1 Y.-Z. WANG,1 C. Y. LIU,2 H. ZHANG,2 AND F. L. CHRISTOFI2
Departments of 1Pharmacology and 2Anesthesiology, The Ohio State University, Columbus, Ohio 43210

Cooke, Helen J., Y.-Z. Wang, C. Y. Liu, H. Zhang, and F. L. Christofi. Activation of neuronal adenosine A₁ receptors suppresses secretory reflexes in the guinea pig colon. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G451–G462, 1999.—The role of adenosine A₁ receptors (A₁R) in reflex-evoked short-circuit current Iₛₑᵥₑ₉ indicative of chloride secretion was studied in the guinea pig colon. The A₁R antagonist 8-cyclopentyltheophylline (CPT) enhanced reflex-evoked Iₛₑᵥₑ₉. Adenosine deaminase and the nucleoside transport inhibitor S-(4-nitrobenzyl)-6-thioinosine enhanced and reduced reflex-induced Iₛₑᵥₑ₉, respectively. The A₁R agonist 2-chloro-N-cyclopentyladenosine (CCPA) inhibited reflex-evoked Iₛₑᵥₑ₉ 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 Iₛₑᵥₑ₉. CCPA reduced the response to a 5-HT pulse without affecting the tetrodotoxin-insensitive Iₛₑᵥₑ₉ responses to carbachol or forskolin. Immunoreactivity for A₁R was detected in the membrane (10% of neurons) and cytoplasm (90% of neurons) of neural protein gene product 9.5-immunoreactive neurons, in glia, and in the muscularis mucosa. A₁R immunoreactivity in a majority of neurons remained elevated in the cytoplasm despite preincubation with adenosine deaminase or CPT. A₁R immunoreactivity colocalized in synaptophysin-immunoreactive presynaptic varicose nerve terminals. The results indicate that endogenous adenosine binding to high-affinity A₁R on submucosal neurons acts as a physiological brake to suppress reflex-evoked Iₛₑᵥₑ₉ 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 is 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 P₁ purinoceptor family of receptors which includes A₁, A₂A, A₂B, and A₃ receptors cloned from human or animal sources (25). Highly specific affinity-purified anti-A₁, A₂A, A₂B, and A₃ 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 A₁ receptors (A₁R) have been identified on isolated myenteric varicosities by ligand binding techniques (6, 7). Biochemical studies indicate that A₁R activation leads to inhibition of acetylcholine and tachykinin release from presynaptic varicose nerve terminals in 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 A₁R inhibition of slow synaptic transmission [slow excitatory postsynaptic potentials (EPSP)] (11, 12). In AH/type 2 neurons, the main postsynaptic action of adenosine is A₁R-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, A₁R 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 presynaptic A₁ sites to inhibit voltage-activated calcium currents and release of acetylcholine, a transmitter involved in nicotinic fast excitatory transmission (1, 2).

A₁R 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 A₁R should be internalized at all times, if indeed A₁R undergo internalization and recycling.
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 mucus or submucosal primary afferents containing substance P, acetylcholine, and glutamate (15, 26, 29). The presence of NK1 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 variocities 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 calomel half cells for measurement of transmural potential difference (PD) and aluminium foil electrodes for passing Isc from a voltage-clamp apparatus. Solution resistance between the PD-sensing bridge and calomel half cells 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- and cAMP-mediated secretion, conventional flux chambers were used. Drugs were added to the mucosal or serosal compartment (10 μl), and Isc, 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
evoke neural reflexes, a 2-mm-wide brush attached to a micromanipulator was lowered to the mucosal surface. Stroking occurred with a forward or backward motion for 1 s. After two to three strokes at 5-min intervals, drugs were perfused either 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 onto the mucosal surface was previously shown to activate 5-HT₃ 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 given following 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 mM 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 microdissection 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 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 labels varicose nerve terminals, or S-100 that labels enteric glial 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 primary antibodies overnight at 4°C. When PGP 9.5 was used to label neurons, the tissue was also exposed to 0.1% Triton X-100 while being incubated with goat serum. The dilution of the antibodies was 1:25–100 for A₁R and 1:50–100 for other proteins. After removal of the primary antibody and washes with PBS, the tissues were simultaneously 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 occur for other G protein-coupled receptors in the gut (35, 40, 41). Two protocols were used to assess the dynamics of A₁R in submucous 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 mM 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 at 37°C, 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 adenosine. 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 appropriate 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 fluorescence between the two channels was negligible (~2%). Specimens 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 average image of two to four consecutive scans was saved as a 512 × 512 RGB tiff image for later analysis. For simultaneous immunofluorescent colabeling of A₁R and S-100 protein or other proteins, the images were acquired as 512 × 512 overlay RGB tiff images displayed as single or dual fluorescence images. 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 qualitative 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 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. Colocalization was then 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 conjugated 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 labeling 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 tissue.

Antisera. Anti-A₁R affinity-purified rabbit polyclonal antibody (Alpha Diagnostic International, San Antonio, TX) to a 14-amino acid sequence corresponding to the third extracellular 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 1SE2E2 (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 on 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 A1 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 I sc. Basal I sc in 64 control groups averaged 4 ± 3 µA/cm² and was not statistically different from zero. The I sc 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, I sc stroking-induced change in I sc groups averaged 4.6 ± 0.5 µA/cm² of the neurally evoked reflex response due to stroking CPT caused a concentration-dependent enhancement A2A receptors on neurons to increase I sc increased over baseline levels by 82% (n = 3 or 4). *P < 0.05.

Role for endogenous adenosine in reflex-evoked I sc. To determine whether endogenous adenosine modulates I sc 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 I sc 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 I sc and thereby also contribute to the CPT response, 8-(3-chlorostyryl)caffeine, a specific A2A receptor antagonist, was added. CPT (0.5 µM) enhanced stroking-evoked I sc 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 I sc (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 I sc (Table 1). On the other hand, when endogenous adenosine levels were increased by adding submicromolar concentrations of S-(4-nitrobenzyl)-6-thioinosine (NBTI), I sc at 0 µM = 63 ± 8 µA/cm² (n = 3 or 4). *P < 0.05.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Baseline I sc, 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</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>9</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-N 6-cylo- pentyladenosine (0.1 µM)</td>
<td>5</td>
<td>-2.2 ± 1.5</td>
</tr>
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Values are means ± SE; n, no. of tissues. I sc, short-circuit current.

Table 2. Modulation of endogenous adenosine levels on stroking-evoked change in short-circuit current (ΔI sc). A: effect of adenosine A1 receptor (A1R) antagonist 8-chlorophenyltheophylline (CPT), I sc at 0 µM = 62 ± 8 µA/cm² (n = 4–6). B: effect of nuclease transport inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBTI). I sc at 0 µM = 63 ± 8 µA/cm² (n = 3 or 4). *P < 0.05.
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...
forskolin evoked a large increase in $I_{sc}$ that was reduced by 0.1 µM CCPA (Fig. 5B, left), whereas in the presence of tetrodotoxin, CCPA had no effect on the forskolin-induced $I_{sc}$ (Fig. 5B, right). Thus CCPA was effective in reducing $I_{sc}$ when the neural pathway to the epithelium 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 $I_{sc}$ response to stroking (Fig. 6A). The residual response after piroxicam treatment, which reflects $I_{sc}$ due to activation of the 5-HT pathway alone, was further reduced by 0.1 µM CCPA (Fig. 6A). Thus CCPA inhibited the 5-HT-mediated pathway. On the other hand, when the 5-HT-mediated pathway was blocked with the 5-HT$_1$P antagonist hydroxytryptophyl-5-hydroxytryptophan amide (HTP), at concentrations that were previously shown to be maximally effective, $I_{sc}$ was significantly reduced (Fig. 6B). The residual response, which reflects $I_{sc}$ due to activation of the prostaglandin-mediated pathway, was further attenuated by CCPA (Fig. 6B). This reduction indicated that CCPA affected submucosal neurons 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-HT$_1$P 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 $I_{sc}$ that was nearly abolished by 0.1 µM CCPA (Fig. 7). Thus CCPA still inhibited the $I_{sc}$ 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 A$_1$R immunoreactivity in submucosal ganglia. The distribution of A$_1$R in submucosal ganglia of the guinea pig colon was further characterized in immunofluorescent colabeling studies with anti-A$_1$R 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 A$_1$R and PGP 9.5 immunoreactivity. A$_1$R immunoreactivity was prominent in many but not all PGP 9.5 immunoreactive cells as shown in Fig. 8, B 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 A$_1$R. Preliminary studies (data not shown; n = 5 animals) showed that overnight fixations of tissues for A$_1$R 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 A$_1$R (Fig. 8C). A 5-h incubation was adopted for all subsequent dual labeling studies involving A$_1$R. Single immunofluorescence labeling studies for A$_1$R revealed a similar distribution profile of A$_1$R immunoreactivity in large cells ranging in neuronal size from 20 to 50 µm (data not shown).

Colocalization of A$_1$R and S-100 immunoreactivity. The distribution of A$_1$R in S-100-immunoreactive glial cells is shown in Fig. 9. Nine tissues were colabeled with S-100 and A$_1$R, 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 A$_1$R immunoreactivity is colocalized in glial cells (Fig. 9, C and D). A$_1$R immunoreactivity was unevenly distributed in glia, with some ganglia having more immunoreactive glia than others. All A$_1$R-immunoreactive cells lacking S-100 immunoreactivity have larger cell diameters and represent submucosal neurons (Fig. 9B). Not all neurons had A$_1$R immunoreactivity.
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 submucosal 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 µM adenosine deaminase (n = 6 tissues, 3 animals) for 1–2 h (Fig. 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 I_sc but inhibits reflex-evoked I_sc 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 I_sc 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 I_sc, respectively.

The finding that the A1R antagonist had little effect on baseline I_sc 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 electrophysiological transport that is not reflected by I_sc. It is only after stroking that the antagonist enhances reflex-evoked I_sc. 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 that this nucleoside is released from nonneural cells as well (23).

The inhibitory effect of adenosine A1R activation on reflex-evoked I_sc 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 sug- gests that they are unlikely to be found in abundance on epithelial cells, because CCPA was ineffective in altering carbachol- or forskolin-evoked I_sc 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 enterochro- maaffin cells by directly activating intrinsic primary affer- ents 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 I_sc or on carbachol- or forskolin- stimulated secretion. However, when there was spontaneous neuronal activity, the CCPA-evoked reduction in I_sc 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 en- zymes 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 submucosal ganglia and are usually located in close apposition to the edges of submucosal neurons, making it exceedingly more difficult to reveal mem- brane 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 neural 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 Iₚ in tissues treated with piroxicam, which blocks the prostaglandin-mediated limb, or with HTP, which antagonizes 5-HT₁P 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 P₂ 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 P₂ purinoceptors on these cells (Cooke, unpublished observations).

That neural A₁R play a significant role in attenuating reflex-evoked Iₚ 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-HT₁P 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 submucous 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.

This work was supported by National Institutes of Health Grants DK-37240 (to H. J. Cooke), DK-44179 (to F. L. Christofi), and National Center for Research Resources Grant 1S10-RR-11434–01 (to F. L. Christofi).

Address for reprint requests: H. J. Cooke, Dept. of Pharmacology, The Ohio State University, 333 W. 10th Ave., Columbus, OH 43210.

Received 22 June 1998; accepted in final form 26 October 1998.

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