Intestinal Arteriolar Responses to Mucosal and Serosal Applications of Adenosine Analogues

Kenneth G. Proctor

Adenosine or its synthetic analogues were topically applied to the intestinal jejunum while steady-state blood flow was calculated in submucosal arterioles using video microscopy. Blood flow increased (220 or 130% of control) with the serosal application of 10^{-4} M N-ethyl carboxamido adenosine (NECA, A_{2}-selective agonist) or 2-chloro adenosine (2CA, nonselective agonist) but not with cyclohexyl adenosine (CHA, A_{1}-selective agonist). The nonselective competitive antagonist, 8-phenyl theophylline, attenuated the response evoked by NECA. The mucosal application of 10^{-4} M CHA caused blood flow decreases (81% of control), but neither NECA nor 2CA evoked a response. These observations suggest a mucosal diffusion barrier, so the concentrations of the analogues were raised one hundredfold. Serosal 10^{-4} M CHA or NECA caused blood flow increases, but the effects were negligible with mucosal application, suggesting that the mucosa was indeed impermeable to these compounds. The responses evoked by 10^{-4} M 2CA were similar on the serosa or mucosa (200-220% of control), submaximal (maximum = 400% of control at 10^{-3} M), and not antagonized by 8-phenyl theophylline or by the cellular uptake inhibitor, nitrobenzyl-6-thio guanosine. In context with earlier studies, > 10^{-4} M 2CA probably evokes vasodilation that is not entirely mediated by extracellular receptors. Serosal adenosine (10^{-4} M) caused submaximal blood flow increases (200% of control) that were not potentiated by nitrobenzyl-6-thio guanosine or another transport inhibitor, dipyridamole. In contrast, mucosal adenosine (10^{-4} M) had no effect on blood flow unless the transport inhibitors were present, and even then, the blood flow increase was lower than that evoked by serosal adenosine. Overall, these results suggest that 1) adenosine caused intestinal arteriolar vasodilation by intracellular and extracellular mechanisms, 2) the extracellular mechanism is mediated, in part, by A_{2}-receptors, and 3) cellular uptake in the mucosa as well as a diffusion barrier probably restricts the passage of adenosine and its analogues from the lumen to the interstitium. (Circulation Research 1987;61:187-193)
room air. Rectal temperature was continuously monitored (Yellow Springs International, Yellow Springs, Ohio) and maintained at 36–38° C with a heat lamp. Carotid arterial blood pressure was continuously monitored with a Gould Statham P231D transducer (Oxford, Calif.) and typically ranged between 80 and 100 mm Hg.

The intestine was prepared as previously described. Briefly, an incision along the antimesenteric border of the jejunum was heat cauterized. Several ties were sutured to the two flaps of tissue. The upper flap was retracted. The lower flap was gently pulled to a flattened sheet over a glass viewing port on a specially designed animal board. A chamber top was lowered onto the preparation so that the tissue was completely enclosed in a controlled environment.

The mucosal (upward facing) and serosal (downward facing) surfaces were each suffused with bicarbonate-buffered Ringer's solutions. Solution temperatures, gas tensions, and pH were maintained by vigorous bubbling with a gaseous mixture of CO₂, 5%–O₂, 5%–N₂, 90% in glass chambers encased in water jackets at 37° C. The glass chambers were connected to the mucosal and serosal suffusion chambers of the animal board by thick-walled rubber tubing.

Tissue temperature was continuously monitored and maintained at 36–38° C by varying the flow rates of the heated suffusate solutions. Isoproterenol was added to the serosal suffusate to suppress spontaneous intestinal motility. The threshold concentration for increasing calculated intestinal blood flow was 1 μM, which is 25 times higher than the maximum concentration used in these experiments.

The microcirculation was transilluminated with white light from a stable Stahl Model 576 DC power supply (Port Chester, N.Y.). Observations were made with long working distance (4–6 mm) objectives [Leitz L-20X, 0.32 numerical aperture (Rockleigh, N.J.), or L-32X, 0.40 numerical aperture], long working distance Leitz L-11 condensor, and a Leitz L-l 11 condensor (0.60 numerical aperture), and a Leitz L-l 1 condensor (0.32 numerical aperture) objectives, the rotating prism velocitometer was custom designed by Micro-Precision, Inc. (Norcross, Ga.) to measure red blood cell velocity. The rotating prism method favorably compares with more conventional methods for measuring red blood cell velocity in the microcirculation. In addition, blood flow changes in submucosal arterioles calculated with this method reflect the whole organ blood flow changes evoked by similar stimuli in the rat, dog, and cat.

Arterial blood pressure, arteriolar diameter, red blood cell velocity, and tissue temperature were continuously recorded on a Gould polygraph (Cleveland, Ohio). The hemodynamic variables were digitized every 5 seconds by a Commodore microcomputer (West Chester, Penn.). Blood flow was computed on-line from the product of a constant, arteriolar diameter, and red blood cell velocity and was recorded on a Dec-writer.

Experimental Protocol

After a 30–60-minute postsurgery stabilization period, a first-order arteriole in the submucosa (40–90 μm resting diameter) was tested for the presence of spontaneous vasomotor tone by the continuous topical application of adenosine (10⁻⁵–10⁻³ M) to the serosa. Complete dose-response data have been published. The typical response was a steady-state increase in submucosal arteriolar diameter and calculated blood flow. Experiments were terminated if motility was excessive, if a preparation lacked vasomotor tone, or if systemic arterial blood pressure decreased below 70 mm Hg.

Thereafter, various amounts of N-ethylcarboxamido adenosine (NECA), 2-chloro adenosine (2CA), adenosine (ADO), or cyclohexyl adenosine (CHA) were added to the mucosal or serosal suffusate chambers to final concentrations of 10⁻⁸–10⁻⁵ M. After a 5–10-minute equilibration period, steady-state blood flow was calculated in a submucosal arteriole. That substance was then washed from the solution for 10–20 minutes, and measurements were repeated at a posttreatment baseline. In some experiments, the mucosal and serosal suffusates contained 10⁻⁵–10⁻⁴ M either 8-phenyl theophylline (8pTHEO), dipryridamole (DIPYRID), or nitrobenzyl-6-thio guanosine (NBTG). Multiple treatments were applied to each animal if the posttreatment baseline was similar to the pretreatment baseline. Otherwise, the experiment was terminated. All drugs were purchased from Sigma Chemical Co., St. Louis, Mo., except NBTG, which was supplied as a gift from Dr. R.A. Olsson at the University of South Florida College of Medicine.

Statistical Analysis

All values are expressed as mean ± SEM. Most comparisons were paired, and all treatments were randomized. Differences were determined with paired or unpaired t tests. Significance was assessed at the 95% confidence interval.

Results

Responses Evoked by Synthetic ADO Analogues

NECA. The threshold concentration for eliciting a response with serosal NECA was 10⁻⁶–10⁻⁵ M, which was below the threshold for 2CA. The left panel of Figure 1 shows responses to equal concentrations (10⁻⁶
M) of NECA, 2CA, and CHA in 7 animals exposed to each compound twice in random sequence, once on the mucosa and once on the serosa. The right panel shows responses at a higher concentration (10^{-4} M) to mucosal and serosal applications of these three compounds in 7-17 animals. These results should be interpreted with caution because the selectivity of the analogues for A2- and A1-receptors is lost at concentrations >10^{-6} M in vitro.12

Serosal 10^{-6} M NECA caused significant increases in submucosal arteriolar diameter (123 ± 5% of control) and calculated blood flow (221 ± 21% of control). In contrast, mucosal NECA had no significant effect on either variable.

The magnitude of the response elicited by serosal 10^{-6} M NECA was probably near maximal for this compound because 10^{-4} M NECA caused no further increases in blood flow or diameter. In 7 animals, 10^{-4} M NECA was applied to the serosa and mucosa in random order. Serosal application caused both diameter and blood flow increases (133 ± 6% of control and 184 ± 17% of control). These values did not differ from the corresponding values during serosal 10^{-4} M NECA. In contrast, even at 10^{-4} M, mucosal NECA had no significant effect on either diameter or calculated blood flow.

Thus, low and high concentrations of the A2-selective agonist, NECA, caused twofold increases in submucosal blood flow but only when the compound was applied to the serosal surface. For comparison, the nonselective agonist, ADO, applied to the serosa elicits dose-related blood flow increases with a threshold at 10^{-5} M and a maximum near 400% of control at 10^{-3} M.11 Therefore, the arterioles shown in Figure 1 were not maximally dilated.

2CA. In 7 animals, serosal 10^{-6} M 2CA caused increases in submucosal arteriolar diameter (111 ± 4% of control) and blood flow (134 ± 10% of control). These changes were significantly lower than those elicited by serosal NECA but were significantly greater than corresponding values during mucosal 2CA (99 ± 3 and 96 ± 8% of control).

The regional difference between the serosa and mucosa was not evident when the concentration of 2CA was increased. In 17 animals, 10^{-3} M 2CA was applied to the serosa and mucosa in random sequence. Serosal application evoked increases in submucosal arteriolar diameter (130 ± 6% of control) and blood flow (218 ± 28% of control). These changes were similar to those evoked by mucosal 2CA (122 ± 4 and 187 ± 12% of control).

In 3 animals, 10^{-3} M 2CA applied to the serosa caused further increases in diameter (160 ± 12% of control) and blood flow (382 ± 81% of control), so the responses shown in Figure 1 were submaximal. However, at high concentrations, 2CA probably evokes responses that are not receptor mediated (see below).

CHA. Serosal 10^{-6} M CHA had no significant effect on diameter or blood flow in 7 animals. In contrast, a mucosal application caused a significant reduction in blood flow (81 ± 5% of control) but not in diameter.

This regional difference between the serosa and mucosa persisted at 10^{-3} M in 8 animals. Serosal CHA caused a significant increase in submucosal arteriolar diameter (122 ± 7% of control) and blood flow (169 ± 13% of control), but mucosal CHA produced no significant effect on either variable.

Thus, at both low and high concentrations, submucosal arteriolar blood flow during mucosal CHA was lower than during serosal CHA.

Effect of Receptor Antagonists on Serosal NECA and 2CA Responses

NECA. Figure 2 shows paired responses in 2 groups of animals. In one group containing 5 animals (left panel), the nonselective competitive antagonist, 8pTHEO, was added to both mucosal and serosal sulfuseates to final concentrations of 10^{-3} M. Serosal 10^{-6} M NECA elicited a blood flow increase (239 ± 40% of control) that was significantly attenuated (156 ± 34% of control) by 8pTHEO.

2CA. In another group containing 4 animals (Figure 2, right panel), serosal 10^{-6} M 2CA elicited a submaximal blood flow increase (205 ± 28% of control) that was comparable to that elicited by 10^{-6} M NECA, but this response was not attenuated by 8pTHEO. The lack of effect of 8pTHEO was also observed on the response evoked by mucosal 2CA. In an additional 3 animals (not shown in Figure 2), mucosal 10^{-4} M 2CA caused similar blood flow increases in the presence (253 ± 72% of control) or absence (186 ± 9% of control) of 8pTHEO.

Thus, 8pTHEO antagonized the blood flow increases caused by 10^{-6} M NECA but not those caused by 10^{-4} M 2CA, even though the increases caused by NECA...
were near maximal and the increases caused by 2CA were submaximal.

Table 1 shows baseline hemodynamic variables in 7 animals during treatment with 10^{-5} M 8pTHEO or the vehicle (bicarbonate-buffered Ringer's solution). There were no significant treatment effects.

**Effect of Uptake Inhibitors on ADO Responses**

**Serosal ADO.** The left panel of Figure 3 shows results from 75 animals in which serosal 10^{-4} M ADO caused increases in diameter and calculated blood flow (131 ± 3% and 207 ± 11% of control). This response was not significantly potentiated by 10^{-5} M DIPYRID or by 10^{-5} M NBGT.

Since serosal 10^{-4} M ADO elicits a blood flow increase that averages approximately half-maximal, the lack of effect of the uptake inhibitors cannot be attributed to maximal vasodilation.

**Mucosal ADO.** In 22 animals, mucosal 10^{-4} M ADO had no effect on submucosal arteriolar diameter or calculated blood flow in the absence of the uptake inhibitors. In 5 animals during DIPYRID, mucosal ADO caused a significant increase in blood flow (114 ± 2% of control). Similarly, in 6 animals during NBGT, mucosal ADO caused significant increases in both diameter (109 ± 1% of control) and blood flow (130 ± 7% of control).

NBGT was less effective on the responses evoked by serosal or mucosal 2CA (data not shown). In 2 animals, serosal 10^{-4} M 2CA caused similar blood flow increases in the absence (144 ± 6% of control) or presence (139 ± 29% of control) of NBGT. In the same 2 animals, mucosal 2CA caused similar blood flow increases in the absence (138 ± 25% of control) or presence (134 ± 31% of control) of NBGT.

The effects of DIPYRID and NBGT on baseline hemodynamic variables are shown in Table 1. Both DIPYRID (n = 5 animals) and NBGT (n = 7 animals) tended to cause decreases in diameter and blood flow, but the changes were not significant.

In summary, cellular uptake inhibitors had some effect on blood flow responses evoked by mucosal ADO, but this response was a small fraction of those evoked by serosal ADO, serosal 2CA, or mucosal 2CA.

**Discussion**

Synthetic ADO analogues applied to the serosa caused increases in calculated submucosal arteriolar blood flow with an order of potency suggesting NECA > 2CA > CHA (Figure 1). The same substances applied to the mucosa produced minimal effects. Similarly, ADO itself evoked vasodilation when applied on the serosa but not on the mucosa (Figure 3). Although mucosal ADO caused vasodilation in the presence of cellular uptake inhibitors, the response was a relatively small fraction of the response evoked by serosal ADO even in the absence of the inhibitors. Based on the logic developed below, the most likely explanation for these observations is that intestinal arterioles are endowed with A2-receptors that are virtually inaccessible to luminal ADO because of a physical and metabolic mucosal diffusion barrier.

<table>
<thead>
<tr>
<th>Drug</th>
<th>BP (mm Hg)</th>
<th>Diam (μm)</th>
<th>BFC (nl/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n = 7)</td>
<td>90 ± 2</td>
<td>60 ± 8</td>
<td>23.0 ± 7.5</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>85 ± 3</td>
<td>61 ± 2</td>
<td>35.7 ± 5.0</td>
</tr>
<tr>
<td>Vehicle (n = 7)</td>
<td>88 ± 4</td>
<td>66 ± 8</td>
<td>15.5 ± 2.2</td>
</tr>
</tbody>
</table>

BP, systemic arterial blood pressure; Diam, submucosal arteriolar diameter; BFC, calculated arteriolar blood flow; n, number of animals; 8pTHEO, 8-phenyl theophylline; DIPYRID, dipryridamole; NBGT, nitrobenzyl-6-thio guanosine.

Drugs were added to mucosal and serosal suffusates to 10^{-5} M and were exposed to tissue for 10–30 minutes before data were collected; all comparisons within a group were paired; vehicle (bicarbonate-buffered Ringer's solution) or drug treatments were applied in random sequence. There were no significant treatment effects within a group.
Critique

It must be emphasized that ADO receptors are operationally defined by the effects of selective agonists on adenylate cyclase rather than by their effects on blood flow per se. It was assumed that ADO receptor stimulation would elicit parallel changes in adenylate cyclase and blood flow because A2-receptor stimulation increases both adenylate cyclase activity and blood flow in the isolated heart, kidney, and brain. Furthermore, ADO and ADO analogues stimulate adenylate cyclase by a receptor-mediated mechanism in the rabbit intestine in vitro, and the effect of ADO on intestinal blood flow is antagonized by theophylline. By the effects of the purines, such as effects on membrane metabolism, depresses the vascular effects evoked by ADO in isolated arteries. In addition, ADO can relax vascular smooth muscle by mechanisms that are not coupled to adenylate cyclase. Therefore, to further test whether the agonist-evoked blood flow change was associated with ADO receptor stimulation, the effect of a competitive antagonist was evaluated.

The methylxanthine 8pTHEO attenuated the blood flow increases evoked by the A2-selective agonist NECA (Figure 2). In identical conditions, the parent compound, theophylline, attenuated the blood flow increase evoked by the nonselective agonist ADO. 8pTHEO has certain advantages over theophylline. Both substances compete for ADO receptor sites and thereby inhibit ADO-induced alterations in adenylate cyclase, but the efficacy of theophylline, and most other methylxanthines, is usually compromised by numerous side effects, e.g., phosphodiesterase inhibition. For example, in the intact skeletal muscle and the intact intestinal microcirculation, irreversible alterations in baseline hemodynamic variables were often produced by the concentrations of topically applied theophylline (10^{-4} M) that were required to attenuate the action of topically applied ADO. In contrast, 8pTHEO is a poor phosphodiesterase inhibitor, the affinity of 8pTHEO for A2- and A1-receptors is 50 times greater than that of theophylline, and the efficacy for inhibiting the effects of ADO is 10 times greater. These desirable properties could explain the lack of effect of topically applied 10^{-6} M 8pTHEO on baseline hemodynamic variables in the intestinal microcirculation.

Based on the data presented in Figures 1 and 2 and on previous studies that have identified A1-receptors in the intestine in vitro, it is reasonable to conclude that the hyperemia elicited by the serosal application of synthetic ADO analogues was mediated by A1-receptors.

Interpretation

The results obtained with the mucosal applications of the various compounds are more difficult to explain. At 10^{-6} M, the responses were minimal compared with those observed with serosal application (Figure 1). These results might be explained by the presence of A2-receptors in the mucosa, but the order of potency did not conform to a pattern that could be attributed to A2-receptor stimulation. Four other possibilities were considered. First, the mucosal response might have been mediated by a mixed population of A1- and A2-receptors; second, the response might have been partially compensated by intrinsic mechanisms that offset the blood flow change; third, the response might have been mediated by the nonreceptor-mediated effects of the purines, such as effects on membrane permeability or sympathetic transmission; and finally, the interstitium might have been inaccessible if the analogues were placed in the lumen.

The passage of intact ADO molecules through the mucosal epithelium into the interstitium is probably restricted, in part, by the activity of a purine uptake mechanism and by extracellular catabolism. Indeed, purines are actively secreted, not absorbed, from the...
lumen and are preferentially transferred from the con-
traluminal to the lumenal side of the epithelium. Thus, in vitro data would predict a formidable metabolic barrier for the passage of ADO from the lumen to the interstitium. This idea of a mucosal metabolic barrier is supported by the observations that the structurally dissimilar cellular uptake inhibitors DIPYRID and NBTG had greater effects on the blood flow responses evoked by mucosal ADO compared with those evoked by serosal ADO (Figure 3).

NBTG is a member of a family of substituted thio-purine nucleosides that interact strongly with the purine transport mechanism in most animal cells. To our knowledge, there have been no previous studies of its effect on the microcirculation. In our hands, NBGT compared favorably to the more thoroughly studied purine transport inhibitor DIPYRID (Table 1, Figure 3). Other investigators have reported nonspecific effects of DIPYRID in many experimental conditions, including phosphodiesterase inhibition. In the intestine, intraarterial DIPYRID caused vasodilation in the fed state but no effect in the fasted state.

In this present study, topical DIPYRID had no significant effects on baseline hemodynamic variables in the resting state (Table 1). There was a tendency to cause vasoconstriction at a concentration (10⁻³ M) that had minimal effects on the response to exogenous ADO applied to the serosa and somewhat larger effects on the response to ADO applied to the mucosa (Figure 3). At the same concentration (10⁻³ M), the effect of NBGT was less prominent on the baseline (Table 1) and more prominent on the responses to ADO (Figure 3).

Several factors could restrict the entry of luminal ADO, and probably other purines, into the interstitium. These factors include active secretion into the lumen, an unusually high adenosine deaminase activity in the mucosa, and the size, charge, and low lipid solubility of the purines (R.A. Olsson and J.W. Daly, personal communication).

A high activity of adenosine deaminase in the mucosa is one explanation for the markedly smaller responses evoked by mucosal ADO (even in the presence of the cellular uptake inhibitors) compared with those evoked by serosal ADO (in the absence of the inhibitors). Nevertheless, metabolic inactivation mechanisms, such as a high adenosine deaminase activity, could not explain the lack of effect of mucosal NECA or CHA (Figure 1); for this reason, additional explanations must be considered.

It is well known that the luminal epithelium is coated with a thick mucus layer that, together with an unstirred water layer, can offer a physical impediment to the passage of substances from the lumen to the interstitium. Since NECA, 2CA, CHA, and ADO have similar sizes (approximate mol wt 300) and low lipid solubilities, a relatively restrictive physical diffusion barrier could explain the observations that serosal NECA evoked near-maximal responses at 10⁻⁴ M but minimal effects on the mucosa, even if the concentration was raised to 10⁻³ M (Figure 1). It should be reemphasized that the responses at the higher concentration should be interpreted with caution because of possible nonspecific actions of these agonists at 10⁻⁴ M.

If NECA or CHA had had diffuse access to intestinal arterioles, current evidence suggests that their vascular effects would have been almost entirely mediated by extracellular receptors. In contrast, 2CA might have evoked vascular responses that were not entirely receptor mediated.

At high concentrations, 2CA can enter cells, under certain conditions, either by disrupting membrane permeability or by a weak tendency to bind to the purine transporter. Once inside a cell, 2CA can be phosphorylated but not further metabolized (J.W. Daly, personal communication). Thus, one interpretation from Figures 1 and 2 is that mucosal or serosal 2CA evoked similar responses at 10⁻³ M (blood flow increases to 200% of control), despite a regional difference at 10⁻⁴ M, because 2CA diffused, or was transported, into cells at the higher concentration. This interpretation is supported by the observation that the responses evoked by 10⁻⁴ M 2CA were not attenuated by 8pTHEO (Figure 2).

These present experiments could not distinguish between the passive diffusion or carrier mediated transport of 2CA into cells because the uptake inhibitor, NBGT, did not attenuate the responses evoked by mucosal or serosal 10⁻³ M 2CA (see “Results”). Indeed, it is possible that 2CA interfered with calcium fluxes or sympathetic transmission as demonstrated with high concentrations of ADO in vitro. Yet, regardless of the mechanism, it is unlikely that 2CA penetrated the mucosal diffusion barrier into the interstitium because 10⁻³ M 2CA evoked a prolonged, reversible hyperemia that was independent of the route of application (Figure 1) and was not antagonized by 8pTHEO (Figure 2).

Implication
If the epithelium/mucus/unstirred water layer is in fact a barrier for the passage of ADO from the lumen to the interstitium, then both the interstitial and intraluminal concentration of ADO might be determined by the activity of the mucosal cells. The concept of different pools of ADO within an organ is not new. In the heart, ADO formation can be extracellular or intracellular and can occur in the myocardial cells, the coronary vascular smooth muscle, or the endothelium. The capillary endothelial cells appear to be a significant barrier for the transport of exogenous ADO from the plasma to the myocardial interstitium, suggesting that pools of interstitial and plasma ADO can be regulated independently. If endothelial and mucosal epithelial cells actively metabolize ADO in the intestine, then the meaning of intestinal ADO dose-response curves might have to be reevaluated. Indeed, for the heart, infused ADO changes plasma concentration far more than it changes interstitial concentration.

In summary, the topical application of three synthetic ADO analogues elicited intestinal blood flow increases that were consistent with the stimulation of
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A_2-receptors but only when the substances were applied to the serosal surface. On the mucosal surface, the substances evoked minimal responses at low concentrations. Some of these results could be attributed to events that were not receptor mediated, but most could be explained by the presence of a mucosal diffusion barrier. ADO itself had no effects when applied on the mucosa unless the tissue was treated with inhibitors of the purine transport system. Even then, the hyperemia responses were less than those evoked by serosal application of ADO in the absence of the inhibitors. In context with other studies, the intestinal mucosa probably is a physical and metabolic barrier for the passage of ADO from the lumen to the interstitium.

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K G Proctor

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