Korean J Physiol Pharmacol
Vol 2: 521–527, August, 1998

cAMP-Dependent Signalling is Involved in Adenosine-Stimulated Cl^- Secretion in Rabbit Colon Mucosa

Sae Ock Oh, Eui Yong Kim[1], Jin Sup Jung, Jae Suk Woo, Yong Keun Kim, and Sang Ho Lee

Department of Physiology, College of Medicine, Pusan National University and [1]Inje University, Pusan 602–739, Korea

An important property of the intestine is the ability to secrete fluid. The intestinal secretion is regulated by a number of substances including vasoactive intestinal peptide (VIP), ATP and different inflammatory mediators. One of the most important secretagogues is adenosine during inflammation. However, the controversy concerning the underlying mechanism of adenosine-stimulated Cl^- secretion in intestinal epithelial cells still continues. To investigate the effect of adenosine on Cl^- secretion and its underlying mechanism in the rabbit colon mucosa, we measured short circuit current (I_{SC}) under automatic voltage clamp with DVC-1000 in a modified Ussing chamber. Adenosine, when added to the basolateral side of the muocsa, increased I_{SC} in a dose-dependent manner. The adenosine-stimulated I_{SC} response was abolished when Cl^- in the bath solution was replaced completely with gluconate. In addition, the I_{SC} response was inhibited by a basolateral Na-K-Cl cotransporter blocker, bumetanide, and by apical Cl^- channel blockers, dephenylamine-2-carboxylate (DPC), 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB), glibenclamide. Amiloride, an epithelial Na^+ channel blocker, and 4,4-diisothiocyanato-stilbene-2,2-disulphonate (DIDS), a Ca^{2+}-activated Cl^- channel blocker, had no effect. In the mucosa pre-stimulated with forskolin, adenosine did not show any additive effect, whereas carbachol resulted in a synergistic potentiation of the I_{SC} response. The adenosine response was inhibited by 10 μM H-89, an inhibitor of protein kinase A. These results suggest that the adenosine-stimulated I_{SC} response is mediated by basolateral to apical Cl^- secretion through a cAMP-dependent Cl^- channel. The rank order of potencies of adenosine receptor agonists was 5'-(N-ethylcarboxamino)adenosine(NECA) > N^6-(R-phenylisopropyl)adenosine(R-PIA) > 2-[p-(2-carbonylethyl)-phenyl-ethylamino]-5'-N-ethylcarboxaminoadenosine(CGS21680). From the above results, it can be concluded that adenosine interacts with the A_{2b} adenosine receptor in the rabbit colon mucosa and a cAMP-dependent signalling mechanism underlies the stimulation of Cl^- secretion.

Key Words: Adenosine, cAMP, Rabbit colon mucosa

INTRODUCTION

An important property of the intestine is its ability to secrete fluid. Fluid secretion into the intestinal lumen is required for a number of intestinal functions including the passage of intestinal contents, digestion and absorption of the nutrients (Barrett, 1993). The importance of intestinal secretion is noted in the pathological conditions such as cystic fibrosis and secretory diarrhea. The primary ion transport mechanism driving fluid secretion in the intestine is Cl^- secretion, which is a key electrolyte transport mechanism in a variety of organs including airway, biliary, renal, and pancreatic systems. During the process of Cl^- secretion, Cl^- is taken up across the basolateral membrane of the cells via Na-K-Cl cotransporters and exits the cells across the apical membrane via Cl^- channels (Barrett, 1993).

The intestinal secretion is regulated by a number of substances including vasoactive intestinal peptide

Corresponding to: Sang Ho Lee, Department of Physiology, College of Medicine, Pusan National University, Ami-Dong 1-10, Suh-Gu, Pusan 602-739, Korea

(VIP) (Racusen & Binder, 1977), ATP (Mason et al, 1991) and inflammatory mediators (Timothy et al, 1993). One of the most important secretagogues is adenosine during inflammation. Neutrophils or eosinophils, togethered by inflammation, secrete 5'-adenosine monophosphate (5'-AMP), which then is degraded by ecto-5'-nuceotidase to adenosine, which then increases Cl^- secretion in the intestinal muscosa (Madara et al, 1993). However, the controversy concerning the underlying mechanism of adenosine-stimulated Cl^- secretion in intestinal epithelial cells continues. Some investigators insist that adenosine-stimulated Cl^- secretion is mediated by the cAMP-dependent pathway (Londos et al, 1980; Strohmeier et al, 1995). While, it was denied by several groups in view of the experimental results that adenosine agonists increase Cl^- secretion at the concentration at which the intracellular cAMP concentration does not increase (Dho et al, 1992; Madara et al, 1992; Madara et al, 1993).

To investigate the underlying mechanim of adenosine-stimulated Cl^- secretion in the rabbit colon mucosa, we examined effects of a variety of Cl^- channel blockers, the interaction of other secretagogues with adenosine, and effects of H-89, a specific protein kinase A inhibitor.

METHODS

Materials

New Zealand White rabbits of both sexes, weighing 1.5~2.0 kg, were used. 5'-(N-ethylcarboxamino) adenosine (NECA), N^6-(R-phenyl-isopropyl) adenosine (R-PIA) and 2-[p-(2-carbonylethyl)-phenylethyl-amino]-5'-N-ethylcarboxaminoadenosine (CGS 21680) were purchased from RBI (Natick MA). cAMP measurement kits were obtained from Amersham (Arlington Heights, IL). Other agents were purchased from Sigma (St. Louis, MO).

Electrical measurement

New Zealand white rabbits (1.5~2.0 kg) were anesthetized with intraperitoneal injection of pentobarbital sodium (120 mg/100 g b.w.) and segments of descending colon were isolated. The outer muscle layers were stripped off by blunt dissection and the partial mucosal strip preparations were mounted vertically in a modified Ussing chamber. Transepithelial voltage was clamped using automatic voltage clamp (DVC-1000, WPI) and short circuit current (I_{SC}) and transepithelial potential difference were recorded. Before experiments, fluid resistance and input resistance were corrected. Transepithelial conductance was calculated from I_{SC} and transepithelial potential difference. Standard Ringer's solution (composition in mM: Na^+ 140, K^+ 5, Ca^{2+} 1.4, Mg^{2+} 1.0, Cl^- 130, HCO_3^- 24, HPO_4^{2-} 1.3, $H_2PO_4^-$ 0.3, glucose 5) was used as the bathing medium unless stated otherwise. Cl^--free solution contained gluconate instead of Cl^-. These solutions were gassed with a mixture of 95% O_2 and 5% CO_2 resulting in a pH of 7.4. The experiments were performed at 37°C.

Measurement of intracellular cAMP concentration

For determination of tissue cAMP levels, epithelia were stripped from the underlying muscle layers with a slide glass and incubated at 37°C in reaction tubes containing 5 ml Ringer's solution stirred by a gas stream of 95% O_2 and 5% CO_2. Incubation was terminated by submerging tissues in ice-cold 6% trichloroacetic acid. The tissues were homogenized and centrifuged (2000 *g*, 15 min, 4°C). After centrifugation, supernatants were extracted four times with water-saturated diethyl ether. Aliquots of the aqueous phase were dried at 50°C under a stream of N_2. Cyclic AMP was then determined using commercially available kits based on competitive binding (Amercham, England). Protein was measured by the method of Bradford (1976).

Data analysis

The data were presented as the mean±S.E. The difference between two mean values was evaluated by Student's *t*-test (unpaired comparison). A value of $p<0.05$ was considered statistically significant.

RESULTS

To examine the effect of adenosine on Cl^- secretion in the rabbit colon mucosa, we observed changes in I_{SC}. The isolated distal colon mucosa absorbs Na^+ by an electrogenic process that accounts for the spontaneous, serosa-positive transepithelial

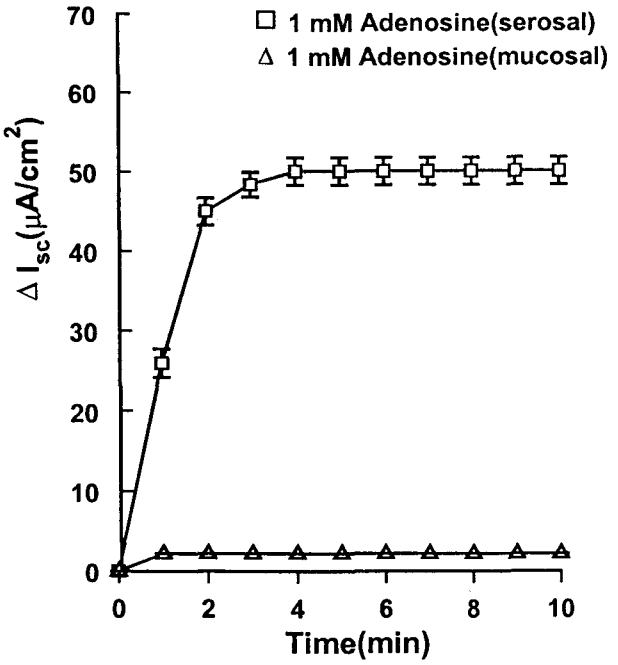

Fig. 1. Effect of adenosine on I_{SC} in the rabbit colon mucosa. The mucosa was mounted in a modified Ussing chamber and allowed to equillibrate for 30 min before exposure to the experimental protocol. Adenosine (1 mM) was added into the mucosal(△) or serosal (□) bathing solution. ΔI_{sc} indicates the difference between the baseline and the peak value of I_{SC} at each time. Values shown are the means±S.E. of 4 experiments.

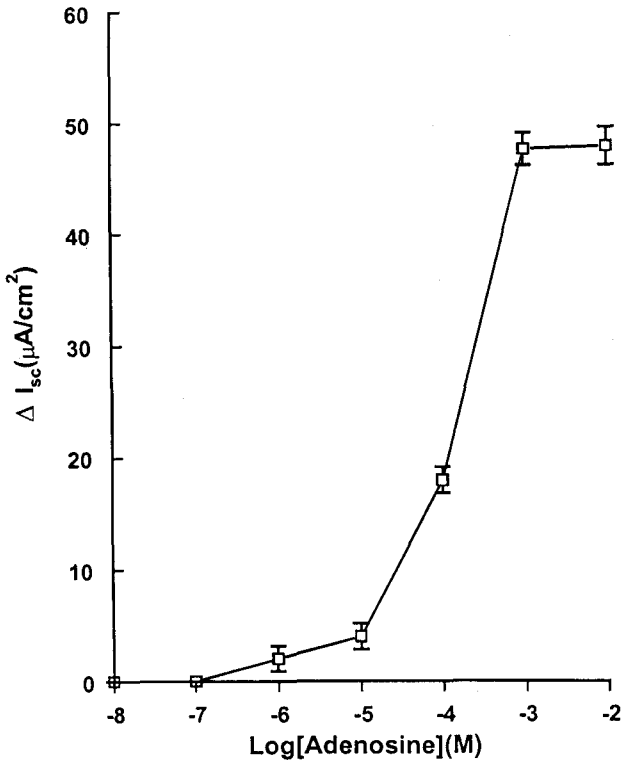

Fig. 2. Dose-dependent changes of the adenosine-stimulated I_{SC} response in the rabbit colon mucosa. Values shown are the means±S.E. of 4 experiments.

electrical potential difference (PD) and the basal I_{SC} across the epithelium. The distal colon mucosa mounted in the Ussing chambers showed the stable basal PD and I_{SC}. In the standard Ringer's solution, the distal colon mucosa had 6.7±3.3 mV of basal PD and 32±5 $\mu A/cm^2$ of basal I_{SC}. The serosal treatment of adenosine increased I_{SC} rapidly, whereas the mucosal treatment had no effect. The adenosine-stimulated I_{SC} response was a biphasic response, consisting of a rapid, transient increase and a following steady-state phase (Fig. 1). The adenosine-stimulated I_{SC} response was dose-dependent (Fig. 2). The maximum response was reached at 1 mM and ED_{50} was 0.3 mM. To determine if the adenosine response is mediated by submucosal nerves tetrodotoxin was treated. Tetrodotoxin did not block the adenosine response (data not shown).

To confirm that the adenosine-stimulated I_{SC} response is mediated by an increase of the transepithelial transport of Cl^-, the following experiments were carried out. The effect of depletion of Cl^- in the standard Ringer's solution on the adenosine response was determined. For the Cl^--depleted solution, sodium gluconate and potassium gluconate were added instead of sodium chloride and potassium chloride respectively in the standard Ringer's solution so that Cl^- was completely substituted by gluconate. The Cl^- depletion inhibited 95.2±4.3% of the adenosine response (Fig. 3). As the transepithelial transport of Cl^- is dependent on the intracellular Cl^- gradient produced by the Na-K-Cl cotransporter, the effect of bumetanide, an inhibitor of this cotransporter, was examined. Preincubation for 10 min with 100 μM bumetanide inhibited 86.3±4.8% of the adenosine response (Fig. 3). To rule out the involvement of Na^+ current in the change of I_{SC}, the effect of amiloride was tested. The mucosal addition of 100 μM amiloride did not affect the adenosine response although the basal I_{SC} decreased from 32±5 to 23±5 $\mu A/cm^2$ (Fig. 3). These results indicate that the adenosine-stimulated I_{SC} response is mediated by the increase of the transepithelial transport of Cl^-.

To determine which type of Cl^- channel is involved in the adenosine response, the effects of

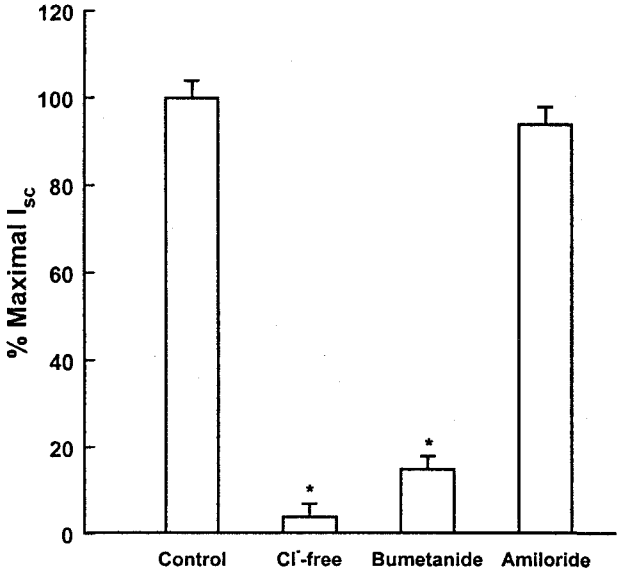

Fig. 3. Dependency of the adenosine-stimulated I_{SC} response on the presence of Cl^- gradient. For the depletion of Cl^-, NaCl and KCl in the soluition were replaced with Na^+- and K^+-gluconate, respectively. Bumetanide (10 μM) or amiloride (0.1 mM) was added into the serosal or mucosal bathing solution 10 min before the exposure to adenosine. *, $p<0.001$ with respect to the control. Values shown are means$\pm$S.E. of 4 experiments.

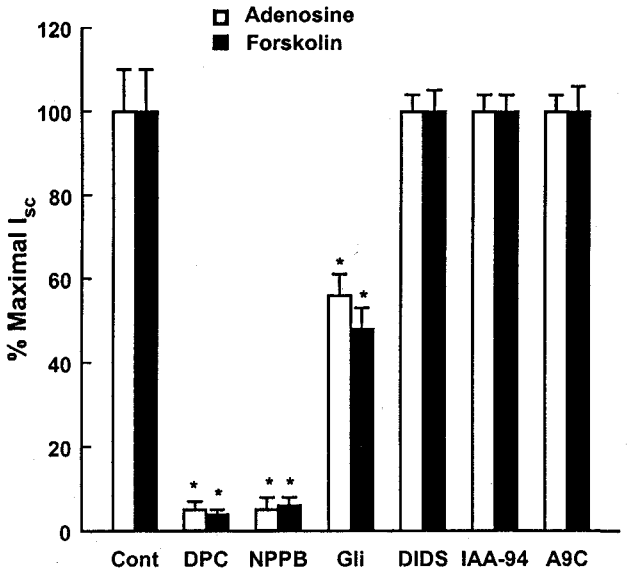

Fig. 4. Effects of Cl^- channel blockers on 1 mM adenosine-(□) or 10 μM forskolin (■)-stimulated I_{SC} responses. Concentrations of inhibitors were : 1 mM DPC, 0.1 mM NPPB, 1 mM glibenclamide(Gli), 0.1 mM DIDS, 0.1 mM IAA-94 and 1 mM A9C. Data are presented as the percent of the control. *, $p<0.001$ with respect to the control. Values shown are means$\pm$S.E. of 3 experiments.

several Cl^- channel blockers were examined. The adenosine response was inhibited by dephenylamine-2-carboxylate (DPC, 1 mM), 5-nitro-2-(3-phenyl-propyl-amino)-benzoate (NPPB, 0.1 mM) and glibenclamide (1 mM) by 95$\pm$2%, 95$\pm$3% and 44$\pm$5%, respectively. However, indanyloxyacetic acid (IAA-94, 0.1 mM), anthracene-9-carboxylate (A9C, 1 mM) and 4,4-diisothiocyanato-stilbene-2,2-disulphonate (DIDS, 0.1 mM) had no effect (Fig. 4). Cystic fibrosis transmembrane regulator (CFTR) might be a candidate to fit this inhibition pattern (Anderson et al, 1992).

To determine whether the adenosine response is mediated by the specific receptor, we tested effects of adenosine receptor antagonists. Caffeine or theophylline blocked the adenosine response (Fig. 5), indicating that the adenosine response is mediated by binding of adenosine to its receptor.

Several well-known agonists for the adenosine receptors were tested to determine which subtype of adenosine receptor was involved. The rank order of the potencies of the adenosine receptor agonists was NECA > 2-[p-(2-carbonylethyl)-phenylethyl-amino]

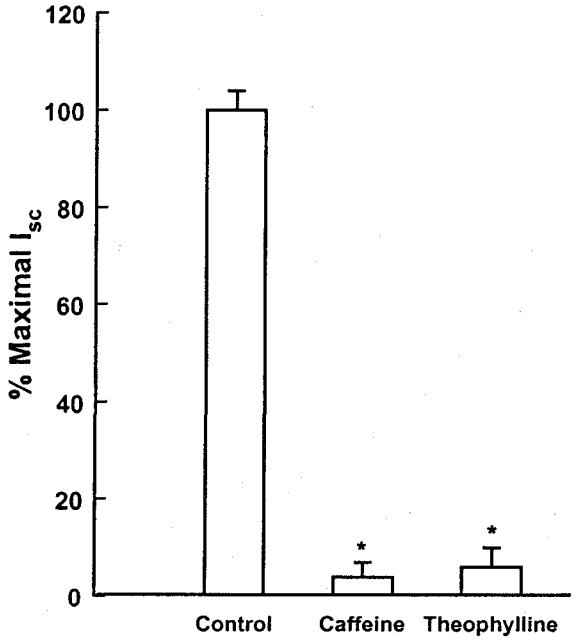

Fig. 5. Inhibition of the adenosine-stimulated I_{SC} response by adenosine receptor blockers. Caffeine (0.1 mM) or theophylline (0.01 mM) was added into the serosal bathing solution before the exposure to adenosine. *, $p<0.001$ with respect to the control value. Values shown are means$\pm$S.E. of 4 experiments.

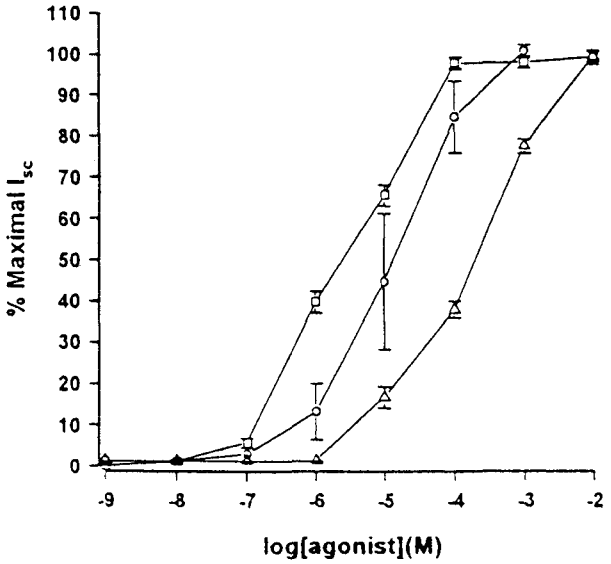

Fig. 6. Dose-response curve for I_{SC} responses stimulated by adenosine receptor agonists. NECA(□), R-PIA(○), and CGS21680(△) were added into the serosal bathing solution. Data are presented as the percent of the maximal I_{sc} response observed in each experiment. Values shown are means±S.E. of 3 experiments

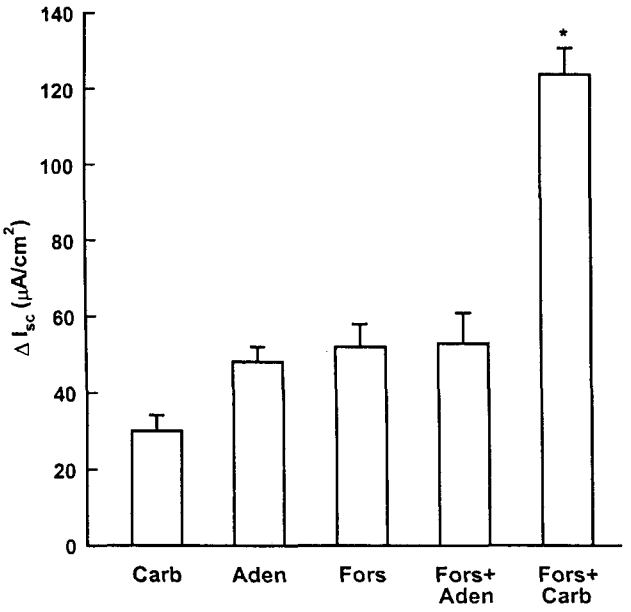

Fig. 7. Interaction of secretagogues in the rabbit colon mucosa. Adenosine (Aden, 1 mM) or carbachol (Carb, 10 μM) was added into the serosal bathing solution 5 min after the addition of forskolin (Fors, 5 μM) into the same side. *, $p<0.001$ with respect to the values in the presence of forskolin alone. Values shown are means± S.E. of 4 experiments.

Table 1. Tissue content of cAMP under control conditions and after 25 min exposure to adenosine. Values shown are means±S.E. of 3 experiments

Condition	cAMP (pmol/mg protein)	Isc ($\mu A/cm^2$)
Basal state	5.1±0.5	32±8
Adenosine 0.3 mM	5.4±0.6	56±5*
Adenosine 1 mM	7.2±0.5*	80±6*

*, $p<0.05$ with respect to the control value.

-5'-N-ethyl-carboxaminoadenosine(CGS21680) > N6-(R-phenylisopropyl)-adenosine (R-PIA). ED_{50} of the agonists were 2.7 μM, 10.5 μM and 0.14 mM, respectively (Fig. 6). A_{2b} adenosine receptor fits this pattern of agonist specificity (Vanhoutte et al, 1994).

To determine whether the adenosine response was dependent on the adenylyl cyclase activity, the adenosine response was examined in the mucosa in which adenylyl cyclase was fully stimulated with 5 μM forskolin. In this preparation, adenosine did not show an additive effect on the I_{SC}. This is in sharp contrast with the effect of carbachol which resulted in synergistic stimulation of the I_{SC}. (Fig. 7). In intestinal epithelial cells, the synergism with cAMP pathway of the carbachol response, which is mediated by Ca^{2+} pathway, was reported by many previous studies although the mechanism was not clearly elucidated (Barrett, 1993). Thus, these results indicate that the action of adenosine is mediated mostly by cAMP pathway but not Ca^{2+} pathway. To confirm the second messenger of adenosine receptor in the rabbit colon mucosa, we measured intracellular cAMP concentration. As presented in Table 1, adenosine failed to increase intracellular cAMP content at 0.3 mM which is ED_{50} to stimulate I_{SC}. At 1 mM, at which concentration it shows maximum stimulation of I_{SC}, adenosine resulted in 1.4 fold increase of cAMP content. As a slight increase of the cAMP concentration can activate protein kinase A, we tested the effect of H-89, an inhibitor of protein kinase A. H-89 (50 μM) inhibited the adenosine and forskolin response by 55.5±7.9% and 45.8±8.8%, respectively, whereas did not affect the carbachol response (Fig. 8).

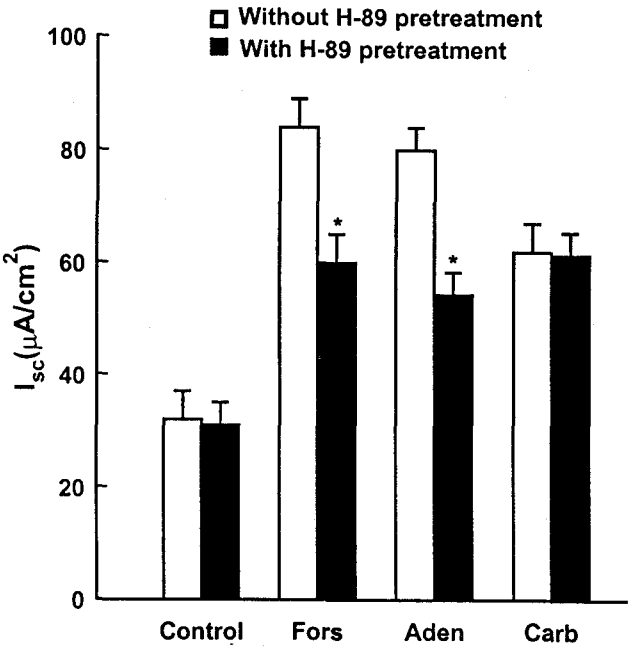

Fig. 8. Effect of H-89 on forskolin-, adenosine- and carbachol-stimulated responses. H-89 was added 10 min before the application of 5 μM forskolin (Fors), 1 mM adenosine (Aden) or 10 μM carbachol (Carb). *, $p < 0.001$ with respect to the control. Values shown are means ± S.E. of 4 experiments.

DISCUSSION

Adenosine, which is derived from mast cell or neutrophil, is an important mediator of diarrhea in the various inflammatory diseases of colon (Madara et al, 1993). However, the underlying mechanism of the action of adenosine is controversial. The present study showed that adenosine interacts with the A_{2b} adenosine receptor and stimulates Cl^- secretion via a cAMP-dependent pathway in the rabbit colon mucosa.

Adenosine increased I_{SC} in a dose-dependent manner from the basolateral side only. Such an increase of I_{SC} was blocked by replacement of the bath solution with Cl^--free solution, Cl^- channel blockers, or bumetanide, but not by amiloride. These results indicate that basolateral to apical Cl^- secretion is responsible for the adenosine-stimulated I_{SC} in this study.

The pharmacological profiles of agonist-induced stimulation showed that the adenosine receptor present in the basolateral membrane of rabbit colon epithelium is the A_{2b} adenosine receptor. Studies using mammalian expression systems indicated adenosine receptors are linked to adenylyl cyclase (Linden et al, 1993; Londos et al, 1980; Pierce et al, 1992). The study using T84, a human colon cancer cell line, revealed that the subtype of adenosine receptor in the intestine was A_{2b} and adenosine increased Cl^- secretion through the cAMP pathway (Strohmeier et al, 1995). However, other investigators suggested that the signal transduction pathway of adenosine receptor was not linked to cAMP, cGMP or intracellular calcium pathway (Dho et al, 1992; Madara et al, 1992; Madara et al, 1993). In those studies, NECA, the most potent adenosine receptor agonist, increased Cl^- secretion at the concentration at which NECA did not increase the intracellular cAMP concentration. In the present study, several results indicate that the adenosine response is mediated by the cAMP pathway. The adenosine response disappeared in the mucosa pre-stimulated with 5 μM forskolin. It suggests strongly that the adenosine response is mediated by the cAMP pathway. The agents which act through the cAMP pathway can not increase the cAMP concentration further more after the pretreatment with 5 μM forkolin because 5 μM forskolin fully increases the cAMP concentraton in intestinal epithelial cells. This result also indicates that Ca^{2+} pathway is not involved in the adenosine response because the Ca^{2+}-mediated response is well known to be potentiated by cAMP pathway (Barrett, 1993).

In the present study, adenosine did not increase the cAMP concentration at ED_{50}, to stimulate I_{SC}. It is hard to explain this discrepancy, however several explanations might be possible. First, the adenosine response may be mediated by a new pathway other than cAMP pathway. For example, the increase of I_{SC} is caused by release of arachidonic acid (Kim & Timothy, 1993). Second, cAMP is rapidly degraded by phosphodiesterase. Thus, it is difficult to measure the cAMP concentration exactly (Barrett et al, 1990). Third, a slight increase of intracellular cAMP can efficiently induce Cl^- secretion (Madara et al, 1992). Fourth, the second messenger system of the adenosine receptor may be multiple like the muscarinic or α-adrenergic receptor (Dagmara & Lal, 1992; Cotecchia et al, 1990). In the present study, H-89, a well-known inhibitor of protein kinase A, inhibited the forskolin and adenosine responses but not the carbachol response. This result suggests that a slight increase of cAMP concentration can activate efficiently protein kinase A in the rabbit colon mucosa.

In conclusion, the results of this study indicate that adenosine increases Cl^- secretion via A_{2b} adenosine receptor and the cAMP pathway is responsible for the signalling mechanism in the rabbit colon mucosa.

ACKNOWLEDGEMENT

This reserch was performed by the support of Pusan National University Fund (1995).

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