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Molecular identification and pharmacological characterization of adenosine receptors in the guinea-pig colon

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1 The aim of this study is to elucidate the role of adenosine in the motor function of the guinea-pig distal colon.

2 To determine whether adenosine A_1 receptors and A_{2B} receptors are expressed in the guinea-pig colon, we employed the reverse transcription-polymerase chain reaction (RT–PCR). The gene expression of A_1 receptor and A_{2B} receptor was found for the first time in the guinea-pig proximal and distal colon.

3 Adenosine A_1 agonist N^6-cyclopentyladenosine (CPA), and A_1/A_2 agonist 5′-N-ethylcarboxamidoadenosine (NECA) concentration-dependently inhibited neurogenic responses to electrical field stimulation ($EC_{50} = 1.07 \times 10^{-8}$ and 2.12×10^{-8} M) in the longitudinal muscle, but A_{2A} agonist 2-p-(2-carboxyethyl)phenylethylamino-5′-N-ethycarboxamido-adenosine (CGS21680) had only a slight inhibitory effect (25.9%, 1 μM). A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 nM: A_1 selective concentration) antagonized responses to CPA and NECA. Furthermore, the affinity order of antagonists at inhibiting the effect NECA was: DPCPX > 8-phenyltheophylline (8-PT: A_1/A_2 antagonist).

4 In the presence of tetrodotoxin (TTX, 0.3 μM), CPA and NECA relaxed myogenic precontraction induced by KCl (50 mM) ($EC_{50} = 1.26 \times 10^{-5}$ and 1.04×10^{-5} M, respectively), but CGS21680 (1 μM) did not cause any relaxation. DPCPX did not affect responses to CPA and NECA at a concentration of 10 nM, but a higher concentration (1 μM) of DPCPX and 10 μM of 8-PT antagonized those responses.

5 These data lead us to the hypothesis that adenosine may mediate relaxation through two different inhibitory receptor subtypes; A_1 receptors on the enteric neuron and A_{2B} receptor on the smooth muscle in the guinea-pig distal colon.

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Abbreviations: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; RT–PCR, reverse transcription-polymerase chain reaction; RT, reverse transcriptase; bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EFS, electrical field stimulation; CPA, N^6-cyclopentyladenosine; NECA, 5′-N-ethylcarboxamidoadenosine; CGS21680, 2-p-(2-carboxyethyl)phenylethylamino-5′-N-ethylcarboxamido-adenosine; 8-PT, 8-phenyltheophylline.

Introduction

The role of adenosine 5′-triphosphate (ATP) and its breakdown product adenosine in the enteric nervous system has been studied by various investigators since Burnstock *et al.* (1970) postulated the presence of purinergic inhibitory nerves modulating the contractility of the gastrointestinal smooth muscle.

Adenosine receptors have been subdivided into four distinct subtypes, A_1, A_{2A}, A_{2B} and A_3, based on the orders of agonist potency and antagonist affinity, G-protein coupling mechanisms, cellular responses and receptor cloning studies (see Collis & Hourani, 1993; Fredholm *et al.*, 1994; Feoktistov & Biaggioni, 1997 for reviews). A_2 receptors appear to be exclusively coupled to the activation of adenyl cyclase, whereas it is now known that A_1 receptors are coupled to the inhibition of adenyl cyclase and moreover can act through other effectors such as the stimulation of phospholipase C, activation of K^+ channels and inhibition of Ca^{2+} channels. Regarding the newly identified A_3 receptors, little is so far known about their signal transduction cascades, and although these receptors are probably coupled to the inhibition of adenyl cyclase, they are insensitive to the selective A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX).

The function of adenosine in the bowel motility is poorly understood (Bailey & Hourani, 1992; Poli *et al.*, 1996; Nicholls *et al.*, 1996; Nicholls & Hourani, 1997; Coupar, 1999), in particular, in the colonic motor function (Bailey & Hourani, 1992). Furthermore, with the exception of A_{2B} receptor, it has not yet been molecularbiologically determined whether adenosine receptor subtypes are present in the colon.

This study was undertaken to investigate whether the mRNA of adenosine A_1 and A_{2B} receptors could be expressed in the guinea-pig colon and whether these adenosine receptor subtypes could affect the motor function of the guinea-pig distal colon.

Methods

Collection of tissues

Male Hartley guinea-pigs weighing 350–460 g were used. Animals were provided with food and water *ad libitum*, and when used for experiments were stunned by a blow to the head

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and exsanguinated. The distal colon was identified as the part of the colon between the hypogastric flexure and the pelvic brim, and the distal part of the distal colon at 5–20 cm proximal from the pelvic brim, was rapidly removed. A segment of the proximal colon was taken 10–15 cm from the ileocecal junction.

Reverse transcription–polymerase chain reaction (RT–PCR) probing for adenosine A_1 receptor and A_{2B} receptor expression

Tissue samples (506–540 mg) were obtained from the distal and proximal colon and the cerebral cortex of guinea-pigs. Each sample was homogenized in 8 ml Trizol Reagent (GIBCO BRL, Grant Island, U.S.A.) to extract total RNA. Homogenates were mixed with 1.6 ml chloroform, incubated for 3 min at room temperature and centrifuged at 10,000 × *g* for 15 min. RNA was precipitated from the supernate by adding the same volume of isopropanol, incubated for 10 min at room temperature, and centrifuged at 10,000 × *g* for 15 min. The RNA pellet was washed with 70% ethanol, centrifuged and resuspended in distilled water. The concentration of RNA was measured spectrophotometrically. Two μg of total RNA were reverse-transcribed to cDNA, by using 4 μl of 20 mM dNTPs (the mixture of all four 2′-deoxynucleoside 5′-triphosphates, Pharmacia, Uppsala, Sweden), 4 μl of 0.009 OD/μl random primer (GIBCO BRL), and 400 U M-MLV reverse transcriptase (RT, GIBCO BRL). The sample (total volume: 40 μl) was incubated at 42°C for 90 min, followed by incubation at 95°C for 5 min and chilling at 0°C. The control sample was processed as above except that the RT was omitted to confirm the absence of contaminating genomic DNA.

The first strand cDNA from the reverse transcription was amplified by polymerase chain reaction (PCR). A 20 μl sample of the diluted cDNA solution (×5) was 30 μl of PCR master mixture, consisting of 1.5 mM $MgCl_2$, 200 μM dNTPs (Pharmacia), 1 μM each primer and 1.25 U Taq DNA polymerase (Toyobo, Osaka, Japan). The initial denaturation was conducted at 95°C for 2 min, followed by primer annealing for 2 min at 60°C, primer extension at 72°C for 2 min, and denaturation at 95°C for 1 min. After the end of the PCR cycles, the reaction mixture was kept at 72°C for 10 min and then brought to room temperature. The PCR product was analysed electrophoretically on 2% agarose gel.

The adenosine A_1 receptor gene of the guinea-pig was amplified for 40 cycles with sense primer nucleotide sequences of 325–348 (5′-GTC AAG ATC CCT CTC CGG TAC AAG-3′) and antisense primer of 702–725 (5′-AGG AAG AGG ATG AGG GCC AGC GAC-3′) of the guinea-pig adenosine A_1 receptor. The adenosine A_{2B} receptor gene of the guinea-pig was amplified for 30 cycles with sense primer of 333–353 (5′-CAG GTA TAA AGG TTT GGT CAC-3′) and antisense primer of 622–639 (5′-AAG CTG CTT GCA GGC CAC-3′) of the rat adenosine A_{2B} receptor sequence. The primer set for the adenosine A_{2B} receptor of the guinea-pig was based on regions that could be expected to be conserved between rats and guinea-pigs, because guinea-pig adenosine A_{2B} receptors have not yet been cloned. The predicted size of the PCR products for target RNAs of adenosine A_1 receptor and adenosine A_{2B} receptor was 401 and 307 base pairs (bp), respectively. The total RNA was also subjected to RT–PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as a housekeeping gene. The sense primer was 756–779 (5′-GAA CGG GAA GCT CAC TGG CAT GGC-3′) and antisense primer was 1052–1074 (5′-TGA GGT CCA CCA CCC TGT TGC TG-3′) of the rat GAPDH nucleotide sequence. The expected product size was 311 bp.

Following RT–PCR, amplified adenosine A_1 or A_{2B} receptor cDNA was digested at 37°C for 1 h with the specific endonucleases (A_1 receptor; *Alu*I, A_{2B} receptor; *Aat*I and *Sac*I, Toyobo). The digestion products were then separated on 2% agarose gel.

Preparation of tissues

Four segments, each ~3 cm in length, were dissected from the anal end of the guinea-pig distal colon. The segment was suspended in the longitudinal direction in Krebs-Ringer solution (in mM): NaCl 120, KCl 6, $MgCl_2$ $6H_2O$ 1.2, $NaH_2PO_4H_2O$ 1.2, $NaHCO_3$ 14.4, $CaCl_2$ $2H_2O$ 2.5 and glucose 11.5 and bubbled with a gas mixture of 5% CO_2-95% O_2 and allowed to stabilize for at least 40 min. A resting tension of 0.5 g was applied to the tissue and force was recorded with load and displacement transducer (UL-10GR: Minebea, Nagano, Japan). The tissue was then exposed to 50 mM KCl to give a moderate stimulation for approximately 90 s, washed and allowed to re-attain baseline tension for 30 min before electrical field stimulation (EFS), KCl precontraction or addition of acetylcholine (ACh).

Electrical field stimulation

EFS was used to investigate the role of adenosine receptors in the myenteric neurons. Bipolar rectangular pulses were passed between two parallel platinum electrodes 1 cm apart. One of the electrodes was set in the lumen of the distal colon. The rectangular pulses were used of 1 ms duration at a frequency of 10 Hz for 1 s every 1 min to evoke contractions. The voltage was set to produce a submaximal contraction. When a steady contraction was observed, effects of adenosine agonists [adenosine A_1 agonist: N^6-cyclopentyladenosine (CPA); A_1/A_2 agonist: 5′-N-ethylcarboxamidoadenosine (NECA); A_{2A} agonist: 2-p-(2-carboxyethyl)phenylethylamino-5′-N-ethylcarboxamido-adenosine (CGS21680)] were evaluated by cumulative application. In the study using adenosine antagonists [adenosine A_1 antagonist: DPCPX; A_1/A_2 antagonist: 8-phenyltheophylline (8-PT)], the tissue was incubated with each antagonist for ~30 min and each was present during the construction of the subsequent concentration-response curve. These antagonists had no effect on the contractions by themselves. The agonist induced changes in the height of the contractions were calculated as percentage of the contraction heights obtained before the application of the agonist.

High KCl-induced precontraction

Preparations were exposed to KCl (50 mM) to evoke a non-neuronal precontraction in the presence of tetrodotoxin (TTX, 0.3 μM). When a stable tonic contraction had been established, the experiments were carried out in the same way as in the EFS study. Responses were measured as decreases in the tension and expressed as percentage relaxation of the KCl-induced tone.

ACh-induced contraction

ACh (0.1 μM) was added to the bath and washed when a peak contraction was observed. This procedure was conducted in a 6 min cycle until a consistent contraction was obtained. The adenosine agonist was added to the bath,

and 3 min later ACh was added again. The agonist-produced changes in the height of the ACh-induced contractions were calculated as percentage of the contraction heights obtained before the application of the agonist.

Data analysis

The results are expressed as mean ± s.e.mean. EC_{50} values were calculated as the concentration inducing 50% relaxation of the agonist in the absence of the antagonist by the linear regression analysis of the logit-log plot of concentration-response curve. Concentration-ratios were calculated from the ratio of the individual EC_{50} value in the absence and presence of antagonist. Apparent pA_2 values were calculated as the negative logarithm of the molar concentration of the antagonist divided by the concentration-ratio -1. Probability values of 0.05 or less were considered statistically significant.

Drugs

The following drugs were purchased from the suppliers indicated: N^6-cyclopentyladenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA), acetylcholine chloride (ACh), and atropine methyl bromide from Sigma Chemical, St. Louis, U.S.A.; 2-p-(2-carboxyethyl)phenylethylamino-5′-N-ethylcarboxamido-adenosine (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and 8-phenyltheophylline (8-PT) from Research Biochemicals International, Natick, U.S.A.; tetrodotoxin (TTX) from Sankyo Co., Tokyo, Japan. CPA, NECA, CGS21680 and TTX were dissolved in distilled water, and DPCPX and 8-PT in dimethyl sulphoxide. The maximal final concentration of dimethyl sulphoxide was 0.1%, which did not have any effect on the responses of the preparation.

Results

RT–PCR for adenosine A_1 receptor and A_{2B} receptor expression

To determine whether adenosine A_1 and A_{2B} receptors are expressed in the guinea-pig colon, we used RT–PCR. mRNA extracted from the guinea-pig cerebral cortex, which is well known to abundantly express A_1 receptors (Meng *et al*., 1994), was used as a positive control tissue to verify that the RT–PCR products corresponded to mRNA encoding A_1 receptors. As shown in Figure 1, RT–PCR analysis showed single expression with almost the predicted product size (401 bp) in both the proximal and distal colon as well as the cerebral cortex. Furthermore, in comparison with the expression of GAPDH in the same tissue, it was speculated that the mRNA of A_1 receptors might be more abundant in the proximal colon than the distal colon.

Likewise, A_{2B} receptor mRNA was detected as single expression with almost the expected product size (307 bp) in both the proximal and distal colon, and relative amounts of the signals were almost the same in these tissues.

No detectable expression was observed when the RT was omitted in the PCR analysis. Moreover, the specificity of each PCR amplification was confirmed by the restriction digest.

Electrically evoked contraction

EFS induced contractions (2.79 ± 0.24 g, $n=15$) in the guinea-pig distal colon which were completely blocked by TTX (0.3 μM) or atropine (1 μM), suggesting that the contractions are due to the excitation of cholinergic neurons. The A_1 agonist, CPA exerted the most potent inhibitory effect ($EC_{50} = 1.07 \times 10^{-8}$ M, $n=4$) on the neurogenic contractions. A potent effect ($EC_{50} = 2.12 \times 10^{-8}$ M, $n=4$) was also observed with the mixed A_1/A_2 agonist NECA, but the selective A_{2A} agonist CGS21680 had little effect (25.9% inhibition at 1 μM, $n=4$). The rank order of agonist potency was CPA ≥ NECA > > CGS21680 (Figure 2). DPCPX (10 nM: A_1 selective concentration, $n=4$) caused a parallel shift to the right of the concentration-response curve to CPA, giving a concentration-ratio of 10.5, corresponding to an apparent pA_2 value of 9.0. DPCPX (10 nM, $n=4$) also gave a parallel rightward displacement of the concentration-response curve to NECA with a concentration-ratio of 7.8, corresponding to an apparent pA_2 value of 8.8, whereas a much higher concentration (1 μM, $n=4$) of A_1/A_2 antagonist 8-PT caused a somewhat

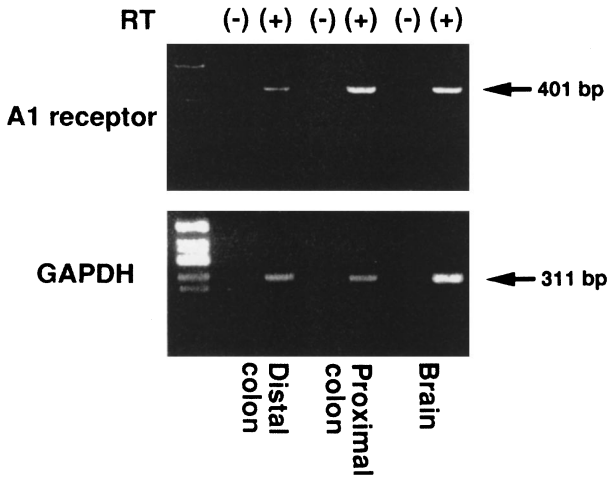

Figure 1 Expression of adenosine A_1 receptor mRNA in the guinea-pig colon as examined by RT–PCR. RT was omitted to confirm the absence of contaminating genomic DNA in control samples (−). mRNA extracted from the cerebral cortex was used as a positive control to verify that the RT–PCR products correspond to mRNA encoding adenosine A_1 receptors. Furthermore, RT–PCR for GAPDH mRNA serves as a housekeeping gene.

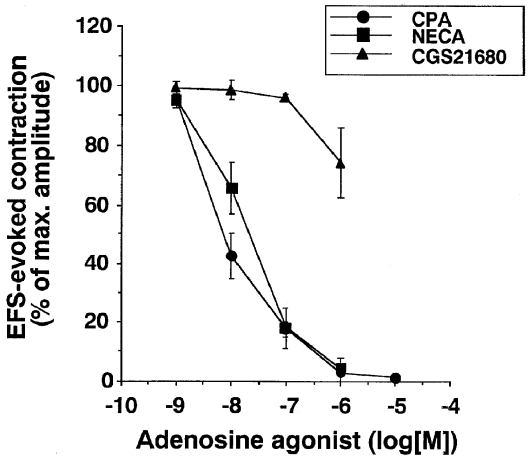

Figure 2 Mean cumulative concentration-response curves for the inhibition of EFS (1 ms pulse duration, 10 Hz for 1 s)-evoked contraction of the guinea-pig distal colon by adenosine agonists. Responses are plotted as percentage of the maximum amplitude of the contraction. Bars indicate s.e.mean. $n=4$ for each agonist.

smaller shift with a concentration-ratio of 3.9, corresponding to an apparent pA_2 value of 6.5 (Figure 3).

High KCl-induced pre-contraction

Inhibitory responses were quantified by pre-contracting the tissue with high K^+ (3.48 ± 0.18 g, $n=39$) before challenge with the purines. CGS21680 (1 μM) did not cause any relaxation of high K^+-induced precontraction in the guinea-pig distal colon, but CPA and NECA relaxed the precontracted distal colon with equi-potency [EC_{50}: 1.26×10^{-5} M ($n=4$) and 1.04×10^{-5} M ($n=6$)] (Figure 4). The nanomolar range of DPCPX (10 nM: A_1 selective concentration) had no effect on the concentration-response curve of CPA ($n=4$) and NECA ($n=5$) (Figures 5 and 6). The micromolar range of DPCPX (1 μM) shifted the concentration-response curve to CPA and NECA to the right. The respective concentration-ratio was 10.5 ($n=5$) and 4.9 ($n=6$), corresponding to an apparent pA_2 value of 7.0 to CPA and 6.6 to NECA (Figures 5 and 6). The relaxation responses to NECA were antagonized by 8-PT (10 μM, $n=5$) with a concentration-ratio of 6.2, giving an apparent pA_2 value of 5.7 (Figure 5).

ACh-induced contraction

ACh produced a submaximal contraction (2.55 ± 0.17 g, $n=23$) in the guinea-pig distal colon. Neither CPA nor NECA at a concentration of 100 nM affected the ACh-induced contractions ($96.7 \pm 1.9\%$, $n=5$ and $101.6 \pm 5.1\%$, $n=5$).

Discussion

A binding study has speculated the existence of adenosine A_1 binding sites using the A_1-selective ligand [^{3}H]-DPCPX in rat duodenum and colon (Peachey *et al*., 1994), but the adenosine

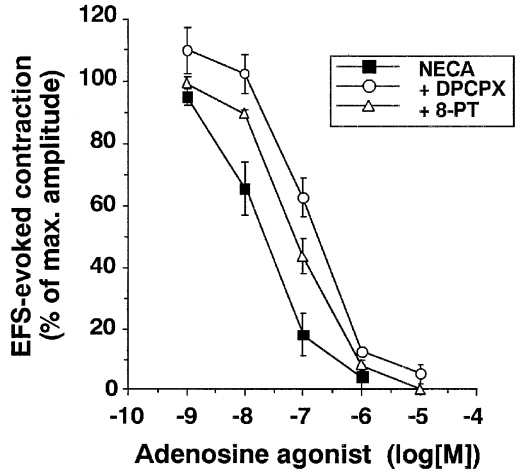

Figure 3 Suppression of EFS (1 ms pulse duration, 10 Hz for 1 s)-evoked contraction of the guinea-pig distal colon by NECA in the absence or presence of DPCPX (10 nM) or 8-PT (1 μM). Each point is the mean with s.e.mean of four determinations.

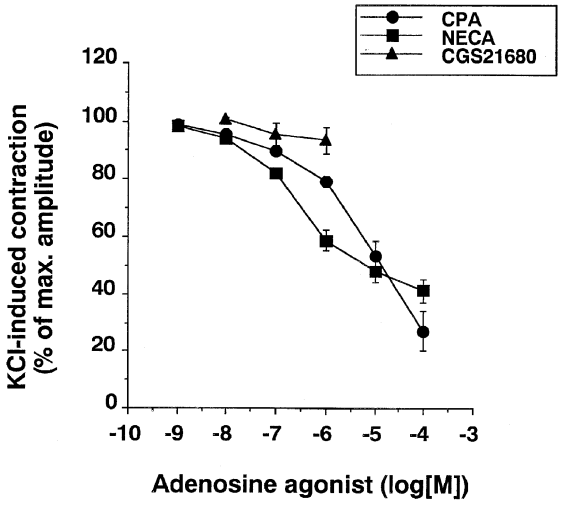

Figure 4 Mean cumulative concentration-response curves for the relaxation of KCl-induced precontraction of the guinea-pig distal colon by adenosine agonist. Responses are plotted as percentage of the maximum amplitude of the precontraction. Bars indicate s.e.mean. $n=4-6$ for each agonist.

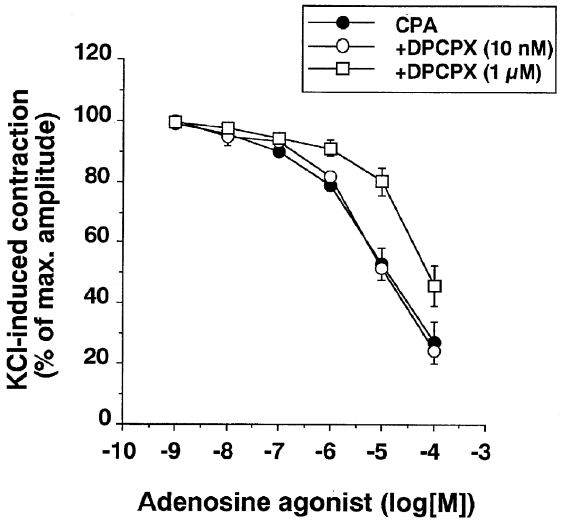

Figure 5 Relaxation of KCl-induced precontraction by CPA in the absence or presence of DPCPX (10 nM or 1 μM). Each point is the mean with s.e.mean of four or five determinations.

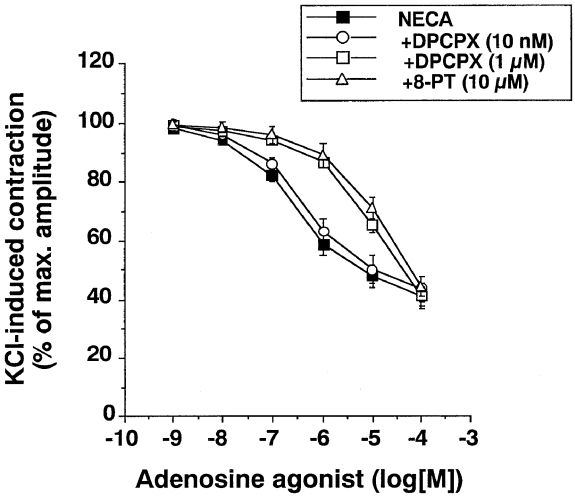

Figure 6 Relaxation of KCl-induced precontraction by NECA in the absence or presence of DPCPX (10 nM or 1 μM) or 8-PT (10 μM). Each point is the mean with s.e.mean of five or six determinations.

receptors, especially in the gastrointestinal tract, have been mainly investigated with functional methods. Recently, the expression of the adenosine receptors has been examined in the rat by use of RT–PCR (Dixon *et al*., 1996) and Northern blot analysis (Stehle *et al*., 1992). The A_1 receptor gene is expressed in the entire brain area and also in a wide variety of peripheral tissues (heart, aorta, spleen, kidney, liver, testis, eye and bladder), but the amount of amplifide product is lower than that in the brain. Very low levels of amplification product are detected in the lung and stomach, while none are observed in the skeletal muscle, jejunum and proximal colon (Dixon *et al*., 1996). By use of Northern blot analysis, A_1 receptor mRNA cannot be found in the gastrointestinal tract (Stehle *et al*., 1992), whereas in contrast, A_1 receptors have functioned in the rat duodenum (Nicholls *et al*., 1996), ileum (Nicholls & Hourani, 1997; Coupar, 1999) and colonic muscular mucosa (Bailey *et al*., 1992). Likewise in the guinea-pig tissues when Northern blot analysis is used, A_1 receptor mRNA is found in the brain, heart, spleen and kidney, but not in the stomach and intestine (Meng *et al*., 1994), even though an A_1 receptor has exerted an inhibitory effect in the duodenum (Poli *et al*., 1996). Therefore, A_1 receptors have been only functionally characterized and it has never been proved that mRNA of A_1 receptors are distributed in the colon. The result of the RT–PCR presented here showed for the first time that A_1 receptor gene expression could be detected in the proximal and distal colon, which might be due to being able to employ 40 cycles for PCR amplification without non-specific signals instead of 30 cycles as used by Dixon *et al*. (1996). This finding can resolve the discrepancy between functional data and molecular biological data on the A_1 receptor in the bowel.

On the other hand, a widespread distribution of A_{2B} receptor mRNA has been shown in the rat CNS and periphery (Stehle *et al*., 1992; Dixon *et al*., 1996). In the rat gastrointestinal tract where A_{2B} receptors can function (Bailey & Hourani, 1992; Nicholls *et al*., 1996), the highest amount of amplification product is observed in samples from the proximal colon, with lower levels in the stomach and lowest levels in the jejunum (Dixon *et al*., 1996). In addition, it has been demonstrated that the A_{2B} receptor is detected throughout the human colon with Northern blot analysis (Strohmeier *et al*., 1995). The present result could confirm an abundant expression of A_{2B} receptor mRNA in the colon.

These molecular biological studies can allow the elucidation of adenosine actions in those tissues where the receptors are obviously expressed. A_1 and A_{2B} receptor expression found in the distal colon of guinea-pig motivated great interest in defining their function and precise location. We thus used the criteria of agonist order of potency to characterize the adenosine receptor subtypes in the distal colon. The present result in the EFS experiment shows that in the distal colon, all the adenosine receptor agonists used inhibited the neurogenic contractions in the order of CPA$\geqslant$NECA$>>$CGS21680, which best complies with the rank order of agonist potency that represents activation of the A_1 receptor: CPA$>$NECA$>>$CGS21680 (Collis & Hourani, 1993; Feoktistov & Biaggioni, 1997). Furthermore, in the previous reports for the EFS experiment, CPA and NECA are potent in activating A_1 receptors with an EC_{50} in the low nanomolar range [CPA: 7.45×10^{-9} M in the rat ileum (Coupar, 1999) and 7×10^{-9} M in the guinea-pig ileum (Nitahara *et al*., 1995), NECA: 1.68×10^{-8} M in the rat ileum (Coupar, 1999)], which is almost consistent with the values of EC_{50} obtained here (CPA: 1.07×10^{-8} M, NECA: 2.12×10^{-8} M).

Another criterion used in this study to differentiate between A_1/A_{2B} receptors was the relative affinities of DPCPX and 8-PT (Collis & Hourani, 1993). DPCPX has about 160 and 60 fold greater affinity than 8-PT for A_1 receptors in the EFS experiment when CPA is used as agonist in the guinea-pig ileum (Nitahara *et al*., 1995) and when NECA is used as agonist in the rat ileum (Coupar, 1999), respectively, whilst DPCPX is approximately 5 fold more potent than 8-PT as an antagonist of adenosine A_{2B} receptor in the guinea-pig cerebral cortex (Alexander *et al*., 1989). In the current studies, DPCPX at the nanomolar range of 10 nM, shifted the concentration-response curve of NECA to the right with an apparent pA_2 value of 8.8, and was much more effective at inhibiting NECA-evoked responses than 8-PT (an apparent pA_2 value of 6.5), which is consistent with the previous reports for A_1 receptor (Nitahara *et al*., 1995; Coupar, 1999). This pA_2 value of 8.8 for DPCPX is similar to the apparent pA_2 value of 9.3 using the same agonist in the EFS experiment of the rat ileum (Coupar, 1999). In addition, the apparent pA_2 value of 9.0 for DPCPX against CPA is in close agreement with the corresponding value for the guinea-pig ileum (a pA_2 value of 9.0, Nitahara *et al*., 1995) and the rat ileum (a pK_b value of 9.2, Coupar, 1999), which further pharmacologically confirms the presence of the A_1 receptor.

None of adenosine agonists modified the post-synaptic contractile responses to exogenous ACh, even at the concentration greatly inhibiting EFS-evoked responses. The result of the present experiment could eliminate the possibility of a post-synaptic A_1 receptor site of action of adenosine agonists. Taken all together, the present findings can demonstrate the presence of functional A_1 receptor subtype at pre-synaptic site coupled negatively to the release of ACh.

The $A2_A$ selective agonist CGS21680 has been useful in differentiating between A_{2A} and A_{2B} receptor subtypes. CGS21680 is virtually ineffective on A_{2B} receptors but is as potent as NECA in activating A_{2A} receptors, with an EC_{50} in the low nanomolar range for both agonists (Feoktistov & Biaggioni, 1997). The present result shows that CGS21680 (1 μM) has no effect on high K^+-induced precontraction in the guinea-pig distal colon, even though NECA behaves as an agonist with an EC_{50} in the micromolar range (1.04×10^{-5} M), thus suggesting the absence of A_{2A} receptor subtype in the distal colon. CPA also concentration-dependently relaxed the precontraction; nevertheless the EC_{50} value of CPA (1.26×10^{-5} M) is not in the nanomolar range, but micromolar range. Accordingly, taking the lack of effect of 10 nM DPCPX (a concentration selective for blocking the A_1 receptor) on the responses to CPA and NECA together, the contribution of the A_1 receptor can be discarded.

However, the order of potency does not fall clearly into either subtypes as NECA and CPA are almost equipotent. The potency order is actually more like that reported for adenosine A_3 receptor subtype (NECA$\geqslant$CPA). It is well known that CGS21680 can activate rat A_3 receptor at approximately 0.5 μM and that the receptor is highly resistant to xanthine antagonists such as DPCPX and 8-PT even at concentrations of 10 and 100 μM (Van Galen *et al*., 1994). In the present study, DPCPX acted as an antagonist against NECA at a dose of 1 μM, indicating that the A_3 receptor subtype does not seem to be involved in the adenosine agonist-mediating relaxation.

Furthermore, DPCPX (1 μM), in a concentration high enough to block A_{2B} receptor (Alexander *et al*., 1989; Nicholls *et al*., 1996), caused a parallel shift to the right of the concentration-response curves to CPA and NECA with apparent pA_2 values of 7.0 and 6.6, respectively, which are in reasonable agreement with the apparent pA_2 values of 6.6 and 6.4 previously reported for A_{2B} receptors in rat duodenum muscularis mucosa (Nicholls *et al*., 1996). 8-PT (10 μM) also

gave a parallel rightward displacement of the concentration-response curve to NECA with an apparent pA_2 value of 5.7, and was 8 fold less potent than DPCPX, which is in good agreement with the previous reports for A_{2B} receptor (about 5 fold, Alexander *et al*., 1989). Taken all together, we can identify the post-synaptic inhibitory adenosine A_{2B} receptor on the longitudinal muscle layer of the guinea-pig distal colon.

Previous functional experiments on the rat A_1 receptors have showed that adenosine agonists relax the intact intestine and the longitudinal muscle through A_1 receptors located on the smooth muscle in the rat ileum (Nicholls & Hourani, 1997), which is a conflicting result with the present findings of the localization of A_1 receptors. The most possible explanation is that the reason might be due to such species differences as observed in a previous report that inhibitory A_1 receptors are situated on cholinergic nerve endings innervating the circular muscle of rat ileum, but not guinea-pig ileum (Coupar, 1999). However, the present result in the guinea-pig distal colon is consistent with the circular responses of the rat ileum rather than the guinea-pig ileum. On the other hand, the rat A_{2B} receptors are located on the smooth muscle and their activation relax the longitudinal muscle of both the distal colon (Bailey & Hourani, 1992) and the duodenum (Nicholls *et al*., 1996), which is in good agreement with the present result. In addition, it has been demonstrated from studies using the guinea-pig small intestine that adenosine can act at pre-synaptic A_1 receptors to suppress synaptic neurotransmission (Christofi & Wood, 1993; 1994), and the release of ACh, substance P and neurokinin A in the myenteric plexus (Broad *et al*., 1992; Nitahara *et al*., 1995; Moneta *et al*., 1997), and twitch responses (Poli *et al*., 1996). Taking the present findings together with the previous results, it could be postulated that two different adenosine receptor subtypes could mediate relaxations in the guinea-pig distal colon; pre-synaptic A_1 receptors on the enteric neurons and post-synaptic A_{2B} receptors on the longitudinal smooth muscle.

The molecularbiological and pharmacological findings in this study raise the possibility that the endogenous substance adenosine could well-regulate the colonic motility through different inhibitory receptor subtypes with different localization in physiological and/or certain pathophysiological conditions.

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