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Functional characterization of the adenosine receptor mediating inhibition of intestinal secretion

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1 Previous studies have shown that the mixed A_1/A_2 adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) inhibits intestinal fluid secretion which is thought to contribute to its antidiarrhoeal effect in the rat. The aim of this study was to characterize the adenosine receptor mediating this antisecretory effect via functional studies using a range of selective agonists and antagonists and by applying the pharmacological criteria of relative agonist and antagonist potencies.

2 Adenosine agonists and antagonists were administered i.v. to anaesthetized rats. Intestinal secretion was then stimulated by i.a. infusion of vasoactive intestinal peptide (VIP, 0.8 $\mu g\ min^{-1}$) and the net fluid transport across the wall of the jejunum was measured by a recirculation technique.

3 The rank order of agonist potency to reduce the response to VIP was: NECA > N^6-cyclopentyladenosine (CPA) > R-N^6-(2-phenylisopropyladenosine) (R-PIA) > S-PIA > chloroadenosine (2-CADO) > 2-phenylaminoadenosine (CV-1808). This order best complies with the rank order of agonist potency that represents activation of the recently described A_{2B} receptor: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1808 > = CGS-21680. The most potent agonists (NECA, CPA and R-PIA) had ED_{50} values in the low microgram range.

4 The anitsecretory action of NECA (submaximal dose of 40 $\mu g\ kg^{-1}$) was antagonized equally (approximately 50%) by the selective adenosine antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 $mg\ kg^{-1}$) and 8-phenyltheophylline (8-PT, 0.1 $mg\ kg^{-1}$). This equipotent activity indicates the presence of an A_2 and not an A_1 receptor.

5 It is suggested that adenosine A_{2B} receptor agonists could be evaluated for potential use as antidiarrhoeal drugs.

Keywords: Adenosine receptors; rat intestine; antisecretory; antidiarrhoeal; vasoactive intestinal peptide (VIP); *in vivo*; fluid transport; NECA; A_{2B} receptor.

Introduction

Two subclasses of adenosine receptors were initially defined on the basis of inhibition (A_1) or stimulation (A_2) of adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation (Van Calker *et al.*, 1979). However, it is now known that adenosine receptors are not linked to adenylyl cyclase activity in all cases (Jacobson, 1990). Structure-activity relationship studies have led to the development of selective agonist and antagonists for A_1 and A_2 receptors (Jacobson *et al.*, 1992) which together with radioactive ligands have created a definitive receptor classification based on agonist potency order and antagonist affinity order (Collis & Hourani, 1993). There is also increasing evidence from biochemical, functional and receptor cloning studies supporting the existence of further receptor subtypes: A_1, A_{2A}, A_{2B} and A_3 (Girdlestone & Watson, 1994).

The A_1 receptor is differentiated from the A_2 receptor by the selective A_1 agonist, N^6-cyclopentyladenosine (CPA) (Lohse *et al.*, 1988) and the selective A_1 antagonist 1,3 -dipropyl-8-cyclopentylxanthine (DPCPX) (Bruns *et al.*, 1987a). The existence of the A_1 receptor is further established since it has been cloned from bovine (Olah *et al.*, 1992), rat (Mahan *et al.*, 1991) and human (Libert *et al.*, 1992) brain. The selective A_2 receptor ligand, CGS-21680, is used to characterize A_2 receptor-mediated tissue responses (Collis & Hourani, 1993).

The possible existence of A_2 receptor subtypes was first introduced by Daly *et al.*, (1983) on the basis of regional differences in affinity orders of adenosine analogues and high and low affinity binding sites for adenosine in the rat brain. Bruns *et al.* (1986) later proposed that the high affinity A_2 receptor be designated A_{2A} and the low affinity receptor be designated A_{2B}. Further studies found that the agonist CGS-21680 (Jarvis *et al.*, 1989) and the antagonist PD-115199 (Bruns *et al.*, 1987b) have high affinity at the A_{2A} compared to the A_{2B} receptor. Unfortunately, selective ligands specific for A_{2B} receptors have not yet been developed. At present the A_{2B} receptor is identified by the low affinity of several A_{2A}-selective ligands where the agonist potency order is: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1801 > = CGS-21680 and antagonist affinity order is DPCPX = 8-PT > = PD-115199 (Collis & Hourani, 1993). Molecular cloning and expression of proteins which correspond to A_{2A} (Furlong *et al.*, 1992) and A_{2B} receptors (Pierce *et al.*, 1992) provides further evidence for the existence of A_2 receptor subtypes.

In 1986, Riberio and Sebastio first suggested a third class of adenosine receptor, the A_3 receptor. However, the existence of this receptor has been challenged (Carruthers & Fozard, 1993). The more readily accepted A_3 receptor is that proposed by Zhou and colleagues in 1992, which has been cloned from rat striatum and is characterized by similar binding affinities of NECA and R-PIA. However, the alkylxanthine antagonists, such as DPCPX at concentrations as high as 100 μM did not significantly bind to this receptor.

Identification of receptor subtypes provides an opportunity to develop therapeutic drugs with selective action. However, functional studies have fallen behind the biochemical studies to date. For instance, studies to determine the therapeutic potential of adenosine receptor agonists in the treatment of diarrhoea would be of value. This is highlighted by the finding that the non-selective adenosine agonist, NECA, is a potent inhibitor of morphine withdrawal diarrhoea in rats (Dionyssopoulos *et al.*, 1992). The diarrhoea that is produced on opiate with-

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drawal in this species is associated with a decrease in intestinal fluid absortion (Chang *et al.*, 1984) and increased intestinal motility (Brown *et al.*, 1988). Recently we have established that NECA exerts its antidiarrhoeal effect by inhibiting both prostaglandin and vasoactive intestinal peptide (VIP)-induced intestinal fluid secretion as well as inhibiting peristalsis (Coupar & Hancock, 1994). The specific aim of this study was to characterize the receptor mediating one of these effects, namely inhibition of intestinal fluid secretion, using a range of metabolically stable adenosine analogues with different potencies and degrees of specifity for adenosine receptor subtypes.

Some of these results have been communicated to the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists (Coupar & Hancock, 1993).

Methods

The method of measuring fluid transport by the rat jejunum follows that described in detail by Coupar in 1985. A brief outline is given below.

Surgical procedure

Hooded Wistar rats of either sex (200–290 g weight) were fasted over night. They were anaesthetized with pentobarbitone sodium (60 mg kg^{-1} s.c.), which has been shown to be appropriate for intestinal secretion experiments (Coupar, 1985). A cannula was introduced into the left jugular vein for administration of the adenosine agonist and/or antagonist in saline (0.1 ml 100 g^{-1}) or in the case of the controls, saline or vehicle. Another cannula was introduced into the left common carotid artery for constant intra-arterial (i.a.) infusion of VIP (0.8 $\mu g\ min^{-1}$) in saline to induce near maximum fluid secretion (Coupar, 1985), or saline as the control to measure the basal absorptive value. The rates of infusion were 40 $\mu l\ min^{-1}$. Mean systemic blood pressure was recorded to monitor the condition of the rats from a side-arm off the carotid cannula by means of a Statham pressure transducer connected to a Neomedix Neotrace recorder.

A recirculation technique was used to measure the net fluid transport rate of the jejunum. A 25 to 30 cm section of the jejunum, starting distal from the Ligament of Trietz, was continuously perfused with 8 ml of an iso-osmotic solution containing (mM): NaCl 148, KCl 5, dextrose 5.5 and phenol red 0.05, as a non-absorbable water marker. The solution was contained in a reservoir maintained at 37°C and recirculated through the lumen of the jejunum for 20 min by a gas-lift column of moistened 5% CO_2 in O_2.

The drug administration sequence for testing adenosine agonists commenced with the i.v. injection of the adenosine agonist or vehicle as the control. This was followed at 5 min with i.a. infusion of VIP, or saline as the control with mean systemic blood pressure recorded. The segment of jejunum was washed before intestinal perfusion was started at 10 min. At 30 min the fluid from the intestine was collected for analysis and the weight of the intestine perfused was measured. The testing of the adenosine antagonists followed a similar order to that of the above, except that the antagonist or its vehicle as the control was injected i.v. 5 min prior to injecting a submaximal dose of NECA (40 $\mu g\ kg^{-1}$) or saline as the control.

VIP was chosen to induce intestinal secretion in the present studies, since it is present in intrinsic enteric nerves that project to the mucosa and thought to be involved in fluid transport control. In addition it is associated with certain neuroendocrine-related diarrhoeas such as pancreatic cholera (Brown & Miller, 1991).

Analytical procedure

Samples were centrifuged and then diluted with buffer and peak absorbances were measured at 560 nm as well as 520 and 600 nm to correct for non-specific interferences as described by Miller & Schedl (1972). The results were expressed as the amount absorbed(+) or secreted(−) in $\mu l\ g^{-1}$ weight of tissue during the 20 min perfusion.

Secretory potency

The secretory potency of VIP was monitored routinely since it showed a slight variation in its effect over the 6 months of experiments. Consequently, separate secretory base lines to VIP were established for each adenosine agonist and the antagonists tested.

Statistical analysis

The rates of water transport are given as means ± s.e.mean. The potency of each adenosine agonist is expressed as an ED_{50} value, which is the concentration that elicits a half-maximal effect on the net water transport between the range of the VIP-induced secretion to the basal level of absorption with a 95% confidence interval using linear regression analysis (Tallarida & Murray, 1987).

Pairs of means were compared for statistical differences using Student's two tailed *t* test and series of means were compared to common controls using Dunnett's t test (Tallarida & Murray, 1987). The criterion for statistical significance was set at $P < 0.05$.

Drugs

The following were used: pentobarbitone sodium (Nembutal, Boehringer Ingelheim, Artarmon, Australia), vasoactive intestinal peptide (VIP; AUSPEP, Melbourne, Australia).

Agonists: chloroadenosine (2-CADO, mol. wt. = 301.7), N^6-cyclopentyladenosine (CPA, mol. wt. = 335.4), 2-phenylaminoadenosine (CV-1808, mol. wt. = 358.4), 5'-N-ethylcarboxamidoadenosine (NECA, mol. wt. = 308.3), **R**-(−)-N^6-(2-phenylisopropyl) adenosine (**R**-(−)-PIA, mol. wt. = 385.4) and **S**-(+)-N^6-(2-phenylisopropyl) adenosine (**S**-(+)-PIA, mol. wt. = 385.4). All were obtained from RBI, Natick, U.S.A.

All agonists and VIP were dissolved in 0.9% w/v saline except CV-1808 and CPA which were dissolved in 1:1 ethanol:saline solution and then diluted with saline to give the required dose. The higher dose of CV-1808 (3125 $\mu g\ kg^{-1}$) was dissolved in 90% ethanol and diluted with saline. The vehicle had no significant effect on VIP-induced secretion ($P > 0.05$, Student's two tailed *t* test).

Antagonists: 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, RBI, Natick, U.S.A.) and 8-phenyltheophylline (8-PT, RBI, Natick, U.S.A.) were dissolved in 1% v/v dimethylsulphoxide 0.75% v/v 1 M NaOH in saline. This vehicle had no significant effect of VIP-induced secretion.

Results

The rate of fluid absorption from the jejunum of rats infused with saline i.a. as a control was 239 ± 24 $\mu l\ g^{-1}$ wet weight in 20 min ($n = 6$) and this was reversed by the i.a. infusion of VIP which induced a large net secretion of fluid into the lumen. The secretory response to VIP was monitored for each drug tested. All adenosine agonists tested, except CV-1808, produced a dose-related reversal of the VIP-induced secretory response (see Figure 1). The most potent agonist, NECA displayed a high efficacy at 100 $\mu g\ kg^{-1}$ but this dose did not fully restore fluid transport to the basal level of absorption (Figure 1, $P = 0.03$, two tailed *t* test). A dose of CV-1808 (750 $\mu g\ kg^{-1}$) failed to inhibit VIP-induced secretion ($P = 0.08$, two tailed *t* test) and even at a higher dose (3125 $\mu g\ kg^{-1}$), CV-1808 caused only a small inhibition (Figure 1, $P = 0.04$, two tailed *t* test). The potency of each adenosine agonist is shown in Table 1.

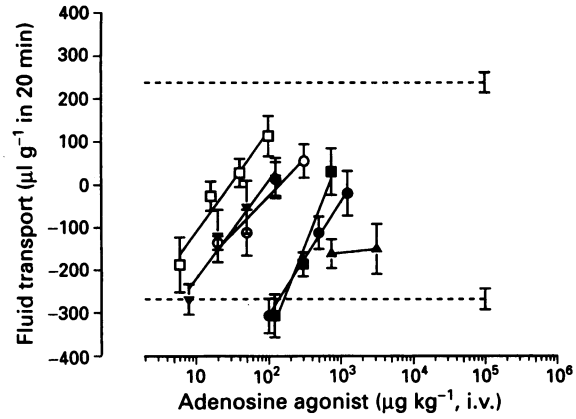

Figure 1 Inhibition by NECA (□), CPA (▼), R-PIA (○) S-PIA (■), 2-CADO (●) and CV-1808 (▲) of intestinal secretion induced by vasoactive intestinal peptide (VIP). The lower non-continuous line indicates the overall net fluid secretion rate (µl g^{-1} wet tissue in 20 min, $n = 21$) in response to i.a. infusion of VIP (0.8 µg min^{-1}). Basal rates of secretion for each adenosine agonist tested are not shown for clarity. The top non-continuous line indicates the absorption rate (i.a. infusion and i.v. injection of saline as controls); s.e.means are indicated. For abbreviations, see text.

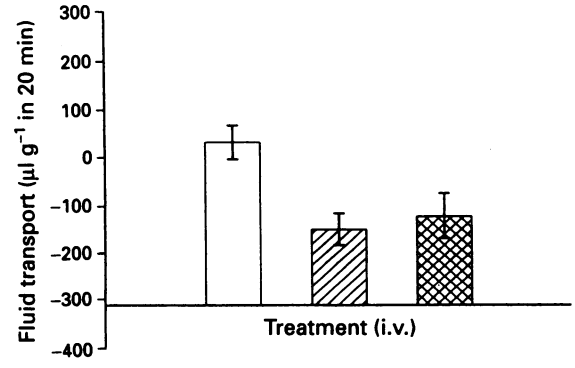

Figure 2 Effect of the adenosine antagonists (0.1 mg kg^{-1}), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (hatched column) and 8-phenyltheophylline (8-PT) (cross hatched column) on the antisecretory action of 5'-N-ethylcarbox-amidoadenosine (NECA, 40 µg kg^{-1}, open column); s.e. means are indicated. The bottom continuous line represents the secretion rate (-316 ± 40 µl g^{-1}) in response to i.a. infusion of VIP (0.8 µg min^{-1}). Both antagonists caused a significant inhibition of NECA ($P < 0.05$, Dunnett's t test).

Adenosine antagonists, 8-PT (0.1 mg kg^{-1}, i.v.) and DPCPX (0.1 mg kg^{-1}, i.v.) significantly inhibited the antisecretory response of NECA (40 µg kg^{-1}, i.v. Figure 2, $P < 0.05$, Dunnett's t test). Neither antagonist affected the basal secretory response to VIP (control secretory response = -316 ± 40 ($n = 7$), DPCPX = -273 ± 54 ($n = 5$), 8-PT = -376 ± 74 ($n = 5$), $P > 0.05$, Dunnett's t test).

Discussion

The potencies and efficacies of both adenosine agonists and antagonists reported in this *in vivo* study suggest that the A_{2B} adenosine receptor is involved in controlling intestinal antisecretory activity in the rat jejunum. This is based on the classification table outlined in the review by Collis & Hourani (1993) which is a proposed framework to aid in the classification of functional adenosine receptors.

Dionyssopoulos *et al.* (1992) found that the adenosine agonists, R-PIA, NECA but not CV-1808 were potent inhibitors of opiate withdrawal diarrhoea in rats. These

Table 1 The potencies of adenosine agonists at inhibiting vasoactive intestinal peptide (VIP)-induced intestinal secretion

Agonist	ED_{50} (µg kg^{-1})	*95% C.I.*, n	*Basal VIP-induced secretion* (µl g^{-1}) ± s.e.mean, n
NECA	21	1.4, 20	-327 ± 22, 7
CPA	74	2.4, 15	-275 ± 31, 10
R-PIA	107	2.3, 25	-285 ± 33, 9
S-PIA	588	1.6, 12	-302 ± 40, 7
2-CADO	1184	2.2, 8	-282 ± 30, 4
CV-1808	>3125	-, 14	-297 ± 33, 9

For abbreviations, see text.

authors concluded that the antidiarrhoeal response was due to the activation of A_1 receptors since at the time R-PIA was classified as a selective A_1 agonist, NECA as a non-selective A_1/A_2 agonist and CV-1808 a selective A_2 agonist (Burnstock & Buckley, 1985; Stone, 1985; Bruns *et al.*, 1986). These results correlate with those of the present study, where NECA had the most potent antisecretory effect and high doses of CV-1808 were ineffective at restoring VIP-induced intestinal secretion to the control level of absorption. An additional feature of the present study is that a greater range of agonists as well as antagonists was tested since the modern classification of adenosine receptors is based on the rank order of both agonist potency and antagonist affinity.

The agonists used in this study inhibited VIP-induced secretion in the order of NECA > CPA > R-PIA > S-PIA > 2-CADO > CV-1808 which best complies with the rank order of agonist potency that represents activation of the recently described A_{2B} receptor: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1808 > = CGS-21680 (see review by Collis & Hourani, 1993). The low potency of 2-CADO in this study could be due to its poor chemical stability (Jacobson, 1990) and the above classification order being derived mainly from *in vitro* techniques. CPA displayed potent antisecretory activity closely following that of NECA. Unfortunately CPA does not appear in the A_{2B} receptor classification but there is no evidence in the literature to suggest that CPA is without activity at the A_{2B} receptor.

The rank order of agonist potency reported in this study next best complies with the A_1 receptor classification where the agonist potency order is: CPA > R-PIA = CHA = > NECA > 2-CADO > S-PIA > CV-1808 > = CGS-21680 (Collis & Hourani, 1993). Both S-PIA and CV-1808 are the least potent, with S-PIA the stronger of the two in both the A_1 and A_{2B} receptor classification which agrees with the results of this study. CPA does appear in the A_1 but not the A_{2B} receptor classification where it is more potent than NECA. However, the results from this study show the reverse of this order. In addition, R-PIA is equipotent or more potent than NECA in the A_1 receptor classification, which is not true for this study since NECA was significantly more potent than R-PIA.

The agonist potency order of the A_{2A} receptor is: CGS21680 = NECA > CV-1808 > = 2-CADO > R-PIA = CHA = CPA > S-PIA (Collis & Hourani, 1993). This order is almost the reverse of the order of the agonists as antisecretory agents, except for NECA which was the most potent. For instance, the least potent agonist in this study CV-1808 is second in line to that of NECA in the A_{2A} receptor classification. The potency of the selective A_{2A} agonist, CGS-21680 was not investigated in this study since the agonists studied did not follow the A_{2A} order of potency but rather the A_{2B} or A_1 where CGS-21680 is the least potent in both classifications. The A_3 receptor was discounted because the agonists R-PIA and NECA are equipotent in the A_3 classification (Collis & Hourani, 1993) whereas NECA inhibits VIP-induced secretion to a significantly greater extent

than R-PIA in this study. In addition, xanthine antagonists, such as those used in this study have low affinity at the A_3 receptor.

The classification of adenosine receptors is also dependent on antagonist affinity order (Collis & Hourani, 1993). The adenosine antagonists, 8-PT and DPCPX were specifically investigated to reinforce the receptor classification based on agonist potencies in this study, since they are able to differentiate between A_1 and A_2 receptors. For instance, binding studies (see review by Van Galen *et al.*, 1992) have established that 8-PT shows a small degree of selectivity for A_1 compared to A_2 sites (K_i = 86 nM at A_1 and 850 nM at A_2) while DPCPX in contrast is highly selective for A_1 sites (K_i = 0.5 nM at A_1 and 340 nM at A_2). The difference between the affinities and selectivity ratios of the two antagonists is also apparent in intact tissues where 8-PT is non-selective for A_1 and A_2 receptors, whilst DPCPX has a 30–50 fold greater affinity for A_1 but is equi-effective with 8-PT at A_2 receptors (Collis *et al.*, 1989). Even though much of the data on these antagonists is derived from *in vitro* studies their use should be valid *in vivo* since in the conscious rat the duration of action of DPCPX is 3–5 h (Collis *et al.*, unpublished results) and 8-PT is up to 5 h (Bowmer *et al.*, 1986). In this study a dose of 0.1 mg kg^{-1} of DPCPX was investigated since both Kellett *et al.* (1989) and Collis *et al.* (1991) established that this low dose was effective at inhibiting the adenosine-mediated responses of bradycardia and diuresis in the rat. DPCPX was thought to block A_1 receptors since a much higher dose of 8-PT was required to achieve a similar inhibition of adenosine-induced responses. In the present study, NECA, the most potent agonist, was inhibited by approximately 50% by both DPCPX and 8-PT at doses of 0.1 mg kg^{-1}. This finding discounts the possibility that the adenosine agonists are inhibiting VIP-induced secretion via the A_1 receptor, since DPCPX failed to exert a more potent inhibitory effect than 8-PT. This equipotent antagonist activity suggests the presence of an A_2 receptor (Collis *et al.*, 1989) and together with the agonist potency order, more precisely indicates the presence of an A_{2B} receptor subtype (Collis & Hourani, 1993). However, at this stage a definitive receptor characterization cannot be completed until the tissue location of these receptors is determined and *in vitro* studies are performed.

An incidental finding of the study was that both DPCPX and 8-PT were free of intrinsic pro-secretory activity at the doses employed. This suggests that endogenous adenosine is not released to inhibit secretion under the conditions of the presently described experiments. However, the possibility that the antagonist doses were not high enough to unmask the presence of endogenous adenosine activity cannot be discounted.

The clinically used opiate antidiarrhoeal drugs such as difenoxin and loperamide have ED_{50} values of 0.23 mg kg^{-1} and 0.5 mg kg^{-1} respectively for inhibiting VIP-induced secretion in the rat jejunum (De Luca & Coupar, 1993). However, in this study the adenosine agonists NECA (ED_{50} = 21 μg kg^{-1}) and CPA (ED_{50} = 74 μg kg^{-1}) prove to be more potent antisecretory drugs since they have ED_{50} values in the microgram range. In addition, De Luca & Coupar (1993) found that the highest dose of both difenoxin (3.1 mg kg^{-1}) and loperamide (1.5 mg kg^{-1}) tested were unable to restore fluid transport further than zero net fluid transport. However, this study shows that the highest dose of NECA investigated (100 μg kg^{-1}) had a higher efficacy compared to the clinically used antidiarrhoeal drugs.

We suggest that adenosine A_{2B} receptor agonists could be evaluated for potential use as antidiarrhoeal drugs. This proposal is based on the present results where selectivity of the A_{2B} receptor was achieved with agonist analogues displaying high potency and efficacy in inhibiting intestinal fluid secretion in the rat.

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