Characterization of the adenosine receptor mediating contraction in rat colonic muscularis mucosae

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1 The objective of this study was to characterize the adenosine receptor mediating contraction in rat isolated colonic muscularis mucosae (RCMM).

2 Sequential additions of the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA; 0.01–10 µM) elicited reproducible, concentration-related contractions in RCMM. The effects of NECA were mimicked by the adenosine A₁ receptor-selective agonists cyclopentyladenosine (CPA), R-phenylisopropyladenosine (R-PIA) and N-[1S, trans)-2-hydroxycyclopentyl] adenosine (GR79236) and by S-PIA (the stereoisomer of R-PIA). The adenosine A₂ agonists N-[(2-methylphenyl)methyl] adenosine (metrifudil) and 2-[p-(2-carboxyethyl)phenethylamine]-5'-N-ethylcarboxamidoadenosine (CGS21680) also produced contractions in RCMM but were 54 and 165 times less potent respectively than NECA. The rank order of agonist potency for contraction of RCMM was CPA ≥ GR79236 = R-PIA ≥ NECA > S-PIA = metrifudil > CGS21680, which is identical to that reported for the inhibition of spontaneous rate in rat isolated right atria and inhibition of lipolysis in rat isolated adipocytes by these same agonists.

3 R-PIA, S-PIA and metrifudil behaved as partial agonists in RCMM.

4 The adenosine A₁ receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) inhibited the contractions produced by all the adenosine agonists tested, with pK_B values between 9.2 and 9.5. The non-selective adenosine antagonist 8-phenyltheophylline (8-PT) antagonized the effects of NECA but also markedly potentiated (by 93.0 ± 10.2% at 3 µM) the maximum contractile response to NECA in RCMM. Neither 8-PT (3 µM) nor DPCPX (0.1 µM) had any effect on the contractions produced by carbachol.

5 The contractile responses to NECA in RCMM were not affected by atropine (1 µM), tetrodotoxin (0.3 µM) or the P₂ antagonist, suramin (100 µM).

6 The present study confirms that contractions to adenosine agonists in the RCMM are mediated via adenosine A₁ receptors.

Keywords: Adenosine A₁ receptors; rat colonic muscularis mucosae; contraction

Introduction

Based on whether adenosine inhibits or stimulates the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in brain cells, adenosine receptors have been divided into two types, namely A₁ and A₂ respectively (Van Calcar *et al.*, 1979). Although it is now known that adenosine receptors can be coupled to second messengers other than cyclic AMP, this receptor classification is still used. More recently, it has been suggested that the A₂ receptor can be further divided into A_{2a} and A_{2b} (Bruns *et al.*, 1986), whilst a putative adenosine A₃ receptor has been cloned recently (Zhou *et al.*, 1992).

In general, stimulation of adenosine A₁ receptors is associated with an inhibition of functional responses. For example, stimulation of adenosine A₁ receptors produces an inhibition of electrically-induced twitch responses in guinea-pig ileum (Paton, 1981), an inhibition of lipolysis in rat adipocyte (Londos *et al.*, 1980) and inhibition of spontaneous rate in rat atria (Kurahashi & Paton, 1986). In contrast, it has been reported recently that adenosine elicited contractions in the rat isolated colonic muscularis mucosae (RCMM; Bailey & Hourani, 1990; Bailey *et al.*, 1992) which were mimicked by the adenosine A₁ receptor-selective agonist cyclopentyladenosine (CPA; Londos *et al.*, 1980) and inhibited by very low concentrations (nM) of the A₁ receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Bruns *et al.*, 1987; Haleen *et al.*, 1987). These data are in keeping with the proposal that contraction in RCMM is mediated via adenosine A₁ receptors (Bailey *et al.*, 1992).

The purpose of the present study was firstly, to extend the

findings of Bailey *et al.* (1992) in RCMM using a wider range of adenosine receptor agonists and secondly, to characterize further the receptor mediating the contractile effect by use of purine receptor blocking drugs. The data are compared with those obtained for the same agonists in two well characterized adenosine A₁ receptor preparations, namely inhibition of noradrenaline-induced lipolysis in rat isolated adipocytes and the inhibition of spontaneous rate in rat isolated right atrium (Londos *et al.*, 1980; Kurahashi & Paton, 1986; Gurden *et al.*, 1993).

Methods

Rat colonic muscularis mucosae (RCMM)

Female Wistar rats (100–200 g) were killed by cervical dislocation, the abdomen was opened and a 2 cm section of distal colon removed. A glass rod (diameter 5 mm) was passed inside the lumen of the colonic section and the outer longitudinal and circular muscle layers were carefully cut through with a scalpel blade and then removed by gentle rubbing with moist cotton wool. The remaining tube of colonic muscularis mucosae (RCMM) was then suspended along its longitudinal axis in an organ bath containing a modified Krebs-Henseleit solution at 32°C gassed with 95% O₂/5% CO₂. The tissues were suspended under an initial tension of 1 g and left to equilibrate for at least 1 h. The ionic composition of the Krebs-Henseleit solution (in mM) was: NaCl 118.5, NaHCO₃ 25.0, KCl 4.7, MgSO₄ 0.6, KH₂PO₄ 1.2, CaCl₂ 1.3 and glucose 11.1.

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Effect of agonists and antagonists

The RCMM were contracted by the addition of a single low concentration of 5'-*N*-ethylcarboxamidoadenosine (NECA, 0.01 μ M). When the peak contractile response was obtained, the tissues were washed with fresh Krebs-Henseleit solution and allowed to recover before adding a higher concentration of NECA. This process was repeated to obtain full sequential concentration-effect curves to NECA.

In agonist studies, two sequential concentration-effect curves to NECA were constructed followed by a further curve to either NECA (control tissues), or a test agonist. In antagonist studies, two control concentration-effect curves to an agonist were constructed followed by a curve in the presence of the antagonist (30 min preincubation).

In some experiments, the effects of NECA or CGS21680 on RCMM pre-contracted with a submaximal concentration of carbachol (1 μ M) in the absence or presence of DPCPX (0.1 μ M) were determined.

The specificity of the adenosine antagonists 8-PT and DPCPX was determined against carbachol-induced contractions. Control cumulative concentration-effect curves to carbachol were constructed, followed by test curves in the presence of the antagonist (30 min preincubation).

Analysis of results

The contractile responses in RCMM are expressed as a percentage (arithmetic mean $\pm$ s.e.mean) of the maximum response obtained in the second (control) concentration-effect curve. Equi-effective molar concentration-ratios (EMCR) and EC_{50} values for agonists were calculated graphically at the 50% response level of the control curve and expressed as geometric means (with 95% confidence limits) where n is the number of experiments. Rightward shifts in agonist curves (concentration-ratios (CR)) by antagonists were also measured from the 50% response level and are expressed as geometric means (with 95% confidence limits). In both agonist and antagonist studies the EC_{50} value from the 'test' curve was compared directly to the EC_{50} value for the second control curve.

The negative logarithm of the apparent dissociation constant for an antagonist (pK_B) was estimated by calculation of the arithmetic mean (with 95% confidence limits) of the individual results for each concentration of antagonist from the equation: $[pK_B = \log (\text{concentration-ratio} - 1) - \log (\text{antagonist concentration})]$.

Drugs

Commercially obtained drugs were: atropine sulphate (Sigma), carbachol chloride (BDH) and tetrodotoxin (Calbiochem) which were dissolved in distilled water; N^6 -(*R*-phenylisopropyladenosine) (R-PIA) and N^6 -(*S*-phenylisopropyladenosine) (S-PIA) were obtained from Boehringer Mannheim and were dissolved in 0.1 N HCl; and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Research Biochemicals Inc.) was dissolved in dimethylsulphoxide. 2-[*p*-(2-Carboxyethyl) phenethyl amine]-5'-*N*-ethylcarboxamidoadenosine (CGS21680) and 8-phenyltheophylline (8-PT) were from Research Biochemicals Inc. and were dissolved in 0.1 N NaOH.

Compounds synthesized by Glaxo Group Research Ltd were: N^6 -cyclopentyladenosine (CPA), N -[1*S*,*trans*]-2-hydroxycyclopentyl adenosine (GR79236), 5'-*N*-ethylcarboxamidoadenosine (NECA) and N -[(2-methylphenyl)methyl] adenosine (metrifudil) which were dissolved in 0.1 N HCl. Suramin was kindly supplied by Bayer UK Ltd and was dissolved in distilled water.

All compounds were initially prepared as stock concentrations of 10 mM.

Figure 1 The effect of repeated 5'-*N*-ethylcarboxamidoadenosine (NECA)-induced contractile concentration-effect curves in rat isolated colonic muscularis mucosae: (O) = curve 1, (●) = curve 2 and (▲) = curve 3. Each point is the mean ($\pm$ s.e.mean) of 12 observations calculated as a percentage of the maximum contraction to NECA obtained in curve 1.

Figure 2 The contractile effects of a range of adenosine agonists in rat isolated colonic muscularis mucosae: (●) NECA ($n = 17$); (□) CPA ($n = 9$); (▽) R-PIA ($n = 11$); (▲) GR79236 ($n = 4$); (△) S-PIA ($n = 8$); (▼) metrifudil ($n = 3$) and (○) CGS21680 ($n = 4$). Each point represents the mean ($\pm$ s.e.mean) calculated as a percentage of the maximum contraction obtained in the second curve to NECA. For abbreviations, see text.

Results

Effect of adenosine receptor agonists

NECA (0.01–10 μ M) produced concentration-dependent contractions in RCMM (Figure 1). The response to each concentration of NECA produced its maximum effect within 3 min. Following the construction of an initial concentration-effect curve to NECA, the second and third curves to the agonist were very reproducible with mean EC_{50} values of 0.18 (0.14–0.23) and 0.14 (0.11–0.16) μ M respectively. The maximum contractions (at 3 μ M) for the second and third curve were 0.35 ± 0.04 g and 0.37 ± 0.05 g respectively.

The contractions produced by NECA in the RCMM were mimicked by CPA, GR79236, R- and S-PIA, metrifudil and CGS21680 (Figure 2). CPA (0.001–0.3 μ M) and GR79236 (0.001–3.0 μ M) were potent and full agonists when compared to NECA in the RCMM. However, R-PIA (0.003–3 μ M),

S-PIA (0.1–100 μM) and metrifudil (0.1–100 μM) behaved as partial agonists with maximum responses of only 76.7 ± 3.0 , 66.1 ± 3.8 and $61.0 \pm 2.5\%$ respectively of that produced by NECA. Since CGS21680 (0.1–100 μM) was so weak, it was not clear if the true maximum response to this agonist was achieved but, at 100 μM , $78.0 \pm 7.9\%$ of the maximum response to NECA was obtained. The EMCR values (where NECA = 1) for these agonists at producing contraction in RCMM are shown in Table 1. The rank order of agonist potency in RCMM was: CPA $\geq$ GR79236 = R-PIA $\geq$ NECA $\gg$ S-PIA = metrifudil $>$ CGS21680.

A submaximal concentration of carbachol (1 μM) produced a maintained contraction of 1.06 ± 0.24 g ($n = 6$) in RCMM. Addition of NECA (10 μM) or CGS21680 (100 μM) produced a further contraction of 0.47 ± 0.131 ($n = 3$) and 0.19 ± 0.04 g ($n = 3$) respectively. DPCPX (0.1 μM) completely abolished the further contractions to NECA or CGS21680 in carbachol contracted RCMM. Under these conditions, no relaxation to these agonists was observed.

Table 1 Relative potencies of adenosine receptor agonists in rat colonic muscularis mucosae

Agonist	EMCR	n
NECA	1.0 [EC ₅₀ = 162 (141–186) nM]	17
CPA	0.19 (0.13–0.27)	9
R-PIA	0.49 (0.34–0.71)	9
GR79236	0.57 (0.40–0.82)	9
S-PIA	40 (33–48)	8
Metrifudil	54 (46–65)	3
CGS21680	165 (102–266)	4

Equi-effective molar concentration ratios (EMCR) together with the EC₅₀ value of NECA, are expressed as geometric mean values, with 95% confidence limits in parentheses and where n = number of preparations. For abbreviations, see text.

Figure 3 The effect of 8-phenyltheophylline (8-PT) on concentration-effect curves to 5'-N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (●) control NECA, $n = 15$; (○) NECA + 8-PT (0.3 μM , $n = 6$); (▽) NECA + 8-PT (1 μM , $n = 4$); (□) NECA + 8-PT (3 μM , $n = 5$). Each point represents the mean ($\pm$ s.e.mean) calculated as a percentage of the control NECA maximum.

Effect of antagonists

The non-selective adenosine A₁/A₂ receptor antagonist, 8-PT, produced concentration-dependent and rightward shifts of the NECA concentration-effect curves (Figure 3), mean CR values for 8-PT of 2.6 (1.7–3.8) and 11.3 (5.3–24.2) being obtained at 1 ($n = 4$) and 3 μM ($n = 6$) respectively. Interestingly, treatment with 8-PT also produced marked and significant increases in the NECA maximum, an increase of $93.0 \pm 10.2\%$ ($P < 0.001$) being observed in the presence of 8-PT at 3 μM .

The adenosine A₁ receptor-selective antagonist, DPCPX (1–10 nM), also produced concentration-dependent and rightward shifts of NECA concentration-effect curves in RCMM (Figure 4), although at 3 and 10 nM significant ($P < 0.05$) reductions in the maximum contractile responses were observed. Mean apparent pK_B values for DPCPX versus NECA of between 9.16 and 9.25 (Table 2) were estimated from these data. DPCPX (1 nM) also antagonized contrac-

Figure 4 The effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on concentration-effect curves to 5'-N-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (●) control NECA, $n = 16$; (○) NECA + DPCPX (1 nM, $n = 5$); (△) NECA + DPCPX (3 nM, $n = 5$); (□) NECA + DPCPX (10 nM, $n = 6$). Each point is the mean ($\pm$ s.e.mean) calculated as a percentage of the control NECA maximum.

Table 2 Antagonist effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) in rat colonic muscularis mucosae (RCMM)

Agonist	[DPCPX] nM (n)	CR	pK _B
NECA	1 (5)	2.67 (1.90–5.20)	9.16 (8.66–9.67)
	3 (5)	5.44 (3.81–7.75)	9.17 (8.98–9.36)
	10 (6)	18.77 (11.64–30.28)	9.25 (9.03–9.47)
CPA	1 (4)	4.45 (2.47–8.00)	9.53 (9.21–9.85)
R-PIA	1 (4)	3.15 (1.16–8.61)	9.28 (8.60–9.96)
GR79236	1 (4)	2.86 (1.99–4.10)	9.26 (9.00–9.52)
S-PIA	1 (4)	3.99 (1.93–8.23)	9.46 (9.02–9.89)
Metrifudil	1 (6)	3.68 (2.03–6.71)	9.40 (9.06–9.74)
CGS21680	1 (6)	3.64 (1.62–8.17)	9.54 (9.26–9.81)

Effective concentration ratios (CR) expressed as geometric means and apparent pK_B values for DPCPX against adenosine receptor agonists in RCMM expressed as arithmetic mean values, with 95% confidence limits in parentheses and where n = number of preparations. For abbreviations, see text.

Table 3 A comparison between rat colonic muscularis mucosae (RCMM), rat atria and rat adipocytes

Agonist	†RCMM	EMCR (NECA = 1) *Rat atria	*Rat adipocytes
NECA	1.0 [EC ₅₀ = 162(141–186) nM]	1.0 [EC ₅₀ = 93(72–119) nM]	1.0 [EC ₅₀ = 20(17–24) nM]
CPA	0.19	0.21	0.21
R-PIA	0.49	0.70	0.54
GR79236	0.57	0.77	0.34
S-PIA	40	25	32
Metrifudil	54	59	56
CGS21680	165	382	253

†This study.

*Reproduced from Gurden *et al.* (1993).

Equi-effective molar concentration ratios (EMCR) calculated from EC₅₀ values relative to NECA. A comparison between the relative potencies of agonist-induced contraction in RCMM, inhibition of rate in rat right atria and inhibition of lipolysis in rat adipocytes. For abbreviations, see text.

tions induced by all the other purine agonists tested in the RCMM with pK_B values ranging between 9.26 and 9.54 (Table 2).

Carbachol (0.01–30 μ M) produced concentration-dependent and reproducible contractions in RCMM with mean EC₅₀ values of 1.99 (1.10–3.45) and 2.60 (1.78–3.85) μ M for consecutive curves ($n = 4$). Neither DPCPX (0.1 μ M) nor 8-PT (3 μ M) produced any significant effect on the responses to carbachol with mean CR values ($n = 4$) of 0.97 (0.74–1.28) and 1.74 (1.19–2.54) respectively compared to the control curve and no change in the carbachol maximum response (98.7 ± 3.0 and $108.3 \pm 2.0\%$ of the control curve).

The contractions of the RCMM induced by NECA (0.01–10 μ M) were not inhibited by atropine (1 μ M), tetrodotoxin (0.3 μ M) or suramin (100 μ M), which produced mean CR values of 1.16 (0.79–1.70), 0.83 (0.49–1.40) and 1.07 (0.82–1.40) respectively with no change in the maximum response to NECA.

Discussion

The present study has shown that adenosine receptor agonists produce concentration-dependent contractions in the rat isolated colonic muscularis mucosae (RCMM). The contractile responses appeared to result from a direct effect of the agonists on the smooth muscle cells and did not involve cholinergic or neuronal stimulation, since the effects of NECA were not affected by pretreatment with atropine or tetrodotoxin. It has been reported that the RCMM contains both P₁ (adenosine receptors) and P₂ receptors (Bailey & Hourani, 1990). The latter study together with the present work suggests that the contractile effects of adenosine agonists are not due to P₂ receptor stimulation, since the P₂ receptor-selective antagonist suramin (Hoyle *et al.*, 1990) produced no inhibitory effect. Based on the potent antagonism of responses to CPA, NECA and adenosine by DPCPX, it was proposed that the receptor mediating contraction in RCMM was of the adenosine A₁ receptor subtype (Bailey *et al.*, 1992). The present study has further characterized the pharmacological properties of this receptor.

The contractions to NECA in RCMM were reproducible, although the maximum response did increase slightly during consecutive curves. The contractile responses to NECA were mimicked by all of the adenosine analogues tested, with CPA, R-PIA and GR79236, three agonists with selectivity for adenosine A₁ receptors (Londos *et al.*, 1980; Gurden *et al.*, 1993) being more potent than NECA. S-PIA, the stereoisomer of R-PIA, and metrifudil were less potent than NECA while the A_{2a} agonist, CGS21680 (Hutchison *et al.*, 1989) was the least potent compound tested. The EC₅₀ value for NECA in producing contraction in RCMM was similar to that reported to produce an inhibition of spontaneous rate

in rat isolated right atria but was approximately 8 fold greater than that for the inhibition of lipolysis in rat isolated adipocytes (Table 3). However, the relative potencies of the agonists (Table 3) were remarkably similar and the rank order of agonist potency (CPA $\geq$ GR79236 = R-PIA $\geq$ NECA $\gg$ S-PIA = metrifudil $>$ CGS21680) was identical in each of these isolated preparations. Interestingly, R-PIA, S-PIA and metrifudil were partial agonists relative to NECA in RCMM, a profile which has not been reported for these agonists in other tissues containing adenosine A₁ receptors. These data may simply reflect a poorer receptor coupling in RCMM, or possibly indicates that there may be a subtle difference between the receptors in RCMM and those in rat atria and rat adipocytes. However, the results with the agonists are generally consistent with contraction in RCMM being mediated via adenosine A₁ receptors.

That the contraction in RCMM is mediated via adenosine A₁ receptors was further substantiated by the effect of DPCPX, an A₁-selective antagonist (Bruns *et al.*, 1987; Haleen *et al.*, 1987). DPCPX produced concentration-dependent, rightward displacements of the NECA concentration-effect curves and mean pK_B values of approximately 9.2 were calculated. In this study, DPCPX at 3 and 10 nM produced a significant reduction in the NECA maximum and thus did not appear to behave as a truly competitive antagonist in the RCMM. However, DPCPX was agonist-independent producing a similar antagonist potency (pK_B 9.2–9.5) irrespective of the adenosine agonist used. The affinity values for DPCPX in RCMM are consistent with those obtained in other preparations containing adenosine A₁ receptors (Bruns *et al.*, 1987; Haleen *et al.*, 1987; Collis *et al.*, 1989). In addition, this antagonism was specific for adenosine since contractions to carbachol were not affected by a high concentration of DPCPX (0.1 μ M). These data indicate that the RCMM contains a population of adenosine A₁ receptors which mediate contraction. Furthermore, since NECA and CGS21680 had no effect in carbachol pre-contracted preparations pre-treated with DPCPX, it is unlikely that adenosine A₂ receptors mediating relaxation are present in RCMM. This is in contrast to the longitudinal muscle of the rat colon where relaxant adenosine A₂ receptors have been reported (Bailey & Hourani, 1992).

Treatment with the non-selective adenosine receptor antagonist, 8-PT (Collis *et al.*, 1985) yielded interesting results in RCMM. 8-PT produced concentration-dependent rightward shifts of the NECA curves with CR values consistent with adenosine-receptor antagonism (i.e. pK_B 6.4–6.6, Collis *et al.*, 1985). Interestingly, 8-PT treatment also produced very large potentiations in the maximum response to NECA, an effect not previously reported in other adenosine receptor containing preparations. In contrast to the results with NECA, 8-PT had no effect on contractions to carbachol in RCMM suggesting that potentiation was specific to adenosine receptor-mediated contraction. The mechanism by

which 8-PT enhanced the contraction to NECA in RCMM remains to be determined.

In conclusion, we have extended the work of Bailey and colleagues (1990, 1992) in order to characterize further the adenosine-mediated contraction in RCMM. The present study has demonstrated an identical rank order of agonist potency in RCMM, rat right atria and rat adipocytes. These findings, together with comparable pK_B estimates for the

inhibition by DPCPX of all agonists tested, confirm that contraction in RCMM is mediated via adenosine A_1 receptors. Since certain agonists were apparently partial with respect to NECA in RCMM, these receptors may be less well-coupled than in the other adenosine A_1 receptor containing preparations. Furthermore, we have observed an unexpected, and as yet unexplained, potentiation of the contractile effects following treatment with the antagonist, 8-PT.

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(Received May 17, 1993

Revised July 21, 1993

Accepted July 22, 1993)