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Characterization of the Prejunctional Adenosine Receptors in the Rat Anococcygeus Muscle

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Abstract—Adenosine receptor agonists inhibited electrically-evoked contractions of the rat isolated anococcygeus muscle. The compounds tested were: N^6-cyclopentyladenosine (CPA), *N*((*S*, *trans*)-2-hydroxycyclopentyl)adenosine (GR79236), the *R*- and *S*-isomers of phenylisopropyladenosine (PIA), 5′-*N*-ethylcarboxamidoadenosine (NECA), ((2-(4-(2-carboxyethyl)phenyl)ethyl)amino)-*N*-ethylcarboxamidoadenosine (CGS 21680) and *N*-((2-methylphenyl)methyl)adenosine (metrifudil). The rank order of agonist potency was: CPA = *R*-PIA = GR79236 = NECA ≫ *S*-PIA > metrifudil > CGS 21680, which is consistent with an effect mediated by adenosine A_1 receptors. A similar rank order of potency was obtained for inhibition of electrically-evoked contractions of the guinea-pig ileum. However, there may be a lower receptor reserve in rat anococcygeus compared with the guinea-pig ileum, since higher concentrations of agonists were necessary to produce effects in the anococcygeus than in the guinea-pig ileum and *S*-PIA behaved as a partial agonist. The effect of NECA was antagonized in rat anococcygeus and guinea-pig ileum by the mixed A_1/A_2 receptor antagonist, 8-phenyltheophylline (pA_2 values of 6·8 and 6·9, respectively). The selective A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), also blocked the inhibitory response to NECA in both tissues. Here, however, the pA_2 values (9·6 and 8·6, respectively) were slightly but significantly different. These values confirm that the prejunctional adenosine receptors of the rat anococcygeus are of the A_1 type, and suggest that they are similar but not necessarily identical to those of the guinea-pig ileum. The differing potencies of DPCPX as an antagonist of NECA between the preparations may reflect a tissue-dependent variation in sensitivity to this antagonist.

Adenosine receptors were originally classified into A_1 and A_2 types on the basis of their effects on intracellular cyclic AMP levels, and subsequently by the effects of selective agonists and antagonists. The adenosine A_1 receptor is generally found to inhibit hormone-sensitive adenylate cyclase (Van Calker et al 1979); it is activated by agonists such as *R*-phenylisopropyladenosine (*R*-PIA), while the xanthine derivative 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) is a potent and selective antagonist (Haleen et al 1987).

Unlike A_2 adenosine receptors, for which there is pharmacological and molecular biological evidence for subtypes designated A_{2a} and A_{2b} (Bruns et al 1987; Furlong et al 1992; Pierce et al 1992; Gurden et al 1993), there is at present no convincing evidence for subtypes of the A_1 receptor (Kennedy et al 1992; Coates et al 1994). However, a recently-cloned novel adenosine receptor, designated the A_3 receptor, was shown to have high affinity for A_1-receptor agonists (Zhou et al 1992) but very low affinity for some xanthine antagonist ligands such as 8-phenyltheophylline.

Stone (1982) demonstrated that electrically-evoked contractions of the rat anococcygeus muscle preparation could be inhibited by adenosine. The receptors mediating the response were classified as A_1 on the basis of the relative potency of adenosine agonists, since selective A_1 antagonists were not available at the time. To our knowledge, the adenosine receptors in this tissue have not been the subject of further study. We have, therefore, re-examined the response of the anococcygeus to a range of adenosine analogues, including the novel selective A_1 agonist GR79236 (Strong et al 1993), the selective A_{2a} agonist CGS 21680 (Jarvis et al 1989), and the modestly-selective A_{2b} agonist, metrifudil (Gurden et al 1993).

The results obtained with the rat anococcygeus preparation were compared with those in the guinea-pig ileum longitudinal muscle preparation, in which the prejunctional adenosine receptor mediating inhibition of contractions has been identified as the A_1 receptor (Paton 1981; Collis 1990; Gurden et al 1993). In the present study, we have additionally determined the potency of the selective A_1 antagonist, DPCPX, and the mixed A_1/A_2 antagonist, 8-phenyltheophylline, in blocking the responses to NECA in both rat anococcygeus and guinea-pig ileum. The results suggest that there may be differences between the adenosine A_1 receptors in the two preparations.

A preliminary account of some of these findings has already been published (Green et al 1993).

Materials and Methods

Methods

The anococcygeus was removed from male AH/A rats, 300–350 g, as described by Gillespie (1972). The proximal part of the ileum of male Dunkin Hartley guinea-pigs, 400–500 g, was removed and 2-cm sections were threaded onto a glass rod; the longitudinal muscle-myenteric plexus was removed by gentle rubbing with a cotton wool bud. The isolated muscle preparations were suspended in 10-mL organ baths containing Krebs solution at 37°C and gassed with 95% O_2–5% CO_2, and stimulated at supramaximal voltage with 1-ms rectangular pulses at 0·2 Hz (for ileum) or 10 Hz for 1 s

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every 30 s (for anococcygeus). Agonist studies in guinea-pig ileum were performed using the method described by Kennedy et al (1992) in which cumulative concentration-response curves to NECA were constructed in every preparation. After a 30-min wash-out period, a second agonist was also tested. One preparation from each animal was again dosed with NECA as the second agonist to check the reproducibility of the response. In rat anococcygeus, only one cumulative concentration-response curve could be obtained from each preparation. Preparations in which the twitch height could be inhibited by less than 50% by NECA (approx. 1 in 20) were considered atypical and were not used. Agonist potency was expressed as an IC50 concentration (concentration inhibiting contractions by 50%).

The direct effect of NECA on the smooth muscle of the rat anococcygeus was tested by pre-contracting the tissue with a concentration of phenylephrine which elicited a sub-maximal response (10^{-8}–3×10^{-7} M), constructing a cumulative concentration-response curve to NECA and then eliciting a further contraction with phenylephrine (10^{-6}–10^{-4} M). For similar experiments in the guinea-pig ileum, a non-cumulative concentration-response curve to acetylcholine (10^{-8}–3×10^{-6} M) was constructed in the absence and presence of NECA.

For antagonist studies in guinea-pig ileum, consecutive concentration-response curves to NECA were constructed in the absence and presence (15-min pretreatment) of antagonist. In rat anococcygeus it was necessary to use paired preparations, one tissue serving as control for the other which received 15-min pretreatment with antagonist. The antagonists used were 8-phenyltheophylline, which blocks both A_1 and A_2 classes of receptor (Bruns et al 1987), and DPCPX, which is selective for A_1 receptors (Haleen et al 1987). Only a single concentration of antagonist was tested on each preparation. Concentration-ratios for agonists were determined at the IC50 level and Schild plots were used to determine pA_2 values for each antagonist (Arunlakshana & Schild 1959). To determine the statistical significance of differences in antagonist potency values, an unpaired Student's *t*-test was performed on the pK_B values calculated from individual concentration ratios using the equation: $pK_B = \log$ (concentration ratio − 1). In further experiments to test the specificity of the antagonist DPCPX, the agonist used was xylazine.

Drugs

Adenosine agonists and antagonists. 5′-*N*-Ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA) and *N*((*S*,*trans*)-2-hydroxycyclopentyl)adenosine (GR79236) were synthesized by Dr F. Ellis of the Medicinal Chemistry Department, Glaxo Group Research Ltd. ((2-(4-(2-Carboxyethyl)phenyl)ethyl)amino)-*N*-ethylcarboxamidoadenosine (CGS 21680), *R*-phenylisopropyladenosine (*R*-PIA), 8-phenyltheophylline and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Research Biochemicals Incorporated, Natick, MA. DPCPX and 8-phenyltheophylline were dissolved in dimethylsulphoxide containing a few drops of 2 M NaOH and diluted in water.

Other drugs. Other drugs were as follows: acetylcholine hydrochloride (Sigma, Poole); atropine sulphate (BDH, Poole); phenylephrine (Sigma); prazosin hydrochloride (Pfizer, Sandwich); tetrodotoxin (Sigma); xylazine hydrochloride (Bayer, Leverkusen, Germany).

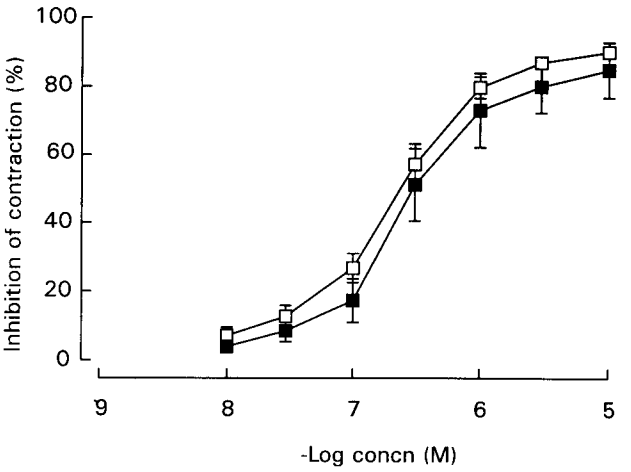

FIG. 1. Similar potency of NECA in inhibiting electrically-evoked contractions in paired preparations of rat anococcygeus muscle from the same animal, represented by □ and ■ (mean with s.e.m. of five preparations).

Results

Nature of the twitch response in the rat anococcygeus

Electrically-evoked contractions of the rat anococcygeus muscle were abolished by both tetrodotoxin (1 μM) and prazosin (10 nM).

Responses to NECA

The non-selective adenosine-receptor agonist, NECA, caused a concentration-dependent inhibition of electrically-evoked contractions of the rat anococcygeus (Fig. 1). The maximal achievable inhibition was usually 80–90%. The mean IC50 value (with 95% confidence limits) for the response was 295 nM (179–485 nM, n = 17). In the guinea-pig ileum the IC50 value for NECA was 28 nM (19–41 nM, n = 27), a value similar to that reported in our previous work (Kennedy et al 1992; Gurden et al 1993).

It was not possible to perform two consecutive cumulative concentration-response curves for NECA in the rat anococcygeus preparation because the tissues did not completely recover, even after a prolonged period of wash-out. However, the responses of paired preparations (right and left anococcygeus muscles) from the same animal were almost identical in sensitivity (Fig. 1), and so the protocol adopted for antagonist studies was to use such pairs, one preparation acting as the control for the other.

In unstimulated anococcygeus preparations from rat, neither NECA nor *R*-PIA (10^{-8}–10^{-4} M) affected the muscle tension (data not shown). NECA also failed to alter muscle tone when the anococcygeus was partially pre-contracted with a submaximal concentration of phenylephrine (control tension = 2640 ± 550 mg; tension in the presence of NECA (10^{-4} M) = 2550 ± 690 mg).

Responses to other adenosine-receptor agonists

The A_1-selective agonists CPA, *R*-PIA and GR79236

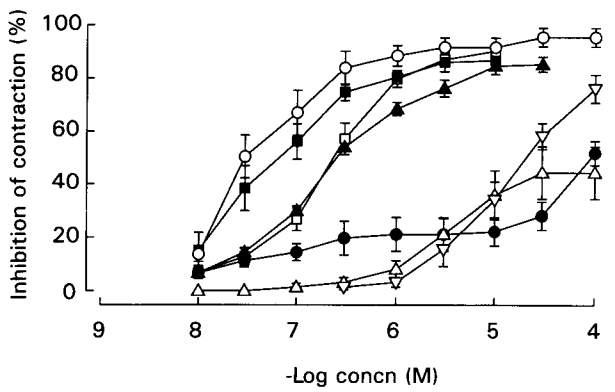

FIG. 2. Concentration-response curves for seven adenosine-receptor agonists in rat anococcygeus muscle. Each curve is the mean with s.e.m. of 4–11 preparations. ○ *R*-PIA, ■ CPA, ▲ GR79236, □ NECA, ▽ metrifudil, △ *S*-PIA, ● CGS 21680.

inhibited electrically-evoked contractions of both tissues at low concentrations (Fig. 2). The *R*-isomer of PIA was 240 times as active as the *S*-isomer (measured at the IC20 level), the *S*-isomer behaving as a partial agonist (Fig. 2).

In contrast to the A_1-selective agonists, the A_{2a}-selective agonist, CGS 21680, and the modestly-selective A_{2b} agonist, metrifudil, were two orders of magnitude weaker (Fig. 2).

The absolute potency of NECA and the relative potency of the other adenosine agonists are shown in Table 1.

Antagonism of NECA by 8-phenyltheophylline and DPCPX

The non-selective A_1/A_2 adenosine-receptor antagonist, 8-phenyltheophylline (10^{-6}–10^{-5} M), antagonized the response to NECA in both guinea-pig ileum and rat anococcygeus. In both tissues, the control concentration-response curves were shifted rightwards with no significant change of maximum response. The pA_2 values calculated from these experiments are shown in Table 2.

The selective A_1-receptor antagonist, DPCPX, also antagonized the responses to NECA in both rat anococcygeus and guinea-pig ileum in an apparently competitive fashion (Figs 3, 4, respectively). The pA_2 value calculated in rat anococcygeus (9·6; Table 2) was significantly higher ($P < 0.01$) than the value of 8·6 obtained in guinea-pig ileum.

Table 1. Absolute and relative potency values for adenosine agonists in rat anococcygeus.

Compound	IC50 (nM)	Equi-effective concentration ratio
R-PIA	35 (12–108)	0·20 (0·05–1·53)
CPA	37 (9–156)	0·30 (0·06–3·01)
GR79236	220 (150–340)	0·39 (0·19–0·78)
NECA	295 (179–485)	1
Metrifudil	9900 (5800–17000)	28 (17–46)
S-PIA*	4300 (2100–8900)	48 (24–90)
CGS 21680	⩾ 100000	⩾ 242

Absolute potency values for 50% inhibition of electrically-evoked contraction (IC50) of adenosine agonists in the rat anococcygeus, and relative potencies (equi-effective concentration ratio) compared with NECA in parallel preparations. Values shown are the geometric mean of at least four experiments with 95% confidence limits in parentheses. * *S*-PIA behaved as a partial agonist (being capable of causing a mean maximal 52% inhibition of contraction) and hence the concentration and relative potency values shown were calculated at the IC20 level.

Table 2. Antagonism by 8-phenyltheophylline and DPCPX of the response to NECA in rat anococcygeus and guinea-pig ileum.

Antagonist	Rat anococcygeus	Guinea-pig ileum
8-Phenyltheophylline		
pA_2 value	6·8 (6·3–7·2)	6·9 (6·6–7·2)
Slope	1·2 (0·9–1·5)	0·9 (0·8–1·0)
n	12	12
DPCPX		
pA_2 value	9·6 (9·2–10·1)	8·6* (8·4–9·0)
Slope	0·9 (0·7–1·1)	1·1 (0·8–1·4)
n	17	12

Values were calculated from the concentration-ratios obtained in n experiments at three antagonist concentrations. Data in parentheses show the 95% confidence limits. * Significant difference in the pA_2 value between the two preparations ($P < 0.05$, unpaired *t*-test comparing individual pK_B values).

Specificity of 8-phenyltheophylline and DPCPX

The α_2-adrenoceptor agonist, xylazine, inhibited evoked

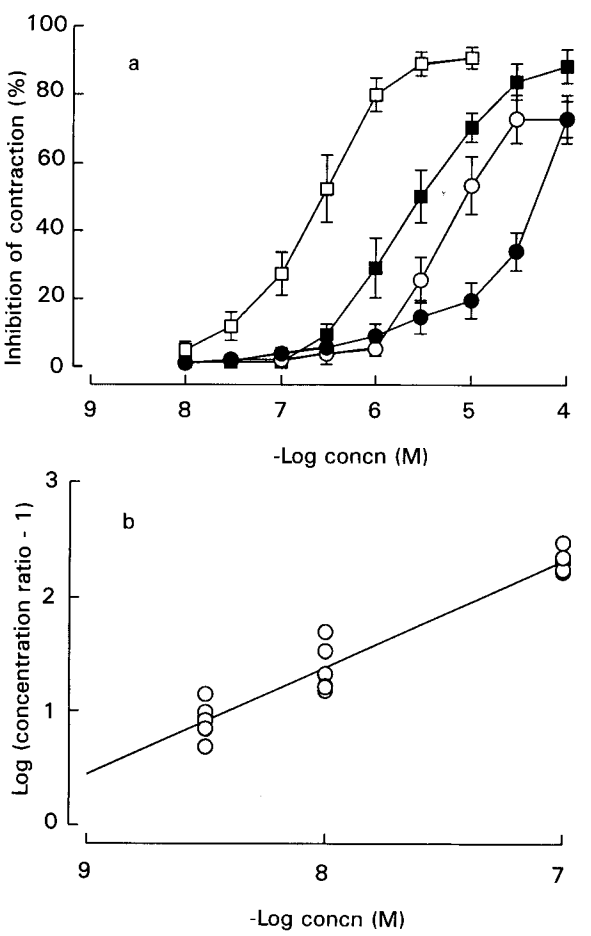

FIG. 3. a. Antagonism by DPCPX of the response to NECA in the rat anococcygeus. Values are the mean (with s.e.m.) for NECA alone (□, n = 17) and in the presence of DPCPX at 3×10^{-9} M (■, n = 6), 10^{-8} M (○, n = 6) or 10^{-7} M (●, n = 5). b. Schild plot for the same data. Each point represents a separate experiment.

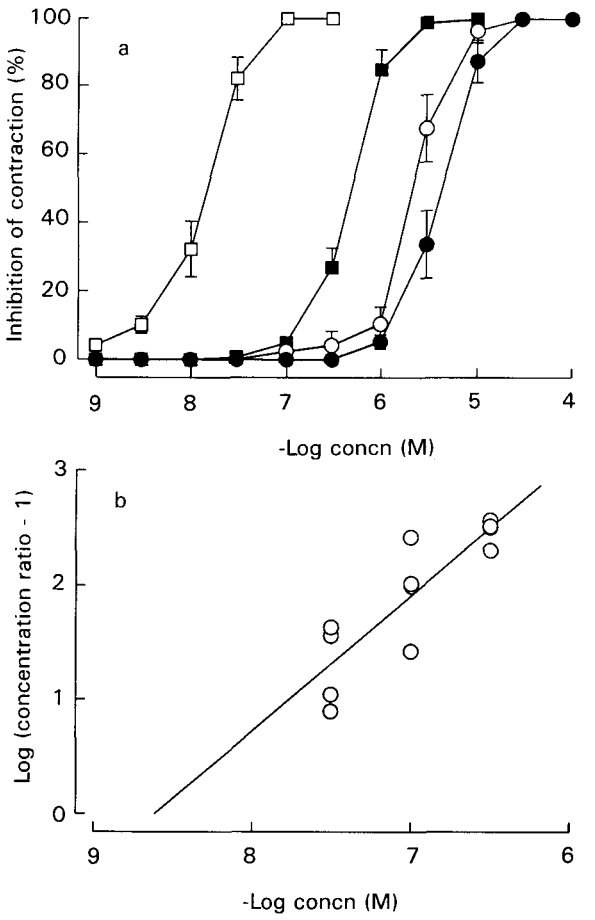

FIG. 4. a. Antagonism by DPCPX of the response to NECA in the guinea-pig ileum. Values are the mean (with s.e.m.) for NECA alone (□, n = 12) and in the presence of DPCPX at 3×10^{-8} M (■, n = 4), 10^{-7} M (○, n = 4) or 3×10^{-7} M (●, n = 4). b. Schild plot for the same data. Each point represents a separate experiment.

contractions in the rat anococcygeus. Neither 8-phenyltheophylline (10^{-6} M) nor DPCPX (3×10^{-8} M) affected the response to xylazine. Concentration ratios for xylazine in the presence of antagonist compared with the same tissues in their absence were 1·5 (0·8–2·6; n = 3) and 1·1 (0·4–3·5; n = 4) for 8-phenyltheophylline and DPCPX, respectively.

Discussion

This study has confirmed that the prejunctional adenosine receptors in both the rat anococcygeus muscle and guinea-pig ileum longitudinal muscle are of the A_1 type, as proposed by Paton (1981) and Stone (1982), respectively. Nevertheless, the receptors do not appear to be identical in their affinity for the substituted xanthine antagonist, DPCPX.

NECA and the other adenosine-receptor agonists tested caused a concentration-dependent inhibition of the electrically-evoked contraction in both the rat anococcygeus muscle and the guinea-pig ileum. The rank order of agonist potency in the rat anococcygeus was: CPA = *R*-PIA = GR79236 = NECA ≫ *S*-PIA > metrifudil > CGS 21680. This is in agreement with that proposed by Gurden et al (1993) to be characteristic of a response mediated by activation of adenosine A_1 receptors. Our data thus confirms and extends that published by Stone (1982). The rank order of agonist potency in the rat anococcygeus, and their relative potencies, were virtually identical to those which we have previously reported for the guinea-pig ileum (Gurden et al (1993), Table 3). However, the absolute potency of NECA and the other adenosine agonists was about an order of magnitude lower in the rat anococcygeus than in the guinea-pig ileum.

Table 3. Comparison of potency values of adenosine agonists in the rat anococcygeus and guinea-pig ileum.

Agonists	Equi-effective concentration ratio	
	Guinea-pig ileum[a]	Rat anococcygeus
R-PIA	1·2 (0·8–1·6)	0·2 (0·05–1·53)
CPA	0·4 (0·3–0·5)	0·3 (0·05–3·01)
GR79236	1·3 (0·8–2·2)	0·39 (0·19–0·78)
NECA	1	1
Metrifudil	51 (31–71)	28 (17–46)
S-PIA	56 (41–75)	48[b] (24–90)
CGS 21680	299 (161–553)	⩾ 242

Values are the equi-effective concentration ratio relative to NECA, determined by comparison in the same tissue (guinea-pig ileum) or in individual preparations (rat anococcygeus), and are the geometric mean of at least four experiments with 95% confidence limits in parentheses. [a] Data from Gurden et al (1993). [b] *S*-PIA behaved as a partial agonist in anococcygeus (intrinsic activity relative to NECA = 0·52) and hence the ratio was calculated at the IC20 level.

This data indicates that prejunctional A_1 adenosine receptors are present on the rat anococcygeus. The lower absolute potency of agonists suggests, however, that the receptor reserve may be lower than in the guinea-pig ileum. Consistent with this supposition is the observation that *S*-PIA behaved as a full agonist in ileum but as a partial agonist in anococcygeus. However, the receptor reserve does not appear to be as low as in the muscularis mucosae of the rat colon, in which *R*-PIA, *S*-PIA and metrifudil all behaved as partial agonists (Reeves et al 1993).

The apparent affinities of two competitive antagonists, 8-phenyltheophylline and DPCPX, are also consistent with the presence of A_1 adenosine receptors in the rat anococcygeus. Published pA_2 values for blockade of A_1-mediated responses by these antagonists are approximately 6·5 for 8-phenyltheophylline and 9 for DPCPX (Reeves et al 1993). In the present study, 8-phenyltheophylline was found to have a pA_2 value of 6·9 for antagonism of NECA in guinea-pig ileum, and 6·8 in the rat anococcygeus. The pA_2 value for DPCPX antagonism of NECA in the guinea-pig ileum was 8·6, compared with 9·6 in rat anococcygeus; these latter values were significantly different. DPCPX is highly selective for the adenosine A_1 receptor (Bruns et al 1987; Haleen et al 1987; Coates et al 1994) and its apparent affinity in both tissues was sufficiently high to support the attribution of the responses to adenosine A_1 receptors. However, the modest difference in pA_2 values may arise from species differences in the amino acid sequence of the A_1 receptor in guinea-pig and rat. Similar small differences in the affinity of DPCPX for the A_1 receptor in rat and guinea-pig have previously been noted (Musser et al 1993), although we have found no apparent differences in the affinity for [^{3}H]DPCPX for receptors in the cerebral cortex of rat and guinea-pig (Coates et al 1994). The high affinity of 8-phenyltheo-

phylline and DPCPX does, however, allow the newly-identified A_3 receptor to be eliminated as a potential mediator of the responses, since this receptor has very low affinity for substituted xanthines (Zhou et al 1992).

Further experiments were conducted to investigate whether NECA had any postsynaptic effects in the two preparations. Stone (1982) found that NECA and adenosine increased the basal tone of electrically-stimulated rat anococcygeus in addition to producing inhibition of twitch height. In contrast, in the present study no contraction was seen when NECA was added to preparations with steady resting tone, nor was there any contractile or relaxant effect in preparations partially pre-contracted with phenylephrine. We conclude that, at least in tissues from the rat strain we have used, all effects of adenosine agonists were presynaptic.

In summary, our data suggest that the adenosine receptors in the rat anococcygeus are prejunctional, of the A_1 subtype, and are similar to those in the guinea-pig ileum. The rat preparation may have a lower receptor reserve, as indicated by the lower potency of agonists and the observation that *S*-PIA behaved as a partial agonist. A modest difference in the pA_2 values for the A_1 antagonist, DPCPX, in rat anococcygeus and guinea-pig ileum, may reflect a tissue-dependent variation in sensitivity to this antagonist.

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