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The binding of 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine to adenosine A_1 receptors in rat smooth muscle preparations

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1 The binding of 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX), an antagonist radioligand selective for adenosine A_1 receptors, was studied in rat duodenum, colon muscularis mucosae and longitudinal muscle, urinary bladder and vasa deferentia.

2 [^{3}H]-DPCPX bound with high affinity to a single site in all membrane preparations studied with the exception of the rat urinary bladder in which no specific binding was detected. The affinity (K_d) of the binding site for [^{3}H]-DPCPX was similar in all membrane preparations, the colon longitudinal muscle (1.18 ± 0.47 nM), colon muscularis mucosae (0.84 ± 0.15 nM), duodenum (1.59 ± 0.18 nM) and vasa deferentia (0.93 ± 0.17 nM). The density of [^{3}H]-DPCPX binding sites was similar in the duodenum (38.8 ± 4 fmol mg^{-1} protein), muscularis mucosae (43 ± 3.5 fmol mg^{-1} protein) and vasa deferentia (43.3 ± 12.2 fmol mg^{-1} protein), but in the longitudinal muscle 6–7 fold more binding sites (295 ± 70 fmol mg^{-1} protein) were identified.

3 Inhibition studies using DPCPX (0.1–100 nM), N^6-cyclopentyladenosine (CPA) (0.1–100 nM), 5'-N-ethylcarboxamidoadenosine (NECA) (2 nM–10 μM) and (**R**)-N^6-phenylisopropyladenosine (**R**-PIA) (1 nM–1 μM) to displace the binding of [^{3}H]-DPCPX at a concentration around the K_d value (1 nM), demonstrated an order of potency of displacement in all tissues of DPCPX ≥ CPA > **R**- PIA > NECA. This potency order is characteristic of an A_1 receptor, indicating that [^{3}H]-DPCPX binds to adenosine A_1 receptors in the rat duodenum, colon and vasa deferentia. Two site analysis revealed that the agonists bind to both a high and low affinity state of the receptor.

4 The existence of A_1 binding sites in the rat vasa deferentia, colon muscularis mucosae and duodenum, and their absence in the urinary bladder, is consistent with previous functional studies. However, in contrast to the findings of the [^{3}H]-DPCPX binding assay, no functional response mediated by adenosine A_1 receptors could be detected by measuring contractile or relaxant responses to CPA in the colon longitudinal muscle. The functional significance of the binding sites in this tissue has therefore yet to be determined.

Keywords: Rat smooth muscle preparations; [^{3}H]-DPCPX; radioligand binding; adenosine A_1 receptor; purinoceptors

Introduction

The P_1-purinoceptors mediating the action of extracellular adenosine were originally divided into two major subclasses, A_1 and A_2, on the basis of their differential selectivity for adenosine and adenosine analogues and because of their opposite effects on adenylate cyclase, with A_1 inhibiting and A_2 stimulating enzyme activity (Van Calker *et al.*, 1979). On A_1 receptors N^6 substituted adenosine analogues such as N^6-cyclopentyladenosine (CPA) and (**R**)-N^6-phenylisopropyladenosine (**R**-PIA) are more potent than 5' substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), whereas on A_2 receptors the reverse is true. Xanthines such as 8-sulphophenyltheophylline (8-SPT) are antagonists at both A_1 and A_2 receptors, whereas the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has nanomolar affinity at A_1 receptors and micromolar affinity at A_2 receptors and thus effectively discriminates between the two types of receptor. A_2 receptors have been further subdivided into high and low affinity subtypes A_{2a} and A_{2b} based on their affinity for [^{3}H]-NECA in radioligand binding studies (Bruns *et al.*, 1986). A_{2a} receptors can be distinguished from A_{2b} on the basis of the selective activity of some 2-substituted adenosine analogues such as 2-[*p*-(carboxyethyl)-phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS 21680) at A_{2a} receptors. More recently, a xanthine-insensitive A_3 receptor has been cloned from rat striatum and has also been shown to be negatively linked to adenylate cyclase (Zhou *et al.*, 1992). For a recent review of adenosine receptor subtypes see Collis & Hourani (1993).

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Numerous agonist radioligands are available for A_1 binding studies and binding affinity at A_1 sites has commonly been assessed by use of [^{3}H]-N^6-cyclohexyladenosine (CHA) or [^{3}H]-CPA as the ligand and brain membrane preparations as a source of binding sites (Bruns *et al.*, 1980; Williams *et al.*, 1986). Until recently, the lack of highly selective A_2 agents has generally meant that specific A_2 binding has been determined by the use of a ligand such as [^{3}H]-NECA, which binds to both A_1 and A_2 receptors, with the binding study carried out in the presence of an A_1 agonist and rat striatum being used as a source of tissue (Bruns *et al.*, 1986; Stone *et al.*, 1988). More recently [^{3}H]-CGS 21680 has been used to identify A_{2a} binding sites in various regions of the brain of both human and rat (Wan *et al.*, 1990), though no agent specific for the low affinity A_{2b} site has yet been developed. The affinity of agonist radioligands used in receptor classification, however, may be subject to complications which can be overcome by the use of selective antagonists. Antagonist radioligands bind with high affinity to both agonist defined coupling states of a given receptor and are thus preferable. To this end [^{3}H]-DPCPX has superseded tritiated A_1 selective agonists, such as [^{3}H]-CPA, for the study of the binding affinity of A_1 receptors. Indeed, [^{3}H]-DPCPX has been shown to bind to a single site commensurate with an A_1 receptor, in rat whole brain membranes (Bruns *et al.*, 1987).

Much of the interest in adenosine receptors has focused on its effects in the central nervous system and has involved the use of radioligand binding assays to characterize receptor subtypes. Studies of the peripheral effects of adenosine have

in contrast, used mainly functional studies and very few successful binding studies for adenosine receptors in peripheral tissues have been reported, although binding has been shown to A_1 receptors in the heart (Leid *et al.*, 1988; Martens *et al.*, 1988; Musser *et al.*, 1993), testis (Cushing *et al.*, 1988), uterus (Schiemann *et al.*, 1990; 1991; Smith *et al.*, 1988), human fat cell (Larrouy *et al.*, 1991) and to A_2 receptors in some vascular tissues (Diocee & Souness, 1987; Moritoki *et al.*, 1990).

We have attempted to develop a receptor binding assay in a number of rat peripheral tissues using the antagonist [^{3}H]-DPCPX to identify the A_1 adenosine receptor, and to study the distribution of these receptors in various smooth muscle preparations. The rat duodenum, vas deferens and colon muscularis mucosae were chosen as these tissues have previously been shown in functional studies to contain A_1 receptors (Nicholls *et al.*, 1992; Bailey *et al.*, 1992; Hourani *et al.*, 1993). The rat urinary bladder and the longitudinal muscle of the colon were chosen as negative controls as these tissues have been shown in functional studies to contain A_{2b} receptors (Nicholls *et al.*, 1992; Bailey & Hourani, 1992).

Some of our findings have previously been published in abstract form (Peachey *et al.*, 1994).

Methods

Membrane preparation

Adult male Wistar albino rats (200–300g), University of Surrey strain, were killed by cervical dislocation. The duodenum and urinary bladder were dissected out as described in Nicholls *et al.* (1990), the distal colon and the vasa deferentia were rapidly removed and placed in ice-cold 50 mM Tris HCl buffer (pH 7.4). The longitudinal muscle and the muscularis mucosae of the colon were separated, as described by Bailey & Hourani (1992). Tissues from 25 animals were pooled, all tissues were cleaned and blotted dry to remove any excess buffer, weighed and roughly scissor chopped. Homogenization was performed with a Polytron homogenizer (three 10 s bursts, mid-speed setting), followed by a Potter homogenizer (15 strokes at 1300 r.p.m.). The homogenates were centrifuged for 20 min at 1000*g*, the pellets discarded and the supernatants recentrifuged at 50,000*g* for 20 min. The resulting pellets were resuspended in 50 mM Tris HCl buffer and incubated with 5 u ml^{-1} adenosine deaminase at room temperature for 30 min before use. Protein estimations were performed according to the method of Lowry *et al.* (1951).

Binding assays

Saturation experiments were performed in triplicate in polypropylene tubes using a total assay volume of 250 µl. A range of [^{3}H]-DPCPX (specific activity 88.2 Ci mmol^{-1}) concentrations (0.05–10 nM) were used and the non-specific binding defined by 1 µM CPA. The reaction was initiated by the addition of homogenate and the mixture incubated for 30 min at temperatures determined to be optimal in pilot studies (duodenum 0°C, colon muscularis mucosae and longitudinal muscle 25°C and vas deferens 35°C). The reaction was terminated by the addition of ice-cold 50 mM Tris HCl buffer followed by three 2.5 ml washes under vacuum filtration over Whatman GF/B filters using a Brandel Cell Harvester. The filters were removed and placed in scintillation vials, solubilised with 20% Triton in toluene, scintilla-

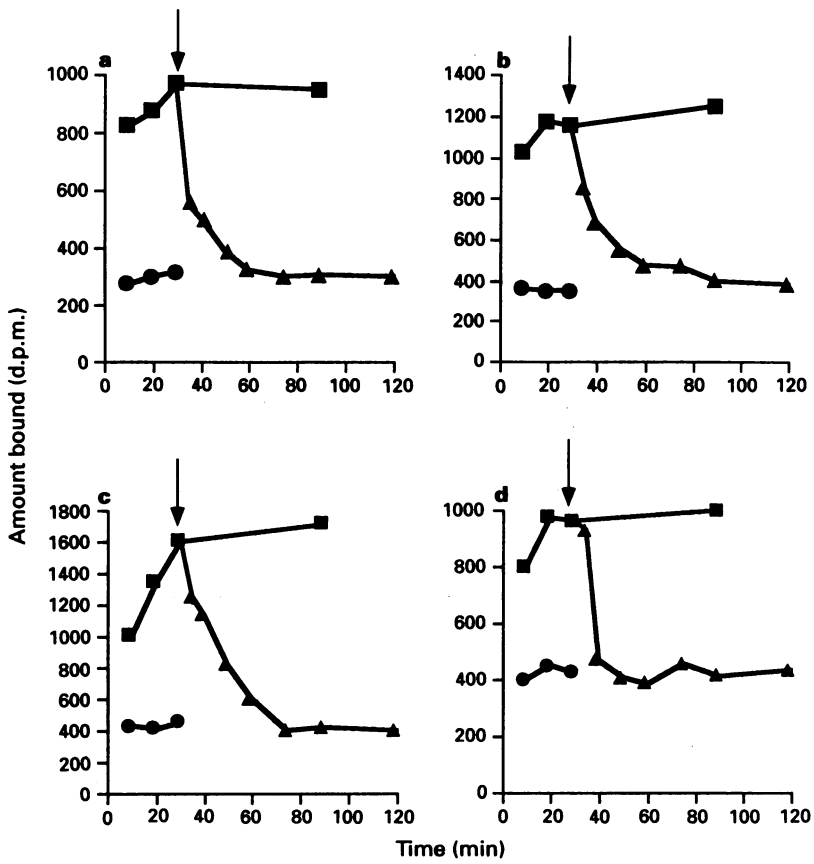

Figure 1 Representative time course experiments on (a) colon longitudinal muscle, (b) colon muscularis mucosae, (c) duodenum and (d) vasa deferentia performed at optimal temperatures (colon 25°C, vasa deferentia 35°C and duodenum on ice), showing total binding (■) and non-specific binding (●) determined in the presence of 1µM CPA; reversibility of binding is shown by the addition of 1µM CPA (↓) at 30 min and the subsequent dissociation (▲) of [^{3}H]-DPCPX. For abbreviations in this and subsequent figure legends, see text.

tion fluid (Unisolve E) added and the tubes left overnight to reduce chemiluminescence prior to counting on a Wallac LKB scintillation counter. The results were analysed using LIGAND (Munson & Rodbard, 1980) and the specific binding was subjected to Scatchard transformation for estimation of B_{max} and K_d. Inhibition studies were performed with a single concentration (1 nM) of [^{3}H]-DPCPX in the presence of increasing concentrations of competing ligands, DPCPX 0.1–100 nM, CPA 0.1–100 nM, **R**-PIA 1 nM–1 μM and NECA 2 nM–10 μM, and the same procedure was followed as outlined above.

Functional assays

Adult male Wistar albino rats (200–250 g), University of Surrey strain, were killed by cervical dislocation and the distal colon rapidly excised and placed in Tyrode buffer (ionic composition, mM: Na^+ 149.1, K^+ 2.8, Ca^{2+} 1.8, Mg^{2+} 2.1, Cl^- 147.5, $H_2PO_4^-$ 0.3, HCO_3^- 11.9 and glucose 5.6), pregassed with 95% O_2, 5% CO_2. The dissection of the longitudinal muscle was carried out as described in Bailey & Hourani (1992). The longitudinal muscle was suspended with the lumen closed, under a resting tension of 1g, in 3.5 ml glass organ bath in gassed Tyrode solution at 32–35°C. Contractions were measured isometrically via an FTO3 force displacement transducer and displayed on a Grass 79D polygraph. The tissues were allowed to equilibrate for 90 min with frequent washing, before the addition of any drugs. Carbachol (CCh) 1 μM was used to contract the tissue and this response was used as a reference contractile response against which the effects of CPA (1 nM–1 μM) in the absence and presence of DPCPX (1 nM) were assessed. The tissues were dosed non-cumulatively and an interval between doses of 15 min was maintained in all cases. To determine whether CPA had any inhibitory or stimulatory effect on the CCh-induced response, the tissues were treated with CPA either for 1 min prior to the addition of CCh, or when the CCh-induced contraction had achieved a stable plateau. The effects of CPA on field stimulation of the longitudinal muscle by trains of pulses (Grass S48 stimulator, train rate 0.03, duration 10 s, stimulation rate 10 Hz for 1 ms, 70 V) via parallel platinum electrodes (0.3 mm diameter, 0.5 cm apart) were also investigated. In experiments to determine the effects of DPCPX on the CPA-induced response, a control concentration-response curve was carried out, after which the tissues were incubated with 1 nM DPCPX for 30 min and then a second concentration-response curve to CPA was obtained.

Materials

CPA, **R**-PIA, NECA and adenosine deaminase (ADA) (Type VI) were obtained from Sigma UK. Ltd, Poole, Dorset; DPCPX from Research Biochemicals, Natick, MA (U.S.A.) and [^{3}H]-DPCPX from NEN DuPont UK. Ltd., Stevenage, Hertfordshire. NECA (10 mM) was dissolved in distilled water, CPA (10 mM), DPCPX (10 mM) and **R**-PIA (10 mM) were dissolved in 20% ethanol and diluted in buffer for use.

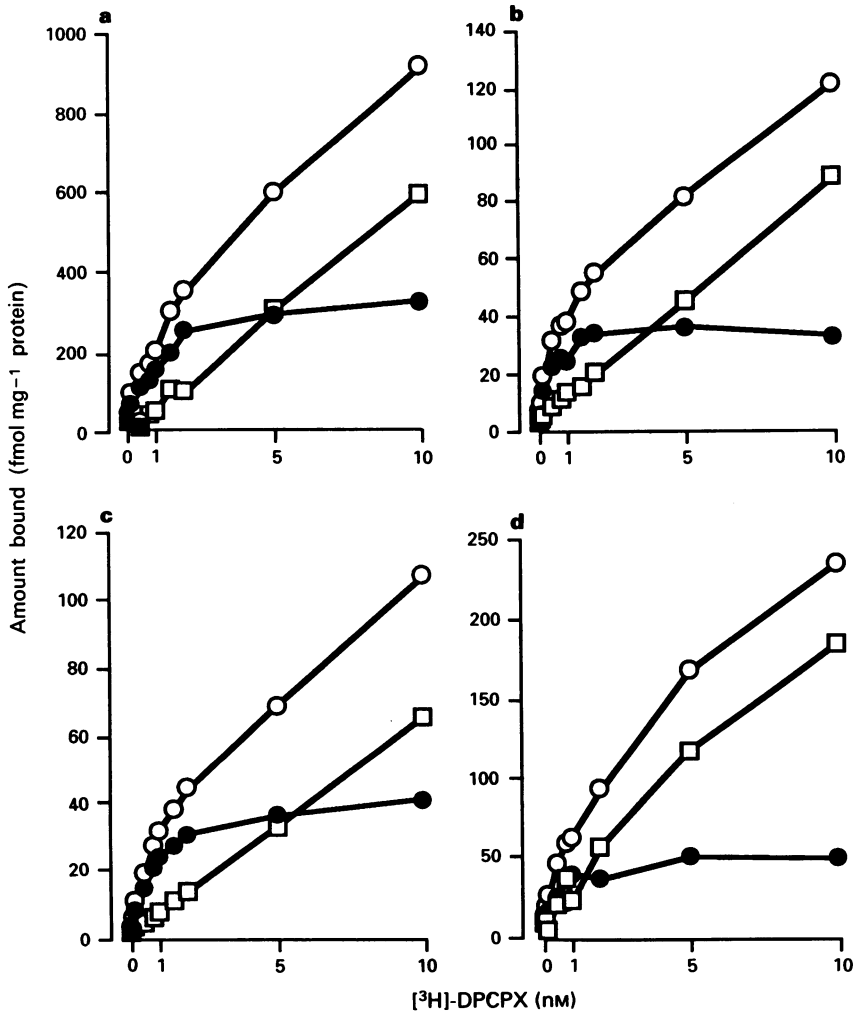

Figure 2 Representative saturation plots performed on (a) colon longitudinal muscle, (b) colon muscularis mucosae, (c) duodenum and (d) vasa deferentia, showing total binding (○), non-specific binding (□) (determined in the presence of 1 μM CPA) and specific binding (●).

All stock solutions were stored frozen at − 18°C. The ADA solution was supplied in 50% glycerol-0.01 M potassium phosphate solution at a concentration of 1000 units in 0.6 ml.

Results

Pilot studies

Pilot studies were undertaken to determine the optimal temperature at which to perform the binding assay on each tissue preparation (results not shown). The optimal temperature for binding was 25°C for the colon, 35°C for the vas deferens and on ice (0°C) for the duodenum. In the duodenum, at higher temperatures the ligand demonstrated a very rapid binding, followed by an immediate loss of binding. At 0°C the reaction was slower but the binding of the radioligand remained at a steady state. A similar loss of binding was observed in the colon at 35°C, but to a lesser extent, allowing the binding assay to be carried out at 25°C. The reason for the loss of binding at 35°C in these tissues in not known, but could be due to some degradation of the radioligand by the gut tissues, which are known to degrade rapidly other purinoceptor ligands (Bailey & Hourani, 1990; 1992; Hourani *et al.*, 1991). Time course studies showed the binding of [^{3}H]-DPCPX to be rapid and reach a steady state within 30 min for each tissue preparation; 30 min was therefore used as the incubation time in the rest of the experiments. On addition of unlabelled CPA (1 μM), [^{3}H]-DPCPX dissociated rapidly from its binding site, with the total amount of [^{3}H]-DPCPX bound returning to a level equivalent to the non-specific binding after 30 min in each tissue (Figure 1). Reproducible binding was obtained only in fresh tissue preparations, freezing homogenate preparations resulted in a decrease in the density of [^{3}H]-DPCPX binding sites.

Saturation experiments

[^{3}H]-DPCPX bound saturably, reversibly and with high affinity to all tissue preparations studied (Figure 2) with the exception of the rat urinary bladder, in which no specific binding of the radioligand was detected (data not shown). Scatchard transformation of specific binding generated monophasic plots (Figure 3) and Hill coefficients were not significantly different from unity, indicating that [^{3}H]-DPCPX binds to an apparently homogenous population of binding sites. The affinity (K_d) of the ligand for each tissue prepara-

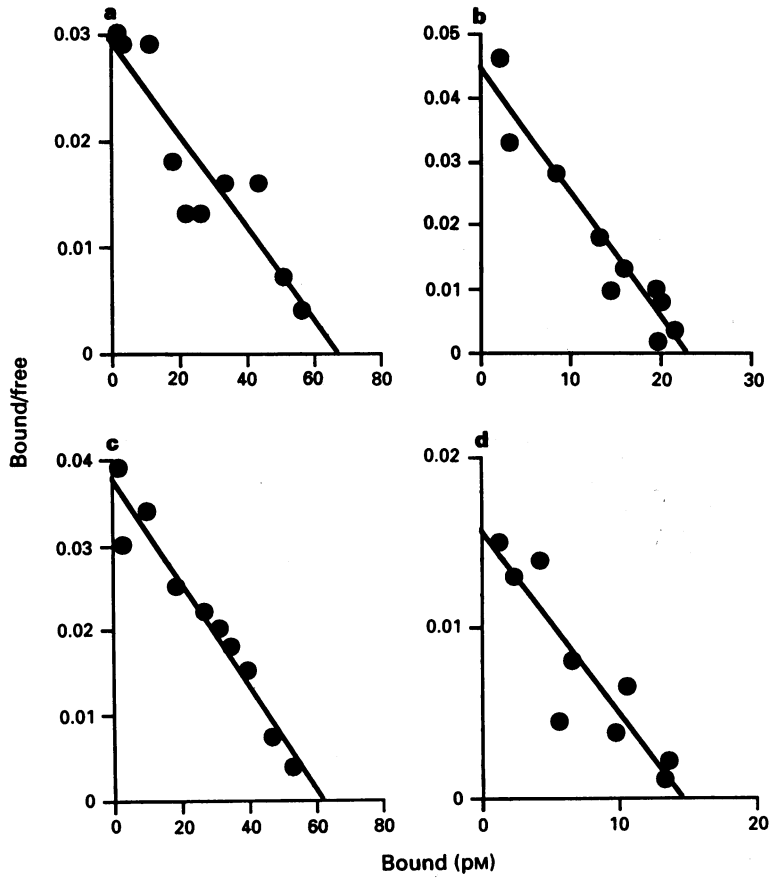

Figure 3 Representative Scatchard plots showing the transformation of the data shown in Figure 2 for the specific binding (●) of [^{3}H]-DPCPX in (a) colon longitudinal muscle, (b) colon muscularis mucosae, (c) duodenum and (d) vasa deferentia.

Table 1 Binding affinity (K_d) and capacity (B_{max}) for 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine in various membrane preparations

	Colon LM	*Colon MM*	*Duodenum*	*Vas deferens*
B_{max} (fmol mg^{-1} protein)	295 ± 70	43 ± 3.5	38.8 ± 4	43.3 ± 12.2
K_d (nM)	1.18 ± 0.47	0.84 ± 0.15	1.59 ± 0.18	0.93 ± 0.17

Values are the mean ± s.e.mean of at least 3 observations.
LM, longitudinal muscle; MM, muscularis mucosae.

tion and the density of binding sites (B_{max}) were determined from individual Scatchard plots (Scatchard, 1949). The affinity of [^{3}H]-DPCPX binding was similar in all membrane preparations with a K_d of ~1 nM (Table 1). The density of binding sites (B_{max}) did not differ significantly between the duodenum, vas deferens and colon muscularis mucosae (Table 1). In the longitudinal muscle of the colon however, there were 6–7 fold more binding sites (Table 1).

Inhibition studies

Inhibition studies were performed with 1 nM [^{3}H]-DPCPX and increasing concentrations of displacing ligands as previously described. Binding was inhibited in a dose-dependent manner by various adenosine receptor ligands, with a rank order of potency of DPCPX ≥ CPA > **R**-PIA > NECA (Figure 4 and Table 2). This rank order of potency is indicative of binding to an A_1 receptor. Concentration-inhibition curves for unlabelled DPCPX versus [^{3}H]-DPCPX were steep with slope factors near unity, whereas the agonists CPA, **R**-PIA and NECA presented with slope factors ranging from 0.8–0.6, suggesting the presence of more than one binding site. Where a better fit was obtained with a two site model than with a one site model, the agonist concentration-inhibition curves were fitted to a two site model using LIGAND (Table 3), resulting in K_d and B_{max} estimates for both a high and low affinity binding site.

Functional assays

Various attempts were made to demonstrate some functional response mediated by A_1 receptors in the longitudinal muscle of the colon (see Figure 5 for representative traces). CPA (1 nM–1 μM) added alone had no direct effect on the longitudinal muscle. Preincubating the tissue with CPA (1 nM–1 μM) for 1 min before the addition of CCh (1 μM) had no effect on the CCh-induced response either in the absence or presence of DPCPX (1 nM). Addition of CPA following contraction of the tissue by CCh was also without effect, except at the higher concentration of CPA (10 μM) when a small relaxation was observed. DPCPX (1 nM) did not modify this effect. Field stimulation of the longitudinal muscle by trains of pulses did not induce contractions but after precontracting the tissue with CCh, field stimulation caused relaxations which were neurogenic in origin as they were inhibited by tetrodotoxin (TTX, 1 μM). Addition of CPA (1 nM–1 μM),

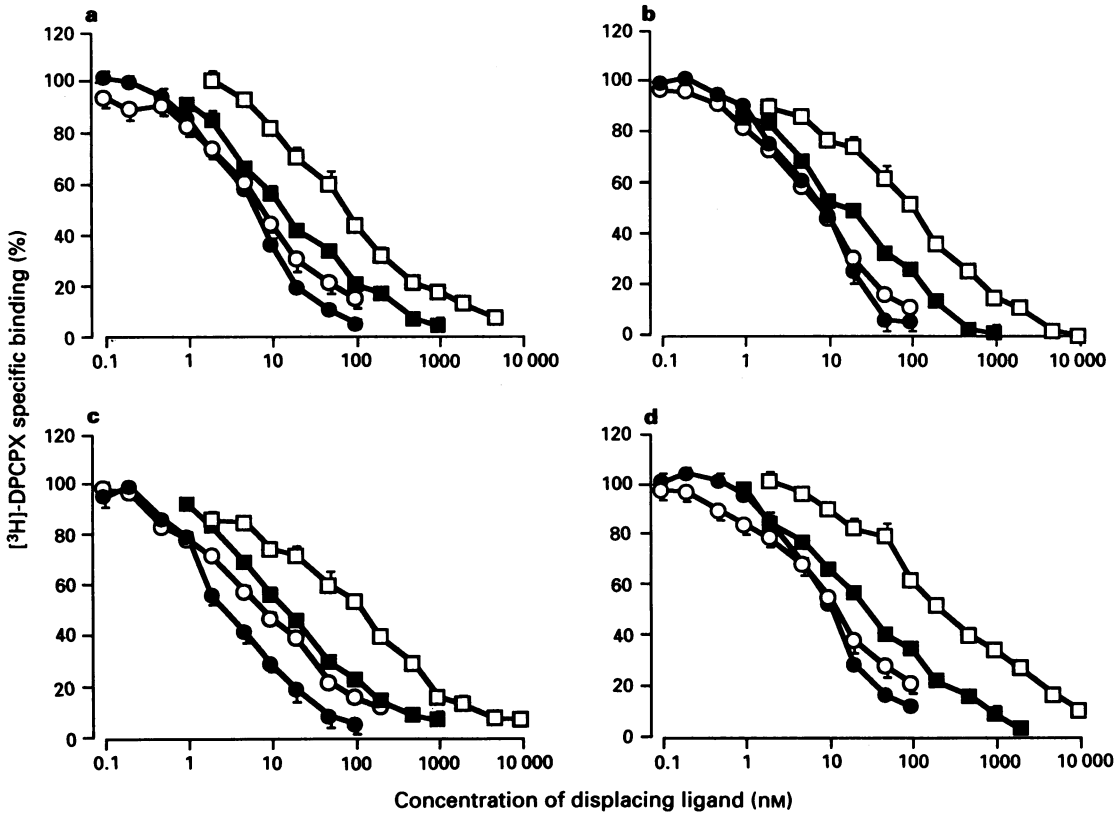

Figure 4 Inhibition of specific [^{3}H]-DPCPX (1nM) binding by (●) DPCPX, (○) CPA, (■) **R**-PIA or (□) NECA in (a) colon longitudinal muscle, (b) colon muscularis mucosae, (c) duodenum and (d) vasa deferentia. Each point is the mean with s.e.mean of at least 5 observations.

Table 2 K_i values (nM) for the inhibition of 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX) binding by various adenosine receptor ligands

	DPCPX		*CPA*		***R**-PIA*		*NECA*	
	K_i	Hill slope	K_i	Hill slope	K_i	Hill slope	K_i	Hill slope
Colon LM	2.95 ± 0.26	1.06 ± 0.03	2.96 ± 0.36	0.81 ± 0.08	9.4 ± 1.67	0.72 ± 0.04	50.09 ± 3.48	0.69 ± 0.04
Colon MM	2.8 ± 0.35	0.99 ± 0.03	3.07 ± 0.62	0.82 ± 0.08	12.22 ± 3.34	0.73 ± 0.05	56.37 ± 12.42	0.62 ± 0.04
Duodenum	1.69 ± 0.28	0.97 ± 0.04	4.45 ± 1.02	0.63 ± 0.02	9.19 ± 1.13	0.69 ± 0.02	67.45 ± 8.4	0.59 ± 0.05
Vas deferens	3.58 ± 0.4	1.09 ± 0.05	3.28 ± 0.64	0.74 ± 0.09	13.62 ± 1.98	0.64 ± 0.03	64.48 ± 9.21	0.58 ± 0.06

Values are the mean ± s.e.mean of at least 5 observations, calculated using a one site model.
LM, longitudinal, MM, muscularis mucosae.

Table 3 Two-site analysis of concentration-inhibition curves for agonists in displacing 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine binding: K_i values (nM) are given for high (K_H) and low (K_L) affinity sites

	CPA			*R-PIA*			*NECA*		
	K_H	K_L	% K_H	K_H	K_L	% K_H	K_H	K_L	% K_H
Colon LM	0.25	41.99	45.52	2.63 ± 0.75	370 ± 270	62.6 ± 4.9	22.62 ± 3.82	4110 ± 1700	74.7 ± 2.3
Colon MM	0.93 ± 0.56	10.84 ± 1.45	55.2 ± 8.5	1.52 ± 0.04	88.1 ± 45.6	55.7 ± 5.7	10.97 ± 6.18	2190 ± 1560	44 ± 7.5
Duodenum	0.17 ± 0.06	31.58 ± 21.46	36.9 ± 6.6	1.97 ± 0.33	43.9 ± 1.4	50.2 ± 12	11.22 ± 6.32	509.6 ± 205.8	51 ± 10.4
Vas deferens	0.43	9.82	37.7	3.04 ± 0.93	30.25 ± 9.07	58.6 ± 12	18.49 ± 7.8	5350 ± 3500	40.7 ± 5.8

% K_H shows the percentage of receptors in the high affinity state.
Not all the competition inhibition experiments with CPA were best described by a two site fit, consequently the number of determinations presented for this agonist are reduced compared with Table 2; Colon LM $n = 1$, Colon MM $n = 3$, duodenum $n = 4$ and vas deferens $n = 2$.

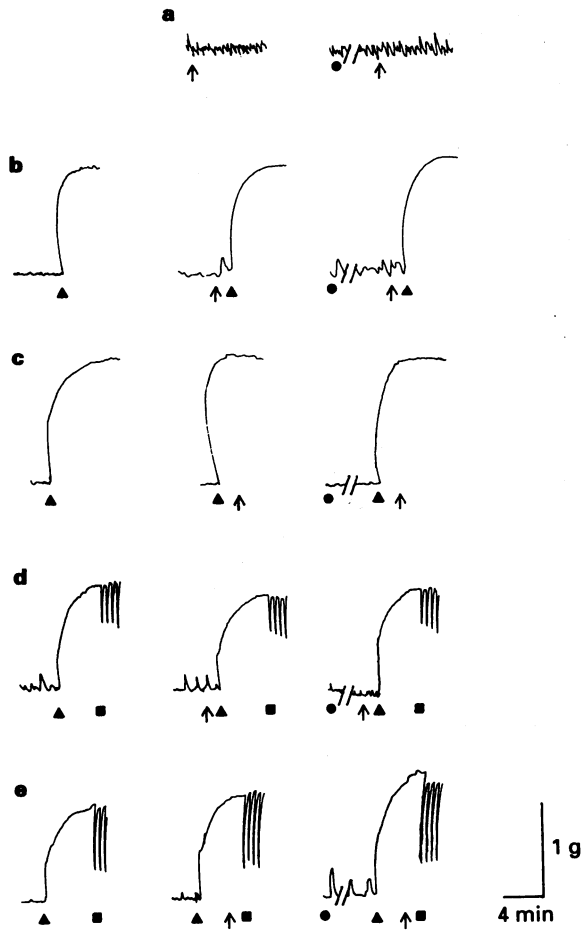

Figure 5 Representative traces showing the responses of the rat colon longitudinal muscle to CPA (↑) (10 nM) in the absence and presence of DPCPX (1 nM) (●); (a) the effects of CPA alone; (b) the effect of preincubation with CPA (1 min) followed by contraction induced by CCh (1 μM) (▲); (c) the effect of precontraction with CCh followed by administration of CPA; (d) the effect on nerve stimulation (■) of CPA added before contraction with CCh (1 μM); (e) the effect on nerve stimulation of CPA added after contraction with CCh (1 μM).

however, had no effect on the nerve induced response either in the absence or in the presence of DPCPX (1 nM).

Discussion

Much of the interest in adenosine binding sites has concentrated on the central nervous system, with binding sites commensurate with adenosine A_1 receptors identified in the brains of a variety of different species including rat, sheep, guinea-pig and cattle (Bruns *et al.*, 1987; Klotz *et al.*, 1991; Leung *et al.*, 1990; Williams & Valentine, 1985). However, limited attention has been paid to adenosine A_1 binding sites in peripheral tissues, although such sites have been identified in myocardial tissue (Leid *et al.*, 1988; Musser *et al.*, 1993), bovine testicular tissue (Cushing *et al.*, 1988), guinea-pig uterus (Schiemann *et al.*, 1990; 1991) and human adipocyte membranes (Larrouy *et al.*, 1991). The limited information regarding adenosine A_1 binding sites in peripheral tissues led us to attempt to develop a radioligand binding assay for A_1 receptors in a variety of rat smooth muscle preparations for which we already had functional data (Bailey *et al.*, 1992; Nicholls *et al.*, 1992; Hourani *et al.*, 1993; Hourani & Jones, 1994). [^{3}H]-DPCPX is an antagonist radioligand with 700 fold A_1 over A_2 selectivity (Bruns *et al.*, 1987), and was thus the ligand of choice to characterize the adenosine A_1 binding sites in smooth muscle.

The present study demonstrated [^{3}H]-DPCPX binding sites on all membrane preparations studied, with the exception of the rat urinary bladder where no specific [^{3}H]-DPCPX binding was detected. However, such a finding was not unexpected as previous functional assays have demonstrated the existence of A_2 receptors only (Nicholls *et al.*, 1992), and furthermore provided a useful method of validation for the assay protocol used. [^{3}H]-DPCPX bound with high affinity to the rat duodenum, colon muscularis mucosae and longitudinal muscle and vasa deferentia and in all cases the affinity of the adenosine A_1 binding site for [^{3}H]-DPCPX was similar, with K_d values in the low nM range. This is consistent with previous studies in a number of tissue preparations where the K_d values have ranged from 0.45 nM for rat brain membranes (Klotz *et al.*, 1990), 1.6 nM for pregnant guinea-pig myometrium (Schiemann *et al.*, 1990) to 3.01 nM for guinea-pig ventricular preparations (Musser *et al.*, 1993). The K_d values for [^{3}H]-DPCPX derived for the duodenum, colon muscularis mucosae and vasa deferentia are also consistent with values previously derived with DPCPX used an an antagonist in functional studies. In these tissues, K_B values in the region of 1 nM were obtained, although Schild plots were not carried out in the duodenum and vas deferens and the slope of the Schild plot was not unity in the colon muscularis mucosae (Nicholls *et al.*, 1992; Bailey *et al.*, 1992; Hourani *et al.*, 1993). The density of binding sites in these tissues was similar with B_{max} values ranging from 38–43 fmol mg^{-1} protein, and this is again consistent with our previous functional studies, where the potency of the A_1 agonist CPA was similar with EC_{50} values of around 0.1 μM for each tissue (Nicholls *et al.*, 1992; Bailey *et al.*, 1992; Hourani *et al.*, 1993). These findings provide evidence that the [^{3}H]-DPCPX binding sites do indeed correspond to A_1 receptors.

Further evidence that [^{3}H]-DPCPX binds to A_1 receptors was established by inhibition experiments using CPA, DPCPX, NECA and R-PIA. In all tissue preparations studied the order of potency of the competing ligands was DPCPX ≥ CPA > R-PIA > NECA, with K_i values in good agreement with those previously reported (Bruns *et al.*, 1987;

Leid *et al.*, 1988) and this order of potency is characteristic of an A_1 receptor (Collis & Hourani, 1993). Competition for binding of [^{3}H]-DPCPX resulted in shallower curves for the agonists than for unlabelled DPCPX suggesting the existence of receptors in both high and low affinity states for the agonists. Indeed when subjected to a two site fit the agonists did display both a high and low affinity binding component of the receptor (Table 3), and such findings are consistent with previous reports demonstrating the existence of multiple affinity binding states for agonists versus [^{3}H]-DPCPX binding, in the brain (Bruns *et al.*, 1987; Klotz *et al.*, 1991), adipocyte membranes (Larrouy *et al.*, 1991) and myocardial tissue (Leid *et al.*, 1988).

Species, tissue and ligand differences, in combination with variations in membrane preparations, make it difficult to compare directly the published binding parameters, such as receptor density (B_{max}), of radioligands for the adenosine A_1 receptor. Porcine brain has a significantly lower density of A_1 binding sites labelled with [^{3}H]-DPCPX than both hamster and sheep brain (Klotz *et al.*, 1991); pregnant guinea-pig myometrium has half the number of A_1 binding sites compared to non-pregnant guinea-pig myometrium (Schiemann *et al.*, 1990; 1991). The density of A_1 binding sites, labelled by [^{3}H]-DPCPX, in the rat duodenum, colon muscularis mucosae and vasa deferentia is less than the B_{max} values reported for brain membranes (0.19–4.09 pmol mg^{-1} protein) (Klotz *et al.*, 1991; Oliveira *et al.*, 1991), but is in agreement with the density of A_1 binding sites in myocardial tissue (8.5–75.9 fmol mg^{-1} protein) (Musser *et al.*, 1993), thereby suggesting that peripheral tissues have a lower density of A_1 receptors. Further evidence for the diversity in density of A_1 receptors between peripheral and central tissues has been provided by Williams & Valentine (1985), who demonstrated a greater number of high affinity A_1 binding sites labelled with [^{3}H]-CHA in the brain membranes than the ileal membranes of guinea-pigs. Surprisingly, we found the highest density of A_1 binding sites in the outer thin walled longitudinal muscle of the rat colon, seven fold higher than the density of binding sites situated on the thick walled inner muscularis mucosae, thereby indicating a heterogeneous density of A_1 binding sites within the structure of the distal colon. Such interstructure variations have been reported elsewhere, as Musser *et al.* (1993) found that in guinea-pig myocardial tissue, the density of A_1 binding sites differed between the atria and ventricles, with the higher density of A_1 binding sites found in the atria.

The presence of A_1 receptors in the rat duodenum, colon muscularis mucosae and vasa deferentia has previously been confirmed by functional assays. We have shown the existence of inhibitory A_1 receptors in the rat duodenum (Nicholls *et al.*, 1992), excitatory A_1 receptors in the colon muscularis mucosae (Bailey *et al.*, 1992) and both inhibitory prejunctional and excitatory postjunctional A_1 receptors in the vasa deferentia (Hourani *et al.*, 1993; Hourani & Jones, 1994). However, the binding of [^{3}H]-DPCPX to the longitudinal muscle of the rat colon was unexpected as we have previously shown the existence of relaxant A_2 receptors only in this tissue preparation (Bailey & Hourani, 1992). The possible existence of previously undetected postjunctional A_1 receptors was explored using the A_1 agonist CPA and the antagonist DPCPX at a concentration (1 nM) selective for A_1 receptors. The finding that CPA had no direct effect on the tissue preparation and no effect on the CCh-induced contractile response, either in the absence or presence of DPCPX, demonstrated the lack of postjunctional A_1 receptors in this smooth muscle preparation. The presence of any prejunctional A_1 receptors was also ruled out, as CPA was without effect on the nerve stimulated responses, again both in the absence and presence of DPCPX. The apparent lack of a functional response to A_1 receptor stimulation is in direct contrast to the findings of the [^{3}H]-DPCPX binding assay performed on this tissue preparation, where a large number of A_1 binding sites were identified. This suggests that the [^{3}H]-DPCPX binding sites identified in the longitudinal muscle of the rat colon correspond to A_1 receptors which cannot be detected by measuring contractile or relaxant responses.

In conclusion, we have successfully developed a [^{3}H]-DPCPX radioligand binding assay using smooth muscle tissue preparations and have demonstrated high affinity, saturable binding of the A_1-selective antagonist [^{3}H]-DPCPX to rat duodenum, vasa deferentia and colon muscularis mucosae, which we have previously shown in functional studies (Nicholls *et al.*, 1992; Bailey *et al.*, 1992; Hourani *et al.*, 1993) to contain A_1 receptors. A high density of binding sites for [^{3}H]-DPCPX was also detected in the longitudinal muscle of the rat colon, although the functional significance of these binding sites has yet to be determined.

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