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Differential development of adenosine A_1 and A_{2b} receptors in the rat duodenum

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1 The development of the adenosine A_1 and A_{2b} receptors inducing relaxation of the rat duodenum was studied by use of a combination of functional and radioligand binding assays on rats aged between 5 and 30 days and compared with results previously found in adult rat duodenum.

2 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX) bound with high affinity to a single site in duodenum preparations from rats aged 20, 25 and 30 days. At 10 and 15 days there was no detectable specific binding of [^{3}H]-DPCPX.

3 The affinity (K_D) of the binding site for [^{3}H]-DPCPX was similar in membrane preparations from 20, 25 and 30 day old animals (1.58-2.27 nM), but the density (B_{max}) of binding sites was found to increase up to 25 days where peak levels (72.0±9.5 fmol mg^{-1} protein) were observed and then decline at 30 days (45.5±2.9 fmol mg^{-1} protein) to levels commensurate with those previously determined in the adult rat duodenum.

4 In duodenum from 10 day old rats no responses to N^6-cyclopentyladenosine (CPA, 1 nM-10 μM) were observed, at 15 days the duodenum responded to the highest concentration of CPA (3 μM) only, and at 20-30 days concentration-related responses were observed, with the potency of CPA increasing with an increase in age. DPCPX (10 nM) abolished the responses to CPA except at the highest concentration of CPA (3 μM) where the response was markedly attenuated, suggesting the presence of an A_1 receptor.

5 In rat duodenum from animals of all ages (5-30 days), concentration-related responses to 5'-N-ethylcarboxamidoadenosine (NECA) were observed. The potency of NECA remained constant with an increase in age, whereas the maximum relaxation response increased from 20% at 5 days to 110% at 25 and 30 days. In the presence of 1 μM DPCPX a right-ward shift in the concentration-response curve to NECA was observed at all ages. In the presence of 10 nM DPCPX, the response to NECA was unaffected in the duodenum from animals aged 10 and 15 days. However, in duodenum from animals aged 20-30 days the concentration-response curve to NECA was shifted to the right suggesting that there is an A_1 component to the action of NECA at these ages. Schild analysis of the effects of increasing concentrations of DPCPX versus NECA on the duodenum from 25 day old animals generated a slope of 0.62 suggesting that NECA acts at A_1 and A_{2b} receptors as in the adult.

6 The A_{2b}-selective analogue, 2-[*p*-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) (10 nM-10 μM) was without effect on the carbachol-contracted duodenum from 15 day old rats and the duodenum from 25 day old rats responded to the highest concentration of CGS 21680 only, suggesting that the A_2 receptors here, as in the adult, are not of the A_{2a} subtype. The adenosine antagonist, 8-phenyltheophylline (8-PT) (10 μM), abolished the inhibitory effects of NECA (100 nM-100 μM) on 10, 15 and 25 day old rat duodenum indicating that the responses to NECA were not mediated via an adenosine A_3 receptor.

7 These results show that adenosine A_1 receptors in rat duodenum are present and functionally viable from day 20 onwards and that the density of A_1 receptors varies with age, increasing up to day 25 and then declining at day 30 to a density commensurate with that found in the adult. The responses to CPA, mediated via the A_1 receptor, increase with age in a similar fashion. In contrast however, the response to NECA was evident from day 5, the earliest age studied, and from days 5-15 NECA acted via the A_{2b} receptor subtype. However, from day 20 onwards NECA acted at a mixed population of A_1 and A_{2b} receptors. These results demonstrate the differential development of the A_1 and the A_{2b} receptors in the rat duodenum.

Keywords: Rat duodenum; ontogeny; A_1 and A_{2b} receptors; [^{3}H]-DPCPX; CPA; NECA

Introduction

The pharmacological actions of adenosine are mediated via specific receptors (P_1 purinoceptors) which were originally subdivided into A_1 and A_2 receptors, based on their differential selectivity for adenosine analogues and their sensitivity to A_1-selective antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). On A_1 receptors, N^6 substituted adenosine analogues such as N^6-cyclopentyladenosine (CPA) are more potent than 5' substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA) and DPCPX has a dissociation constant in the nanomolar range, whereas on A_2 receptors 5' substituted adenosine analogues are more potent and DPCPX has a dissociation constant in the micromolar range. A_2 receptors have been further subdivided into A_{2a} and A_{2b}, with the adenosine analogue 2-[*p*-(carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) being more potent at the A_{2a} receptor (for review see Collis & Hourani, 1993; Fredholm *et al.*, 1994). Recently, a receptor which binds both the A_1 selective agonist [^{125}I]-(N^6-(4-aminophenyl)-ethyladenosine (APNEA) and [^{3}H]-NECA but not [^{3}H]-DPCPX has been cloned and termed the A_3 receptor (Zhou *et al.*, 1992).

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The A_1 receptor has been studied extensively by use of radioligand binding assays. Many agonist radioligands, for example [^{3}H]-CPA, [^{3}H]-N^6-cyclohexyladenosine (CHA) or [^{3}H]-(**R**)-N^6-phenylisopropyladenosine (**R**-PIA) are available for the study of this receptor subtype and have been used to identify A_1 receptors in both central (Bruns *et al.*, 1980; Williams *et al.*, 1986) and peripheral tissues (Williams & Valentine, 1985). The development of the antagonist radioligand, [^{3}H]-DPCPX, with 700 fold selectivity for A_1 receptors compared to A_2 receptors (Bruns *et al.*, 1987), has overcome the problems associated with agonist radioligands and is thus the ligand of choice to characterize these sites. Radioligand binding studies using [^{3}H]-DPCPX have identified A_1 binding sites in the brain of a variety of different species (Lohse *et al.*, 1987; Klotz *et al.*, 1991; Oliveira *et al.*, 1991) and in peripheral tissues A_1 receptors have been identified in myocardial tissue (Musser *et al.*, 1993), uterine membranes (Schiemann *et al.*, 1990; 1991) and adipocyte membranes (Larrouy *et al.*, 1991). More recently, we have developed a radioligand binding assay using [^{3}H]-DPCPX to study the distribution of A_1 receptors in a variety of adult smooth muscle preparations, and A_1 binding sites were identified on the rat duodenum, vasa deferentia and both the longitudinal muscle and muscularis mucosae of the distal colon (Peachey *et al.*, 1994). In adult rat duodenum, the K_D value for [^{3}H]-DPCPX derived for the duodenum was found to be in good agreement with values derived by use of DPCPX as an antagonist in functional studies, where K_B values in the region of 1 nM were obtained (Nicholls *et al.*, 1992).

The ontogenetic profile of the P_1-purinoceptor on the rat duodenum has indicated that these receptors are present from birth (Nicholls *et al.*, 1990). Adenosine exerted an inhibitory action on neonatal rat duodenum and the potency of adenosine increased with age up to day 25. However, subsequent studies in the adult rat duodenum revealed the presence of both the A_1 and A_{2b} subtypes of the P_1-purinoceptor, both receptor subtypes unusually mediating an inhibitory response

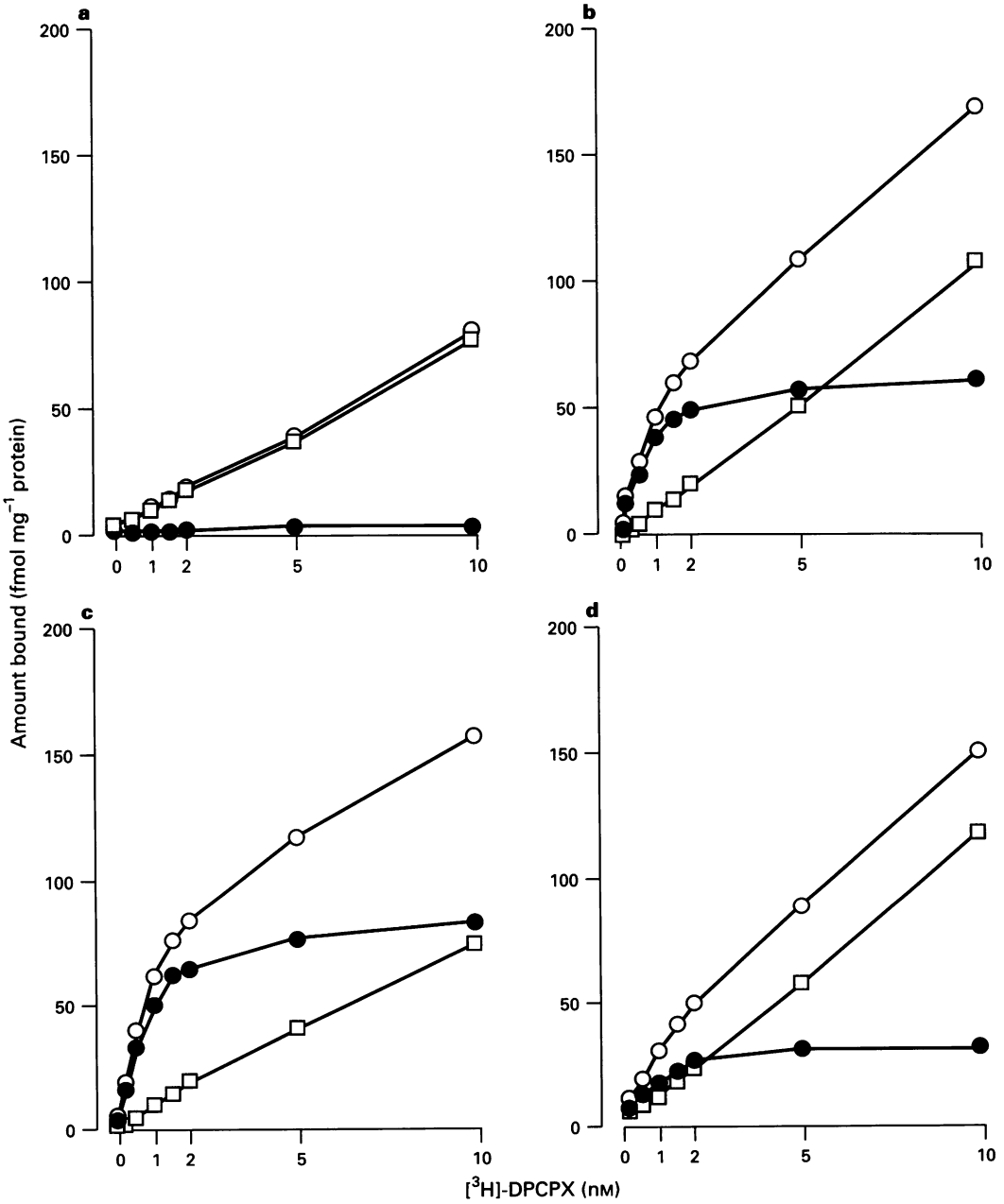

Figure 1 Representative saturation plots performed on rat duodenum from animals aged (a) 15 days, (b) 20 days, (c) 25 days and (d) 30 days, showing total binding (○), non-specific binding (□) (determined in the presence of 1 μM CPA) and specific binding (●).

(Nicholls *et al.*, 1992). However, the ontogenetic profile of the P_1-purinoceptor previously constructed did not distinguish between the A_1 and A_2 receptor as adenosine can act via both subtypes. It was therefore not possible to tell from this profile whether the inhibitory response to adenosine, observed from postnatal day 5 to 25, was due to action at more than one receptor subtype, or whether the receptors exhibited differential development. The aim of this study was therefore, to extend the radioligand binding study developed for the adult rat duodenum, in combination with age-matched functional studies, to follow the ontogenetic profile of the A_1 receptor subtype in neonatal rat duodenum. In addition, the development of the A_{2b} receptor subtype was followed using functional assays, as no radioligand selective for this subtype is as yet available.

Methods

Tissues

Male Wistar albino rats (University of Surrey strain) aged 5, 10, 15, 20, 25, 30 days old and adult were used throughout these experiments. The day of birth was designated as day 1 and animals were culled to litters of 8–10 pups to each female to maintain a standard litter size. Animals were housed as mixed sexes. The animals were killed by cervical dislocation and in each case the duodenum was dissected out by cutting at the base of the pylorus and 0.5 cm from this point, cleared of any connective tissue and washed through with buffer.

Membrane preparation

Tissues were placed in ice-cold 50 mM Tris/HCl (pH 7.4) buffer. Tissues from at least 15 age-matched animals were pooled for each assay. All tissues were blotted dry to remove excess buffer, weighed and roughly scissor chopped. Homogenization and centrifugation were performed as outlined in Peachey *et al.* (1994). The resulting pellet was resuspended in 50 mM Tris/HCl and incubated with 5 uml^{-1} adenosine deaminase for 30 min at room temperature before use. Protein estimations were performed according to the method of Lowry *et al.* (1951).

Binding assays

Saturation experiments were only undertaken on 15, 20, 25 and 30 day old duodenum and were performed according to the method already established for adult duodenum (Peachey *et al.*, 1994). Eight concentrations of [^{3}H]-DPCPX (0.05–10 nM) (specific activity 109 Ci $mmol^{-1}$) were used, the non-specific binding was defined by 1 μM CPA and the assay mixture incubated on ice for 30 min. The limited homogenate obtained from 10 day old neonates prevented saturation assays from being performed, therefore homologous displacement assays were undertaken. A single concentration of [^{3}H]-DPCPX (1 nM) was used in the presence of increasing concentrations of unlabelled DPCPX (0.1–100 nM) and incubations performed as outlined previously (Peachey *et al.*, 1994). Results were analysed by LIGAND (Munson & Rodbard, 1980) giving measures of ligand affinity (K_D) and receptor density (B_{max}).

Functional assays

Functional assays were performed according to the method of Nicholls *et al.* (1990). Tissues were mounted with the lumen sealed in 3.5 ml organ baths containing Krebs solution (mM composition: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 2.5 and glucose 11), gassed with 95% O_2/5% CO_2 and maintained at 35–36°C at resting tensions of 0.5 g (20–30 days) and 0.2 g (<20 days). Tissues were allowed to equilibrate for 1 h prior to the addition of any drugs and responses were recorded isometrically via an FTO3 force displacement transducer and displayed on a Grass 79D polygraph. Inhibitory responses to CPA (1 nM–10 μM) and NECA (100 nM–30 μM) were quantified by precontracting the tissue with 0.1 μM carbachol (CCh) and challenging with the purine 1 min later. In the duodenum from both adult and 15 day old rats this concentration of carbachol results in a 40% contractile response. Contractions to CCh were measured from the peak of spontaneous activity to the peak of CCh response and the relaxations to CPA and NECA were determined as the reduction in this peak height and expressed as % inhibition of CCh response. Concentration-responses were obtained non-cumulatively and a dose cycle of 15 min maintained. Concentration-response curves to CPA were constructed in the absence and the presence (30 min incubation period) of 10 nM DPCPX and response curves to NECA were determined in the absence and presence of 10 nM and 1 μM DPCPX. In the case of animals aged 20 and 25 days, the responses to NECA were also examined in the presence of 100 nM and 3 μM DPCPX. The effect of NECA (100 nM–100 μM) on the CCh-contracted duodenum from 10, 15 and 25 day old rats was also examined in the presence of the P_1 purinoceptor antagonist 8-phenyltheophylline (8-PT, 10 μM). The response of 15 day old rat duodenum to the A_{2a}-selective adenosine agonist, CGS 21680 (10 nM–10 μM) was also assessed.

Responses to CPA were calculated as IC_{50} values (the concentration of CPA giving a 50% reversal of the CCh-induced contraction) and the potency of CPA was expressed as pIC_{50} values, the negative $\log_{10}$ of the IC_{50} value. In the case of animals aged 15 and 20 days the response to CPA was extrapolated to given an estimation of the IC_{50} value. Responses to NECA were calculated as EC_{50} values, referring to the concentration of NECA which results in a response half the size of the maximal response at that age, rather than 50% relaxation. At 5, 10, 15, 20, 25 and 30 days, EC_{50} values were therefore calculated at 10%, 15%, 30%, 50%, 55% and 55% relaxations respectively, and in all cases the potency of NECA was expressed as a pD_2 value. At 25 days a Schild plot of the effect of DPCPX on the NECA-induced response was constructed, dose-ratios being calculated relative to the pooled mean EC_{40} values of the control responses to NECA to minimize the interday variation observed with the NECA-induced response curve. The EC_{40} was used in this case because DPCPX sometimes caused a decrease in the maximum response of the tissue.

Materials

CPA, NECA, 8-PT and adenosine deaminase (ADA) (Type VI) were obtained from Sigma UK. Ltd, Poole, Dorset; DPCPX and CGS 21680 from Research Biochemicals, Natick,

Table 1 Binding affinity (K_D) and capacity (B_{max}) for [^{3}H]-DPCPX in rat duodenum

	Age (days)					
	10	*15*	*20*	*25*	*30*	*Adult*
B_{max} (fmol mg^{-1} protein)	ND	ND	58.5±7.8	72.0±9.5	45.5±2.93	38.8±4.0
K_D (nM)	ND	ND	1.58±0.62	2.27±0.7	1.66±0.37	1.59±0.18

Values are the mean±s.e.mean of at least 3 observations. ND not detectable. Adult values obtained from Peachey *et al.* (1994).

MA (U.S.A.) and [^{3}H]-DPCPX from NEN DuPont UK. Ltd., Stevenage, Hertfordshire. CPA (10 mM) was dissolved in 20% ethanol and DPCPX (10 mM) was dissolved in 20% ethanol or 6 mM NaOH containing 6% dimethyl sulphoxide (DMSO), all stock solutions were diluted in buffer for use, 8-PT (1 mM) was dissolved in 0.02 M NaOH containing 1% ethanol and CGS 21680 (10 mM) was dissolved in 10% DMSO. 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

Binding assays

[^{3}H]-DPCPX bound with high affinity to 30, 25 and 20 day old rat duodenum (Figure 1). Scatchard transformation of the specific binding generated monophasic plots and Hill coefficients were not significantly different from unity, indicating that [^{3}H]-DPCPX binds to an apparently homogeneous population of binding sites. The affinity (K_D) of [^{3}H]-DPCPX for the duodenum from 30, 25 and 20 day old animals was similar (1.58–2.27 nM) (Table 1) and these values were not significantly different from the K_D value previously determined for adult duodenum (1.59 nM) (Peachey *et al.*, 1994). The density (B_{max}) of [^{3}H]-DPCPX binding sites in duodenum increased up to day 25 where peak levels were observed and decreased thereafter by 40% to levels equivalent to those found in the adult (Table 1, Figure 3). At 10 and 15 days only very low levels of specific binding were detected thus ligand affinity and receptor density were unable to be quantified.

Functional assays

At day 10 no response to CPA (10 nM–3 μM) was observed (results not shown), at day 15 a response to 3 μM CPA only was observed with no response at concentrations below this. At days 20, 25 and 30 CPA relaxed the CCh-contracted rat duodenum (Figure 2), the potency of CPA increasing with an increase in age (Figure 3). At all ages studied the maximum response to CPA was not achieved, but the increase in potency of CPA appeared to reflect a leftward shift in the concentration-response curve with an increase in age. At 15 and 20 days the response to CPA was extrapolated to give an estimate of potency from which pIC_{50} values could be calculated, derived from the concentration causing 50% reversal of CCh-induced contraction rather than 50% of the max-

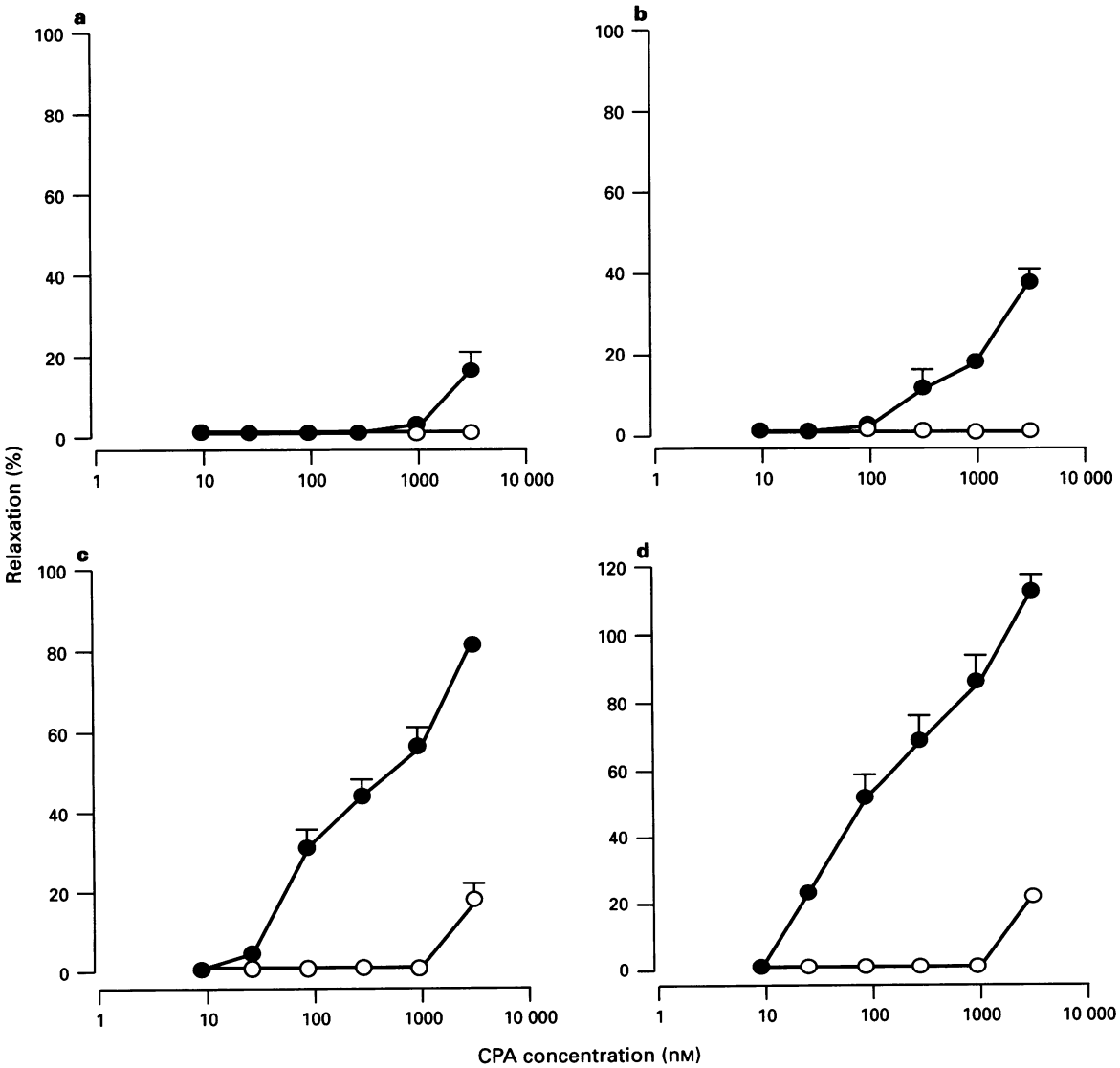

Figure 2 The effects of CPA (10 nM–3 μM) in the absence (●) and presence (○) of DPCPX (10 nM) on rat duodenum from animals aged (a) 15 days, (b) 20 days, (c) 25 days and (d) 30 days. Responses are expressed as the % inhibition of the contraction induced by 0.1 μM carbachol. Each point is the mean with s.e.mean of at least 6 observations.

imum response. Although these values are not exact, they give an indication of the potency of the adenosine analogue. DPCPX (10 nM) abolished the inhibitory responses induced by CPA (10 nM – 1 μM) while at the highest concentration of CPA (3 μM), the inhibitory response was greatly attenuated (Figure 2).

Inhibitory responses to NECA (100 nM – 30 μM) were observed from day 5 onwards (Figure 4), and full concentration-response curves could be obtained with the maximum response to NECA increasing with an increase in age (Figures 4 and 5), whereas the potency of NECA expressed as pD_2 values remained constant (Figure 5). DPCPX (1 μM) gave a seven fold shift in the concentration-response curve to NECA at days 5 – 25, and at day 30 a 30 fold shift was observed. In the presence of DPCPX (10 nM), the concentration-response curve to NECA in 20, 25 and 30 day old rat duodenum shifted to the right giving a dose ratio of 4.78, 6.7 and 6.7 respectively, whereas at 10 and 15 days, the response to NECA was unaffected in the presence of 10 nM DPCPX (Figure 6). In the presence of increasing concentrations of DPCPX the response to NECA in animals aged 25 days shifted to the right, dose-ratios being calculated relative to the pooled mean EC_{40} values of the control responses to NECA and Schild analysis of the effects of DPCPX versus NECA gave a plot with a slope of 0.62 (Figure 7). As the slope was much less than unity no pA_2 could be calculated. 8-PT (10 μM) abolished the inhibitory effect of NECA (100 nM – 100 μM) on the CCh-contracted duodenum from rats aged 10, 15 and 25 days, and CGS 21680 (10 nM – 10 μM) was without effect on the duodenum from 15 day old rats and 25 day old rat duodenum responded to 10 μM CGS 21680 only (results not shown).

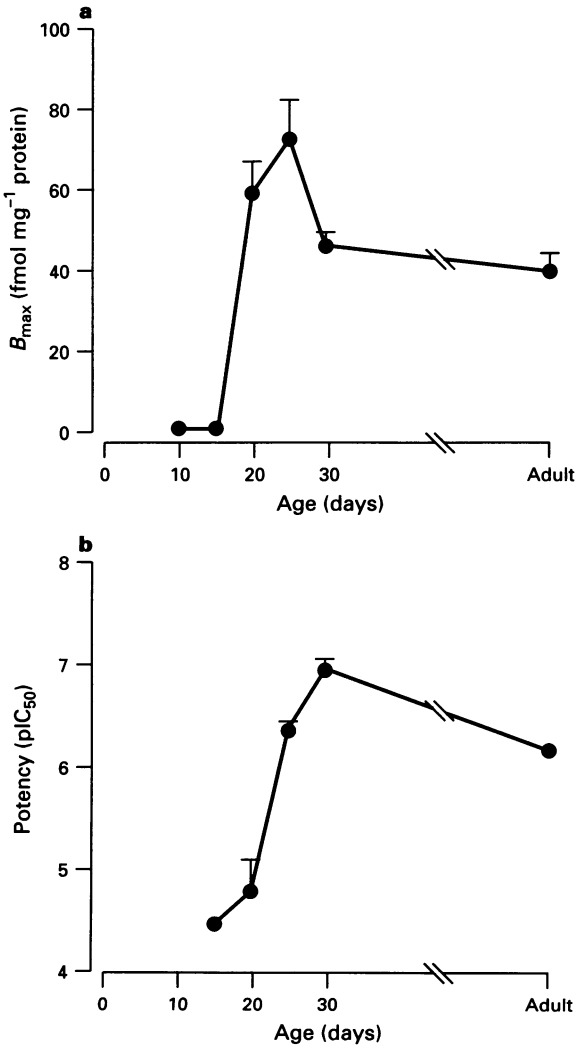

Figure 3 Variations in (a) density (B_{max}) of A_1 receptors and (b) potency (pIC_{50}) of CPA with age in rat duodenum. Potency is expressed as pIC_{50} (negative $\log_{10}$ of concentration producing 50% reversal of CCh-induced contraction), and for points at 15 and 20 days graphs were extrapolated to 50% and therefore are not exact values. Each point is the mean with s.e.mean of least 3 determinations. The points for adults were obtained from data previously published (a) Peachey *et al.* (1994), (b) Nicholls *et al.* (1992).

Discussion

Previous studies on neonatal rat duodenum have shown P_1 purinoceptors to be present from postnatal day 5 onwards. Adenosine relaxed the CCh-contracted duodenum from day 5, the earliest age studied, with potency values increasing with age up to day 25 (Nicholls *et al.*, 1990). However, adenosine can act via either an A_1 or an A_2 receptor and the adult rat duodenum contains a mixture of A_1 and A_{2b} receptors which both cause relaxation (Nicholls *et al.*, 1992). In view of this, it was not possible to differentiate clearly the development of the A_1 and A_2 receptors from the ontogenetic profile of the P_1 purinoceptor already constructed, and indeed it was not known whether in neonatal rat duodenum the receptors are of the same subtype as those identified in the adult. Thus, this study attempted to follow the development of the A_1 receptor in the rat duodenum, using functional assays and the A_1 receptor ligand binding assay method developed for adult rat duodenum (Peachey *et al.*, 1994), and also the development of the A_2 receptor by studying functional responses.

The present study demonstrated [^{3}H]-DPCPX binding sites to be present from day 20 onwards. The density of binding sites increased up to postnatal day 25, where peak levels were observed, and decreased thereafter to levels commensurate with those already established in the adult duodenum (Peachey *et al.*, 1994). The K_D values generated for [^{3}H]-DPCPX binding sites in neonatal rat duodenum did not differ significantly from the K_D value previously generated for adult rat duodenum and suggests that the observed differences in receptor density are not due to binding to another receptor system. Furthermore, the similiarity in K_D values suggests that the [^{3}H]-DPCPX binding sites identified in neonatal rat duodenum correspond to an A_1 receptor. The lack of specific binding observed at days 10 and 15 compared with day 20 does not suggest a failure in methodology but rather suggests that either the A_1 receptors are not present, or are present at levels too low to be measured accurately by the traditional binding assay method. At 10 days the very small size of the tissue prevented a full saturation assay from the being performed, therefore homologous displacement assays were undertaken, but as with the binding observed at 15 days, specific binding was not consistently detectable.

Functional assays showed that the inhibitory response of the neonatal rat duodenum to CPA varied with age. At 10 days no response to CPA was observed, at 15 days the duodenum responded to the higher concentrations of CPA whereas at days 20, 25 and 30 the response to CPA was more pronounced. DPCPX (10 nM) attenuated the inhibitory responses to CPA at these ages suggesting that CPA is acting via an A_1 receptor. The potency of CPA was shown to increase with an increase in age shown by the increase in pIC_{50} values, although these vlaues only give an indication of relative potency as it was not possible to define a maximal response. In addition, at 15 and 20 days the pIC_{50} value was obtained from an extrapolated concentration-response curve. However, the developmental profiles of the A_1 receptor identified by both functional and radioligand binding studies are similar, an increase in both the number of receptors and the inhibitory response of the duodenum to CPA observed with an increase in age. At 20 days however, the increase in potency of CPA appears to lag behind

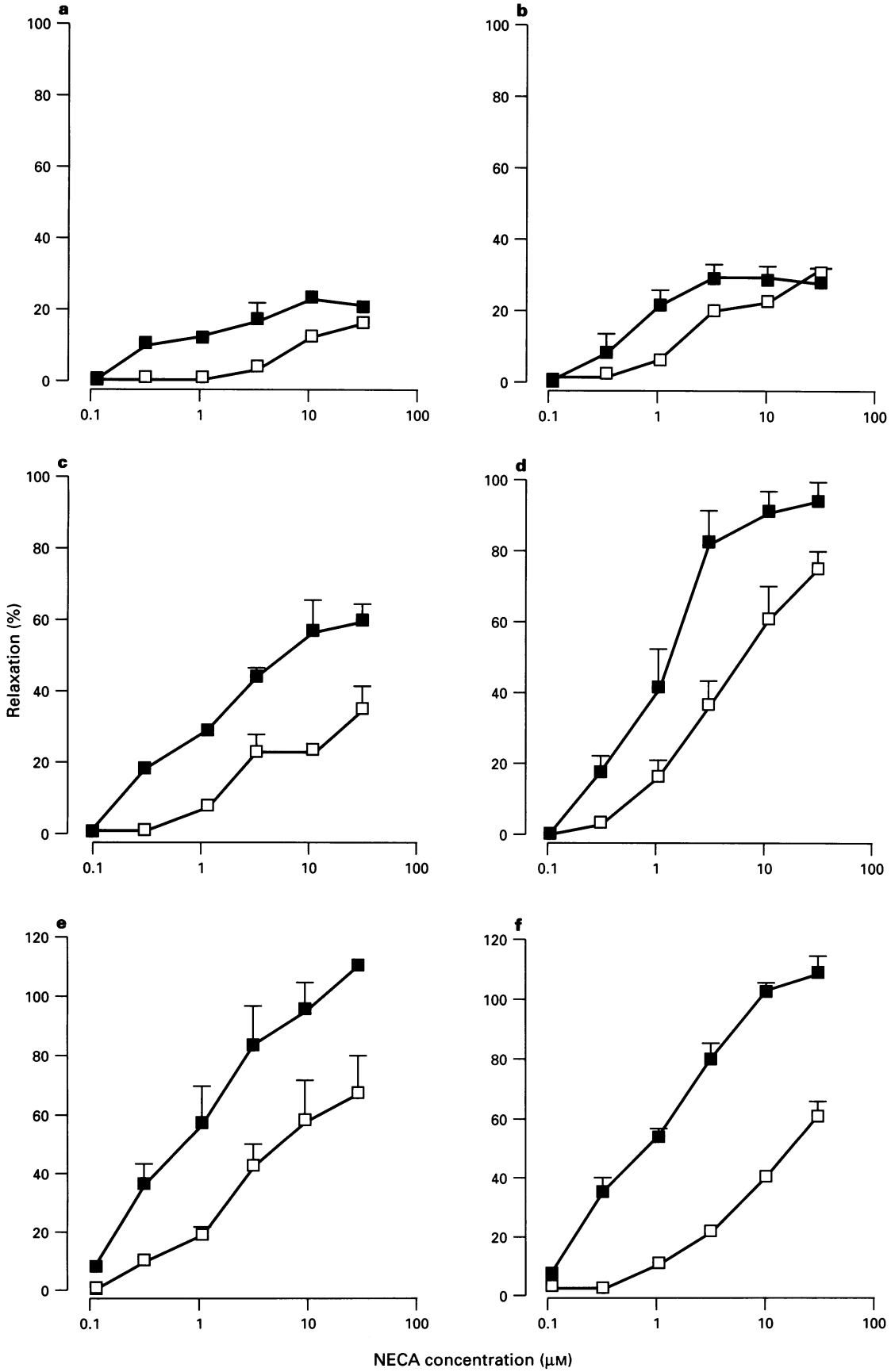

Figure 4 The effects of NECA (100 nM – 30 μM) in the absence (■) and presence (□) of DPCPX (1 μM) on rat duodenum from animals aged (a) 5 days, (b) 10 days, (c) 15 days, (d) 20 days, (e) 25 days and (f) 30 days. Responses are expressed as the % inhibition of the contraction induced by 0.1 μM carbachol. Each point is the mean with s.e.mean of at least 3 observations.

the increase in receptor density, suggesting that although the receptors are present and can be identified by the binding of [^{3}H]-DPCPX to the duodenum, not all of the receptors are functionally coupled at this age. A similar link between potency and number of receptors has been observed in the rat aorta where the number of binding sites for [^{3}H]-NECA has been shown to parallel the functional response to adenosine in aging rats (Moritoki *et al.*, 1990). Furthermore, A_1 receptors measured in rat forebrain membranes by [^{3}H]-CHA have been shown to be detectable from birth; however, inhibition of binding by the GTP analogue GppNHp was observed only from day 6 postpartum suggesting that adenosine receptors are present, but are not functionally coupled in very young neonates (Morgan & Marangos, 1987).

The finding that adenosine relaxed neonatal rat duodenum from day 5 onwards (Nicholls *et al.*, 1990) in combination with the demonstration that adenosine relaxes the adult rat duodenum via A_{2b} receptors (Nicholls *et al.*, 1992) suggested that the response to adenosine from day 5–15 is an A_{2b} effect. This is consistent with the results in this present study showing that A_1 receptors are not present at this age. Indeed, functional responses to NECA (100 nM–30 μM) were evident from day 5 onwards, and the potency of NECA remained constant ($EC_{50} \sim 1$ μM) with an increase in age, being similar to that previously generated for adult rat duodenum (EC_{50} 0.4 μM, Nicholls *et al.*, 1992). Furthermore, the A_{2a}-selective agonist, CGS 21680, was without effect on the CCh-contracted 15 day old rat duodenum and at 25 days only the highest concentration of CGS 21680 (10 μM) had an effect on the tissue indicating that the A_2 receptor in the neonate is A_{2b} as previously shown for the adult (Nicholls *et al.*, 1992). In addition, in the separated layers of the rat duodenum CGS 21680 was without effect in the muscularis mucosae and in the longitudinal muscle it was much less potent than adenosine, indicating that the A_2 receptors are of the A_{2b} subtype (Nicholls *et al.*, 1996). This is further corroborated by the high density of expression of A_{2b} receptors demonstrated in the rat intestine (Stehle *et al.*, 1992). The A_1/A_2 antagonist, 8-PT (10 μM) abolished the inhibitory responses to NECA suggesting that NECA is not acting via an A_3 receptor, as it is known that the rat A_3 receptor is highly resistant to certain xanthine antagonists including 8-PT even at concentrations of 100 μM (van Galen *et al.*, 1994). Moreover, the relaxations induced by NECA are not xanthine-insensitive responses as have been seen with high concentrations of agonists in tissues such as guinea-pig trachea (Brackett & Daly, 1991), aorta (Collis & Brown, 1983; Martin, 1992) and taenia caecum (Prentice *et al.*, 1995).

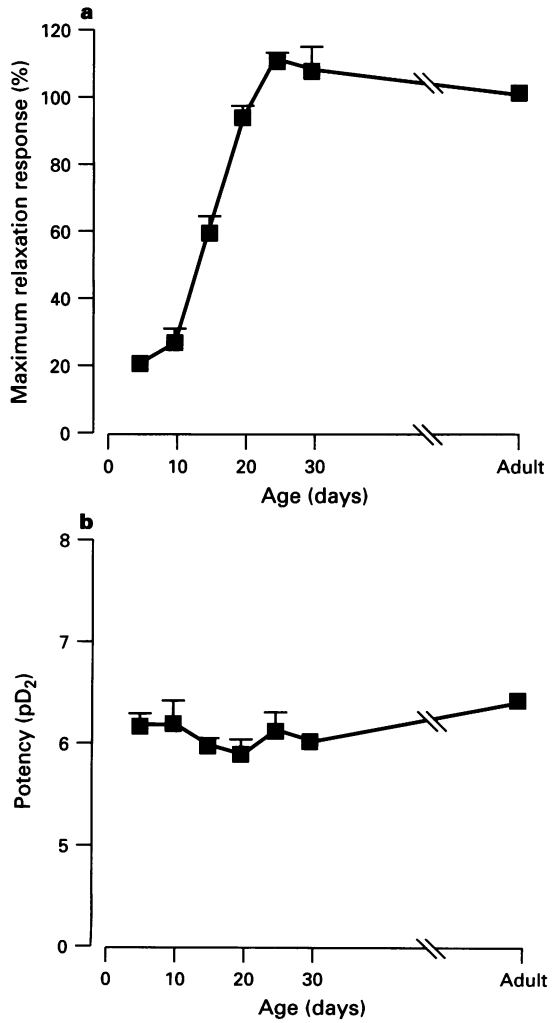

Figure 5 Variations in (a) maximum inhibitory response observed at 30 μM NECA and (b) potency of NECA with an increase in age in rat duodenum. Potency is expressed as pD_2 values (the negative $\log_{10}$ of the concentration of NECA required to produce half maximal response, see Methods). Each point is the mean with s.e.mean of at least 3 determinations. The points for adults were obtained from data previously published by Nicholls *et al.* (1992).

DPCPX (1 μM) shifted the response to NECA to the right at all ages, consistent with the presence of A_2 receptors. However, in the presence of 10 nM DPCPX the concentration-response curve to NECA (30 nM–10 μM) shifted to the right in duodenum from 20, 25 and 30 day old rats only, suggesting that at these ages but not at days 10 and 15, NECA also acts partly via an A_1 receptor. Shifts in the concentration-response curve to NECA in the duodenum from animals aged 25 days were observed in the presence of 10 nM–3 μM DPCPX, when compared with the pooled control responses to NECA, and Schild analysis of the effects of DPCPX versus NECA revealed a slope much lower than unity (0.62), indicating that NECA acts at a mixed population of receptors. Similar results were obtained in the isolated longitudinal muscle of the adult rat duodenum where it was concluded that NECA acts at a mixed population of A_1 and A_{2b} receptors (Nicholls *et al.*, 1996). An additional complication in interpreting results with DPCPX in the whole duodenum is that as well as the relaxant A_1 and A_{2b} receptors present on the longitudinal muscle, there are also contractile A_{2b} receptors in the muscularis mucosae although the responses of the whole duodenum to NECA are qualitatively similar to those observed on the longitudinal muscle (Nicholls *et al.*, 1996). The lack of effect of 10 nM DPCPX on the response to NECA observed in rats aged 10 and 15 days supports the results obtained from both the radioligand binding assays and the functional studies using CPA where no A_1 receptors were detected before 20 days. Thus, prior to day 20 NECA acts solely via the A_{2b} receptor, whereas from day 20, NECA can exert its effects via both the A_1 and A_{2b} receptors. This could explain the increase in maximum response to NECA at days 20–30 compared to the earlier ages, although there is also an increase at day 15 compared to day 10, which cannot be explained in this way. Another explanation for an increase in maximum response could be an increase in the number of A_{2b} receptors, although we could not investigate this directly due to the lack of a reliable A_{2b} binding assay.

The presence of the A_{2b} receptor on neonatal rat duodenum from day 5 onwards suggests that this receptor subtype is more important in the early stages of development than the A_1 receptor which is not evident until day 20. In contrast, in the rat vas deferens, we have recently established that the prejunctional A_1 and the postjunctional A_2 receptors are present at the earliest ages tested (day 15 and day 10 respectively); however, the postjunctional A_1 receptor on the rat vas deferens, identified by both functional and radioligand binding assays, was

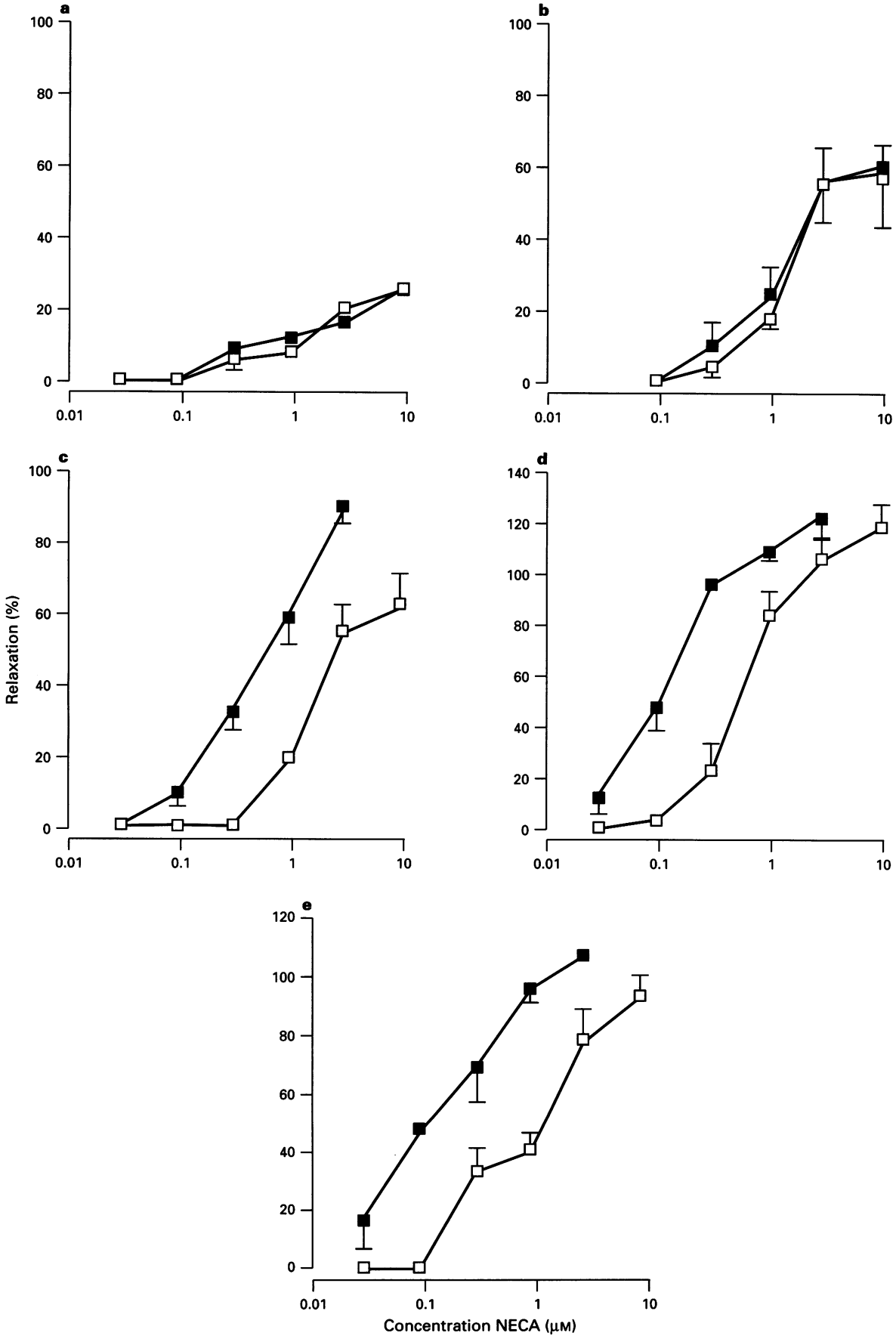

Figure 6 The effects of NECA (30 nM – 10 μM) in the absence (■) and presence (□) of DPCPX (10 nM) on rat duodenum from animals aged (a) 10 days, (b) 15 days, (c) 20 days, (d) 25 days and (e) 30 days. Responses are expressed as the % inhibition of the contraction induced by 0.1 μM carbachol. Each point is the mean with s.e.mean of at least 4 observations.

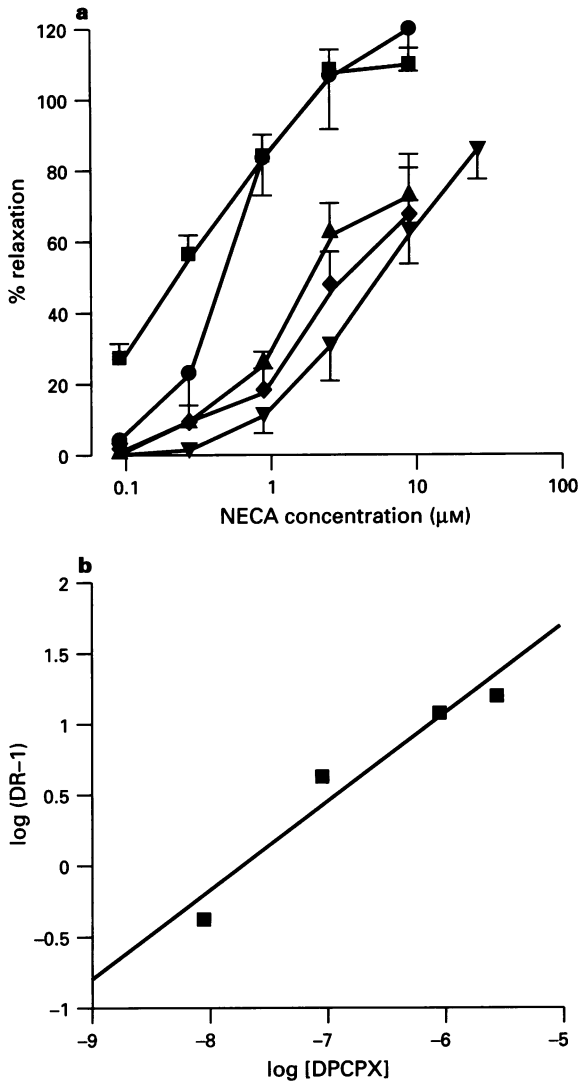

Figure 7 (a) The response of 25 day old rat duodenum to NECA in the absence (■) and presence of increasing concentrations of DPCPX: 10 nM (●), 100 nM (▲), 1 μM (◆) and 3 μM (▼) and (b) Schild plot showing the effect of DPCPX versus NECA (slope 0.62) dose-ratios being calculated relative to the pooled mean EC_{40} values of the control responses to NECA. Each point is the mean with s.e.mean of at least 4 observations.

evident only from day 20 onwards (Peachey *et al.*, 1996). In neonatal rat colon muscularis mucosae, responses to adenosine via A_1 receptors were evident from day 5 onwards (Bailey *et al.*, 1992; Hourani *et al.*, 1993). In the central nervous system the development of A_1 receptors follows the patterns of postnatal neurogenesis, with A_1 receptors being identified from the earliest ages studied, embryonic day 18 and postnatal day 1 respectively (Marangos *et al.*, 1982; Geiger *et al.*, 1984). Therefore, the ontogenetic profile of the A_1 receptors differs between different tissues of the rat and this suggests that the relative importance of this receptor subtype during development is tissue-dependent. The increase in density of A_1 receptors in the rat duodenum from day 15 to day 25 observed in the present study suggests that some process occurs during this phase which stimulates the A_1 receptors. It is possible that this process is involved in the physiological maturation associated with weaning (Henning, 1981). Indeed in this study the rat pups were weaned between 20 and 21 days and we have recently shown the environmental stimulus of weaning directly affects the development of another G-protein coupled receptor, the δ opioid receptor (Muhammad & Kitchen, 1993; Kitchen *et al.*, 1994; 1995).

In conclusion, we have shown that the A_1 receptor on neonatal rat duodenum is present from day 20 onwards. Radioligand binding studies using [^{3}H]-DPCPX showed binding to sites commensurate with A_1 receptors from 20 days, and the density of binding sites peaked at 25 days and then declined at 30 days to a level previously established for adult duodenum. Functional assays revealed responses to CPA to be present from 20 days, and these results were consistent with those obtained from radioligand binding assays. In contrast, the A_{2b} receptor was present from day 5 onwards as shown by the inhibitory action of NECA on the carbachol-contracted duodenum, thereby demonstrating the differential development of the P_1-purinoceptor subtypes on the rat duodenum.

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