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The ontogenetic profiles of the pre- and postjunctional adenosine receptors in the rat vas deferens

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1 The ontogenetic profiles of the prejunctional A_1 and postjunctional A_1 and A_2 receptors on the rat vas deferens were investigated, using a combination of functional and radioligand binding assays to follow the A_1 receptors and functional assays alone to follow the development of the A_2 receptors.

2 The prejunctional A_1 receptor, assessed by the inhibitory action of N^6-cyclopentyladenosine (CPA) (3 nM-3 μM) on nerve-mediated contractions, was present from day 15 onwards, day 15 being the earliest age at which nerve-mediated contractions could be detected. The potency of CPA was constant across the ages studied, with pD_2 values ranging from 6.4-7.1, not significantly different from that previously observed in adult rat vas deferens.

3 The postjunctional A_2 receptors, assessed by the inhibitory action of 5'-N-ethylcarboxamidoadenosine (NECA) (10 nM-30 μM) on KCl-induced contractions were present from day 10 onwards, day 10 being the earliest age at which responses to KCl could be observed. The potency of NECA remained constant with an increase in age, with potency values, expressed as pEC_{25} values, ranging from 6.5-7.0.

4 The postjunctional A_1 receptor displayed a different development profile from that of the prejunctional A_1 and postjunctional A_2 receptors. Postjunctional A_1 receptors were identified by the enhancement of KCl-induced contractions by CPA (10 nM-0.3 μM). At 10 and 15 days, CPA failed to enhance KCl-induced contractions. From day 20 to day 40, this enhancement increased with an increase in age and the level of enhancement achieved statistical significance from day 30.

5 Radioligand binding studies using 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX) revealed binding sites characteristic of A_1 receptors on the vas deferens from rats aged 20 days onwards. The density (B_{max}) of A_1 receptors expressed relative to protein content was greatest at day 20 (153 $\pm$ 33 fmol mg^{-1} protein) and declined at day 30 (43.9 $\pm$ 3.7 fmol mg^{-1} protein) to a level commensurate with that previously determined in adult rat vas deferens (43.3 $\pm$ 12 fmol mg^{-1} protein). However, when expressed relative to tissue wet weight little variation in receptor density was observed between these ages (B_{max} 0.13 $\pm$ 0.02 fmol mg^{-1} wet weight at 20 days; 0.17 $\pm$ 0.01 fmol mg^{-1} wet weight at 30 days). The binding affinity (K_D) remained constant with an increase in age and was similar to the K_D value previously generated for adult rat vas deferens ($\sim$1 nM). At ages 10 and 15 days no reproducible binding could be detected.

6 These results show the differential development of the adenosine receptors on the rat vas deferens with postjunctional A_1 receptors demonstrating delayed development, while prejunctional A_1 and postjunctional A_2 receptors were present from the earliest ages studied. In addition, comparison of binding studies and functional studies suggests that the binding studies detect only the A_1 receptors present on the smooth muscle and not those present on the nerve terminals.

Keywords: Rat vas deferens; ontogeny; adenosine receptors; radioligand binding; [^{3}H]-DPCPX

Introduction

The extracellular effects of adenosine are mediated by specific cell surface receptors classified as P_1- and P_2-purinoceptors, recognising adenosine and adenosine 5'-triphosphate (ATP) respectively (Burnstock, 1978). The P_1 receptors have been further subdivided into A_1 and A_2 receptors and these receptor subtypes can be clearly distinguished by the potency of various adenosine analogues, N^6-substituted adenosine analogues such as N^6-cyclopentyladenosine (CPA) being more potent at A_1 receptors whereas 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA) are more potent at A_2 receptors. The antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) is highly selective for A_1 receptors with a dissociation constant (K_D) around 1 nM, whereas at A_2 receptors it has a K_D around 1 μM and thus effectively discriminates between the two receptor subtypes. The A_2 receptor has been further subdivided into high affinity A_{2a} and low affinity A_{2b} receptors, each of which has been cloned as has the previously unrecognised A_3 receptor (for review see Collis & Hourani, 1993). In general in smooth muscle A_1 receptors are said to exist prejunctionally where they act to inhibit transmitter release, whereas A_2 receptors are postjunctional and inhibit contraction (Burnstock, 1990; Kennedy, 1990). In addition, A_1 receptors have been shown to mediate the contractile effects of adenosine in some smooth muscle preparations such as the rat colon muscularis mucosae (Bailey *et al.*, 1992).

The rat vas deferens is a tissue which has been widely used for adenosine research. It has been assumed that the inhibitory actions of adenosine on the nerve-induced contractions are mediated via a prejunctional A_1 receptor where adenosine acts to inhibit transmitter release (Paton, 1981). However, we have recently identified an inhibitory postjunctional A_2 receptor and have shown that adenosine and NECA but not CPA act primarily via this receptor, as shown by the inhibitory action of NECA and adenosine on contractions induced by ATP and noradrenaline (Hourani *et al.*, 1993a). Further support for the existence of this inhibitory A_2 receptor was provided by the high concentrations of DPCPX required to antagonize the

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effects of both NECA and adenosine. Another study has also demonstrated the presence of an excitatory postjunctional A_1 receptor on the rat vas deferens (Hourani & Jones, 1994). Low concentrations (<1 μM) of the A_1 adenosine agonists CPA and (**R**)-N^6-phenylisopropyladenosine (**R**-PIA) were shown to enhance contractions induced by ATP, and this effect was sensitive to nanomolar concentrations of DPCPX confirming the involvement of A_1 receptors.

The ontogeny of responses to adenosine in the rat vas deferens has also been studied (Hourani *et al.*, 1993a). Nerve stimulated contractions were detectable from day 15, and adenosine inhibited these contractions, the potency of adenosine decreasing with an increase in age. However, in this study no attempt was made to distinguish between the A_1 and the A_2 receptor subtypes and therefore the possibility of differential development of the receptors was not explored. However, in the rat duodenum we have recently shown that the A_1 and A_2 receptors develop at different times, with the A_2 receptor present from day 5 and the A_1 receptor evident from day 20 only (Peachey *et al.*, unpublished observation).

We have recently established a radioligand binding assay to identify adenosine A_1 receptors in a number of adult rat smooth muscle preparations including the rat vas deferens (Peachey *et al.*, 1994). Using the A_1-selective radioligand [^{3}H]-DPCPX we have identified high affinity binding sites commensurate with A_1 receptors in the adult rat vas deferens, as shown by a K_D value in the low nM range and inhibition studies using adenosine receptor ligands which yielded a potency order characteristic of an A_1 receptor.

As part of a continuing project to study the development of receptors in smooth muscle we have followed the ontogenetic profile of the pre- and postjunctional A_1 and postjunctional A_2 receptors in the rat vas deferens by studying the response to the A_1-selective analogue, CPA and the A_2 agonist, NECA, using traditional functional assay methods. In addition, we have followed the ontogenetic profile of A_1 receptors using binding assays with [^{3}H]-DPCPX as the radioligand to complement the functional studies on A_1 receptors.

Some of our findings have been presented previously in abstract form (Peachey *et al.*, 1995).

Methods

Animals and tissue preparation

Isolated vasa deferentia from male Wistar albino rats (University of Surrey strain) aged 10, 15, 20, 25, 30 and 40 days old and adult (> 60 days, 200–250 g) were used in these experiments. The day of birth was designated as day 1 and animals were culled to litters of eight to ten pups (mixed sexes) to each mother to maintain a standard litter size. The animals were killed by cervical dislocation. In each case whole vasa deferentia were dissected out by cutting just below the epididymis and above the urethra and cleared of any connective tissue.

Functional assays

The vasa deferentia were mounted in 4 ml organ baths containing Mg^{2+}-free Krebs of the following composition (mM): NaCl 118, $NaCO_3$ 25, KH_2PO_4 1.2, KCl 4.8, $CaCl_2$ 2.5, glucose 11, gassed with 95% O_2/5% CO_2, and maintained at 37°C. Mg^{2+}-free Krebs was used as omitting Mg^{2+} enhances neurogenic contractions and allows nerve-mediated responses to be detected earlier in postnatal development. A resting tension of 0.5 g was applied and tissues were allowed to equilibrate for 90 min, during which time the Krebs buffer was replaced every 20 min. Responses were recorded isometrically via an FTO3 force displacement transducer and displayed on a Grass polygraph model 79D.

The presence of prejunctional A_1 receptors was assessed by the inhibitory effects of CPA (3 nM–3 μM) on nerve-mediated contractions (field stimulation; Grass S48 stimulator, twin pulses of 1 ms duration, 75 ms delay, 70 V) via parallel platinum electrodes. To measure the inhibitory effects of CPA, the tissues were field-stimulated at a frequency of 0.1 Hz and CPA was added to the bath when the response to the nerve stimulation had stabilized and maintained in contact with the tissue until maximal inhibition of contraction was observed. The inhibition was expressed as % reduction of the nerve mediated response, and potency estimates calculated as EC_{50} values and expressed as pD_2 values (negative $\log_{10}$ of the EC_{50} value). To investigate the effects of DPCPX (10 nM) on the inhibition of nerve-mediated contractions by CPA, control concentration-response curves to CPA were constructed followed by incubation with the antagonist for 30 min prior to obtaining a second concentration-response curve to CPA. In the case of animals aged 15 days however, tissue fragility prevented two concentration-response curves from being constructed on one tissue preparation, therefore experiments in the absence and presence of DPCPX were performed in parallel. The effect of CPA on the nerve-mediated response was studied from day 15 onwards, day 15 being the earliest age at which nerve-mediated responses could be detected. In adult and 15 day old vas deferens, nerve-mediated responses were neurogenic in origin as responses were abolished in the presence of tetrodotoxin (1 μM). The presence of the excitatory postjunctional A_1 receptor was assessed as outlined in Hourani & Jones, (1994), with minor modifications. The effects of CPA (10 nM–300 nM) on the contractile response induced by submaximal concentrations of KCl (35 mM) were assessed. KCl was used as the contractile agent in preference to ATP as it was found that responses to KCl were evident from postnatal day 10, whereas responses to ATP were evident only from 15 days (Hourani *et al.*, 1993a). Tissues were preincubated with CPA for 1 min before the addition of KCl, and responses were expressed as the % enhancement by CPA of the KCl-induced contraction in the absence of CPA. A 1 min preincubation with CPA was used as this was previously found to be optimal for the detection of enhancement of contractions (Hourani & Jones, 1994). The inhibitory effects of NECA (10 nM–30 μM) and high concentrations of CPA (1–30 μM) on the contractile response induced by KCl (35 mM) were assessed to study the postjunctional inhibitory A_2 receptor. NECA or CPA were incubated with the tissue for 1 min before the addition of KCl, responses were expressed as % inhibition of the KCl-induced contraction and potency estimates were expressed as pEC_{25} values (the negative $\log_{10}$ of the EC_{25} value). In all cases responses were obtained non-cumulatively and a dose cycle of 10 min maintained. Statistical analysis across the ages was carried out by one-way ANOVA followed by Duncan's New Multiple Range *post-hoc* test. For the inhibitory effect of CPA on the nerve-stimulated response the pD_2 value was compared, for the inhibitory effect of CPA and NECA on KCl-induced contractions the pEC_{25} value was compared and for the enhancement by CPA of KCl-induced contractions the % enhancement at 0.3 μM CPA (the concentration giving maximal enhancement) was compared. For statistical analysis of the enhancement by CPA, a Student's paired *t* test was used at each age to compare the contractions induced by KCl in the absence and presence of CPA. For statistical analysis of the effect of DPCPX (10 nM) on the inhibition of nerve-mediated contractions by CPA, the pD_2 value for CPA in the absence and presence of antagonist was compared by Student's *t* test.

Membrane preparation for binding assays

Tissues were placed in ice-cold 50 mM TrisHCl (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 in Peachey *et al.* (1994). The resulting pellet was resuspended in 50 mM TrisHCl and incubated with 5 u ml^{-1} adenosine deaminase for 30 min at room temperature before use. Protein estimations were performed according to the method of Lowry *et al.* (1951).

Saturation binding assays

Saturation experiments were performed in duplicate in polypropylene tubes using a total assay volume of 250 μl. Saturation assays were undertaken only on 15, 20, 25 and 30 day old vas deferens and were carried out according to the method of Peachey *et al.* (1994). Eight concentrations of [^{3}H]-DPCPX (0.05–10 nM) (specific activity 109 Ci mmol^{-1}) were used and the non-specific binding was defined by 1 μM CPA. 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 above. Results were analysed using LIGAND (Munson & Rodbard, 1980) and the specific binding subjected to Scatchard transformation (Scatchard, 1949) for estimation of K_D and B_{max}.

Materials

NECA, CPA and adenosine deaminase (ADA) (TypeVI) 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. CPA (10 mM) was dissolved in 20% ethanol and DPCPX (10 mM) was dissolved in either 20% ethanol or 6% dimethylsulphoxide containing 6 mM NaOH and all drugs were diluted in buffer for use. 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

Functional assays

CPA inhibited the nerve-mediated contractions of the vas deferens from day 15 onwards, day 15 being the earliest age at which nerve-mediated contractions could be detected. The inhibitory effect of CPA was similar at all ages studied with pD_2 values between 6.4 and 7.1, and these values were not significantly different from that determined in the adult ($P>0.05$) (Figure 1a). DPCPX (10 nM) caused a significant ($P<0.05$) rightward shift in the dose-response curve to CPA at day 15 and day 25, with dose-ratios of 3.5 and 8.0 respectively corresponding to apparent pA_2 values of 8.4 and 8.9 (Figure 2). NECA (10 nM–30 μM) inhibited the contractions induced by KCl (35 mM) from day 10 onwards, the earliest age at which responses to KCl could be detected, with pEC_{25} values between 6.5 and 7.0 and these were not significantly different from adult at any age (Figure 1b). In contrast, CPA (10 nM–0.3 μM) enhanced the contractions induced by KCl from day 20 onwards (Figure 3), with the maximal enhancement observed at 0.3 μM CPA and the response to this concentration of CPA was therefore used to compare potency at different ages. No enhancement of the KCl-induced response by CPA was observed at days 10 and 15 (Figure 3), and the enhancement of KCl-induced contractions by 0.3 μM CPA achieved statistical significance from day 30 ($P<0.05$). At this concentration of CPA (0.3 μM), the % enhancement of the KCl-induced contractile response increased with an increase in age up to day 40, and until day 35 the level of enhancement was significantly different from that found in the adult ($P<0.05$) (Figure 1c). At concentrations >0.3 μM CPA inhibited contractions induced by KCl (Figure 3), and the potency of CPA for this effect was not significantly different from the adult at any age, and was always at least 10 fold lower than that of NECA (Figure 1b).

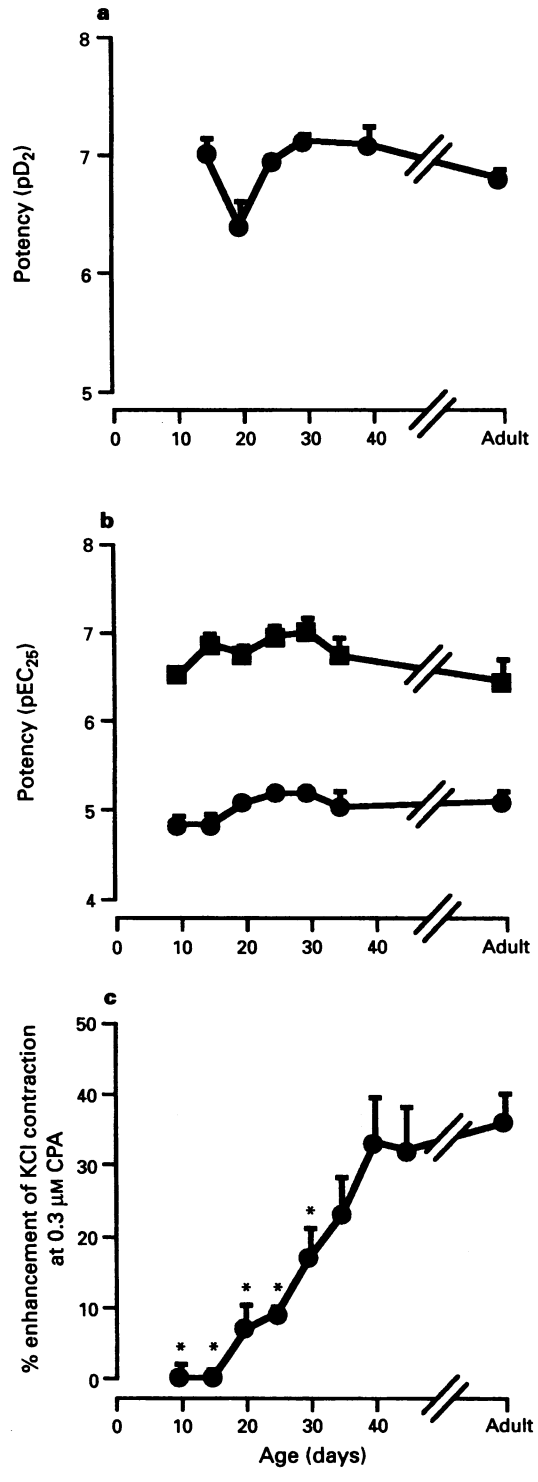

Figure 1 Variations in the responses of the rat vas deferens with age. (a) Variation in the potency of CPA as an inhibitor of nerve-stimulated contractions expressed as pD_2 values, showing the development of the prejunctional A_1 receptor. (b) Variations in the potency of NECA (■) and CPA (●) as inhibitors of KCl-induced contractions, expressed as pEC_{25} values, showing development of the postjunctional A_2 receptor. (c) Variation in the % enhancement of KCl-induced contraction by CPA (0.3 μM), showing development of the postjunctional A_1 receptor. Each point is the mean with s.e.mean of as least four determinations. Statistical analysis was performed by one-way ANOVA and Duncan's New Multiple Range *post-hoc* test, *$P<0.05$ significantly different from adult values.

Binding assays

At days 10 and 15 no specific binding to [^{3}H]-DPCPX could be detected. At day 20, specific binding was observed (Figure 4),

but transformation of the specific binding yielded Scatchard plots with poor regression coefficients ranging from 0.37–0.74. However, measures of affinity (K_D) and density (B_{max}) of receptors derived from these plots resulted in values (K_D 0.74±0.09 nM; B_{max} 0.11±0.01 fmol mg^{-1} wet weight) not dissimilar from those determined at 25 days (K_D 0.6±0.03 nM; B_{max} 0.17±0.03 fmol mg^{-1} wet weight). From day 25 [^{3}H]-DPCPX bound with high affinity to the vas deferens from neonatal rats (Figure 4b). Scatchard transformation of the specific binding yielded monophasic plots and Hill coefficients which were not significantly different from unity, indicating that [^{3}H]-DPCPX binds to an apparently homogeneous population of receptors. The affinity of [^{3}H]-DPCPX for the vas deferens from animals aged 25 (0.6±0.03 nM) and 30 (0.91±0.2 nM) days was similar and values were not significantly different to the K_D value previously determined for adult vas deferens (0.93±0.2 nM) (Peachey *et al.*, 1994). The protein content of the tissue homogenate from 20 day old animals (69.1±10.9 μg protein ml^{-1} homogenate) was approximately half that from animals aged 25 days (174.3±35.2 μg protein ml^{-1} homogenate) and when receptor density was expressed relative to protein content [^{3}H]-DPCPX binding decreased three fold from day 20 to day 30 (Figure 5a). However, when expressed per wet weight of tissue the density of A_1 receptors showed a small increase from day 20 to day 30 (Figure 5b).

Discussion

Previous ontogenetic studies of the P_1-purinoceptors on the rat vas deferens revealed adenosine to be more potent in the neonate than the adult, with the potency of adenosine in inhibiting nerve mediated contractions decreasing with an increase in age (Hourani *et al.*, 1993a). However, no attempt was made to characterize the receptors at which adenosine acted at the various ages. In contrast in this study, we have shown the potency of the A_1-selective adenosine agonist, CPA, as an inhibitor of the nerve-mediated contractions, to be constant across the ages studied. Furthermore, the low concentrations of DPCPX (10 nM) required to antagonize this effect confirmed that CPA was acting at a prejunctional A_1 receptor. In addition, CPA in the present study was more potent than adenosine had been in the study of Hourani *et al.* (1993a), consistent with an action at the prejunctional A_1 receptor. The constant potency of CPA across the ages studied and the early appearance of this functional response suggests that this inhibitory prejunctional A_1 receptor is as important in the neonate as it is in the adult vas deferens.

For investigation of the postjunctional A_1 and A_2 receptors in the present study, KCl was used as the contractile agent as responses to KCl were evident from day 10 onwards, whereas

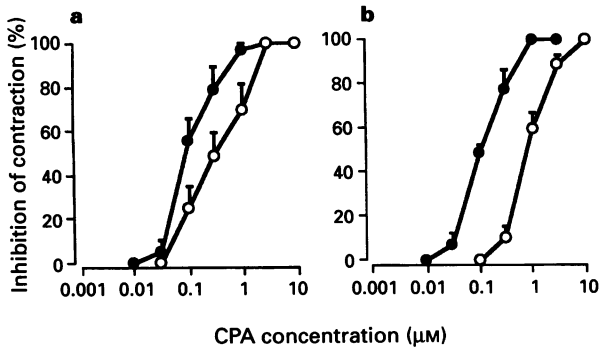

Figure 2 Effect of CPA (0.1–10 μM) in the absence (●) and presence (○) of DPCPX (10 nM) on the nerve-stimulated contractions of the vas deferens from animals aged (a) 15 days and (b) 25 days. Each point is the mean with s.e.mean of at least four determinations.

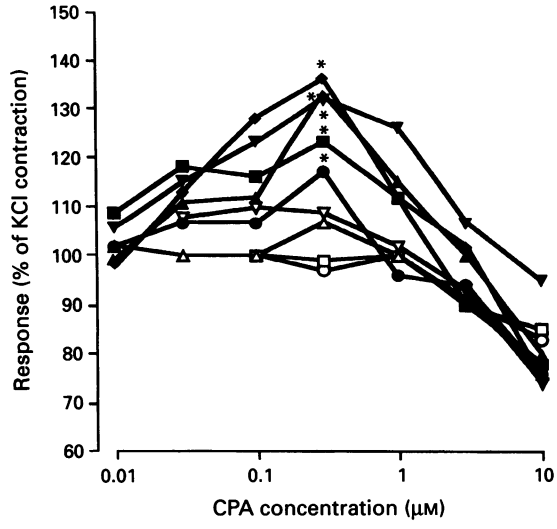

Figure 3 Effect of preincubation with CPA for 1 min on the contractile response induced by KCl (35 mM) at 10 days (○), 15 days (□), 20 days (△), 25 days (▽), 30 days (●), 35 days (■), 40 days (▲), 45 days (▼) and adult (◆) rat vas deferens. Each point is the mean of at least four determinations. Statistical analysis was performed by Student's paired *t* test to compare the contractions induced by KCl in the absence and presence of 0.3 μM CPA, $*P<0.05$. Error bars have been removed for clarity.

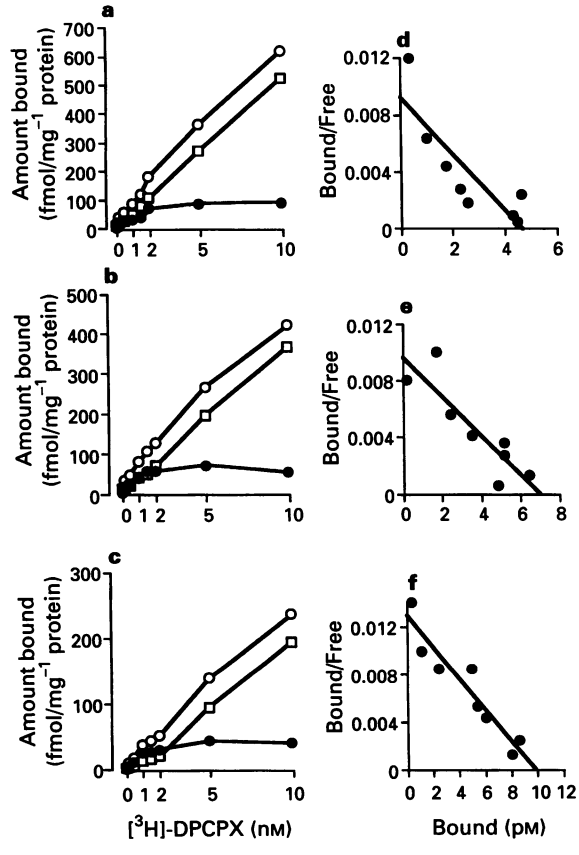

Figure 4 Representative saturation plots and Scatchard transformation of [^{3}H]-DPCPX binding to vas deferens from animals aged (a) 20 days, (b) 25 days and (c) 30 days, showing (○) total, (□) non-specific and (●) specific binding. In all cases the non-specific binding was defined by 1 μM CPA. Scatchard transformation of the specific binding (●) from (a), (b) and (c) are shown in (d), (e) and (f) respectively.

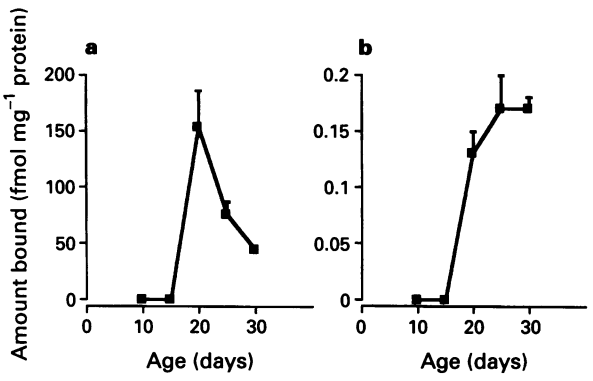

Figure 5 Variation in the density of adenosine A_1 receptors expressed as amount bound relative to (a) protein content of homogenate (fmol mg^{-1} protein) and (b) wet weight of tissue (fmol mg^{-1} wet weight) against age. Each point is the mean of at least three determinations and the vertical bars show s.e.mean.

responses to both ATP and noradrenaline (NA), the two co-transmitters in this tissue, vary with age (Hourani *et al.*, 1993a), and we wished to avoid the complications associated with these factors when constructing developmental profiles for the adenosine receptor subtypes. Our results with NECA show that the ontogenetic profile of the inhibitory postjunctional A_2 receptor is similar to that for the inhibitory prejunctional A_1 receptor as the potency of NECA remained constant across all ages studied and was similar to that observed in the adult. High concentrations of CPA (>0.3 μM) also inhibited the KCl-induced contractions presumably due to activation of the postjunctional A_2 receptor, with NECA being more potent than CPA at all ages (see Figure 1b). The ontogeny of the postjunctional A_2 receptor in the rat vas deferens follows a similar pattern to that observed in the rat duodenum (Peachey *et al.*, unpublished observation), and urinary bladder (Nicholls *et al.*, 1990) where responses to NECA and adenosine respectively, acting via A_2 receptors (Nicholls *et al.*, 1992), were observed from day 5 onwards.

The presence of an excitatory postjunctional A_1 receptor has been established in the adult rat vas deferens (Hourani & Jones, 1994). The lack of enhancement of the KCl-induced contractile response by low concentrations of CPA (10 nM – 0.3 μM) on the vas deferens from 10 and 15 day old rats in the present study indicates the absence of this A_1 receptor, whereas from day 20 onwards, enhancement of KCl-induced contractions mediated via this excitatory postjunctional A_1 receptor were detectable, achieving statistical significance by day 30. Responses to CPA increased with an increase in age and levels of enhancement not significantly different from that observed in the adult were achieved by day 35, implying that this receptor subtype is more important in the later stages of development. The late development of this stimulatory postjunctional A_1 receptor may explain why the overall inhibitory effect of adenosine on nerve stimulation was observed to decrease with age in our previous study (Hourani *et al.*, 1993a). Binding with [^{3}H]-DPCPX to rat vas deferens of animals aged 10–15 days also failed to identify binding sites commensurate with an A_1 receptor. From day 20 onwards however, [^{3}H]-DPCPX binding to an A_1 receptor was observed as shown by a K_D value in the low nM range, equivalent to the K_D value previously established for adult rat vas deferens (0.93 ± 0.17 nM) (Peachey *et al.*, 1994). The density of A_1 receptors expressed relative to protein content varied with age, being greatest at day 20 and declining at day 30 to levels equivalent to that previously established for adult rat vas deferens (43.3 ± 12.2 fmol mg^{-1} protein) (Peachey *et al.*, 1994). However, the increase in protein content of the tissue homogenate from 20 days to 30 days resulted in the apparent decrease in receptor density over this period, as when expressed in terms of the wet weight of the tissue, a small increase in receptor density was observed between the ages (see Figure 5b). The lack of variation in the ligand affinity with age indicates that the observed differences in receptor density are not due to binding to another receptor system. Overall there is a similarity in the profile of the postjunctional A_1 receptor identified by functional studies and the A_1 receptor identified by radioligand binding studies, in that they both exhibit delayed development. This suggests that the [^{3}H]-DPCPX binding assay identifies the postjunctional A_1 receptor only and not those A_1 receptors present on the nerve terminals. The reasons for this apparent preferential binding to the postjunctional A_1 receptors are unknown, but it may simply be a consequence of the greater mass of smooth muscle than nerve tissue present in the vas deferens. The fact that the density of A_1 binding sites expressed as fmol mg^{-1} protein was greater at day 20 than in the adult, whereas the functional response to CPA was still increasing at this age and achieved statistical significance only by day 30, may imply that there is a lag between appearance of the receptors and their functional coupling. A similar lag has been observed in the neonatal rat duodenum where the potency of CPA appeared to lag behind the density of A_1 receptors at 20 days (Peachey *et al.*, unpublished observation).

The relative rates of development of the A_1 receptor in smooth muscle preparations would appear to be tissue-dependent. In the muscularis mucosae of the rat colon we have shown the A_1 receptor to be present from day 5 onwards (Hourani *et al.*, 1993b), whereas in the rat duodenum the A_1 receptors were evident only from day 20 onwards (Peachey *et al.*, unpublished observation). In the present study we have shown that the prejunctional A_1 receptor is present from day 15 onwards, whereas the postjunctional A_1 receptor identified by functional and radioligand binding studies is detectable from day 20. There is no evidence at present in the literature to indicate that there are subtypes of the A_1 receptor and thus it is likely that some process occurs around day 20 which stimulates the development of these postjunctional receptors. It has been reported that the process of sexual maturation in the male rat starts at around 4 weeks of age, with an increase in the number of Leydig cells per testes from the fourth to the tenth week of age, and a corresponding rise in plasma testosterone concentration (Pahnke *et al.*, 1975). The appearance of the postjunctional A_1 receptors on the vas deferens at post-natal day 20, although earlier than the reported start of sexual maturation in the rat, may have some involvement in this process.

In conclusion, we have shown that the prejunctional A_1 and the postjunctional A_2 receptors on the rat vas deferens are present from day 15 and day 10 respectively, the earliest ages at which responses can be measured, and that the potency of the adenosine analogues acting at these receptor subtypes is constant with an increase in age. In contrast, the excitatory postjunctional A_1 receptors exhibit delayed development. The A_1 binding sites identified with [^{3}H]-DPCPX were present only from day 20, and this suggests that this binding assay detects only the postjunctional A_1 receptors and not those present on the nerve terminals. This finding may have important implications for the interpretation of radioligand binding studies in peripheral tissues.

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