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Differential ontogeny of adenosine receptors in the longitudinal muscle and muscularis mucosae of the rat isolated duodenum

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Abstract

The ontogeny of P_1 purinoceptors in the separated layers of the rat duodenum was investigated using functional assays. In the longitudinal muscle N^6-cyclopentyladenosine (CPA) caused relaxations from day 20 that were inhibited by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (10 nM) indicating an action via adenosine A_1 receptors. 5′-*N*-Ethylcarboxamidoadenosine (NECA) caused relaxations at day 15 that were inhibited by DPCPX (1 μM) while 2-*p*-(2-carboxyethyl)phenylethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) was almost inactive, indicating an action at adenosine A_{2B} receptors. From day 20 NECA was inhibited by DPCPX (10 nM) but was not antagonised by DPCPX (1 μM) to the extent expected for an adenosine A_1 receptor, suggesting activation of adenosine A_1 and adenosine A_{2B} receptors. In the muscularis mucosae, CPA and NECA caused contractions from day 10 inhibited by DPCPX (1 μM) while CGS 21680 was less potent, indicating activation of adenosine A_{2B} receptors. These results show that adenosine A_{2B} receptors are present early in the postnatal period, whereas adenosine A_1 receptors develop after day 20.

Keywords: Duodenum longitudinal muscle, rat; Duodenum muscularis mucosae, rat; P_1 purinoceptor; Development; Ontogeny; Adenosine receptor.

1. Introduction

The pharmacological actions of adenosine and its analogues on smooth muscle preparations are mediated via receptors known as P_1 purinoceptors (Burnstock, 1978). There are at least four major classes of P_1 purinoceptors, adenosine A_1, A_{2A}, A_{2B} and A_3 which have been cloned and can be distinguished in pharmacological and binding studies (for reviews, see Collis and Hourani, 1993; Fredholm et al., 1994; Olah and Stiles, 1995). On adenosine A_1 receptors N^6-substituted analogues of adenosine such as N^6-cyclopentyladenosine (CPA) are generally more potent than 5′-substituted analogues such as 5′-*N*-ethylcarboxamidoadenosine (NECA), whereas on adenosine A_2 receptors NECA is generally more potent than CPA. The antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has nanomolar affinity at adenosine A_1 receptors and is at least 100-fold selective for adenosine A_1 receptors over adenosine A_2 (Yakel et al., 1993; Collis and Hourani, 1993; Brackett and Daly, 1994; Coates et al., 1994; Fredholm et al., 1994; Van Galen et al., 1994). Adenosine A_2 receptors have been further subdivided into adenosine A_{2A} and adenosine A_{2B} with the adenosine analogue 2-*p*-(2-carboxyethyl)phenylethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) being more potent at the adenosine A_{2A} receptor (for reviews see Collis and Hourani, 1993; Fredholm et al., 1994). On adenosine A_3 receptors, 5′ and N^6 substituted analogues are both potent, but unlike adenosine A_1 and adenosine A_2 receptors the rat adenosine A_3 receptor is resistant to methylxanthine antagonists such as DPCPX (Zhou et al., 1992; Van Galen et al., 1994).

Previous studies on the rat duodenum have shown a mixture of adenosine A_1 and adenosine A_{2B} receptors both mediating relaxation (Nicholls et al., 1992). In more recent studies we have investigated P_1 purinoceptors in the two layers of the rat duodenum which contract in the longitudinal plane, the longitudinal muscle and the muscularis mucosae. In the longitudinal muscle there are adenosine A_1 and adenosine A_{2B} receptors both mediating relaxations and in the muscularis mucosae there are adenosine A_{2B} receptors mediating contractions (Nicholls et al.,

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1996). The relaxant responses mediated by adenosine A_1 receptors and the contractile responses mediated by adenosine A_{2B} receptors are very unusual as these receptors usually mediate contraction and relaxation respectively. However relaxant responses to adenosine analogues in the longitudinal muscle and contractile responses in the muscularis mucosae have also been found in other areas of the gastrointestinal tract such as in the rat colon, but in this tissue relaxation is mediated via adenosine A_2 receptors and contraction via adenosine A_1 receptors, as expected (Bailey et al., 1992; Bailey and Hourani, 1990, 1992). The ontogeny of P_1 purinoceptors in the whole rat duodenum has been investigated using adenosine (Nicholls et al., 1990), and the aim of the present study was to investigate the development of P_1 purinoceptors in the separated layers of the rat duodenum using more selective agonists and antagonists to determine what subtypes of P_1 purinoceptors are present and functional in neonatal tissues and whether they develop at different times.

2. Materials and methods

2.1. Pharmacological studies

Adult male Wistar rats (> 60 day) and male neonatal rats 10, 15, 20, 25, 30, or 40 days old were killed by cervical dislocation. The day of birth was designated as day 1, and animals were culled to litters of ten rats per mother to maintain a standard litter size. Neonatal rats were weaned at day 20.

The duodenum was dissected out by cutting at the base of the pyloris and a length of 1.5 cm (adult) or 0.75 cm (neonates) was used, cleared of any connective tissue and placed over a glass rod. The longitudinal muscle was removed by making a longitudinal cut and gently rubbing the layer with moist cotton wool, and the remaining thick walled tube contained the muscularis mucosae. The tissues were mounted in 4 ml organ baths containing Krebs of the following composition; 1.2 mM $MgSO_4$, 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 2.5 mM $CaCl_2$, 11 mM glucose, gassed with 95% O_2: 5% CO_2, and maintained at 37°C. A resting tension of 1 g (adult), 0.5 g (15–40 days) or 0.3 g (10 days) was applied to the tissues, and responses were measured isometrically with a Grass FT03 transducer and recorded on a Grass model 79D polygraph after incubation for 60 min. Concentration-response curves were obtained non-cumulatively using half $\log_{10}$-unit concentration intervals, with a 10–15 min dose cycle. Responses of the longitudinal muscle were quantified by precontracting the tissue with carbachol (0.3 μM) before challenge with the drugs and relaxations were expressed as % reduction of the carbachol contraction. This concentration of carbachol was chosen because it gave reproducible sustained contractions corresponding to about 75% of the maximal response in both adults and neonates (day 20). The contraction induced by carbachol (0.3 μM) in tissues from neonatal animals was not significantly different from that induced in tissues from adult rats when expressed as tension (g) per wet weight of tissue (g), being 0.11 ± 0.08, 0.13 ± 0.05 and 0.21 ± 0.05 g/g at days 15, 20 and adult respectively. Responses of the muscularis mucosae were quantified by adding the drug directly to the bath and contractions were expressed as % of contraction induced by KCl (35 mM). The contraction induced by KCl (35 mM) in tissues from neonatal animals was not significantly different from that induced in tissues from adult rats when expressed as tension (g) per wet weight of tissue (g), being 3.2 ± 1.3, 3.3 ± 1.1 and 2.5 ± 1.2 g/g at days 10, 20 and adult respectively. In the case of DPCPX, concentration-response curves to agonists were obtained in the same tissue before and after incubation for 30 min with the antagonist. In our previous studies using adult tissues, control $\log_{10}$ concentration-response curves were not significantly different from curves repeated after 30 min in the presence of solvent corresponding to the highest concentration of DPCPX used (Nicholls et al., 1996).

The potency of each adenosine agonist in causing relaxations in the longitudinal muscle was expressed as the negative $\log_{10}$ of the molar concentration of the agonist producing 35% reduction of carbachol contraction (pEC_{35}). The potency of each agonist in causing contractions in the muscularis mucosae was expressed as the negative $\log_{10}$ of the molar concentration of the agonist producing 40% of the KCl response (pEC_{40}). The potency values of pEC_{35} for the longitudinal muscle and pEC_{40} for the muscularis mucosae were calculated by linear regression of the linear portions of the $\log_{10}$ concentration-response curves and these points were chosen because in each case they represent midpoint responses of the curves obtained. Concentration ratios were derived from EC_{35} or EC_{40} values, and apparent pA_2 values were calculated as the negative logarithm of the molar concentration of the antagonist divided by concentration ratio − 1. The EC_{35} or EC_{40} values in the absence and presence of antagonist were compared using Student's t test. Log_{10} concentration-response curves in the absence and presence of antagonist were tested for parallelism using Student's t test to compare the slopes of the fitted lines, and unless stated otherwise the lines were parallel. Statistical analysis across the ages was carried out using one-way analysis of variance (ANOVA) followed by Duncans New Multiple Range post-hoc test, and for comparison of the potencies of NECA across the ages in the presence and absence of DPCPX (1 μM) two-way ANOVA was used.

2.2. Drugs

DPCPX, CGS 21680 and CPA were obtained from Research Biochemicals (Natick, MA, USA), and all other drugs were obtained from Sigma (Poole, UK). The buffer salts were of analytical grade and were obtained from

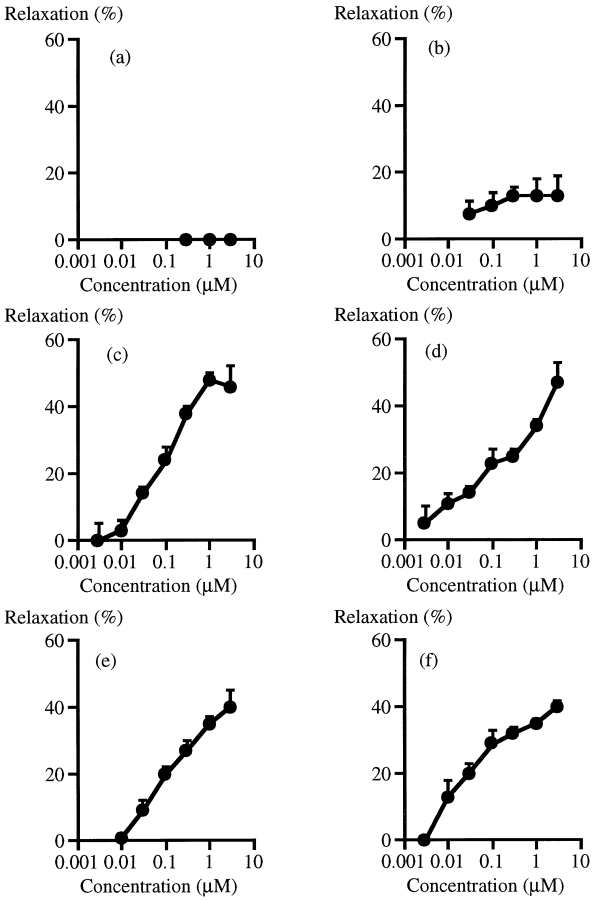

Fig. 1. Relaxant responses to CPA (●) in the carbachol-contracted rat duodenum longitudinal muscle at various ages. *a*: 15 day; *b*: 20 day; *c*: 25 day; *d*: 30 day; *e*: 40 day; *f*: adult (> 60 day). Results are expressed as % reduction of the carbachol-induced (0.3 μM) contraction. Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

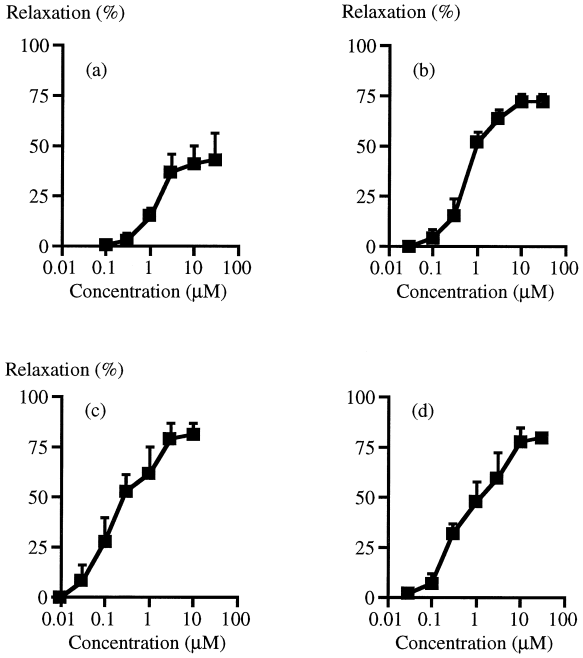

Fig. 3. Relaxant responses to NECA (■) in the carbachol-contracted rat duodenum longitudinal muscle at various ages: *a*: 15 day; *b*: 20 day; *c*: 25 day; *d*: adult (> 60 day). Results are expressed as % reduction of the carbachol-induced (0.3 μM) contraction. Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

Fisons (Loughborough, UK), or BDH (Poole, UK). CPA (10 mM) was dissolved in 20% ethanol, DPCPX (1 mM) was dissolved in 6% aqueous dimethylsulphoxide (DMSO) containing NaOH (6 mM) and CGS 21680 (10 mM) was dissolved in 7% ethanol. After dilution to a final bath concentration corresponding to the highest concentration

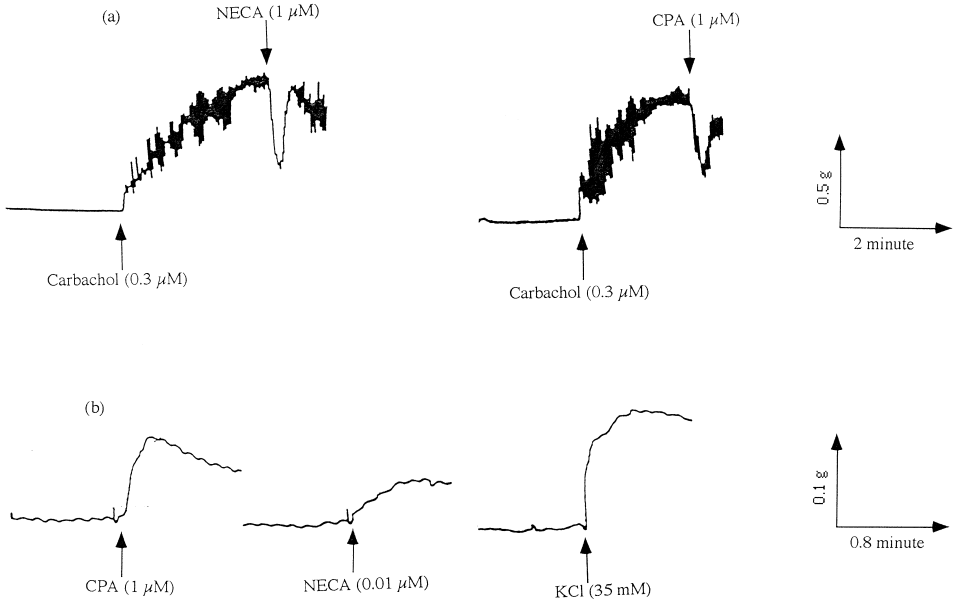

Fig. 2. Representative traces showing (*a*) effects of NECA and CPA in causing relaxation on carbachol-contracted (0.3 μM) neonatal (25 day) rat duodenum longitudinal muscle and (*b*) effects of NECA, CPA and KCl in causing contraction in neonatal (20 day) rat duodenum muscularis mucosae.

of drug used the solvents had no effect on the tissue, causing neither contraction nor relaxation.

3. Results

3.1. Rat duodenum longitudinal muscle

In the neonatal rat duodenum longitudinal muscle, CPA caused a relaxation in a concentration-dependent manner from day 25 onwards (Fig. 1; see Fig. 2 for representative trace). At day 15 no relaxations to CPA were observed, whereas small relaxations to CPA (less than 15%), were seen at day 20 (Fig. 1). Relaxations to NECA were observed in this tissue from day 15 onwards (the earliest age it could be studied due to the fragility of the tissue) (Fig. 3; see Fig. 2 for representative trace). The maximum relaxation achieved by NECA at day 15 (approximately 50%) in this tissue was less than that observed for NECA at days 20, 25 or in adult preparations (approximately 75%) (Fig. 3). CGS 21680 also caused a weak relaxation (less than 15%) in this tissue at day 15 but only at concentrations greater than 10 μM (Fig. 4a).

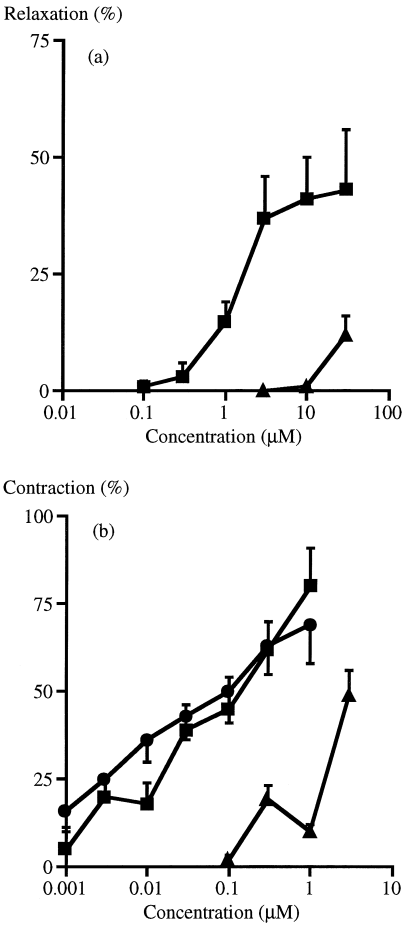

Fig. 4. *a*: relaxant responses to NECA (■) and CGS 21680 (▲) in the carbachol-contracted rat duodenum longitudinal muscle at day 15; *b*: contractile responses to NECA (■), CPA (●) and CGS 21680 (▲) in the rat duodenum muscularis mucosae at day 20. Contractile responses are expressed as % of contraction induced by KCl (35 mM) and relaxant responses are expressed as % reduction of the carbachol-induced (0.3 μM) contraction. Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

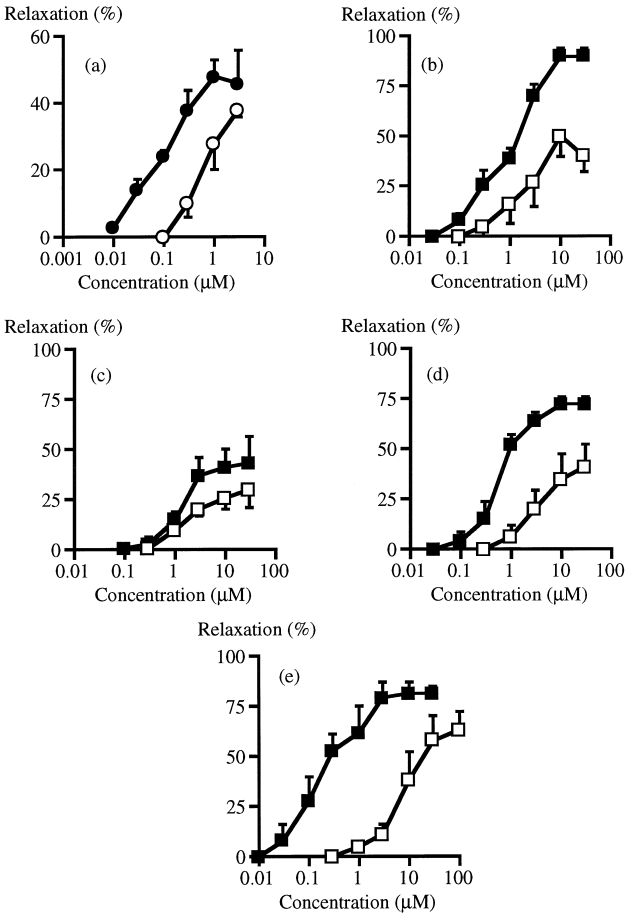

Fig. 5. Effect of DPCPX on the relaxant responses to CPA and NECA in the carbachol-contracted rat duodenum longitudinal muscle. *a*: relaxant responses to CPA in the absence (●) and presence (○) of DPCPX (10 nM) at day 25; *b*: relaxant responses to NECA in the absence (■) and presence (□) of DPCPX (10 nM) at day 25; *c*, *d* and *e*: relaxant responses to NECA in the absence (■) and presence (□) of DPCPX (1 μM) at days 15, 20 and 25 respectively. Relaxant responses are expressed as % reduction of the carbachol-induced (0.3 μM) contraction. Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

DPCPX (10 nM) significantly ($P < 0.05$) antagonised the response to CPA at day 25 (Fig. 5a), giving a concentration ratio of 18.4 ± 6.5, and an apparent pA_2 value of 8.9 ± 0.5. DPCPX (1 μM) abolished the response to CPA at day 25 (results not shown). Because responses to CPA at days 15 and 20 were absent or weak the effect of DPCPX against this agonist at these ages could not be tested. DPCPX (10 nM) significantly ($P < 0.05$) antagonised the response to NECA at day 25 (Fig. 5b), giving a concentration ratio of 14.1 ± 5.3 corresponding to an apparent pA_2 value of 8.9 ± 0.3, however in the presence of the antagonist the concentration-response curves were not

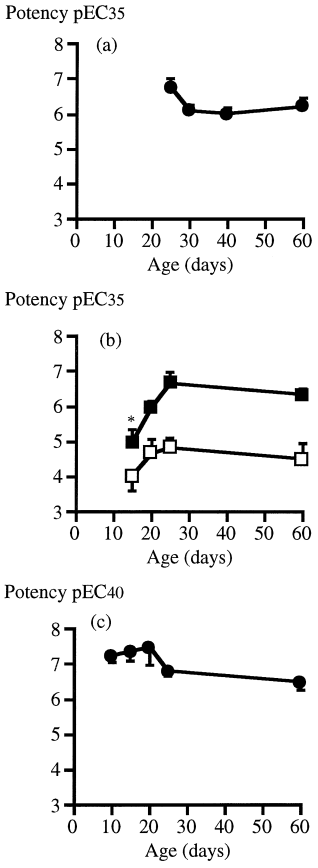

Fig. 6. Variation with age in potency of (*a*) CPA causing relaxations (●) in the rat duodenum longitudinal muscle, (*b*) NECA causing relaxations in the rat duodenum longitudinal muscle in the absence (■) and presence (□) of DPCPX (1 μM) and (*c*) CPA causing contractions (●) in the rat duodenum muscularis mucosae. The potency of CPA and NECA causing relaxations in the longitudinal muscle was expressed as pEC_{35}. The potency of CPA in causing contractions in the muscularis mucosae was expressed as pEC_{40}. The potency value for NECA causing relaxations in the adult rat duodenum longitudinal muscle in the presence of DPCPX (1 μM) was obtained from our previous work (Nicholls et al., 1996). Each point is the mean of 3–5 determinations and the vertical bars show S.E.M. *, indicates significantly different from the adult value; $P < 0.05$, one-way ANOVA followed by Duncans New Multiple Range post-hoc test.

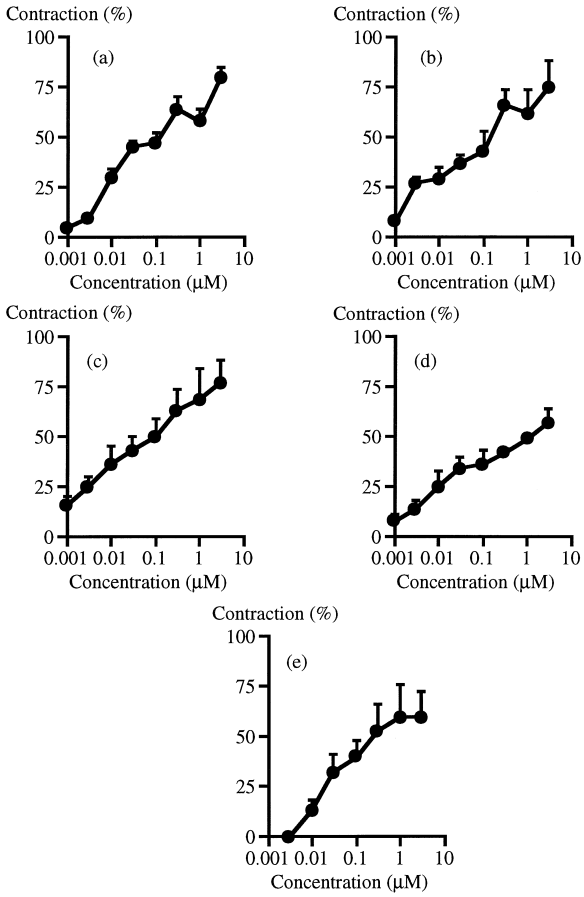

Fig. 7. Contractile responses to CPA (●) in the rat duodenum muscularis mucosae at various ages. *a*: 10 day; *b*: 15 day; *c*: 20 day; *d*: 25 day; *e*: adult (> 60 day). Results are expressed as % of contraction induced by KCl (35 mM). Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

parallel and a suppression of the maximal response was observed. The responses to NECA at days 15, 20 and 25 were also antagonised by a higher concentration of DPCPX (1 μM) (Fig. 5c, d, e), giving concentration ratios of 4.6 ± 2.3, 49.3 ± 18.3 and 144.9 ± 56.3, corresponding to apparent pA_2 values of 6.8 ± 0.1, 7.5 ± 0.2 and 8.0 ± 0.2 respectively. Apart from day 15 the antagonism by DPCPX (1 μM) was significant ($P < 0.05$).

The potency of CPA in causing relaxations (expressed as pEC_{35}) after day 25 was not significantly different from the potency in the adult at any age ($F(3,13) = 3.238$, $P > 0.05$, one-way ANOVA) (Fig. 6a). The potency of NECA (expressed as pEC_{35}) in causing relaxations increased with age ($F(3,11) = 7.649$, $P < 0.05$, one-way ANOVA), being significantly different from the adult value

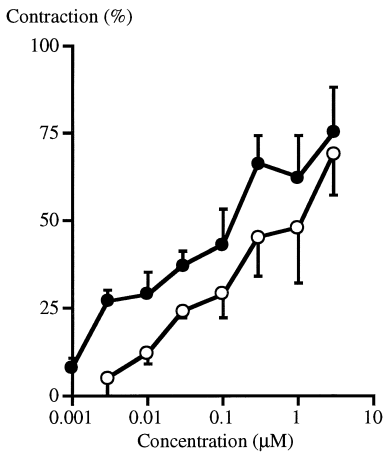

Fig. 8. Contractile responses to CPA in the 15 day muscularis mucosae in the absence (●) and presence (○) of DPCPX (1 μM). Contractile responses are expressed as % of contraction induced by KCl (35 mM). Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

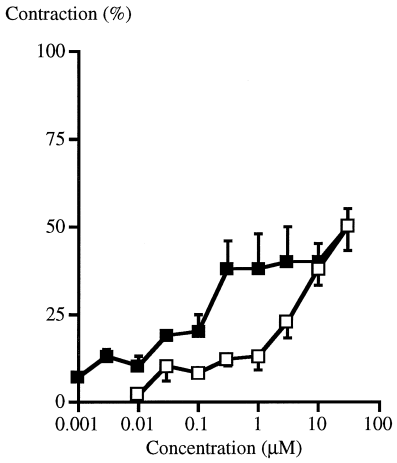

Fig. 9. Contractile responses to NECA in the 20 day muscularis mucosae in the absence (■) and presence (□) of DPCPX (1 μM). Contractile responses are expressed as % of contraction induced by KCl (35 mM). Each point is the mean of 3–5 determinations and the vertical bars show S.E.M.

at day 15 ($P < 0.05$, Duncan New Multiple Range test) and not significantly different from the adult from day 20 ($P > 0.05$, Duncan New Multiple Range test) (Fig. 6b). However the potency of NECA in causing relaxations in the presence of DPCPX (1 μM) did not vary with age ($F(3,12) = 0.894$, $P > 0.05$, one-way ANOVA) (Fig. 6b). When variations in the potency of NECA with age in the absence and presence of DPCPX (1 μM) were compared using two-way ANOVA, a significant effect of age ($F(3,23) = 4.633$, $P < 0.05$) and of treatment ($F(1,23) = 36.241$, $P < 0.01$) was found with no interaction effect.

3.2. Rat duodenum muscularis mucosae

In the neonatal muscularis mucosae, CPA caused contractions in a concentration-dependent manner from day 10 onwards (the earliest age it could be studied due to the fragility of the tissue) (Fig. 7; see Fig. 2 for representative trace). DPCPX (1 μM) significantly ($P < 0.05$) antagonised the response to CPA at days 15 (Fig. 8) and 10, giving concentration ratios of 9.6 ± 3.5 and 6.8 ± 2.7, corresponding to apparent pA_2 values of 6.7 ± 0.2 and 6.6 ± 0.3 respectively. The potency of CPA in causing contractions (expressed as pEC_{40}) did not vary with age ($F(4,17) = 2.114$, $P > 0.05$, one-way ANOVA) (Fig. 6c).

CGS 21680 and NECA also caused a contraction in the neonatal tissue at day 20 (Fig. 4b; see Fig. 2 for representative trace), and the potency order of agonists was NECA = CPA ≫ CGS 21680 ($pEC_{40} = 7.4 \pm 0.2$, 7.4 ± 0.6, 5.6 ± 0.1 respectively, $n = 3–5$). Responses to NECA at day 20 were significantly ($P < 0.05$) antagonised by DPCPX (1 μM) with a concentration ratio of 7.3 ± 5.1, corresponding to an apparent pA_2 of 6.8 ± 0.3 (Fig. 9).

4. Discussion

Using the adult rat duodenum longitudinal muscle we have previously shown that CPA activates adenosine A_1 receptors and NECA activates a mixture of adenosine A_1 and adenosine A_{2B} receptors to mediate relaxation (Nicholls et al., 1996). In the longitudinal muscle CPA caused a relaxation in a concentration dependent manner after day 20, with small relaxations being observed at day 20 (less than 15%), but no relaxations at day 15. DPCPX (1 μM) abolished the response to CPA at day 25 and DPCPX (10 nM) antagonised it with an apparent pA_2 of 8.9, suggesting that CPA was solely acting via adenosine A_1 receptors as previously observed for the adult tissue (Nicholls et al., 1996). The potency of CPA in causing relaxations was not significantly different from adult from day 25 onwards (see Fig. 6a), suggesting that, once the adenosine A_1 receptor has developed (between days 20–25) there is no further increase in the receptor population with age. Alternatively it is possible that there are further changes in receptor number but that they are exactly balanced by opposing changes in receptor-effector coupling, but this seems less likely.

NECA also caused a relaxation in neonatal rat duodenum longitudinal muscle, but was active earlier, from day 15 onwards. At day 15, responses to NECA were antagonised by DPCPX (1 μM) with an apparent pA_2 of 6.8, showing the activation of only adenosine A_2 receptors by NECA at this age and confirming the lack of functional adenosine A_1 receptors. The potency of NECA increased with age, with NECA being least potent at day 15 and equipotent with adult by day 20 (see Fig. 6b), and the concentration-response curve to NECA at day 15 reached a lower maximal response (50%) than at days 20 or 25 (75%). This increase in potency and maximal response between days 20 and 25 of the NECA concentration-response curves may be due to the development of adenosine A_1 receptors (as shown by results with CPA) which are also activated by NECA. In our previous work on the adult tissue, DPCPX (1 μM) antagonised the response to NECA with an apparent pA_2 value of 8.1, and the Schild plot slope was much lower than unity, suggesting that NECA activates a mixture of adenosine A_1 and adenosine A_2 receptors (Nicholls et al., 1996). The results with DPCPX (1 μM) at days 20 and 25 giving apparent pA_2 values of 7.5 and 8.0, respectively, suggests that at these ages too NECA may activate a mixture of adenosine A_1 and adenosine A_2 receptors. This was further confirmed by the fact that at day 25 as in the adult (Nicholls et al., 1996) DPCPX (10 nM) antagonised the response to NECA with an apparent pA_2 of 8.9, indicating an adenosine A_1-mediated effect of NECA at this age. The results with NECA therefore confirm the results with CPA suggesting that at day 15 there are no functional adenosine A_1 receptors but at around day 20 an adenosine A_1 receptor population begins to develop. The potency of NECA in causing

relaxations in the presence of DPCPX (1 μM) (to abolish any adenosine A_1-mediated effect) was not significantly different at any age from the potency in the adult (see Fig. 6b) suggesting that the adenosine A_2 receptor is functionally fully developed by day 15. CGS 21680 also caused some relaxations (less than 15%) in the rat duodenum longitudinal muscle at day 15, but only at concentrations greater than 10 μM, suggesting that the adenosine A_2 receptor in the neonates is of the adenosine A_{2B} subtype as is the case in the adult (Nicholls et al., 1996). Our earlier results in the whole rat duodenum (Nicholls et al., 1990) showing that adenosine was less potent in the neonates (below day 15) than in the adult are similar to our present results with NECA suggesting that adenosine, like NECA, may activate adenosine A_{2B} receptors at day 15 and below and a mixture of adenosine A_1 and adenosine A_{2B} receptors from day 20 onwards.

In the neonatal rat duodenum muscularis mucosae CPA, NECA and CGS 21680 all caused contractions with a potency order of CPA = NECA $\gg$ CGS 21680 (at day 20), similar to the situation in the adult tissue where all the purines were reported to activate adenosine A_{2B} receptors to cause contractions (Nicholls et al., 1996). Since contractions via adenosine A_{2B} receptors rather than adenosine A_1 receptors are unexpected and CPA was equipotent with NECA, the ontogeny of this contractile adenosine A_{2B} receptor was followed in this tissue using CPA instead of NECA. This would also allow us to see if at any age there was an adenosine A_1 receptor-mediated component in this tissue. However DPCPX (1 μM) antagonised the response to CPA and NECA in neonatal tissues with an apparent pA_2 value for DPCPX in the micromolar range, confirming that CPA activated only adenosine A_{2B} receptors in the neonatal tissues as is the case for the adult (Nicholls et al., 1996). The potency of CPA in causing contractions was not significantly different from adult throughout the ages (see Fig. 6c), suggesting that the contractile adenosine A_{2B} receptor population is functionally fully developed as early as day 10 in this tissue.

In conclusion, the contractile adenosine A_{2B} receptor in the rat duodenum muscularis mucosae and the relaxant adenosine A_{2B} receptor in the rat duodenum longitudinal muscle are present as early as days 10 and 15 respectively (the earliest ages which could be studied), whereas the relaxant adenosine A_1 receptor in the rat duodenum longitudinal muscle appears between days 20–25. The ontogeny of adenosine receptors in other smooth muscle preparations such as in the rat colon and in the rat vas deferens has also been studied. In the rat colon muscularis mucosae contractile adenosine A_1 receptors have been shown to be present from day 5 onwards (Bailey et al., 1992; Hourani et al., 1993). In the rat vas deferens inhibitory pre-junctional adenosine A_1 receptors and the post-junctional adenosine A_{2B} receptors have been shown to be present from as early as days 15 and 10 respectively, the earliest ages which could be studied, whereas the excitatory post-junctional adenosine A_1 receptors have been shown to develop much later on, after day 20, and has been linked to the start of sexual maturation of the rat (Peachey et al., 1996). Similarly, the late development of the relaxant adenosine A_1 receptor in the rat duodenum longitudinal muscle between days 20–25 may be due to the physiological maturation of the gut associated with weaning which takes place at day 20. Overall therefore the ontogeny of adenosine receptors differs between different smooth muscle preparations suggesting that the roles of adenosine in controlling smooth muscle function vary with age.

Acknowledgements

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