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Neuropharmacology 50 (2006) 690–704
NEURO PHARMACOLOGY
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Postnatal development of P2 receptors in the murine gastrointestinal tract

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Received 20 July 2005; received in revised form 28 October 2005; accepted 28 November 2005

Abstract

The actions of purine and pyrimidine compounds on isolated segments of the mouse intestine were investigated during postnatal development. The localization of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_2$ and $P2X_3$ receptors were examined immunohistochemically, and levels of expression of $P2Y_1$, $P2X_1$ and $P2X_2$ were studied by Western immunoblot. From day 12 onwards, the order of potency for relaxation of longitudinal muscle of all regions was 2-MeSADP $\geq$ α,β-meATP $\geq$ ATP = UTP = adenosine, suggesting $P2Y_1$ receptors. This was supported by the sensitivity of responses to 2-MeSADP to the selective antagonist MRS 2179 and $P2Y_1$ receptor immunoreactivity on longitudinal muscle and a subpopulation of myenteric neurons. A further α,β-meATP-sensitive P2Y receptor subtype was also indicated. ATP and UTP were equipotent suggesting a $P2Y_2$ and/or $P2Y_4$ receptor. Adenosine relaxed the longitudinal muscle in all regions via P1 receptors. The efficacy of all agonists to induce relaxation of raised tone preparations increased with age, being comparable to adult by day 20, the weaning age. During postnatal development the contractile response of the ileum and colon was via $P2Y_1$ receptors, while the relaxant response mediated by $P2Y_1$ receptors gradually appeared along the mouse gastrointestinal tract, being detectable in the stomach from day 3 and in the duodenum from day 6. In the ileum and colon relaxant responses to 2-MeSADP were not detected until days 8 and 12, respectively. 2-MeSADP induced contractions on basal tone preparations from day 3, but decreased significantly at day 12 and disappeared by day 20. At day 8, contractions of colonic longitudinal muscle to ATP showed no desensitisation suggesting the involvement of $P2X_2$ receptors. Immunoreactivity to $P2X_2$ receptors only was observed on the longitudinal muscle of the colon and ileum from day 1 and on a subpopulation of myenteric neurons from day 3. These data suggest that $P2Y_1$ receptors undergo postnatal developmental changes in the mouse gut, with a shift from contraction to relaxation. Such changes occur 1 week before weaning and may contribute to the changes that take place in the gut when the food composition changes from maternal milk to solid food.
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Keywords: Mouse; Postnatal; Adenosine triphosphate; P2X; P2Y; Gut

1. Introduction

Purine nucleotides and nucleosides play an important role in the modulation of both secretory and motor functions in the gastrointestinal (GI) tract. In 1970, Burnstock and colleagues proposed adenosine 5′-triphosphate (ATP) as a transmitter involved in non-adrenergic, non-cholinergic (NANC), nerve-mediated responses of smooth muscle in the gastrointestinal tract (Burnstock et al., 1970). Since then evidence has accumulated in support of the hypothesis that ATP is a NANC transmitter in the enteric nervous system (Burnstock, 2001a).

Burnstock (1978) proposed that receptors selective for adenosine and adenosine monophosphate (AMP) be

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0028-3908/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2005.11.015

designated P1-purinoceptors and those selective for ATP and adenosine diphosphate (ADP) designated as P2-purinoceptors. P1 receptors have been further subdivided into A_1, A_{2A}, A_{2B} and A_3. P2 receptors have been divided into two families (Abbracchio and Burnstock, 1994), currently seven mammalian P2X ionotropic ligand-gated ion channel receptors, $P2X_{1-7}$ and eight P2Y metabotropic G protein-coupled receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ have been identified (Khakh et al., 2001; Abbracchio et al., 2003).

Both adenosine and ATP receptors participate in the regulation of GI motility. Adenosine can directly activate receptors located on smooth muscle (Nicholls et al., 1996; Kadowaki et al., 2000), or act prejunctionally to suppress the release of acetylcholine and substance P (Moneta et al., 1997; Kadowaki et al., 2000).

In most species, ATP acts via P2 receptors to produce relaxations of smooth muscle in the GI tract, although there are reports of ATP causing contraction of smooth muscle in some regions of the gut (Burnstock, 2001a). Recent pharmacological and morphological evidence has been provided for the presence of $P2Y_1$ receptors on myenteric and submucosal NANC inhibitory neurons mediating relaxation of the longitudinal muscle in the mouse gut, via nitric oxide (NO) and ATP acting on P2Y receptors located on smooth muscle (Giaroni et al., 2002). In the mouse gut, ATP can produce relaxation via $P2Y_1$ receptors, but also via $P2Y_2$ receptors and an unidentified α,β-methylene ATP (α,β-meATP)-sensitive P2Y receptor, located on smooth muscle. Activation of $P2X_2$ receptors mediates contraction of the mouse colon smooth muscle (Giaroni et al., 2002).

Ontogenetic studies of the development of purinergic effects in the GI tract have demonstrated that these are present during prenatal development (Burnstock, 2001b). NANC responses have been demonstrated in the rat stomach (Ito et al., 1988) and in the mouse and rabbit small intestine (Gershon and Thompson, 1973) during foetal development. In addition, intense quinacrine fluorescence, which is indicative of high levels of ATP, has been detected in enteric neurons of the rabbit stomach and ileum before birth (Crowe and Burnstock, 1981).

There are reports indicating that developmental changes occur in purinergic signalling in the small intestine after birth. In the rat duodenum, changes in purinergic signalling have been observed in the third postnatal week, corresponding to the weaning period (Furukawa and Nomoto, 1989; Nicholls et al., 1990; Irie et al., 1994; Hourani, 1999). ATP has been shown to produce relaxation of the longitudinal muscle in the neonatal rat duodenum via P2Y receptors, which were fully developed by day 25 (Brownhill et al., 1997). In the same study, P2Y receptors mediating contraction have been described in the muscularis mucosae before day 20, but subsequently this effect was mediated by P2X receptors.

In the present study, we examined the presence and function of P2 receptors in the neonatal mouse gut during postnatal development, using pharmacological, morphological and biochemical approaches. To this end, we studied the effect of purinoceptor agonists and antagonists on the longitudinal muscle of the stomach fundus, duodenum, ileum and colon obtained from animals between days 3 and 12 after birth and at day 20, which corresponds to the weaning period. The presence and localization of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_2$ and $P2X_3$ receptors was studied with immunohistochemical methods in the colon and ileum between days 1 and 20. Further, the levels of expression of $P2Y_1$, $P2X_1$ and $P2X_2$ receptors were investigated by Western immunoblotting in the colon between days 1 and 20. This study was carried out on the GI tract of the mouse, partly because only scarce information is available concerning the postnatal development of purinergic transmission in the gut of this species, but also because the later development of the mouse, being born physiologically very immature, makes such information of particular interest. The increasing availability of P2 receptor knockout mice also make such control information desirable.

2. Materials and methods

2.1. Animals

Principles of good laboratory animal care were followed and animal experimentation was in compliance with specific national (U.K.) laws and regulations. Neonatal male and female mice (1–12 days old; strain C57/BL10) were killed by cervical dislocation, whereas 20 days old and adult mice were killed by carbon dioxide and death was ensured by cervical dislocation according to Home Office (UK) regulations covering Schedule One procedures. After the abdominal cavity had been opened, the gut was rapidly removed and placed in a beaker with a physiological saline solution. The day of birth was designated as day 1, neonatal mice were weaned at day 20.

2.2. Pharmacological studies

Pharmacological studies had been carried out from day 3 onwards. The stomach was opened along the longitudinal axis of the greater curvature, pinned flat and longitudinal smooth muscle strips (1 mm in width, 3 mm in length) were dissected from the anterior fundus wall (upper part). The duodenum was dissected out at the base of the pylorus and a longitudinal length of 5 mm was used; 1 cm longitudinal segments of the distal ileum, approximately 2 cm oral to the ileo-caecal junction and the distal colon were also removed. Intestinal segments were flushed and cleaned of connective tissue. Silk ligatures were applied to each end of the strip; one end was attached to a rigid support and the other to a Grass FT03C force displacement transducer. Tissues were mounted in 10 ml organ baths, continually gassed (95% O_2/5% CO_2) and containing Krebs solution of the following composition (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7; and $CaCl_2$, 2.5; pH 7.3. Experiments were carried out at 32 ± 1 °C, as this temperature reduced spontaneous activity. Mechanical activity was recorded using the software PowerLab Chart for Windows (Version 4; ADInstruments New South Wales, Australia). An initial load of 0.2 g was applied to the stomach fundus strips and 0.5 g for the other regions; tissues were allowed to equilibrate for 60 min prior to the start of experiments.

For each tissue, a concentration–response curve to the muscarinic agonist carbachol (CCh) was constructed in order to obtain an EC_{50} concentration, found to be approximately 2 μM for the stomach fundus and 5 μM for the duodenum, ileum and colon. This concentration was used to induce sustained contractions for the measurement of relaxation responses. Concentration–response curves for the different purinoceptor agonists were constructed cumulatively on preparations where tone had been induced with CCh. In preparations obtained from animals between days 8 and 12, the concentration–response curves to 2-MeSADP (a $P2Y_{1,12}$ and $P2Y_{13}$ receptor agonist) were repeated in the presence of the $P2Y_1$-selective antagonist MRS 2179 (1 μM) and curves to ATP in the presence of the non-selective adenosine antagonist, 8-*p*-sulfophenyl-theophylline (8-*p*SPT, 30 μM). In the duodenum,

between days 8 and 12, curves to 2-MeSADP were repeated in the presence of nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM), then in the presence of L-NAME and L-arginine (L-Arg, 5 mM). In all regions of the mouse gut examined, between days 8 and 12, the effect of a submaximal dose of ATP (300 μM), 2-MeSADP (3 μM) and the $P2X_1$ and $P2X_3$ receptor agonist α,β-meATP (30 μM) was evaluated in the absence and presence of the neurotoxin tetrodotoxin (TTX, 1 μM). In the same age group, the effect of a submaximal dose of ATP (300 μM) was evaluated in the absence and presence of L-NAME (100 μM).

In ileal and colonic specimens obtained from 3-, 6-, 8- and 20-day-old mice, contraction concentration–response curves to ATP and 2-MeSADP were constructed in a cumulative fashion on basal tone. Between days 8 and 12, the curves to 2-MeSADP were repeated in the presence of MRS 2179 (30 μM), whereas curves to ATP in the colon, at day 8, were repeated in the presence of the non-selective P2X receptor antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM) and suramin (100 μM) All antagonists were allowed to equilibrate for 20 min before concentration–response curves were repeated.

2.3. Immunohistochemistry

Sections (12 μM) of 1-, 3-, 8-, 12- and 20-day-old and adult mouse ileum and colon were cut using a cryostat (Reichert Jung CM1800) and collected on gelatin-coated slides and air-dried at room temperature. The slides were stored at −20 °C.

The avidin–biotin (ABC) technique was employed according to the protocol described by Llewellyn-Smith et al. (1993). The sections were left at room temperature for at least 10 min and then fixed in 4% formaldehyde (0.1 M phosphate buffer, pH 7.4) containing 0.2% saturated solution of picric acid for 4 min.

Endogenous peroxidase was blocked with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Non-specific binding sites were blocked by a 60-min incubation with 10% normal horse serum (NHS) in phosphate-buffered saline (PBS) containing 0.05% merthiolate.

The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_2$ and $P2X_3$ receptor antibodies (rabbit) were diluted to 2.5 μg/ml with 10% NHS in PBS containing 0.05% merthiolate and 0.2% Triton X-100. $P2X_1$, $P2X_2$ and $P2X_3$ primary antibodies were kindly donated by Roche Bioscience (Palo Alto, CA, USA); $P2Y_1$, $P2Y_2$, $P2Y_4$ primary antibodies were purchased from Alomone (Alomone Laboratories, Jerusalem, Israel). The specimens were then incubated overnight at room temperature with the primary $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_2$ and $P2X_3$ antibodies. The secondary antibody was a biotinylated donkey anti-rabbit IgG diluted 1:500 in 1% NHS in PBS containing 0.05% merthiolate for 2 h, followed by the ExtrAvidin peroxidase conjugate used at 1:1000 for 60 min. After washing, a nickel-diaminobenzidine enhancement technique was used to visualise the reaction product. The specimens were dehydrated in isopropanol and air dried for 30 min before mounting in DPX (VWR International, Poole, UK).

The following control experiments were performed to establish a specific immunoreaction: omission of the primary antibody, replacement of the primary antibodies with rabbit pre-immune IgG or absorption of the primary antibodies with an excess of their homologous peptide antigen.

Photographs of the immunohistochemical sections were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, San Jose, CA, USA) and prints were made with an Epson Stylus Photo 810 printer.

2.4. Western blot

Colonic segments were pooled from 3–4 animals, minced with scissors (1–2 mm in length) and homogenized in ice-cold isolation buffer [MOPS–sucrose buffer containing 25 mM MOPS, 10 mM $MgCl_2$, 8% w/v sucrose, a cocktail of protease inhibitors Complete 1:50 (Roche Diagnostics GmbH, Mannheim Germany), pH 7.4] with an Ultra-Turrax homogenizer. The crude homogenate was centrifuged at 1000 × *g* for 10 min, at 4 °C. The supernatant was collected and centrifuged again at 40,000 × *g* for 10 min, at 4 °C. The resulting pellet was successively suspended in protein extraction reagent T-PER (Pierce, Rockford, IL) containing 1:50 Complete protease inhibitor cocktail, incubated in ice for 15 min, sonicated and centrifuged at 10,000 rpm for 5 min and processed for Western-blot analysis. Aliquots of the sample were used for protein assay (Bradford, 1976), the remaining was boiled for 2 min after dilution with sample buffer (Laemmli, 1970) and centrifuged at 10,000 rpm for 5 min. Proteins (100 μg) were electrophoresed on 10% SDS–PAGE and electroblotted to Immobilon-P transfer membranes (Millipore). Membranes were then incubated with $P2Y_1$, $P2X_1$, $P2X_2$ primary antisera, diluted 2.5 μg/ml with a TBST solution [composition: 20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5] containing 0.2% blotting grade blocker, at 4 °C overnight. After washing with TBST for 30 min, appropriate secondary antisera (labeled with alkaline phosphatase, BioRad, Hercules, CA) were incubated at room temperature for 30 min. Antibody/substrate complex was visualized on an X-ray film (X-Omat-AR film, Eastman Kodak Company, Rochester, NY) by chemiluminescence (Immun-Star, BioRad). Signal intensity was quantified by densitometric analysis using Multi-Analyst software (BioRad).

Pre-stained molecular mass markers (250–10 kDa range, Precision Plus Protein Standards, BioRad) were used to determine the molecular weight (MW) of immunoreactive bands. Specificity of each primary antibody was evaluated by omission of the primary antibody and by absorption of the primary antibodies with an excess of their homologous peptide antigen.

2.5. Drugs

L-Arg, α,β-meATP, ATP, carbamyl-β-methyl choline chloride (carbachol), ExtrAvidin-horseradish peroxidase, glucose oxidase, hydrogen peroxide, 2-MeSADP, L-NAME, normal horse serum, PPADS, saturated picric acid solution, merthiolate (thimerosal), 8-*p*SPT suramin, TTX and UTP were purchased from Sigma; MRS 2179 was obtained from Tocris, Bristol, UK. Formaldehyde stabilized with 10% methanol was obtained from Analar, BDH; biotinylated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch, PA, USA. Stock solutions were prepared in distilled water. The volume added to the organ bath to produce the final concentration was not in excess of 100 μl.

2.6. Statistical analysis

Relaxant responses were expressed as mean percentage reduction of the CCh-induced contraction (at EC_{50} concentration) ± standard error of the mean (S.E.M.) of (*n*) animals and contractile responses were expressed as mean maximum tension developed in mg ± S.E.M. (*n*). The potency of the agonists in causing relaxation or contraction was expressed as the negative $\log_{10}$ of the molar concentration of the agonist producing 25% of the response ($p[A]_{25}$), calculated by non-linear regression analysis of the individual log concentration–response curves, by means of the software Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA). Significance was tested by analysis of variance (ANOVA) followed by Bonferroni's test or by two-way ANOVA followed by Tukey's test, or by Student's *t*-test, as appropriate. A probability of $P < 0.05$ was taken as significant for all statistical analyses.

3. Results

3.1. Relaxation

3.1.1. Stomach

2-MeSADP, α,β-meATP, UTP, ATP and adenosine relaxed the CCh-contracted mouse stomach fundus at day 3 at high concentrations of agonists (> 1 μM) and at lower concentrations (10–100 nM) from day 6. The order of potency for 2-MeSADP was: 3 days = 6 days < 8 days < 20 days = adult (significant difference between groups $P < 0.05$ or less) (Fig. 1a). Concentration–response curves to 2-MeSADP in 8–12-day-old animals were significantly inhibited ($P < 0.05$) by MRS 2179 (1 μM) (Fig. 1b). The potency order for α,β-meATP was:

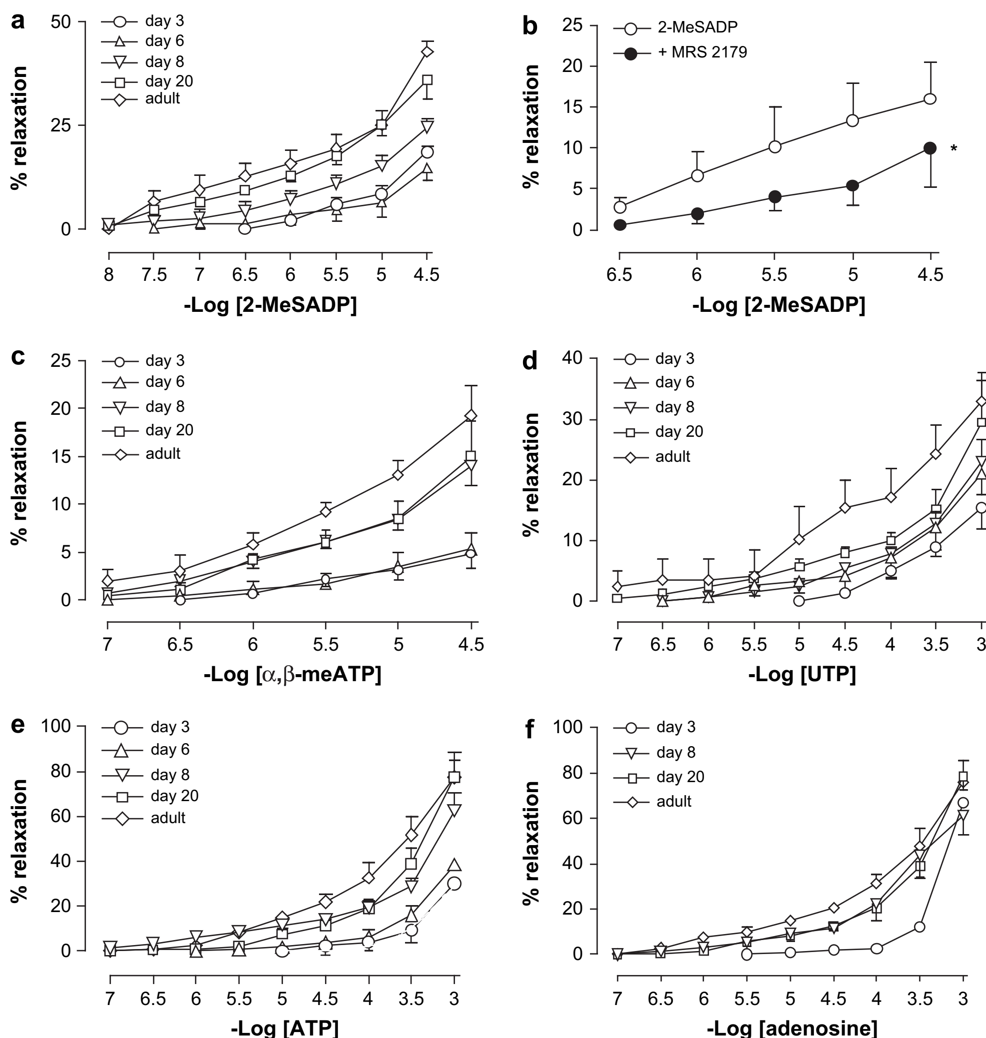

Fig. 1. Relaxation of mouse stomach fundus longitudinal muscle to P2 agonists at different days of postnatal development and in adults on raised tone preparations (carbachol, 2 μM). (a) Cumulative concentration–response curves to 2-MeSADP; (b) relaxation to 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (c) concentration–response curves to α,β-meATP; (d) UTP; (e) ATP; (f) adenosine. All symbols represent mean% relaxation ± S.E.M. (unless masked by symbol; $n = 5–8$ for each agonist). Significance was tested by two-way ANOVA $^*P < 0.05$.

3 days = 6 days < 8 days = 20 days < adult ($P < 0.001$ or less) (Fig. 1c). The potency order for UTP was: 3 days < 6 days = 8 days < 20 days < adult ($P < 0.05$) (Fig. 1d). The potency order for ATP was: 3 days = 6 days < 8 days = 20 days < adult ($P < 0.05$ or less) (Fig. 1e). The order of potency for adenosine was: 3 days < 8 days = 20 days < adult ($P < 0.05$) (Fig. 1f). Potency values, expressed as pA_{25} (the concentration of agonist that produced 25% relaxation) are given in Table 1. The agonist potency order for the stomach fundus at days 8 and 20 was: 2-MeSADP > α,β-meATP > ATP = UTP = adenosine, whereas in the adult: 2-MeSADP > α,β-meATP = ATP= UTP = adenosine.

3.1.2. Duodenum

ATP, UTP and adenosine relaxed the CCh-contracted mouse duodenum at day 3 at concentrations of agonists > 50 μM. Responses to all the agonists could be detected by day 6 at lower concentrations (10 nM–100 nM). The order of potency for 2-MeSADP was: 6 days < 8 days = 20 days = adult ($P < 0.001$) (Fig. 2a). Concentration–response curves to 2-MeSADP in 8–12-day-old animals were significantly inhibited ($P < 0.001$) by MRS 2179 (1 μM) (Fig. 2b). The potency order for α,β-meATP was: 6 days < 8 days = 20 days < adult ($P < 0.001$ or less) (Fig. 2c). The potency order for UTP was: 3 days < < 6 days < 8 days = 20 days = adult ($P < 0.001$ or less) (Fig. 2d). The potency order for ATP was: 3 days < < 6 days = 8 days = 20 days = adult ($P < 0.001$ or less) (Fig. 2e). The order of potency for adenosine was: 3 days < < 8 days = 20 days = adult ($P < 0.001$ or less) (Fig. 2f). Potency values for all agonists are given in Table 1. The potency order for the mouse duodenum at day 8, 20 and in adult was 2-MeSADP = α,β-meATP > ATP = UTP = adenosine.

3.1.3. Ileum

In the mouse ileum, ATP, UTP and adenosine relaxed the CCh-contracted longitudinal muscle between days 3 and 6,

Table 1
Ontogenic profile of the potency of different purinoceptor agonist-induced relaxation of preparations of mouse stomach and duodenum, pre-contracted with carbachol (2 μM for stomach, 5 μM for duodenum) expressed as $p[A]_{25}$ values (concentration that induced 25% relaxation)

Region	Agonist	3 days	6 days	8 days	20 days	Adult
Stomach	ATP	3.13 ± 0.05**	3.31 ± 0.08*	3.71 ± 0.19	3.86 ± 0.20	4.37 ± 0.21
	Adenosine	3.40 ± 0.09**	–	3.94 ± 0.17	3.88 ± 0.08	4.31 ± 0.13
	2-MeSADP	<25%	<25%	4.49 ± 0.11	5.00 ± 0.14	5.00 ± 0.23
	α,β-meATP	<25%	<25%	<25%	<25%	<25%
	UTP	<25%	<25%	2.90 ± 0.25	3.16 ± 0.15	3.48 ± 0.13
Duodenum	ATP	3.23 ± 0.13***	5.74 ± 0.43	5.76 ± 0.20	5.88 ± 0.39	5.70 ± 0.11
	Adenosine	3.84 ± 0.13**	–	6.01 ± 0.34	6.00 ± 0.60	5.66 ± 0.44
	2-MeSADP	–	5.35 ± 0.43*	7.05 ±0.14	7.53 ± 0.31	7.07 ± 0.37
	α,β-meATP	–	5.18 ± 0.12***	6.14 ± 0.28**	6.07 ± 0.15**	7.28 ± 0.14
	UTP	3.33 ± 0.31***	4.69 ± 0.11	5.66 ± 0.14	5.68 ± 0.19	5.56 ± 0.35

Post hoc comparisons (Bonferroni's multiple comparison test) for potency differences across ages are shown compared to adult value for each agonist. Values are mean ± S.E.M. of at least five experiments. <25% indicates where relaxation was less than 25% of carbachol contraction. – indicates no response or data not available. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. adult.

at concentrations of agonist > 10 μM. By day 8 all agonists except α,β-meATP (day 12) induced relaxation at lower concentrations (10–100 nM). The order of potency for 2-MeSADP was: 6 days < 8 days < 12 days < 20 days = adult ($P < 0.05$ or less) (Fig. 3a). Concentration–response curves to 2-MeSADP in 8–12-day-old animals were significantly inhibited ($P < 0.001$) by MRS 2179 (1 μM) (Fig. 3b). The potency order for α,β-meATP was: 12 days < 20 days = adult ($P < 0.001$) (Fig. 3c). The potency order for UTP was: 3 days < 6 days < 8 days < 20 days = adult ($P < 0.05$ or less) (Fig. 3d). The potency order for ATP was: 3 days = 6 days < 8 days < 20 days = adult ($P < 0.001$ or less) (Fig. 3e). The order of potency for adenosine was: 3 days < < 8 days = 20 days = adult ($P < 0.001$) (Fig. 3f). Potency values are given in Table 2. The potency order for all agonists in the ileum at day 12 was: 2-MeSADP > α,β-meATP = adenosine > ATP = UTP, whereas at day 20 and in adult animals the order was: 2-MeSADP > α,β-meATP = adenosine = ATP = UTP.

3.1.4. Colon

ATP, UTP and adenosine relaxed the CCh-contracted mouse colon at day 3 at high concentrations of agonists (> 5 μM). By day 6, α,β-meATP and UTP induced relaxation at lower concentrations (10–100 nM) and by day 8 ATP and day 12 2-MeSADP also induced relaxations. The potency order for 2-MeSADP was: 12 days = 20 days = adult (Fig. 4a). Concentration–response curves to 2-MeSADP in 8–12-day-old animals were significantly ($P < 0.001$) inhibited by MRS 2179 (1 μM) (Fig. 4b). The potency order for α,β-meATP was: 6 days < 8 days = 20 days = adult ($P < 0.001$) (Fig. 4c). The potency order for UTP was: 3 days < 6 days < 8 days = 20 days = adult ($P < 0.001$) (Fig. 4d). The potency order for ATP was 3 days < 8 days < 12 days = 20 days = adult ($P < 0.001$) (Fig. 4e). The order of potency for adenosine was: 3 days < < 8 days = 20 days = adult ($P < 0.05$) (Fig. 4f). Potency values are given in Table 2. The order of potency for the different agonists at day 12, 20 and adult was: 2-MeSADP = α,β-meATP > ATP = UTP = adenosine.

Between days 8 and 12, TTX (1 μM) significantly reduced the relaxation induced by a submaximal concentration of ATP (300 μM) and 2-MeSADP (3 μM), but had no effect against a submaximal concentration of α,β-meATP (30 μM) in each region of the gut studied (Fig. 5). L-NAME (100 μM) induced a significant ($P < 0.001$) inhibition of the concentration–response curve to 2-MeSADP in the duodenum; the inhibitory effect was reversed by L-Arg (5 mM). In contrast L-NAME (100 μM) did not affect the relaxant response to ATP (300 μM) either in the stomach fundus, duodenum, ileum and colon (data not shown). Concentration–response curves to ATP, from days 8–12, were significantly ($P < 0.001$) inhibited by 8-*p*SPT (30 μM) in each region of the GI tract examined (data not shown).

3.2. Contraction

At day 3, 2-MeSADP and ATP induced a concentration-dependent contraction of the mouse ileum and colon longitudinal muscle at basal tone. In both regions, contractile concentration–response curves to 2-MeSADP decreased with increasing age, being highest at days 3 and 6 and significantly reduced at days 8 and 12 ($P < 0.0001$) (Figs. 3g and 4g) and undetectable by day 20. In the ileum, contractile responses to ATP also decreased with age, being undetectable by day 20 (Fig. 3i). In the colon, contractile responses to ATP decreased with age, but contractile responses remained in the adult (Fig. 4i). At day 8, the contractile effect of 2-MeSADP, both in the ileum and colon, was significantly inhibited by MRS 2179 ($P < 0.01$ and $P < 0.001$, respectively) (Figs. 3h and 4h). $p[A]_{25}$ values for 2-MeSADP- and ATP-induced contractions in the ileum and colon were not significantly different at all age groups and are reported in Table 3.

In the colon, at day 8, PPADS (30 μM) and suramin (100 μM) caused a significant inhibition of the concentration–response curves to ATP ($P < 0.0001$ for both antagonists; data not shown). In this experimental group, repeated administration of ATP (300 μM; $n = 4$) at intervals of 8 min,

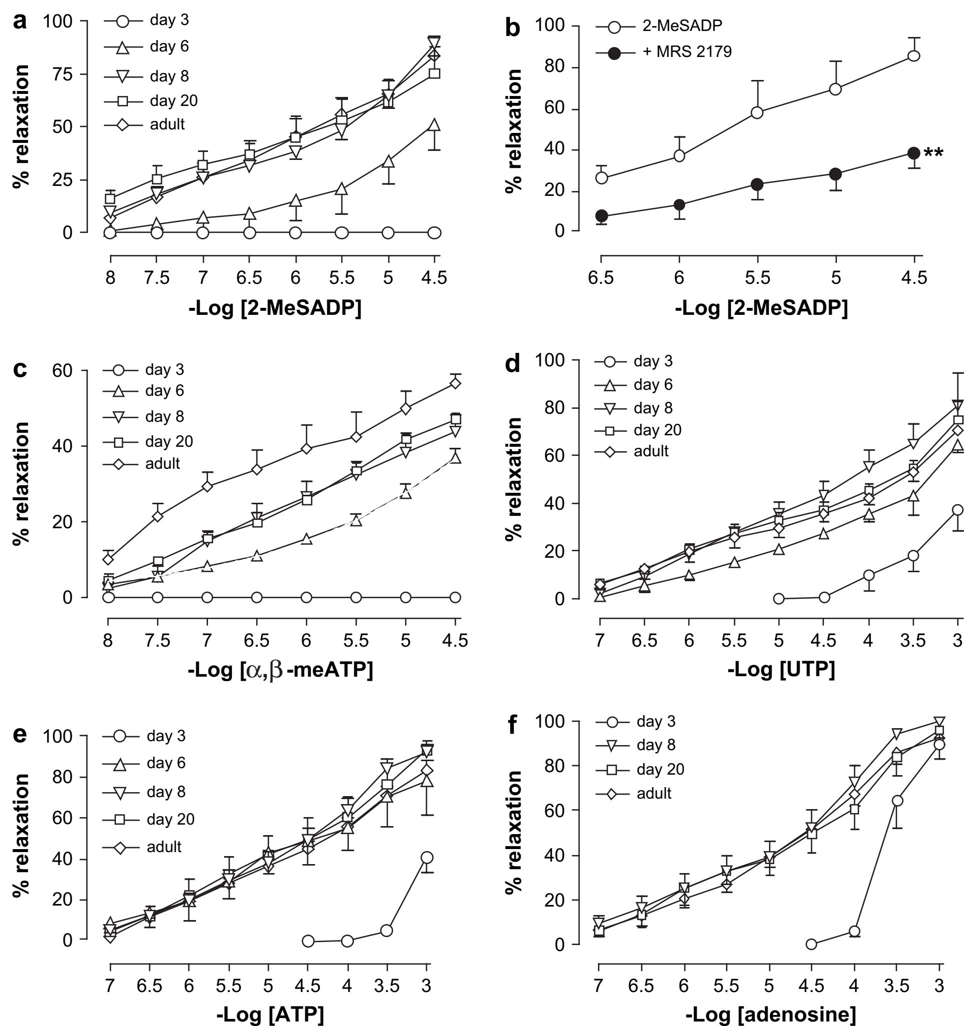

Fig. 2. Relaxation of mouse duodenum longitudinal muscle to P2 agonists at different days of postnatal development and in adults on raised tone preparations (carbachol, 5 μM). (a) Cumulative concentration–response curves to 2-MeSADP; (b) relaxation to 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (c) concentration–response curves to α,β-meATP; (d) UTP; (e) ATP; (f) adenosine. All symbols represent mean % relaxation ± S.E.M. (unless masked by symbol; $n = 5$–8 for each agonist). Significance was tested by two-way ANOVA $^{**}P < 0.01$.

after which time the tension had return to baseline, did not result in a reduction of the response to the agonist (data not shown). The contractile response to a single concentration of ATP (1 mM; $n = 3$) was not affected by TTX (1 mM; data not shown).

3.3. Development of response

The functional studies with 2-MeSADP (via $P2Y_1$ receptors) revealed that there was a gradual development of a relaxation response along the intestinal tract from stomach to colon, starting at day 3 in the stomach, day 6 in the duodenum, day 8 in the ileum and reaching the colon by day 12. In contrast, a contractile response in the ileum and colon, present before the development of relaxation, declined with age. By day 8 in the ileum and day 12 in the colon contractile responses had declined and a relaxation response had developed. This change in response is summarised in Table 4.

3.4. Immunohistochemistry

Immunoreactivity to $P2Y_1$ receptors was present in the longitudinal smooth muscle of the ileum and colon from day 1 after birth (Fig. 6a). The staining was strong and diffuse in the younger animals (3 and 1 days old) and became more defined in animals older than 8 days. $P2Y_1$ receptor immunoreactivity was detected also in the soma of a subpopulation of myenteric neurons of the ileum from day 3 onwards. In the ileum of animals older than 12 days, immunostaining for $P2Y_1$ receptors was weaker in smooth muscle cells and stronger in the ganglia and interconnecting fibres of the myenteric plexus. In the colon of animals older than 8 days, immunoreactivity to $P2Y_1$ receptors was prevalently localized to muscle cells of the muscularis propria and muscularis mucosae, whereas weak immunoreactivity was observed in myenteric neurons. In adult mouse ileum staining for $P2Y_1$ receptors of weaker intensity with respect to that observed in the cytoplasm of myenteric neurons, could be evidenced also in nerve

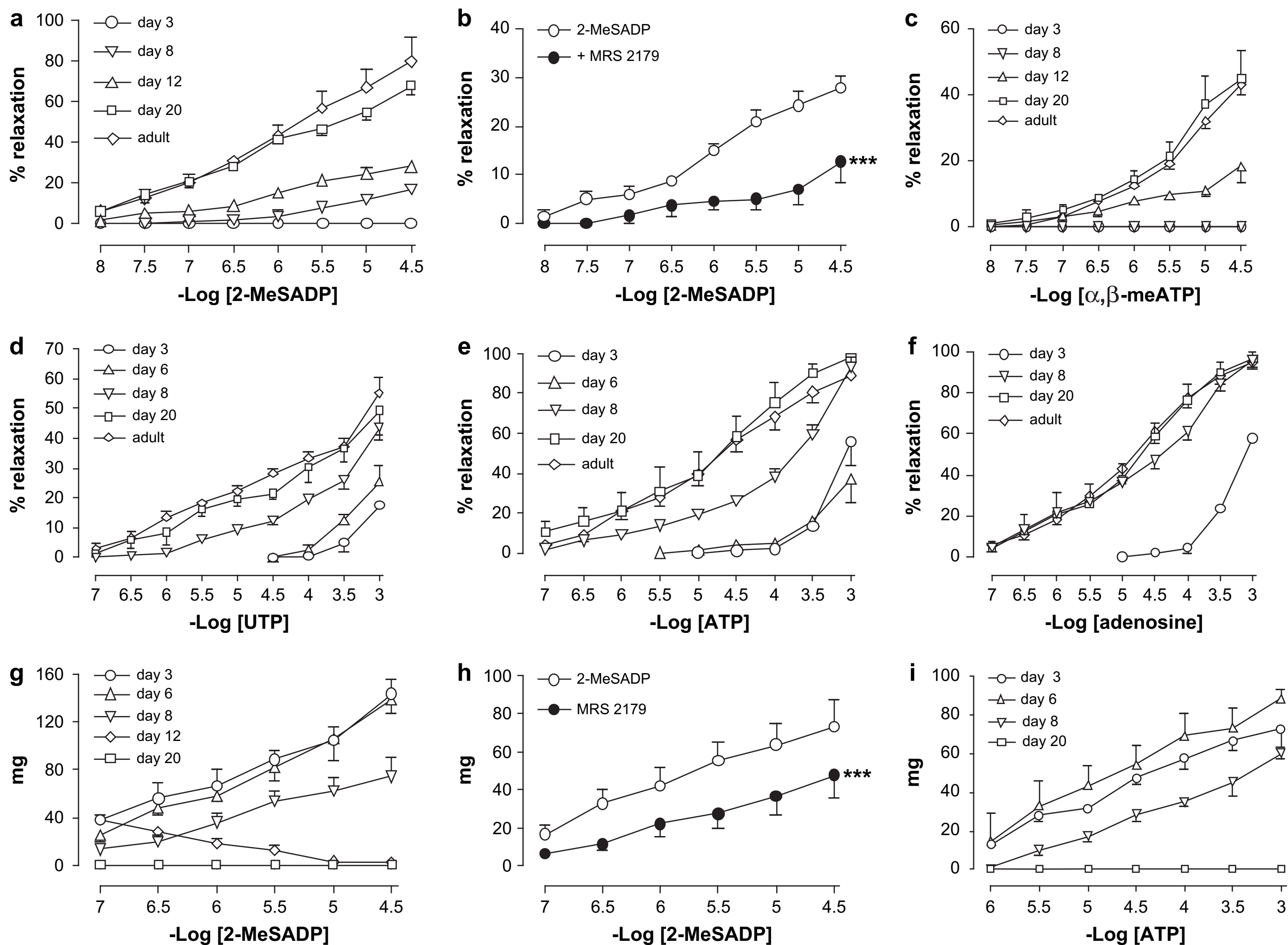

Fig. 3. (a–f) Relaxation of mouse ileum longitudinal muscle to P2 agonists at different days of postnatal development and in adults on raised tone preparations (carbachol, 5 μM). (a) Cumulative concentration–response curves to 2-MeSADP; (b) relaxation to 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (c) concentration–response curves to α,β-meATP; (d) UTP; (e) ATP; (f) adenosine. (g–i) Contractile effects of P2 agonists on basal tone of the mouse ileum at different postnatal ages expressed as mg tension developed. (g) Cumulative concentration–response curves to 2-MeSADP; (h) 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (i) concentration–response curves to ATP. All symbols represent mean ± S.E.M. (unless masked by symbol; $n = 5–8$ for each agonist). Significance was tested by two-way ANOVA. $^{***}P < 0.0001$.

fibres innervating the circular muscle layer. $P2Y_1$ immunoreactivity was also detected in the soma of a subpopulation of submucosal neurons from day 20. $P2Y_2$ receptor immunoreactivity was also observed in the smooth muscle from day 1, the intensity of staining was stronger in younger animals (8 days and younger) and was of a lower intensity than that seen for $P2Y_1$ receptors (Fig. 6b). $P2Y_4$ immunoreactivity was present in the longitudinal muscle from day 1; it was of much lower intensity than that seen for $P2Y_1$ and $P2Y_2$ receptors at all ages. Some weak immunoreactivity to $P2Y_4$ was also occasionally seen in the cytoplasm of neurons within ganglia of the myenteric plexus from day 8 and of the submucosal plexus from day 20. Intense immunoreactivity to $P2Y_4$ receptor was also observed on blood vessels in the ileum and colon from day 8.

$P2X_1$ receptor immunoreactivity was not found in the smooth muscle of the mouse ileum and colon at any age, but it was observed on smooth muscle of blood vessels within the GI tract from day 8 (Fig. 6e, left-hand panel) and in the soma of a subpopulation of myenteric neurons from day 1 (Fig. 6e, right-hand panel). Immunoreactivity to $P2X_1$ receptor was found also in the cytoplasm of neurons within submucosal ganglia and in nerve fibres innervating the circular muscle layer from day 20. Small spots of immunoreactivity indicating staining in nerve endings were observed in the circular muscle layer of adult preparations.

$P2X_2$ receptor immunoreactivity was present in the longitudinal smooth muscle of the colon from day 3 and of the ileum from day 8. Immunostain for $P2X_2$ receptors was also present in the soma of a subpopulation of myenteric neurons from day 3 (Fig. 6g). Weak staining for $P2X_2$ receptors appeared in the cytoplasm of neurons within the submucosal plexus in the colon from day 8 and in the ileum from day 12. Nerve fibres innervating the mucosa and the circular muscle layer were evident only in adult ileum and colon preparations. In the ileum, immunoreactivity to $P2X_2$ receptors was prevalently localized to enteric ganglia with respect to smooth muscle.

Table 2
Ontogenic profile of the potency of different purinoceptor agonist-induced relaxation of preparations of mouse ileum and colon, pre-contracted with carbachol (5 μM) expressed as $p[A]_{25}$ values

Region	Agonist	3 days	6 days	8 days	20 days	Adult
Ileum	ATP	3.38 ± 0.25***	3.30 ± 0.16***	4.60 ± 0.13*	5.81 ± 0.45	5.71 ± 0.19
	Adenosine	3.50 ± 0.14***	–	5.70 ± 0.12	5.58 ± 0.39	5.71 ± 0.14
	2-MeSADP	–	<25%	<25%	6.71 ± 0.21	6.77 ± 0.25
	α,β-meATP	–	–	–	5.40 ± 0.14	5.29 ± 0.14
	UTP	<25%	3.02 ± 0.21***	3.59 ± 0.10**	4.32 ± 0.34	4.78 ± 0.12
Colon	ATP	3.34 ± 0.04***	–	4.04 ± 0.21**	5.06 ± 0.22	5.19 ± 0.36
	Adenosine	3.45 ± 0.13***	–	5.34 ± 0.20	5.14 ± 0.32	5.15 ± 0.31
	2-MeSADP	–	–	–	7.23 ± 0.21	6.96 ± 0.09
	α,β-meATP	–	4.52 ± 0.27***	6.15 ± 0.18	6.35 ± 0.22	6.60 ± 0.18
	UTP	2.83 ± 0.25**	3.62 ± 0.18	4.82 ± 0.49	4.44 ± 0.14	5.29 ± 0.25

Post hoc comparisons (Bonferroni's multiple comparison test) for potency difference across ages are shown compared to adult value for each agonist. Values are mean ± S.E.M. of at least five experiments. <25% indicates where relaxation was less than 25% of carbachol contraction. – indicates no response or data not available. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. adult.

Focal and weak immunoreactivity to $P2X_3$ receptor was found in the soma of a subpopulation of myenteric neurons from day 1, staining became more evident from day 3 (Fig. 6i). Strong staining for $P2X_3$ receptor appeared also in the soma of a subpopulation of neurons within submucosal ganglia and in the interconnecting fibres between myenteric ganglia, from day 20. In ileal and colonic preparations obtained from adult animals, $P2X_3$ immunoreactivity was also found in sparse fibres innervating the circular muscle layer. The staining was specific for all P2 receptors studied since pre-absorption of the primary antibody resulted in no observed immunoreactivity (Fig. 6b,d,f,h,j). The ontogenic profile of the distribution of P2 receptors in the mouse ileum and colon is summarised in Table 5.

Positive immunostaining for $P2Y_1$, $P2Y_2$ and $P2X_2$ receptors has been visualised within the mucosae of the GI tract of the mouse during postnatal development. The presence of these receptors has been reported previously (see Burnstock and Knight, 2004).

3.5. Western blot

In the adult mouse colon, Western blot analysis of $P2Y_1$ receptor revealed three principal bands at 78, 100 and 200 kDa. The $P2Y_1$ receptor antibody has already been described to recognize a band at 200 kDa in spinal cord astrocytes (Suadicani et al., 2003), and at 100 kDa in human platelets (information provided by the commercial manufacturer, Alomone Labs). 78 and 200 kDa immunoreactive bands were detectable from day 1 after birth. The density of both bands significantly increased with increasing age being lowest at day 1 and reaching values not different from adults by day 8. The density of the 100 kDa band could be measured only in adult (Fig. 7Aa,Ab).

In the adult mouse colon, $P2X_1$ antibody revealed a principal band at 45 kDa, as already described for human platelets (Scase et al., 1998). The 45 kDa band was detectable from day 1 after birth and its intensity increased with an increase in age, being lowest at day 1 and reaching values not significantly different from adult by day 8 (Fig. 7Ba). In the adult mouse colon, $P2X_2$ antibody revealed a principal band at about 50 kDa, which corresponds to the molecular weight of native protein (Lynch et al., 1999). In the mouse colon, the 50 kDa band was detectable from day 1 after birth and its intensity slightly, but not significantly, increased from day 1 to adult (Fig. 7Bb).

4. Discussion

This study has investigated the changing roles of multiple P2 receptor subtypes on the longitudinal muscle of the mouse GI tract during postnatal development. A striking feature of postnatal development is that a relaxant response mediated by $P2Y_1$ receptors gradually develops along the length of the GI tract from the first week after birth, detectable in the stomach from day 3 and in the duodenum from day 6, while in the ileum and colon relaxation responses could not be detected until days 8 and 12, respectively. The relaxation responses in all regions resembled that seen in the adult by day 20. In contrast $P2Y_1$ receptor-mediated contractions were prominent in the ileum and colon from day 3, but had decreased significantly by day 12 and disappeared by day 20. In a developmental study of the rat duodenum, ATP and 2-MeSATP induced relaxations from day 2 and the potency increased with age, being similar to the adult by day 25 (Nicholls et al., 1990), suggesting that in the rat relaxation responses post weaning (approximately day 21 in the rat) are comparable to that seen in the adult, as also seen in this study.

4.1. $P2Y_1$ receptors

Between days 6 and 12, 2-MeSADP, α,β-meATP, ATP, UTP and adenosine all induced relaxation of the longitudinal muscle in all regions. 2-MeSADP was the most potent agonist, suggesting the involvement of $P2Y_1$ receptors (Harden et al., 1998; King et al., 1998). $P2Y_1$ receptors are activated by endogenous ATP and ADP; ADP is the most potent natural agonist and 2-MeSADP is a selective agonist (Ralevic and Burnstock, 1998). The presence of $P2Y_1$ receptors is supported by the sensitivity of 2-MeSADP responses to the

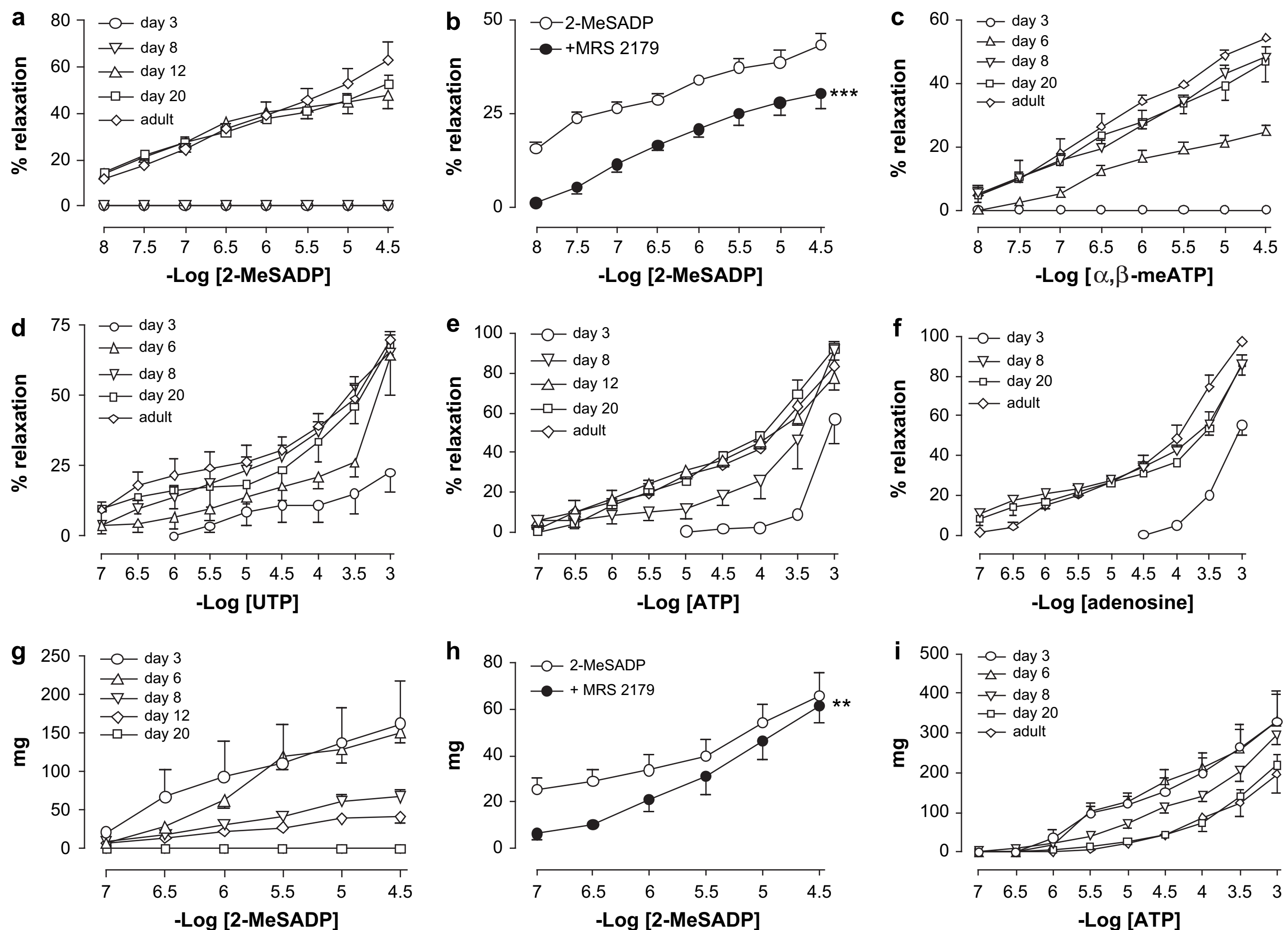

Fig. 4. (a–f) Relaxation of mouse *colon* longitudinal muscle to P2 agonists at different days of postnatal development and in adults on raised tone preparations (carbachol, 5 μM). (a) Cumulative concentration–response curves to 2-MeSADP; (b) relaxation to 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (c) concentration–response curves to α,β-meATP; (d) UTP; (e) ATP; (f) adenosine. (g–i) Contractile effects of P2 agonists on basal tone of the mouse colon at different postnatal ages expressed as mg tension developed. (g) Cumulative concentration–response curves to 2-MeSADP; (h) 2-MeSADP in the absence and presence of MRS 2179 (1 μM); (i) concentration–response curves to ATP. All symbols represent mean ± S.E.M. (unless masked by symbol; $n = 5–8$ for each agonist). Significance was tested by two-way ANOVA $^{**}P < 0.01$, $^{***}P < 0.0001$.

selective $P2Y_1$ antagonist, MRS 2179 (Camaioni et al., 1998). The longitudinal smooth muscle of the ileum and colon have been found to intensely stain for $P2Y_1$ receptors from day 1 after birth; in addition, immunoreactivity to $P2Y_1$ receptors was visualised on a subpopulation of myenteric neurons from day 3. These data suggest that, in the neonatal mouse intestine, $P2Y_1$ receptors are located on myenteric neurons and on smooth muscle, and this is consistent with the partial inhibition of ATP- and 2-MeSADP-induced relaxations by TTX in all regions examined. During postnatal development, $P2Y_1$ receptor immunoreactivity was found in the cell bodies of myenteric neurons of the mouse gut, whereas no detectable immunoreactivity was seen on nerve terminals innervating smooth muscle. Staining for $P2Y_1$ receptors, of weaker intensity than that observed on cell somas, could be observed in nerve fibres impinging on smooth muscle only in the adult mouse gut. These observations suggest that $P2Y_1$ receptors appear developmentally earlier on cell bodies than on nerve terminals and that purine agonist action is mainly localized to post-synaptic $P2Y_1$ receptors in the neonatal mouse gut. Sensitivity of 2-MeSADP-induced relaxations to L-NAME, which inhibits the activity of NOS (Rees et al., 1990), suggests that $P2Y_1$ receptors are located on NANC neurons in enteric ganglia, and mediate relaxation partly via the release of NO. In the neonate mouse gut, as in the adult (Giaroni et al., 2002), $P2Y_1$ receptors may represent the principle receptor subtype involved in NANC relaxation mediated by NO and ATP.

At basal tone, 2-MeSADP and ATP induced contractions between days 3 and 6 in the ileum and colon. 2-MeSADP was the most potent agonist and its inhibition by MRS 2179 supports the involvement of $P2Y_1$ receptors. This response was strongest in the first week after birth, declined in the second week, and was undetectable by day 20. After day 20, ATP mediated a contractile response only in the colon. Thus our data suggests that in the neonatal mouse ileum and colon responses to $P2Y_1$ receptor activation shift from contraction to relaxation. There are reports suggesting a similar shift from a contractile to an inhibitory effect of purines in the rat

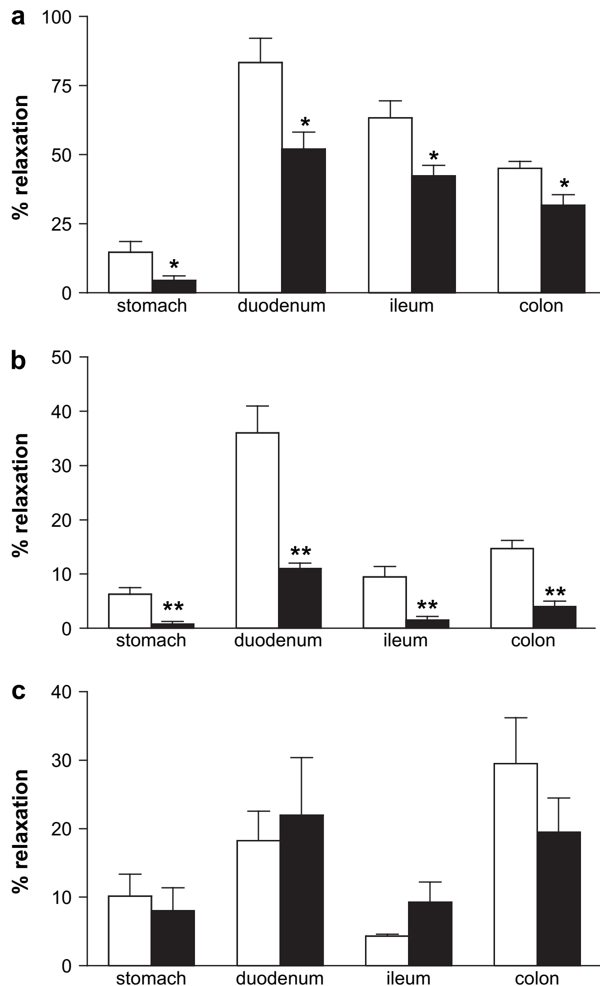

Fig. 5. Relaxation of preparations of mouse GI tract pre-contracted with carbachol (2 μM for stomach, 5 μM other regions) induced by (a) ATP (300 μM), (b) 2-MeSADP (3 μM) and (c) α,β-meATP (30 μM) in the mouse gut between days 8 and 12, in the absence (empty column) and presence (full column) of TTX (1 μM). Note that TTX inhibited responses to ATP and 2-MeSADP, but not to α,β-meATP. Values are given as mean ± S.E.M. of at least four experiments. *$P < 0.05$, **$P < 0.01$ vs. agonist in the absence of TTX following a paired Student's *t*-test.

duodenum around the third postnatal week, corresponding to the weaning period (Furukawa and Nomoto, 1989; Nicholls et al., 1990; Hourani, 1999). The response of the duodenum to the ganglion stimulant, nicotine, switches from contraction by a cholinergic mechanism to relaxation by a NANC mechanism during the weaning period (Irie et al., 1994).

The presence of $P2Y_1$ receptors in the neonatal mouse gut was further confirmed by Western blot analysis revealing specific immunoreactivity bands for the receptor from day 1, whose intensity increased with an increase in age. Furthermore, changes in the pattern of the bands to $P2Y_1$ receptors, but not those of $P2X_1$ and $P2X_2$ receptors, were seen between neonatal and adult tissues, with the appearance of a 100 kDa band in adults. The different pattern of $P2Y_1$ immunoreactive bands may arise from alternative splicing, post-translational modifications such as glycosylation and phosphorylation or oligodimerization of the receptor (Lynch et al., 1999;

Table 3
Ontogeny of the contractile response to 2-MeSADP and ATP on preparations of mouse ileum and colon at basal tone

	Ileum		Colon	
	ATP	2-MeSADP	ATP	2-MeSADP
3 days	5.16 ± 0.11	6.65 ± 0.16	4.78 ± 0.19	6.14 ± 0.44
6 days	5.77 ± 0.31	6.32 ± 0.13	4.60 ± 0.11	6.24 ± 0.13
8–12 days	4.99 ± 0.12	6.06 ± 0.21	4.43 ± 0.08	6.11 ± 0.20
20 days	–	–	4.32 ± 0.07	–
Adult	–	–	4.02 ± 0.13	–

Values are given as mean ± S.E.M. of at least 4 experiments. – indicates no response.

Yoshioka et al., 2002; Zhong et al., 2004). The distinctive expression patterns in $P2Y_1$ receptor levels may contribute to the development of different responses (relaxation and contraction) to $P2Y_1$ receptor agonists seen during postnatal development in the mouse intestine.

The striking switch from a contractile response to one of relaxation in response to $P2Y_1$ receptor stimulation has also been shown in the rat gut (Hourani, 1999) during the weaning period. This might contribute to the development of changes that take place in the gut when the food source and composition change from being liquid and rich in fat, to being solid and rich in carbohydrate (Henning, 1981).

The mechanism behind this switch from $P2Y_1$ receptors mediating contraction to relaxation, may relate to the cell types on which the receptors are expressed or to changes in the coupling of the receptor. $P2Y_1$ receptors, like all P2Y receptors are coupled to G proteins (mainly $G_{q/11}$) which either activates phospholipase C and releases intracellular calcium or affects adenylate cyclase and alters cAMP levels (King et al., 2000). Coupling of the same P2Y receptor to distinct G proteins and signalling pathways provides the possibility of agonist-specific signalling involving distinct active conformations of the receptor. For example, activation of $P2Y_{11}$ receptors by ATP results in a rise in cAMP and inositol triphosphate and cytosolic calcium, whereas stimulation by UTP produces calcium mobilization without inositol triphosphate or cAMP increase (White et al., 2003). Responses to 2-MeSADP were inhibited by L-NAME, while responses to ATP were not, implying that in vivo, ADP and ATP do not act primarily on the same receptors. Whether an alteration in the signalling cascade of the $P2Y_1$ receptor is occurring or the response depends on the agonist activating the receptor during development of the gut would require further detailed studies. An alternative,

Table 4
Summary of relaxation and contractile responses to 2-MeSADP via $P2Y_1$ receptors in the developing mouse stomach, duodenum, ileum and colon

Day	Stomach	Duodenum	Ileum	Colon
3	↓		↑	↑
6	↓	↓	↑	↑
8	↓	↓	↓ and ↑	↑
12	↓	↓	↓	↓ and ↑
20	↓	↓	↓	↓
Adult	↓	↓	↓	↓

↓ Represents a relaxation response, ↑ represents a contractile response.

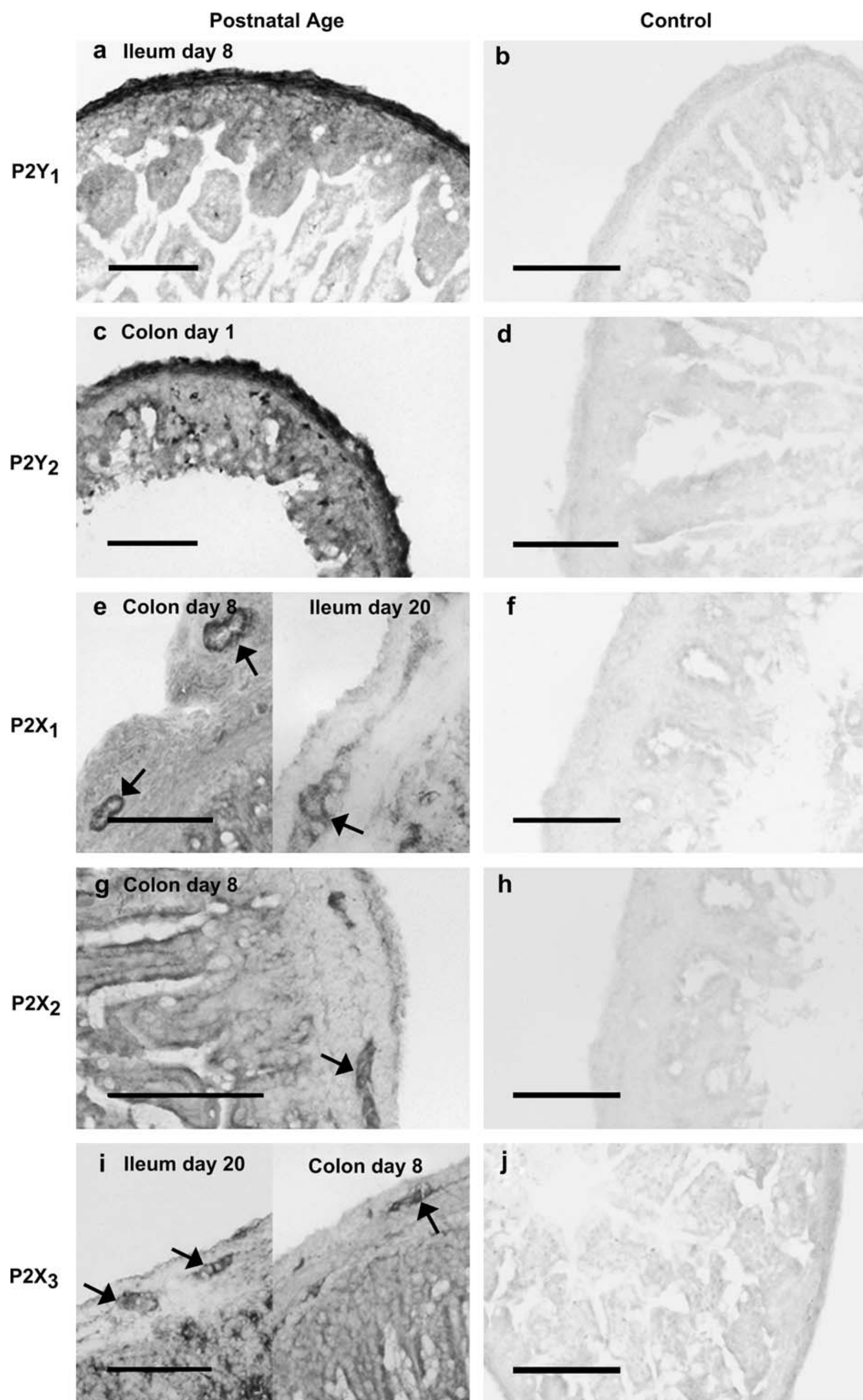

Fig. 6. Sections of mouse ileum and colon showing positive staining for P2Y and P2X receptors on longitudinal muscle and myenteric neurons at different ages. (a) $P2Y_1$ receptors, ileum day 8, positive staining on the longitudinal muscle; (b) negative control following pre-absorption of the primary antibody; ileum day 1; (c) $P2Y_2$ receptors, colon day 1, positive staining on the longitudinal muscle; (d) negative control following pre-absorption of the primary antibody; ileum day 8; (e) $P2X_1$ receptors, left-hand panel colon day 8, positive staining on vascular smooth muscle (arrows); right-hand panel ileum day 20, positive staining on myenteric neurones (arrow); (f) negative control following pre-absorption of the primary antibody, colon day 3; (g) $P2X_2$ receptors, colon day 8, positive staining on myenteric neurons (arrow); (h) negative control following pre-absorption of the primary antibody, colon day 3; (i) $P2X_3$ receptors, left-hand panel ileum day 20, right-hand panel colon day 8, positive staining on myenteric neurons (arrows); (j) negative control following pre-absorption of the primary antibody, ileum day 1. All scale bars represent 500 μm.

Table 5
Ontogenic profile of P2 receptor immunoreactivity in the mouse ileum and colon

	Age (days)																	
	1			3			8			12			20			Adult		
	LM	N	V	LM	N	V	LM	N	V	LM	N	V	LM	N	V	LM	N	V
$P2Y_1$	++	−	−	++	+/−	−	++	+	−	++	+	−	++	++	−	++	++	−
$P2Y_2$	+	−	−	+	−	−	+/−	−	−	+/−	−	−	+/−	+	−	+/−	+	−
$P2Y_4$	+/−	−	−	+/−	−	−	+/−	+	+	+/−	+	+	+/−	+	+	+/−	+	+
$P2X_1$	−	+/−	−	−	+/−	−	−	+	+	−	+	+	−	+	+	−	+	+
$P2X_2$	−	−	−	+/−	+/−	−	+	+	−	+	+	−	+	+	−	+	+	−
$P2X_3$	−	+/−	−	−	+	−	−	++	−	−	++	−	−	++	−	−	++	−

(++) Strong immunoreactivity; (+) weak immunoreactivity; (+/−) very weak immunoreactivity. LM, longitudinal muscle; N, neurons; V, vascular muscle.

and probably more likely, explanation may lie in the localization of the $P2Y_1$ receptors during development. At days 1–3, $P2Y_1$ receptors were found to be localized on smooth muscle in the ileum and colon when the response to 2-MeSADP on basal tone induced contraction. By day 8, $P2Y_1$ receptor immunoreactivity was also localized on myenteric neurons and this coincided with the beginning of a shift from contractile to relaxation responses. By day 20 there was strong staining on myenteric neurons and the contractile response to 2-MeSADP was gone. Thus the presence of $P2Y_1$ receptors on myenteric neurons may underlie the switch in response.

4.2. Other P2Y receptor subtypes

The four regions of the neonatal mouse GI tract were also found to relax to UTP and α,β-meATP, both of which are inactive at $P2Y_1$ receptors (Ralevic and Burnstock, 1998), indicating the presence of further receptor subtypes on the longitudinal muscle. The activity of UTP (being equipotent with ATP) suggests the presence of $P2Y_2$ and/or $P2Y_4$ receptors (King et al., 2000). $P2Y_2$ immunoreactivity was found on smooth muscle, whereas $P2Y_4$ was very weak. Furthermore, UTP and ATP were equipotent in mediating relaxation of all regions from day 8, suggesting that a $P2Y_2$ receptor may be already operative in the first weeks after birth. The activity of α,β-meATP suggests the presence of a P2Y receptor with an atypical pharmacological profile, as described for the guinea-pig taenia-coli, caecum and colon (Brown and Burnstock, 1981; Manzini et al., 1986; Bültmann et al., 1996; Zagorodnyuk et al., 1996). Inhibitory responses to α,β-meATP were not affected by TTX, suggesting a postjunctional smooth muscle location for this receptor. Generally, inhibitory responses to both UTP and α,β-meATP increased with an increase in age, such that responses were equivalent to those from adults by day 20. On the whole these data suggest that in the neonatal mouse gut the weaning period is critical for a full development of the relaxation response to $P2Y_2$ receptors and to the α,β-meATP-sensitive P2Y receptor.

4.3. $P2X_2$ receptors

In the neonatal mouse colon, ATP-induced contractions might also involve $P2X_2$ receptors from the first week after birth, as suggested by the lack of desensitisation of the response at day 8. The lack of sensitivity of ATP-induced contractions to TTX suggests that the majority of excitatory P2 receptors are located on smooth muscle. Immunoreactivity to $P2X_2$, but not to $P2X_1$ receptors, is present on smooth muscle cells of the longitudinal muscle layer from day 3 after birth. In addition, as seen in the adult mouse, rat and guinea-pig gut, $P2X_2$ receptors are located on subpopulations of myenteric and submucosal neurons and in the neonatal mouse gut (Vulchanova et al., 1996; Castelucci et al., 2002; Giaroni et al., 2002; Xiang and Burnstock, 2004). Recent studies carried out on the small intestine of $P2X_2$ receptor gene knockout mice suggests that $P2X_2$ receptors, located on myenteric interneurons and motor neurons, contribute to fast excitatory post synaptic potentials in neuronal pathways underlying peristalsis (Ren et al., 2003). In addition, $P2X_2$ receptors may be involved in sensory transmission in the gastrointestinal tract as since $P2X_2$ receptor immunoreactivity was present both on intrinsic sensory neurons in the guinea pig and rat gut (Castelucci et al., 2002; Xiang and Burnstock, 2004) and on terminals of extrinsic sensory neurons associated with myenteric ganglia in the mouse gut (Castelucci et al., 2003).

In contrast to $P2Y_1$ receptors, Western blot analysis of $P2X_2$ receptors did not reveal significant changes either in protein levels or immunoreactivity patterns, suggesting that $P2X_2$ receptors do not undergo substantial biochemical modifications during postnatal development in the mouse gut. In addition, $P2X_2$ receptor-mediated contractions persist during maturation since responses to ATP at day 20 were similar to those obtained in adult. Previous investigations demonstrated the presence of a predominant $P2X_2$ receptor excitatory to adult colon smooth muscle (Giaroni et al., 2002).

In the rat gut major changes do not seem to occur in the colon as they do in the mouse, although NOS activity increases during the first 20 postnatal days and then falls after weaning (Brown and Tepperman, 1997).

4.4. $P2X_3$ receptors

$P2X_3$ receptors have also been shown to have a role in the control of sensory transmission in the GI tract. In the mouse small intestine, $P2X_3$ receptors, located on intrinsic sensory myenteric neurons, participate in neural pathways underlying peristalsis, since mice lacking the $P2X_3$ receptor subunit have impaired distension-evoked reflexes (Bian et al., 2003).

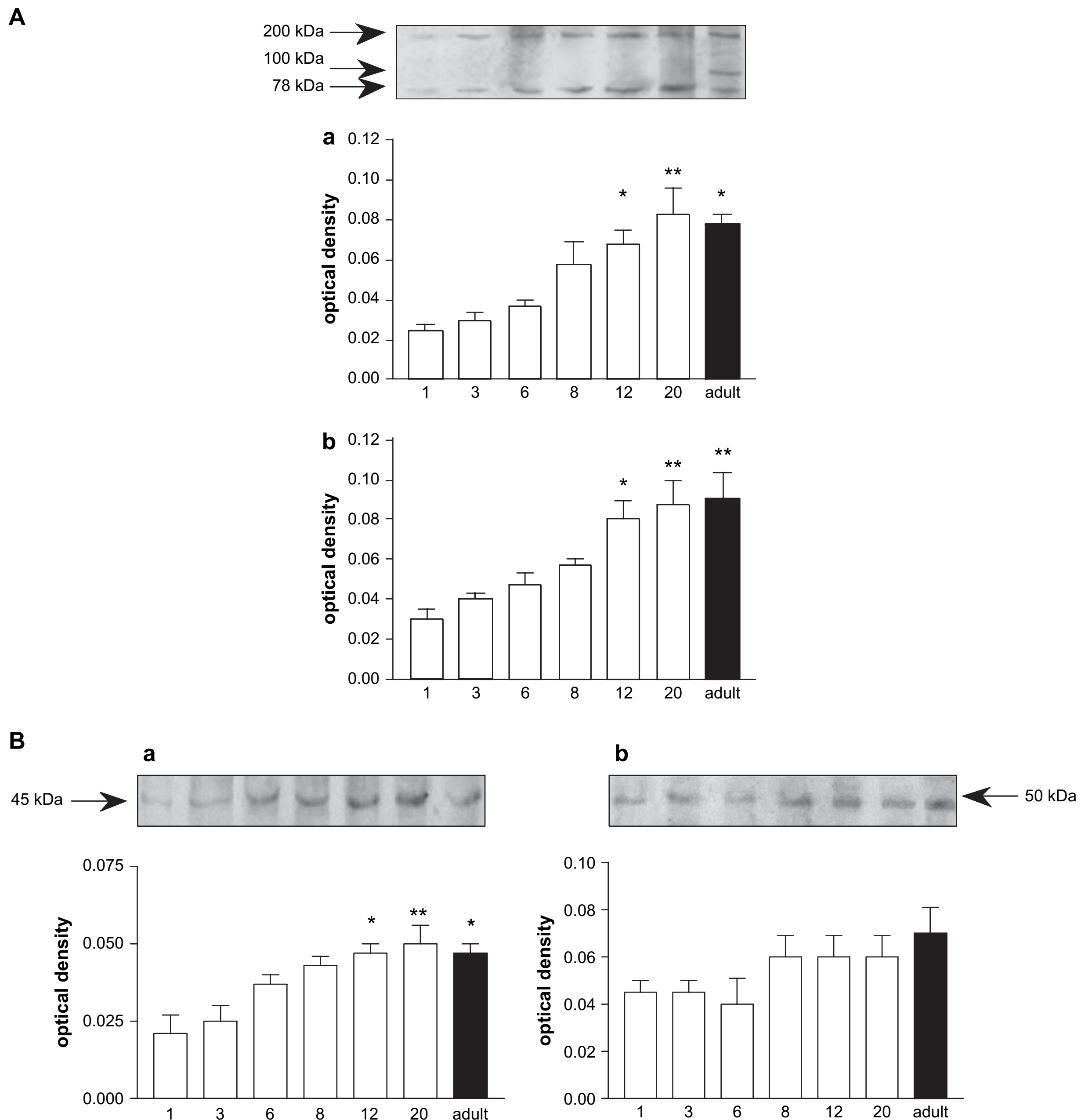

Fig. 7. (A) Western blot analyses of $P2Y_1$ receptors in the mouse colon at different days of postnatal development and in adults. Numbers at margin of blots indicate relative molecular weight of the protein in kDa. (a) Optical densities of 78 kDa and (b) 200 kDa bands on different days of postnatal development and in adult. Each column represents the mean of 4–6 experiments ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$ vs. day 1 by one-way ANOVA followed by Bonferroni's test. (B) Western blot analyses of (a) $P2X_1$ and (b) $P2X_2$ receptors in the mouse colon, at different days of postnatal development and in adult. Number at margin of blots indicates relative molecular weight of the proteins in kDa. Optical densities of respective bands on different postnatal days and in adult are shown below the Western blots. Each column represents the mean of 4–6 experiments ± S.E.M. $^*P < 0.05$ vs. day 1 by one-way ANOVA followed by Bonferroni's test.

The relevance of $P2X_3$ receptors in the modulation of neurotransmission in the mouse gut is confirmed in this study by the presence of immunoreactivity to $P2X_3$ receptors in subpopulations of myenteric neurons from postnatal day 1.

4.5. P1 receptors

The study of the pharmacological effects of ATP may be complicated by its breakdown by ectonucleotidases to adenosine, which retains its own action on P1 receptors (Moody et al., 1984; Kadowaki et al., 2000). In the neonatal mouse gut, ATP-induced relaxation may be partly mediated via P1 receptor activation as indicated by the sensitivity of ATP effect to the P1 receptor antagonist, 8-*p*SPT. The ability of adenosine to relax the longitudinal muscle of the gut suggests that P1 receptors are functional during postnatal development. In the duodenum, ileum and colon, responses to adenosine increased with age, reaching responses comparable to that of the adult by day 8, suggesting that in the mouse gut responses to P1 receptor activation might appear earlier than those to P2 receptor

activation. In addition, potency values of adenosine were similar at all ages for each region examined. In the rat duodenum, adenosine-induced relaxation of longitudinal muscle from day 5 had a potency similar to that in the adult (Nicholls et al., 1990).

In conclusion, pharmacological, morphological and biochemical investigations have shown the presence of a dominant $P2Y_1$ receptor, mediating relaxation of the longitudinal muscle in the neonatal mouse gut partly through its action on NANC inhibitory neurons which release NO and ATP. In addition to $P2Y_1$ receptors located on smooth muscle, inhibitory α,β-meATP-sensitive P2Y receptors and $P2Y_2$ receptors are also present from the first two weeks after birth. Relaxation following metabolism of ATP to adenosine, acting via P1 receptors, has also been demonstrated. In the mouse GI tract, inhibitory responses after purine receptor activation increase with an increase in age and are fully developed by day 20, the weaning age. In addition, major changes involving $P2Y_1$ receptors in the ileum and colon occur between the second and the third week after birth; these are characterized by a shift from a contractile to a relaxation response and by changes in the levels and pattern of receptor expression. These changes occur 1 week before weaning from maternal milk to solid food. In addition, the presence of $P2X_2$ receptors mediating a contractile response, which persists from the neonatal age to adult in mouse colon, has been demonstrated.

Acknowledgements

The authors are very grateful to Dr H.Z. Ruan and Mr T. Robson for technical assistance with the immunohistochemical staining.

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