

Changes in purinergic signalling in developing and ageing rat tail artery: Importance for temperature control

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Received 14 June 2005; received in revised form 24 August 2005; accepted 30 August 2005

Abstract

This study aimed to examine the expression and function of P2 receptors of the rat tail and mesenteric arteries during maturation and ageing (4, 6 and 12 weeks, 8 and 24 months). Functional studies and receptor expression by immunohistochemistry revealed a heterogeneous phenotype of P2 receptor subtypes depending on artery age. The purinergic component of nerve-mediated responses in the tail artery was greater in younger animals; similarly responses to ATP and α, β -meATP and the expression of P2X₁ receptors decreased with age. Contractile responses to 2-MeSADP decreased with age, and were absent at 8 and 24 months; P2Y₁ receptor expression followed this pattern. UTP-induced contractions and P2Y₂ receptor expression also decreased with age. The mesenteric artery contracted to UTP, responses at 4 and 6 weeks were larger than at other ages although P2Y₂ receptor expression did not significantly differ with age. 2-MeSADP induced relaxation of the mesenteric artery, responses being greatest at 6 weeks and decreased thereafter, which was mimicked by the P2Y₁ receptor immunostaining. We speculate that the dramatic changes in expression of P2 receptors in the rat tail artery, compared to the mesenteric artery, during development and ageing are related to the role of the tail artery in temperature regulation.

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Keywords: Purinergic; P2X; P2Y; Ageing; Vessels; Signalling

1. Introduction

It is now recognised that extracellular purines (adenosine, adenosine diphosphate (ADP) and adenosine 5'-triphosphate (ATP)) and pyrimidines (uridine diphosphate (UDP) and uridine 5'-triphosphate (UTP)) are important extracellular signalling molecules that mediate diverse effects on many biological processes (see [Ralevic and Burnstock, 1998](#); [Burnstock and Knight, 2004](#)). Currently, seven mammalian P2X ionotropic ligand-gated ion channel receptors (P2X_{1–7}) and eight P2Y metabotropic G protein-coupled receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄) have been identified ([Burnstock, 2004](#)).

There is much evidence showing the existence of sympathetic purinergic co-transmission involving noradrenaline

(NA) and ATP in a variety of blood vessels, including the tail artery of the rat ([Sneddon and Burnstock, 1985](#)) and the mesenteric artery of the guinea pig and rat ([Ishikawa, 1985](#); [Sjöblom-Widfeldt, 1990](#)). The sympathetic nerves supplying different blood vessels release a variable ratio of NA:ATP, the purinergic component being relatively minor (~10%) in the rat tail artery ([Bao et al., 1993](#)), whereas in the rabbit splenic artery, nerve-mediated responses are largely purinergic ([Ren and Burnstock, 1997](#)). In the rabbit mesenteric artery ATP is the sole excitatory transmitter, although NA is released and contributes to feedback inhibition ([Ramme et al., 1987](#)).

P2 receptors are widely distributed in the cardiovascular system and are important in the regulation of vascular tone ([Boarder and Hourani, 1998](#)). The P2X₁ receptor is the principal P2X receptor subtype expressed on most vascular smooth muscles ([Valera et al., 1994](#); [Collo et al., 1996](#)) and [Bo and Burnstock \(1993\)](#) reported that the smooth muscle of the rat tail artery has a high density of P2X₁ receptors.

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P2X₂ receptor immunoreactivity has been visualised on smooth muscle cells of rat mesenteric, renal and pulmonary arteries, although at a lower density than P2X₁ (Hansen et al., 1999). In addition, P2X₄ receptors have also been visualised in rat aorta and vena cava as well as coronary, pulmonary, renal and femoral arteries (Soto et al., 1996; Nori et al., 1998).

Metabotropic P2Y receptors also participate in the control of vascular tone. At least four P2Y receptor subtypes mediate the vascular effects of extracellular nucleotides, namely P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors. P2Y₁ receptors are activated by the endogenous ligands ADP and ATP; in contrast P2Y₂, P2Y₄ and P2Y₆ receptors are activated by uridine nucleotides. UTP was found to be equipotent with ATP at evoking contraction of the tail artery (Evans and Kennedy, 1994), suggestive of two populations of P2 receptors (McLaren et al., 1998), the second population being G protein-coupled P2Y₂ and P2Y₄ receptors.

P2Y₁ receptors are distributed on both vascular smooth muscle and endothelium. When present on the endothelium they mediate vasodilatation via endothelial nitric oxide (NO; Moncada et al., 1991) and also by the generation of endothelium-dependent hyperpolarising factor (EDHF; Malmjö et al., 1999; Stanford et al., 2001). Smooth muscle P2Y₁ receptors also produce vasodilatation of a number of blood vessels, including the rabbit mesenteric artery (Mathieson and Burnstock, 1985; Burnstock and Warland, 1987) and vasoconstriction of others (Knight et al., 2003; Steinmetz et al., 2003). Similarly P2Y₂ receptors in the vasculature are either present on the endothelium, such as in the rat and golden hamster isolated mesenteric arterial beds (Ralevic and Burnstock, 1996a, b), where they mediate vasodilatation via NO synthesis and release, or on smooth muscle as in the rat and bovine middle cerebral artery (Miyagi et al., 1996) where they mediate contraction. In the rat tail and femoral arteries, UTP has been shown to produce vasoconstriction (Saijag et al., 1990).

Ageing produces changes in vascular smooth muscle cells and endothelial cells (Wei, 1992). These changes include thickening of the media and enlargement of the lumen diameter, increased stiffening and an accompanying reduction in arterial distensibility of larger vessels (see Moreau et al., 1998; Laurant et al., 2004). Generally, there is a reduced tendency for endothelium-dependent relaxation and endothelial release of NO with increasing age (Hynes and Duckles, 1987). Relaxations to histamine decline during ageing in the rat mesenteric artery (Moritoki et al., 1986), as do relaxations to ACh, partly due to reduced hyperpolarisation by EDHF (Marín, 1995). Endothelium-dependent relaxations to ATP and acetylcholine (ACh) are impaired in carotid arteries from old and hypertensive rats (Hongo et al., 1988). Endothelium-independent vasodilatations are also modified with age (Docherty, 1990; Marín, 1995).

There is a reduction during ageing in the maximum contractile responses induced by NA in rat, rabbit and guinea-pig aortae, dog and monkey mesenteric arteries, dog cerebral arteries and rat tail arteries (Fouda and Atkinson, 1986; Marín, 1995). In contrast, contractions to NA are increased with age in isolated dog iliac, carotid, renal and mesenteric arteries (Cox et al., 1976), ear artery from lambs and ewes (Wyse et al., 1977) and rat aorta (Olah and Rahwan, 1987). The

isolated rabbit aorta (Hayashi and Toda, 1978) and rat femoral and carotid arteries (Duckles et al., 1985) are unaffected by increasing age.

Considerable plasticity during development and ageing of the autonomic nervous system in terms of co-transmitter expression and function is apparent. Hormones, disease, surgery and trauma can also underlie plasticity in the autonomic nervous system (Burnstock, 1997). Changes in the structure of neurons and nerves (Cowen, 1993) and on adrenergic neurotransmission in the sympathetic nervous system occur with age. Neuron structure can change, as well as a reduction in neuron number and loss of nerve fibres. Dhall et al. (1986) showed developmental changes in perivascular noradrenergic and peptide-containing nerves in the guinea pig; noradrenergic nerve density peaked 4 weeks after birth whilst nerves containing VIP, CGRP and SP peaked at birth and declined thereafter in the mesenteric and carotid arteries.

The rat tail artery is important for both balance and thermoregulation. Body appendages, such as the tail, are important heat exchange sites where increases in blood flow cause heat dissipation. Skin blood flow results in heat loss from the surface and this is modified by a sympathetically mediated mechanism. The tail of the rat functions as a heat-loss organ since it lacks hair covering, has a large surface area to volume ratio and is highly vascularised with arteriovenous anastomoses (Gordon, 1990). Up to 20% of total body heat loss can occur via the tail.

The aim of this study was to examine the expression and function of P2 receptors in the rat tail and mesenteric arteries during maturation and ageing. Rats attain sexual maturity at 2–3 months of age; they show rapid growth up to 8–9 months, which becomes stable by about 12 months. Twenty-four months is a commonly accepted age for senescence in rats (Weihe, 1987). For this study young rats of 4 and 6 weeks old were used to show any changes occurring in the early life of a rat, 12-week-old rats were used to reflect any changes as sexual maturity was reached, 8-month-old adult rats whose rapid growth was beginning to slow down and old (senescent) rats aged 24 months were used.

2. Methods

2.1. General procedures

Male Sprague–Dawley rats, aged 4, 6 and 12 weeks, 8 and 24 months, were killed according to Home Office (UK) regulations covering Schedule one procedures—by asphyxiation with CO₂ and subsequent cervical dislocation to confirm death. The tail and mesenteric arteries were dissected free, carefully cleaned of adhering fat and excessive connective tissue. The arteries were cut into rings approximately 4 mm in length and mounted horizontally for tension recording by inserting two tungsten wires through the lumen of the vessels; one was attached to a rigid support and the other to a Grass FT03C force-displacement transducer. Each arterial segment was suspended in a 10-ml organ bath containing modified Krebs solution of the following composition (mM): NaCl, 133; KCl, 4.7; NaHCO₃, 16.4; MgSO₄, 0.6; NaH₂PO₄, 1.4; glucose, 7.7 and CaCl₂, 2.5; pH 7.3. The solution was continuously gassed with 95% O₂, 5% CO₂ maintained at 37 ± 1 °C. Separate ring preparations of tail artery were used to study the effect of electrical field stimulation (EFS) and the action of exogenously applied agonists. Control frequency- and concentration–response curves were carried out to ensure reproducibility of responses.

EFS of the tail artery was applied via a pair of platinum electrodes 6 mm in length positioned either side of the preparation. The software PowerLab Chart for Windows (version 4; ADInstruments, Australia) was used to record mechanical activity. Preparations of tail and mesenteric artery were preloaded with 1 g tension and allowed to equilibrate for at least 60 min prior to the start of the experiment. The contraction due to a standard concentration of KCl (120 mM) was noted for each preparation at the end of each experiment.

2.2. Frequency–response curves

Control frequency–response curves to EFS were constructed for tail artery rings (80 V, 0.1 ms, 1–64 Hz, 1 s stimulation every 5 min) from each age group. The frequency–response curves were repeated in the presence of prazosin (1 μ M) to isolate the purinergic component, or in the presence of suramin (100 μ M) to isolate the noradrenergic component of nerve-mediated responses. The curves were then repeated in the presence of both prazosin (1 μ M) and suramin (100 μ M) and finally in the presence of tetrodotoxin (TTX; 1 μ M) to identify if any part of the response was due to direct smooth muscle stimulation.

2.3. Concentration–response curves

Cumulative concentration–response curves were constructed for NA (0.01 μ M–1 mM) on tail and mesenteric arteries. EC₅₀ concentrations were determined for each vessel from each age group and pD₂ values calculated (–Log EC₅₀).

Non-cumulative concentration–response curves were constructed for ATP (0.1 μ M–1 mM) and α , β -meATP (0.01 μ M–100 μ M) on tail arteries and the time interval between applications of ATP and α , β -meATP was 15–20 min with repeated washouts to avoid desensitisation. Cumulative concentration–response curves were also constructed for 2-MeSADP (10 μ M–100 μ M) on the tail artery and for UTP in both the tail and mesenteric arteries (0.01 μ M–1 mM, tail artery; 0.1 μ M–1 mM, mesenteric artery).

The NA EC₅₀ concentration from each tail and mesenteric artery from each age group was used to induce tone. ACh was added cumulatively (0.01 μ M–1 mM) to pre-constricted vessels to determine the integrity of the endothelium. On raised tone preparations of tail and mesenteric artery cumulative concentration–response curves to 2-MeSADP (0.01 μ M–100 μ M) were constructed. The response to a single concentration of SP (3 μ M) was examined on raised tone preparations of mesenteric arteries.

In order to determine whether P2Y₁ receptors were located on the endothelium or smooth muscle of the mesenteric artery, concentration–response curves to 2-MeSADP were repeated in the presence of L-NAME (100 μ M) in vessels from 6-week-old animals.

2.4. Immunohistochemistry

Sections of tail and mesenteric arteries from each age group were embedded unfixed in Tissue-Tek and rapidly frozen by immersion in isopentane (pre-cooled in liquid nitrogen) at –70 °C for 2 min. Sections (12 μ m) of tail artery and mesenteric artery were cut using a cryostat (Reichert Jung CM1800), thaw-mounted on gelatine-coated slides and air-dried at room temperature. The avidin–biotin complex (ABC) technique was used according to the protocol described by Llewellyn-Smith et al. (1993).

The sections were fixed in 4% formaldehyde (0.1 M phosphate buffer, pH 7.4) containing 0.2% picric acid for 2 min. Endogenous peroxidase activity was blocked with 50% methanol containing 0.4% hydrogen peroxide (H₂O₂) for 10 min. Non-specific binding sites were blocked by a 20-min incubation with normal horse serum (NHS) in phosphate-buffered saline (PBS) containing 0.05% merthiolate. The sections were incubated with the primary antibodies (P2X_{1–7} primary antibodies were kindly donated by Roche (Roche Palo Alto, CA, USA); P2Y primary antibodies were obtained from Alomone Labs, Jerusalem, Israel) in a humidified chamber overnight with 10% NHS in PBS containing 0.05% merthiolate and 0.2% Triton. The P2Y₄ primary antibody was used at a dilution of 1:100; the P2X₁, P2X₄, P2X₇, P2Y₁ and P2Y₂ primary antibodies were used at dilutions of 1:200; the P2X₂, P2X₃, P2X₅ and P2X₆ primary antibodies were used at dilutions of 1:400.

Subsequently the sections were incubated with the secondary antibody, biotinylated donkey anti-rabbit immunoglobulin G (IgG), at a dilution of 1:500 in 1% NHS in PBS containing 0.05% merthiolate for 1 h. This was followed by incubation with ExtrAvidin-horseradish peroxidase used at 1:1000 in PBS containing 0.05% merthiolate for 30 min. All incubations were carried out at room temperature and separated by 3 \times 5-min washes in PBS (except after incubation with 50% methanol containing 0.4% hydrogen peroxide, which was followed by 3 \times 2-min washes in PBS). Finally a nickel-diaminobenzide enhancement technique was used to visualise the reaction product as follows. A colour reaction solution containing 0.05% 3,3'-diaminobenzide (DAB), 0.04% nickel ammonium sulphate, 0.1 M sodium phosphate, 0.004% NH₄Cl, 0.2% glucose and 0.1% glucose oxidase was applied to the sections and left to react for 5 min. The sections were then washed, dehydrated with iso-propanol and mounted with Eukitt (BDH, Poole, UK), and examined with a light microscope. Omission of the primary antibody or preabsorption of the primary antibody with the relative P2X or P2Y receptor peptides was performed as a control to establish a specific immunoreaction. Photographs of DAB immunohistochemical staining were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Pictures were processed using a graphics package (Adobe-Photoshop 5.5 software on an Apple Power-Macintosh G3) and prints were made with an Epson Stylus Photo 700 printer.

The level of immunoreactivity was estimated and assigned to the following categories: + + +, strong expression; + +, moderate expression; +, weak expression; \pm , barely detectable expression; –, no expression.

2.5. Drugs used

ACh, α , β -meATP (lithium salt), ATP, DAB, ExtrAvidin-horseradish peroxidase, glucose oxidase, H₂O₂, L-NAME, 2-MeSADP, NA, prazosin, saturated picric acid solution, SP, suramin, merthiolate, TTX and UTP were obtained from Sigma Chemical Co. (Poole, UK). NHS was obtained from Gibco; formaldehyde stabilised with 10% methanol was obtained from Analar, BDH; biotinylated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch, PA, USA.

All drugs were prepared in distilled water, except NA that was dissolved in ascorbic acid (0.1 mM). The volume added to the organ bath to produce the final concentration was not in excess of 100 μ l. All antagonists were incubated for 20 min.

2.6. Statistical analysis

Responses to EFS of the tail artery from each age group are expressed as mean % contraction of the maximum response \pm S.E.M. (*n*). Contractile responses to ATP, α , β -meATP, UTP, 2-MeSADP, NA, KCl and the maximum contraction to NS (in the absence of any blocking agents) are expressed as tension developed in mg \pm S.E.M. (*n*). Vasodilator responses to ACh, SP and 2-MeSADP are expressed as mean % relaxation of the NA-induced contraction \pm S.E.M. (*n*).

Statistical significance of frequency–response curves and concentration–response curves was tested by a two-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) followed by a post hoc test (Tukey's), to test if the curves from different age groups were significantly different from each other. Unless otherwise stated significance between consecutive ages is quoted. The KCl contractions and the pD₂ values for NA and ACh were compared using a one-way ANOVA followed by a post hoc (Bonferroni's) test. A probability of *P* < 0.05 was considered significant for each ANOVA test.

3. Results

3.1. Responses to nerve stimulation in the tail artery

EFS of the tail arteries from each age group induced sympathetic nerve-mediated, frequency-dependent contractions

that were TTX (1 μM) sensitive. In the presence of suramin (100 μM) alone (Fig. 1a–e), contractile responses induced by EFS in all age groups were significantly inhibited ($P < 0.05$ or less). When the frequency–response curves were repeated in the presence of suramin and prazosin (1 μM) contractions were almost completely inhibited for each age group ($P < 0.001$). Conversely, when the curves were carried out in the presence of prazosin (1 μM) alone the contractile responses was also significantly reduced ($P < 0.001$) in vessels from all age groups (Fig. 2a–e). When the frequency–response curves were repeated in the presence of both antagonists, contractions were almost completely inhibited for each age group ($P < 0.001$).

The maximum contractions developed, as measured in mg, by each age group, in the absence of any blocking agents are shown in Table 1. The maximum contraction developed by vessels from 4-week-old animals was significantly less ($P < 0.05$) than the other age groups.

Differences in the proportions of the purinergic and noradrenergic components were observed between the various age groups. The proportion of the purinergic (suramin-sensitive) component of nerve-mediated responses in the tail artery decreased significantly with age (Fig. 3a). Conversely, the proportion of the noradrenergic (prazosin-sensitive) component of nerve-mediated responses in the tail artery increased significantly with age (Fig. 3b).

3.2. Responses to exogenously applied agonists in the tail artery

NA (0.01 μM –1 mM) produced concentration-dependent, reproducible contractions of the isolated rat tail artery and concentration–response curves were constructed (Fig. 4a). Responses to NA from the 24-month-old animals were greater than those from the other age groups that did not differ with age. pD_2 values ($-\log \text{EC}_{50}$ concentration) were calculated and are shown in Table 1. Following a one-way ANOVA, the pD_2 value for vessels from 4-week-old rats was less than that for 24-month-old vessels ($P < 0.01$).

ATP (0.1 μM –1 mM) and α, β -meATP (0.01 μM –100 μM) both induced concentration-dependent, transient contractions of the rat tail artery. Concentration–response curves were constructed for both agonists. The order of potency for ATP (Fig. 4b) and α, β -meATP (Fig. 4c) was: 4 weeks > 6 weeks = 12 weeks = 8 months > 24 months following a two-way ANOVA ($P < 0.05$ or less).

2-MeSADP (10 μM –100 μM) produced concentration-dependent contractions of the rat tail artery and concentration–response curves were constructed (Fig. 4d). In the younger animals (4 weeks and 6 weeks) contractions were not maintained and it was necessary to apply 2-MeSADP non-cumulatively. The order of potency was: 4 weeks > 6 weeks > 12 weeks following a two-way ANOVA ($P < 0.001$). 2-MeSADP failed to induce contraction in tail arteries from 8- and 24-month-old animals.

UTP (0.01 μM –1 mM) elicited concentration-dependent contractions of the rat tail artery from which concentration–response curves were derived (Fig. 4e). The order of potency

for UTP was: 4 weeks > 6 weeks > 12 weeks = 8 months > 24 months following a two-way ANOVA ($P < 0.01$ or less).

On raised tone (NA, EC_{50} concentration) preparations of tail artery, ACh (0.01 μM –1 mM) induced concentration-dependent relaxations in all age groups examined with the exception of vessels from 4-week-old animals. This was due to the small size of the vessels. The maximum relaxations to ACh are given in Table 1.

The contractile capacity of the rat tail artery from each age group was examined by the addition of KCl (120 mM) (see Table 1). The contraction from tail arteries from 4-week-old animals was significantly less ($P < 0.001$) than that induced in the 12-week, 8- and 24-month-old animals.

3.3. Responses to exogenously applied agonists in the mesenteric artery

NA (0.01 μM –1 mM) induced concentration-dependent contractions of the isolated rat mesenteric artery and concentration–response curves were constructed (Fig. 5a). Responses to NA were greater in 8-month mesenteric arteries than the other age groups following a two-way ANOVA ($P < 0.001$). pD_2 values were calculated and are shown in Table 1. There was no significant difference in the pD_2 values for any of the age groups tested.

UTP (0.1 μM –1 mM) induced concentration-dependent contractions of the rat mesenteric artery and concentration–response curves were constructed (Fig. 5b). The order of potency for UTP was: 4 weeks = 6 weeks > 12 weeks = 8 months > 24 months, when compared using a two-way ANOVA test ($P < 0.05$ or less).

On raised tone (NA, EC_{50} concentration) preparations of mesenteric artery, 2-MeSADP (0.01 μM –100 μM) induced concentration-dependent relaxations of vessels from each age group tested (Fig. 5c) with the following order of potency: 6 weeks > 4 weeks = 12 weeks > 8 months > 24 months, following a two-way ANOVA ($P < 0.001$).

In the presence of L-NAME (100 μM), relaxations to 2-MeSADP in the mesenteric artery from 6-week-old animals were not significantly different from relaxations induced by 2-MeSADP in the absence of L-NAME (100 μM) (data not shown).

A single concentration of SP (3 μM) induced vasodilatation of pre-constricted (NA, EC_{50} concentration) mesenteric arteries at each age group tested with the exception of 24-month-old animals where SP failed to induce relaxation (see Table 1). The order of potency for SP was: 4 weeks > 6 weeks > 12 weeks > 8 months following a one-way ANOVA ($P < 0.05$).

On raised tone preparations (NA, EC_{50} concentration), ACh (1 nM–1 mM) induced concentration-dependent relaxations (Fig. 5d). The order of potency for ACh was: 6 weeks = 4 weeks > 12 weeks > 8 months > 24 months. Significance was tested by a two-way ANOVA ($P < 0.01$ or less).

KCl (120 mM) induced contractile responses in each age group (see Table 1). The contractions produced in mesenteric arteries from 4-week-old animals were significantly smaller ($P < 0.05$) than those from 8- and 24-month-old animals.

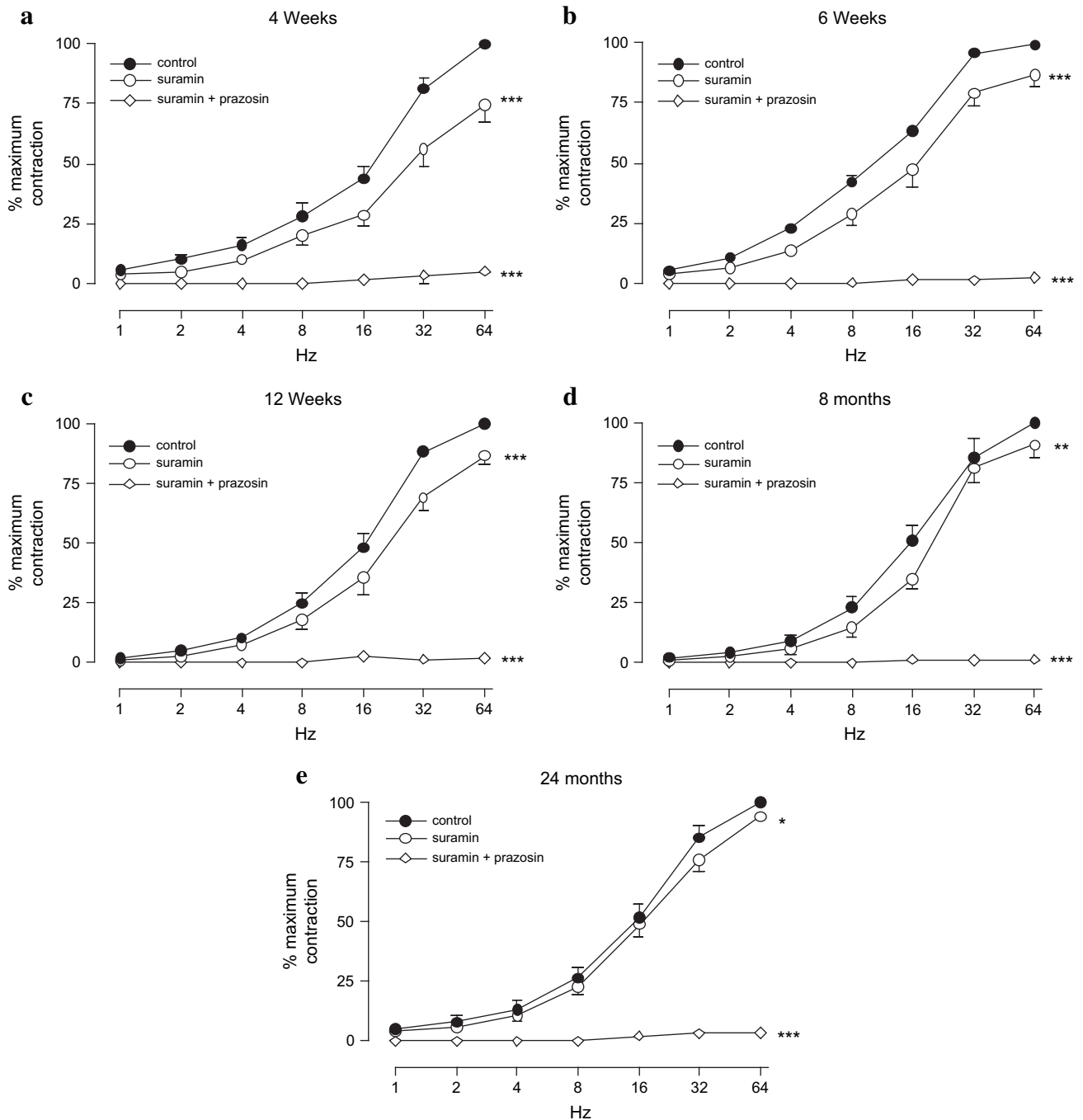


Fig. 1. Frequency–response curves (80 V, 0.1 ms, 1–64 Hz, 1 s stimulation every 5 min) in the absence (control) and presence of suramin (100 μ M), then in the presence of both suramin (100 μ M) and prazosin (1 μ M) on the rat tail artery of vessels of different age. (a) Four-week-old animals ($n = 5$); (b) 6-week-old animals ($n = 5$); (c) 12-week-old animals ($n = 8$); (d) 8-month-old animals ($n = 5$) and (e) 24-month-old animals ($n = 5$). All data are mean \pm S.E.M. (n), expressed as % of the maximum contraction. Statistical significance was tested by 2-way analysis of variance, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.4. Immunohistochemistry

Immunohistochemistry was performed to investigate the pattern of P2X and P2Y receptor protein expression in rat tail and mesenteric arteries taken from animals aged 4, 6, 12 weeks, 8 and 24 months. The level of immunoreactivity for P2X and P2Y receptors was estimated for the tail and mesenteric arteries at different age groups, and is shown in Tables 2 and 3.

3.4.1. Tail artery

P2X₁ receptor specific immunoreactivity was associated with the smooth muscle layer in the rat tail artery. The results show an inverse relationship between staining intensity and animal age. Substantial P2X₁ receptor immunoreactivity was observed in arterial sections from the 4-week-old animals (Fig. 6a). Less intense staining was present in the 6-week arterial sections (Fig. 6c). Twelve-week sections showed weak

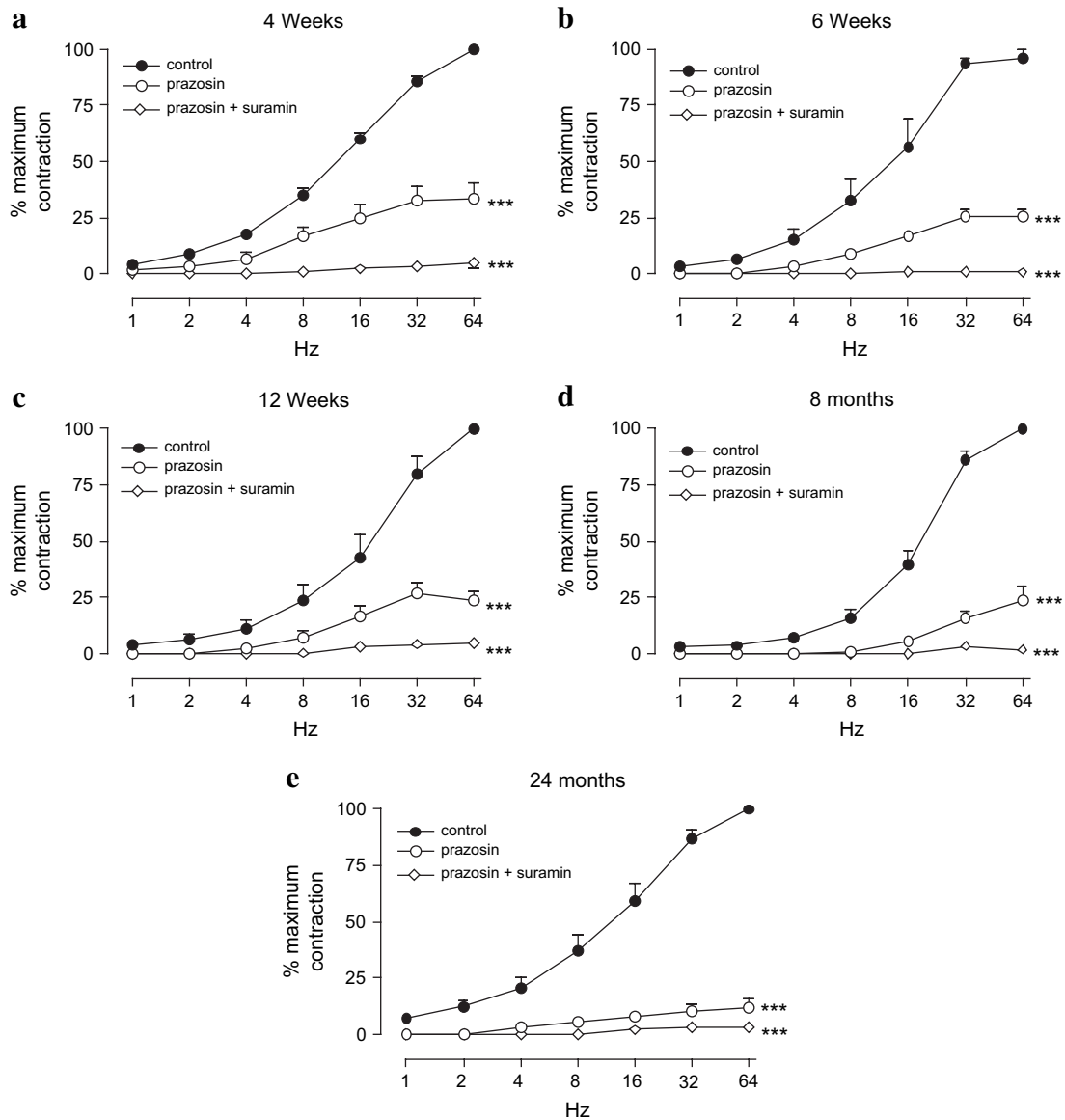


Fig. 2. Frequency–response curves (80 V, 0.1 ms, 1–64 Hz, 1 s stimulation every 5 min) in the absence (control) and presence of prazosin (1 μ M), then in the presence of both prazosin (1 μ M) and suramin (100 μ M) on the rat tail artery of vessels of different age. (a) Four-week-old animals ($n = 7$); (b) 6-week-old animals ($n = 5$); (c) 12-week-old animals ($n = 5$); (d) 8-month-old animals ($n = 5$) and (e) 24-month-old animals ($n = 5$). All data are mean \pm S.E.M. (n), expressed as % of the maximum contraction. Statistical significance was tested by 2-way analysis of variance, *** $P < 0.0001$.

P2X₁ immunoreactivity (Fig. 6e) whilst in the 8- and 24-month sections immunoreactivity was barely detectable (Fig. 6g,i).

Weak P2X₄ receptor specific immunoreactivity was found in the smooth muscle and moderate P2X₄ immunoreactivity in the endothelial layer of 4-week and 6-week tail arteries. P2X₄ immunoreactivity was barely detectable in the smooth muscle in the other age groups and absent from the endothelium in the other age groups (figure not included).

Immunoreactivity for P2X₂, P2X₃, P2X₅, P2X₆ and P2X₇ was not detected in the tail artery for any of the age groups.

P2Y₁ receptor specific immunoreactivity was associated with both the smooth muscle and endothelial layers of tail arteries. At 4 weeks there was very intense P2Y₁ immunoreactivity in the smooth muscle and endothelial layers (Fig. 7a). The intensity of staining declined with increasing age of the

vessel, so that at 6 weeks the staining was moderate in both layers (Fig. 7c), weak at 12 weeks in both layers (Fig. 7e), and undetectable in the smooth muscle and barely detectable in the endothelium at 8 months and undetectable in both layers at 24 months (Fig. 7g,i).

P2Y₂ receptor specific immunoreactivity was observed in the tail artery smooth muscle layer. The staining was strong at 4 weeks (Fig. 8a), moderate at 6 weeks (Fig. 8c) and 12 weeks (Fig. 8e), and declined slowly thereafter (Fig. 8g,i).

Some P2Y₄ receptor expression was also detected in the smooth muscle of the tail artery. Immunoreactivity was weak in 6- and 12-week vessels, and barely detectable in 4-week and 8- and 24-month vessels (figure not included).

Staining was absent if the primary antibody was omitted from the staining protocol. These controls were used as

Table 1
pD₂ values and maximum responses for agonists on the rat tail and mesenteric arteries from animals of different age

Tail artery	4 weeks	6 weeks	12 weeks	8 months	24 months
NS maximum contraction (mg)	359.7 ± 40.2 (10)	689.2 ± 61.1 (10)	801.4 ± 92.4 (11)	772.5 ± 64.4 (10)	781.7 ± 85.4 (10)
NA pD ₂	5.73 ± 0.06 (6)	5.57 ± 0.11 (5)	5.48 ± 0.15 (5)	5.38 ± 0.20 (6)	5.17 ± 0.07 (5)
ACh maximum relaxation (%) (endothelium-dependent)	No relaxation	29.0 ± 3.3 (3)	35.5 ± 3.5 (4)	23.7 ± 4.1 (6)	22.4 ± 3.3 (6)
KCl contraction (g)	0.46 ± 0.03 (14)	0.65 ± 0.06 (8)	0.69 ± 0.06 (14)	0.74 ± 0.08 (9)	0.73 ± 0.08 (5)
<i>Mesenteric artery</i>					
NA pD ₂	5.92 ± 0.16 (5)	5.53 ± 0.06 (6)	5.66 ± 0.12 (6)	5.58 ± 0.17 (7)	5.38 ± 0.11 (5)
SP % Relaxation (endothelium-independent)	48.9 ± 5.0 (6)	34.1 ± 3.3 (5)	21.8 ± 1.7 (5)	8.2 ± 1.4 (7)	No relaxation
KCl contraction (g)	0.49 ± 0.06 (9)	0.65 ± 0.05 (8)	0.64 ± 0.05 (8)	0.93 ± 0.12 (9)	0.86 ± 0.09 (5)

Data expressed as mean ± S.E.M. (number of animals).

comparison for P2X- and P2Y-specific immunoreactivity (see Figs. 6b,d,f,h,j, 7b,d,f,h,j and 8b,d,f,h,j).

3.4.2. Mesenteric artery

Moderate P2X₁ receptor-specific immunoreactivity was associated with the smooth muscle layer of mesenteric arteries from rats of all ages studied, with the exception of arteries from the 24-month-old rats where staining was less intense. P2X₄ was weakly expressed in the smooth muscle layer of 4-week, 12-week and 8-month arteries and moderately expressed in the smooth muscle layer of 6-week arteries. There was also a very low level of P2X₄ expression in the endothelium of 12-week and 8- and 24-month vessels. Some P2X₅

receptor immunoreactivity was detected in the smooth muscle, weakly in 4-week vessels but barely detectable in the remaining four groups (figures not included). P2X₂, P2X₃, P2X₆ and P2X₇ receptor immunoreactivity was not detected.

P2Y₁ receptor immunoreactivity was detected in the smooth muscle and endothelial layers of the rat mesenteric artery. Expression was moderate in the smooth muscle and weak in the endothelium of 4-week arteries (Fig. 9a). Immunoreactivity was strong in the smooth muscle and moderate in the endothelium of 6-week-old vessels (Fig. 9c). Weak staining in the smooth muscle and endothelium was seen in vessels from 12-week (Fig. 9e) and 8-month-old rats (Fig. 9g), while staining was barely detectable in vessels from 24-month-old rats (Fig. 9i).

Moderate P2Y₂ receptor immunoreactivity was present in the smooth muscle and endothelium of the rat mesenteric artery. There was no observable difference in the staining intensity between the various age groups (Fig. 10a,c,e,g,i) although staining of the endothelium was more pronounced in vessels from the 8- and 24-month-old rats.

Weak P2Y₄ immunoreactivity was present in the smooth muscle layer of the rat mesenteric artery of all age groups, except for vessels from 24-month-old rats, where the staining was barely detectable.

P2X and P2Y receptor staining was absent if the primary antibody was omitted from the staining protocol. These controls were used as comparisons (Figs. 9b,d,f,h,j and 10b,d,f,h,j).

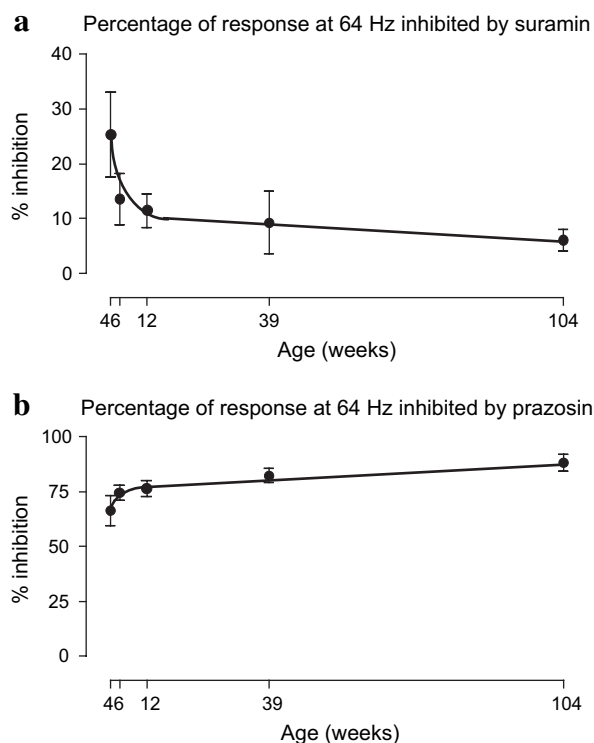


Fig. 3. Figure showing the percentage of the response to electrical field stimulation of the rat tail artery at 64 Hz inhibited by (a) suramin (100 μM; n = 5–8) and (b) prazosin (1 μM; n = 5–7) at different ages. Note the percentage of the response at 64 Hz inhibited by suramin decreases with age, while the percentage inhibited by prazosin increases with age.

4. Discussion

This study has shown that the function and expression of P2X and P2Y receptors of the rat tail and mesenteric artery alter with maturation and age. In general the trend is for the sensitivity and expression of P2X and P2Y receptors to decrease as the age of the animal increases. However, there were significant differences between the two vessels examined that include transient maturational increases in the expression of selected receptors in the mesenteric artery.

Bao et al. (1993) previously showed that the purinergic component contributed about 10% to the EFS-induced contractile response of the rat tail artery, and this was confirmed in the present study. The relative proportions of the purinergic

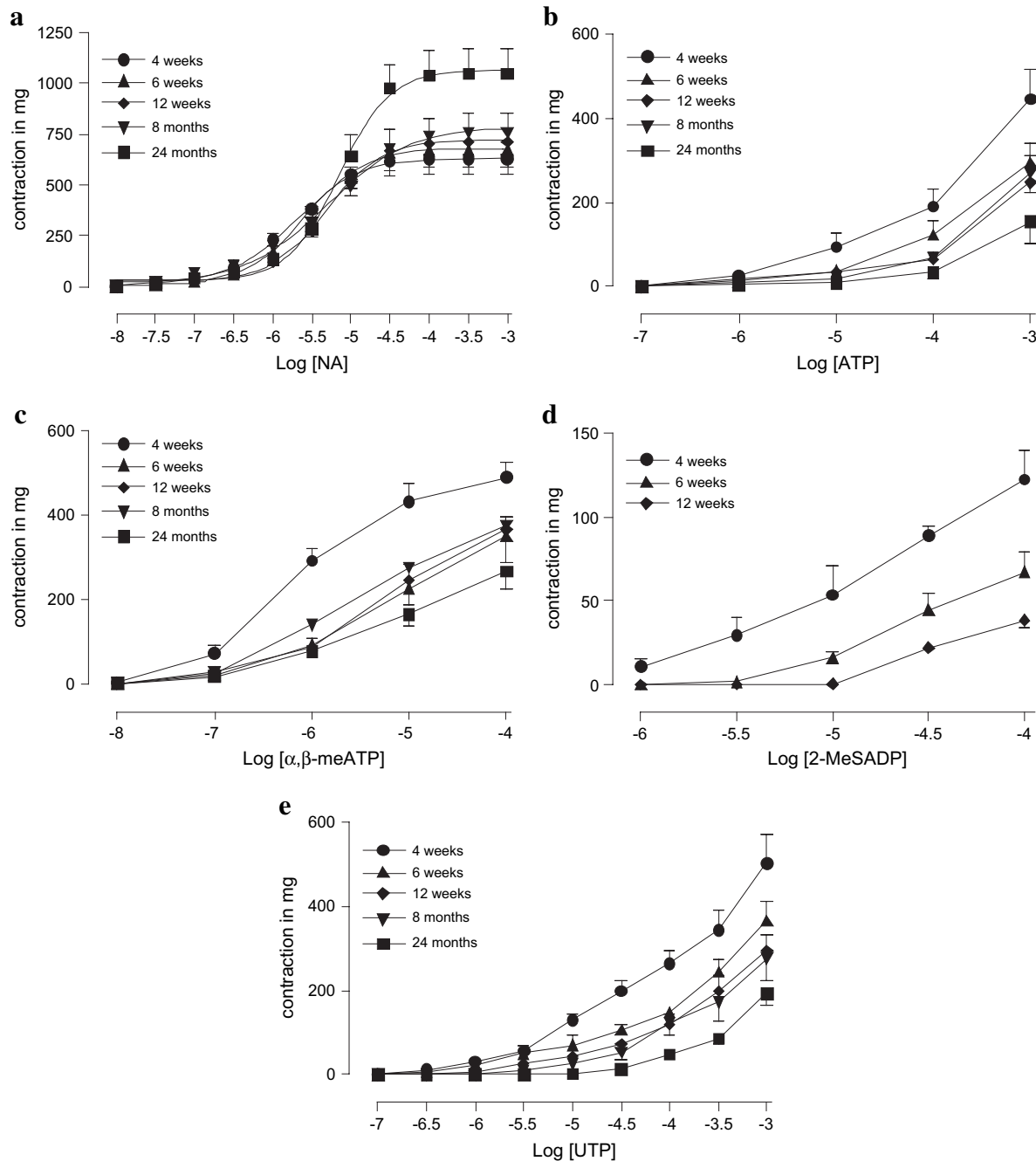


Fig. 4. Concentration–response curves for exogenously applied agonists on the rat tail artery from different aged animals. (a) Cumulative concentration–response curves for NA (10 nM–1 mM; $n = 5-6$). (b) Non-cumulative concentration–response curves for ATP (0.1 μ M–1 mM; $n = 5-6$). (c) Non-cumulative concentration–response curves to α,β -meATP (10 nM–100 μ M; $n = 5-6$). (d) Non-cumulative (4 and 6 weeks) and cumulative (12 weeks) concentration–response curves to 2-MeSADP (1–100 μ M; $n = 5$). (e) Cumulative concentration–response curves for UTP (0.1 μ M–1 mM; $n = 5-6$). All data are mean \pm S.E.M. (n) expressed as mg tension developed.

and non-purinergeric components varied with age; the purinergeric component was largest at 4 weeks ($\sim 25\%$), decreasing in size as the vessel aged ($\sim 10\%$ in 8-month vessels and $< 10\%$ in 24-month vessels).

In the present study there was little change in the responses to NA with age, although there are many examples in the literature that show increased NA responses in older vessels, such as in the dog iliac, carotid, renal and mesenteric (Cox et al., 1976), sheep ear arteries (Wyse et al., 1977) and

rat aorta (McAdams and Waterfall, 1986; Olah and Rahwan, 1987). The greater maximal contraction to NA after 6 weeks and the increase in the noradrenergic component of nerve-mediated responses seems to imply that there is an increased sensitivity of α -adrenoceptors, and not an alteration in the ability of the older vessels to constrict since the contractions to KCl, while smaller in the younger animals (4 and 6 weeks old) were consistent in the older animals (12 weeks and older). Unaltered KCl contraction with increasing age has

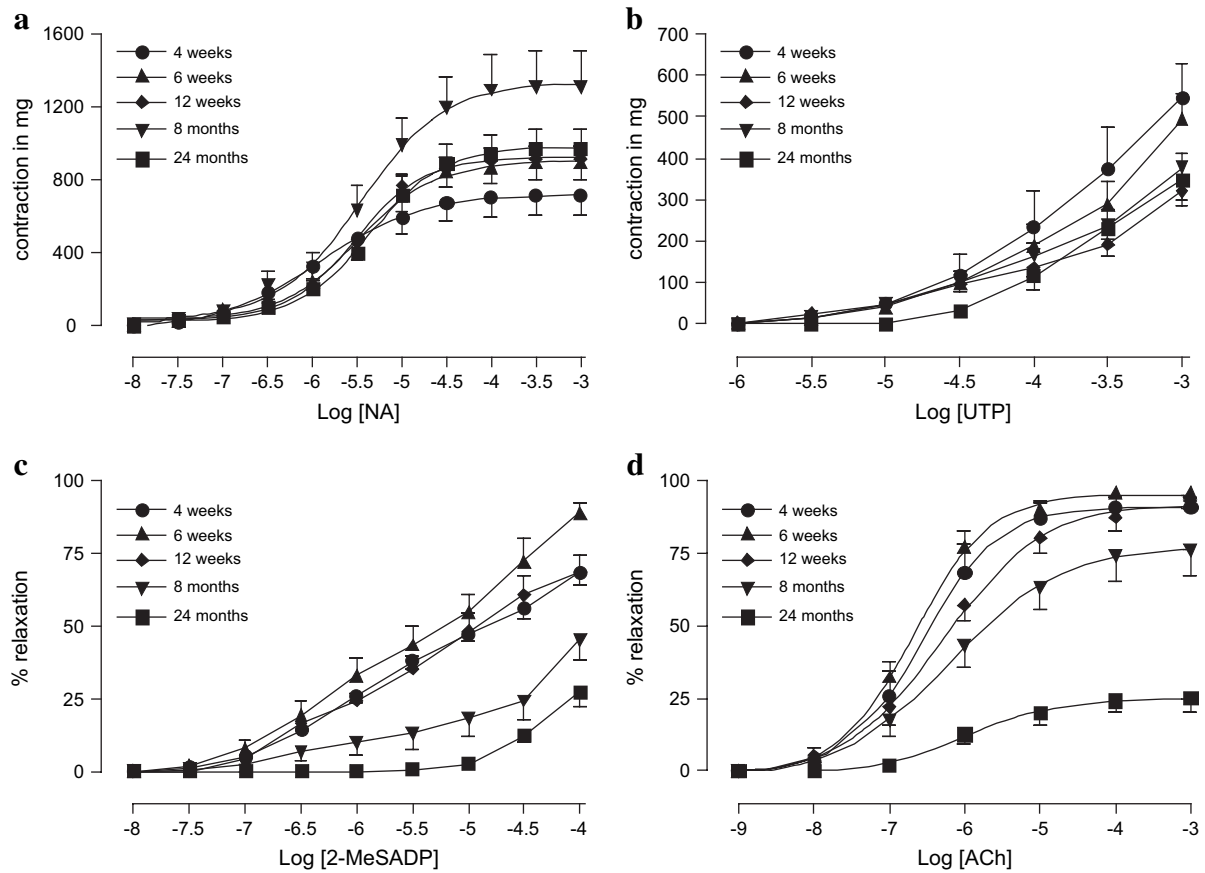


Fig. 5. Concentration–response curves for exogenously applied agonists on the rat mesenteric artery from different aged animals. (a) Cumulative concentration–response curves for NA (10 nM–1 mM; $n = 5–7$). (b) Cumulative concentration–response curves for UTP (1 μ M–1 mM; $n = 5–6$). (c) Cumulative concentration–response curves to 2-MeSADP (10 nM–100 μ M; $n = 5–6$). (d) Cumulative concentration–response curves for ACh (1 nM–1 mM; $n = 5–7$). All data are mean \pm S.E.M. (n) expressed as mg tension developed for NA and UTP and percentage relaxation of NA (EC_{50} concentration) pre-constricted vessels for 2-MeSADP and ACh.

been previously reported for the rat aorta (Wanstall and O’Donnell, 1989) and rabbit basilar and mesenteric arteries (Stewart-Lee and Burnstock, 1991; Brizzolara et al., 1994).

The P2X₁ receptor is the principle P2X receptor subtype expressed on smooth muscle of most blood vessels (Valera et al., 1994; Collo et al., 1996) and both ATP and α,β -meATP produced concentration-dependent, transient contractions of

the rat tail artery, α,β -meATP being more potent than ATP. This agonist potency profile suggests the presence of P2X₁ or P2X₃ receptors; however, immunohistochemical staining demonstrated only P2X₁ receptor expression, precluding the latter. There was a marked difference in sensitivity to ATP and α,β -meATP depending on the age of the vessel. The potency of response to both agonists was greatest in the

Table 2
Immunostaining of P2X and P2Y receptors on sections of tail artery from rats 4, 6 and 12 weeks, 8 and 24 months old

	4 weeks		6 weeks		12 weeks		8 months		24 months	
	SM	END	SM	END	SM	END	SM	END	SM	END
P2X ₁	+++	–	++	–	+	–	±	–	±	–
P2X ₂	–	–	–	–	–	–	–	–	–	–
P2X ₃	–	–	–	–	–	–	–	–	–	–
P2X ₄	+	++	+	++	±	–	±	–	±	–
P2X ₅	–	–	–	–	–	–	–	–	–	–
P2X ₆	–	–	–	–	–	–	–	–	–	–
P2X ₇	–	–	–	–	–	–	–	–	–	–
P2Y ₁	+++	+++	++	++	+	+	–	±	–	–
P2Y ₂	+++	–	++	–	++	–	+	–	±	–
P2Y ₄	±	–	+	–	+	–	±	–	±	–

SM, smooth muscle; END, endothelium. +++, strong expression; ++, moderate expression; +, weak expression; ±, barely detectable expression; –, no expression.

Table 3
Immunostaining of P2X and P2Y receptors on sections of mesenteric artery from rats 4, 6 and 12 weeks, 8 and 24 months old

	4 weeks		6 weeks		12 weeks		8 months		24 months	
	SM	END	SM	END	SM	END	SM	END	SM	END
P2X ₁	++	–	++	–	++	–	++	–	+	–
P2X ₂	–	–	–	–	–	–	–	–	–	–
P2X ₃	–	–	–	–	–	–	–	–	–	–
P2X ₄	+	–	+++	–	+	±	+	±	+	±
P2X ₅	+	–	±	–	±	–	±	–	±	–
P2X ₆	–	–	–	–	–	–	–	–	–	–
P2X ₇	–	–	–	–	–	–	–	–	–	–
P2Y ₁	++	+	+++	++	+	+	+	+	+	+
P2Y ₂	++	+	++	+	++	+	++	++	++	++
P2Y ₄	+	–	+	–	+	–	+	–	±	–

SM, smooth muscle; END, endothelium. + + +, strong expression; ++, moderate expression; +, weak expression; ±, barely detectable expression; –, no expression.

4-week vessels and their potency decreased with increasing age. This supports and extends the earlier finding that the purinergic component of the responses to nerve stimulation in the rat tail artery decreased with age, that has also been shown previously (Bao et al., 1989), which implies that the effect is post-junctional rather than an effect on transmitter release. The nerve stimulation and pharmacological investigations suggest alterations either in sensitivity of the P2X₁ receptor to the effect of agonists, or in receptor expression. Our immunohistochemical results suggest the latter. A similar reduction in P2X₁ receptor mRNA expression with ageing has been demonstrated in the rat basilar artery (Miao et al., 2001). A reduction in responses to ATP and α,β -meATP with age has also been reported for the rat mesenteric artery (Konishi et al., 1999).

The contractile activity of 2-MeSADP, a P2Y₁ receptor agonist, suggests the presence of P2Y₁ receptors on the tail artery. Immunohistochemical staining confirms the presence of P2Y₁ receptors on both smooth muscle and endothelium. The potency of 2-MeSADP was greatest in 4-week vessels and decreased with age, with a failure to induce contractions in the 8- and 24-month vessels. This is probably due to the absence of P2Y₁ receptors on smooth muscle from the tail arteries of the older animals as demonstrated by the immunohistochemical investigations. P2Y₁ mRNA expression was also found to decrease with age in the rat basilar artery (Miao et al., 2001). The presence of P2Y₁ receptors mediating vasoconstriction is by no means unusual. Human small renal resistance arteries constrict in the presence of exogenous ADP (Steinmetz et al., 2003) and the rat renal artery exhibits a P2Y₁ receptor-mediated constriction (Knight et al., 2003). 2-MeSADP is also an agonist at the P2Y₁₂ receptor, and a report has been published where 2-MeSADP acting via P2Y₁₂ receptors induced contraction of the human internal mammary artery (Wihlborg et al., 2004). Although there is immunohistochemical evidence that 2-MeSADP is acting via P2Y₁ receptors in this study, that 2-MeSADP is also acting via P2Y₁₂ receptors cannot be discounted at this time.

UTP also induced contractions of the rat tail artery via P2Y₂ receptors, the presence of which has been shown previously on the smooth muscle of the rat tail and femoral arteries

(Saiag et al., 1990) and in the mesenteric beds of rats and hamsters (Ralevic and Burnstock, 1996a, b). In the present study, UTP potency, like that of 2-MeSADP, was greater in 4-week tail arteries and decreased with age up to 12 weeks. After this age, potency remained stable until 8 months and then decreased in the 24-month vessels; this pattern of reduced responsiveness was matched by changes in the immunohistochemical staining for P2Y₂ receptors.

The effect of maturation and ageing on P2Y receptor function and expression was also investigated on the mesenteric artery of the rat. The presence of an intact endothelium in the mesenteric arteries was confirmed, since application of ACh to pre-constricted vessels induced concentration-dependent relaxation. Interestingly, ACh was most potent in the younger two age groups and then potency decreased with age. A reduction in relaxations induced by ACh in the rabbit aorta with ageing has been reported (Ragazzi et al., 1995) and both ageing and hypertension resulted in diminished endothelium-dependent relaxations in an *in vivo* study of the rat (Tominaga et al., 1994). The reduction in ACh-mediated vasodilatation of the isolated mesenteric artery of the rat with age was concluded to be due to reduced activity of NOS combined with an increase in endothelium-derived constricting products, such as thromboxane A₂ and prostaglandin H₂ (Matz et al., 2000).

2-MeSADP induced relaxation of the rat mesenteric artery via P2Y₁ receptors. Although there was no significant difference between responses to 2-MeSADP at 4, 6 and 12 weeks, there was a tendency for responses to be greater at 6 weeks. The responses were significantly less at 8 and 24 months. Immunohistochemical staining confirmed the presence of P2Y₁ receptors on both the smooth muscle and endothelial layers of the mesenteric artery. L-NAME did not significantly affect responses to 2-MeSADP; it would appear that P2Y₁ receptor activation does not mediate vasodilatation via the release of NO but by EDHF (Malmjö et al., 1999, 2002).

A single concentration of SP (3 μ M) also induced vasodilatation and it was found to be more potent in young vessels, and SP potency decreased with age. This has also been reported for the guinea pig mesenteric and carotid arteries (Dhall et al., 1986), where peptidergic nerve density peaked at birth and

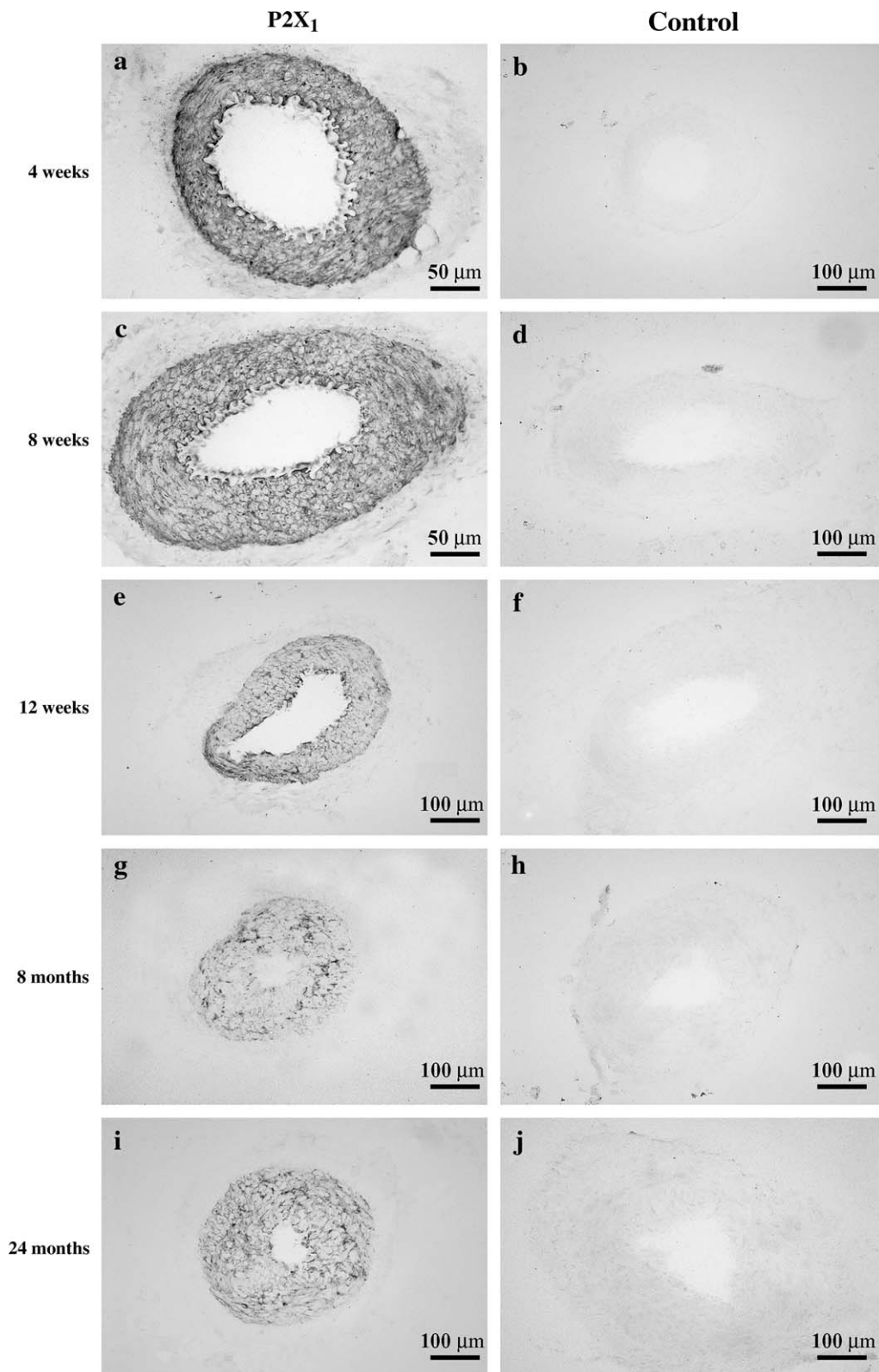


Fig. 6. P2X₁ receptor immunostaining of the rat tail artery on vessels of different age accompanied by a control consisting of the omission of the primary antibody to P2X₁ receptors on consecutive sections of vessel. (a, b) Four-week-old animals; (c, d) 6-week-old animals; (e, f) 12-week-old animals; (g, h) 8-month-old animals; (i, j) 24-month-old animals. Specific immunoreactivity for P2X₁ receptors was present in the smooth muscle of the tail artery although the intensity of staining decreased with increasing age of the vessel.

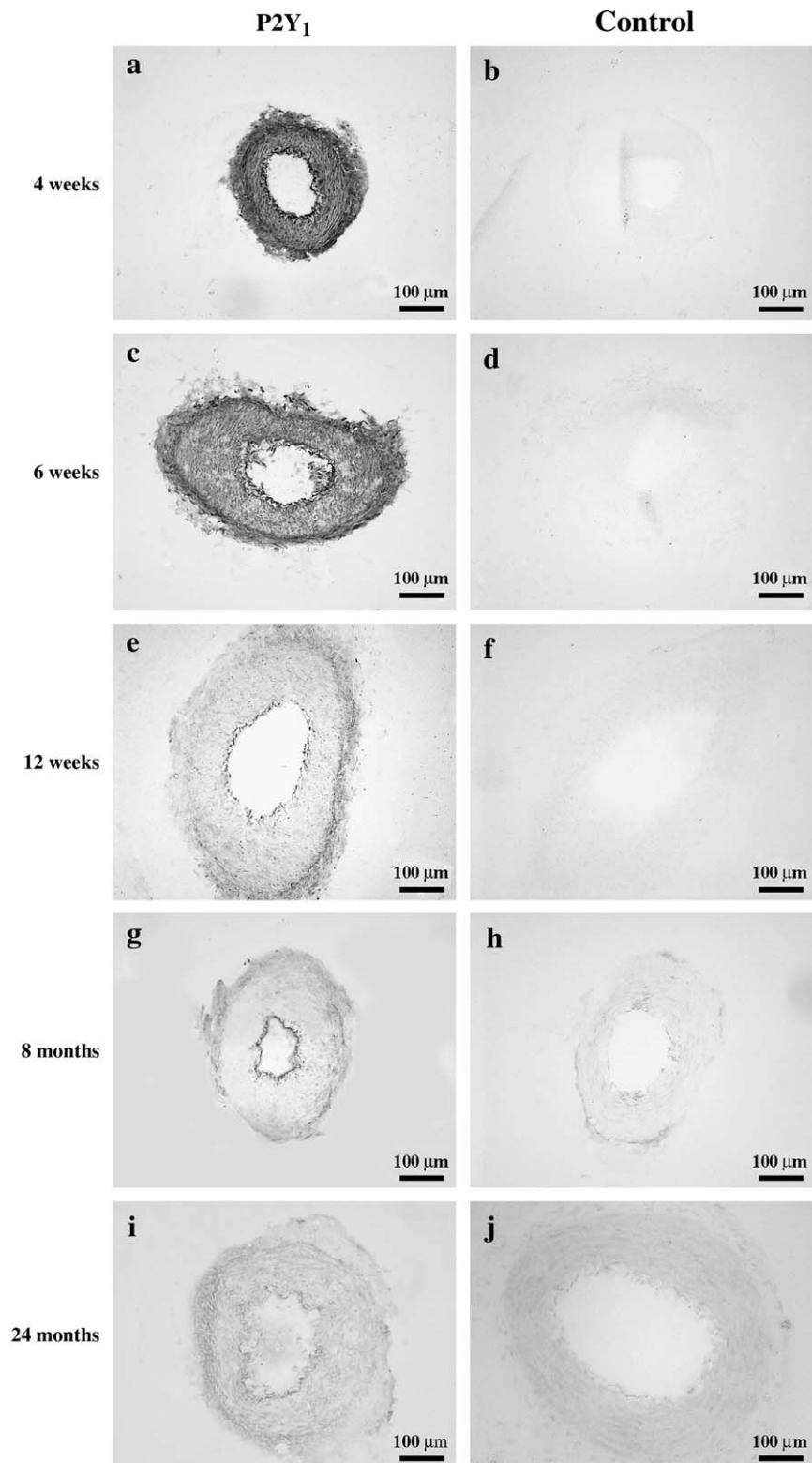


Fig. 7. P2Y₁ receptor immunostaining of the rat tail artery on vessels of different age accompanied by a control consisting of the omission of the primary antibody to P2Y₁ receptors on consecutive sections of vessel. (a, b) Four-week-old animals; (c, d) 6-week-old animals; (e, f) 12-week-old animals; (g, h) 8-month-old animals; (i, j) 24-month-old animals. Specific immunoreactivity for P2Y₁ receptors was present in the smooth muscle and endothelium of tail arteries from 4-, 6- and 12-week-old animals and absent from the smooth muscle of 8-month-old vessels. No staining was seen in tail arteries of 24-month-old animals.

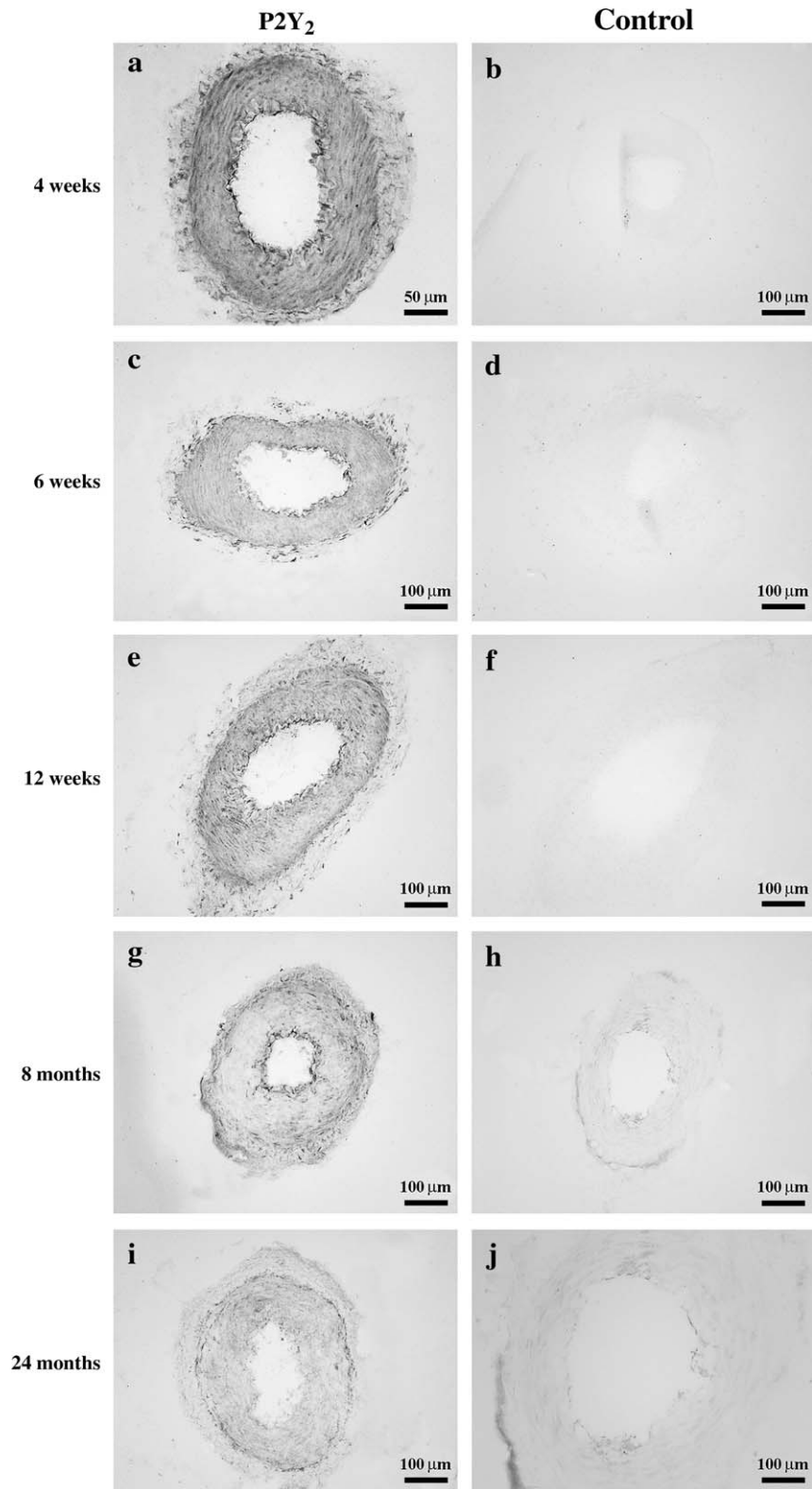


Fig. 8. P2Y₂ receptor immunostaining of the rat tail artery on vessels of different age accompanied by a control consisting of the omission of the primary antibody to P2Y₂ receptors on consecutive sections of vessel. (a, b) Four-week-old animals; (c, d) 6-week-old animals; (e, f) 12-week-old animals; (g, h) 8-month-old animals; (i, j) 24-month-old animals. Specific immunoreactivity for P2Y₂ receptors was present in the smooth muscle of the tail artery although the intensity of staining decreased with increasing age of the vessel.

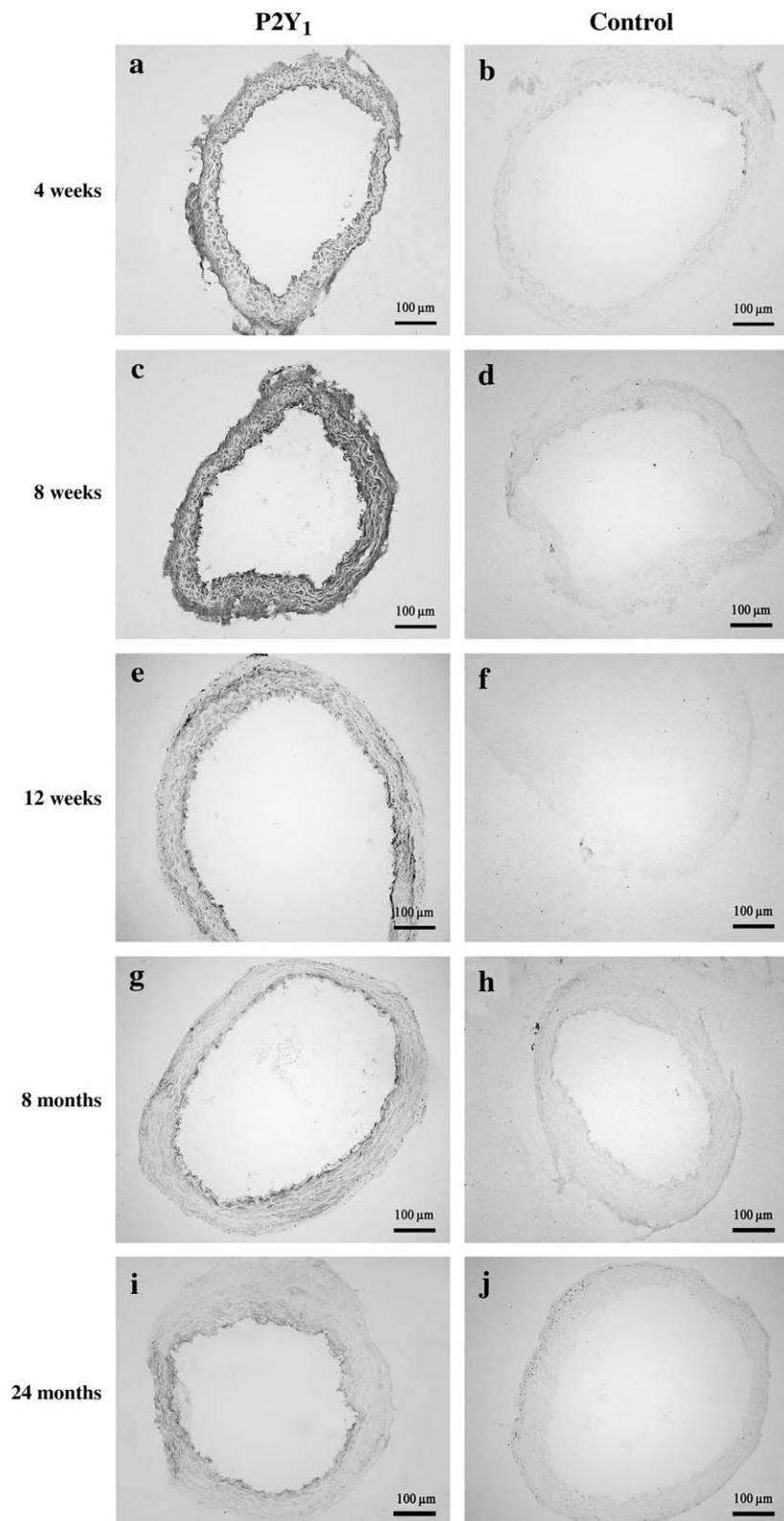


Fig. 9. P2Y₁ receptor immunostaining of the rat mesenteric artery on vessels of different age accompanied by a control consisting of the omission of the primary antibody to P2Y₁ receptors on consecutive sections of vessel. (a, b) Four-week-old animals; (c, d) 6-week-old animals; (e, f) 12-week-old animals; (g, h) 8-month-old animals; (i, j) 24-month-old animals. Specific immunoreactivity for P2Y₁ receptors was present in the smooth muscle and endothelium of the mesenteric artery. Intensity of staining was greater in vessels from 6-week-old animals and less intense in the other vessels.

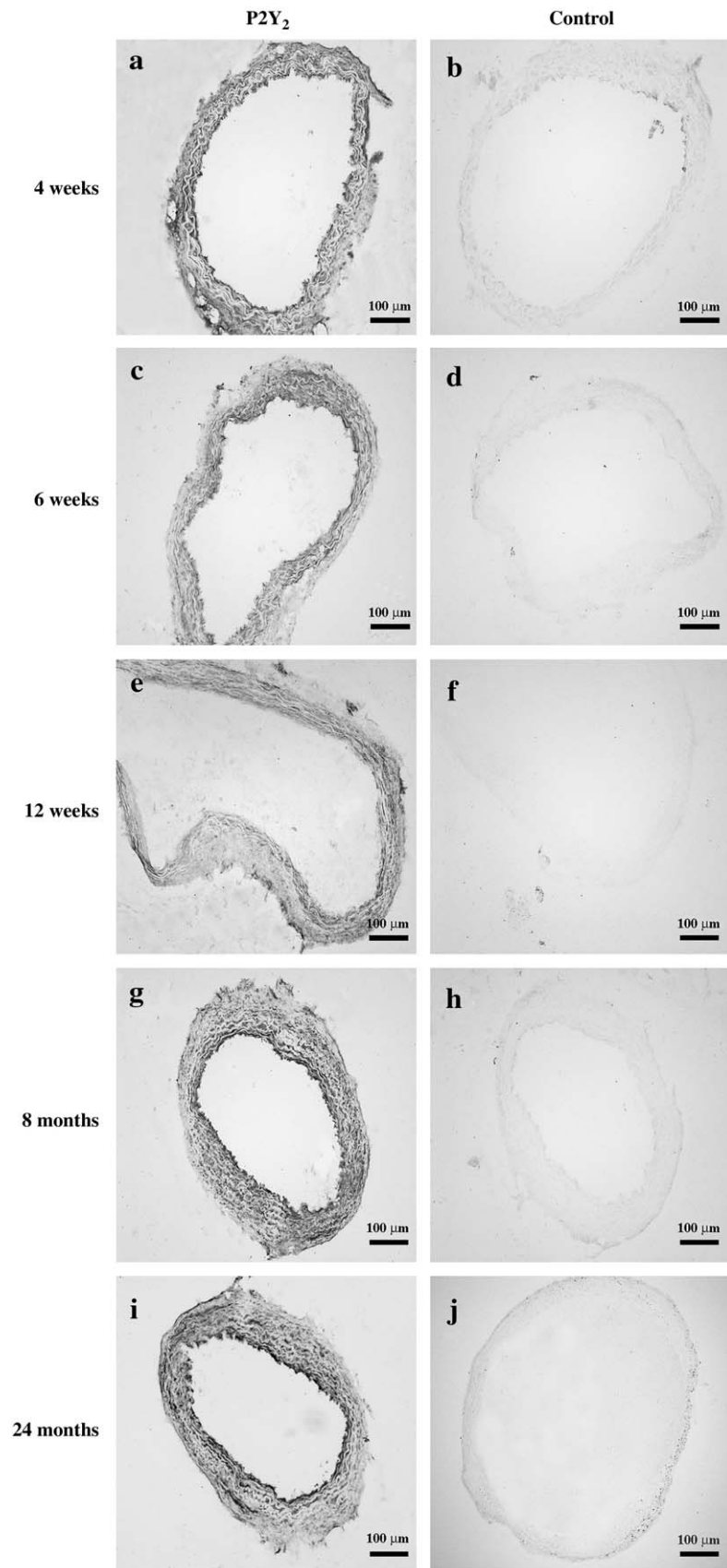


Fig. 10. P2Y₂ receptor immunostaining of the rat mesenteric artery on vessels of different age accompanied by a control consisting of the omission of the primary antibody to P2Y₂ receptors on consecutive sections of vessel. (a, b) Four-week-old animals; (c, d) 6-week-old animals; (e, f) 12-week-old animals; (g, h) 8-month-old animals; (i, j) 24-month-old animals. Specific immunoreactivity of a similar intensity for P2Y₂ receptors was present in the smooth muscle and endothelium of the mesenteric artery at each age, with a greater intensity of staining in the endothelium of 8- and 24-month-old vessels.

declined thereafter. Decreased sensitivity to SP may be due to a down-regulation of SP receptors on the endothelium or may be to decreased innervation by peptide-containing nerves. The latter has been demonstrated in the guinea pig mesenteric and carotid arteries (Dhall et al., 1986), where peptidergic nerve density peaked at birth and declined thereafter.

In the rat mesenteric artery, UTP induced vasoconstriction and this has also been demonstrated in the mouse mesenteric artery (Vial and Evans, 2002), although in other vessels such as pulmonary arteries of the rabbit and piglet, UTP induces vasodilatation (McMillan et al., 1999; Konduri et al., 2004). The presence of P2Y₂ receptors, which mediate this response, on the rat mesenteric artery vascular smooth muscle was confirmed by immunohistochemical staining. UTP was found to be slightly more potent in the younger experimental groups (4 and 6 weeks) than the older groups. However, P2Y₂ receptor immunoreactivity was found not to change with age, resembling results of other studies in the rat aorta and carotid artery where neither P2Y₂ receptor mRNA expression (Miao et al., 2001) nor ATP relaxations in endothelium-intact aortic rings (Koga et al., 1992) changed with age.

The presence of the additional P2 receptor subtypes (P2X₄, P2X₅ and P2Y₄) on the smooth muscle and endothelium of the tail and/or mesenteric artery has been demonstrated by immunohistochemical staining. These subtypes are expressed on other vessels such as the rat aorta, vena cava and mesenteric bed (Soto et al., 1996; Gitterman and Evans, 2000). The role of these receptors remains unclear although P2X₄ receptors appear to be involved in cell adhesion and gap junction formation (Burnstock, 2002; Glass et al., 2002).

The results from this study have demonstrated heterogeneous purinergic control of vascular tone in the rat tail and mesenteric arteries. The presence of a greater purinergic component of nerve-mediated responses in the tail artery of young rats might suggest that extracellular nucleotides play an important role in the development and maturation of neuromuscular function. This may be related to the normal physiological functions of the tail such as thermoregulation and balance. Tail heat-loss and blood flow increases with ambient temperature (Rand et al., 1965) as well as with increasing body temperature (Raman et al., 1987). By directing blood into the tail, heat can be lost, while conversely restricting blood flow to the tail reduces heat loss in the cold. Younger animals are smaller with a larger surface area to volume ratio and therefore lose heat faster. They also have very little excess body fat and so heat is less readily conserved. In addition, younger animals tend to be far more active than their older counterparts and this increased activity generates heat, which then needs to be dissipated.

Young animals are vulnerable to predation and susceptible to harsh environmental conditions. Hypothermia and infection can be life threatening. Hyperthermia, whether caused by environment, exercise or infection, can be fatal. Senescent animals are also vulnerable since in old animals there is a loss of muscle mass, which has an impact on body temperature and thermoregulation (Kenny and Buskirk, 1995). Immune systems become weaker. Thus the decrease in the contribution

of purines to the control of vascular tone during ageing may relate to the animal becoming less vulnerable as they grow and gain weight, increasing their surface area to volume ratio and percentage body fat.

Heterogeneity in the receptors mediating vascular control of the mesenteric artery could also be related to its normal physiological functions. The mesenteric artery supplies the intestines with blood. For the first 4 weeks of postnatal life, rats are fed with maternal milk and are then weaned onto a diet of solids. The greater purinergic control of vascular tone and the greater general propensity to vasodilatation in the mesenteric artery of young rats could be a reflection of a change in nutritional needs and diet as the rat grows rapidly up to an age of 8 to 9 months.

The heterogeneity of P2Y receptors in both arteries could be related to their trophic roles. Activation of P2Y₂ and/or P2Y₄ receptors stimulates vascular smooth muscle proliferation that may have implications in the development of atherosclerosis and hypertension (Erlinge, 1998). ATP and UTP released from endothelial cells can act via P2Y₁, P2Y₂ and P2Y₄ receptors to stimulate endothelial and smooth muscle cell proliferation (Burnstock, 2002). During postnatal development and rapid growth of the rat, these trophic roles could be very important and help explain the greater P2Y₁ and P2Y₂ receptor expression.

In conclusion, considerable heterogeneity in the function and expression of P2X and P2Y receptors in the rat tail and mesenteric arteries occurs with age, in general there is a trend for reduced activity and expression of P2 receptors during maturation and age, and this may be related to the normal physiological functions of the vessels.

Acknowledgements

The authors thank Dr H.-Z. Ruan for invaluable assistance with immunohistochemistry, and Mr Tim Robson for excellent technical assistance.

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