



Vasoconstriction of intrapulmonary arteries to P2-receptor nucleotides in normal and pulmonary hypertensive newborn piglets

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1 The vasoconstrictor responses of isolated intrapulmonary arteries (IPA) to P2-receptor agonists was investigated during adaptation to extrauterine life in the normal piglet and the effect of pulmonary hypertension was studied following exposure of newborn animals to chronic hypobaric hypoxia (51 kPa) for 3 days.

2 At resting tone, α,β -methyleneATP (α,β -meATP) (P2X-receptor agonist) contracted intrapulmonary arteries from adult, but not immature pigs, and repeated application desensitized the response.

3 Adenosine 5'-triphosphate (ATP) induced endothelium-independent relaxation at low concentrations at all ages, a variable contractile response to high concentrations developed by 3 days, becoming larger and consistent by 14 days of age.

4 Uridine 5'-triphosphate (UTP) evoked a contractile response in normal intrapulmonary arteries from foetal to adult life, the magnitude of the response increasing with age. Endothelial removal and pre-incubation with N^w-nitro-L-arginine methyl ester (L-NAME) (100 μ M) increased the contractile response of adult vessels.

5 Pre-incubation with α,β -meATP (100 μ M), increased the contractile response to UTP in both newborn and adult vessels. ATP-induced relaxations were reduced in newborn vessels but there was no effect on the responses of adult vessels.

6 Responses to UTP, ATP and α,β -meATP of intrapulmonary arteries from newborn piglets exposed to chronic hypobaric hypoxia for 3 days were normal.

7 In summary, UTP elicited marked vasoconstriction of porcine IPA at all ages. UTP and ATP responses were consistent with activation of the P2Y₄-receptor recently identified in vascular smooth muscle by others. α,β -meATP induced a small vasoconstriction in the adult probably *via* the P2X₁-receptor. Responses remained normal in neonatal pulmonary hypertension.

Keywords: Pyrimidine; purine; pulmonary artery; neonatal hypertension; vasoconstriction

Abbreviations: Ach, Acetylcholine; ANOVA, analysis of variance; ATP, adenosine 5'-triphosphate; IPA, intrapulmonary artery; KCl, potassium chloride; L-NAME, N^w-nitro-L-arginine methyl ester; α,β -meATP, α,β -methylene adenosine 5'-triphosphate; PAP, pulmonary arterial pressure; PGF_{2 α} , prostaglandin F_{2 α} ; PPHN, persistent pulmonary hypertension of the newborn; PSS, physiological salt solution; UTP, uridine 5'-triphosphate

Introduction

The pulmonary vascular resistance is high *in utero* and remains elevated in persistent pulmonary hypertension of the newborn (PPHN), a clinical syndrome most commonly associated with hypoxic lung disease (Haworth, 1979; 1993). Whether or not nucleotides play a role in maintaining a high resistance in either of these circumstances is not known.

ATP can produce either relaxation or contraction depending upon the P2-receptor subtype(s) present in the tissue and the tone of the vessel investigated (Burnstock, 1997), suggesting that the pulmonary arterial response to ATP might change with age in normal pulmonary arteries as the vascular resistance falls after birth due to maturation of vascular reactivity and structure (Allen & Haworth, 1998; Hall & Haworth, 1987; Haworth *et al.*, 1987; Levy *et al.*, 1995; Liu *et al.*, 1992). At low vascular tone, vasoconstriction to ATP is the dominant response, mediated by P2X-receptor(s), partly *via* the P2X₁ subtype on smooth muscle and partly by P2X subtypes which have not yet been clearly identified (Benham & Tsien, 1987; Neely *et al.*, 1991; 1996). Currently there are seven recognized P2X-receptors, including the non-selective ion pore P2X₇ (formerly P2Z) (Burnstock, 1997). Transcripts of receptor

mRNA for the P2X_{1,2} and 4-receptor sub-units have been co-localized by *in situ* hybridization in adult rat aortic and pulmonary arterial smooth muscle (Nori *et al.*, 1998). The metabolically stable ATP analogue α,β -meATP evoked a contractile response in the isolated guinea-pig taenia coli and then desensitized P2X-receptors, without significant vasodilator activity (Kasakov & Burnstock, 1983; Kennedy & Leff, 1995). ATP induces a contraction in isolated adult human and rat pulmonary artery and α,β -meATP can block the response suggesting that these agonists act *via* a common receptor (Liu *et al.*, 1989a,b). However, in the isolated perfused adult rat lung, vasoconstriction to ATP was only partially inhibited by α,β -meATP (Rubino & Burnstock, 1996). Pyrimidines (uridine nucleotides) are also known to evoke α,β -meATP-insensitive vasoconstriction of some systemic and pulmonary vessels (Juul *et al.*, 1993; Matsumoto *et al.*, 1997; Ralevic & Burnstock, 1991; 1998; Saiaj *et al.*, 1987; 1990; von Kugelgen & Starke, 1990). They can be released from the vascular endothelium of rabbit thoracic aorta, and from platelets, suggesting physiological sources (Goetz *et al.*, 1971; Saiaj *et al.*, 1995). Pyrimidine-induced vasoconstrictions reported for the adult rat small pulmonary artery and adult rabbit coronary artery were thought to be mediated by members of the G-protein coupled, P2Y-receptor family, which can activate phospholipase C

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through G_{q11} and subsequently stimulate a rise of intracellular calcium (Burnstock 1997; Nicholas *et al.*, 1996; Hartley *et al.*, 1998; Matsumoto *et al.*, 1997). Recently, adult rat aorta smooth muscle has been shown to express P2Y_{2,4, and 6}, subtypes, which are known to be activated by pyrimidines (Harper *et al.*, 1998).

Persistent pulmonary hypertension of the newborn is a potentially fatal condition, which results in abnormal remodelling of the intrapulmonary arteries during the first few days of life (Allen & Haworth, 1986; Haworth, 1979; 1993). The marked hypertrophy of the IPA smooth muscle cells has been taken to indicate an increase in contractile response to different stimuli, including hypoxia. The role of purines and pyrimidines in this condition is unknown. In an experimental study using the perfused adult rat lung, the vasoconstrictor response to acute alveolar hypoxia could not be attributed to up-regulation of α,β -methylene-sensitive P2X-receptors (McCormack *et al.*, 1989).

In the present study, the response of normal, isolated porcine IPA to cumulative doses of ATP, α,β -meATP and UTP was investigated from foetal to adult life. We also studied P2X-receptor desensitization by α,β -meATP on the response to ATP and UTP. The effect of neonatal pulmonary hypertension was determined in the IPA of newborn piglets which had been exposed to chronic hypobaric hypoxia for 3 days (Tulloh *et al.*, 1997).

Materials

Preparation of tissue

Piglets were produced by pregnant Large White sows at term. Animals were killed by an intraperitoneal injection of sodium pentobarbitone (Expiral 100 mg kg⁻¹) when 5 min – 3 h old and at 3 and 14 days of age. In addition, heart-lung blocks from foetal (5 days pre-term), 3, 14 day and adult animals (9-months-old) were delivered from a recognized tissue supplier in cold physiological salt solution. Normal newborn piglets were exposed to chronic hypobaric hypoxia (51 kPa) for a period of 3 days in a hypobaric chamber. Piglets exposed to hypoxia were regularly provided with fresh water and piglet feed (Sow Milk Equivalent, SCA Nutrition Ltd., U.K.) *ad libitum*, in addition to tube feeding as required. The chamber was illuminated and maintained at a temperature of 25°C (Tulloh *et al.*, 1997). The treatment of all animals followed the guidelines set down in the British Home Office regulations and in the 'Principles of Laboratory Animal Care' (National Institutes of Health, publication number 80/23, revised 1978).

Organ bath experiments

The main intrapulmonary conduit artery (IPA) was dissected from the middle third region of a lower lobe, placed in calcium containing physiological salt solution (PSS: NaCl 119; KCl 4.7; NaHCO₃ 25; MgSO₄ 1.2; KHPO₄ 1.2, CaCl₂ 2.5, glucose 11 mM) and cleaned of lung parenchyma and connective tissue. The vessel was cut into rings 2–4 mm long, external diameter range of 1.5 mm (foetal) to 3.5 mm (adult). The endothelium was removed from selected rings by mechanical rubbing with a metal tool. Two horizontal tungsten wires (120 μ m diameter) were inserted through the vessel lumen and then mounted for isometric force recording in 5 ml organ baths. Isometric force data was recorded in a digital format using Chart software on a MacLab computer system. The

rings were allowed to stabilize for at least 40 min, during which time the PSS was replaced once and the tension gradually increased to 1000 mg, in vessels from all animals. Contraction to 30 mM potassium chloride (KCl) established the presence of smooth muscle function in all preparations. In order to verify that the endothelium was intact or had been removed effectively, in animals aged 3 days or more acetylcholine (ACh) (1–10 μ M) was added in the presence of a stable contraction to PGF_{2 α} (10 or 30 μ M), to detect endothelium-dependent relaxation (Liu *et al.*, 1992). Conduit porcine IPA from younger or pulmonary hypertensive animals do not relax in response to ACh (Tulloh *et al.*, 1997). A contractile response to a second bolus of KCl (30 mM) was determined, against which responses could be evaluated (in combination with the tension remaining after 100 μ M papaverine). The KCl was then washed out, allowing the baseline to stabilize again. A cumulative-concentration response curve to either α,β -meATP (0.01–100 μ M), ATP (0.001–30 mM) or UTP (0.001–30 mM) was then carried out in rings from normal animals, from foetal to adult life, and in rings from neonatal hypoxic animals.

In order to investigate the role of the endothelium in the response to UTP, IPAs with endothelium from adult pigs were pre-incubated with either L-NAME (100 μ M) or indomethacin (10 μ M), both inhibitors being added before carrying out a cumulative-concentration response study to UTP. The effect of P2X-receptor desensitization upon the responses was investigated in normal newborn and adult porcine IPAs. Twenty minutes after carrying out a cumulative dose-response study with α,β -meATP, a 100 μ M bolus of α,β -meATP was added to confirm a P2X-receptor block. Then in the continued presence of α,β -meATP, a cumulative dose response study to either ATP or UTP was performed on the same vessel. Finally, in each experiment, a bolus of papaverine (100 μ M) was given in order to remove all remaining tone. The change in tension between the peak of the contractile response to 30 mM KCl and the relaxation to 100 μ M papaverine was taken as the measure of greatest contractile activity, as 100% against which the response to P2-agonists was assessed.

Drugs

The drugs were purchased from Sigma and dissolved in distilled water unless stated otherwise: acetylcholine (hydrochloride salt), adenosine 5'-triphosphate (di-sodium salt), indomethacin, L-NAME, papaverine (hydrochloride salt), prostaglandin F_{2 α} (Sigma and Cayman Chemical Company (made up in absolute ethanol), uridine 5'-triphosphate (sodium salt), α,β -methylene adenosine 5'-triphosphate (from RBI).

Statistical analysis

Excel (version 7a) and SPSS (version 6.1.3) PC were used to do the following data analysis. The effect of age on the contractile response to 30 mM potassium chloride (including the basal tone revealed by giving 100 μ M papaverine) was assessed using a one-way analysis of variance (ANOVA) with *post-hoc* Bonferroni testing. One-way ANOVA was used for each P2-receptor agonist to determine the concentration at which the effect of age was greatest. Bonferroni *post-hoc* testing was used to identify between which age groups the responses seen at this concentration were significantly different. A two-sample Student *t*-test was used to test the following: the influence of the endothelium on responses; the effect of L-NAME and indomethacin on the response to UTP in the adult; the effect of P2X-receptor desensitization by pre-incubation with α,β -

meATP on the response to ATP and UTP. The effect of exposure to chronic hypobaric hypoxia from birth for 3 days was compared to normal age-matched data using a two-sample Student *t*-test.

Results

Responses of intrapulmonary arteries from normal pigs

A contractile response to a bolus addition of potassium chloride (KCl) was present at all ages and the increase in tension was similar in IPA from foetal life until 14 days of age (Figure 1), but was significantly greater in normal adult vessels ($P < 0.05$). Removing the endothelium did not change the response at any age.

In IPA from normal foetal, newborn and 3 day-old piglets, cumulative addition of ATP induced a concentration-dependent relaxation which was independent of the endothelium (Figure 2a). In addition, small transient contractions were evoked at similar high concentrations in the 3 day-old, but not in younger animals. By contrast, in normal 2 week-old and adult pigs ATP-induced only a small relaxation, with a greater contractile response at high concentrations. Thus, there was a significant reversal from a pre-dominantly relaxant response to a contractile one as age increased ($P < 0.05$).

In IPA from piglets of all ages, cumulative addition of UTP induced a contractile response in vessels with and without endothelium (Figure 2b). The response in rings without endothelium increased significantly with age and was significantly greater in the adult than in foetal animals ($P < 0.05$). In adult IPA the contractile response was significantly greater in rings without endothelium ($P < 0.05$). Representative traces of ATP- and UTP-cumulative concentration response curves in newborn and adult IPA are presented (Figure 3a,b,c,d).

Cumulative-addition of α,β -meATP only induced a significant response in adult IPA, when a small transient contraction was evoked at $10 \mu\text{M}$ (Figure 2c). Removing the endothelium had no significant effect on the response at any age. In the normal adult porcine IPA the rank order of vasoconstrictor activity was $\text{UTP} > \text{ATP} > \alpha,\beta\text{-meATP}$.

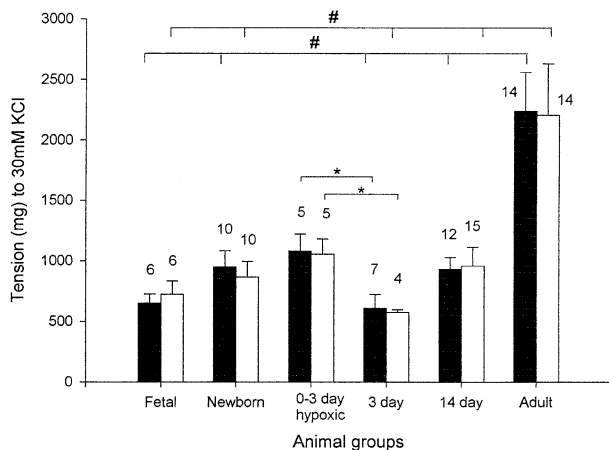


Figure 1 The response of isolated porcine intrapulmonary arteries to 30 mM potassium chloride, at different ages, in normal animals and in those exposed to hypoxia, with and without endothelium. Each column shows the mean \pm s.e.mean. The number of animals used is indicated above each column. Solid columns indicate arteries with endothelium and empty columns those without. # $P < 0.05$ ANOVA, * $P < 0.05$ Student *t*-test.

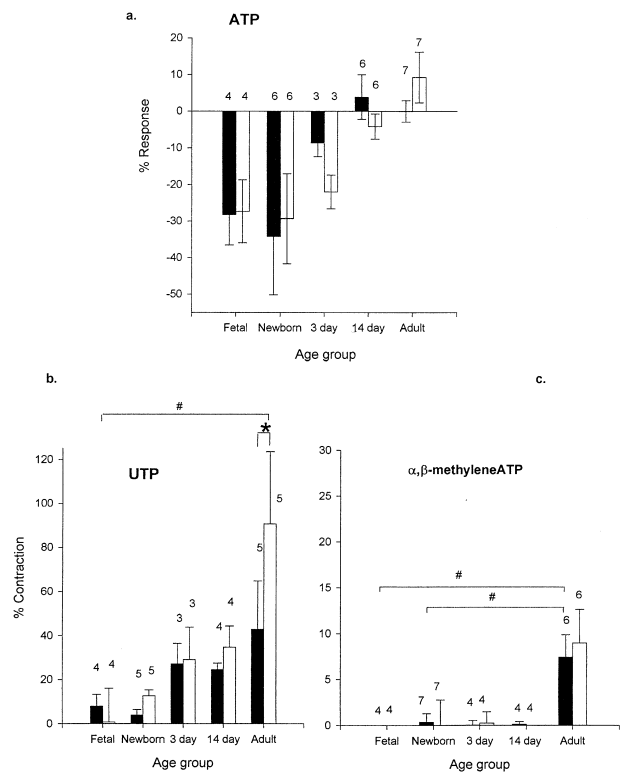


Figure 2 Histograms showing the mean response to (a) ATP, 20 mM; (b) UTP, 20 mM; (c) α,β -meATP, $10 \mu\text{M}$, at different ages. The data shown for each agonist gave the most significant (ANOVA) change in response with age and was derived from cumulative concentration-response curves. Columns show mean \pm s.e.mean. Solid columns indicate arteries with endothelium and empty columns those without. The number of animals used is indicated above each column. # $P < 0.05$ ANOVA, * $P < 0.05$ Student *t*-test.

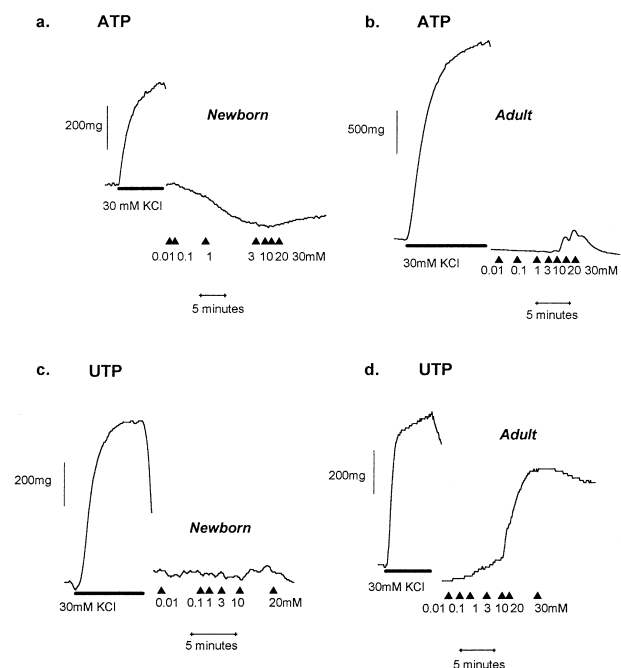


Figure 3 Representative traces for the responses of isolated porcine intrapulmonary arteries, with endothelium, to a bolus of 30 mM KCl and cumulative doses of either ATP or UTP at resting tension. Responses are shown for ATP (a, b) and UTP (c, d) in newborn and adult vessels respectively.

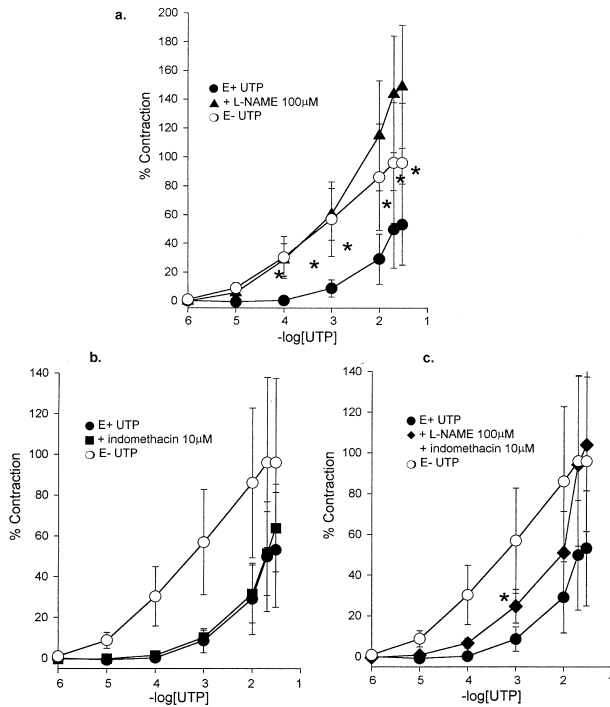


Figure 4 Porcine intrapulmonary arteries from four normal adults with and without endothelium exposed to UTP, and in vessels with endothelium following pre-incubation with (a) L-NAME (100 μ M), (b) indomethacin (10 μ M) or (c) L-NAME (100 μ M) and indomethacin (10 μ M), showing the mean response \pm s.e.mean. * P < 0.05 between the treated vessels and the response of vessels with endothelium, Student t -test.

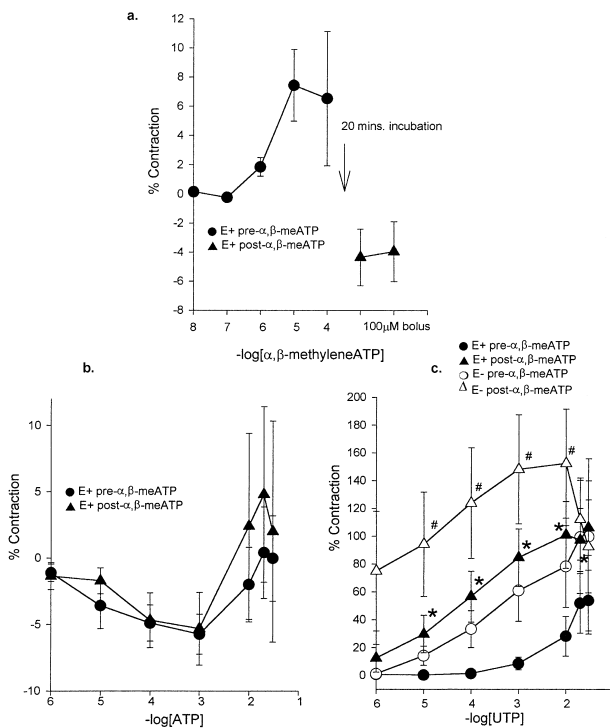


Figure 5 The effect of P2X-receptor desensitization by pre-incubation with (100 μ M) α,β -meATP in isolated intrapulmonary arteries with endothelium from normal adult pigs. Effect on the response to (a) α,β -meATP ($n=6$); (b) ATP ($n=6$); (c) UTP ($n=5$), showing the mean response \pm s.e.mean. Empty symbols denote vessels without endothelium. 'n' indicates the number of animals used and applies to subsequent legends. * With and # without endothelium, P < 0.05 between treated and untreated vessels.

Effect of nitric oxide synthase and prostaglandin synthase inhibitors in adult porcine IPAs

The resting tone did not change significantly following incubation with either 100 mM L-NAME (increased by $22 \pm 12\%$) or 10 μ M indomethacin (decreased by $15 \pm 6\%$) or with a combination of the two (increased by $4 \pm 7\%$). The effect of the synthase inhibitors was assessed from the newly established baseline. The significant increase in UTP-induced contraction following removal of the endothelium from the normal adult pig IPA was mimicked by pre-incubation with L-NAME (100 μ M) (Figure 4a). Pre-incubation with indomethacin (10 μ M) had no effect (Figure 4b), while a combination of the two inhibitors reduced the effect of L-NAME given alone (Figure 4c).

P2X-receptor desensitization experiments

In IPA from normal adult animals a 20 min pre-incubation with 100 μ M α,β -meATP, followed by a repeated dose of 100 μ M α,β -meATP desensitized the contractile response in vessels with or without endothelium (Figure 5a). In adult vessels, the response to ATP was unchanged by pre-incubation

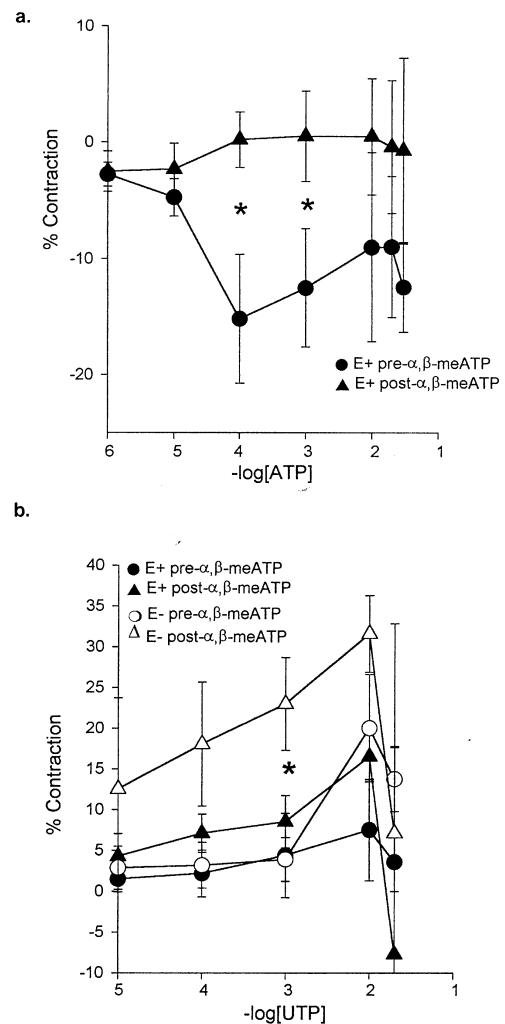


Figure 6 The effect of P2X-receptor desensitization by pre-incubation with (100 μ M) α,β -meATP in isolated intrapulmonary arteries with endothelium from normal newborn pigs. Effect on the response to (a) ATP ($n=3$); (b) UTP ($n=3$) and (c) UTP ($n=3$) without endothelium, showing the mean response \pm s.e.mean. * P < 0.05.

with α,β -meATP (Figure 5b). UTP-induced contractions displayed a significantly augmented potency and magnitude in IPA with and without endothelium (Figure 5c).

In normal newborn piglets, the same protocol prevented the normal ATP-induced relaxation response ($P < 0.05$ Figure 6a). After pre-incubation with α,β -meATP, UTP appeared to induce an increased contractile response but this was only statistically significant in vessels in which the endothelium had been removed (Figure 6b).

Responses of intrapulmonary arteries from piglets with neonatal pulmonary hypertension

The response to a bolus addition of KCl in IPA from animals exposed to chronic hypobaric hypoxia from birth for a period of 3 days was similar to the normal response at birth and greater than that in the normal at 3 days of age (Figure 1) ($P < 0.05$). Removal of the endothelium did not alter this observation.

The response to ATP and α,β -meATP in IPAs from the hypoxic piglets was similar to that in normal age-matched healthy animals (Figure 7a,b). The contractile response to UTP was more similar to the normal response at birth than the normal at 3 days of age with (Figure 7c), or without (data not shown) endothelium.

Discussion

The present study has demonstrated that under resting conditions the contractile response of the porcine conduit intrapulmonary artery to P2-receptor agonists increased significantly between foetal and adult life. The contractile response was low in the perinatal animals suggesting that the

agonists tested may not have physiological importance in the presence of the high pulmonary tone present at this age. A contractile response to UTP was evident in the normal foetal and newborn preparations, much earlier than ATP-induced contraction. UTP also had a greater contractile effect than either ATP or α,β -meATP at all ages. The explanation for the increase in contractile response to the different nucleotides with age is unclear. The response to purines is generally tone-dependent, vasoconstriction being favoured at low tone, such as is found in IPAs of mature normal animals. But the response to other contractile agonists also increases with age (Levy *et al.*, 1995) and could be related to a progressive increase in smooth muscle cell contractile myofilament volume density (Hall & Haworth, 1987).

α,β -meATP only constricted adult porcine IPA and the responses were transient, and tachyphylactic, as described in the isolated IPAs of man and the adult rat (Liu *et al.*, 1989a, b). Following reports of P2X_{1,2,4}-receptor mRNA located on adult rat IPA smooth muscle it may be suggested that α,β -meATP acts at the P2X₁-receptor subtype in the present study (Nori *et al.*, 1998). It was interesting to note the absence of any contractile response in the IPAs of younger piglets, because the rat tail artery shows a greater P2X-receptor mediated vasoconstriction in the young rat than in the adult (Bao *et al.*, 1989). In the adult porcine IPA, α,β -meATP evoked a smaller contractile response at lower concentrations than either ATP or UTP. α,β -meATP has been shown to desensitize certain subtypes of P2X-contractile receptors (P2X₁ and 3) (Burnstock, 1997). In the present study, α,β -meATP reduced the relaxation response to low concentrations of ATP in the newborn pig IPA, and actually increased the contractile response to UTP. In the adult porcine IPA, the ATP-induced contractile responses were not blocked and the UTP-induced responses were increased by pre-incubation with α,β -meATP. Other workers have also found the contractile response to ATP was not inhibited following P2X-receptor desensitization by α,β -meATP in systemic vessels (Juul *et al.*, 1993; Maguire *et al.*, 1996; O'Connor *et al.*, 1990) and potentiation of UTP-induced vasoconstriction has been reported in the isolated adult rat pelvic artery (Maguire *et al.*, 1996). In addition, α,β -meATP reduced ATP induced vasoconstriction of perfused adult rat lungs but it did not inhibit the UTP response (Rubino & Burnstock, 1996). α,β -meATP has been shown to be an inhibitor of ectoATPase activity on bovine aortic endothelial cells (Chen & Lin, 1997). EctoATPase inhibition may increase the levels of ATP and UTP and therefore increase the contractile response at low tone (Zimmerman, 1996).

UTP evoked a marked vasoconstriction of IPA at all ages in the present study. The role of UTP in the cardiovascular system, normal or abnormal, is uncertain. High concentrations are present in platelets and UTP can be released from the endothelium under conditions of increased shear stress and hypoxia (Saiag *et al.*, 1995), suggesting the involvement of UTP in high flow and hypoxia-induced vasoconstriction *in vivo*. Ours is the first study to demonstrate a vasoconstrictor action for UTP which was greater than that of purines, as it was throughout life in porcine IPA. The observation that UTP induces a greater contractile response in the adult porcine IPA without endothelium would suggest UTP-stimulated release of an EDRF (Purkiss *et al.*, 1994). This was confirmed in the present study with L-NAME. A pyrimidine-preferring P2Y receptor (G-protein coupled receptor superfamily) is thought to mediate the contractile response of the adult rabbit coronary artery induced by UDP and UTP (Matsumoto *et al.*, 1997). In the present study UTP evoked an α,β -meATP-insensitive vasoconstriction, suggesting action at a P2Y₂-

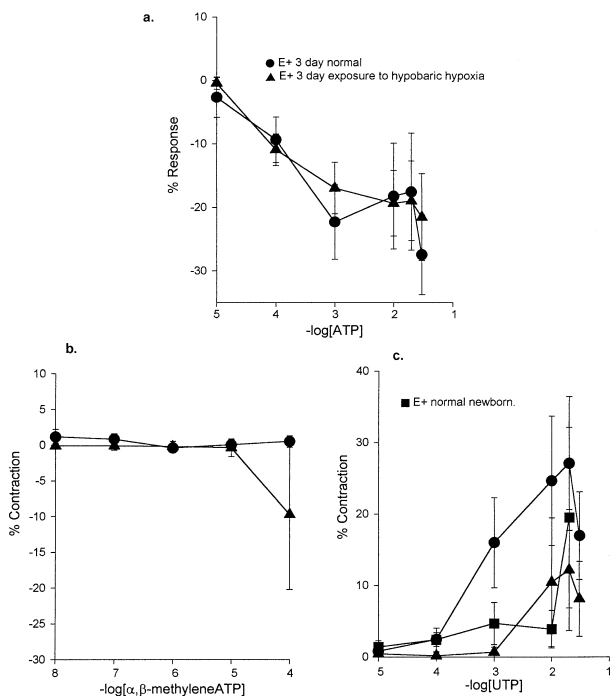


Figure 7 The effect of chronic pulmonary hypertension induced by hypoxia from birth for a period of 3 days on the response of isolated intrapulmonary arteries, with endothelium, to (a) ATP ($n=4$); (b) α,β -methyleneATP ($n=3$); (c) UTP ($n=3$), showing the mean response \pm s.e. mean, normal newborn, normal 3 day-old both with endothelium. * $P < 0.05$.

receptor, recently identified in adult rat small pulmonary artery (Hartley *et al.*, 1998). However, the observation that ATP seemed to act as a partial agonist also evoking an α,β -meATP-insensitive vasoconstriction would lead us to conclude that the responses to both UTP and ATP were mediated by a P2Y₄-receptor. This conclusion is based on receptor expression studies by Nicholas *et al.* (1996), demonstrating that UTP was more potent than ATP at the P2Y₄-receptor, while both nucleotides were equipotent at the P2Y₂ and that the P2Y₆ was only potently activated by UDP.

Chronic hypoxia produces vascular remodelling of the intrapulmonary arteries in the newborn piglet and other species including man, which involves an increase in smooth muscle (Allen & Haworth, 1986; Haworth, 1993). However, in both present and previous studies on chronically hypoxic piglets, the increase in smooth muscle was not associated with an increase in the contractile response to potassium chloride (Tulloh *et al.*, 1997). Hypoxic exposure prevented the normal postnatal reduction in response from taking place. The responses evoked by P2-receptor agonists were also similar to those of vessels from normal piglets at birth, prior to entering the hypobaric chamber. Reports from our own group and others make it clear that the vasoconstrictor response to other agonists is not always enhanced following exposure to chronic

hypoxia (Tulloh *et al.*, 1997; Porcelli & Bergman, 1983; Karamsetty *et al.*, 1995; Maclean *et al.*, 1996).

Chronic hypobaric hypoxia may have remodelled the IPA in such a way as to produce a 'pre-contracted, high tone vessel' which would favour a relaxation response, judging by the tone-dependent nature of the responses evoked by P2-agonists. An alternative explanation would be that the smooth muscle sensitivity to ATP induced relaxation was up-regulated to balance the vasoconstricting effect of hypoxia *in vivo*. An initial up-regulation of IPA vasodilatation to EDRF and activation of K⁺_{-ATP} channels has been shown in response to acute hypoxia, prior to a secondary loss of vasodilatation (Wadsworth, 1994).

The present study suggests that the P2X₁-receptor mediates the α,β -meATP vasoconstriction response in the adult. However, UTP was found to be a more potent vasoconstrictor than ATP at all ages, suggesting the α,β -meATP-insensitive contractile responses were mediated by the P2Y₄-receptor subtype. Neonatal pulmonary hypertension induced by chronic hypoxia did not affect these conclusions.

We thank Dr P.J. Boels for his pharmacological and statistical advice. This work was funded by the British Heart Foundation.

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(Received January 13, 1999

Revised June 10, 1999

Accepted July 6, 1999)