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

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1 The vasodilator responses of isolated intrapulmonary arteries (IPA) to P2-receptor agonists were investigated during adaptation to extrauterine life in the piglet. The effect of pulmonary hypertension on the normal response was determined after exposing newborn animals to chronic hypobaric hypoxia (51 kPa) for 3 days.

2 Adenosine 5′-triphosphate (ATP), 2-methylthioATP (2-meSATP), adenosine 5-O-(2-thiodiphosphate) (ADPβS) and uridine 5′-triphosphate (UTP) induced a relaxation in normal newborn piglet IPA pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The relaxations were not affected by removal of the endothelium. The responses to ATP and ADPβS increased significantly with age.

3 The relaxation responses of IPA to ATP, 2-meSATP and ADPβS continued to increase normally after birth in an hypoxic environment.

4 The results of the study show that vasodilatation of porcine intrapulmonary vessels to nucleotides increased during development from foetus to adult; that the vasodilatation to purines was mediated by P2Y-receptors on the vascular smooth muscle rather than on the endothelium; and that the P2Y-receptor mediated relaxation of IPA remained normal in the pulmonary hypertensive neonate.

Keywords: Purine; pyrimidine; pulmonary artery; neonatal hypertension; vasodilatation; vasoconstriction

Abbreviations: ACh, acetylcholine; ADPβS, adenosine 5′-O-(2-thiodiphosphate); ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; IPA, intrapulmonary artery; KCl, potassium chloride; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; 2-meSATP, 2-methylthio adenosine 5′-triphosphate; PAP, pulmonary arterial pressure; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PPHN, persistent pulmonary hypertension of the newborn; PSS, physiological salt solution; UTP, uridine 5′-triphosphate

Introduction

The high pulmonary arterial pressure during foetal life falls after birth as the intrapulmonary arteries (IPA) adapt to extrauterine life, involving changes in vascular structure and reactivity (Hall & Haworth, 1987; Haworth & Hislop, 1981; Levy *et al*., 1995; Liu *et al*., 1992). Endothelium-dependent relaxation is poor, even absent at birth, maturing during the first 2 weeks of life (Abman *et al*., 1991; Liu *et al*., 1992). Endothelium-independent relaxation to exogenous nitric oxide and sodium nitroprusside is present at birth; however it is significantly less than it is by 3 weeks of age (Liu *et al*., 1992; Shaul *et al*., 1993). The role of purines in the newborn and transitional circulation is uncertain. ATP relaxes mature isolated intrapulmonary arteries from man and several other species (Greenberg, 1987; Liu *et al*., 1989a,b; Purkiss *et al*., 1994), and it also relaxes the isolated IPA of 10–17 day-old piglets (Perez-Vizcaino *et al*., 1996). Purines may be important at birth since blood levels of ATP are increased by exposing the foetal lamb to high oxygen levels, and infusion of the di-sodium salt of ATP into the foetal lamb reduces the pulmonary vascular resistance to a level comparable to that measured after birth (Konduri *et al*., 1997).

When the pulmonary circulation fails to adapt normally to extrauterine life, the pulmonary arterial pressure (PAP) remains high. Pulmonary hypertension of the newborn (PHN), contributes to morbidity and mortality in a number of pathological conditions, most commonly in hypoxic lung disease (Haworth, 1993). In the chronically hypoxic newborn piglet both endothelium-dependent and -independent relaxation is impaired (Allen & Haworth, 1986; Tulloh *et al*., 1997). In the adult rat pulmonary artery chronic hypoxia reduces the endothelium-dependent relaxation to ATP (Rui & Cai, 1991). An ATP-$MgCl_2$ infusion reduced the raised PAP in a man suffering from chronic obstructive pulmonary disease (Gaba & Prefaut, 1990) and has been used successfully, but infrequently, to treat children with pulmonary hypertension secondary to congenital heart defects (Brook *et al*., 1994). ATP also reduced the PAP in acutely hypoxic piglets aged 3–15 days and lambs aged 10–16 days (Konduri & Woodward, 1991; Paidas *et al*., 1988).

The purpose of the present study was to clarify the role of purine-induced vasodilatation in the neonate by studying the response to nucleotides in isolated porcine IPA from foetal to adult life. In order to classify the receptor responsible a rank order of agonist potency was determined involving ATP (native purinergic ligand), 2-meSATP, ADPβS ($P2Y_1$-agonist), α,β-methyleneATP (α,β-meATP), ($P2X_1$- and $P2X_3$-agonist), and UTP ($P2Y_{2,4}$-agonist) (Abbrachio & Burnstock, 1994; Harden *et al*., 1998).

The effect of chronic pulmonary hypertension on the relaxant response to purines in newborn piglet IPA was assessed in vessels taken from piglets which had been exposed to chronic hypobaric hypoxia for 3 days, a procedure known to produce pulmonary hypertension in this model (Allen & Haworth, 1986; Tulloh *et al*., 1997).

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Methods

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 when 3, 6 and 14 days old, having been maternally fed. In addition, heart-lung blocks from foetal (5 days pre-term), 3, 6 and 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 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).

Pharmacology organ bath experiments

The main intrapulmonary conduit artery (IPA) was dissected from the middle third region of a lower lobe and placed in calcium containing physiological salt solution (PSS) (mM): NaCl 119, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $KHPO_4$ 1.2, $CaCl_2$ 2.5, glucose 11. Lung parenchyma and connective tissue was removed. 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 by mechanical rubbing with a metal tool. Two horizontal tungsten wires (120 μm diameter) were inserted through the vessel lumen and mounted for isometric force recording in 5 ml organ baths. Isometric force data was recorded in a digital format using a Chart software package on a MacLab computer system. The rings were allowed to stabilize for at least 40 min, during which time the PSS was replaced and the tension gradually increased to 1000 mg in all animal groups. Contraction to 30 mM potassium chloride (KCl) established the viability of the preparation in all experiments. 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 μM) was added to a stable contraction to $PGF_{2\alpha}$ (10 or 30 μM). Previous studies have shown that conduit porcine IPAs from younger animals do not relax in response to ACh (Liu *et al*., 1992). A bolus of 30 μM $PGF_{2\alpha}$ was used to product a monophasic, stable pre-contraction. This concentration had previously been found to be effective in this preparation (Liu *et al*., 1992; Tulloh *et al*., 1997). P2-nucleotide agonists were then added in a cumulative manner. A bolus of sodium nitroprusside 100 μM was also applied before the addition of 100 μM papaverine, to remove all remaining tone. The change in tension between the peak of the contractile response to $PGF_{2\alpha}$ and the relaxation response to papaverine was taken as 100% against which the response to P2-agonists was assessed. In order to confirm the structural integrity of the endothelium and to confirm that the endothelium had been removed in denuded preparations, IPA from each group were fixed in 2.5% gluteraldehyde and stained with haematoxylin and eosin. These studies gave us positive confirmation.

Table 1 Responses to $PGF_{2\alpha}$ (including the loss of tone due to papaverine, 100 μM) in isolated IPA from normal and pulmonary hypertensive (PH) pigs

	Contraction (30 μM $PGF_{2\alpha}$–100 μM papaverine) *Milligrams tension* Mean±standard error (number of animals used)	
Age groups	*With endothelium*	*Without endothelium*
Normal foetal	925±248 (4)	1028±160 (5)
Normal newborn	1052±133 (14)	1008±145 (11)
Normal 3 day	637±81 (17)	531±49 (16)*
Normal 6 day	512±71 (8)	371±66 (5)*
Normal 14 day	643±251 (7)	441±74 (6)*
Normal adult	1067±119 (24)	1515±198 (16)
Birth - 3 day P H.	1306±157 (12)#	1032±138 (14)#

*Indicates a significant difference from the adult response; # indicates a significant difference from the 3 day old normal age-matched control, $P<0.05$.

Drugs

The drugs were purchased from Sigma and dissolved in distilled water unless stated otherwise: acetylcholine (hydrochloride salt), adenosine 5′-O-(2-thiodiphosphate) (tri-lithium salt), adenosine 5′-triphosphate (di-sodium salt), papaverine (hydrochloride salt), prostaglandin $F_{2\alpha}$ (Sigma and Cayman Chemical Company) (made up in absolute ethanol), sodium nitroprusside, uridine 5′-triphosphate (sodium salt), α,β-methylene adenosine 5′-triphosphate (from RBI), and 2-methylthio adenosine 5′-triphosphate (tetra-sodium salt) (from RBI).

Statistical analysis

Excel (version 7a) and SPSS (version 6.1.3.) for PC were used to carry out the following data analysis. The effect of age on the response to $PGF_{2\alpha}$ was assessed using one-way analysis of variance (ANOVA) with *post-hoc* Bonferroni testing. Full-concentration relaxation response curves were not obtained to P2-agonists in this study, within conventional, practical dose ranges. Therefore, it was not possible to generate an EC_{50} value from logistic curve fitting in order to compare agonist relaxation responses, as is customary.

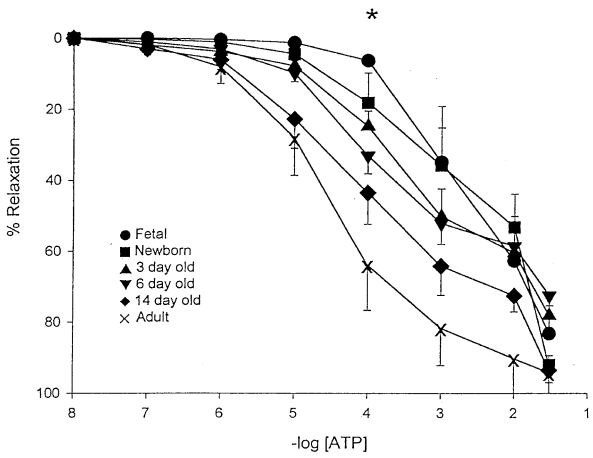

Figure 1 Relaxation response to ATP in isolated porcine intrapulmonary arterial rings pre-contracted with 30 μM $PGF_{2\alpha}$, during normal adaptation to extrauterine life: foetal ($n=3$), newborn ($n=8$), 3 day-old ($n=6$), 6 day-old ($n=8$), 14 day-old ($n=7$), adult ($n=10$), showing mean±s.e.mean. Removing the endothelium did not affect the responses (data not shown). '*n*' indicates the number of animals used in this and all subsequent legends. *Indicates the concentration at which age had the most significant effect (ANOVA, $P<0.05$).

An alternative analytical approach was sought. One-way ANOVA was used to determine the influence of age at each concentration of ATP in order to identify the concentration at which age had the most significant effect. One way ANOVA (with *post-hoc* Bonferroni testing) was then used at this concentration of ATP, ADPβS and 2-meSATP in order to find out if age had any effect. The activity of each agonist within an age group was compared by two-sample Student *t*-tests. For α,β-meATP and UTP, the concentration of agonist that gave the greatest contractile response in normal adult porcine IPA was the concentration at which the comparison was made between the adult and younger age groups, and between each of the younger age groups. The effect of age on the response to these agonists was assessed by one-way ANOVA. The effect of the endothelium was assessed using two-sample Student *t*-tests. The effect of exposure to chronic hypobaric hypoxia from birth for 3 days was studied by comparison with data from normal age-matched controls, using a two sample Student's *t*-test.

Results

Organ bath experiments

The effect of normal development and neonatal pulmonary hypertension on the pre-contractile agonist used in the present study, prostaglandin $F_{2\alpha}$ Intrapulmonary arteries (IPA) from animals of all ages studied contracted to 30 μM $PGF_{2\alpha}$ (Table 1). The response appeared to decline at 3 days of age before increasing significantly between 14 days and adulthood. The later increase in response was only significant in IPA without endothelium ($P<0.05$). The high newborn level of response to $PGF_{2\alpha}$ was seen in IPA from piglets exposed to chronic hypobaric hypoxia from birth for a period of 3 days (pulmonary hypertensive) ($P<0.05$). Removal of the endothelium did not affect the contractile response in any group studied.

The response of pre-contracted intrapulmonary arteries from normal piglets to P2-receptor agonists ATP produced concentration-dependent relaxations in IPA pre-contracted with $PGF_{2\alpha}$ (30 μM) from both foetal and newborn animals (Figures 1 and 2a). The increase in relaxation with age was greatest at 100 μM ($P<0.05$) (Figure 1). The relaxation response to ATP was significantly greater in the adult than at birth or 3 days of age ($P<0.05$) (Figure 3). At low tone ATP > 10 mM evoked transient contractions in IPA from normal pigs older than 3 days of age (Figure 2b).

2-meSATP produced concentration-dependent relaxations in rings from newborn animals which did not change significantly with age (Figure 3). A small, transient contraction at < 100 μM was recorded in two newborns (of four studied), one 3 day-old (of four studied), and one adult animal (of six studied).

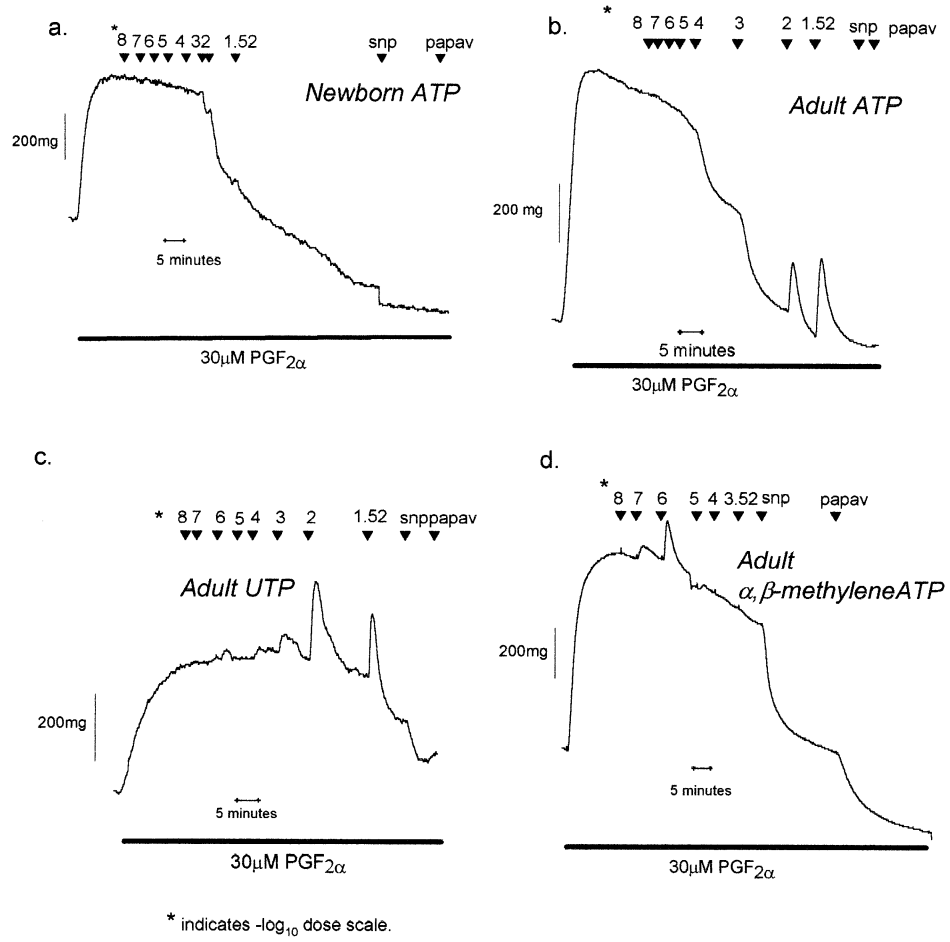

Figure 2 Representative traces of responses of isolated porcine intrapulmonary arteries, pre-contracted with 30 μM $PGF_{2\alpha}$. (a) ATP in a normal newborn piglet, (b) ATP in an adult, (c) UTP in an adult and, (d) α,β-meATP in an adult (note desensitization with higher concentrations). s.n.p. = sodium nitroprusside; papav = papaverine.

ADPβS produced concentration-dependent relaxations in rings from newborn animals. This analysis includes data from the three foetal animals in which the responses did not differ from those in the newborn animals, and therefore the data were pooled. The relaxation response increased significantly between newborn and adult life ($P<0.05$) (Figure 3). A small, transient contraction was evoked at $\geqslant 100$ μM, in three of seven adult preparations studied. Removing the endothelium did not influence the response to ATP, 2-meSATP or ADPβS at any age (data not shown).

In the normal newborn piglet IPA, UTP-induced an endothelium-independent relaxation which was similar to the newborn response to ATP (Figure 4, data from rings without endothelium are not shown). Analysis includes data from two foetal animals in which the responses did not differ from those in the newborn animals. In older animals the response reversed to show transient contractions (Figures 2c and 4). The greatest transient contractile response was seen consistently in normal adult porcine IPA in response to 10 mM UTP.

High concentrations (100 μM) of $\alpha\beta$-meATP induced variable relaxations in IPA from younger piglets (data not shown). In IPA from normal adult pigs α,β-meATP evoked a transient, concentration-dependent contractile response which was maximal at 1 μM (Figures 2d and 4). This concentration had no significant effect in IPA from normal and 3 day-old piglets. Removing the endothelium did not affect the contractile response to UTP or α,β-meATP, at any age (data not shown).

The effect of neonatal pulmonary hypertension on the response of pre-contracted intrapulmonary arteries to P2-receptor agonists
In comparison with age-matched controls the maturation of the relaxation response to ATP, 2-me*S*ATP and ADPβS was not changed by exposure to chronic hypobaric hypoxia from birth for 3 days (Figure 5a,b,c).

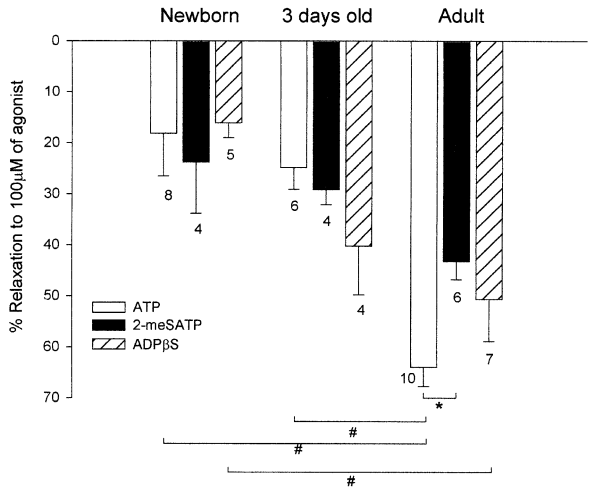

Figure 3 The responses of isolated porcine intrapulmonary arteries with endothelium from normal newborn, 3 day-old and adult animals to 100 μM of ATP, 2-meSATP and ADPβS, pre-contracted with 30 μM $PGF_{2\alpha}$. Data taken from cumulative concentration-response curves at 100 μM. Each column represents the mean $\pm$ s.e.mean. The number of animals used is indicated under each column. $\#P<0.05$ ANOVA, $*P<0.05$ *t*-test.

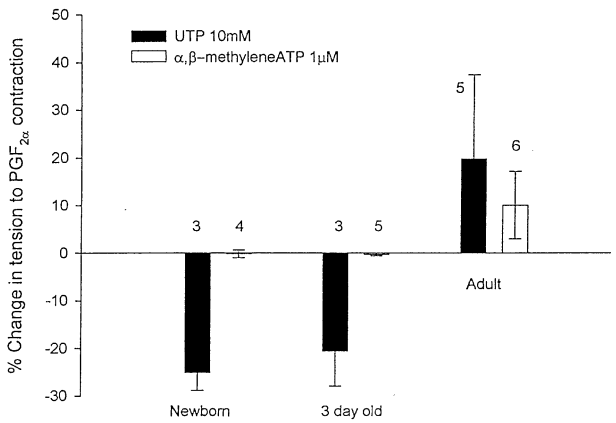

Figure 4 The response of isolated intrapulmonary arteries with endothelium from normal newborn, 3 day-old and adult animals to UTP, and α,β-meATP, pre-contracted with 30 μM $PGF_{2\alpha}$. Data taken from cumulative concentration-response curves at 10 mM for UTP and 1 μM for α,β-meATP. These concentrations evoked the greatest response in the adult vessels. Columns represent the mean $\pm$ s.e.mean. Removing the endothelium had no significant effect. The number of animals used is indicated above each column.

Discussion

In the present study, we have shown that the relaxation response to ATP in intrapulmonary arteries (IPAs) from normal foetal and newborn pigs was similar, increased with postnatal age, and was endothelium-independent at all ages. The response to ATP increased significantly between 3 days of age and adulthood. The relaxant effect of ATP demonstrated on the porcine foetal IPA is in accord with perfusion studies of

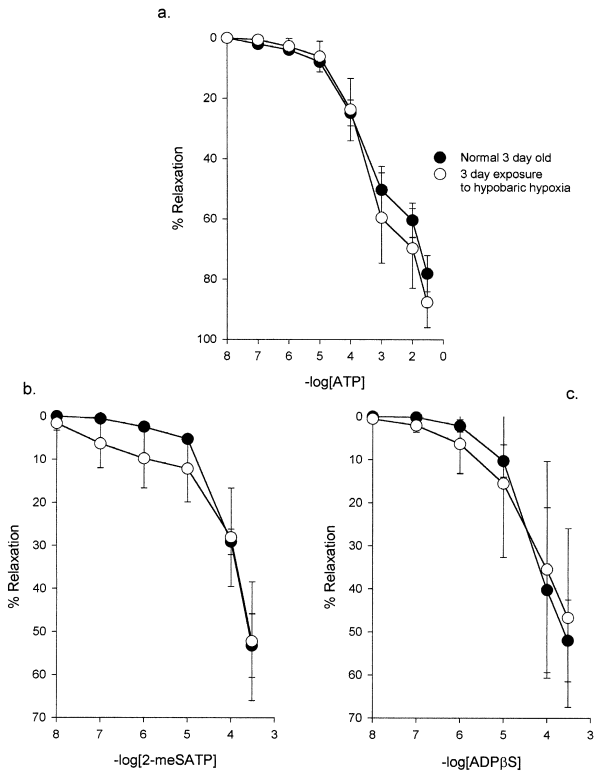

Figure 5 The effect of pulmonary hypertension following exposure to chronic hypobaric hypoxia for 3 days from birth on isolated porcine intrapulmonary arteries, compared with age matched normals, in vessels with endothelium. Plots show the mean $\pm$ s.e.mean response of arteries, pre-contracted with 30 μM $PGF_{2\alpha}$, to (a) ATP (PH, $n=5$, normal, $n=6$); (b) 2-methylthioATP (PH, $n=5$, normal, $n=4$); (c) ADPβS (PH, $n=4$, normal, $n=4$).

the intact newborn ovine lung in which ATP produced a decrease in pulmonary vascular resistance, but the site of action was not defined (Konduri & Woodard, 1991). ADP-induced endothelium-dependent vasodilatation in isolated ovine IPA was also reported to increase with age (Abman *et al*., 1991).

In the present study the relaxations of conduit porcine IPAs to ATP, ADPβS and 2-meSATP were endothelium-independent at all ages. The potency of the ATP-induced relaxation in large conduit IPA from 2 week-old pigs in this study was similar to the endothelium-independent relaxation in the smaller IPA from 10–17 day-old normal piglets reported by other investigators (Perez-Vizcaino *et al*., 1996). ATP and 2-meSATP were also found to induce an endothelium-independent relaxation response in adult rabbit main PA and small human IPAs (Liu *et al*., 1989a; Qasabian *et al*., 1997). However, relaxation was endothelium-dependent in the large IPA of man and rat (Greenberg, 1987; Liu *et al*., 1989b). The observations on the human vessels support the suggestion that the cellular distribution of ATP-receptors depends on the size and location of the pulmonary vessel studied, as well as species. In the systemic circulation the response to ATP and its analogues can be endothelium-independent but it is predominantly endothelium-dependent (Boeynaems & Pearson, 1990; Brizzolara & Burnstock, 1991; Corr & Burnstock, 1994; De-Mey & Vanhoutte, 1981; Kennedy & Burnstock, 1985). Purine- and pyrimidine-selective P2Y-selective P2Y-receptors have been cloned from bovine aortic endothelial cells, and mRNA for $P2Y_{2,4\text{ and }6}$ has been detected in vascular smooth muscle cells (Chang *et al*., 1995; Henderson *et al*., 1996; Harper *et al*., 1998; Hartley *et al*., 1998).

Conventionally, the receptor mediating the vasodilator response to ATP has been classified as a P2Y-receptor subtype, based on the rank order of analogue agonist potency (Abbrachio & Burnstock, 1994). We saw a clear agonist rank order for the relaxation response in adult animals, (ATP = ADPβS $\geqslant$ 2-meSATP > UTP > α,β-meATP). The rank order was less clear in the younger animals, where the contractile responses seen in adult animals were largely absent, and all agonists tested induced a relaxation response to a greater or lesser extent. The rank order of potency for inducing vasodilatation in the present study indicates a P2Y-receptor stimulated vasodilatation which becomes more effective with age (Abbrachio & Burnstock, 1994). In support of a $P2Y_1$-mediated relaxation response by ATP, the stable $P2Y_1$-receptor agonist analogue ADPβS produces similar vasodilatation (Zimmerman, 1996; Harden *et al*., 1998). We found that the potency of the relaxation response did increase for ADPβS with age, but did not increase for 2-meSATP, which may indicate the presence of two P2Y-receptor subtypes on porcine IPA smooth muscle. It must be acknowledged that the ATP induced responses in this study may be due in part to enzyme produced metabolites such as ADP and adenosine. However, ADP was recently found to be the most potent endogenous agonist for the platelet $P2Y_1$-receptor (Leon *et al*., 1997).

A single *in vivo* study is in accordance with our observation in that a P2Y-receptor was implicated in the ATP-induced decrease in pulmonary vascular resistance in the foetal lamb, using the P2Y-receptor antagonist cibacron blue (Konduri *et al*., 1997).

Pyrimidine-preferring receptors mediating vasodilatation are a recognized part of the P2Y-receptor family ($P2Y_{2,4,6}$) (Burnstock, 1997). In the newborn porcine pulmonary artery, UTP and ATP produced similar relaxations. It would seem possible, in young porcine IPA, that both ATP and UTP could act at a receptor subtype which recognises both agonists equally, such as the $P2Y_2$-receptor recently reported on adult rat small pulmonary smooth muscle (Harper *et al*., 1998; Nicholas *et al*., 1996). Organ bath studies of isolated adult rabbit pulmonary artery have shown endothelium-dependent vasodilatation to UTP (Qasabian *et al*., 1997) and other studies have shown that UTP can induce the release of nitric oxide from normal adult porcine IPA (McMillan *et al*., 1999). However, UTP did not induce a contractile response in the adult rabbit study, in contrast to the findings in the present porcine study. It would appear that the contractile response of porcine IPA to UTP may be masking a relaxation response in the adult vessels.

The mechanisms by which P2Y receptors mediate relaxation of vascular smooth muscle were not addressed in this study but it is accepted that members of this receptor superfamily activate G-proteins linked to phospholipase C producing an increase in intracellular calcium levels. We are not the first to report the vasodilatory action of P2 agonists on vascular smooth muscle (Perez-Vizcaino *et al*., 1996; Liu *et al*., 1989a; Qasabian *et al*., 1997; Brizzolara & Burnstock, 1991; Corr & Burnstock, 1994; Kennedy & Burnstock, 1985). The $P2Y_2$-receptor, present in vascular smooth muscle, when expressed in *Xenopus* oocytes can increase inward rectifying potassium currents (Mosbacher *et al*., 1998; Harper *et al*., 1998). In addition, the $P2Y_1$ and $P2Y_{11}$ receptors are, in theory, potential relaxant P2 receptors. The action of the $P2Y_1$ receptor on platelets has been linked to adenylate cyclase (Hechler *et al*., 1998) and the $P2Y_{11}$ receptor stimulates cyclic AMP production (Communi *et al*., 1997).

The present study showed that IPA isolated from newborn animals with pulmonary hypertension maintain the ability to relax to P2Y-receptor agonists, the response increasing in the hypobaric chamber as is normal between birth and 3 days of age. By contrast, in the same animal model of neonatal pulmonary hypertension, endothelium-dependent relaxation to ACh was abolished and the response to certain endothelium-independent agonists, such as nitric oxide was attenuated (Tulloh *et al*., 1997). The pulmonary arterial pressure (PAP) of both normal and hypoxic neonatal animals can be reduced *in vivo* by relatively low, but effective doses of ATP which do not reduce the systemic arterial pressure (Konduri & Woodard, 1991; Paidas *et al*., 1988). ATP has also been used successfully in the management of neonatal pulmonary hypertensive crises, as recently as 1994 (Brook *et al*., 1994).

In summary, the presence of a functioning P2Y receptor in the normal foetus and neonate indicates that ATP could help initiate the fall in pulmonary arterial pressure occurring at birth and help maintain pulmonary vasodilatation during the first weeks of extrauterine life, without metabolism to adenosine (Konduri *et al*., 1997; Konduri & Woodard, 1991). In the chronically hypertensive newborn piglet the retention of a relaxation response suggests that it may be appropriate to treat pulmonary hypertensive newborn infants with a stable ATP analogue to complement, or even substitute, treatment with nitric oxide (NO) or an NO donor. The P2Y-agonist analogue ADPβS, which mimicked the ATP responses in this study, displays an increased resistance to degradation and was effective in stimulating P2Y-receptor mediated responses in the adult rat and dog after oral administration (Hillaire-Buys *et al*., 1993). Such a compound producing a sustained plasma concentration of a potent vasodilator agonist would help to achieve a sustained reduction in PAP.

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