The Role of Purine Receptors in the Control of Pulmonary Vascular Reactivity in the Normal and Hypertensive Newborn.

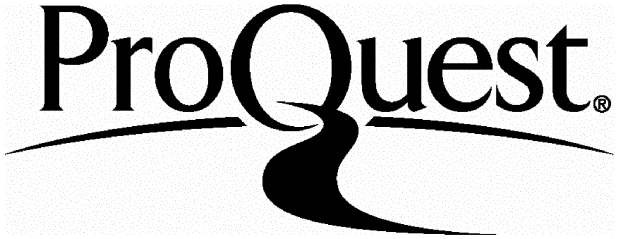
Thesis submitted for the degree of

Doctor of Philosophy

by

Michael Ross M^{c}Millan B.Sc. (Honours).

Institute of Child Health, University of London (1998).



Abstract of the thesis.

The porcine model of pulmonary development was used to investigate the role of purines and pyrimidines in the adaptation of the normal pulmonary circulation to extrauterine life {160,8}. The effect of neonatal pulmonary hypertension (PHN) was studied by exposing animals to chronic hypobaric hypoxia (50.8kPa.) {172,7}. *In vitro* force measurements of isolated vessels demonstrated a $P2Y_1$-purine receptor mediated endothelium-independent relaxation in precontracted newborn intrapulmonary arteries (IPA) , which increased with age and was not affected by PHN. ATP induced an endothelium-independent relaxation in intrapulmonary veins (IPV) which was unaffected by age. At resting tone, ATP induced a relaxation in IPA from neonatal piglets, developing a contractile response in the mature animal with a rank order of potency, uridine 5'-triphosphate ≥ ATP >> α,β-methyleneATP. However, in the porcine intrapulmonary vein (IPV) the order of potency was α,β-methyleneATP >> UTP > ATP. Desensitisation experiments indicated that in both IPA and IPV, ATP and UTP were acting at a different contractile receptor(s) than α,β-methyleneATP. PHN reduced the IPA contractile response to ATP and UTP. Radioligand binding on frozen lung sections demonstrated that [^{35}S] dATPαS bound to a $P2Y_1$-purine receptor but that [^{35}S] UTPαS bound to a non-selective nucleotide-receptor binding site on the media of the IPA. The normal transient increase in binding of both ligands at 3 days of age was reduced by PHN.

Vessels studied from a small number of normal and pulmonary hypertensive children gave similar results to those in porcine vessels, except that the P2Y-relaxation of IPA was largely endothelium-dependent and that [^{35}S] dATPαS bound selectively to a P2X-purine receptor on the media of the IPA from a normal 8 year old child.

In conclusion, the *in vitro* findings from the present study support the clinical impression that infusion of a purine will tend to cause vasodilatation of a high resistance pulmonary circulation such as that normally present at birth and found in PHN, and indicate a therapeutic potential for these compounds.

Dedication :

to my parents for instilling my freedom of thought and confidence to question and to my wife, Jo, for her patience and consistent faith in my ability.

Acknowledgements :

I would like to thank Prof.S.G Haworth and Prof. G. Burnstock for their supervision; Dr. P.J. Boels for sharing his analytical, theoretical and computing knowledge ; Mrs.J.Deutsch for her efforts in providing tissue during the present study; Dr.A.A.Hislop for comments regarding the autoradiography sections of the thesis; Dr.B.King for advice in relating my findings to recent emerging reports; Dr.A.Rubino for her advice on purine research in the pulmonary circulation.

I would also like to thank the British Heart Foundation for their financial support of the study.

List of Contents

Table of Figures, diagrams and tables. **Page Number.**

Chapter 1 - Background.

Chapter 2 - Materials and Methods.

Chapters of Results
Chapter 3.

Chapter 4.

Chapter 5.

Chapter 6.

Chapter 7.

Chapter 8.

Preface.

This thesis will begin with an introduction to the clinical questions addressed by the study, including an account of what is understood about adaptation of the normal pulmonary circulation to extrauterine life and the clinical syndrome of persistent pulmonary hypertension. The vasomotor control of blood vessels is discussed in general, before specifically describing the control of the pulmonary circulation. This is followed by an introduction to the field of purinergic research, including an historical backround, information about the role and distribution of purines and their classification. The choice of the experimental animal model selected is assessed.

A description of the materials and methods follows. The results from the study are divided into 6 chapters, including : the vasodilating actions of the purines, the contractile responses and autoradiographic ligand binding studies, each chapter incorporating data from a small amount of human tissue, and lastly comparative studies of porcine intrapulmonary veins. The discussion of the thesis begins with a critical analysis of the methods used , followed by a discussion of the conclusions of the present study. It ends with suggestions for future studies.

Glossary of Abbreviations and Terminology.

A23187 : *calcium-ionophore*
Acetylcholine: *ACh*
Adenosine 5'-diphosphate : *ADP*
Adenosine 5'-monophosphate : *AMP*
Adenosine 5'-O-(3-thiotriphosphate) : *ATPγS*
Adenosine 5'-triphosphate : *ATP*
Adenosine 5'-O-(2-thiodiphosphate) : *ADPβS*
Adenylyl-(β,γ-imido)diphosphate : *AMP-PNP*
Analysis of variance : *ANOVA*
Atrial natriuretic peptide : *ANP*
Attomols/mm^2 : *amols/mm^2 (unit of density)*
Caclitonin gene-related peptide : *cGRP*
Chronic hypobaric hypoxia : *CHH*
Cyclic adenosine 5'-monophosphate : *cAMP*
Cyclic guanosine 5'-monophosphate : *cGMP*
[^{35}S]-deoxyadenosine 5'-[α-thio] triphosphate : *[^{35}S]-dATPαS*
Diacylglycerol : *DAG*
Endothelium-derived relaxing factor : *EDRF*
Extracorporeal membrane oxygenation : *ECMO*
Guanine nucleotide-binding protein : *G-protein*
Hypoxic vasoconstriction : *HPV*
Institute of Child Health : *ICH*
Inositol 1,4,5-triphosphate : *IP_3*
International Union of Pharmacologists: *IUPHAR*
Intrapulmonary artery : *IPA*
Intrapulmonary vein : *IPV*
N^G-monomethyl -L-arginine : *L-NMMA*
α ,ß-methylene adenosine 5'-triphosphate : *α,β-MeATP*
2-Methylthio adenosine 5'-triphosphate : *2-MeSATP*
Myosin light chain kinase : *MLCK*
Nitric oxide : *NO*
Nitric oxide synthase : *NOS*
N^ω-nitro-L-arginine : *L-NA*
Non-adrenergic non-cholinergic : *NANC*
Persistent pulmonary hypertension of the neonate: *PPHN*
Pertussis toxin : *PTX*
Physiological salt solution : *PSS*
Phospholipase (C or D or A_2) : *PLC, PLD, PLA_2*
Potassium chloride : *KCl*
Pulmonary hypertension : *PH*
Pulmonary hypertension of the newborn: *PHN*
Prostacyclin : *PGI_2*
Prostaglandin $F_{2\alpha}$: *$PGF_{2\alpha}$*
Protein kinase (C or A) : *PKC, PKA*
Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid : *PPADS*
Serotonin : *5-HT*
Sodium nitroprusside : *SNP*
Thromboxane A2 : *TXA_2*
Transposition of great arteries : *TGA*
U-46619 : *TXA_2-mimetic*
Uridine 5'-triphosphate : *UTP*
[^{35}S] -Uridine 5'-triphosphate αS: *[^{35}S] UTPαS*
Vasoactive intestinal peptide : *VIP*
Ventricular septal defect : *VSD*

Chapter 1 : Background

Introduction to the present study.

There has been much clinical interest taken in the underlying cause and management of pulmonary hypertension especially in newborn infants with the persistent fetal circulation syndrome. The pathogenesis of this condition is still not understood and treatment of difficult cases depends upon prolonged treatment with nitric oxide. There is no other drug therapy available which is selective for the pulmonary circulation and does not have an adverse effect on the systemic circulation.

Research into purines has advanced rapidly in recent years and it has become clear that they have activity in the pulmonary circulation. It is therefore appropriate to assess their role during early pulmonary neonatal development as the pulmonary vascular resistance is falling after birth and in relation to neonatal pulmonary hypertension.

The purpose of the present study was to investigate the role of purine receptors in determining pulmonary vascular reactivity in the normal and pulmonary hypertensive newborn piglet. Isolated elastic intrapulmonary arteries and veins were taken from normal fetal pigs (5 days preterm), and normal piglets aged < 5 mins. - 3hrs. (newborn), 3, 6 and 14 days of age and adults aged 9 months. Corresponding tissue was also collected from pigs with pulmonary hypertension which had been exposed to hypobaric hypoxia (50.8 kPA) for 3 days, starting at birth, 3 or 14 days of age.

This study aims to address the following aspects of purinergic activity using the porcine model:

(a) P2X- and P2Y-purine receptor nucleotide agonist activity at raised and resting tone.

(b) The influence of the endothelium and the effect of putative receptor blockers upon purine-induced relaxation and contraction.

(c) The possible mechanisms of purine-induced relaxation and contraction.

(d) The distribution and density of P2X- and P2Y-purine binding sites using radioactive autoradiography.

It was hoped that by combining the information collected from each aspect, a picture of purine involvement in pulmonary vascular function would become more clear, and therefore the possibility of therapeutic application may become evident.

General principles of vasomotor control

This section gives an overview of the physiological systems which together modulate vascular smooth muscle tone, and introduces terminology and concepts used throughout the thesis concerning vasomotor control. The discussion is then extended to address the pulmonary circulation.

The mechanisms involved in the involuntary control of vascular tone include neural, smooth muscle, endothelial and blood borne components. A dual regulation of vascular tone results from interactions of perivascular nerve and endothelial cell associated factors. The Dale principle of one nerve, one transmitter was the accepted dogma for neurotransmission until 1976 {107}. This led to the classification of nerves according to the identification of the dominant neurotransmitter in nerve terminal vesicles and the derivation of the functional groups of sympathetic (adrenergic, noradrenaline-containing), parasympathetic (cholinergic, acetylcholine-containing) and peptidergic (containing peptides such as substance P and c-GRP) nerves. In 1976 Geoffrey Burnstock brought together the accumulated evidence available in the literature for the release of multiple functional transmitters, which is encapsulated in the term "co-transmission", from nerve terminals at smooth muscle junctions {63}. This philosophy has been found to be applicable to most nervous systems investigated and has advanced the understanding of nervous communication. The theory of "chemical coding", based upon the notion of variable combinations of the transmitters released in turn determining the outcome of nerve stimulation is a product of this work {138}. This extends to primary afferent and sensory-motor nerves which had been identified as containing combinations of neurotransmitters and neuromodulators {70}.

In general perivascular nerve terminals are confined to the adventitial-medial border, but sometimes in larger vessels penetrate further than the outer layers of the smooth muscle media. The translocation distance from nerve terminal to endothelium would seem to be too great to make the endothelium a likely target due to degradation and dilution of transmitters in the biomass. In addition to classical transmitters, evidence has been found for others such as vasoactive intestinal peptide (VIP), substance P, angiotensin, calcitonin gene-related peptide

(cGRP), nitric oxide and ATP{59,70}. However, not all transmitters that are co-released act as transmitters but rather as modulators. Transmitters (or their metabolites) can act on prejunctional terminal receptors to modulate the release process of many transmitters, such as adenosine and noradrenaline on P1-purinoceptors and α_2-adrenoceptors respectively, decreasing exocytosis {114,72}. But in some instance the interactions are more complicated. VIP for example, by prejunctional action reduces release of noradrenaline and ATP , but also acts at postjunctional receptors to augment the response to the same agonists {70}. The target cells with the closest proximity are smooth muscle cells , where the transmitters may bind to specific receptors on the cell membrane.

Receptors for transmitters usually fall into one of two superfamilies: ligand-gated ion-channels or 7-transmembrane GTP-protein linked receptors. Following activation, both of these receptor systems can initiate intracellular transduction-cascades which can produce either vasoconstriction or dilatation depending upon the agonist involved, the prevailing vascular tone and the environment the vessel is subject to (temperature, gas tensions). The cascades ultimately regulate the phosphorylation state of the enzymes, protein kinase A and C, myosin light chain kinase (MLCK) and phosphatases which together regulate the activity of the contractile filaments.

From the luminal direction, mediators of vascular tone can be found to originate from plasma (endocrine/autocrine factors), platelets (5-HT, ATP and ADP), smooth muscle (prostaglandins, ATP,ADP), and the endothelium (ATP, nitric oxide, prostaglandins, 5-HT, endothelin, vasopressin, substance P) which produce a combined effect {315,243}.
Until the 1980's the endothelium was seen as a translocation-regulating barrier with anti-thrombotic properties and as a site of degradation and uptake. The pivotal role of the endothelial cell in mediating vascular tone was only appreciated after the discovery that dilators such as nitric oxide (NO) were synthesised by these cells {137,310}. Endothelium-derived relaxation factors (EDRF's) are either released into the blood as autocrine factors, or diffuse into the smooth muscle. NO (or a transitional-stable species) stimulates smooth

muscle guanyl cyclase inducing an accumulation of c'GMP, altering the phosphorylation state of the contractile filaments and producing vasodilatation. There is not a great abundance of acetylcholine (ACh) in the blood stream, but local vasoactive agents such as bradykinin and ATP have both been reported to stimulate EDRF release {150,209 ,43}. Local stimuli such as shear stress and hypoxia can alter the release of substances (ATP, endothelin) from endothelial cells, possibly by an action through sensory afferents, which then act in an autocrine fashion on the vessel wall {39,40,38,272,273}.

Vasomotor control of the pulmonary vessels.

The mechanisms previously described for regulating vascular tone in general may equally be applied to the vessels of the pulmonary circulation.

In the lung perivascular sympathetic, parasympathetic and sensory-motor nerves have been identified, functionally and histologically. The nerves usually contain the classical neurotransmitters noradrenaline {264,143,153,198,278,386,396} and ACh {258}. However, there are exceptions such as the adult rat which has no adrenergic innervation of the pulmonary circulation {264}. In addition, there are neuropeptides such as vasoactive intestinal peptide (VIP), substance P and calcitonin-gene related peptide (cGRP) {419,10,250} which also act as neurotransmitters, as do purines such as adenosine 5'-triphosphate (ATP) and adenosine {199,200}and nitric oxide {242,365}. The transmitters listed above include vasoconstrictor and vasodilator substances some of which are involved in sensory-motor reflexes. These substances, can be co-localised in nerve terminals and are co-released. Noradrenaline released from sympathetic nerves acts at constricting α_1-adrenoceptors, but at the same time an underlying activation of vasodilating β-adrenoceptors occurs as well {197}. ACh may act at both prejunctional M2 muscarinic receptors to inhibit further release of ACh but also at post-junctional constricting M3 muscarinic receptors on the smooth muscle {274}. Adenosine and ATP act at prejunctional P1- and P2-purine receptors which reduce the release of noradrenaline {373}. Other cell types may release vasoactive substances into the pulmonary circulation. Neuroendocrine cells in the pulmonary arteries of

adult humans have been found to release serotonin (5-HT), which may induce a vasodilatation or vasoconstriction, at low or high concentrations respectively {99}.

In contrast to agonists being supplied from the exterior of the vessel by nerves, the endothelium produces a host of vasoactive substances as mentioned in the general vasomotor section, and stimuli such as stress, shear and hypoxia can alter the endothelial function. The involvement of basal EDRF release in the regulation of vascular tone is less certain in pulmonary arteries than in the systemic vessels {119}. Inhibitors of nitric oxide synthase (NOS) have been shown to remove the dampening effect of NO on the effect of vasoconstrictors such as noradrenaline released from nerves and prostaglandin $F_{2\alpha}$ from the bloodstream {239,324,119}. Nerve stimulation itself has been found to indirectly stimulate the release of endothelium-derived relaxation factors (EDRFs) by activating K^+-channels {70}. Paradoxically, NO has recently been suggested to operate via voltage operated and calcium-dependent K^+-channels {429}. The release of neurotransmitters may also result from axon-reflexes which involve sensory-motor nerves releasing transmitters such as ATP, cGRP and substance P {70}.

The unique environment of pulmonary vessels at the gaseous interface can be associated with features specifically geared towards the function of the pulmonary vascular bed. In order to maintain physiological levels of O_2 and CO_2 in the blood stream , the vascular bed must react appropriately so as to optimise ventilation and perfusion in response to changes in the gaseous environment. This is achieved when the vessels constrict following an hypoxic stimulus in contrast to the vasodilatation of systemic vessels. A rise in pulmonary arterial pressure to hypoxia was first reported by von Euler *et al* in 1946, in the cat {404}. Hypoxic vasoconstriction results in a redistribution of the blood flow to better ventilated segments, away from underventilated regions. The mechanisms underlying hypoxic pulmonary vasoconstriction (HPV) of arteries and veins would seem to involve a combination of many mediators and pathways, the end effect of which depends on the duration of the hypoxia. Inhibiting the action of cyclooxygenase and lipoxygenase products has been found to reduce

adult porcine arterial hypoxic vasoconstriction {270}. An intact endothelium increased the hypoxic vasoconstrictor response of the main pulmonary artery in the adult pig suggesting a role for the endothelium in the hypoxic response {188}. Endothelial factors have been associated with both of the HPV contractile phases of small and large PA in the adult rat {426}. Hypoxia has been shown to reduce basal levels of nitric oxide from cultured bovine pulmonary arterial endothelial cells and cGMP levels in arterial rings {407}. However, basal cGMP levels in arterial rings from newborn piglets after exposure to chronic hypobaric hypoxia were not reduced {399}.

The pulmonary artery relaxation response to PGI_2 is reduced following hypoxia, attributed to receptor down-regulation {368}. The haemostatic balance between TXA_2 (vasoconstrictor) and PGI_2 (vasodilator) has been an established part of systemic vasomotor regulation and can be applied in the pulmonary circulation. TXA_2- metabolites were high, while PGI_2- metabolites were reduced in children with congenital heart defects and either primary or secondary pulmonary hypertension {4,90}.

Chronic hypoxia-induced pulmonary hypertension has been linked to increased levels of catecholamines and increased or premature sympathetic innervation leading to increased vasoconstriction {205, 10}. Chronic hypoxia in adult rats produced an increased 5-HT-induced pulmonary vasoconstriction of isolated PA and *in vivo* administration augmented the pulmonary vascular remodelling and acute hypoxic pulmonary vasoconstriction {249,125}. HPV in the adult rat and human were found to be significantly dependent on the presence of calcium {190,426}. Pulmonary arterial smooth muscle cell depolarisation induced by hypoxia through inhibition of Ca^{2+}-dependent- and Ca^{2+}-independent-K^+-channels may initiate HPV, in the adult dog and rat {407,342}.

The fetal pulmonary vascular bed exists in a constricted state, possibly as a response to chronic hypoxia. The postnatal fall in pulmonary arterial pressure which occurs during adaptation to extrauterine life will be discussed in the next section.

Normal adaptation of the pulmonary circulation to extrauterine life.

A high resistance to blood flow is present in the fetal pulmonary circulation, because the intrapulmonary arteries are constricted . Blood from the right ventricle is directed through the ductus arteriosus and so bypasses the lungs. At birth the lungs expand and the blood vessels of the lung must dilate rapidly to lower resistance and allow an increase in pulmonary blood flow to produce efficient oxygenation of the blood. The change in pressure distribution between systemic and pulmonary circulations and an increase in arterial oxygenation saturation causes the ductus to close {181}.

In the normal lung, both human and porcine, structural adaptation to extrauterine life involves the entire length of the intrapulmonary arterial pathway {159,176,160,8}. At birth, thick interdigitating endothelial cells and brick-like immature smooth muscle cells show an immediate increase in surface : volume ratio and "spread" within the vessel wall to increase lumen diameter and lower resistance. Adaptation does not involve a reduction in the amount of vascular smooth muscle, as had been supposed. Rapid remodelling is probably facilitated by the relative lack of fixed type I collagen which is synthesised mainly after birth {159,176,160,271,8}. At birth, all pulmonary vascular smooth muscle cells are structurally immature with synthetic organelles predominating {159,176,8,155}. Their contractile myofilament concentration increases rapidly during the first six months of life. The pulmonary arteries and veins are innervated in the healthy human infant. Nerves extend to the respiratory units {10}. Sympathetic nerves containing tyrosine hydroxylase and neuropeptide Y were found in both vessel types. Vasoactive intestinal peptide occur only in arteries and somatostatin is only abundant in parasympathetic nerves of proximal veins. The innervation of the respiratory units arteries increases with age, more so than in the veins{10}. Innervation of the vasculature of the porcine lungs increases dramatically during the first weeks of life, involving sympathetic and peptide containing nerves{419}.

NO is thought to be a factor in reducing basal tone{119}. The nitric oxide pathway has been found to be less effective in the newborn than in later life, but it does play a role at birth. Basal NO release increases during late gestation in the pulmonary bed of the lamb and inhibiting nitric oxide synthase with L-NA prevented the oxygen-stimulated increase in flow to the pulmonary circulation in fetal lambs {369,280}. Ovine and porcine fetal and newborn {131,241} intrapulmonary arteries show a basal release of EDRF, but endothelium-dependent relaxation appears to be relatively poor at birth. In isolated porcine intrapulmonary arteries taken from newborn animals acetylcholine-induced relaxation does not occur until 60 hours of life {241}. Recent work has demonstrated a low binding density of muscarinic receptors on the extra-and intrapulmonary artery at birth {A.A.Hislop *personal communication* 1997}. This rapidly increases during the first 3 days of life. A large surge in ACh-induced relaxation then occurs from this point until ten days of age when the response began to decline gradually to a lower adult level by three months of age {241}. The m1 mRNA rather than the m3 mRNA predominates during the first week of life. M1 and M3 receptors have been shown to mediate acetylcholine induced relaxation of adult human IPA {297}. At birth acetylcholine stimulation had no effect on porcine arterial smooth muscle basal c'GMP accumulation , but significantly increased the accumulation in adult vessels. The relatively high basal level of c'GMP in newborn porcine arteries was associated with an abundance of endothelial NOS at birth, which was maximal at 2-3 days and then decreased gradually to the smaller amount normally present in adult vessels {186}. Intrapulmonary arteries from newborn lambs relaxed to the calcium ionophore A-23187, (sodium nitroprusside) SNP and NO in the presence of indomethacin and propranolol {380,3}.

Bradykinin induced an endothelium-dependent vasodilatation, via nitric oxide (NO), of fetal ovine pulmonary arteries and induced the same reduction in pulmonary arterial pressure in perfused lung preparations from one and seven day old piglets {146,321}. However, several other investigators have shown a greatly reduced response to both acetylcholine and bradykinin in fetal rabbit and immature porcine pulmonary arteries as compared with the adult response{425,2,448}. Endothelium-dependent vasodilatation to ADP of IPA was also found to be poor in fetal lambs but increased significantly in postnatal animals {3}. The

vasodilatation induced by endothelin-1, mediated partially by NO acting through the ET_B-receptor, was found to be greatest in the 1 day old perfused porcine lung {321}. At this age an increase in total binding of [I^{125}] endothelin-1 to the intrapulmonary artery corresponds with, and can be attributed to, transient binding to ET_B-receptors on the endothelium of elastic porcine intrapulmonary arteries {187}. Infusion of prostacyclin (PGI_2) induced pulmonary vasodilatation in intact fetal lambs, suggesting a role for PGI_2 during extrauterine adaptation {151}. Fetal and newborn rats display a lower clearance of PGE_1, which induces vasodilatation, than adult animals {303}.

Intrapulmonary artery smooth muscle cells were capable of relaxing in response to the NO donor SNP at all age groups {241}. The response increased with age in the pig but not the sheep {241,3}. Direct relaxation of intrapulmonary artery smooth muscle cells by activation of K^+-$_{ATP}$ channels is thought to occur in ovine fetal pulmonary vasodilatation . However, there have been contradictory reports as to the involvement of NO as a mediator in this response {80,96}. Vasodilatation induced by K^+-$_{ATP}$ channels occurs readily in isolated newborn porcine pulmonary arteries and the response does not increase with age {321}.

The site of vascular resistance and regulation of blood flow in the pulmonary circulation has been associated with the arterial segment, and especially the microvessels {332,329,158}. The contribution of the intrapulmonary veins to total pulmonary vascular resistance appears to vary with species and age. In the ferret the contribution of the IPV is similar to that of the IPA {333}. The contribution of the IPV to total pulmonary vascular resistance was found to be greater in young rabbits than in the adult animals {331}. The role of EDRF in controlling basal tone has been found to be greater for IPV than IPA in both newborn and adult lambs, suggesting that resistance may at least be partially regulated by the venous segment of the pulmonary circulation {379,142}. Intrapulmonary veins from newborn lambs displayed maximal relaxation to A-23187, SNP and NO with a greater sensitivity than the arteries, in the presence of indomethacin and propranolol {380}. IPV also relaxed more than IPA to both ACh (cGMP stimulator) and pentoxifylline (cAMP stimulator) in the fetal lambs{220}.

In the newborn piglet, the pulmonary arterial endothelium appeared to have a predominantly contractile effect as $PGF_{2\alpha}$ and KCl evoked a greater contractile response in intrapulmonary arteries which had endothelium than in those without endothelium {233}. After 3 days of age this relationship was reversed . Contractions induced by phenylephrine were greater in the absence of the endothelium at all ages. However ,the endothelium did not influence histamine-induced contractions at any age {233}and the sensitivity to KCl did not change as age increased {425} The magnitude of the contractions induced by phenylephrine increased with age, possibly because the α_1-adrenoceptor density was greater in the intrapulmonary artery of the adult sheep as compared to the fetal animal {241,233,367}. Plasma levels of endothelin-1 were high at birth and then decreased rapidly in piglets, even though vasoconstrictive ET_A-receptors are present at all ages {187}. These findings suggest that endothelin may help to maintain a high pulmonary vascular resistance *in utero*. In the normal human infant, mature pulmonary haemodynamics are believed to be established by six to eight weeks of life {161}. This has also been shown to be the case for the pig {174,154}.

Abnormal adaptation of the pulmonary vasculature to extrauterine life.

The fall in pulmonary arterial pressure associated with normal adaptation to extrauterine life does not occur in all human newborns, resulting in persistent pulmonary hypertension. Failure to adapt normally to extrauterine life has been studied in several species by exposing the newborn animal to hypoxia. When newborn pigs were kept in a hypobaric environment from birth the endothelial and smooth muscle cells retained their fetal shape and spatial relationships so that arteries failed to dilate normally {7,9}. Within three days, smooth muscle cells myofilament concentration was abnormally high and the cells had deposited excessive collagen (predominantly type I) and elastin around themselves, as had adventitial fibroblasts. These changes appeared to fix the porcine vessels in an incompletely dilated state. The vessels of hypoxic calves showed increased levels of mRNAs for type I and IV collagen and elastin {103,381}. The decreased lumen in hypoxic rats (8-36 days of age) was associated with increased extracellular matrix but reduced collagen concentration in the young animals {268}. Ligating the ductus arteriosus of fetal lambs results in remodelling of the pulmonary arteries {24}. In the porcine lung the magnitude of the structural response depended upon the age at initial exposure to hypoxia, even a short period of normoxia after birth was protective. In adult rats structural remodelling of intrapulmonary arteries resulting from chronic hypobaric hypoxia was found to be reversible {267}. Intrapulmonary veins can become remodelled in certain pathological conditions, such as mitral stenosis, but is less evident in hypoxic pulmonary hypertension {175,408,52}.

A similar pattern of remodelling with thick walled peripheral pulmonary arteries is seen in babies who die with PPHN {172}. Healthy humans , living at high altitude (chronic hypoxia) have a higher pulmonary arterial pressure than those living at sea level due to remodelling of the pulmonary vasculature. This situation was identified in Indian natives of Bolivia and China in whom non-invasive and invasive measurements were made and postmortem tissue analysed {177}. Isolated pulmonary arteries from patients with end-stage chronic obstructive lung disease have been shown to have a reduced endothelium-dependent vasodilatation response to ACh and adenosine diphosphate {118}. Species other than man have been

studied at altitude to investigate pulmonary hypertension, as a comparative and indirect approach to the human condition {398,15,120,178}.

The maturation of the EDRF pathway was prevented by exposure to chronic hypoxia from birth in piglets. In young animals first exposed to hypoxia at 3 days of age the newly established relaxation response was blunted. The raised pulmonary blood pressure of calves allowed to adapt normally for 4 hours and then exposed to chronic hypobaric hypoxia was not reduced by infusion of ACh *in vivo* {120}. However, a similar study in calves found vasodilatation to ACh and attributed their result to their being a greater active vascular tone *in vivo* {304}. EDRF relaxation and associated c'GMP levels were reduced in the adult rat pulmonary artery after 4-5 weeks exposure to hypoxia {343}. A reduction in pulmonary artery relaxation in response to ACh (and isoproterenol) was also found in adult rats when pulmonary hypertension was induced over 14 days not by hypoxia but a bolus dose of monocrotaline{12}.

In piglets with PPHN, smooth muscle cell relaxation to exogenous nitric oxide and Zapronast was reduced despite stimulating an increase in c'GMP levels {399}.

Intrapulmonary arteries from lambs with persistent pulmonary hypertension failed to relax to A-23187 and had reduced relaxation responses to SNP and exogenous NO. However, in vessels of the same animals the relaxation response to 8-bromo c'GMP was not significantly different from that in normal age-matched animals . This would suggest that at least part of the dysfunction lies between the activation of nitric oxide synthase and regulation of the smooth muscle contractile filaments {399}.

The amounts of PGI_2 and PGE_2 (prostaglandin E2) released from intrapulmonary arterial rings isolated from pulmonary hypertensive calves were reduced {15}. Exogenous PGE_1 and PGI_2 reduces the pulmonary arterial pressure and resistance in hypertensive juvenile pigs and in children with primary and secondary pulmonary hypertension {323,76,20, 303,102,206}. Plasma endothelin levels increase in the chronic hypoxia adult rat and in the newborn infant with PPHN {407,345}. A similar increase in endothelin-1 plasma levels was found to be associated with an increase in the vasoconstrictor ET_A-

receptor subtype density on IPA from pulmonary hypertensive neonatal piglets (296).
In adult rats, 72 hours of acute exposure to hypoxia resulted in an augmented relaxation associated with K^+-channel induced hyperpolarisation and blockade of Ca^{2+}- ion channels in the pulmonary artery {343} and with increased NO activity {407}. This may be attributed to an autoregulation response, where short term dilator activity increases in an attempt to compensate for hypoxic vasoconstriction.
Exposing newborn piglets to chronic hypoxia for 3 days from birth increased the vasodilatation of pulmonary resistance arteries induced by activation of K^+-$_{ATP}$ channels {41}. Levcromakalim has also been shown to inhibit hypoxic vasoconstriction in adult rat pulmonary resistance arteries {426}. The initial acute phase of pulmonary hypertension induced by monocrotaline administration was associated with a transient upregulation of vasoconstriction by potassium chloride, angiotensin II and norepinephrine{12,327}. However, it has been found that the acute hypoxic vasoconstriction event in the adult guinea pig does not initiate or determine the extent of remodelling by chronic hypoxia {393}.

Studies comparable to those done carried out using IPA have also been done with intrapulmonary veins (IPV). In adult rats with monocrotaline-induced pulmonary hypertension the relaxation of isolated IPV to A-23187, SNP and NO was not significantly different from their age-matched controls {380}. Intrapulmonary vein prostaglandin production was not reduced , in contrast to the IPA{15}. It would seem that the relaxation capacity of the IPV remains protected in a pulmonary hypertensive circulation. However, venoconstriction was found to be responsible for thromboxane-induced pulmonary hypertension in neonatal lambs, and in adult rats the contractile response to hypoxia was greater in the pulmonary veins than in the arteries {424,428}. An increase in the sensitivity of IPV from adult sheep to cyclo-oxygenase products underlies the increase in the hypoxic vasoconstriction response following a period of hypoxia. Oxygen radicals may be responsible for increasing the reactivity of the IPV in this study {371}.
Despite learning more about the structural and functional abnormalities associated with PPHN, clinical management of sick newborns remains unsatisfactory.

Persistent pulmonary hypertension of the newborn (PPHN): the clinical syndrome.

The pulmonary vasculature of newborn infants is notoriously labile even in the presence of an anatomically normal heart. In the presence of congenital heart disease the outcome of palliative or corrective surgery in newborn and young infants can be determined by the reactivity of the pulmonary vascular bed. In the presence of an anatomically normal heart, the clinical course of babies with PPHN is variable and the mortality is between 20-50% {133}. A persistent fetal circulation is the manifestation of many underlying abnormalities {161}. The most common cause of PPHN in the newborn with delay or failure of the pulmonary vascular bed to adapt to extrauterine life is hypoxia, either intrauterine, intrapartum or postpartum.

Babies who have an hypoxic delivery and die during the first two days of life with persistent pulmonary hypertension in the presence of an anatomically normal heart die with an unadapted pulmonary arterial wall structure {173}. In those who live longer, secondary changes develop with medial hypertrophy and the differentiation of smooth muscle cells in smaller, more peripheral arteries than is normal for age. The findings are similar to those in babies dying of idiopathic persistent pulmonary hypertension {172,328}.

The aim of clinical intervention in patients with PPHN is to produce a haemodynamic situation which will allow natural or surgical correction of an abnormality to proceed. The method of achieving this will be dependent on the patient's responsiveness to treatment which in turn will be determined by the underlying cause of the raised pulmonary arterial pressure. The development of a range of strategies has been important in reducing the mortality rate from the 20-50% reported in the recent past to a better outcome for the 1 in 1000 newborns with PPHN {281}.

Diagnosis and signs of the underlying problem may be achieved by a combination of clinical judgement, chest X-ray, cross-sectional echocardiography and Doppler studies.

The obvious point of intervention is to target the underlying disease / defect causing the raised pulmonary arterial pressure. In lung disease, ventilation with 100% O_2 may reduce alveolar hypoxia due to perinatal asphyxia or parenchymal disease and administration of buffers can reduce metabolic acidosis. A jet system of administering oxygen has been developed which increases the likelihood of gases reaching the underventilated regions of the lung {161}. Exogenous surfactant (liquid or partial-liquid) may help reverse atelectasis due to reduced surfactant function. A combination of problems may indicate the need for high frequency ventilation but this may be linked to sensorineural hearing loss, and poor developmental outcomes indicates caution as to the degree of ventilation used {281}. Pulmonary hypoxic vasoconstriction is usually severe and a range of vasodilating drugs has been used. These include tolazoline, sodium nitroprusside, prostacyclin, prostaglandin D_2, prostaglandin E_1, adenosine 5'-triphosphate and adenosine. However, these substances display a lack of pulmonary-selectivity and have various unwanted side-effects. Infusion of PGI_2 reduces the pulmonary arterial resistance in children with primary and secondary hypertension and enhances the selective effects of ventilation with 100% oxygen but may also induce systemic hypotension {76,20,75}. Whilst, calcium-channel blockers such as nifedipine do reduce the pulmonary arterial pressure, they have unfavourable systemic vascular and cardiac effects {293,423}. Extracorporeal membrane oxygenation (ECMO) has been shown to benefit critically ill babies with PPHN who have systemic hypotension and possibly allows surfactant function time to develop {National ECMO study in progress}. Unfortunately, the need for systemic heparinization increases the risk of intracranial haemorrhage.

Dysfunction of the vasodilator properties of the pulmonary arterial endothelium in the postpartum period has been thought to be involved in the pathogenesis of PPHN. Currently nitric oxide gas (NO) is the most effective therapeutic available . Being an inhaled gas, the effect of NO is restricted to the pulmonary circulation, as it is rapidly metabolised by haemoglobin {318}. NO has been shown to be successful in treatment of PPHN caused by hypoxia or hypercarbia {282,217}. NO requires access to the affected regions of the lung, which is not always possible if severe parenchymal disease is present. In these cases it has

been found that high frequency ventilation or surfactant treatment used as an adjunct to NO can improve effectiveness depending upon the extent of the parenchymal disease {346,218}. Clinical trials addressing the dosage regimes of NO are in progress in order to determine the optimal dose of NO to be given in specific circumstances. NO can be toxic, particularly to the brain when metabolised and may also reduce surfactant function {281}. Cyclic GMP phosphodiesterase inhibitors increase the effectiveness of NO but are not yet in routine clinical use. Treatment for pulmonary hypertension in infants was shown not to affect the long-term development of neurophysiological or cardiorespiratory function of the infants involved {27}. However, a recent study of nitric oxide and high frequency ventilation has shown that children treated successfully for persistent pulmonary hypertension can have long-term medical complaints {218}.

Adenosine 5'-triphosphate has been mentioned in this section as a vasodilator in infants pulmonary hypertension . Little research has been done to support the use of ATP in this disease or the possibility of systemic hypotension at high concentrations and action at receptors which are distributed throughout the various tissues of the body. The following section gives a background to the pharmacology of ATP (purines) and will be the basis of the present study.

Purinergic pharmacology.

Historical background

ATP is a primitive biological molecule which has been identified as both as an intracellular energy source and as an extracellular signaller during evolution {73}. Adenine based substances were first observed to elicit a physiological response in a mammalian system when extracts from different tissue, shown to contain adenylic acid, were injected into the circulation of various animals {121}. In each case a transient sinus bradycardia was recorded. Subsequently, adenosine was found to mimic the effect of the heart extract. The intracellular role of adenine based substances was not discovered until 1941 when it was shown than ATP had a central metabolic role {235}.

A non-adrenergic non-cholinergic (NANC) nerve transmission was first reported in the guinea -pig taenia coli and urinary bladder but the nature of the transmitter was uncertain {60}. However, in keeping with earlier reports that exogenous ATP could stimulate smooth muscle {189,57,13}, Burnstock *et al* produced evidence to suggest that the inhibitory substance released from non-adrenergic intrinsic nerves of the guinea -pig taenia coli was "ATP or a related nucleotide" {61}. Since that time , it has become clear that ATP is a co-transmitter in sympathetic, parasympathetic and sensory nerves {189,68}

The term "purinergic transmission" was proposed by Burnstock in 1972 and since that time purine pharmacology has evolved to become an important area of research which encompasses an array of physiological processes {62,305}. It has remained a challenge to apply a relevant receptor-classification system. The system is still being revised to accommodate new information about purine pharmacology.

Receptor classification .

Classification of purinergic receptors has proven to be complex and difficult to achieve. Responses induced by different purines indicated a possible subdivision of the receptors involved. It was proposed that the family of nucleoside-receptors with the greatest sensitivity

for adenosine, operating through adenylate cyclase and blocked by methylxanthines, would be called P1-purinoceptors (Table 1, Table 2). In parallel, a family of nucleotide-receptors with the greatest sensitivity for ATP and ADP and influencing prostaglandin synthesis, were called P2-purinoceptors {64}.

In 1985 it was proposed that the P2-purinoceptors should be divided into excitatory -P2X and inhibitory-P2Y families of receptor, based on the assumption that one receptor could not explain the variety of responses induced by ATP in a variety of tissues{65} (Table 3). Two structural analogues of ATP were found to be particularly useful in distinguishing the receptor responsible. α,β-meATP evoked excitatory responses in rat vas deferens where 2-methylthioATP did not {211,149}. 2-methylthioATP relaxed guinea-pig taenia, a tissue which did not respond to α,β-meATP. In addition α,β-meATP was found to desensitise only the ATP excitatory responses, by an intracellular phosphorylation process {244}. As more reports of purine induced responses were collated, it was noted that the traditional agonist rank orders of potency were beginning to vary from those taken to indicate the involvement of a definitive P2X- or P2Y- receptor. Instead, receptor subtypes were described on the basis of functional agonist selectivity data, incorporating new chemically modified purinergic agonists as they were developed, and pyrimidines yielding P2-X,-Y,-U,-Z,-T,and -D (Table 4).

In 1991 Dubyak concluded that purinoceptors were either receptor operated ion channels called P2X-purinoceptors {25,50,21,401), GTP binding protein- linked P2Y-purinoceptors {48,122,19}, or a non-selective ion channel known as P2Z, a theme acknowledged by Abbrachio and Burnstock in their 1994 re-classification proposal {1}. Most receptor classifications had been addressed only from a functional standpoint using available agonists and antagonists in classical pharmacological experiments. However, in 1993 the $P2Y_1$- and $P2Y_2$- receptors were cloned {412,247}followed by the P2X-purine receptor in 1994 {50,401} signalling the involvement of molecular biology in classifying receptor subtypes and a role in identifying new receptors. The receptors existing in 1994 were regrouped as $P2X_{1-4}$ and $P2Y_{1-7}$ by considering analogue activity, transduction mechanisms and the structural motifs of cloned receptors {1}. The rationale was to implement a more logical

system of nomenclature which had to include those receptors already identified but in addition be able to accommodate the growing purine- and pyrimidine-selective receptors being described {230,348}. It was also suggested (Purines '96 Symposium in Milan) that new receptors should only be classified if the clone could be expressed in a mammalian cell line (an unpopular suggestion considering that the first cloned receptor was from chick brain {412}, due to the growing number of receptor subtypes which had been suggested only on the basis of their being interspecies heterogeneity of base sequence. The current situation described in Tables 5 and 6 has been produced by Prof. G. Burnstock's group who will be co-ordinating receptor classification in an attempt to avoid the confusion that has occurred in the past.

Table 1. First subdivision of purine receptors proposed in 1978.

	P1	P2
Agonist potencies	Adenosine > AMP > ATP	ATP > AMP > adenosine
Xanthine antagonism	Yes	No
ANAPP3 antagonism	No	Yes
Localization	Presynaptic and postsynaptic	Mainly postsynaptic
Activity of derivatives	L-adenosine inactive	L-ATP active
	8-Bromoadenosine inactive	8-BromoATP active
	2'-Deoxyadenosine inactive	2'-DeoxyATP active
Function		
Transmitter release	Reduced release	Little effect on release
Direct on smooth muscle	Relaxation	Relaxation or contraction
Adenylate cyclase activity	Increased or decreased in some tissues	No effect
K+ permeability	Increased in some tissues	Increased in some tissues
Ca^{++} permeability	Decreased	Increased
Activation of cyclo-oxygenase	No	Yes
Cardiac activity	Negative inotropy (atria)	Positive or negative inotropy (ventricle)

From Ref. 382. Stone.T.W. (1989). Purine receptors and their pharmacological roles. *Advance in Drug Research..*, 18, 292-429.

Table 2. P_1-purinoceptor subtypes.

Nomenclature	A_1	A_{2a}	A_{2b}	A_3	A_4
Selective Agonists	N^6-Cyclopentyladenosine N^6-Cyclopentyladenosine	CGS-21680	NECA	APNEA N^6-Benzyl NECA	CV-1808
Selective antagonists	CPX CPT DPCPX 8-Cyclopentyltheophylline XAC	CP66713 KF17837 8-(3-Chlorostyryl) caffeine	CPX Alloxazine	1-ABOPX	CGS-15943
Transduction mechanisms	↓cAMP ↑IP_3, ↑K^+, ↓Ca^{2+}	↑cAMP	↑cAMP	↓cAMP ↑IP_3	↑K^+
Radioligands	[^{3}H]-CHA [^{3}H]-CPX [^{3}H]-R-PIA [^{3}H]-DPCPX	[^{3}H]-CGS-21680 [^{3}H]-NECA [^{125}I]-PAPA-APEC	[^{125}I]-PAPA-APEC	[^{3}H]-APNEA [^{125}I]IABA	[^{3}H]-CV-1808
Gene	a1 (rdc7 canine)	a2A (rdc8 canine; DT35 rat)	a2B (RFL9rat)	a3 (R226 rat)	
Structural information					
Amino acids	326 aa human 7TM 326 aa rat P25099 7TM 326 aa canine P11616 7TM 326 aa bovine P28190 7TM 328 aa rabbit 7TM	409 aa human P29274 7TM 410 aa rat P30543 7TM 412 aa canine P11617 7TM	328 aa human P29275 7TM 332 aa rat P29276 7TM	318 aa human 7TM 320 aa rat P28647 317 aa sheep 7TM	
Molecular mass (kDa)	37	45	36	36	
G-protein-coupling	$G_{i(1-3)}$	G_S	G_S	-	
Distribution	Brain (highest in cortex hippocampus, cerebellum, testis, adipose tissue, heart, kidney	Brain (highest in striatum, nucl. accumbens, tuberculum, olfactorium)	Wide. High in gastro-intestinal tract.	Testis. Wide in some species	

APNEA: N^6-2-(4-aminophenyl)ethyladenosine; R-PIA: N^6-(R-phenylisopropyl)-adenosine; CHA: N^6-Cyclohexyladenosine; 1-ABOPX: 3-(3-Iodo-4-aminobenzyl)-8-(4-oxyacetate)-phenyl-1-propyl xanthine; CPT: 8-Cyclopentyl-1,3-dimethylxanthine; NECA: 5'N-ethyl-carboxamidoadenosine; CPX: Cyclopentyl-1,3-dipropylxanthine; PAPA-APEC: 1-[6-Amino-2-[[-[4[3-[[2-[[4-aminophenyl)acetyl]amino]-ethyl]amino]-3-oxopropyl]phenyl]ethyl]amino]-9H-purin-9-yl]-1-deoxy-N-ethyl-β-D-ribofuranuronamide. (ref. 71. Burnstock.G. (1995). Current state of purinoceptor research. *Pharmaceutica Acta Helvetiae*. 69, 231-242).

TABLE 3 **Proposed subclassification of P2 purinoceptors in 1985.**

Name	***P2X purinoceptor family***	***P2Y purinoceptor family***	***P2Z***
Type	Ligand-gated channel	G protein-coupled	Non-selective pore
General agonist profile	α,β-meATP>β,γ-meATP>ATP≈2-MeSATP≈ADP	2-MeSATP>ATP=ADP>α,β-meATP≥β,γ-meATP	ATP^{4-}
Antagonists	α,β-meATP desensitisation Suramin Selectively blocked by PPADS ANAPP3	Suramin Reactive blue 2	Oxidized ATP

α,β-meATP, α,β-methylene ATP; β,γ-methylene ATP; 2-MeSATP, 2-methylthioATP; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; ANAPP3, 3-*O*-3[4-azido-2-nitrophenyl)amino] proprionyl ATP.

(ref 1. Abbrachio.M.P., Burnstock.G. (1994). Purinoceptors : are there families of P2X and P2Y purinoceptors. *Pharmacol Ther.*, 64, p445-475).

TABLE 4 **P_2 Purinoceptor: Characteristics and Subclassification in 1991.**

Receptor	P_{2X}	P_{2Y}	P_{2u} (P_{2n})	P_{2t}	P_{2z}	P_{2D}
Type	Intrinsic ion channel (Na^+,K^+,Ca^{2+})	G-protein-coupled (IP_3/Ca^{2+}/DAG)	G-protein-coupled (IP_3/Ca^{2+}/DAG)	G-protein-coupled (IP_3/Ca^{2+}/DAG/ cAMP)	Nonselective pore	G-protein-coupled (IP_3/Ca^{2+}/DAG)
Agonist profile	αβ–meATP≥βγ–me ATP>ATP≥ 2-meSATP≈ADP	2-meSATP»ATP» αβ–meATP	UTP≥ATP» 2-meSATP	2-ADP	ATP^{4-}	ApxA
Antagonist	Desensitisation by αβ–meATP; blocked by suramin, by ANAPP3 and PPADS	Blocked by suramin and by reactive blue 2		ATP	Oxidised ATP	

(ref. 71. Burnstock.G. (1995). Current state of purinoceptor research. *Pharmaceutica Acta Helvetiae*. 69, 231-242).

TABLE 5 **Classification of subtypes of P2X purinoceptor family on the basis of molecular & functional characteristics in 1997.**

P2 receptor subtype	*Tissue*	*Activity*	*Properties*	*References*	*Genbank/EMBL accession no.*
$P2X_1$	Vas deferens (rat)	2-MeSATP>ATP>α,β-meATP	$I_{Na/K/Ca}$	401	X80477
	urinary bladder (human)	ATP > α,β-meATP	$I_{Na/K/Ca}$	401	X83688
	urinary bladder (mouse)	(nd)	(nd)	415	X84896
$P2X_2$	PC12 cells (rat)	2-MeSATP>ATP (α,β-meATP inactive)	$I_{Na/K}$	50	U14414
					L43511
$P2X_{2}$-1 (short form)	Cochlea (rat)	(nd)	(nd)	192	L43511
$P2X_3$	DRG cells (rat)	2-MeSATP>ATP>α,β-meATP	$I_{Na/K}$	83	X90651
	DRG cells (rat)	ATP>2-MeSATP>α,β-meATP	$I_{Na/K/Ca}$	234	X91167
$P2X_4$	Hippocampus (rat)	ATP>2-MeSATP>α,β-meATP	$I_{Na/K}$	37	X91200
	DRG cells (rat)	ATP active, α,β-meATP inactive	$I_{Na/K/Ca}$	58	X87763
	Neurons (rat , human)	ATP> >2-MeSATP>CTP> α,β-meATP>dATP	$I_{Na/K/Ca}$	378	X93565
	Neurons (rat)	ATP>2-MeSATP>> α,β-meATP	$I_{Na/K}$	366	U32497
	Endocrine tissue (rat)	ATP>ADP>2-MeSATP>> α,β-meATP	$I_{Na/K}$	410	U47031

Table 5 continued over page.

(Table 5 continued...)

$P2X_5$	Neurons (rat)	ATPγS≥ATP≥2-MeSATP >>ADP (α,β-meATP inactive)	$I_{Na/K/Ca}$	91	X92069
$P2X_6$	Neurons (rat)	2-MeSATP=2-cl-ATP=ATP> ATPγS>>ADP (α,β-meATP inactive)	$I_{Na/K/Ca}$	91	X92070
P2X7 (=P2Z)	Brain & macrophage (rat)	BzATP>ATP>2-MeSATP>ADP UTP inactive	$I_{Na/K}$ then pore formation	385	X95882
P2Z (or P2X-like)	Macrophage (mouse)	(i) BzATP>ATP>UTP (ii) ATP>UTP>BzATP	(i) $I_{Na/K}$ (ii) I_{Ca}	298	

(nd), not determined; DRG, dorsal root ganglion; 2-MeSATP, 2-methylthioATP; α,β-meATP, α,β-methylene ATP; BzATP, 3'-O-(4-benzoyl)benzoyl ATP; dATP, 3'deoxyATP.

{ref.305 and 74}

TABLE 6 **Classification of subtypes of P2Y purinoceptor family on basis of molecular and functional characteristics in 1997.**

P2 receptor subtype	*Tissue*	*Activity*	*Properties*	*References*	*Genbank/EMBL accession number*
$P2Y_1$	Brain (chick)	2-MeSATP>ATP>ADP (UTP inactive)	PLCβ/$InsP_3$/Ca^{2+}	412	X73268
	Brain (turkey)	2-MeSATP>ADP>ATP (UTP inactive)	PLCβ/$InsP_3$/Ca^{2+}	129	U09842
	HEL (human)	(nd)	(nd)	14	*U42029/30
	Brain (human)	2-MeSATP>ADP>ATP	PLCβ/$InsP_3$/Ca^{2+}	361	-
	Insulinoma cells (mouse)	(nd)	(nd)	395	U22829
	Insulinoma cells (rat)	2-MeSATP>2-Cl-ATP> ATP (α,β-meATP inactive)	PLCβ/$InsP_3$/Ca^{2+}	395	U22830
	Placenta (human)	(nd)	(nd)	231	Z49205
	Endothelium (bovine)	2-MeSATP>ATP >>UTP	PLCβ/$InsP_3$/Ca^{2+}	180	X87628
	Prostate and ovary (human)	2-MeSATP>ATP=ADP	PLCβ/$InsP_3$/Ca^{2+}	202	-
$P2Y_2$	NG108-15cells (mouse)	ATP = UTP > > 2-MeSATP	PLCβ$InsP_3$/Ca^{2+}	247	L14751
	CT/43 cells (human)	ATP = UTP > > 2-MeSATP	PLCβ$InsP_3$/Ca^{2+}	311	U07225
	Lung (rat)	ATP = UTP	PLCβ$InsP_3$/Ca^{2+}	340	U09402
	Bone (human)	(nd)	(nd)	47	-
	Pituitary (rat)	(nd)	(nd)	87	L46865
	Wistar Kyoto strain (rat)	(nd)	(nd)	252	U56839
$P2Y_3$	Brain (chick)	ADP>UTP>ATP=UDP	PLCβ/$InsP_3$/Ca^{2+}	415	X98283

(Table 6 continued over page.

(Table 6 continued....)

P2Y$_4$	Placenta (human)	UTP =UDP>ATP =ADP	PLCβ/InsP$_3$/Ca^{2+}	94	X91852
	Brain (rat)	(nd)	(nd)	414	-
	Chromosome X (human)	UTP>UDP ATP inactive	PLCβ/InsP$_3$/Ca^{2+}	294	U40223
P2Y$_5$	HEL cells (human)	ADP>ATP>UTP	(nd)	228	U41070
	Lymphocytes (chicken , human)	ATP>ADP>2-MeSATP >> α,β-meATP = UTP†	(nd)	416	P32250 (or L06109)
P2Y$_6$	Aortic smooth muscle (rat)	UTP>ADP=2-MeSATP>ATP	PLCβ/InsP$_3$Ca^{2+}	305	D63665
	Placenta & spleen (human)	UDP>UTP>ADP>2-MeSATP>>ATP	PLCβ/InsP$_3$Ca^{2+}	93	X97058
P2Y7	HEL (human)	ATP>ADP=UTP	(nd)	5	U41070
P2Y8 (tentative)	Neural plate (xenopus)	ATP=UTP=ITP=CTP=GTP	PLCβ/InsP$_3$Ca^{2+}	44	-

(nd), not determined; 2-MeSATP, 2-methylthioATP; 2-Cl-ATP, 2-chloroATP;α,β-meATP, α,β-methylene ATP, PLCβ, phospholipase Cβ, InsP$_3$, inositol 1,4,5-triphosphate.

*Direct submission to Genbank of long and short form of gene encoding the same receptor protein. † Radioligand binding . {ref.305, 74}.

P2-receptor transduction systems

As previously stated, the receptors stimulated by purines represent members of two superfamilies: ligand-gated ion-channels (P2X) and 7 transmembrane GTP-binding protein coupled receptors (P2Y) {122}. In 1983 ATP was shown to be able to directly open membrane ion-channels of neural cells, and the same has since been demonstrated in gland and smooth muscle cells, resulting in membrane depolarisation {201,227}.
The ligand-gated ion channels are permeable to cations such as $Na^+$and K^+ but predominantly Ca^{2+}, displaying close homology to the inward rectifying potassium channel {50,401}. Variation between tissues has been noted for the ability of a maintained concentration of ATP to produce a desensitisation of the inward current and secondly, for the ability of α,β-meATP to mimic ATP in some tissues. By combining these properties with affinity for ATP and sensitivity to α,β-meATP the P2X-receptor subtypes identified to date can be assigned to one of three phenotypes: high ATP affinity and sensitivity to α,β-meATP, rapidly inactivating ($P2X_{1\ and\ 3}$); lower ATP affinity and sensitivity to α,β-meATP with little inactivation ($P2X_{2,4,5\ and\ 6}$); lowest ATP affinity ($P2X_7$ = P2Z). Only mRNA for $P2X_1$ has been isolated from vascular smooth muscle {6}. The increasing number of documented excitatory responses induced by other nucleotides such as the pyrimidines are not included in the current scheme (Table 1). This is partially due to controversy concerning the occurrence of a specific UTP-specific receptor or a less specific nucleotide receptor mediating pyrimidine -evoked contractile responses {348,355,406}.

P2Y-vasodilating receptors on endothelial cells in systemic vessels have been described, stimulating a rise in intracellular calcium concentration, by a mobilisation of calcium stores and ion influx. This has been associated with the release of nitric oxide from endothelial cells. Activation of phospholipase C (PLC) by stimulation of a G protein-coupled system results in the transient production of prostacyclin, preceded by a rise in intracellular calcium {316,78,42,286,361}. The ability of purines to induce prostaglandin release has been linked to the polyphosphate tail moiety structure {55}. PLC activity produces diacyclglycerol which

in turn can activate protein kinase C (PKC). PKC, along with calcium-dependent calmodulin kinases phosphorylates a cascade of proteins involved in smooth muscle relaxation. PKC additionally activates phospholipase D (PLD) hydrolysing phosphatidylcholine. PKC has been shown to be involved in the desensitisation of P2Y-purine selective receptors but not in the desensitisation of UTP-induced responses mediated by P2Y receptors {421, 341,325}. The absence of an effect on cAMP levels by a purine was one of the original criteria for a P2-rather than a P1-receptor mediated response. However, there have been several instances where P2-agonists have produced a response through modulation of cAMP, including the recently identified endogenous $P2Y_1$-receptor expressed on human C6 glioma cells {362}. P2Y-purine receptor agonists have also been found to increase cAMP in bovine vascular smooth muscle cells {387}. An increase in permeability to K^+ ions was originally used to classify a P2-receptor, but a purinergic response has been shown to occur in vascular endothelial cells but without consequence on smooth muscle relaxation {214,51}.

Rabbit thoracic aorta endothelial cells have been shown to release and take up uridine nucleotides, raising the possibility of vascular tone regulation by pyrimidines {357}. Recently a P2Y (P2U)-receptor mediating G-protein linked release of NO has been found to be selectively stimulated by UTP {230,245}. The activation of a Cl^- inward current and the release of IP_3-sensitive intracellular Ca^{2+} stores has been associated with the P2U-receptor in isolated rat pulmonary arterial myocytes {157,165}. P2Y-receptors have been identified with differing degrees of selectivity for pyrimidines, including the $P2Y_6$ -receptor subtype which was cloned from mature rat aortic smooth muscle cells (295). This receptor subtype has a preference for UDP over UTP and is coupled to PLC and mobilisation of intracellular calcium stores induced by the accumulation of 1,4,5-inositol triphosphate (IP_3) {81}.

Hyperpolarization by K^+-ion efflux and activation of Na^+/H^+ ion exchangers can also be involved in P2T-receptor signal transduction{193}. In neuronal tissue ATP has been shown to block the m-current of a K^+-channel and inhibit accumulation of cAMP by a PTX-sensitive adenylate cyclase in a range of cell types independently of IP_3-sensitive Ca^{2+} stores {122}. An example of where the two purine receptor superfamilies overlap can be found in the rat cardiac myocyte which has a 7 transmembrane G_s-coupled receptor apparently directly linked to an L-type Ca^{2+}-channel {360,31}.

Recent developments in purine research .

The use of agonist rank orders of potency as a means of receptor classification has been losing support as more analogues are tested in more tissues/species under different conditions and are found to exhibit varying degrees of activity dependent on metabolic degradation. This was highlighted in a recent review regarding activity at P2X-contractile receptors where 2-meSATP can be more potent than α,β-meATP ($P2X_{1-3}$), questioning the use of these two agonists as the cornerstones of the recent classification system {216}. However, the variation in activity of agonists in different preparations has been suggested to be too great for metabolic stability alone to be responsible. Partial agonism effects at receptors other than those the agonist was thought to be selective for may be a more suitable explanation {300}. The challenge for the future is to correlate receptor primary sequences to the physiological function performed by a given receptor type {163}. When after revision those receptors identified by molecular biology have been allocated to their correct subtype, a certain degree of uniformity and agreement in the basic agents used to classify receptors will be required if we are to continue to use an agonist-rank order of activity to classify receptor subtypes. Such an approach in combination with the use of selective antagonists and novel agonists for improving the division of functional receptor subtypes ought to be successful. To date molecular biology has been invaluable for filtering newly identified receptors into the current classification scheme. However, the real potential must be to utilise information collected concerning receptor primary sequences and tertiary structure to enable rational design of specific agonists and antagonists. It may be found that the non-nucleotide structures are

pharmacologically effective, which would reduce non-specific activity of nucleotides due to their ubiquitous occurrence.

Functional classification has been made worse by the lack of receptor antagonists which withstand the test of time. Recent studies show that NF023, a derivative of the purine non-specific P2 antagonist suramin can specifically inhibit P2X-mediated contractions of the rabbit saphenous artery {447}. Another selective P2 -inhibitor, ARL 67085, has been developed by Astra Charnwood. This inhibitor is now to be taken through the development process as an anti-thrombotic agent. It appears to exhibit greater efficacy than aspirin and reduced bleeding times when compared to GP IIb/IIa receptor antagonists {195}. But both NF023 and ARL 67085 are receptor antagonists which target the ligand binding site, recognising the negatively charged motif inherent to the nucleotides. Greater specificity might be achieved by influencing receptor state in a more subtle way. Allosteric modulation is a possible indirect route making use of variation in the consensus regions of different receptors of the same functional family. Such an approach may also encourage the development of non-nucleotide based ligands both as agonists and antagonists and subsequently increase the likelihood of clinical development {132,49}.

The use of agents directed to purine receptors but not structurally based upon purines may hold the key to increased selectivity, especially agonists. Some tissues and even cells are heterogenous for purine receptor families and subtypes and therefore the net effect of an agonist may be more important than its selectivity. It has been recognised that separate P2Y-receptors with specificity for purines and pyrimidines can co-exist on the same cell, such as the endothelial cell {84}.

In the years following the first subclassification of P2-receptors into P2Y and P2X doubt has been cast on the validity of using some of the agents used to classify the receptors. α,β-meATP was originally identified as an agonist stable to hydrolysis which displayed selectivity for the P2X-excitatory purine ionotropic receptor family {65} and ,more recently, as a trititated radioligand to localise the receptor binding sites in the mature lung of cat and human {34}. However, others have shown that α,β-meATP can also produce vascular relaxation which is only partially endothelium-dependent {238,237,215,53}. It has also been

suggested that α,β-meATP acts by being a non-selective cation channel blocker {108}. It is now appreciated that nucleotide analogues of ATP are not consistently stable to hydrolysis {216}. For example β,γ-methyleneATP is metabolised to adenosine in a range of tissues to varying degrees {382,216}. P2-purine receptor antagonists have been developed from dyes whose selectivity has always been questionable. Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) , originally described as a P2X-antagonist but recently shown to block aortic endothelial cell P2Y-receptors {430,446,422,263,56}. Reactive blue, supposedly P2Y-selective has non-selective properties repeatedly documented {67,339}. In addition to these problems, some receptor antagonists quoted in the literature, such as PPADS, suramin, reactive blue and agonists such as α,β-meATP, ATPγS and AMP-PNP will also inhibit ectoATPase activity and therefore increase the availability of purines in different tissues {191,100,216, 86,85}. Advances are being made with the continued development of agents such FPL 67156, a selective ectoATPase inhibitor {101}.

These examples demonstrate that the extent to which purinergic chemistry has developed so far demands that caution be exercised when inferring any conclusions from studies with established and newly developed pharmacological agents.

Despite the lack of specificity currently associated with agents acting at P2-nucleotide receptors, their therapeutic potential has been considered. Some of the areas targeted include diabetes (P2Y), cancer ($P2X_7(z)$), cystic fibrosis ($P2Y_2$), pulmonary hypertension (P2Xu), gastro-intestinal dysfunction (P2Y), renal failure (P2X and P2Y), central nervous disorders and nociception ($P2X_3$) {1,302}.

Purinergic pulmonary vascular control

Interest in the action of purines on the adult pulmonary circulation has been extensive, providing a source of information for designing experiments to investigate the neonate. There are few studies concerning how the age of an animal affects purine-induced vascular responses. The contractile response evoked by electrical nerve stimulation of the central caudal artery of the rat was found to have a greater purinergic α,β-meATP-sensitive

component in the young animal {16}. It has also been shown that both the endothelium-dependent and -independent relaxation response decreased in the rabbit aorta from between 4 and12 months of age, but only the independent response diminished in rats between 4 and 14 weeks of age{88,219}. In the rabbit study, it was thought that the findings could be explained by a decrease in smooth muscle function with age.

For an agent to be considered as having a physiological role in a tissue it must have a natural source. The increased levels of ATP in the postnatal pulmonary circulation of the lamb have been associated with release from platelets {226}. Purines have been shown to be released in combination with noradrenaline from nerves in the adult rabbit pulmonary artery {212,213,277} by high KCl and oubain, which also caused non-neuronal release. Purinergic neurotransmission has been reported in the adult rat and rabbit {383,199,200}. The basal level of purine release has been found to be greater in the periphery than in the main arteries of the adult rabbit lung and increased by α_1-adrenoceptor stimulation {390,389}. Hypoxia abolishes adrenergic contractions evoked by nerve stimulation in the rabbit pulmonary artery, and may also reduce the levels of co-released purines {248}. Purines have been shown to be released from cells in the vessel wall other than nerve terminals. Increased flow in the adult rat lung increased the levels of circulating ATP released from the endothelium {169}. Increased levels of purine nucleotides have been shown to increase intracellular calcium levels in bovine pulmonary endothelial cells, regulated by ectoATPase activity {275}. It should be noted that both adenosine and ATP acting at P1 and P2 prejunctional receptors have been found to reduce transmitter release in normal adult rabbit pulmonary artery {196,108}.
For an agent to be able to repeatedly produce an effect there must be a method of removing the agent from the site of action. The pulmonary circulation is capable of almost completely metabolising vascular ATP and ADP and 60% of AMP in one passage through the lung {30,353,104,105,82,363,116}. The main breakdown-products are adenosine, inosine and AMP, indicating ATPase and ADPase activity. The pulmonary arterial endothelium has been identified as the site of nucleoside phosphorylase {113} and ecto-ATPase activity {116,322}.

Adenosine produced by metabolism of ATP inhibits further neurotransmitter release from nerves by the action at pre-junctional P1-purinoceptors {384}.

The first functional investigation of the lung and purines reported that ATP produced tone-dependent responses, constricting a resting vascular bed but dilating one which was preconstricted {352}. The next report on this subject concluded that an ATP blocker (2,4-xylenol) had no significant effect on pulmonary hypoxic vasoconstriction {171}, the unresolved phenomenon at the centre of the present study {407,403,418}. That ATP may regulate pulmonary vascular tone was demonstrated by showing that ATP induced prostacyclin (PGI_2) release from isolated piglet lungs{179} .

Purines have been shown to be capable of constricting and dilating blood vessels in a tone-dependent fashion. The potential constricting properties of ATP on intrapulmonary vessels are of particular interest because of the vasoconstrictor response of the vessel to hypoxia. At resting tone, P2X-purinoceptors have been reported in the adult human intrapulmonary artery and have also been found in the intrapulmonary artery and veins of the mature rat {237,238}, though in the cat adenosine resulting from metabolism of ATP also induced vasoconstriction at P1 (A1)-purinoceptors {287,291,71}. A P2X-mediated rapid transient influx of calcium has been found to be stimulated by both ATP and other P2 nucleotide analogues in isolated adult rat pulmonary arterial myocytes {157}. In isolated perfused rat lungs it was reported that purines and pyrimidines produced similar increases in pulmonary pressure {348}. It was suggested that a novel pulmonary pyrimidino-receptor population was present, which might have a role in hypoxic vasoconstriction {168}. The involvement of the P2X-purinoceptor in acute hypoxic vasoconstriction was investigated in mature rats but it was concluded that purines were not responsible for the response{262,266}. The non-selective adenosine receptor antagonist 8-phenyltheophylline was found to abolish acute hypoxic vasoconstriction in adult rats *in vivo* {392}.

Purine induced vasodilatation has been reported in a range of systemic vessels at raised tone and in general the response is dependent on the presence of the endothelium, and the release of PGI_2 {43} and /or EDRF {115}. An endothelium-dependent P2Y-purinoceptor vasodilatation of the intrapulmonary artery has previously been reported in the mature guinea-pig and rat {240,170,238,359}, but reports are inconsistent for the mature human and cat with respect to endothelial-dependency {179,237,287,289,265}. However, recent reports found purine nucleotide-induced vasodilatation was endothelium-independent in neonatal lamb isolated IPA and adult rabbit main PA{319,326}. The mobilisation of intracellular calcium stores preceding PGI_2 release in bovine pulmonary arterial endothelial cells is greatest following stimulation by the ionised species of ATP^{4-} and UTP^{4-} {246}. ATP-induced vasodilatation of the adult rat pulmonary circulation is inhibited by blocking K^+-$_{ATP}$ channel hyperpolarisation {169}. Metabolism of ATP to adenosine acting at P1 (A2) -purinoceptors has been suggested, at least in part, as the vasodilating mechanism in a number of studies {377, 290, 287, 288, 238}. Adenosine has been reported to act on smooth muscle A2-receptors inducing vasodilatation of adult human intrapulmonary arteries {261}.

Endothelium-independent vascular relaxation has been said to be the exception to the rule for P2Y-induced responses. But as the number of examples grow the mechanism underlying the direct ATP-relaxation response becomes more interesting. For vessels such as the intrapulmonary artery, an endothelium-independent mechanism may be crucial in addressing the reversal of hypoxic vasoconstriction resulting from a pathological condition.

Endothelium-independent P2Y-purinoceptor mediated relaxations have previously been reported in the adult rabbit mesenteric artery{257}, portal vein{215}, hepatic artery {53} and canine coronary artery {97,98} and the $P2Y_6$ was cloned from rat aorta smooth muscle cells {81}. Relaxation of the adult rabbit aorta has been attributed to the direct action of ATP and adenosine on the smooth muscle cells: a response assigned to the P3- "nucleotide" receptor {89}. But another study on the same vessel concluded that ATP acts partly through the endothelium {88}.

Some progress has been made in understanding the pathway(s) which are responsible for the vasodilatation induced by P2Y-receptors in the lung. Mobilisation of intracellular stores and

an influx of calcium was induced by ATP in isolated mature porcine and bovine pulmonary endothelial cells {193,203,417}. Phospholipase C (PLC) activation via stimulation of separate P2Y- and P2U-receptors was indicated by the accumulation of inositol phosphate (IP_3) induced by ATP, UTP and adenine analogues. The PLC activation was insensitive to pertussus toxin {84}. ATP can also activate phospholipase D (PLD), stimulating the release of PGI_2 {256}. Calmodulin-dependent phospholipase A_2 (PLA_2) was activated by ATP, UTP and adenine analogues, inducing prostacyclin release {245}. The receptors involved specifically recognised the fully ionised species of the nucleotides {246}. ATP evoked an accumulation of c'GMP in rabbit extrapulmonary arteries which had an intact endothelium, in the presence of indomethacin {204}. ATP was equipotent with ADP, and ATPγS was also active in releasing PGI_2 from 0-2 day old piglet lungs {179}. The vasodilating effect of ATP in rabbit intact extrapulmonary artery was abolished by moderate acute hypoxia, with an associated reduction in c'GMP accumulation{204}.

There have been several *in vivo* studies addressing the response of the pulmonary circulation to purines in animals and humans. In newborn animals, an infusion of ATP-$MgCl_2$ reduces the raised pulmonary arterial pressure of lambs and piglets at concentrations significantly lower than those which induce systemic hypotension {130,223,306,308,221,225}. Pulmonary vasodilatation to ATP-$MgCl_2$ infusion was shown to be partially mediated by nitric oxide, in the neonatal lamb {131}. We do not know whether birth induces a rise of plasma ATP in the newborn pig, but increasing the pulmonary plasma levels of ATP or adenosine in the fetal lamb to the higher level found after birth, caused a fall in pulmonary resistance to the lower postnatal level {222}. Similarly, increasing the level of oxygen in the fetal lamb pulmonary circulation, increased the ATP level to that seen after birth, and the pulmonary vascular resistance decreased by 3-fold {224}. ATP-$MgCl_2$ infusion does not reopen the ductus arteriosus of 3-day old piglets . Were ductal closure to occur *in utero* it would increase pulmonary vascular pressure{307}.

Acute administration of ADP to young calves was found to produce a greater rise in pulmonary arterial pressure in animals which had been exposed to hypobaric hypoxia than

normal animals {338}. Both the rise in PAP and the vascular remodelling shown to occur following chronic administration, were attributed as secondary to ADP platelet activation, not hypoxia. The raised PAP resulting from U46619 in the neonatal lamb was successfully reduced by ATP infusion {131}. ATP has also been shown to be effective in adult patients (46-77 years) with moderate to severe chronic obstructive airways disease who were pulmonary hypertensive. Studies on the mechanism of action indicated that inhibition of hypoxic pulmonary vasoconstriction was responsible for the fall in pulmonary resistance {141}. It was possible that adenosine resulting from the metabolism of ATP may have been responsible for these responses. However, adenosine was shown to be ineffective in these patients {139,140}, despite effectively blocking hypoxic vasoconstriction in the dog{266}. The raised PAP in neonatal lambs from was reduced by ATP-infusion has been used in the management of pulmonary hypertensive crises in children, where infusions had some clinical success without the side-effects of more traditional treatments such as tolazoline {54,145}. ATP has also been routinely used to control blood pressure during cardiopulmonary bypass {92}. Additional advantages of ATP-infusion include myocardial protection (109) and restoration of endothelial function post-hemorrhagic shock {409}.

There is now a growing body of evidence suggesting that purines do have a physiological role in the control of pulmonary vascular reactivity in several species , including man, in both normal and pathological conditions. It is against this background that the present study on the newborn porcine pulmonary circulation has been designed.

Chapter 2 - Materials and Methods

Materials

Choice of an animal model in which to study the role of purines in the normal and hypertensive newborn pulmonary vasculature.

Pulmonary hypertension is caused by a variety of insults but is commonly caused by hypoxia, both in the neonate and adult with chronic obstructive airways disease {182}.

In order to investigate the clinical condition of persistent pulmonary hypertension of the newborn we required a model fulfilling the following criteria:

(a) adaptation of the normal pulmonary circulation to extrauterine life should be similar to that in the human infant.

(b) the insult giving rise to pulmonary hypertension should be similar to that experienced by the sick infant.

(c) the disorder produced by experimental persistent pulmonary hypertension should be similar to the disease found in human infants.

Alternative models of pulmonary hypertension.

As an alternative to using hypobaric hypoxia {185}, the inspired oxygen concentration can be reduced to produce the same pathological (ie. Endothelial cell structure) result and abnormal haemodynamic condition {343,135,18,370}. Other approaches include the use of drugs known to have a pulmonary hypertensive effect . These include using repeated doses of indomethacin (twice a day for 3 weeks) {269}, giving the thromboxane A2 agonist U46619, commonly used to raise pulmonary tone *in vivo* or in isolated preparations {28,314} and using synthetic platelet activating factor {301}. In addition to the physiological agents available, pulmonary hypertension can be induced by glass bead and air embolism{323,320} or chemicals such as monocrotaline ,which may raise plasma serotonin {12,210}. Bacteria such

as group B streptococcus produce pulmonary hypertension in newborn piglets {26}. A persistent fetal circulation can be induced by partially compressing the umbilical cord or ligating the ductus arteriosus of fetal lambs {24,380,376}. A period of intrauterine hypoxemia has been used to induce persistent pulmonary hypertension in newborn rats and rabbits {144,303}. To assess the effects of alveolar hypoxia *in vivo* , invasive vascular pressure measurements can be made or non-invasive techniques can be used such as arteriography to analyse changes in vessel dimensions {11}.

Why the model we decided upon was considered the most suitable.

It has been possible to record the series of physiological and structural changes which occur during normal adaptation to extrauterine life and in the presence of a persistent fetal circulation in the newborn pig. The rate at which the pulmonary and systemic arterial blood pressures in normal piglets change during the first hours and days of life is similar to that seen in the human infant {174,110}. Hypoxia has been identified as a common cause of pulmonary hypertension and therefore our approach of exposing newborn animals to hypobaric hypoxia would appear to be physiologically appropriate {182}. Of the species investigated at altitude, the pig and calf develop the most severe pulmonary hypertension {120,398}. The pig has been chosen rather than the cow because of the greater litter size and because the newborn pig is similar in size to the human newborn. The observations generally used to confirm the presence of pulmonary hypertension in the experimental newborn animal include systemic arterial oxygen desaturation which indicates the magnitude of the right to left shunt through fetal channels in the presence of a high pulmonary vascular resistance ,and after death, the degree of right ventricular hypertrophy and muscularisation of the intrapulmonary arteries. Newborn piglets with PPHN are desaturated, have an increased heart weight ratio [weight of right ventricle / (weight of left ventricle + septum)] and an increase in pulmonary arterial wall thickness{159, 7}. The model is also effective in reproducibly inducing pulmonary hypertension in animals which have first had an opportunity to adapt normally to extrauterine life, for 3 or 14 days before exposure to chronic hypobaric hypoxia.

The practical disadvantages of the porcine developmental model are that the Large White pig is an expensive animal model with a relatively long period of gestation. Studies have been made using isolated pulmonary vessels{262}, perfused lungs {128}and whole animals in a variety of species {312}. In the present study the decision was made to study isolated intrapulmonary arteries and veins, to see if there was heterogeneity of the response in these vessels at any one age, and to find out if the responses changed with age in one or all segments , and if so if they changed at a different rate in intrapulmonary arteries and veins. These factors would be difficult to study in a whole lung preparation.

Sources of healthy porcine tissue

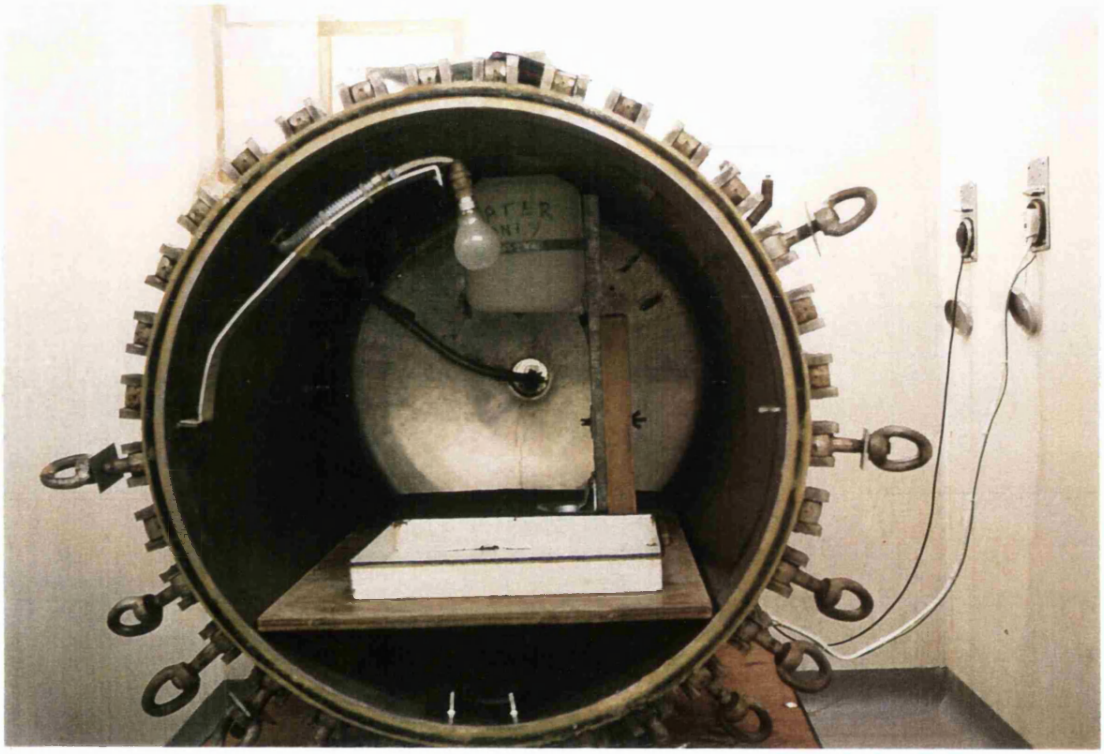
Pregnant Large White pigs were brought to the ICH from approved farms. Each animal had a past history which indicated that a large litter could be expected of approximately 10 piglets. The sows were admitted 2 weeks prior to the calculated farrowing date, to allow them to recover from the journey to reduce stress. They were placed in a pen with ample straw and sawdust bedding and given water *ad libitum*. The room temperature was regulated and lighting followed natural daytime periods. Feeding was restricted near the farrowing date and antibiotics given to reduce the chances of infection (ie. milk fever). Husbandry farrowing indicators were used to follow the progress of the sow and when the time for delivery was approaching the sow was placed in a farrowing crate for her own safety and to avoid injury to piglets after birth. When the farrowing was imminent the animal was checked frequently throughout the day and night. After the birth, a red light or ceramic bulb kept the piglets warm for the first 24 hours. In addition, fetal and young animal tissues were obtained as heart lung blocs. Tissue from adult animals was obtained from an abattoir.

Induction of pulmonary hypertension by chronic hypobaric hypoxia.

Immediately the animal was born the umbilical cord was cut, the piglet's airways were cleared and the animal dried with a towel. Then a period of approximately 20 minutes was allowed for the piglets to obtain some colostrum from the sow.

Normal piglets were exposed to chronic hypobaric hypoxia (50.8kPa) for a period of 3 days: either from birth to maintain the high fetal pulmonary arterial pressure or from 3 days or 14 days (PHN). The chamber was opened 3 times a day for cleaning and replenishing food and water, carried out as rapidly as possible to ensure near continuous exposure to hypobaric pressure (Fig. 1).

Fig 1. Photograph of the hypobaric chamber used in the study to induce chronic pulmonary hypertension.



Source of Human tissue.

Human tissue was collected from children who had been treated at Great Ormond Street Hospital for Sick Children, London. It was not always possible for the tissue to be stored in cold physiological salt solution , due to the time of day or hospital staff taking the samples.

Table 1. Clinical details of children from whom tissue was studied.

Specimen number	Details of child
Normals	
3680	8 years old, donor, mild asthmatic. Accidental death.
4095	5 years old. Tuberous sclerosis, previous heart transplant. Heart failure from acute rejection. Normal PA histology.
4183	4 month old. TGA/VSD, pulmonary arterial banding, switch followed by myocardial infarct. ECMO. Normal pulmonary arterial presssure.
Pulmonary hypertensive	
3508	16 years old. Primary PHN, lung removed during transplantation.
4039	16 years old. Primary PHN, lung removed during transplantation.
4066	15 years old. PHN secondary to complex congenital heart disease, lung removed during heart / lung transplantation.
3954	36 hours old. PHN, congenital lung dysplasia and hypoplasia.
4087	4 month old. PHN, congenital lung dysplasia and hypoplasia.
4139	10 days old. PHN, congenital lung dysplasia and hypoplasia.

Animal sacrifice and gross dissection of the heart lung block.

All animals were sacrificed by an intraperitoneal injection of Expiral (barbiturate), approximately 0.7mg/kg body weight. The weight and gender of all animals was recorded . The lungs were immediately removed and transported in calcium containing physiological salt solution (PSS) from the on-site animal facility to the adjacent laboratory.

Methods.

Isolation of vessels from the lung.

The main intrapulmonary conduit artery and/or vein was dissected from the middle third region of a lower lobe and placed in calcium containing physiological salt solution in a Petri dish (Fig.2).

Lung parenchyma and connective tissue was removed, under a dissecting microscope. The ends of the vessels were discarded where pinning may have caused damage during dissection. The vessel was then cut into rings 2-4 mm long. At the start of the entire study a calibrated microscope graticule in the eye-piece was used to measure the length. However, after a period of experience in judging lengths the graticule was not required. If required the endothelium could be removed by mechanical rubbing with a metal tool on a wetted finger (Fig.3).

Fig. 2 The section of intrapulmonary artery studied is highlighted (blue) in this photograph of a postmortem arteriogram of a porcine lung.

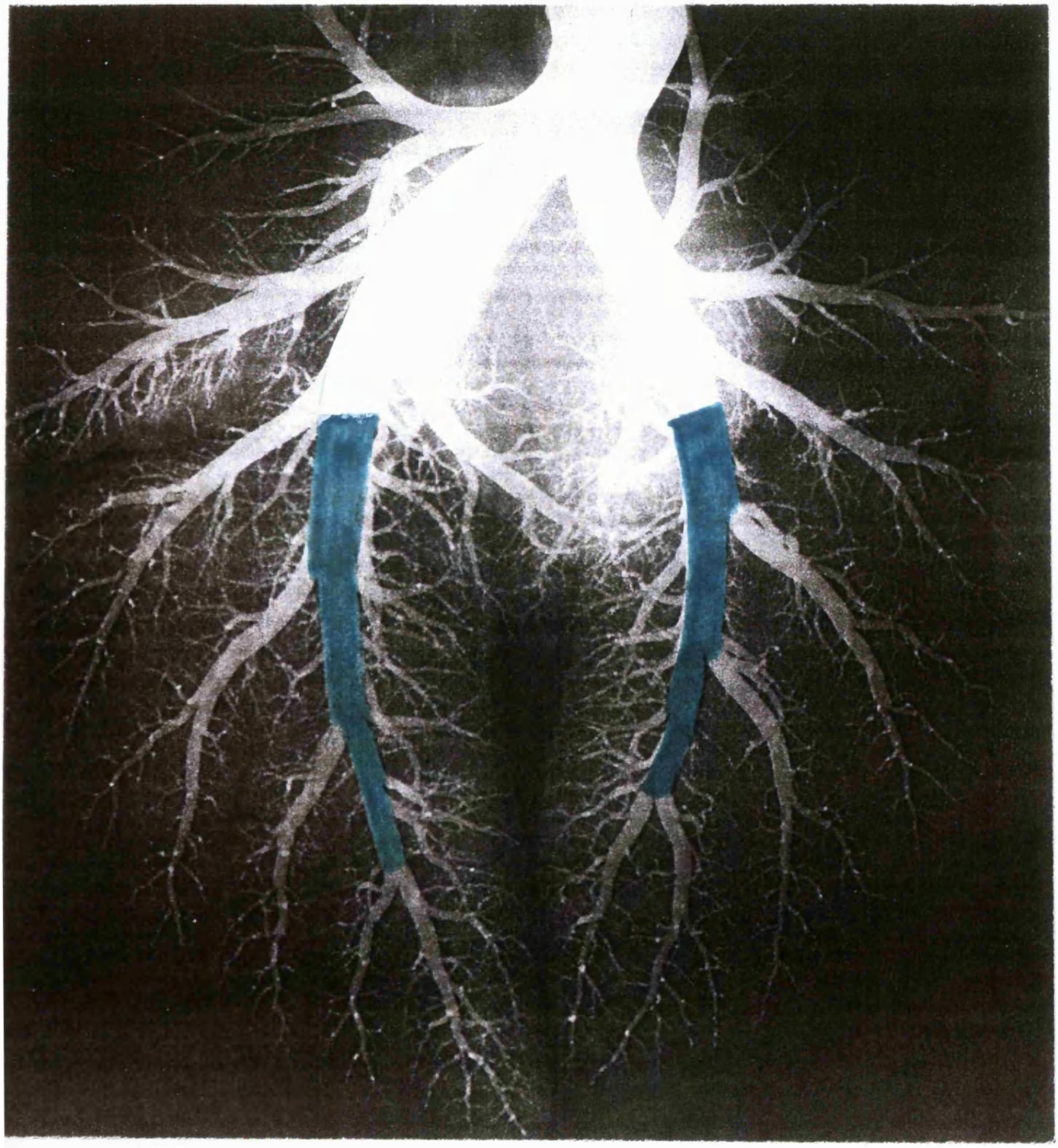
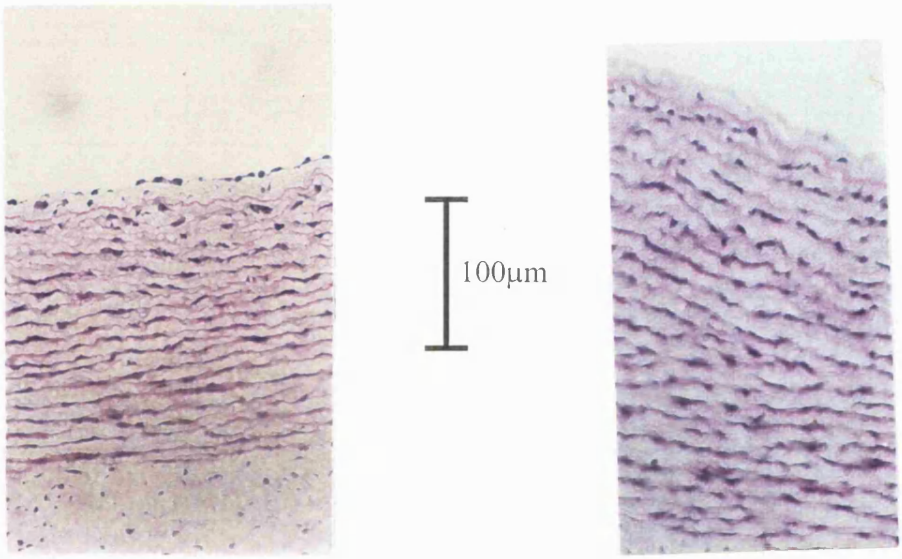


Fig.3 Representative photomicrographs showing the presence or absence of the IPA endothelium in a normal newborn and 3 day old animal, in a 3 day old animal exposed to hypoxia from birth and in a normal adult. Stained with Haematoxylin and eosin (x10 magnification).

Newborn piglet IPA

with endothelium

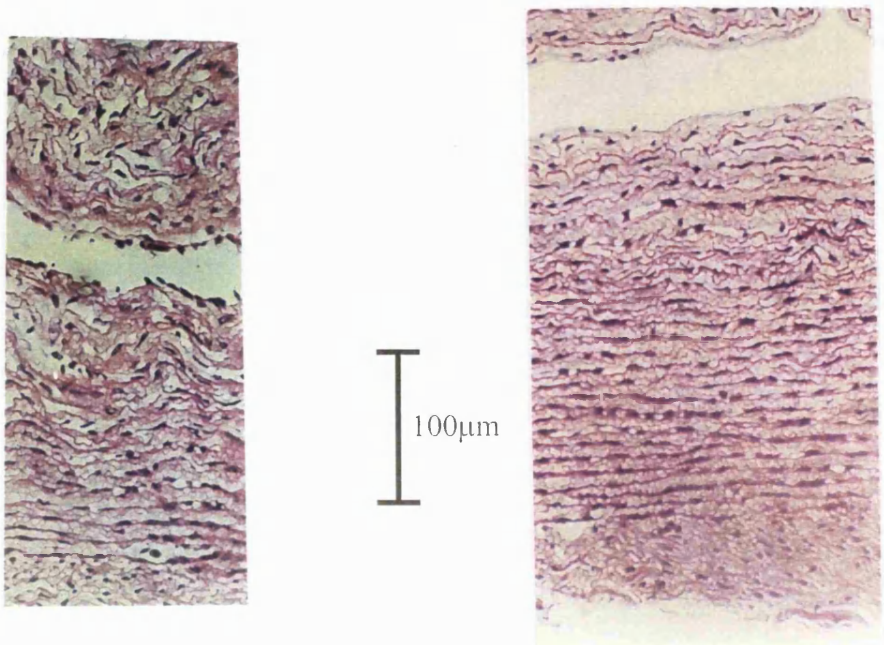
without endothelium



3 day old normal piglet IPA

with endothelium

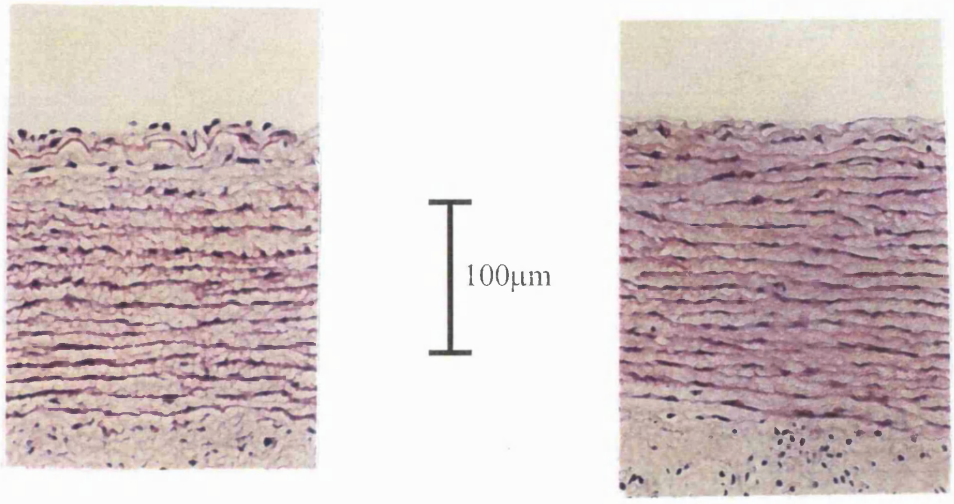
without endothelium



IPA from piglet exposed to 3 days of CHH from birth

with endothelium

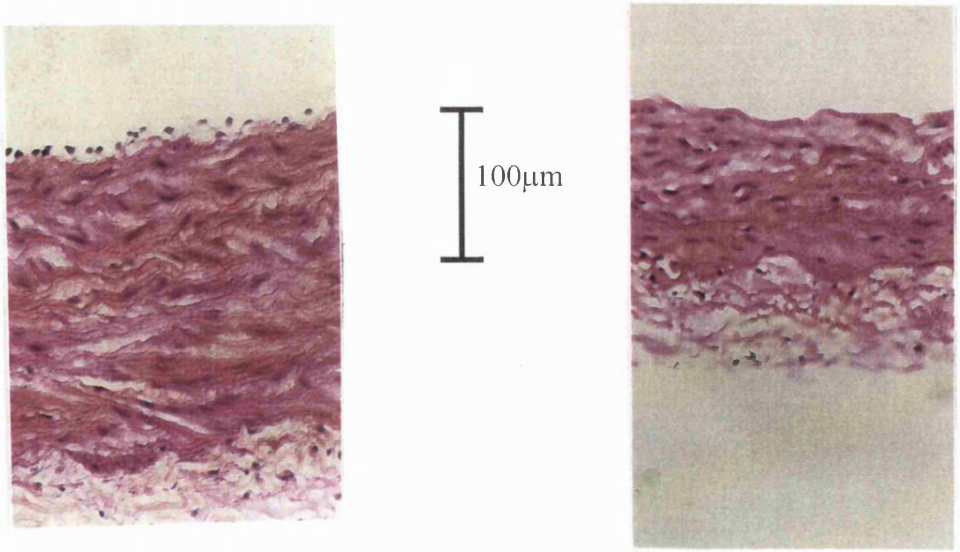
without endothelium



Adult pig IPA

with endothelium

without endothelium.



Organ chamber pharmacology

To prepare the vessel ring for isometric force recording two tungsten wires (120μm diameter) were inserted through the vessel lumen. Vessels were suspended in 5ml organ baths {29} containing PSS with calcium. The rings were allowed to stabilise for 40 minutes to one hour, during which time the PSS was replaced once and the tension gradually increased to 1000mg for arteries and 300mg for veins in all animal groups. Only one ring was studied from any one animal for a given experiment. The notation "n" therefore refers to the number of animals studied. At the end of each experiment all residual active tension was removed by the addition of 100μM papaverine.

1. Preliminary studies of the preparations:

(a) Assessing the viability of the preparation.

30mM KCl was found to be an effective concentration for evoking a standard contractile response in all groups. This was found to apply equally well to the intrapulmonary veins.

(b) Assessing the integrity/absence of the endothelium.

At the beginning of each experiment a stable contraction was obtained by giving a bolus of $PGF_{2\alpha}$ (10 or 30μM). Acetylcholine (1-10μM) was then added in order to verify that the endothelium was intact or had been removed effectively and that the agonist-induced endothelium-dependent relaxation was as expected for animals aged 3 days or more {241}.

2. Studies on the relaxant effects of purines.

(a). Protocol for obtaining cumulative agonist concentration-response curves.

A bolus of 30μM $PGF_{2\alpha}$ was used to produce a monophasic, stable precontraction, to which agonists were added in a cumulative manner. This dose of $PGF_{2\alpha}$ had previously been shown to produce a maximal contractile response in all age groups, including animals exposed to chronic hypobaric hypoxia {241,399}. Dose-response curves were constructed for each of the following agonists:

ATP, 2-methylthioATP, ADPβS, α,β-methyleneATP and the pyrimidine nucleotide UTP .

ATP-derivatives have in the past been reported to have a greater resistance to degradation than ATP, thereby reducing activity at the P1-nucleoside receptor(s) {1,216}. It was decided that in order to target P2-nucleotide receptors and develop a rank order of potency, these agents were the most suitable.

A cumulative concentration-response was carried out from 10^{-8}M to $3x10^{-4}$ M, except for ATP and UTP where the greatest concentration used was 30mM. Whenever possible, paired studies of intact and denuded rings from the same animals were performed.

Additional experiments were carried out with ATP after precontraction with 30mM KCl, in order to investigate the role of the precontractile agonist on any maturational changes observed to ATP and other agonists.

100μM sodium nitroprusside was added after a cumulative concentration-response study had been constructed in the precontracted rings. 100% relaxation was taken as the condition of the precontracted vessel after the addition of papaverine 100μM at the end of the experiment. Following the construction of a full dose-response curve, the difference between the $PGF_{2\alpha}$ tension and that obtained after giving papaverine could be determined and taken to equal a 100% relaxation response. Data points referring to the % response to a concentration of drug could then be determined. These data were then plotted and it was possible to calculate an EC_{50} by fitting a curve, using regressional analysis, to quantify the response.

(b). Inhibitors used to investigate the involvement of endothelium-derived relaxing factor and cyclooxygenase products.

Either 30μM L-NMMA or 10μM indomethacin was added for at least 20 minutes, or for the length of time taken for the effect of the inhibitor on the baseline tone to have stabilised prior to precontraction with $PGF_{2\alpha}$.

The effect of L-NMMA was tested upon the ATP relaxation response in newborn and adult porcine intrapulmonary arteries. The effect of L-NMMA was tested upon the 2-methylthioATP relaxation response in adult porcine intrapulmonary arteries. The effect of indomethacin was tested upon the ATP relaxation response in newborn porcine intrapulmonary arteries.

3. Studies on the contractile effects of purines / pyrimidines

(a). Protocol for obtaining cumulative agonist concentration-response curves.

Cumulative concentration-response curves were constructed for the following agonists at resting tone: ATP (0.1-30mM), UTP (0.1-30mM), α,β-methyleneATP (0.1-100μM). A 100% response was taken as the contraction to KCl 30mM less the tension after giving 100μM papaverine.

(b). Use of putative receptor antagonists.

100μM α,β-methyleneATP was added for at least 20 minutes or for the length of time taken for the effect of the antagonist on the baseline tone to have stabilised. α,β-methyleneATP has been reported to desensitise P2X-receptors after initially activating them. Conventionally, P2X-receptor desensitisation is routinely confirmed by repeated addition of α,β-methyleneATP and this approach was adopted in the present study. The effect of α,β-methyleneATP was tested upon the response to ATP or UTP in newborn and 14 day old control porcine intrapulmonary arteries.

4. (a) Acquisition of data, analysis and presentation of data obtained from organ chamber pharmacology studies.

Raw data were recorded using a Grass ink pen recorder at the start of the study. However, the majority of organ bath studies were recorded in a digital format using Chart on a MacLab computer system. This package provides a spreadsheet into which selected data points can be collected. The data sets can be "pasted" directly to Excel for manipulation. All graphs were created using the Sigmaplot package. Sigmaplot provided the regressional analysis curve fitting required for EC_{50} determinations.

Representative traces were created selecting a relevant section of a recording and processing the data points through Excel. These data points were then used by Sigmaplot to recreate the recording trace in a format which allowed text to be added.

(b) Statistical analysis

SPSS version 7 and Excel version 5 for PC as used to perform the following analysis.

The effect of age upon agonist responses in precontracted vessels was assessed by one-way ANOVA with Bonferroni post-hoc testing of EC_{50} data , except for the bolus effect of acetylcholine which was assessed by two sample unpaired Student t-test assuming unequal variance. The effect of age upon the contractile response at resting tension was assessed by one-way ANOVA with Bonferroni post-hoc testing of maximal effect. The effect of removing the endothelium and incubation with blocking agents was assessed by two sample unpaired Student t-test assuming unequal or equal variance (as required) of EC_{50} and maximal effect data.

The effect of CHH upon responses in precontracted vessels was assessed by one-way ANOVA with Bonferroni post-hoc testing of EC_{50} data. The effect of CHH upon contractile responses at resting tension was assessed by two sample unpaired Student t-test assuming unequal variance, of maximal effect data.

5. Studies of P2X- and P2Y-purine receptor binding sites.

The methods described in this section are designed to prepare tissue for use in radioligand binding studies in order to establish the distribution of specific binding by two radioligands which might be predicted to bind to P2-nucleotide receptors across the elastic IPA, present on lung sections. Further, the protocols employed will make it possible to draw conclusions as to the possible receptor class associated with the radioligand binding. The effect of age and persistent pulmonary hypertension on the binding and receptor class will also be determined.

(a) Preparation of tissue sections for use in radioligand binding studies.

This process outlined below was used to prepare frozen sections of porcine lung, which could then be used for ligand binding experiments.

Fresh lung tissue was obtained from normal newborn, 3 day old and adult pigs as well as from piglets exposed to 3 days of chronic hypobaric hypoxia from birth, which had persistent pulmonary hypertension. The tissue was collected shortly after death except that from adult animals which was delayed by the 4 hours taken for transportation from the abattoir.

1-2cm^3 blocks were cut from the lower lobe, in the same region used for isolating vessels for organ chamber pharmacology. The tissue cube was then orientated on cork discs and embedded in OCT (Raymond Lamb, London). The embedded tissue was then snap frozen for 1 minute in isopentane, which was cooled in a bath of liquid nitrogen. Blocks were stored in a -70°C freezer. When required 10μm serial sections were cut from the frozen blocks of tissue using a cryostat at -20°C, and the sections allowed to melt onto a glass slide coated with Vectabond (Vector Laboratories, Peterborough,UK.). The sections were then stored in slide boxes with silica gel and returned to a -70°C freezer until required.

(b) General method for radioligand binding study to frozen lung sections.

This section describes the protocol used throughout all the radioligand binding studies in the present study.

When required, frozen sections were allowed to return to 25°C, and preincubated in 50mM Trizma/HCl pH7.4 and 0.1% bovine serum albumin buffer for 15 minutes. Excess buffer was

drained from the sections and wiped from the slides to leave an area around each section to which radioactive solutions could be added in 50-75μl volumes. The sections were incubated with the radioactive solution in buffer for 20 minutes at 27°C in large, covered petri dishes. Non-specific binding was assessed on adjacent sections by co- incubation with excess non-radioactive ligand, and then comparing the density to that of the binding using the radioactive ligand alone.

The binding reaction was quenched by two 5 minute washes in buffer (4°C) followed by a rapid rinse in distilled water (4°C). The sections were rapidly dried with cool air and excess water on the slides evaporated at room temperature. In a dark room, the slides were then arranged in film cassette boxes. C^{14} standard samples on a slide were included in each cassette which would produce areas of graded density which were later used for density quantification of the radioligand binding. A photographic film (Hyperfilm™ - ^{3}H, Amersham, UK.) was placed on top, taking care to place the emulsion covered surface in direct contact with the sections and that the slides were flat. The cassettes were placed in black light-proof bags and kept in a fridge at 4°C for the exposure period of 2.5 days. In preparation for developing and fixing the films, both developer and fixative were filtered to remove debris and used at 20°C. The films were carefully removed from the cassettes, avoiding scratching the emulsion surface and immersed in developer for 4 minutes. Excess developer was removed by a rapid rinse with cold tap water before a 5 minute period in fixative. At this point a dark room environment was not required. Excess fixer was removed by a 10 minute wash in cold tap water followed by 10 minutes under a running source of cold tap water. The films were then dried in a heated cabinet and placed in a protective envelope. To allow confirmation of where binding was occurring, sections from each animal were fixed in Bouins picro fixative (1 hour 4°C), washed in phosphate buffered saline (3 x 20 minutes 4°C) and stained with an elastic van Gieson stain.

(c) Experimental method for confirming the binding conditions and concentrations to be used.

From the published work on purine-radioligands, 1-10nM of the radioactive ligand was used for binding assays on cell membranes and tissue sections {32,37,36,413,95}. We decided to use 1nM in an attempt to be economical with the ligands. The published protocols for studying purine-radioligand binding sites refer to an excess concentration of 100μM for the non-radioactive agonist. This appeared to be a high concentration to use for a biochemical approach such as autoradiography. These high concentrations were also required for inducing responses in the functional organ chamber experiments in the present study.

In order to check that a density of measurable binding would be produced by the relatively small dose of 1nM of radioactive ligand , sections of lung from adult animals were chosen. This group had been found to have the greatest sensitivity to the purine agonists in the organ chamber studies and it was postulated they would provide the greatest opportunity for binding.

1nM of the $P2Y_1$-purine receptor radioligand [^{35}S]-deoxyadenosine 5'-[α-thio] triphosphate ([^{35}S]deoxyATPαS) in Triz buffer was co-incubated with either 10^{-9},10^{-8},10^{-7},10^{-6},10^{-5},10^{-4} M 2-meSATP (a P2Y agonist in the present study) for 20 minutes on sections of lung tissue. No work has been published regarding ligand binding to a pyrimidine P2X-receptor. Therefore, 1nM of [^{35}S]-UTPαS in Triz buffer was co-incubated with either 10^{-9},10^{-8},10^{-7},10^{-6},10^{-5}, 10^{-4} M UTP (predominantly a P2X agonist in the present study) for 20 minutes on sections of lung tissue. The general method described in the previous section was then followed. The minimum concentration needed to significantly prevent radioactive binding was established. A 20 minute incubation period with radioligand had been previously described in the literature and was sufficient to produce significant binding in the present study also. A time course of binding levels was not established as the 20 minute was successful.

This study determined that 1nM of radioactive ligand produced measurable binding and that 100μM was required to prevent the specific binding. With this information the degree of specific binding was determined in sections from normal newborn and 3 day old and from

3 day old piglets with persistent pulmonary hypertension, as well as sections from a normal 8 year old child. This was done for both radioligands using the general methods described.

(d) Experimental method for classifying binding sites and associating them with receptor subtypes.

This protocol was based upon the use of the rank order of agonist potencies described in the organ chamber pharmacology study. The ability of an agonist to competitively displace the radioligand binding was taken as a measure of activity at a receptor and using this theory a rank order of displacement could be produced for each radioligand. In addition, the effect of combining non-radioactive ligands was tested, each still at 100μM.

1nM of [^{35}S]deoxyATPαS or [^{35}S]-UTPαS radioligand was co-incubated with either 2-methylthioATP, α,β-meATP or UTP alone or with 2-methylthioATP + α,β-meATP, 2-methylthioATP + UTP, or α,β-meATP + UTP. The density of binding to the intrapulmonary arteries on the lung sections was quantified and used to compare the effect of each agonist combination.

(e) Acquisition, analysis and presentation of radioligand binding data

The radioactivity associated with the radioligands interacts with silver grains on the photographic film to produce a black point. The distribution of black dots reflects firstly the location of the radioligand and secondly, the number of black dots in a given area (density, referred to as grey levels) indicates the amount of radioligand present. The pattern of black dots on the films were selected and collected by the Neotech Image Grabber software (Apple Macintosh System) and stored on disk. Quantification of the grey levels was performed on these stored images with the Optilab software package (Apple Macintosh System). A range of C^{14}-standards was included on each film. The densities associated with these samples were used to construct a standard-density curve relating grey levels to binding activity recorded as attomole/mm^2 (amols/mm^2) of film . With this curve the ligand binding density could be given a value, the greater the value the greater the binding. All images to be compared were

collected by the Image Grabber on the same day under the same conditions of contrast and brightness. The level of specific binding associated with each combination of radioactive and excess non-radioactive agonist was taken to be the difference between the binding density produced by [1nM] radioligand in Triz buffer and the density remaining after co-incubation with excess non-radioactive agonist(s). The binding produced by [1nM] radioligand in Triz buffer, was taken to represent 100% density for each group of animal tissues on a film. This allowed the density data from each section to be expressed as a percentage value and removed inter-film differences ie. absolute amols/mm^2 . The % density data could then be collated from all films and used to establish data sets of experimental groups not done on the same films.

The density data was manipulated using Excel version 5 to produce mean data for statistical analysis (SPSS software version 7) and graphs were created using Sigmaplot (version 3) .

(f) Statistical analysis of radioligand binding studies.

SPSS version 7 PC was used to perform the following analysis.

The effect of age upon the level of specific binding by radioligands was assessed by one-way ANOVA with Bonferroni post-hoc testing. The effect of chronic hypobaric hypoxia on specific binding was tested with a two sample unpaired Student t-test assuming equal variance.

6. List of Drugs used.

The drugs used in this study are listed in alphabetical order. They were purchased from Sigma unless otherwise indicated. All the drugs were dissolved in distilled water unless otherwise stated.

Acetylcholine (hydrochloride salt).

Adenosine 5'-O-(2-thiodiphoshate) (trilithium salt).

[^{35}S]-deoxyadenosine 5'-[α-thio] triphosphate (from Dupont NEN) (10mM Tricine-NaOH buffer (1), pH 7.6, and 1mM dithiothreitol).

Indomethacin (made up in absolute ethanol or equivalent concentration of Na_2C0_3).

N^G-monomethyl -L-arginine.

α ,ß-methylene adenosine 5'-triphosphate (from RBI).

2-Methylthio adenosine 5'-triphosphate (tetra sodium salt) (from RBI).

Papaverine (hydrochloride salt).

Composition of Physiological salt solution (PSS):

NaCl 119 ; KCl 4.7 ; $NaHCO_3$ 25 ; $MgSO_4$ 1.2; $KHPO_4$ 1.2, $CaCl_2$ 2.5, glucose 11(mM).

Prostaglandin$F_{2\alpha}$ (made up in absolute ethanol) .

Prostaglandin$F_{2\alpha}$ (Cayman Chemical Company).

Uridine 5'-triphosphate (sodium salt).

[^{35}S] -Uridine 5'-triphosphate αS (from Amersham) (triethylammonium salts stabilised with 20mM dithiothreitol).

Chapters of Results

Chapter 3. Vasoconstrictor and vasodilator responses of intrapulmonary vessels to potassium chloride, prostaglandin $F_{2\alpha}$ and acetylcholine from normal and pulmonary hypertensive pigs and children.

Summary.

1. Intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs were isolated and mounted with or without endothelium for *in vitro* isometric force recording. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (50.8kPa) from birth, or after 3 and 14 days of age for 3 a day period.

2. Intrapulmonary arteries (IPA) were isolated from the lungs of three children with out pulmonary hypertension (aged 4 months & 5 & 8 years) and six children with pulmonary hypertension which contributed to their death (aged 36 hours, 10 days, 4 months, and three aged 15-16 years old). The vessels were isolated and mounted with or without endothelium for *in vitro* isometric force recording.

3. The contractile response of IPA and intrapulmonary veins (IPV) to potassium chloride (KCl) [30mM] was present for piglets of all ages and increased with age.

4. The contractile response of IPA and IPV to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [30μM] declined after birth and then increased again after 6 days of age.

5. Acetylcholine (ACh) [1μM] induced a significant endothelium-dependent relaxation of IPA precontracted with $PGF_{2\alpha}$ [10μM] after 3 days of age. In contrast, a significant relaxation to Ach was seen even in IPV from fetal animals.

6. Exposure to CHH for 3 days from birth and 3 days of age abolished the ACh-induced relaxation in IPA while reducing the response in the 14-17 day old group. The ACh-induced relaxation was not altered in the IPV.

7. IPA from normal and pulmonary hypertensive children contracted to both KCl and $PGF_{2\alpha}$. IPV from a baby with PPHN also contracted to KCl and $PGF_{2\alpha}$. ACh induced an endothelium-dependent relaxation of IPA precontracted with $PGF_{2\alpha}$ from normal children.

The IPA relaxation response to ACh was affected in 2 of 5 pulmonary hypertensive children, the IPV relaxation response to ACh was present.

8. The observations made from the present study are consistent with previously reported data from the porcine model used for studying normal pulmonary adaptation and neonatal pulmonary hypertension.

Introduction.

The porcine model of normal pulmonary vascular development has been used to study the reactivity of pulmonary vessels to constrictor and dilator agonists during the period of adaptation of extrauterine life {41,241,233,260}. The maturation of the porcine pulmonary circulation has been shown to be similar to that of the human {159,176,8,174}. The abnormal reactivity associated with persistent pulmonary hypertension of the human neonate has also been reproduced using the porcine model by exposing neonatal piglets to chronic hypobaric hypoxia for a period of 3 days {399,328,7}. Before any new piece of research can be incorporated into the established picture of neonatal adaptation it must be shown that within the degrees of experimental error the vessels isolated during the period of the present study displayed a reactivity which has been established by past studies using the porcine model . In this chapter the aim of the study was to define and then discuss the pattern of reactivity to agonists used in the present study and commonly used in previous investigations.

Methods.

Material: intrapulmonary arteries and veins from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (CHH) (50.8kPa) from birth, or after 3 and 14 days of age for 3 a day period. Human tissue was studied when possible (Table 1).

Methods : vessels were isolated and mounted with or without endothelium for in vitro isometric force recording. For details of the protocols used please refer to section 1 of the Chapter 2.

Table 1. Clinical details of children from whom tissue was studied.

Specimen number	Details of child
Normals	
3680	8 years old, donor, mild asthmatic. Accidental death.
4095	5 years old. Tuberous sclerosis, previous heart transplant. Heart failure from acute rejection. Normal PA histology.
4183	4 month old. TGA/VSD, pulmonary arterial banding, switch followed by myocardial infarct. ECMO. Normal pulmonary arterial presssure.
Pulmonary hypertensive	
3508	16 years old. Primary PHN, lung removed during transplantation.
4039	16 years old. Primary PHN, lung removed during transplantation.
4066	15 years old. PHN secondary to complex congenital heart disease, lung removed during heart / lung transplantation.
3954	36 hours old. PHN, congenital lung dysplasia and hypoplasia.
4087	4 month old. PHN, congenital lung dysplasia and hypoplasia.
4139	10 days old. PHN, congenital lung dysplasia and hypoplasia.

Results.

Responses of intrapulmonary arteries from normal and PH pigs.

Intrapulmonary arteries (IPA) from animals of all ages studied contracted to 30mM potassium chloride, with no influence of the endothelium (Fig. 1 A). The magnitude of the response increased gradually during the first 2 weeks of life and the adult response was significantly greater than that at 2 weeks of age($p<0.05$). Removing the endothelium significantly reduced the contractile response to KCl at 3 days of age ($p<0.01$). Intrapulmonary arteries (IPA) from animals of all ages studied contracted to 30μM $PGF_{2\alpha}$ (Fig.1 B). The response declined

between birth and 6 days of age before increasing with an increase in age. Removing the endothelium had no significant effect.

Exposure to chronic hypobaric hypoxia (CHH) from birth has significantly interrupted the maturation of the response to both KCl and $PGF_{2\alpha}$ (Fig.2 A, B). Removing the endothelium did not significantly reduce the contractile response to KCl in contrast to the 3 day old age-matched control. The contractile response to $PGF_{2\alpha}$ was significantly greater with and without endothelium following CHH than 3 day old aged-matched normal responses ($p<0.05$). Exposure from 3 days of age significantly decreased the response to KCl when compared to either 3 or 6 day old normal responses ($p<0.05$). The contractile response to $PGF_{2\alpha}$ was only significantly less than the 3 day old normal after removing the endothelium ($p<0.05$). Exposure to CHH from 14 days increased the contractile responses to both KCl and $PGF_{2\alpha}$, but not significantly.

A small endothelium-dependent relaxation to acetylcholine (1μM) was only present in precontracted IPA isolated from animals after birth (6 of 18 animals), and was significant after 3 days of age ($p<10^{-6}$) (Fig. 3 A.). A contractile response was observed in rings without endothelium. The dilating response was greatest at 3 days of age, while the contractile response increased with age. Exposure to CHH from birth or 3 days of age was associated with a contractile response not a relaxant response. The relaxation response of the 14 day old group was reduced, but not significantly.

Responses of intrapulmonary veins from normal and PH pigs.

Only a small number of IPVs were studied from each age group, but a pattern emerged. IPV from animals of all ages contracted to 30mM KCl,. The response increased with age and did so more rapidly than for the IPA (Fig.4 A). IPV from animals of all ages contracted to 30μM $PGF_{2\alpha}$ and the response increased with age (Fig.4 B). IPV still contracted to KCl and $PGF_{2\alpha}$ following exposure to CHH (Fig.5 A, B). 1μM Acetylcholine produced an endothelium-dependent relaxation of precontracted vessels at all ages, including the fetal animals (Fig.6 A). Exposure to CHH did not affect the relaxation response to ACh.

Responses of intrapulmonary vessels from normal and PH children.

IPA isolated from children with and without pulmonary hypertension contracted to KCl (30 and 125 mM) and $PGF_{2\alpha}$ [30μM] (Fig.7 A,B,C). ACh induced an endothelium-dependent relaxation of IPA precontracted with 30μM $PGF_{2\alpha}$ from three normal children (Fig.8 A-C). The response to ACh in IPA from children with PH was less consistent within the group. The IPA from a 36 hour old baby with PPHN, and two 15 year old with relaxed to ACh showing some endothelial-dependency (Fig.9 A,C,D). However, the IPA with endothelium from 10 day old and an 8 year old children with PH contracted to ACh (Fig.9 B, E).

A pair of IPVs isolated from a 36 hour baby with PPHN contracted to 30mM KCl and 10μM $PGF_{2\alpha}$ (Fig.7D), while ACh induced a dose-dependent relaxation in each ring, despite an attempt to denude one of them *(the denuded ring was used for studying the effect of UTP at resting tone, Chapter 8, Fig.8 B)* (Fig.9A).

Discussion.

The data agree with the general conclusions drawn by other workers using the same porcine model with the same agonists (241,233). The general conclusions were that the contractile ability of the IPA increased with age, reflected by an increased response to KCl, as previously shown {233}. The development of the contractile response to the receptor-coupled physiological agonist $PGF_{2\alpha}$ with age was also as previously found {241,233,123}. The decrease in contractile response to $PGF_{2\alpha}$ between 3 and 6 days of age in the normal piglets occurs when the vasodilating responsiveness of the IPA to dilators such as ACh has been found to be greatest {241,233}.

In the neonatal piglet, CHH-induced pulmonary hypertension (PH) was found to further reduce the IPA contractile response to KCl and $PGF_{2\alpha}$ in this period, which has previously been reported {399}. The responses seen in the small number of mature animals exposed to chronic hypoxia were not reduced in the present study, in contrast to previous work {399}. PH-induced by CHH in the neonatal piglet has been shown to cause a reduction in IPA endothelium-dependent vasodilatation to ACh, as previously shown {399}.

The reactivity of intrapulmonary veins (IPV) from various species has previously been addressed by other groups {428,371,424,330,379,333,142}. By using the same agonists in IPV and IPA a comparative study of the role played by the different segments of the pulmonary circulation both during adaptation to extrauterine life and in pulmonary hypertension has been achieved. The pattern of contractile response by the IPV to both KCl and $PGF_{2\alpha}$ was similar to that seen in the IPA, suggesting that the smooth muscle cells of both vessel types may undergo the same internal reorganisation with age. The observation that IPV endothelium-dependent vasodilatation to ACh was similar *in utero* and after birth, in contrast to the IPA, would suggest that the venous segment may play a modulating rather than a primary role in increasing postpartum pulmonary blood flow. A greater relaxation of IPV than IPA in response to ACh has previously been found in the fetal lamb {220}. It was interesting to find that PHN did not reduce the IPV endothelium-dependent relaxation of either the porcine or human response to ACh, in contrast to the IPA. The pulmonary veins are similar to the systemic arteries, carrying oxygenated blood and would logically be the vessel segment to sense changes blood oxygen levels and respond to perfusion-ventilation mismatching within the lung. Intrapulmonary venoconstriction would reduce the rate at which the blood may leave the respiratory units and increase the time in contact with limited oxygen available thus optimising systemic arterial blood saturation. Venoconstriction has been said to occur in pulmonary hypertension induced by both thromboxane mimetics and hypoxia {424,330}.

It was hoped that the responses of human vessels to various agonists would resemble those of porcine vessels. This would allow the application of knowledge from the porcine model to both normal and pulmonary hypertensive children. The similarities in the structures and haemodynamic findings between the two species, in the normal and in the pulmonary hypertensive were the basis for originally using the porcine model {155,159,8,7,172}. The number of human cases available for study was too small to draw any definitive conclusions but in the present study the observations from these and other cases studied by our group are so far reassuring.

It is important that the results obtained with an untried pharmacological agent should arise from a study which is comparable to those previous studies using the same model, before the data becomes part of the growing picture of adaptation to extrauterine life in the normal and pulmonary hypertensive pig. This requirement was satisfied for the present study.

A.

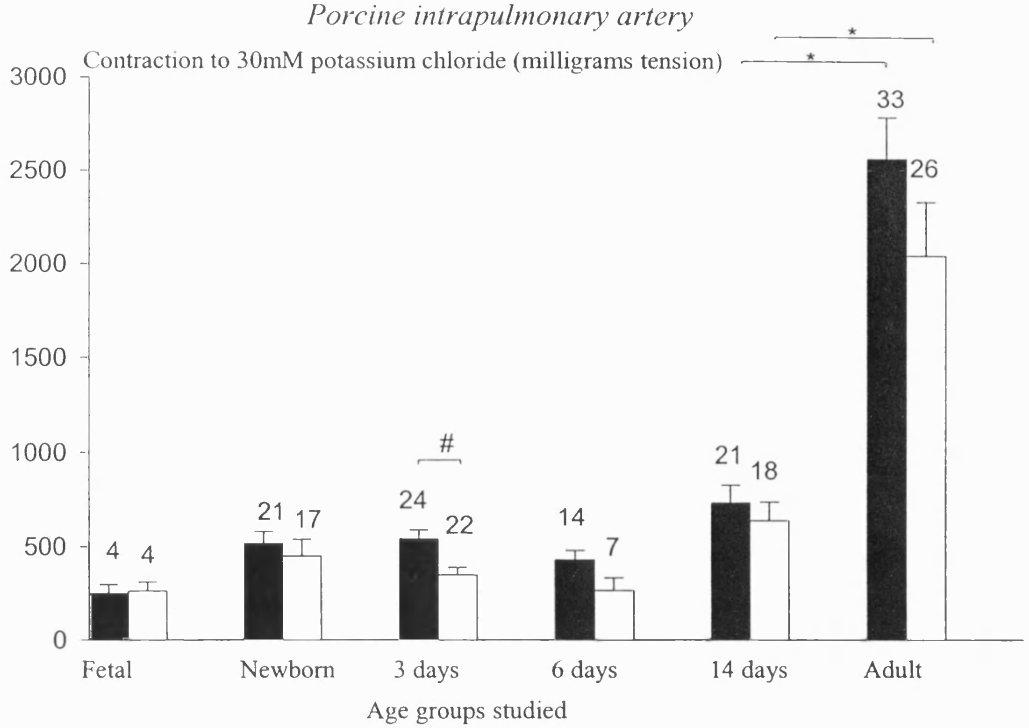
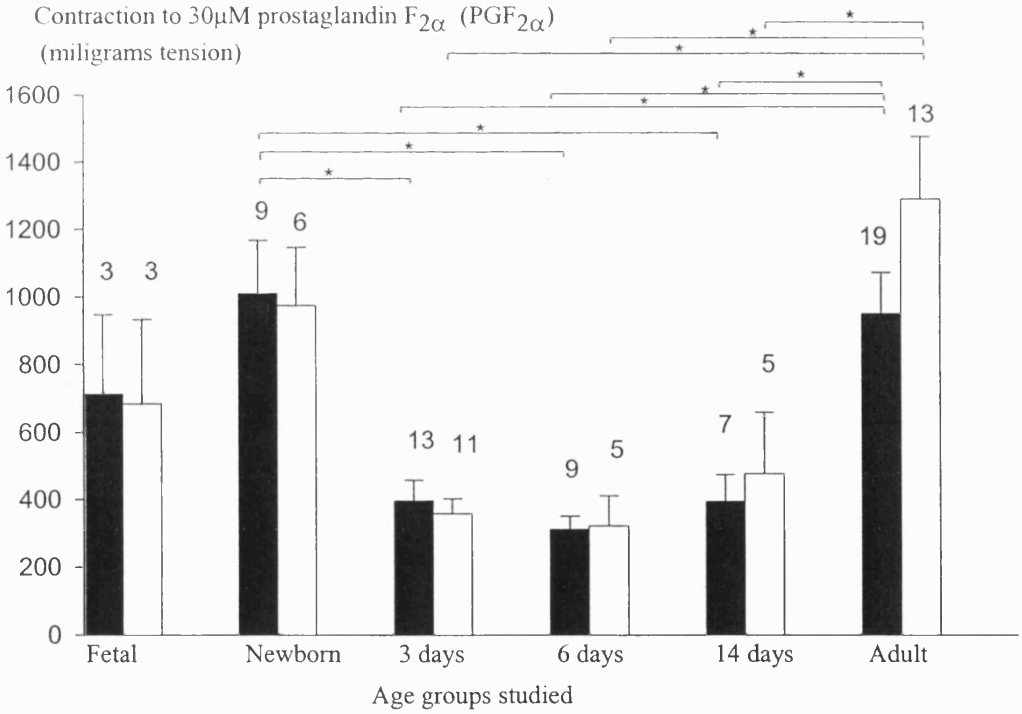


Fig. 1

(A) The absolute force generated by intrapulmonary arteries (IPA), from normal pigs, in response to 30mM KCl did not change significantly during the first 2 weeks of life. There was a significant increase between 2 weeks and adult life. Removing the endothelium significantly reduced the response in the 3 day old animals.
* indicate significant difference at the $p<0.05$, # $p<0.01$ levels. Error bars indicate sem.

B.

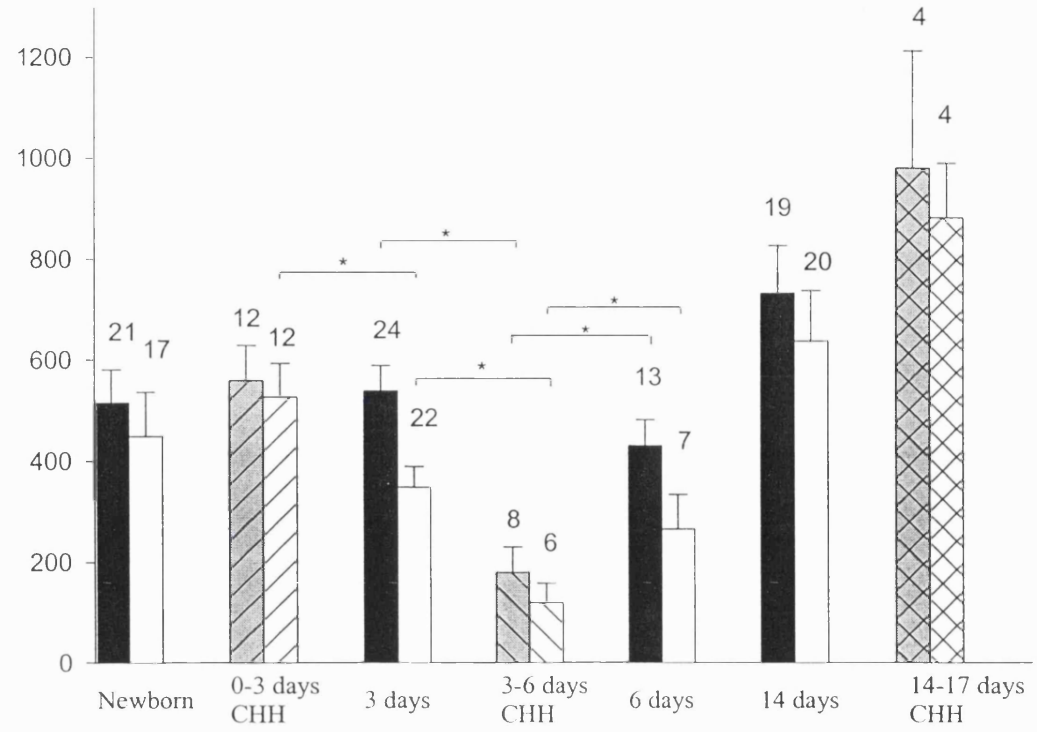


(B) The contractile response to 30μM $PGF_{2\alpha}$ of IPA from normal pigs decreased significantly after birth. The response then increased from 6 days of age significantly when compared to the adult animal response.
*indicates a significant difference at the $p<0.05$ level. Error bars indicate sem.

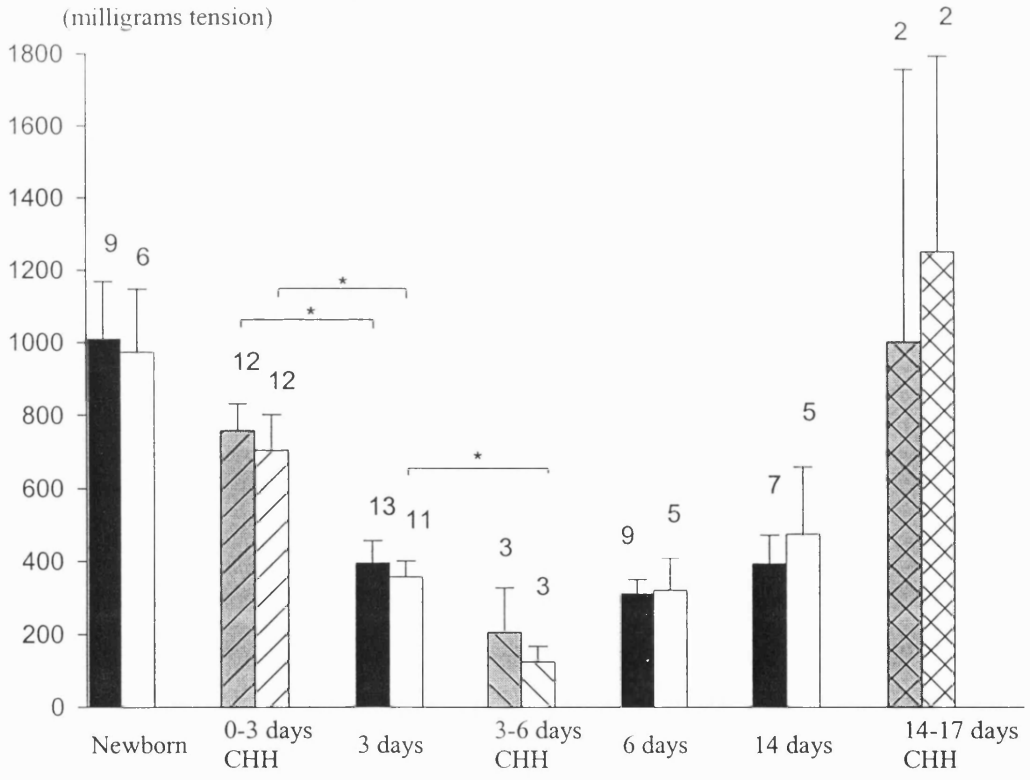
Key: Solid bars represent IPA vessels with endothelium, empty bars represent vessels where the endothelium has been removed. This key applies to all subsequent graphs in this chapter. Numbers above bars indicate the number of animals within the data set.

Fig. 2

A. Contraction to 30mM potassium chloride (milligrams tension)

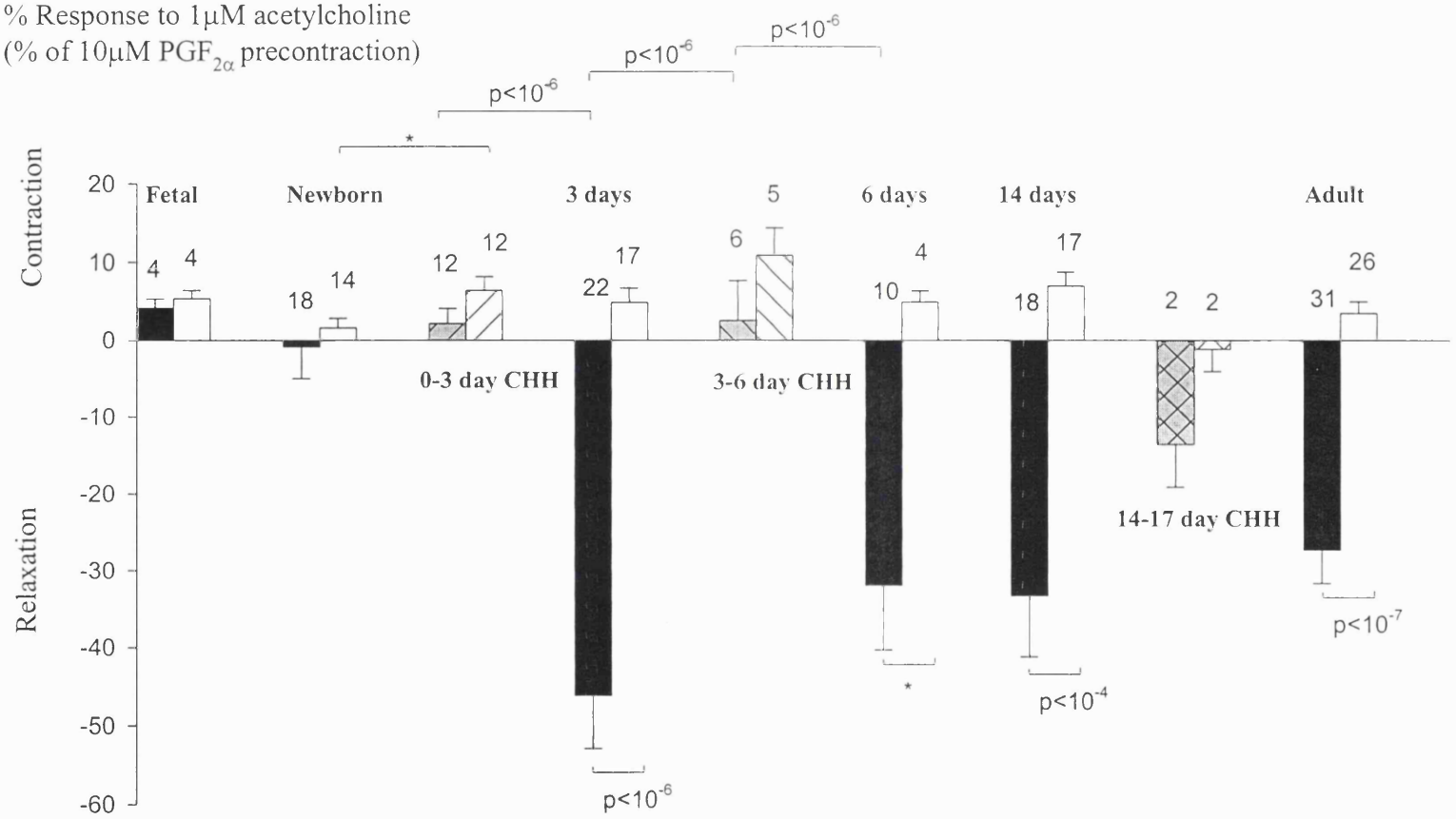


B. Contraction to 30μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (milligrams tension)



(A) Exposure to chronic hypobaric hypoxia (CHH) for a period of 3 days from birth has altered the IPA contractile response to (A) 30mM KCl and (B) 30μM $PGF_{2\alpha}$, compared to the normal age-matched responses. Exposure from birth and from 3 days of age generally reduced the response to both agonists . Exposure from 14 days increased the response, but not significantly.

* indicates a significant difference at the $p<0.05$ level. Error bars indicate sem (standard deviation where n=2).

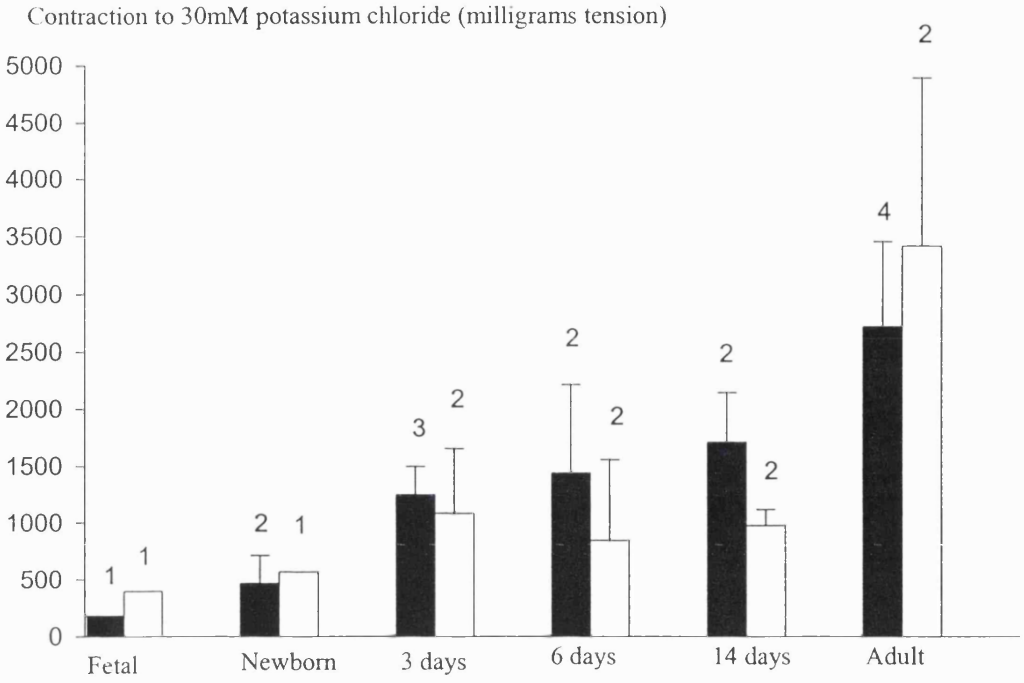


Porcine intrapulmonary artery- a significant relaxation response to acetylcholine [1μM] was first seen with endothelium at 3 days of age. Removing the endothelium promoted a contractile response at all ages. Exposure to chronic hypobaric hypoxia (CHH) from birth and from 3 days of age produced a small contraction in vessels with endothelium while significantly increasing the contraction in vessels without endothelium.

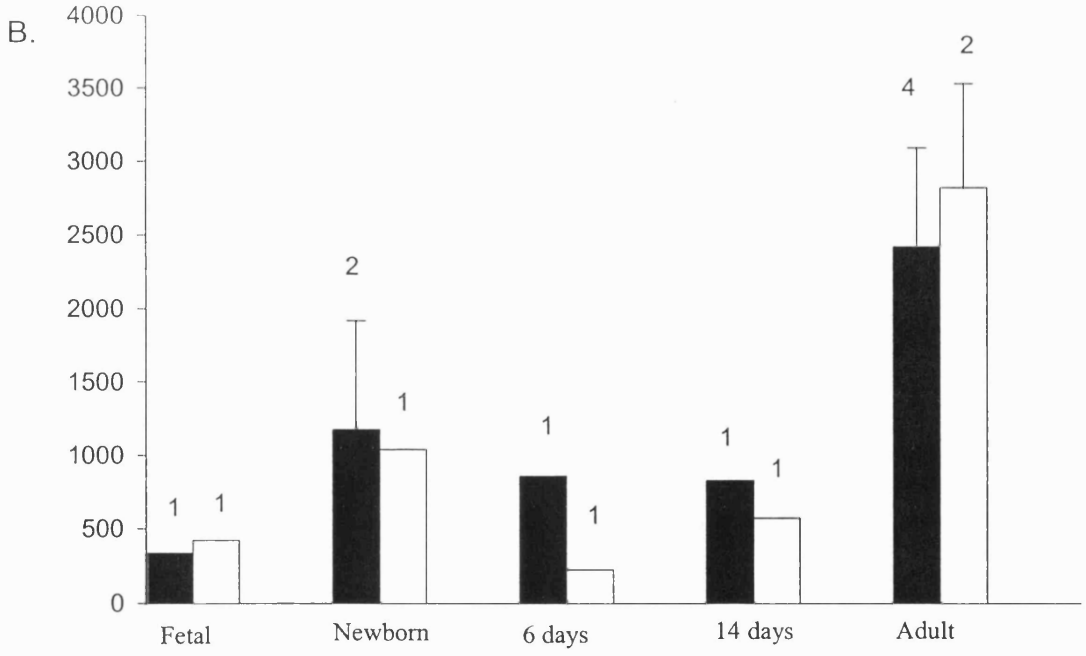
Exposure to CHH from 14 days of age significantly reduced the endothelium-dependent relaxation response.
Error bars indicate sem (standard deviation where n = 2). * indicates significant difference at the $p<0.05$ level.

Fig.3

A.

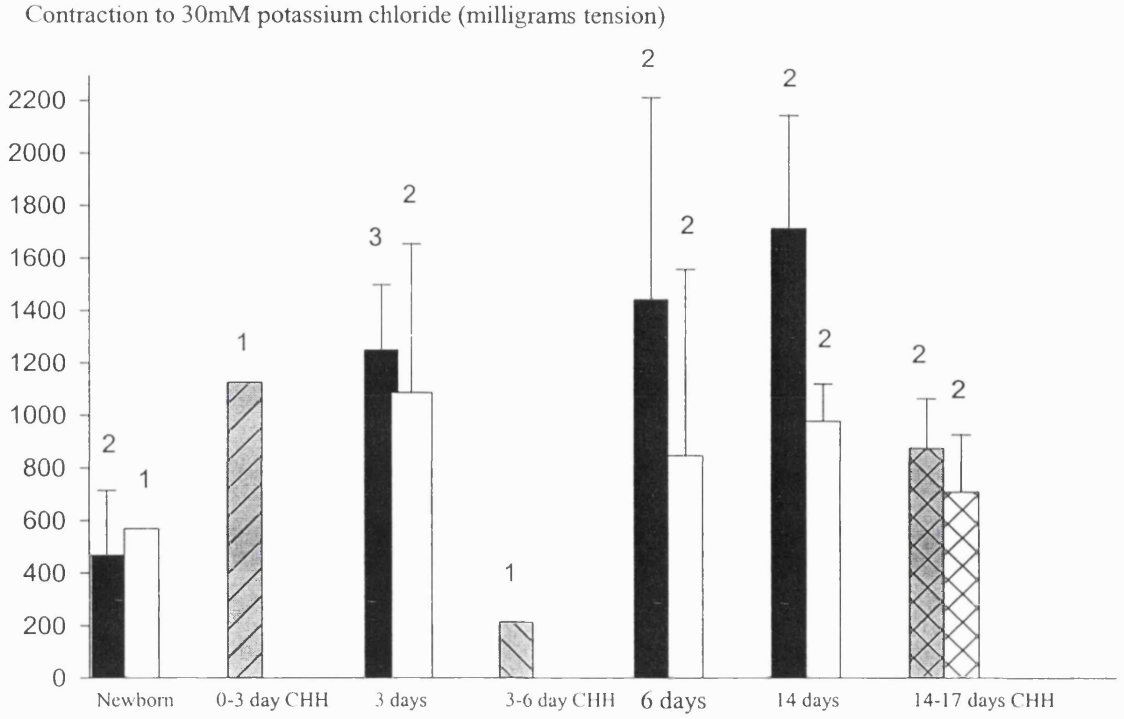


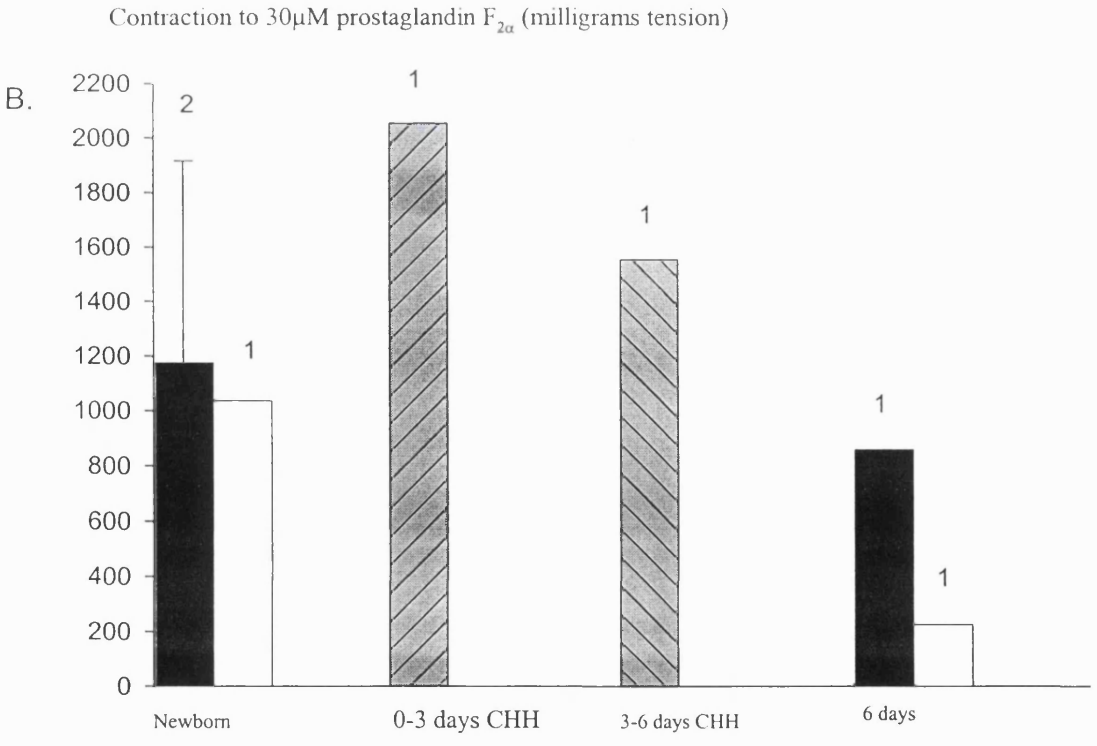
B.



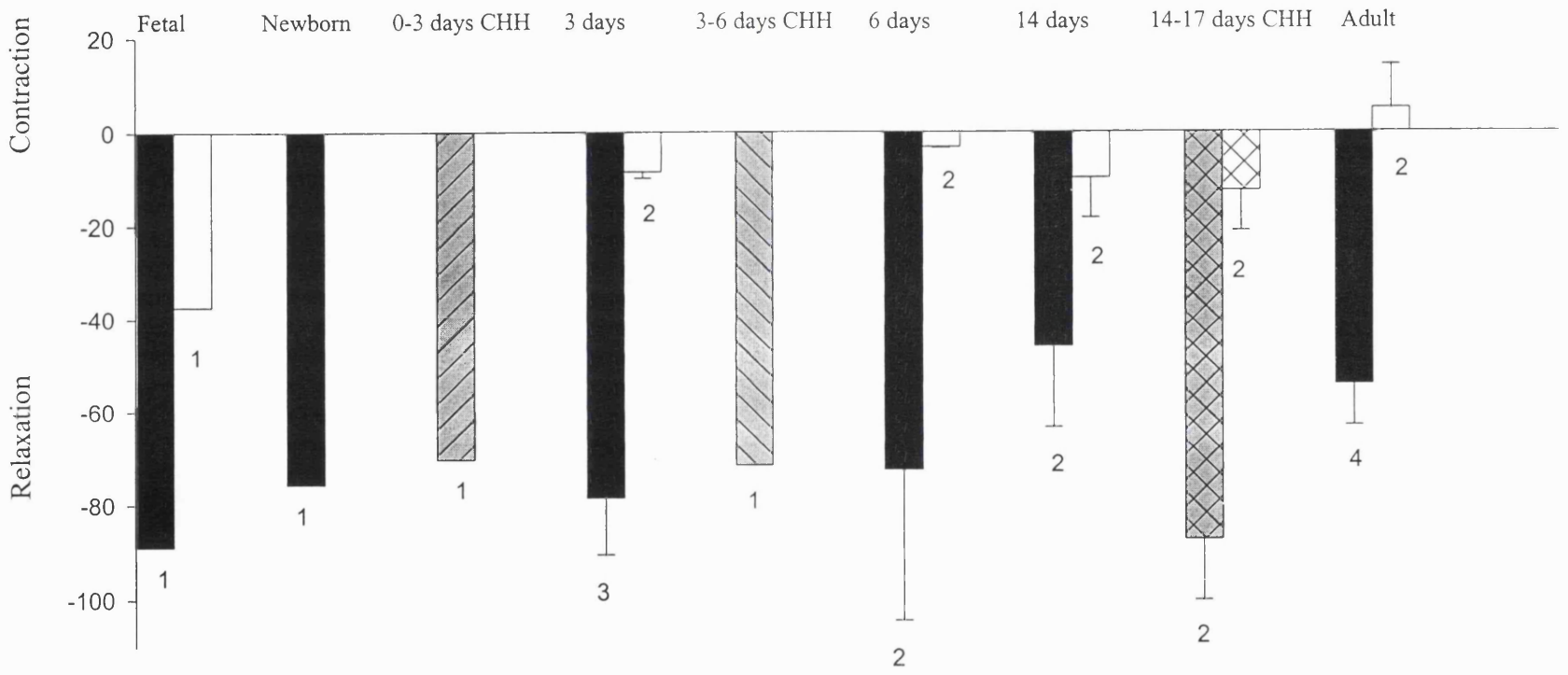
IPV from normal pigs of all ages contracted to (A) 30mM KCl and (B) 30μM $PGF_{2\alpha}$, the response increasing with age. Error bars represent standard deviation (standard error where n = 3).

A.



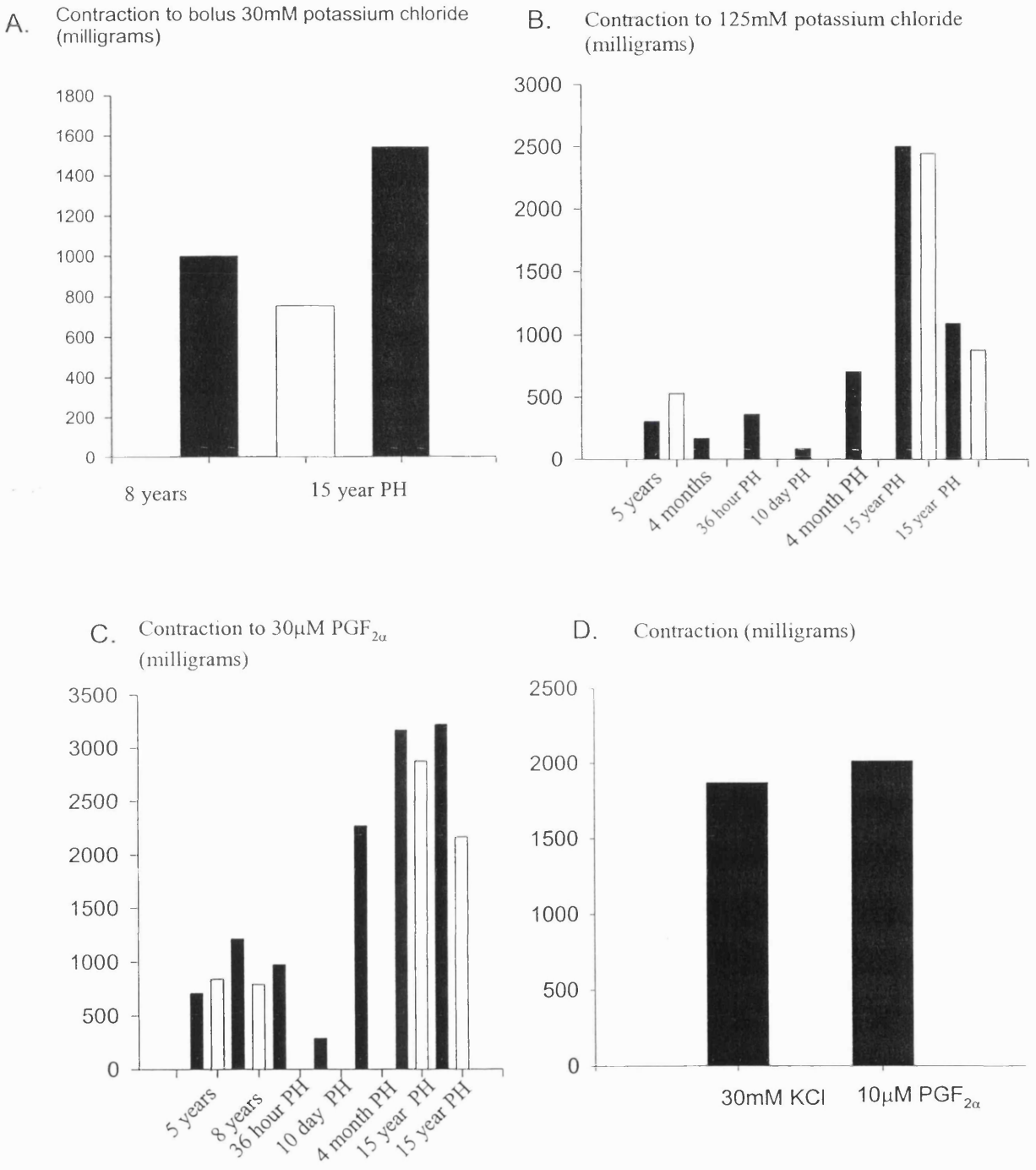


IPV isolated from pulmonary hypertensive nenatal piglets contracted to (A) 30mM KCl and (B) 30μM $PGF_{2\alpha}$. Error bars represent standard deviations (standard error where n = 3).



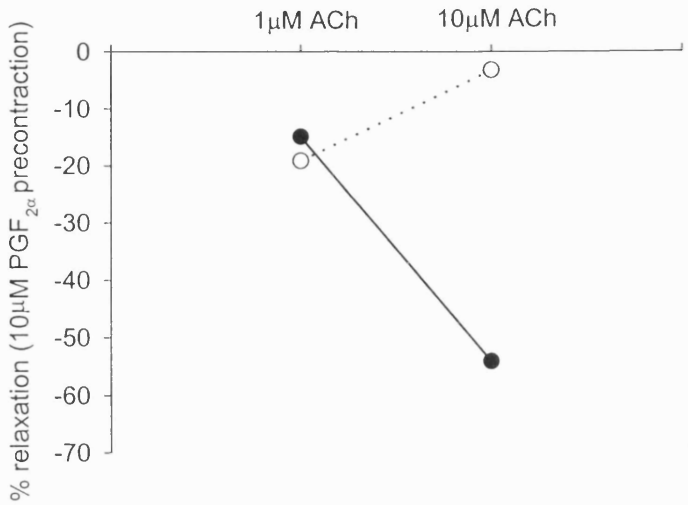
Porcine intrapulmonary veins - acetylcholine [1μM] induced an endothelium-dependent relaxation which was greatest in the young, including the fetal animals. The response in vessels from animals exposed to CHH was similar to that seen in normal age-matched animals.
Error bars represent standard deviation (standard error where n = 3).

Figure



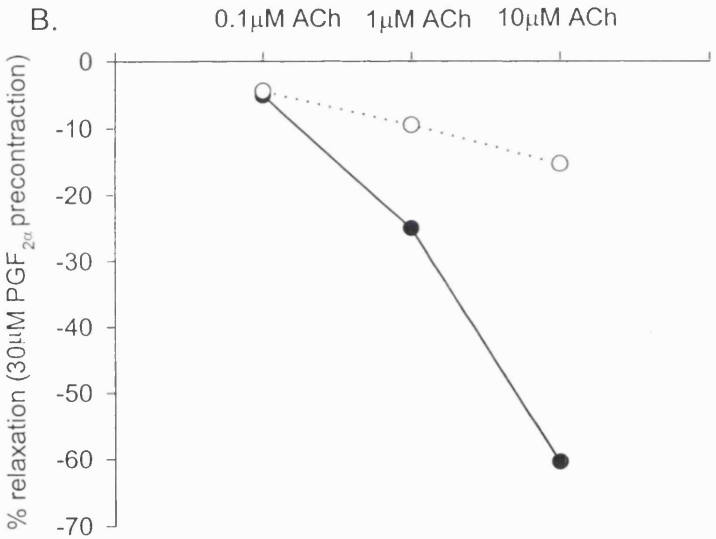
Intrapulmonary arteries (IPA) isolated from normal and pulmonary hypertensive (PH) children contracted to (A) 30mM potassium chloride (B) 125mM potassium choride and (C) prostaglandin $F_{2\alpha}$ [30μM]. The IPV from a 36 hour old baby with PPHN (D) contracted to both KCl and $PGF_{2\alpha}$.

A.



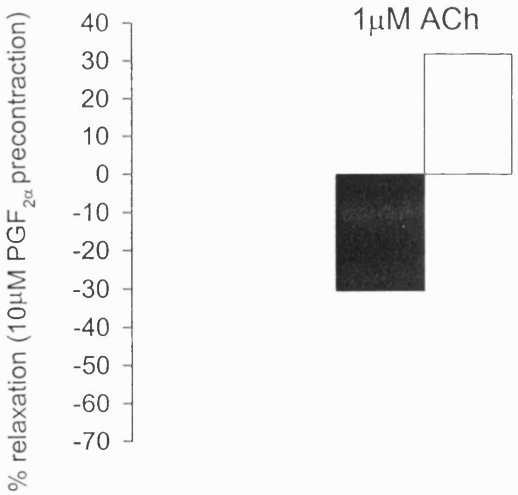
A. The response of precontracted IPA from a 4 month old normal child, to cumulative additions of ACh.

B.



B. The response of precontracted IPA from a 5 year old normal child, to cumulative additions of ACh.

C.

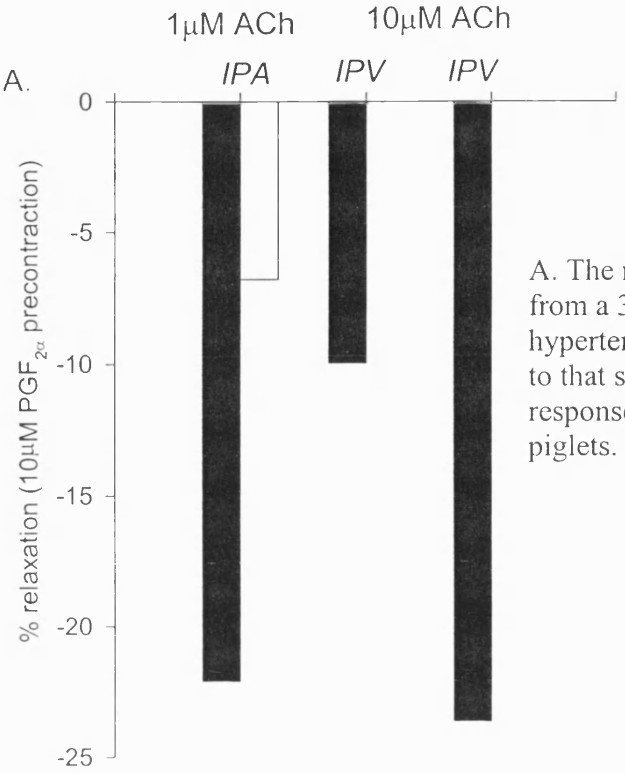


C. The response of precontracted IPA from an 8 year old normal child, to ACh.

In each case (A,B,C) the ACh relaxation response showed endothelial dependence.

Fig.8. Response of IPA from normal children of different ages to ACh.

A.



A. The response of precontracted IPA and IPV from a 36 hour old baby with persistent pulmonary hypertension, to ACh. The IPA response was similar to that seen in the normal child and piglet. The IPV response was similar to that seen in the normal and PH piglets.

B.

B. The response of precontraced IPA with endothelium from a 10 day old pulmonary hypertensive child, to ACh. The endothelial vasodilatation to ACh is absent.

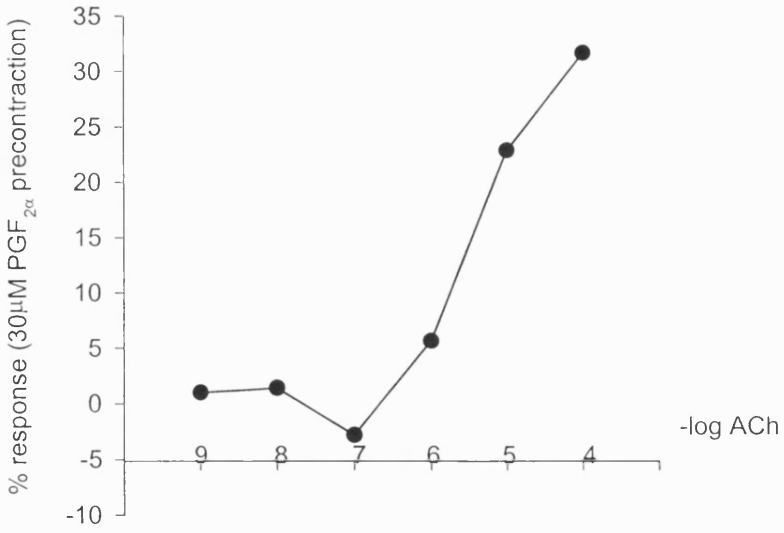
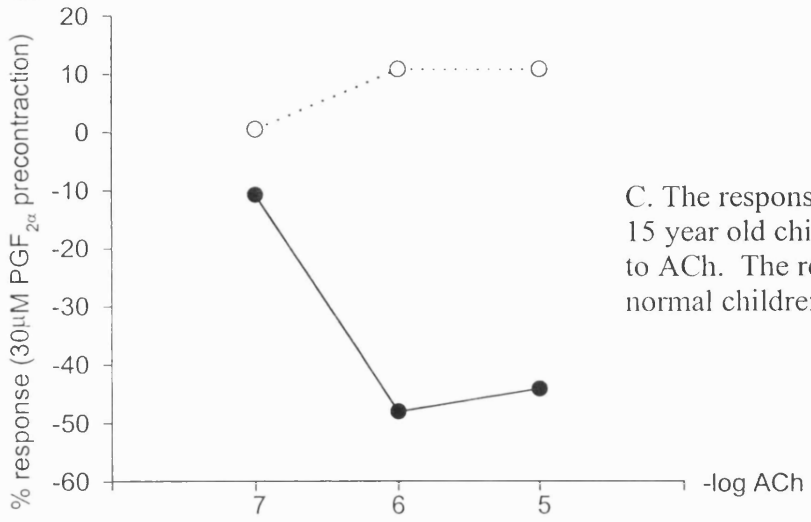


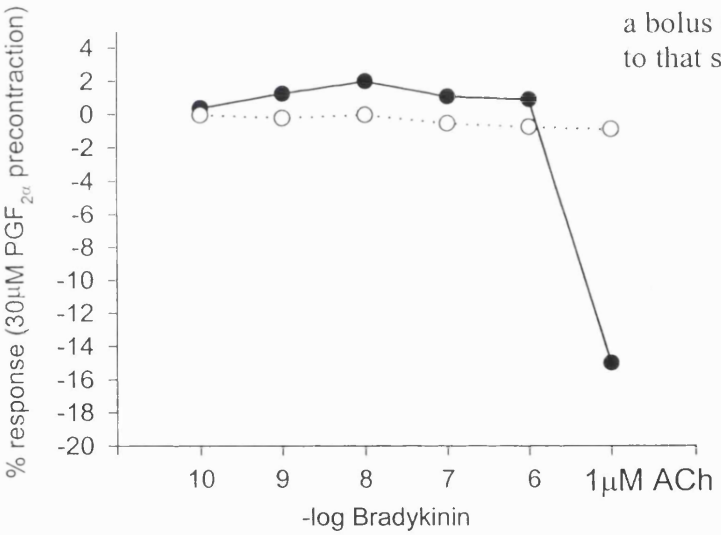
Fig.9. Response of IPA and IPV from pulmonary hypertensive children of different ages to ACh and bradykinin.

C.



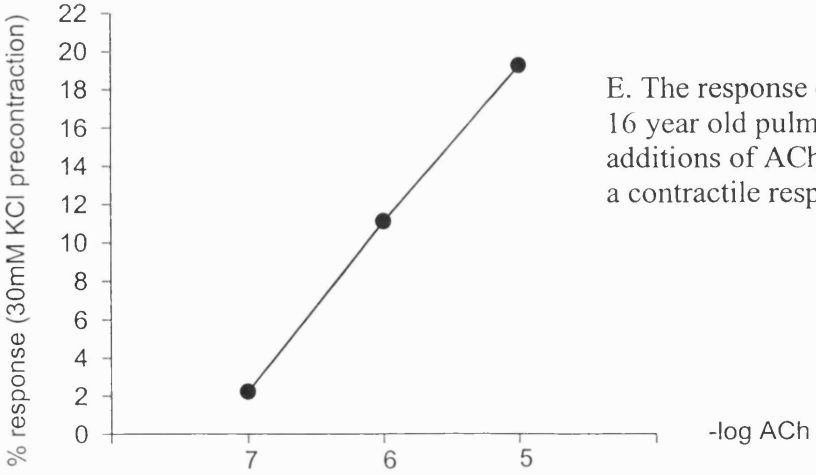
C. The response of precontracted IPA from a 15 year old child with pulmonary hypertension, to ACh. The response is similar to that seen in the normal children.

D.



D. The response of precontracted IPA from a 16 year old with pulmonary hypertension, to first a cumulative addition of bradykinin, then a bolus of ACh. The response to ACh was similar to that seen in the normal children.

E.



E. The response of precontracted IPA from another 16 year old pulmonary hypertensive, to cumulative additions of ACh. In this case, ACh only induced a contractile response.

Chapter 4. ATP-induced relaxation responses of precontracted intrapulmonary arteries from normal and pulmonary hypertensive pigs and children.

Summary.

1. Intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs were isolated and mounted with or without endothelium for *in vitro* isometric force recording. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (50.8kPa) (CHH) from birth, 3 or 14 days of age, for a period of 3 days. IPA were studied from an 8 year old normal and a 15 year old pulmonary hypertensive child.

2. ATP-induced relaxation in normal fetal and newborn piglet IPA precontracted with $PGF_{2\alpha}$ [30μM]. The sensitivity of the relaxation response increased significantly between birth and 14 days of age ($p<0.05$). The response did not increase with age when vessels were precontracted with 30mM potassium chloride.

3. Exposure to CHH from birth and 3 days of age had no significant effect on the IPA relaxation response to ATP, but the response was augmented by exposure from 14 days of age.

4. Removing the endothelium had no significant effect on the response of IPA to ATP precontracted with $PGF_{2\alpha}$, in vessels from both normal and pulmonary hypertensive animals.

5. The nitric oxide synthase inhibitor N^G-monomethyl-L-arginine acetate, L-NMMA [30μM], increased the sensitivity of the relaxation response to ATP in IPA, precontracted with $PGF_{2\alpha}$ [30μM], in 2 of 3 newborn piglets, but had no effect upon the adult relaxation response. Inhibiting cyclooxygenase synthase with indomethacin [10μM] significantly augmented the sensitivity of the relaxation response in ATP of IPA, precontracted with $PGF_{2\alpha}$ [30μM], from newborn piglets.

6. ATP-induced transient contractions in $PGF_{2\alpha}$ precontracted IPA from older pigs, but not in those from fetal or newborn animals. The contractile response was evoked in a greater

proportion of animals as the age of the pigs increased. The magnitude of the contractions also increased with increase in age.

7. In children ATP induced an endothelium-independent relaxation response in IPA precontracted with $PGF_{2\alpha}$ from a normal 8 year old. ATP also relaxed IPA precontracted with KCl from a 15 year old pulmonary hypertensive child.

8. In summary, in the present study ATP induced an endothelium-independent relaxation of fetal, neonatal and mature porcine IPA, and in the IPA from a normal child. This suggests that ATP might be involved in the fall in pulmonary arterial pressure during adaptation to extrauterine life. The ATP induced relaxation of the intrapulmonary arteries was resistant to the effects of chronic hypoxia-induced pulmonary hypertension, even in newborn piglets. This continued effectiveness may have therapeutic implications for children suffering from pulmonary hypertension in the newborn period.

Introduction.

The neonatal pulmonary circulation is labile during adaptation to extrauterine life at a time when the vessels are undergoing a dramatic transformation in morphology and function. The physiological basis of these changes is to facilitate a reduction in the pulmonary arterial resistance and therefore allow efficient gas exchange in the lungs {159,174}. Pulmonary hypertension (PH) of the newborn is potentially fatal, due to a failure of the pulmonary circulation to adapt to extrauterine life {328}. The most common cause of persistent pulmonary hypertension in the newborn (PPHN) is chronic hypoxia {161}. The problem appears to be localised to the arterial segment. In the systemic circulation, the small arterial vessels have been recognised as the site of vascular resistance {372}. By extrapolating from the work on the systemic circulation and considering the results of pressure measurements using servonulling micropuncture and wedge pressure techniques in the lung {158,166}, the arterial segment of the pulmonary circulation has been identified as the major, but not the sole, site of pulmonary vascular resistance {331,332,333}. It is therefore logical that attention should be focused on the intrapulmonary artery (IPA) when discussing the postnatal fall in pulmonary vascular resistance.

The majority of studies investigating pulmonary arterial vasodilatation in the newborn period have been concerned with the nitric oxide (NO) pathway. Endothelium-dependent vasodilating agonists such as acetylcholine and bradykinin, which stimulate nitric oxide synthase, produce a relatively poor relaxation response during the first days of extrauterine life {241,425,448}. Endothelium-independent agonists such as NO and sodium nitroprusside are more effective, as are K^+-$_{ATP}$ channel agonists in relaxing IPA from newborn animals {241, 41}.

By exposing newborn piglets to chronic hypobaric hypoxia (CHH, 50.8kPa) the haemodynamic abnormalities and structural remodelling associated with PPH in the human infant are produced after 3 days. This experimental model also shows that PPH delays the normal postnatal development of endothelium-dependent relaxation to agonists such as acetylcholine and bradykinin {399 120}. It also attenuates endothelium-independent relaxation to nitric oxide, a response which usually matures during the first 2 weeks of life.

The maturation of ATP-induced relaxation has not been studied previously in newborn intrapulmonary arteries. ADP has been shown to induce an endothelium-dependent vasodilatation in neonatal lambs, but ATP itself produced endothelium-independent relaxation in neonatal piglets {3,319}. ATP induces endothelium-dependent vasodilatation by stimulating the release of EDRF and prostacyclin from intrapulmonary arteries of the mature rat and human {238,237}. But, purine-nucleotides have also been shown to induce a vasodilatation of IPA from various species including humans which was endothelium-independent {237,265,326}. The involvement of purines at birth was indicated when exposing fetal lambs *in vivo* to increased oxygen levels led to an increase in the pulmonary arterial plasma levels of ATP, released from fetal erythrocytes {226}. Increasing the fetal level of circulating ATP to the postnatal level produces a fall in pulmonary arterial pressure mediated by P2Y-purine receptor(s) {222,226}. This would suggest a physiological role for purines in the neonatal period. It has been suggested that ATP reduced PAP in adult human patients with chronic obstructive lung disease and secondary PH was due to inhibition of hypoxic vasoconstriction {141}.

ATP also causes a reduction in the PAP of adult humans and animals with pulmonary hypertension {140,141,139,130,225}. Clinically, infusions of ATP have been successful in treating pulmonary hypertension of infants secondary to congenital heart defects and adults during cardiac surgery {54,145}. The effect of chronic hypobaric hypoxia on the response of the newborn to ATP has not previously been investigated.

In the present study, intrapulmonary arteries, with and without endothelium, were removed from normal pigs before and at birth and during early development and the responses to cumulative doses of ATP were investigated. In addition, the reactivity of the IPA isolated from piglets exposed to chronic hypobaric hypoxia between birth and 14 day of age was studied.

Methods.

Material: intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (CHH) (50.8kPa) either from birth, or after 3 or 14 days of age, for a period of three days. Human tissue was studied when possible (Table 1).

Methods : vessels were isolated and mounted with or without endothelium for *in vitro* isometric force recording. The effect of ATP was studied in IPA precontracted with $PGF_{2\alpha}$ at different ages and between species. For further details of the protocols used please refer to sections 1 and 2 of Chapter 2.

Table 1. Clinical details of children from whom tissue was studied.

Specimen no.	Details of child
Normal	
3680	8 year old, donor, mild asthmatic. Accidental death.
Pulmonary hypertensive	
3508	16 year old, Primary PHN, lung removed during transplantation.

Results.

Responses of intrapulmonary arteries from normal pigs.

The natural purinergic ligand, ATP, produced a concentration-dependent relaxation in IPA precontracted with $PGF_{2\alpha}$ [30μM] from both fetal and newborn animals, indicating that birth had no effect upon the response (Fig.1A). At the beginning of the present relaxation study the concentration-range applied to the newborn rings was the same as that used by other investigators in studies of mature vessels {238,237}. However, it was soon apparent that the maximum concentration should be increased to obtain a more complete relaxation response curve in the younger age groups. This was implemented early in the study.

Increasing cumulative doses of ATP induced sustained relaxations which did not reverse between doses. Full sigmoidal curves could be fitted to the dose-response relationships for IPA from older animals and the EC_{50} values determined. In younger animals complete dose-responses were not seen because the highest practical concentrations of agonist were insufficient. However, sigmoidal curves were fitted by using non-linear regressional analysis and EC_{50} values were determined by assuming that a 100% relaxation was possible in the young animals as it had been found to be in the older animals. There was no indication that the maximal relaxation response was potentially less than 100% in the IPA from younger pigs (Table 2). The EC_{50} value is that which concentration of agonists which would produce a 50% effect, in this case vasodilatation. The greater the value the smaller the potency of the agonist.

Table 2. EC_{50} for the relaxation response to cumulative-ATP in normal porcine IPA precontracted with $PGF_{2\alpha}$.

Animal age group	With endothelium EC_{50} ± sem	Without endothelium EC_{50} ± sem
Fetal	6 mM ± 3 (3)	2 mM ± 2 (3)
Newborn	24 mM ± 13 (7)	11 mM ± 5 (4)
3 days old	4 mM ± 2 (6)	5 mM ± 2 (7)
6 days old	4 mM ± 2 (8)	5 mM ± 2 (5)
14 days old	1 mM ± 1 (8)*	2 mM ± 1 (6)*
Adult	62 μM ± 16 (10)*	38 μM ± 20 (5)*

Sem = standard error of the mean. EC_{50} data was derived from curves fitted on the assumption that Emax was 100% in all cases. One pair of results from a newborn animal was not used to derive an EC_{50} as the relaxation response curve obtained was insufficient to fit a sigmoidal curve. (n) = number of animals from which the data is derived. * indicates a significant difference from the newborn group at a significance level of $p<0.05$.

The sensitivity of the relaxation response to ATP increased with increase in age, the response at 14 days and older was significantly greater than in the newborn ($p<0.05$). Removing the endothelium did not influence either the relaxant or contractile component of the ATP response at any age (Fig.1B). In older animals transient contractions were induced by ATP at concentrations ≥ 1mM , but this did not occur in the IPA from fetal or newborn pigs (Fig.2). The proportion of animals within each age group showing a contractile response increased with increase in age (Table 3). The magnitude of the contractile response also increased gradually with age.

Table 3. The percentage of precontracted ($PGF_{2\alpha}$ [30μM]) IPA, each from a different animal, which contracted to ATP.

Normal	Fetal		Newborn		3 days		6 days		14 days		Adult	
	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-
%	0 : 0		0 : 0		83 : 71		75 : 80		60 : 60		100 : 100	
Pulmonary hypertensive					0-3 days		3-6 days		14-17 days			
%					20 : 20		14 : 14		50 : 50			

E+ indicates data for IPA with endothelium and E- indicates data for IPA without endothelium.

The sensitivity of the ATP induced relaxation response in arteries precontracted with 30mM KCl did not increase with increase in age, in contrast to the increase of sensitivity observed when $PGF_{2\alpha}$ was used to precontract the vessels (Fig.3, Table 4).

Table 4. EC_{50} data for ATP-induced relaxation of porcine IPA precontracted with 30mM potassium chloride.

Age group	with endothelium	without endothelium
fetal - 6 days old	18 mM ± 4 (9)	4 mM ± 2 (3)
14 days - adult	5 mM ± 3 (8)	7 mM ± 6 (5)

Mean data ± standard error , (x) = number of animals.

Responses of intrapulmonary arteries from PH pigs.

The maturation of the relaxation response to ATP was not significantly influenced by exposure to CHH (50.8kPa) for 3 days, starting either at birth or at 3 days or 14 days of age, when compared to age-matched controls. (Fig 4 A,B,C). In the older animals first exposed at 14 days of age the sensitivity to ATP was significantly greater than was normal for age

(Table 5). Removing the endothelium did not significantly affect the relaxation response to ATP of IPA precontracted with $PGF_{2\alpha}$ from either normal or pulmonary hypertensive pigs (Fig. 1B & Fig.4 A-C, Table 2 & 5).

Table 5. The effect of removing the endothelium from IPA isolated from pulmonary hypertensive animals upon the response to ATP.

PH animals	with endothelium. EC_{50} (± sem (n))	without endothelium. EC_{50} (± sem (n))
Exposed to CHH from birth - 3 days	5.82mM ± 3.43 (5)	3.88mM ± 2.761 (5)
Exposed to CHH from 3-6 days	9.93mM ± 4.91 (8)	1.943mM ± 4.513 (6)
Exposed to CHH from 14-17 days	65.9μM ± 21.4 (4)	55μM ± 50 (4)

Sem = standard error of the mean. EC_{50} data was derived from sigmoidal curves fitted with the assumption that Emax was 100% in all cases. (n) = number of animals the data is derived from.

Inhibition of nitric oxide synthase (NOS) with L-NMMA [30μM] increased the sensitivity of the relaxation response in IPA with endothelium from 2 of the 3 newborns but had no effect in the adult group (Fig.5 A,B). Indomethacin inhibition of cyclooxygenase synthase had no effect on the ATP-induced relaxation in IPA from newborn pigs (Fig.5 C,D).
ATP induced an endothelium-independent relaxation of IPA from an 8 year old normal child (Fig.6 A,B). ATP also relaxed the IPA from a pulmonary hypertensive child, which had been precontracted with KCl (Fig.6 C).

Discussion.

In the present study, the elastic intrapulmonary arteries (IPA) ,precontracted with $PGF_{2\alpha}$, from adult pigs relaxed to ATP by an endothelium-independent mechanism. Because only a few *in vitro* studies of isolated pulmonary arteries (PA) from mature animals have previously been carried out, it has been difficult to compare the findings of the present study with those of other investigators.

Firstly, the PA have been isolated from different regions of the pulmonary arterial circulation. In the adult rat and rabbit the pulmonary trunk as it arises from the heart was studied , while in the guinea-pig the extrapulmonary PA to each lung was used. Both large and small PA have been studied from within the human lung {238,326,240,152,237}. One could suggest that they are all PA but it may be predicted that the reactivity and function of a PA outside the lung would differ from one located in close proximity to the gaseous interface. The site of pulmonary vascular resistance has been localised to the small arteries in the adult lung , but this may not be so in the fetal lung and so the site of vascular resistance may change during adaptation to extrauterine life {158,166}.

Secondly, the same preconstricting agonist has not been used in any two studies previously reported and has even varied within a species. Phenylephrine (acting via calcium influx and release of intracellular stores), a thromboxane analogue U-44069 and $PGF_{2\alpha}$ (both acting via inositol 1,4,5-triphosphate (IP_3) / diacyglycerol (DAG)) and 5-hydroxytryptamine (5-HT) (acting via cAMP) have all been used, involving different mechanisms to increase vascular tone {3,238,152, 326, 240, 237}.

Thirdly, previous studies represent relaxation responses as a percentage of the tension developed by the preconstricting agonist, but in the present study papaverine [100μM] was used to remove all active tension in the IPA. Therefore, it is almost impossible to compare the data given by other investigators with our own. Papaverine was used in the present study because it was postulated that age may influence the degree of active tone present at different ages during postnatal development and that this possibility should be taken into consideration when calculating responses.

Despite these variations in methodology, the sensitivity of the relaxation response to ATP in PA with endothelium is remarkably similar in the different studies, including findings in the adult porcine IPA in the present study. However, differences appear between the studies when the sensitivity of the relaxation response to ATP of PA without endothelium is analysed. In the present study, the response to ATP was entirely endothelium-independent at all ages in both normal and pulmonary hypertensive animals and normal human IPA. It has been

suggested that the cellular distribution of ATP-receptors depends on vessel size, and that the variation in results within a species might be accounted for by differences in the location of the vessel studied within the vascular bed {237}. The sensitivity of the relaxation response to ATP in large and small adult human IPA with endothelium was found to be similar but removing the endothelium significantly reduced the effect of ATP in large IPA, and had no effect on the small vessels {237}. But, 5-HT and phenylephrine were used to precontract the large IPA whilst $PGF_{2\alpha}$ was used for the small IPA, and this could explain the difference in response. However, the relaxation to ATP was endothelium-independent for the extrapulmonary artery of the adult rabbit but endothelium-dependent for the adult human PA, when phenylephrine used in both studies {326,152}. The response to ATP has also been found to be endothelium-independent in the adult rabbit hepatic artery, hepatic portal vein, mesenteric artery, aorta, coronary artery, the isolated canine femoral artery, guinea-pig coronary artery and the human forearm vascular bed {257,215,53,97,98,115,214,344}. By contrast, the ATP vasodilatation of the canine hindquarters, the adult rat thoracic aorta and mesenteric artery were found to be endothelium-dependent {265, 336, 350,420}. Some vessels showing an endothelium-independent response also displayed a degree of endothelium-dependency such as the adult rabbit hepatic artery {335}. Other endothelium-independent agonists cause a greater relaxation of the IPA from newborn pigs than do endothelium-dependent agonists, in the same animal model. Exogenous nitric oxide can relax IPA from the newborn, whilst receptor mediated endothelium-dependent relaxation by acetylcholine and the non-receptor mediated relaxation to the calcium ionophore A23187 was absent until about 2 days of age {241}. ANP and activation of K^{+}-$_{ATP}$-channels by levcromakalim relaxed IPA from newborn pigs by an endothelium-independent pathway as in the present study {260,41}. All the agonists described may play a part in reducing the pulmonary arterial pressure during adaptation to extrauterine life and ATP induced relaxation might also contribute to this process .

The lack of an inhibitory effect by L-NMMA or indomethacin would argue against nitric oxide or cyclooxygenase products being mediators of the ATP-induced relaxation in the newborn. The enhancement of the relaxation response by L-NMMA in the newborn vessels

was unexpected if, as suggested in the literature NO can mediate ATP-induced vasodilatation {115}. However, by blocking the basal release of NO it is possible that the basal tension in the IPA increased, allowing more active tension for ATP to relax.

The effect of adaptation to extrauterine life and postnatal age upon the IPA reactivity to ATP has not been studied before. Relaxation to ATP of the IPA from fetal pigs was similar to that seen in the newborn animals. Other workers have reported a similar potency for ATP-induced endothelium-independent IPA vasodilatation in neonatal piglets {319}. In the present study, after birth the sensitivity of the relaxation response increased during the first week of life and by 14 days was significantly greater than the newborn. A further 20 fold increase in the sensitivity was found when comparing the 14 day old and adult animal. The same age-dependent response pattern was found for the ADP-induced endothelium-dependent vasodilatation in isolated ovine IPA {3}. In some instances in the systemic arteries the response changed with age but unlike the present findings in the pulmonary circulation, the endothelium-independent relaxation decreases with age. The ATP endothelium-independent relaxation of the rat aorta decreases with age, while the endothelium-dependent response was unaffected {219}. But in the rabbit aorta, the endothelium-dependent response was less in vessels from adult than from younger rabbits {88}. This may reflect a redistribution of receptors, a reduction in receptor number or receptor efficacy, expressed on the vascular smooth muscle cells with age. As the thickness of the blood vessel wall increases with age, the physiological supply of ATP from the endothelium and nerves may become more remote from the smooth muscle and result in downregulation of the response.

Preincubation with indomethacin in newborn IPA had no effect on the ATP-induced relaxation, suggesting a lack of involvement by cyclooxygenasae products in the response. P2Y-receptors have been associated with the production of prostacyclin (PGI_2) in the neonatal porcine lung and human umbilical vein endothelium {179,78}. Thromboxane A_2 (TXA_2) has been shown to mediate vasoconstriction by ATP and adenosine in systemic and pulmonary arteries{374,287,291}.

Observing a relaxation response to ATP is not conclusive evidence for the response being mediated by receptors of the P2-nucleotide family. The possibility that adenosine mediates the relaxation response to ATP must always be a consideration. ATP has been shown to be rapidly metabolised to adenosine by ectoATPase degradation enzymes located on endothelial and smooth muscle cells {317,106,431}. This process has been found to be particularly rapid in the pulmonary circulation {116, 82, 363}. Adenosine could then act either at P1-nucleoside-receptors mediating vasoconstriction (A1) or vasodilatation (A2) on pulmonary artery smooth muscle cells {261}. The rate of metabolism and clearance of purines varies in different tissues and species {216,79}. These processes could therefore conceivably vary during the development of a vascular bed, resulting in maturation of a response. Alternatively, ATP and adenosine from metabolism could operate at the same receptor, distinct from P2- and P1-receptor families. Studies using adenosine-receptor antagonists suggest that a novel "P3"-nucleotide receptor may also be present on the smooth muscle cells of the adult rabbit aorta, which is sensitive to both ATP and adenosine {89}.
When KCl was used to precontract IPA in the present study, the ATP induced relaxation was similar at all ages. The increase in the sensitivity of the relaxation response to ATP with age when $PGF_{2\alpha}$ was used to precontract the intrapulmonary arteries may involve K^+-channel(s) maturation. Transient contractions to high concentrations of ATP have previously been reported in mature pulmonary arteries and may be due to ATP acting at a P2X-receptor or adenosine at P1 (A1)-receptor(s), both of which are located on vascular smooth muscle {291,53,262}. The nature of the ATP contractile response is fully addressed in Chapter 6 of the present study. In the knowledge from previous work that the newborn porcine IPA only contracts to certain agonists it may be suggested that certain receptor-coupled mechanisms leading to vasoconstriction mature with age, including the P2-receptors {233}.

We have shown that the normal relaxation response of IPA from normal fetal and newborn pigs to ATP was similar, only improving after birth. These findings would suggest that the act of birth may initiate maturation of the response, although we may be witnessing a gradual maturation starting early in fetal life which continues after birth.

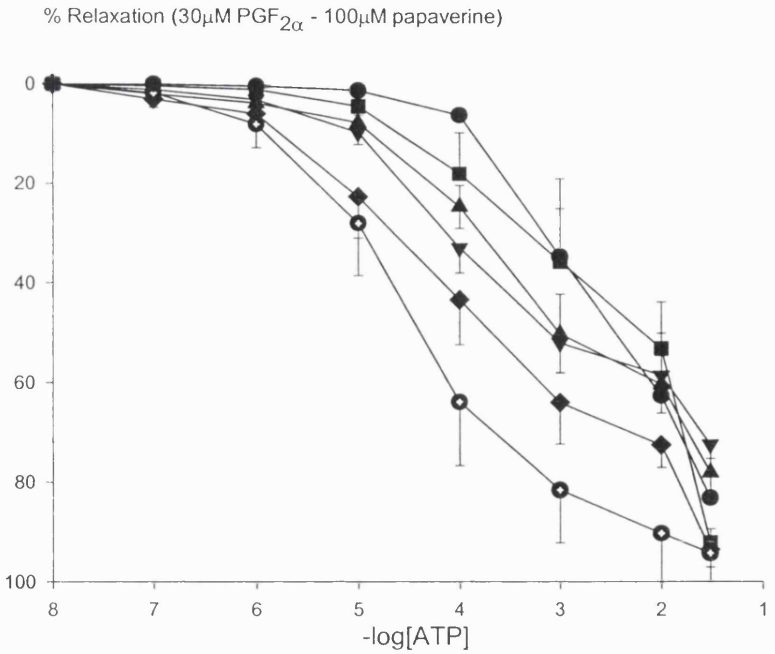
The present study has shown that the endothelium-independent relaxation to ATP in newborn porcine intrapulmonary arteries was not impaired by chronic hypoxic pulmonary hypertension at any time during the first two weeks of life, despite the vascular remodelling which has occurred on exposure to CHH {7,9} . The response to ATP was unaltered in the presence of persistent pulmonary hypertension when compared with the response in vessels from normal 3 day old animals. This was in contrast to endothelium-dependent and -independent responses involving the NO pathway {399}. ACh -induced endothelium-dependent relaxation normally develops during the first 3 days of life which is prevented by exposure to CHH and the response normally established by 2 days of age is inhibited by exposure to chronic hypobaric hypoxia at 6 or 14 days of age. Endothelium-independent relaxation in response to NO was also attenuated {399}. This inhibition of relaxation, despite a normal increase in agonist stimulated cGMP levels, has been attributed to a reduction in the ability of cGMP to cause relaxation of vessels from CHH animals, such as a higher threshold of cGMP-induced relaxation {399}. A 3 day period of chronic hypoxia did not impair the ATP-induced endothelium-independent relaxation response of the porcine IPA precontracted with $PGF_{2\alpha}$. This would indicate that even in a labile vascular system such as the neonatal pulmonary circulation, the mechanism by which ATP relaxation operates was resistant to a period of remodelling by hypoxia. ATP-induced relaxation was also evident in IPA isolated from a pulmonary hypertensive child precontracted with KCl.

In the normal animal, after 2 weeks the reactivity of the porcine IPA is similar to that the adult vessel, where the pulmonary arteries function at a relatively low tone and pressure {154}. A 3 day period of chronic hypobaric hypoxia at this age still results in pulmonary hypertension, inhibition of ACh-endothelium dependent relaxation and attenuation of the response to NO. We were surprised therefore when the sensitivity of the ATP-induced relaxation in this age group was augmented. ATP has been shown to produce a tone-dependent dual response where it is more likely that ATP will induce vasoconstriction at low tone and vasodilatation at raised tone. In the precontracted IPA from normal 14 day old pigs, a relaxation response occurred at lower concentrations removing the $PGF_{2\alpha}$-induced tone. At the lower tone ATP induces transient contractions as the concentration increases,

predicted for a tone-dependent agonist which produces a response which is the resultant of action at vasodilator and vasoconstrictor receptors. In IPA taken from piglets exposed to CHH at 14 days of age, ATP produced a greater relaxation of IPA even at higher concentrations where contractions would have be predicted in normal. It therefore seems possible that a loss of contractile reactivity to ATP has led as an increase in relaxation.

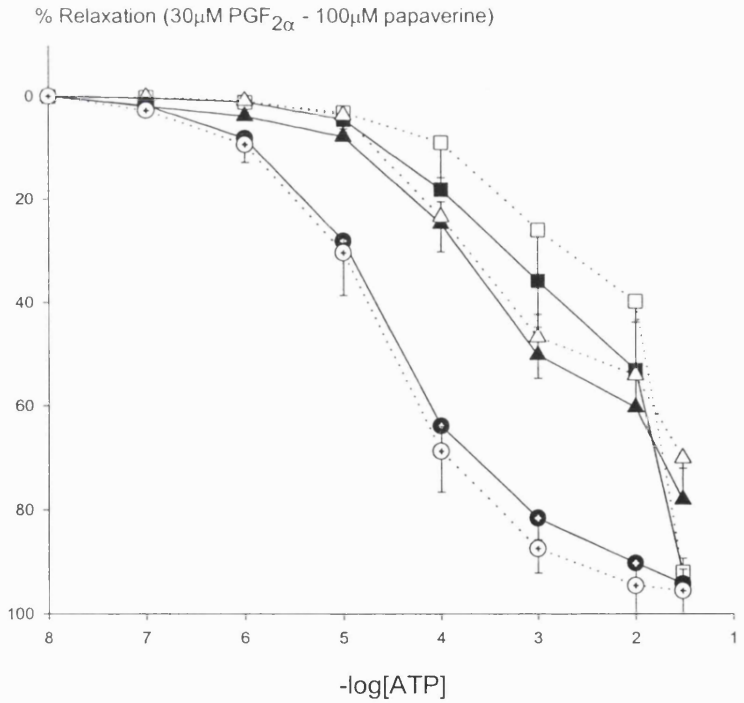
The findings in the present *in vitro* study on isolated porcine IPA suggest that ATP-mediated vasodilatation may play a large part in the normal fall in pulmonary vascular resistance which occurs at birth. This conclusion is supported by the increase in ATP plasma levels at birth and the studies of fetal and newborn lambs *in vivo* showing a reduction in pulmonary arterial resistance following infusion of ATP and adenosine at postnatal blood concentrations {221,224,226}. The present study has produced two findings which have implications for the future use of ATP in the clinical management of pulmonary hypertension, not only in the newborn but also in older children. Firstly, IPA isolated from both newborn and older pulmonary hypertensive piglets relaxed in response to ATP. Secondly that even at high concentrations of ATP a vasoconstrictor response does not occur in IPA from infants. This is important since accurate, consistent titration of a drug in a sick newborn is not always possible due to changing haemodynamics. The findings in the present study would indicate that a sudden rise in pulmonary arterial pressure due to an excessive amount of ATP would be unlikely to happen. Infusions of ATP cause pulmonary-specific vasodilatation at doses which have no systemic effects in neonatal and older animals with pulmonary hypertension induced by hypoxia and drugs (306,308,225} .

A.



Relaxation response to ATP in intrapulmonary arterial rings with endothelium precontacted with 30μM $PGF_{2\alpha}$, from pigs during adaptation to extrauterine life ((fetal (n=3 ●), newborn (n=8 ■), 3 day old (n=6 ▲), 6 day old (n=8 ▼), 14 day old (n=7 ◆), adult (n=9 ✪)) . The sensitivity to ATP increased with increase in age. Error bars = sem.

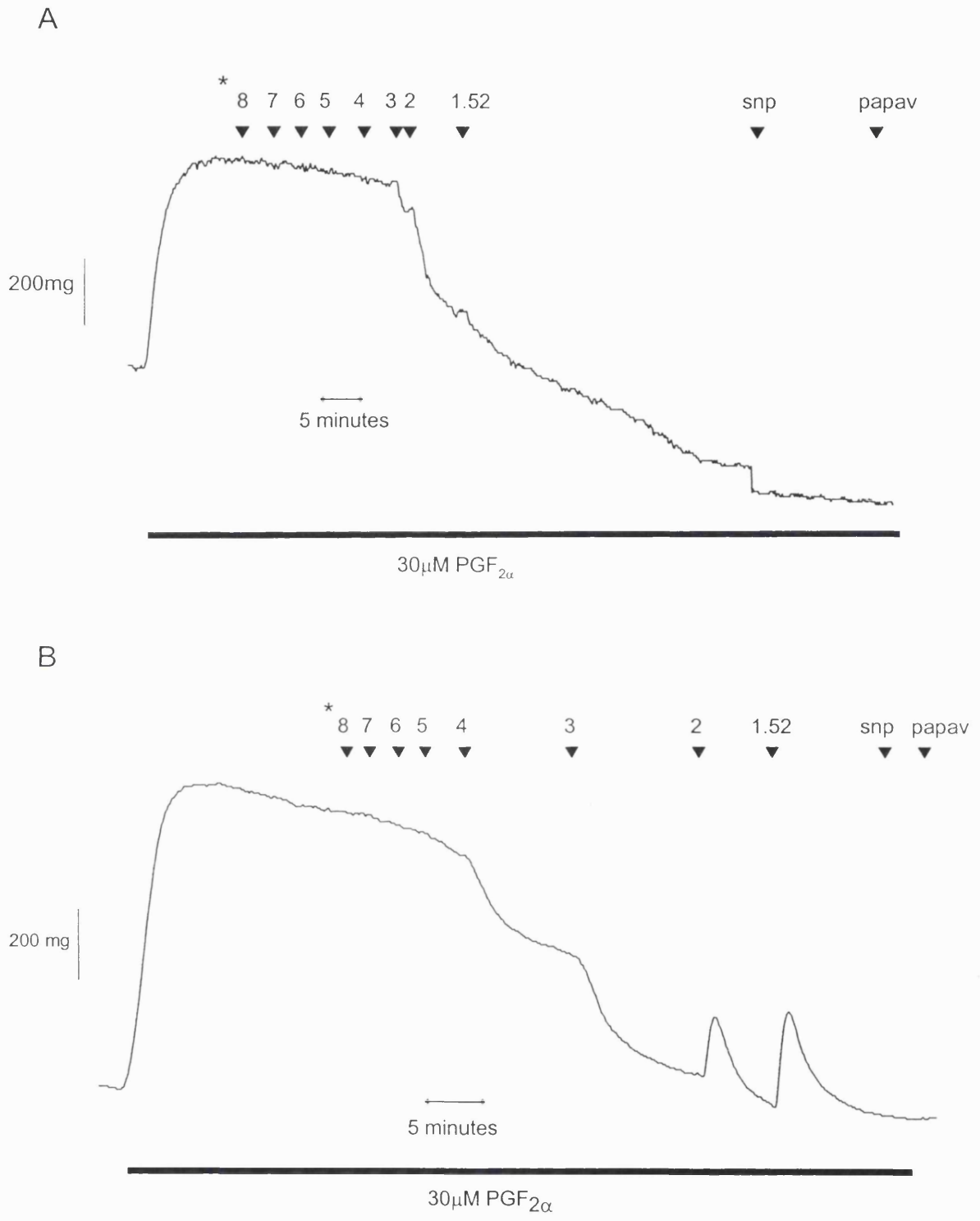
B.



Relaxation response to ATP in porcine intrapulmonary arterial rings with (solid symbol and line) and without (empty symbol and broken line) endothelium, precontracted with 30μM $PGF_{2\alpha}$.

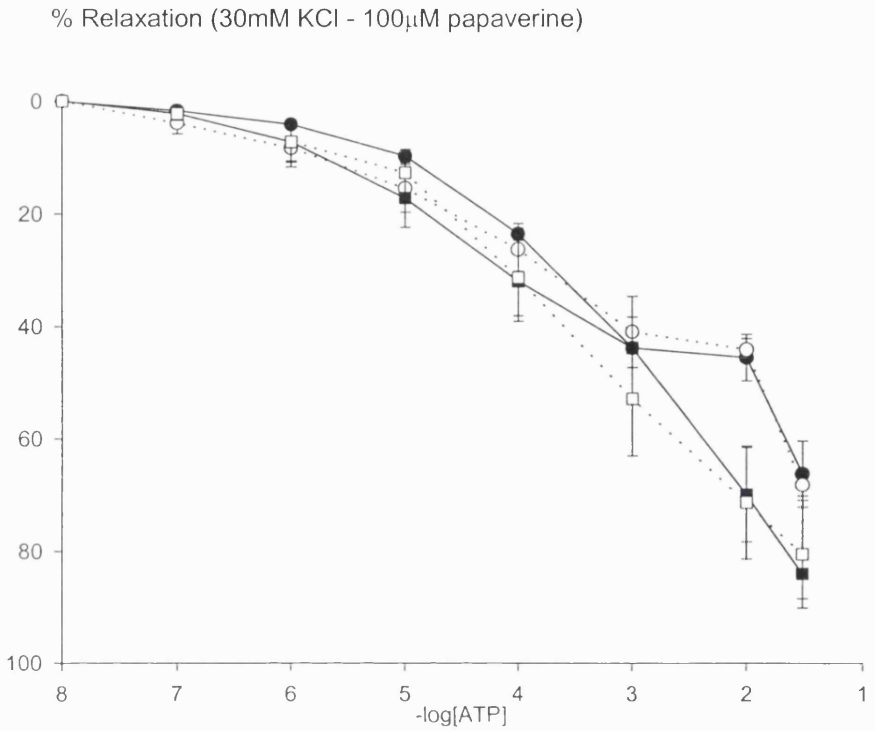
The rings were isolated from pigs as newborns (n=8 ■ and 5 □), at 3 days of age (n=6 ▲ and 7 Δ) and as adults (n=9 ✪ and 5 ⊕).

The endothelium did not influence the response at any age. Error bars = sem.



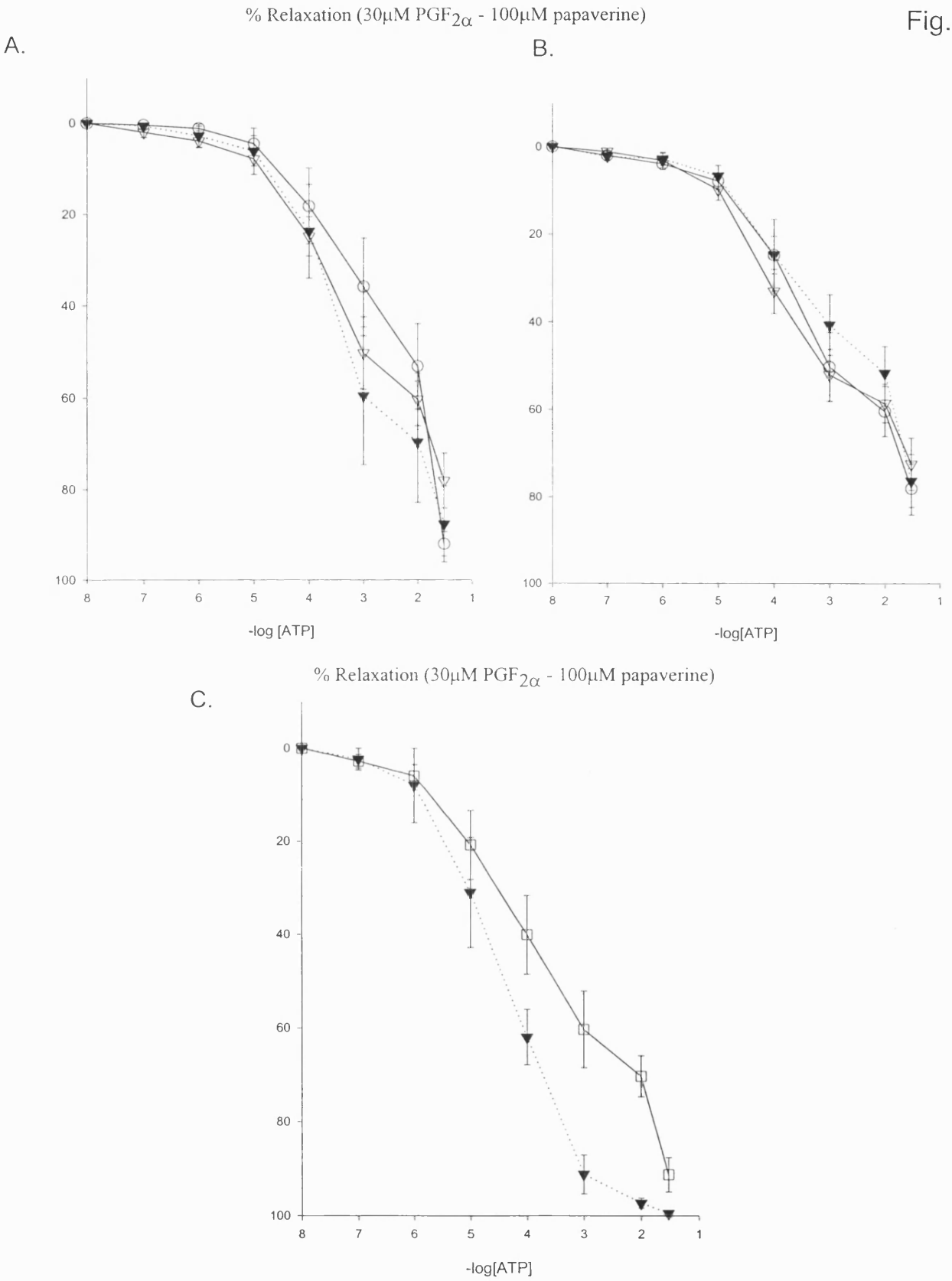
Representative traces of the response to ATP in newborn (A) and adult (B) porcine intrapulmonary arteries, with endothelium, precontracted with 30μM $PGF_{2\alpha}$. At high concentrations a transient contraction could be induced in older animals. Snp, sodium nitroprusside 100μM; papav, papaverine 100μM. * indicates -log values.

A.



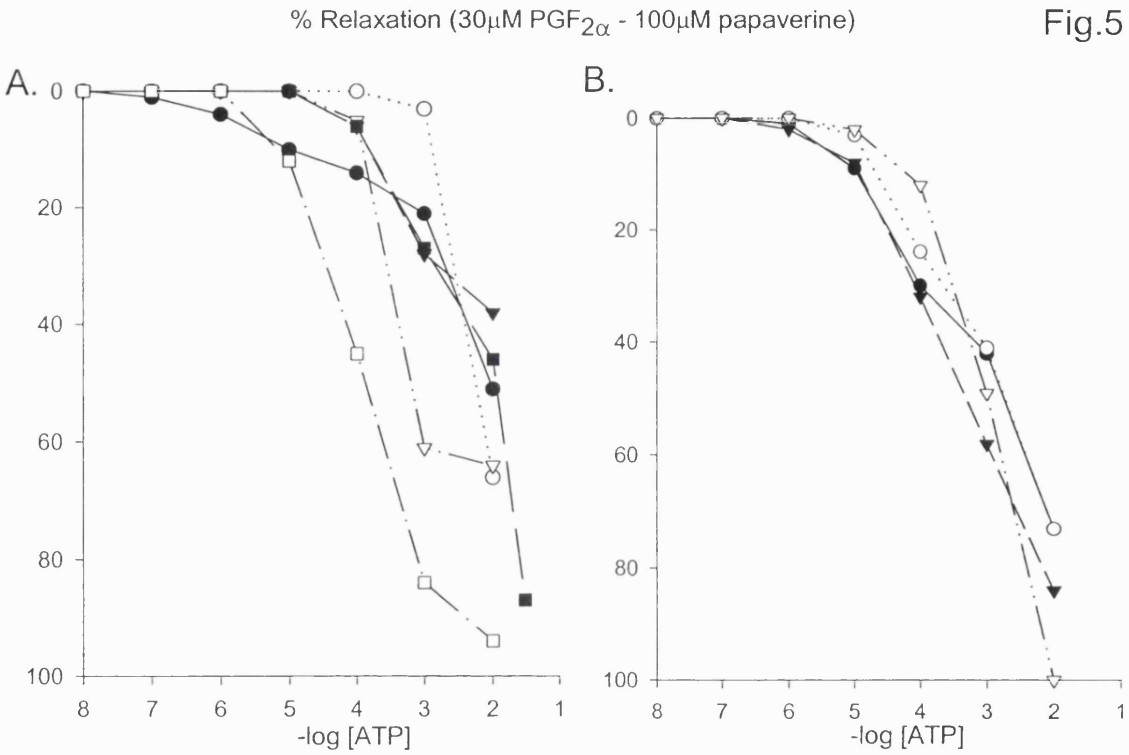
Concentration-response curves to ATP in porcine intrapulmonary arteries precontracted with 30mM KCl. Removing the endothelium did not significantly influence the relaxation response (with, A & B without endothelium). Newborn (n=1), 3 day normal (n=4/2), 6 day normal (n=4/1) ●, 14 day normal (n=3/3) and adult normal (n=5/2)■.
The sensitivity of the relaxation response did not increase with an increase in age.
Bars = sem if n 3, standard deviation if n=2.

Fig. 4

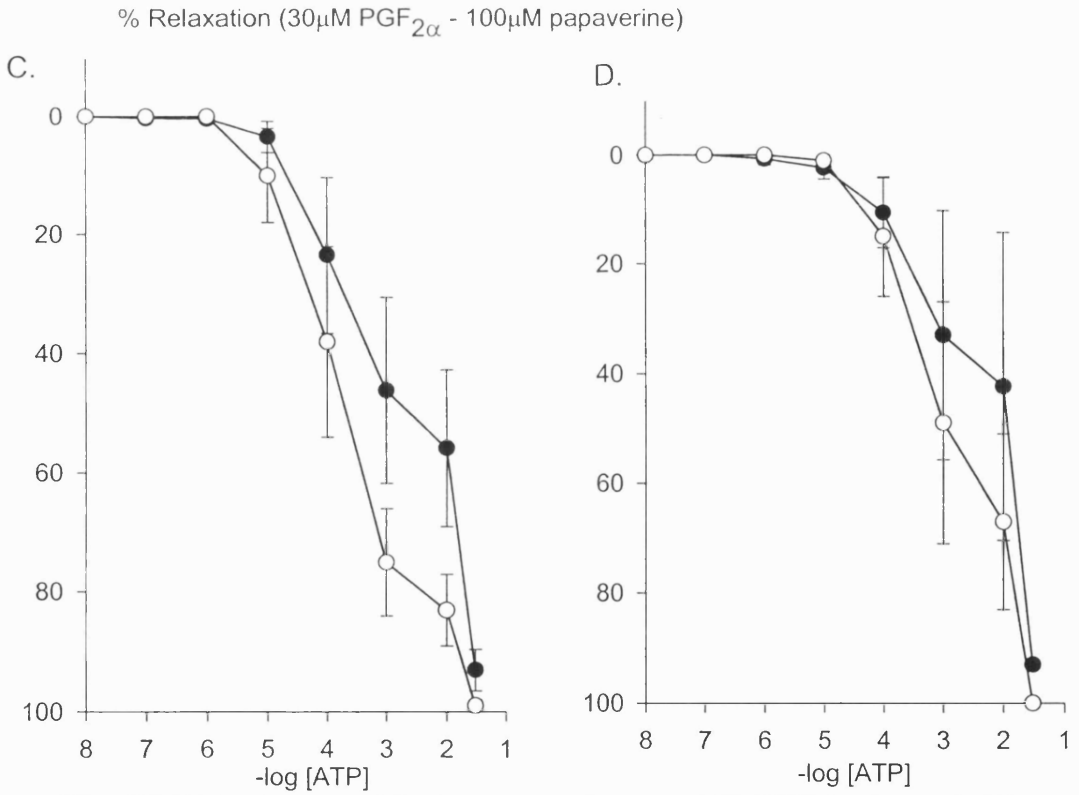


The effect of pulmonary hypertension (PH) on intrapulmonary arterires, with endothelium, precontracted with 30μM $PGF_{2\alpha}$, following exposure to chronic hypobaric hypoxia for 3 days from (A) birth n=5 ▼ (normal newborns n=6 O, normal 3 day old n=6∇); (B) from 3days n=5 ▼ (normal 3 day old n=5 O, normal 6 day old n=8 ∇); (C) and from 14 days n=4 ▼ (normal 14 day old n=8□) on the ATP-induced relaxation.

Fig.5



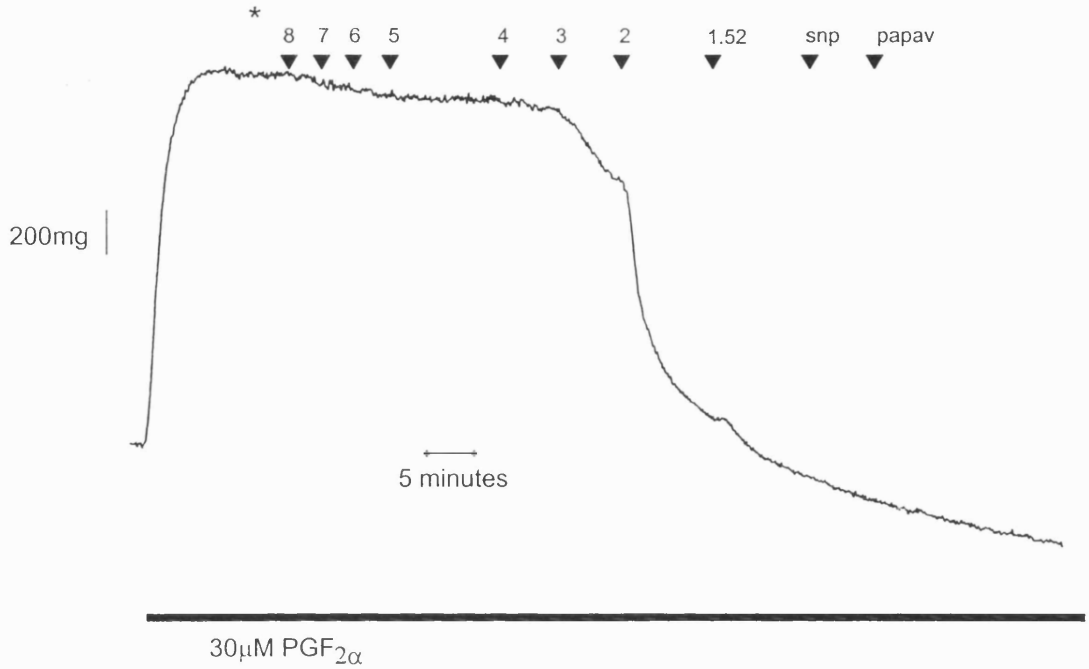
The effect of NOS inhibitor L-NMMA [30μM] on the ATP-induced relaxation of individual precontracted (30μM $PGF_{2\alpha}$) intrapulmonary arteries with endothelium. (A) 3 normal newborns and (B) 2 normal adult pigs.
Solid symbols, without L-NMMA. Empty symbols, with L-NMMA.



Effect of 10μM indomethacin upon ATP-induced relaxation of precontracted (30μM $PGF_{2\alpha}$) newborn porcine IPA, with (C, n=5) and without (D, n=3) endothelium. Indomethacin did not significantly increase the relaxation response to ATP.
Solid symbols, without indomethacin. Empty symbols, with indomethacin. Bars = sem.

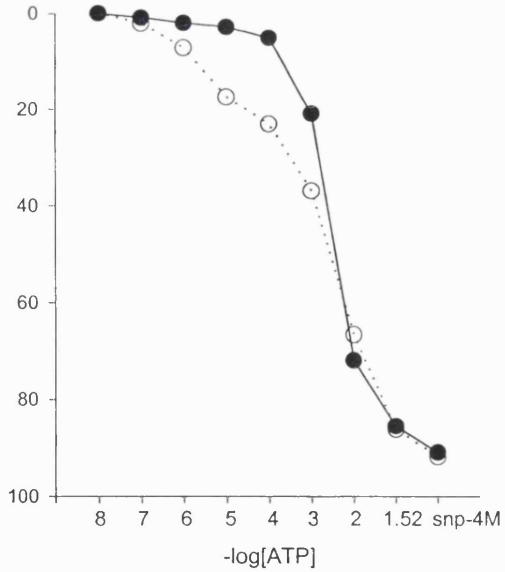
Fig.6

A



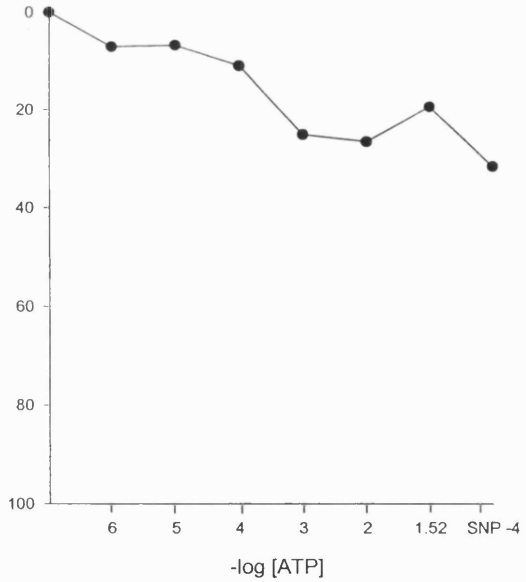
Trace of the response induced by ATP (A) of IPA from an eight year old normal child. IPA with endothelium was precontracted with 30μM $PGF_{2\alpha}$. * -log values.

B. % Relaxation (30μM $PGF_{2\alpha}$ - 100μM papaverine)



Dose-response to ATP of IPA precontracted with 30μM $PGF_{2\alpha}$ from an 8 year old normal child. A gradual decline of $PGF_{2\alpha}$ tension accounts for the apparent greater relaxation in the ring without endothelium (empty symbols).

C. % Relaxation (30mM KCl bolus - 100μM papaverine)



C. Dose-response to ATP in a precontracted (30mM KCl) IPA with endothelium ,from a 15 year old child with pulmonary hypertension. The original recording was extremely noisy, but there did appear to be a relaxation response.

Fig.6. Response of IPA from a normal (A) , and a pulmonary hypertensive child (B) to cumulative additions of ATP.

Chapter 5. P2-nucleotide receptor agonist induced relaxation responses of intrapulmonary arteries from normal and pulmonary hypertensive pigs and children.

Summary.

1. Intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours) ,animals aged 3, 6, 14-17 days and adult pigs were isolated and mounted with or without endothelium for *in vitro* isometric force recording. Pulmonary hypertension was produced in newborn piglets (PHN) by exposing them to chronic hypobaric hypoxia (CHH)(50.8kPa) for 3 days from birth. IPA were studied from the lungs of two children with no cardiopulmonary disease (aged 5 & 8 years) and six children with pulmonary hypertension contributing to death (aged 36 hours, 10 days, 4 months and three aged 15-16 years old).

2. In the porcine lung, 2-methylthioATP (2-meSATP) and adenosine 5-O-(2-thiodiphosphate) (ADPβS) relaxed normal pulmonary arteries independently of the endothelium at all ages studied . The sensitivity of relaxation to ADPβS increased from birth to 3 days of age, and was significantly greater in the adult. The sensitivity of relaxation to 2-meSATP appeared to increase with age, but the change was not statistically significant. PHN did not significantly affect the relaxation response to 2-meSATP, but reduced the relaxation response to ADPβS, when the findings were compared with those in normal age-matched animals. Removing the endothelium did not significantly affect the relaxation response to 2-meSATP or ADPβS in vessels taken from PH animals.

3. In the porcine IPA, the poor relaxation response evoked by α,β-methyleneATP (α,β-meATP) and UTP did not change significantly with age. α,β-meATP and UTP evoked transient contraction responses and the magnitude of the response increased with age . Removing the endothelium significantly increased the potency of the relaxation response to UTP. PHN resulted in the loss of relaxation to α,β-meATP in 2 of the 4 animals studied. The response to UTP was not investigated in PHN.

4. In the human studies ADPβS relaxed IPA from 5 and 8 year old normal and 10 day-old and 4 month-old hypertensive children , with a degree of endothelial-dependency in both groups.

The P2Y-purine receptor agonist 2-meSATP relaxed IPA from an 8 year old normal child and removing the endothelium reduced the response.

5. These findings suggest that a $P2Y_1$-receptor mediates the IPA vasodilatation to P2-agonists in the neonate and may play a role in achieving or maintaining a low pulmonary arterial pressure in the transitional postnatal pulmonary circulation, as well as in regulating vascular tone in the mature lung. The same rank order of P2-receptor agonist-induced relaxation potency was found for animals exposed to CHH, suggesting that a $P2Y_1$-receptor was still functioning. The present study showed that IPA isolated from the lungs of children were still viable 20 hours postmortem for *in vitro* experiments. ATP produced an endothelium-independent relaxation, similar to that seen in the porcine vessels. However, both P2Y-selective agonists , ADPβS (P2Y) and 2-meSATP ($P2Y_1$) , were found to relax, in part, via an endothelium-dependent pathway which was reduced in older children with pulmonary hypertension .

Introduction.

The high pulmonary arterial pressure of the fetal circulation normally falls during adaptation to extrauterine life. But agonist-induced endothelium-dependent vasodilatation to acetylcholine (ACh) does not mature until 2 or 3 days of age in the porcine intrapulmonary artery (IPA) (See Chapter 3) {241,425}. Endothelium-dependent vasodilatation to ACh, bradykinin, ADP is also immature in neonatal lambs and rabbits {448,369,379,3}. Newborn IPA smooth muscle dilates in response to agonists such as exogenous nitric oxide and sodium nitroprusside, although the response is significantly less at birth than at older ages {241}.

In the present study we have shown that ATP relaxes newborn effectively by an endothelium-independent mechanism (See chapter 4). The purpose of this chapter is to characterise the receptor subtype mediating the response during normal development and in PHN. The study is then extended to compare the findings in comparable vessels taken from normal children and those with pulmonary hypertension with the porcine findings. There have been no *in vitro* studies regarding P2Y-receptor(s) in IPA from newborn or neonatal animals.

The original division of P1- and P2-receptors depended to a large extent upon the rank order of potency of ATP and associated purine metabolites. This approach was also used to divide P2-purine receptors into P2Y and P2X subtypes, incorporating new synthetic purine analogues which displayed receptor-specificity in some tissues. Recently pyrimidines, such as UTP and UDP, have been found to be potent agonists at certain P2Y-receptor agonists, provoking the inclusion of these nucleotides in classification studies. This method of classification is still currently accepted in the absence of specific receptor antagonists, and is used in concert with molecular biological system of classification.

In the present study we determined the effect ATP (native purinergic ligand) ,2-meSATP ($P2Y_1$-agonist,), ADPβS (P2Y-agonist), α,β-meATP ($P2X_{1 \text{ and } 3}$-agonist), and UTP ($P2Y_{2,4,6}$) on isolated porcine intrapulmonary arteries from normal pigs during early postnatal life. The agonists for the present study were selected because of their generally accepted receptor selectivity and, apart from UTP, an increased stability to metabolism {48,71, 95, 101, 134, 215,216, 363}. This strategy aims to reduce the possibility that purine metabolite activity at P1-receptors is responsible for responses recorded. 2-meSATP has been used by other workers to classify P2Y-receptors, located on vascular endothelial and smooth muscle cells or expressed on isolated cells {326,238,412}. ADPβS has been used less extensively but has been shown to induce an endothelium-dependent relaxation of canine coronary artery through the release of NO and possibly by membrane hyperpolarization {279,184}. ATP, 2-meSATP and ADPβS each induced an endothelium-dependent relaxation of precontracted aorta from newborn piglets (255,148). α,β-meATP was identified as an agonist, which displays selectivity for the P2X-contractile purine ionotropic receptor family, particularly $P2X_1$ {65}. More recently, [^{3}H] α,β-meATP, has been used to localise and classify P2X-receptor binding sites in the mature lung of cat and human {34}. The $P2Y_4$ and $P2Y_6$-receptors have been defined receptors mediating vascular relaxation, where UTP and UDP respectively is the most potent agonist, while UTP was equipotent with ATP at the $P2Y_2$ {74}. Previously, UTP vasodilatation has been studied in systemic vessels, but was recently investigated in adult rabbit main pulmonary artery (334,326}.

UTP has been shown to induce endothelium-dependent relaxation of the adult rabbit pulmonary artery via NO release and it stimulates PGI_2 release from cultured aortic endothelial cells {326,245}. UTP-induced vasoconstriction occurs in vessel of many types but it is not clear whether there is a specific pyrimidine-contractile receptor {406}. It has been suggested that the contractile action of UTP in the pulmonary vasculature of the adult rat occurs via a novel ionotropic receptor, following antagonist studies{168,348}.
P2Y-receptor mediated vasodilatation of PA has been reported to be both endothelium-dependent and -independent. By using the P2-purine receptor selective agonist 2-meSATP, a P2Y-purine receptor mediated relaxation has been identified on the endothelium of IPA from adult rats {238}. However, using the same approach a P2Y-vasodilating receptor has been identified on the smaller IPA vessels from adult humans {237}. Both ATP and ADP have been found to induce an endothelium-dependent relaxation of large IPA *in vitro*, isolated from adult humans {152}.

Pulmonary hypertension , in particular PHN, contributes to morbidity and mortality in a number of pathological conditions. Persistent fetal circulation may be due to one or more factors, including hypoxia. Rarely it can be idiopathic. The pulmonary circulation fails to adapt to extrauterine life and the pulmonary arterial pressure remains high. If the situation is not corrected early, then a degree of abnormal vascular remodelling may make the condition irreversible {161}. As well as there being a reduction in the activity of certain vasodilator substances, it has been suggested that the increased PAP may also be due to an increase in response to contractile agonists {399}.
Current clinical management entails mechanical ventilation with a high concentration of O_2 , and vasodilator drugs such as inhaled nitric oxide or a prostacyclin infusion. There are a few reports that central line infusions of ATP have been successful in controlling a high pulmonary arterial pressure (PAP) in infants and adults following intracardiac repair {54, 145}. Other workers have also found that ATP infusions reduce the PAP of adults with pulmonary hypertension associated with chronic respiratory disease, while adenosine is ineffective {140}.

The aim of this study was to elucidate P2-receptor activity in the immature lung, normal and hypertensive, identifying the types of receptor involved by applying the current IUPHAR guidelines after compiling a rank order of agonist potency {74}. The study was extended to compare the reactivity of porcine IPA and human IPA to P2-nucleotides, the vessels having been isolated from similar segments of the pulmonary arterial tree. A model of PPHN was produced by exposing newborn piglets to chronic hypobaric hypoxia (50.8kPa) for 3 days {399}.

Methods.

Material: intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (CHH) (50.8kPa) from birth, or after 3 for 3 a day period. Human tissue was studied when possible (Table 1).
Methods : vessels were isolated and mounted with or without endothelium for *in vitro* isometric force recording. The response of IPA precontracted with $PGF_{2\alpha}$ to P2-receptor agonists, 2-meSATP, ADPβS, α,β-meATP and UTP were studied from normal and pulmonary hypertensive pigs. The responses to 2-meSATP and ADPβS were also studied in IPA from normal and pulmonary hypertensive children. For further details of the protocols used please refer to sections 1 and 2 of Chapter 2.

Table 1. Clinical details of children from whom tissue was studied.

Specimen number	Details of child
Normals	
3680	8 year old, donor, mild asthmatic. Accidental death.
4095	5 year old. Tuberous sclerosis, previous heart transplant. Heart failure from acute rejection. Normal PA histology.
Pulmonary hypertensive	
4039	16 years old. Primary PHN, lung removed during transplantation.
4066	15 years old. PHN secondary to complex congenital heart disease, lung removed during heart / lung transplantation.
4087	4 month old. PHN, congenital lung dysplasia and hypoplasia.
3954	36 hours old. PHN, congenital lung dysplasia and hypoplasia.
4139	10 days old. PHN, congenital lung dysplasia and hypoplasia.

Results.

Responses of intrapulmonary arteries from normal pigs.

The response of fetal and newborn IPA to all agonists studied was similar and therefore data from these animals has been combined and is referred to as "newborn". 2-meSATP produced concentration-dependent relaxations in rings from newborn animals (Fig.1A). The sensitivity of the IPA to 2-meSATP relaxations appeared to be greater in the older animals, but the difference was not statistically significant (Fig.1 B,C). A small, transient contraction at ≥100μM was recorded in two newborns (of 4 studied), one 3 day old control (of 4 studied), and one adult animal (of 6 studied). The relaxation response was endothelium-independent for all ages except at 14 days ($p<0.05$) (Table 2). L-NMMA had no significant effect upon the relaxation to 2-meSATP in the adult pig IPA, younger animals were not studied (Fig.1 D). ADPβS produced a concentration-dependent relaxation in rings from newborn animals (Fig.2 A). A small, transient contraction was evoked at ≥100μM in three of seven adults studied (Fig.2 B). The sensitivity and magnitude of the relaxation response increased with

age (Fig.2 C). The EC_{50} was significantly less in vessels from adults than from the newborn ($p<0.05$) (Table 2). The relaxation response was endothelium-independent at all ages.

The P2X-agonist, α,β-meATP induced a relaxation in vessels from the newborn animals and a small transient contraction in vessels from the younger animals (Fig.3 A). In adult vessels the same agonist produced a larger transient contraction in the μM range of agonist, followed by a secondary relaxation (Fig.3 B). There was no significant change in the relaxation response with age and removing the endothelium did not significantly influence the responses to α,β-meATP (Fig.3 C).

At all ages the pyrimidine nucleotide, UTP, induced a transient contraction beginning at large micromolar doses of UTP and followed by a secondary relaxation at >1mM (Fig.4 A,B). In the 3 day old and adult, but not the newborn, removing the endothelium appeared to increase the relaxation response to UTP, but the change was not statistically significant (Fig.4 C).

To establish a rank order of agonist potency based on EC_{50} data (Table 2), each agonist was used in newborn, normal 3 day old and normal adult animal (Fig.5). The rank order of potency was then tabulated for each age group (Table 3).

Table 2. EC_{50} for the relaxation response to cumulative-doses of purines and UTP in **normal** porcine IPA.

	2-MeSATP		ADPβS		α,β-meATP		UTP	
Age group	e+	e-	e+	e-	e+	e-	e+	e-
Newborn	1176 ± 588 (4)	803 ± 336 (4)	2036 ± 541 (5)	8000 ± 4357 (5)	>> 1000 (4) #	>> 1000 (3) #	$2.4 \pm 0.2 \times 10^5$ (3)	$2.6 \pm 0.9 \times 10^5$ (3)
3 day old	339 ± 61 (4)	276 ± 26 (4)	659 ± 404 (4)	8431 ± 8330 (3)	>>1000 (4)	>> 1000 (3) #	$4.6 \pm 3.1 \times 10^5$ (3)	$3.2 \pm 1.4 \times 10^5$ (3)
6 day old	199 ± 115 (3)	467 ± 183 (3)	-	-	-	-	-	-
14 day old	489 ± 111 (4)*	200 ± 60 (3)	-	-	-	-	-	-
Adult	167 ± 32 (6)	563 ± 341 (6)	322 ± 252 (5) **	193 ± 68 (5)	>> 1000 (6)	>> 1000 (5)	$1.5 \pm 0.6 \times 0^4$ (5)	$1.3 \pm 0.7 \times 10^4$ (4)

sem = standard error of the mean. EC_{50} data (μM) was derived from curves fitted on the assumption that Emax was 100% in all cases.

(n) = number of animals from which the data was derived. The EC_{50} data for ATP and graphs are presented in Section 1 Chapter 1, Table 1 and Fig.2. * indicates a significant difference between IPA with and without endothelium $p<0.05$. ** indicates significant difference from the newborn group, $p<0.05$.

Table 3 : The agonist rank order of potency for inducing a relaxation response based upon derived EC_{50} data of intrapulmonary arteries from **normal** pigs.

Group	Agonist order of relaxation activity
Newborn	2-meSATP > ADPβS > UTP >> α,β-meATP
3 day control	2-meSATP ≥ ADPβS > α,β-meATP > UTP
Adult control	2-meSATP = ADPβS >> UTP >> α,β-meATP

The rank order at each age would suggest that the dominant receptor group responsible for the relaxation response was a purine-selective P2Y-receptor {1}. However, all the agonists tested were capable of inducing a relaxation, albeit after a transient contraction in some cases.

Responses of intrapulmonary arteries from piglets with PHN.

Exposure to hypobaric hypoxia from birth for a period of 3 days did not significantly affect the relaxation response of IPA to 2-meSATP when compared to the response seen in age-matched control animals (Fig.6 A). But the relaxation response to ADPβS in the two animals studied did not appear to be as great as that seen in the 3 day old age-matched normal animals (Fig.6 B). Removing the endothelium did not significantly affect the relaxation response to 2-meSATP or ADPβS. The relaxation response to α,β-meATP , which was present in 3 of 4 normal newborns and all of the normal 3 day old animals tested, was absent in the IPA with endothelium from 2 of 4 PPH animals (Fig.6 C). The sensitivity of the responses was represented by EC_{50} values. These are given in Table 4 to describe the effect of age and removing the endothelium and used to produce the rank orders of potency in Table 5.

Table 4. The effect of pulmonary hypertension upon EC_{50} for the relaxation response to cumulative-purine agonists in IPA with endothelium.

P2 agonist	With endothelium	Without endothelium
2-meSATP	398μM ± 118 (3) @	753μM ± 436 (3) @
ADPβS	562μM ± 306 (2)#	435μM ± 181 (2) #
α,β-meATP	2828μM ± 2434 (3) @	$1.95x10^{7}$μM ± $1.25x10^{7}$ (4)

The EC_{50} data is given in μM units. # indicates a standard deviation calculated. @ indicates that an EC_{50} was only determined for 3 of 4 animals due to an inability to fit a representative curve.

Table 5. The rank order of agonist potency for P2-purinergic agonists in IPA from piglets with PH.

Exposed to CHH from birth from 0-3 days	ADPβS > 2-meSATP > α,β-meATP
Normal newborn	2-meSATP > ADPβS >> α,β-meATP
Normal 3 day old	2-meSATP ≥ ADPβS > α,β-meATP

Responses of intrapulmonary arteries from normal children

ADPβS-induced a relaxation of IPA from a 5 year old child, which was both concentration- and endothelium-dependent and which reversed rapidly after each dose (Figs.7 A-C). ADPβS also produced a concentration-dependent relaxation of the IPA from an eight year old child , while 2-meSATP induced a small but significant relaxation which was greater in the presence of the endothelium (Fig.8) (Table 6). The maximal relaxation response to ADPβS was significantly greater in the IPA from the 8 year old child than in those from the 5 year old child.

Table 6. EC_{50} data for the relaxation response of the IPA from an 8 year old normal child to P2-purine receptor agonists.

P2-agonist	EC_{50} with endothelium	EC_{50} without endothelium
2-methylthioATP	1 mM	17 mM
ADPβS	23 μM	not done

A sigmoidal curve could not be fitted to the ADPβS dose-response in the 5 year old normal child, therefore no EC_{50} for could be calculated.

Responses of intrapulmonary arteries from children with pulmonary hypertension.

A bolus addition of ADPβS (100μM) relaxed the IPA ,with endothelium, from a 36 hour old child who had died with persistent pulmonary hypertension (Fig.9 A). ADPβS also induced a concentration-dependent relaxation of the IPA from a 10 day old child (Fig.9 B). The bolus of ADPβS was more effective than the full dose-response regime. The IPA with endothelium isolated from a 4 month old child with pulmonary hypertension also relaxed to a 100μM bolus of ADPβS (Fig.9 C). A cumulative dose-response to ADPβS produced a significant relaxation of the IPA isolated from two 15 year old children who had died with pulmonary hypertension (Fig.10). Removing the endothelium reduced the relaxation response found in one of these cases. The relaxation response to ADPβS appeared to decrease as the age of the children increased (Fig.11).

Discussion.

The present study has shown that 2-meSATP, ADPβS , α,β-meATP and UTP can induce relaxation in isolated newborn porcine IPA, irrespective of the presence or absence of the endothelium. [The response to ATP with respect to age was discussed previously in Chapter 4]. Conventially, the receptor mediating the response to ATP has been classified as a P2Y-receptor subtype based on the rank order of analogue agonist potency. However, the P2Y-receptor family are distinguished from P2X-receptors by virtue of being G-protein coupled receptors. This attribute was not investigated in the present study or in many other published

reports. Therefore, until the receptor is cloned and can be established as a member of the G-protein coupled receptor superfamily it is possible that the receptor could be what is currently recognised as a P2X-receptor. It has recently been suggested that the converse may be true, where a vasoconstrictor response (usually a P2X-receptor associated response) is mediated by a P2Y-pyrimidine-preferring receptor, based only on the rank order of agonist potency {259}.

The rank order of potencies for inducing vasodilatation from the present pharmacological studies indicates the presence of a $P2Y_1$-purine receptor in these vessels, the response to activation of these receptors increasing with age {65,74}.

Relaxations to 2-meSATP (a $P2Y_1$ selective agonist) were present at all ages in the porcine vessels but the sensitivity did not increase with age, in contrast to the ATP and ADPβS responses. In the present study the observation that the 2-meSATP EC_{50} did not change with age, and remained the most potent of the agonists tested, would suggest that the ligand binding site is mature at birth. The difference in sensitivity with age may indicate that the P2Y-receptor matures, developing an increased efficacy for certain agonists, with age. But the increased responsiveness with age seems to be linked to the transduction mechanism stimulated by the precontracting agonist, because the response to ATP did not increase in IPA precontracted (and therefore depolarised) with KCl (see chapter 4). ATP and ADPβS may both bind to the receptor in a such a way as to stimulate mechanisms involving K^+-ion flux, which 2-meSATP does not. All three agonists induced an endothelium-independent vasodilatation response, in the present study. The $P2Y_1$-receptor has been isolated from bovine endothelial cells and functional studies indicate the existence of P2Y-vasodilating receptors on vascular endothelium, but not on smooth muscle cells {180,325,300}. However, 2-meSATP and ATP were both found to induce a relaxation response in adult rabbit main PA and human small IPA, displaying no endothelial dependency {326,237}.

In the neonate UTP and ATP produced relaxations of the IPA with a similar EC_{50}, but UTP became much less effective than ATP in the adult. The pyrimidine-preferring $P2Y_6$ receptor

has been isolated from rat aortic smooth muscle and is also expressed in adult rat lung tissue{81}. However, organ bath studies of adult rat mesenteric artery and adult rabbit pulmonary artery have shown vasodilatation mediated by pyrimidine-preferring receptors with much more potent EC_{50} values than those demonstrated in the present study {326,334}. This would suggest that the UTP-induced relaxation response in the present study was not via a pyrimidine-preferring receptor. In porcine IPA it may be that the low vasodilatation sensitivity to UTP is reflecting the greater contractile activity of UTP than ATP, which masks a more sensitive UTP relaxation. However, UTP-induced vasodilatation in the adult rabbit main pulmonary artery was found to be completely endothelium-dependent in contrast to the present study {326}. This difference may be due to species or that the vessels were isolated from different parts of the pulmonary vasculature or it might reflect the use of different precontracting agonists. The P2-agonists in the present study induced not only vasodilatation but also vasoconstriction of the porcine IPA.

A clear $P2Y_1$- receptor agonist rank order was established for the relaxation response in adult animals as the contractile response induced by P2-agonists at P2X-receptors matured. But, the rank order was less clear in the younger animals, where the contractile responses are largely absent and relaxations were produced by all the agonists tested to varying degrees. The functional presence of fetal / neonatal pulmonary vasodilator P2Y-purine receptors would indicate that ATP could help initiate a fall in pulmonary perfusion pressure after birth and help maintain the pulmonary vasodilatation during extrauterine adaptation, without its being metabolised to adenosine.

Despite selective activity at the contractile $P2X_{1 \text{ and } 3}$-receptors, the α,β-meATP -induced relaxations were similar to those observed in the present study in systemic vessels such as the portal vein {215} and hepatic artery {53} of mature rabbits and in the pulmonary artery of the adult rat {238}. In the portal vein it was thought that the response was "unlikely" to be a P2Y-purine receptor stimulated relaxation {215}. Unfortunately, no mechanism for this relaxation phenomenon has been put forward.

There are reports of transient contractions to ADPβS in aorta of the spontaneously hypertensive rat and human urinary bladder, and they were found in the present study {309,46}. This may be due to non-specific activity at P2X-receptor(s). α,β-meATP has previously been reported to constrict mature pulmonary vessels at P2X-purine receptors {77,199,238, 237, 262,291,348}. In the present study the contractile response to both α,β-meATP and UTP of porcine IPA precontracted with $PGF_{2\alpha}$, increased with age. Other workers have found an increase in response with age using other agonists such as KCl and phenylephrine, for the IPA in the same porcine model {241,233}. The lack of a potent constrictor response in the neonate to P2-agonists might suggest that the contractile P2X-receptors are not involved in maintaining the high IPA tone *in utero*. However, the finding that the contractile reactiveness of the IPA to P2-agonists increases in the mature animal might indicate their involvement in vasomotor control.

The present study showed that IPA isolated from newborn animals with PH maintain the ability to relax to P2-purinergic receptor agonists. The vasodilatation to each P2-agonist was not altered when compared to that in normal age-matched animals. Other workers using this porcine model of PHN have found that endothelium-dependent relaxation to ACh is abolished and the response to endothelium-independent agonists, such as nitric oxide is reduced {399}. We cannot explain the slight reduction in purine-induced endothelium-independent relaxation but a P2Y-receptor was still indicated by the vasodilating activity of ATP, 2-meSATP and ADPβS combined with the lack of vasodilatation by the P2X-receptor agonist α,β-meATP. It is possible that the absence of an endothelial factor in the P2Y-purine receptor mediated relaxation may explain the resistance to CHH. The absence of a relaxation response to α,β-meATP was not due to an increase in opposing contractile activity, because transient contractions such as the one recorded from one IPA from a normal 3 day old animal were not found of any of the IPA from the pulmonary hypertensive animals. UTP was not studied in the PHN group because the potent relaxation response in the normal animals was classified as being mediated by a purinergic $P2Y_1$-receptor, not a pyrimidine-preferring receptor.

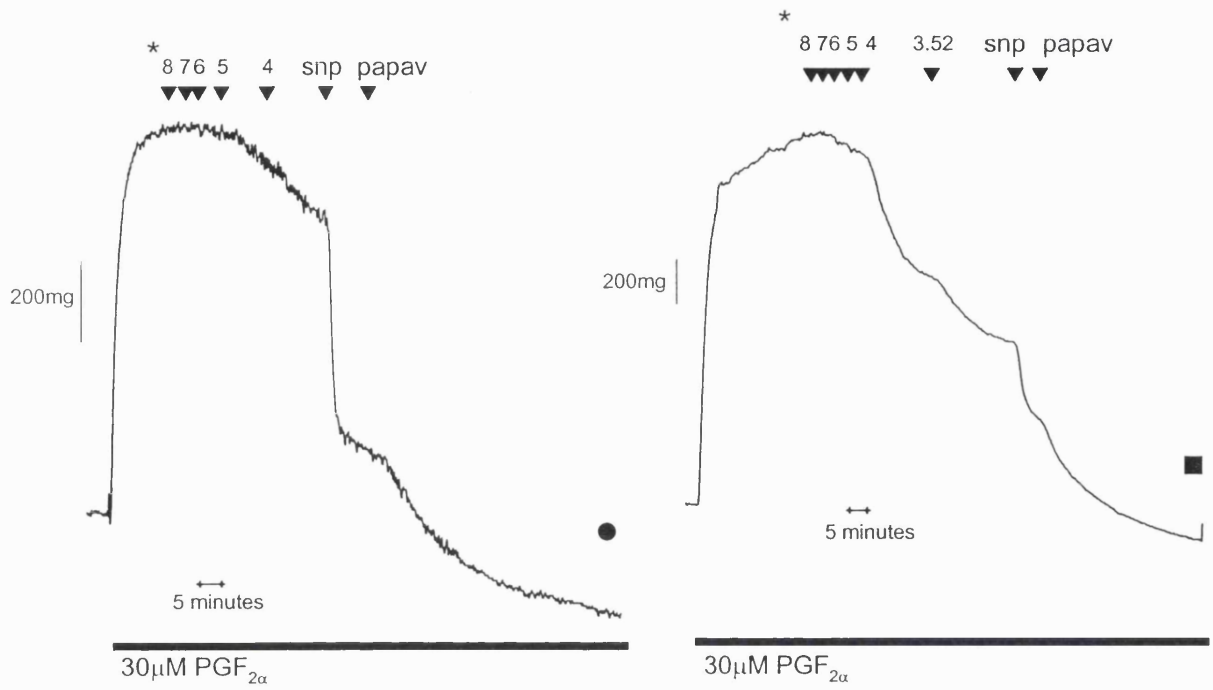
The present study has highlighted certain areas of similarity and contrast between the responses of human and porcine IPA. In IPA from normal children the P2Y-purine nucleotide agonists ADPβS and 2-meSATP induced endothelium-dependent relaxation, but the responses were endothelium-independent in the pig. This may reflect a difference in the size, location and histological type of vessel taken from the human lungs as compared to the pig. The variation in endothelial-dependence in different pulmonary arterial segments and in different species was discussed in Chapter 4. The relaxation response tended to be greater in IPA from younger children, which was the opposite trend to that seen in the pig, but with only a few human cases this is a very preliminary observation. The IPA from children with pulmonary hypertension relaxed to ADPβS, as did the hypertensive porcine IPA. It has been suggested that children with pulmonary hypertension, the earlier the therapeutic intervention and less chronic the pathology the more reversible the situation {54}. The response of the IPA from the teenage pulmonary hypertensive children was significantly reduced when compared to the response of a normal age-matched child. However, the greatest relaxation to a bolus of ADPβS was seen in the IPA from a 36 hour old pulmonary hypertensive baby. The vessels in the children who had survived with pulmonary hypertension for many years presumably showed established pulmonary vascular disease, whilst the 36 hour old case was possibly the most labile.

ATP has been shown to be effective in reducing the raised PAP of adult patients with chronic obstructive pulmonary disease and pulmonary hypertension, where adenosine was ineffective {139,140}. The drop in pulmonary resistance was thought to be due to inhibition of hypoxic pulmonary vasoconstriction rather than increasing blood flow to other regions of the lung {141}. This would support the use of P2Y-receptor agonists to alleviate raised PAP induced by hypoxia.

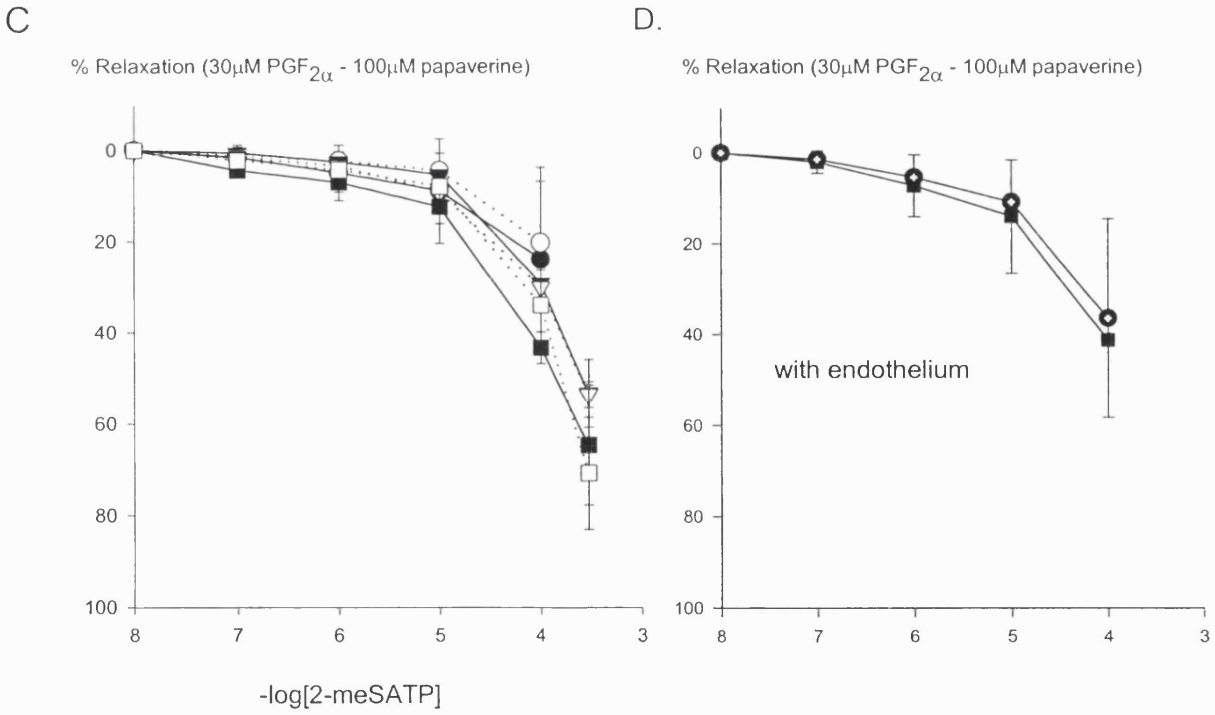
In conclusion, the present *in vitro* study has shown that the vasodilating action of ATP infusion in children with pulmonary hypertension may be mediated by a P2Y-purine receptor on the IPA and helps justify the clinical use of ATP. There are reports of ATP being used

successfully in the management of neonatal pulmonary hypertensive crises, without the side-effects of more traditional treatments such as tolazoline {54,145}. The differences in activity of the P2-agonist purine nucleotides tested in the present study (ADPβS and 2-meSATP) suggest that the development of a purinergic drug with specific action on the pulmonary circulation might be possible. In addition, other workers have found that some analogues display an increased resistance to degradation and oral viability which would could be used to produce a sustained concentration in the blood, reducing the need for continuous intravenous infusion {183}. However, a drug with a short half life may be required, particularly in newborn infants, as the condition of the child alters. But the difficulties associated with attempting to apply results from immature piglets pigs to the human infant should always be borne in mind, despite the similarities in the postnatal development of the pulmonary circulation of these two species.

Fig.1

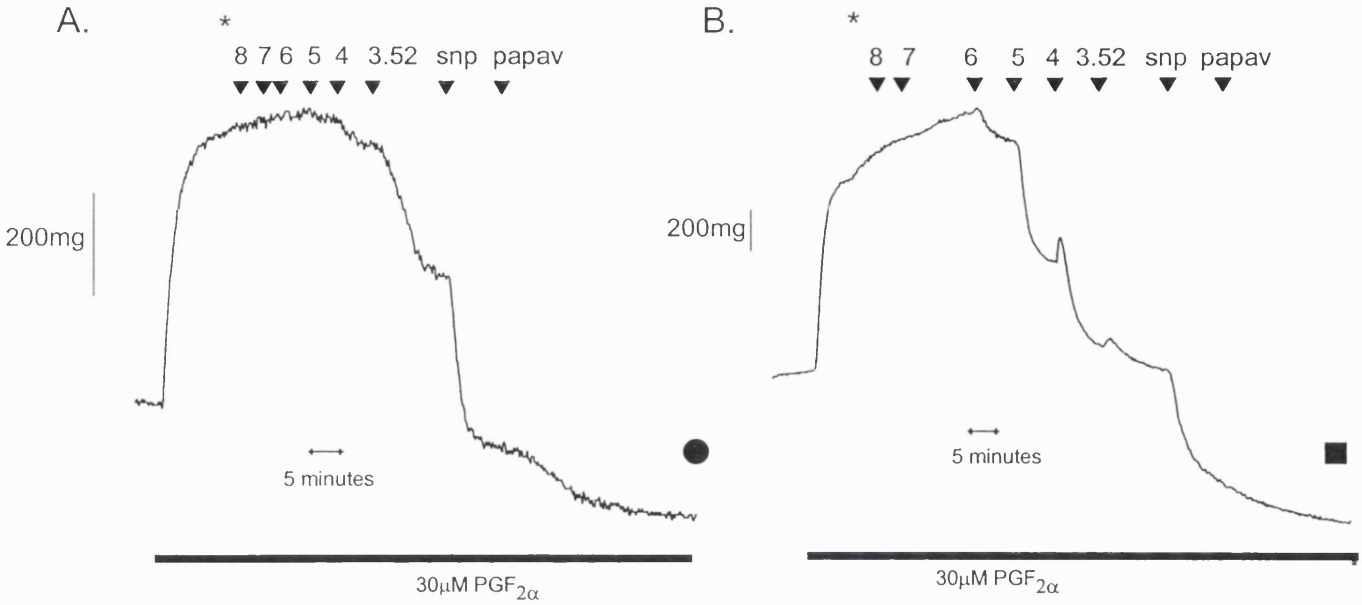


Representative traces of 2-meSATP-induced responses in IPA precontracted with 30μM $PGF_{2\alpha}$ from (A) newborn and (B) adult porcine intact IPA. snp, sodium nitroprusside 100μM,papav, papaverine 100μM. * indicates -log numbers.



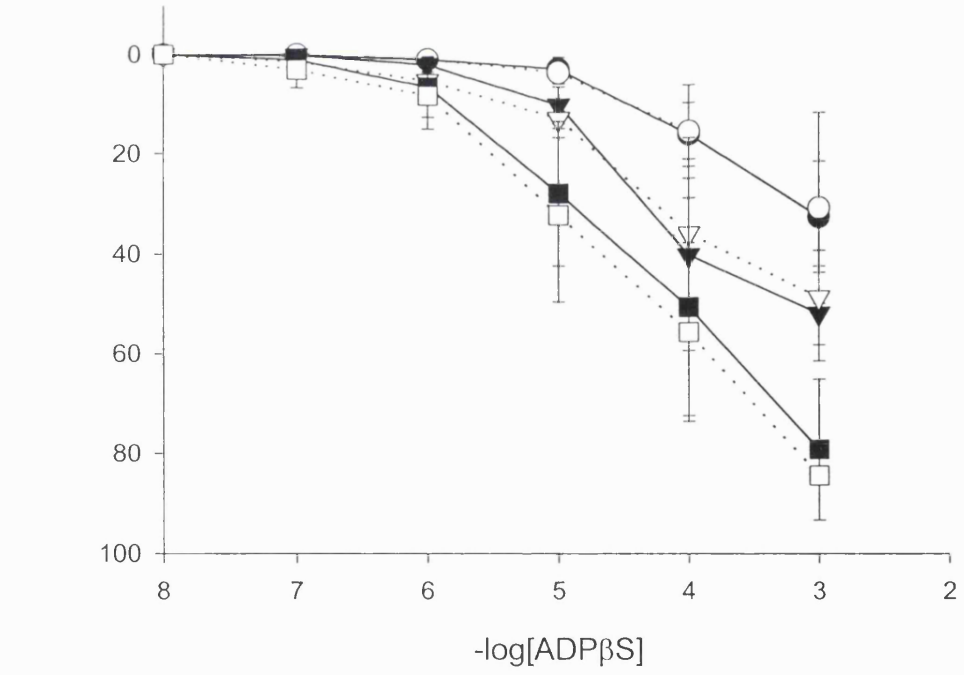
(C) Response to 2-meSATP (P2Y-purine receptor agonist) of porcine IPA precontracted with 30μM $PGF_{2\alpha}$. There was no significant effect of age or the endothelium upon the relaxation response. (newborn n=4/4 ● ;3 day old n=4/4 ▼;adult n=6/6 ■). (D) The effect of 30μM L-NMMA (nitric oxide synthase inhibitor) on the adult porcine response (n=2 ✪).
Bars =sem (standard deviation where n=2).

Note: Empty symbols indicates data for IPA with the endothelium removed. The notation "n= 4/4" for example, indicates the number of animals studied with / without endothelium. This applies to all figures in this chapter unless stated otherwise.

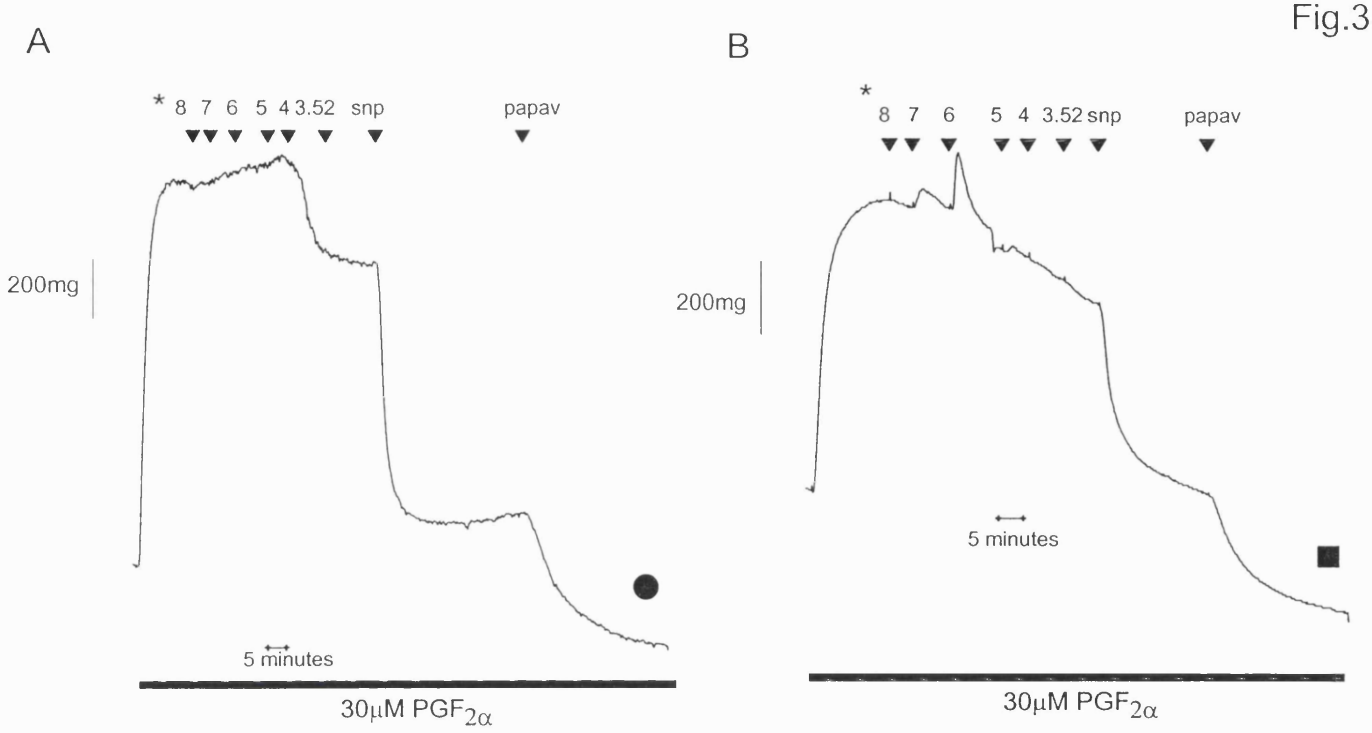


Representative traces of ADPβS-induced responses of IPA precontracted with 30μM $PGF_{2\alpha}$ from a (A) normal newborn and (B) normal adult pig. Snp, sodium nitroprusside ; papav, papaverine 100μM. * indicates -log numbers.

C

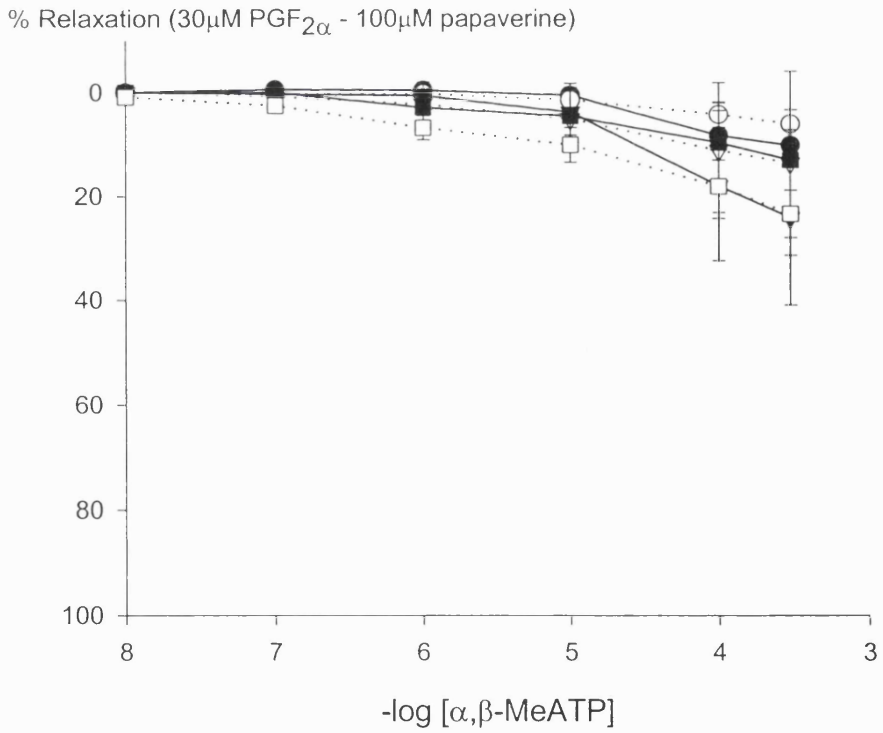


(C) Response to ADPβS (P2Y-purine receptor agonist) of porcine IPA precontracted with 30μM $PGF_{2\alpha}$. Responses with (solid symbols) and without (empty symbols) endothelium. (newborn n=5 (2 fetal)/5 (2 fetal)●;3 day old n=4/3▼ ;adult n=7/5■). The sensitivity to ADPβS increased with increase in age, but removing the endothelium had no significant effect on the relaxation response. Bars = sem.

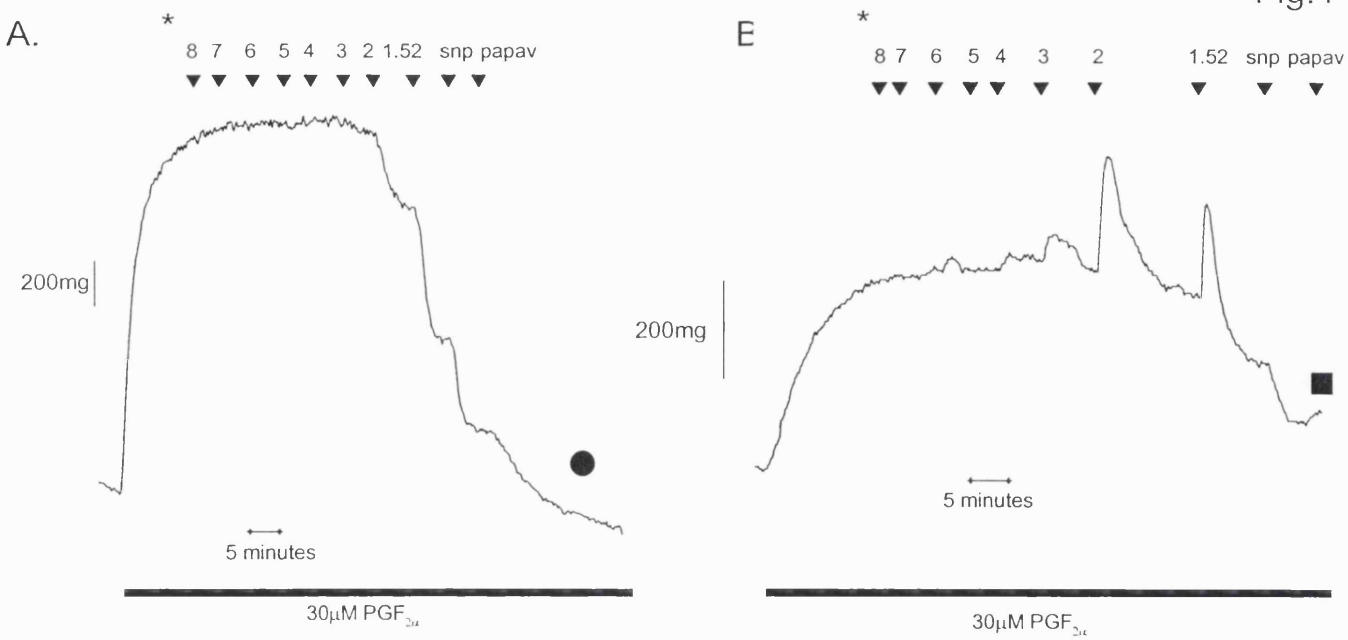


Representative traces of α,β-meATP-induced responses in IPA precontracted with 30μM $PGF_{2\alpha}$ from a (A) normal newborn and (B) normal adult pig. Snp, sodium nitroprusside 100μM; papav, 100μM papaverine. * indicates -log values.

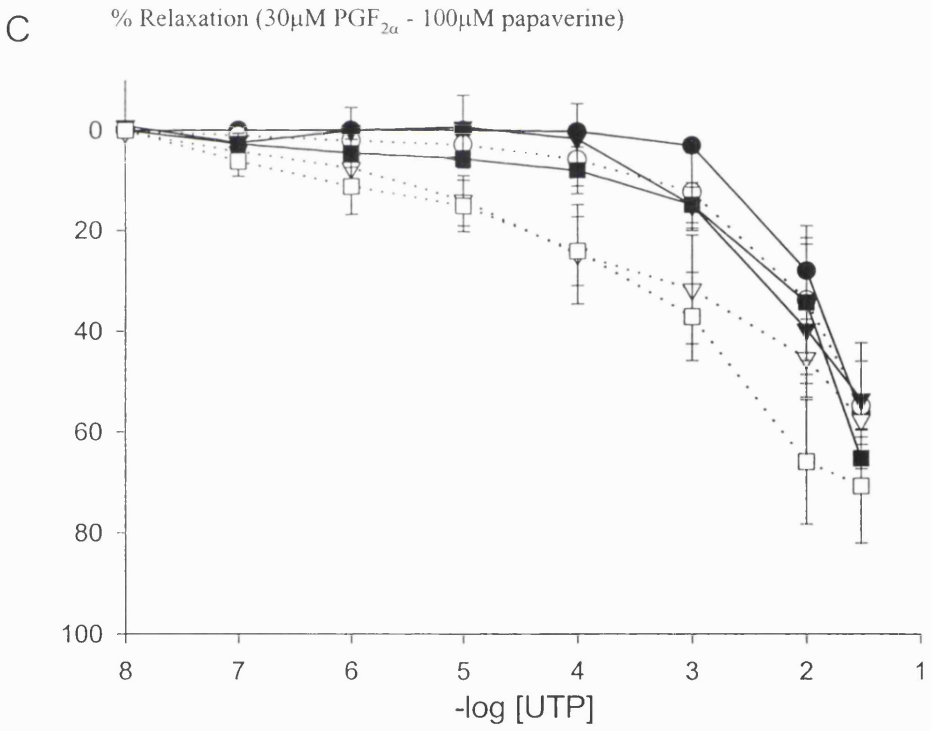
C



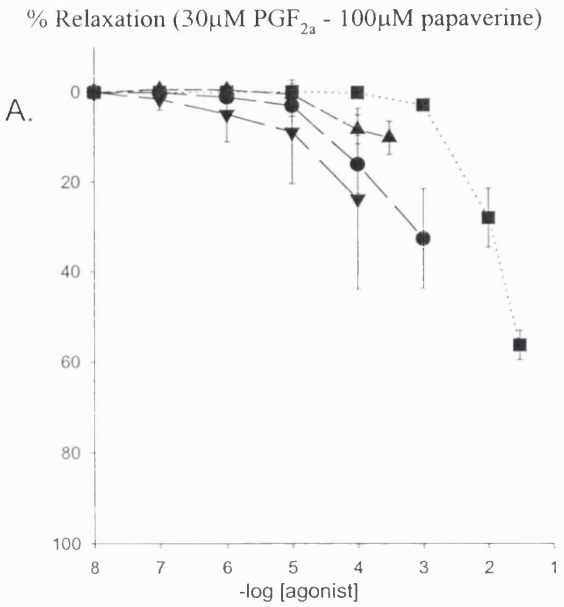
(C) Response to α,β-meATP (P2X-purine receptor agonist) of the porcine IPA precontracted with 30μM $PGF_{2\alpha}$. Responses with (solid symbols) and without (empty symbols) endothelium. There was no significant effect of age (newborn n=4/3● ;3 day old n=5/4 ▼;adult n=6/5 ■) or the endothelium upon the relaxation response. However, a transient contractile response had developed by adulthood (B). Bars = sem.



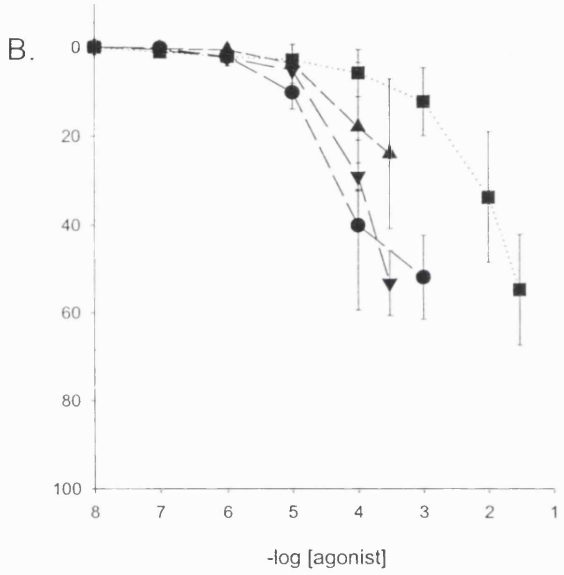
Representative traces of UTP-induced responses in IPA precontracted with 30μM $PGF_{2\alpha}$ from a (A) normal newborn and (B) normal adult pig. Snp , sodium nitroprusside 100μM; papav, papaverine 100μM. * indicates - log numbers.



(C) The response to UTP of porcine IPA precontracted with 30μM $PGF_{2\alpha}$, with (solid symbols) and without (empty symbols) endothelium. The EC_{50} for IPA without endothelium decreased from 3 days to adulthood. (newborn n=3(1 fetal)/3 (2 fetal)● ;3 day old n=3/3▼;adult n=5/4 ■). By 3 days of age a transient contractile response developed.

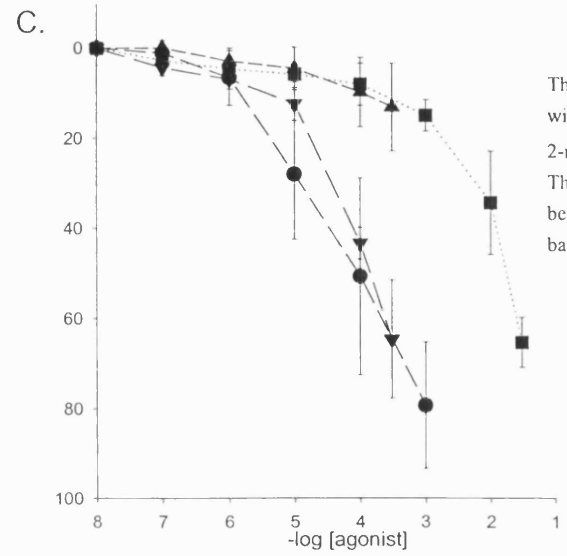


The relaxation responses of IPA with endothelium from normal newborn piglets, precontracted with 30μM $PGF_{2\alpha}$ to determine a rank order of potency for :2-meSATP n=4 ● ;ADPβS n=5 ▼ ; α,β-meATP n=4 ▲ ;UTP n=3 ■ .The rank order would support a dominant P2Y-purinergic receptor population. Error bars = sem.



The relaxation responses of IPA with endothelium from normal 3 day old piglets, precontracted with 30μM $PGF_{2\alpha}$ to determine the rank order of potency for : 2-meSATP n=4● ;ADPβS n=4▼ ;α,β-meATP n=5 ▲ ;UTP n=3 ■ . The order would support a dominant P2Y-purinergic receptor population, even moreso than in the newborn group.

Error bars = sem.

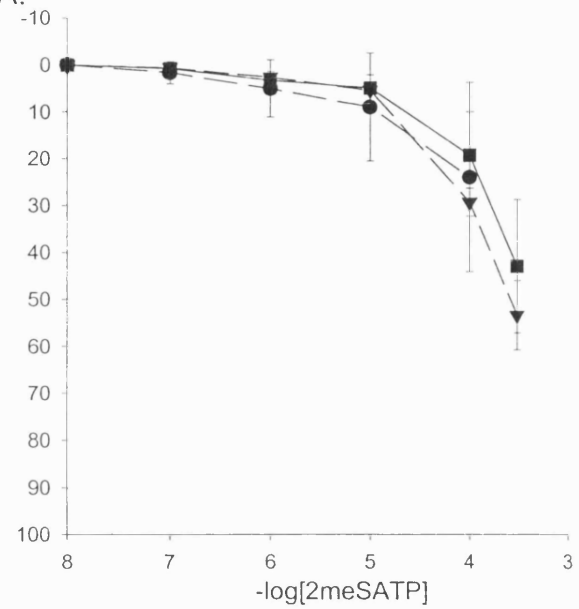


The relaxation responses of IPA with endothelium from normal adult pigs, precontracted with 30μM $PGF_{2\alpha}$ to determine the rank order of potency for : 2-meSATP n=6 ●;ADPβS n=7 ▼ ;α,β-meATP n=6 ▲ ;UTP n=5 ■ . The order would support a dominant P2Y-purinergic receptor population, which is best fulfilled in the adult group.

bars = sem.

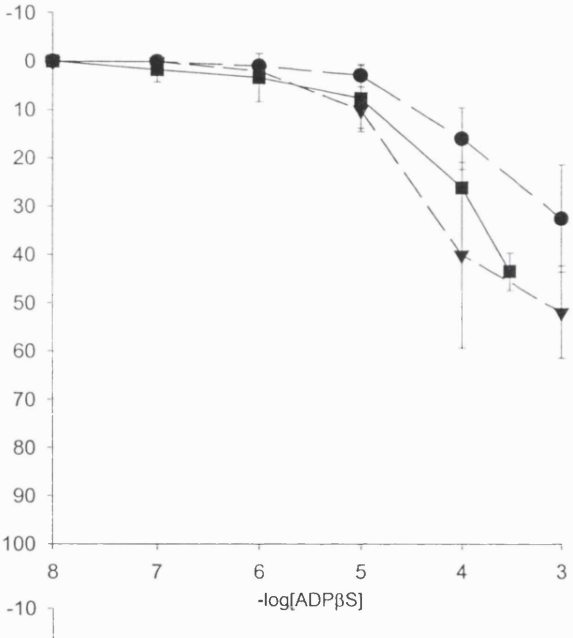
Fig.5. Response of IPA from normal newborn (A), 3 day old (B) and adult (C) pigs, to P2-receptor agonists.

A.



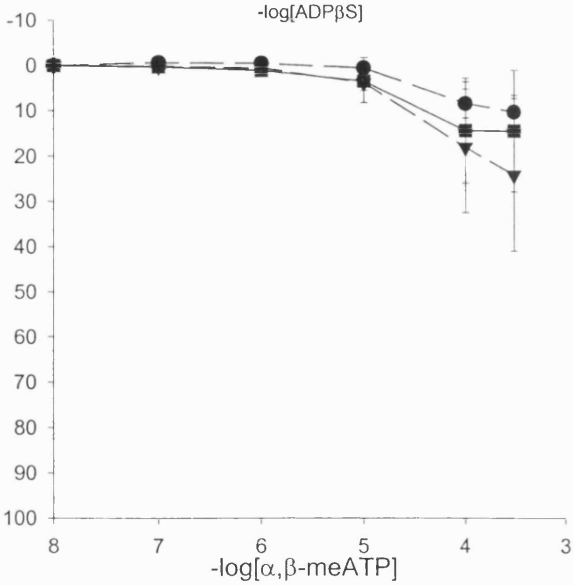
The effect of persistent pulmonary hypertension (PPH) upon 2-meSATP (P2Y-purine receptor agonist)-induced relaxation of IPA with endothelium, precontracted with 30μM $PGF_{2\alpha}$. Vessels were isolated from piglets exposed to CHH for a period of 3 days from birth.
(PPH n=4 ■ ;normal newborn n=4 ● ;normal 3 dayl n=4 ▼).
A period of CHH did not significantly affect the relaxation response when compared to age-matched controls. Bars = sem.

B.



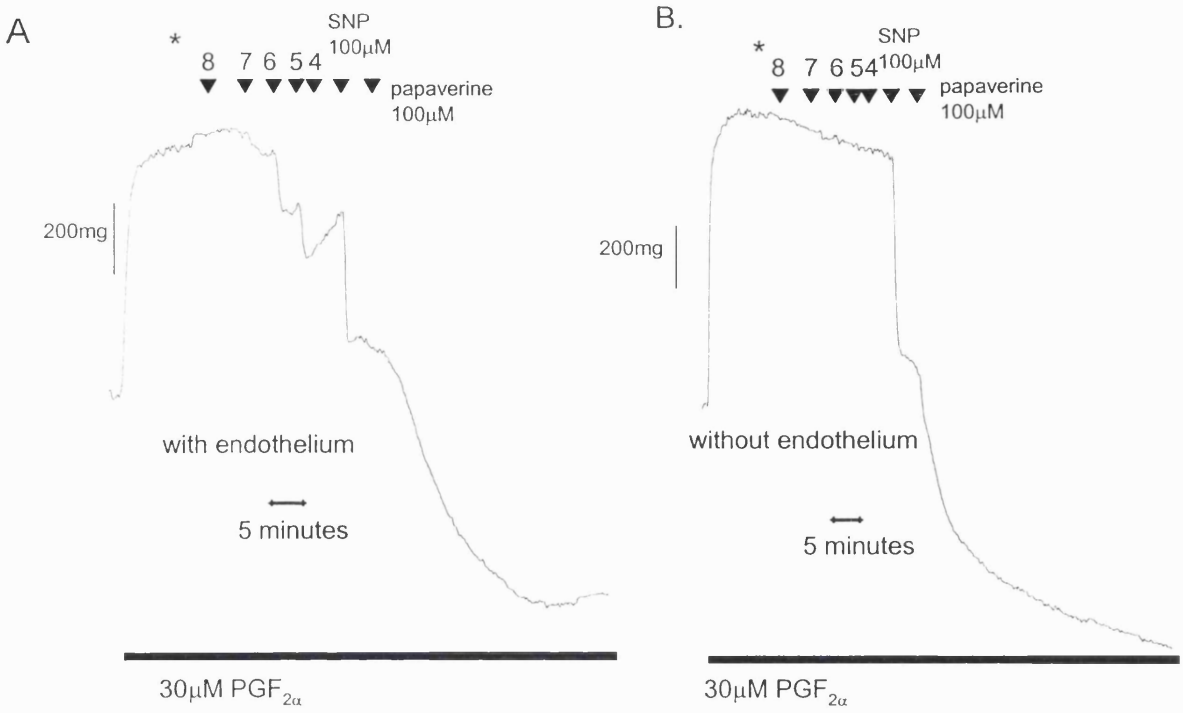
The effect of persistent pulmonary hypertension (PPH) upon ADPβS (P2Y-purine receptor agonist) -induced relaxation of IPA with endothelium, precontracted with 30μM $PGF_{2\alpha}$. Vessels were isolated from 2 piglets exposed to CHH for a period of 3 days from birth,.
(PPH n=2 ■ ;normal newborn n=5 ● ;normal 3 day n=4 ▼). A period of CHH partially reduced the relaxation response when compared to age-matched controls. Bars = sem (standard deviation n=2).

C.

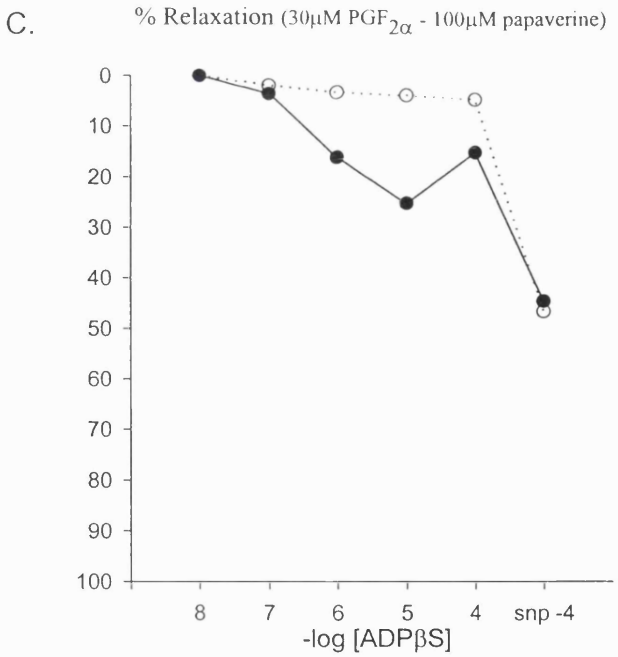


The effect of persistent pulmonary hypertension (PPH) upon α,β-meATP (PX-purine receptor agonist) -induced relaxation of IPA with endothelium, precontracted with 30μM $PGF_{2\alpha}$. Vessels were isolated from piglets exposed to CHH for a period of 3 days from birth.
(PPH n=4 ■ ;newborn n=4 ● ;3 day normal n=5 ▼). A period of CHH reduced the relaxation response when compared to age-matched controls.
Bars = sem.

Fig.6. Response of IPA from piglets exposed to CHH from birth, to P2-receptor agonists.



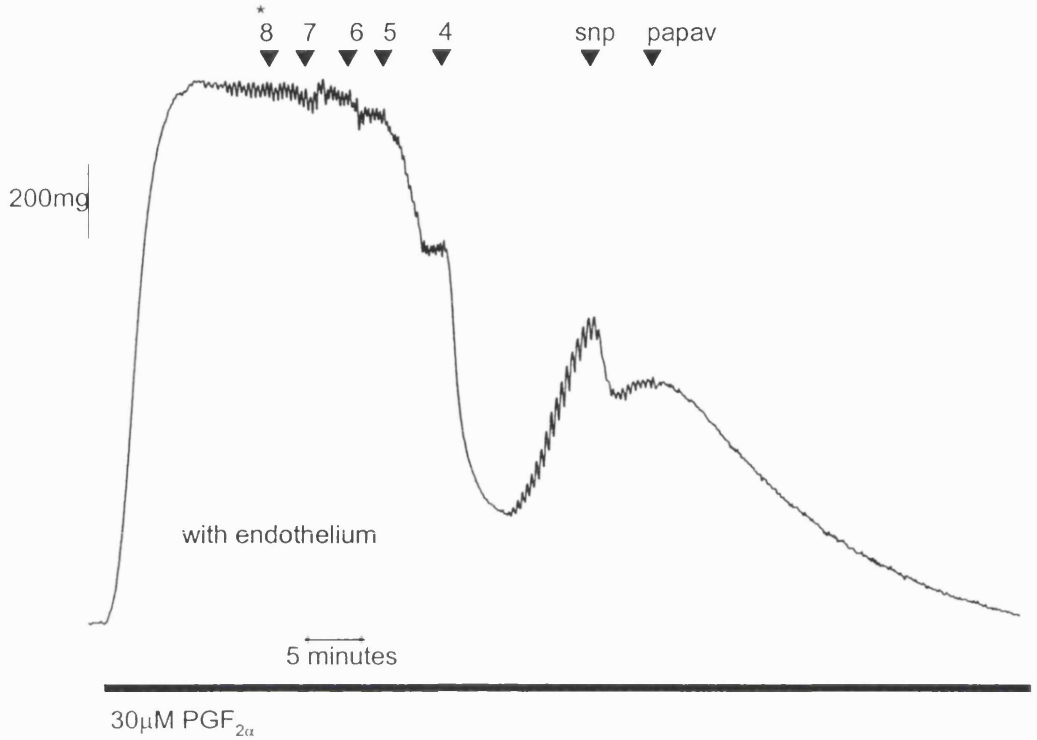
Traces of the response to cumulative ADPβS of IPA isolated from a 5 year old child without pulmonary hypertension. IPA with (A) and without (B) endothelium was precontracted with 30μM $PGF_{2\alpha}$. ADPβS induced an endothelium-dependent relaxation. 100μM SNP had a significant effect. * -log values.



(C). Dose-response to ADPβS of IPA precontracted with 30μM $PGF_{2\alpha}$, from a 5 year old normal child. The relaxation response was endothelium-dependent (solid symbols).

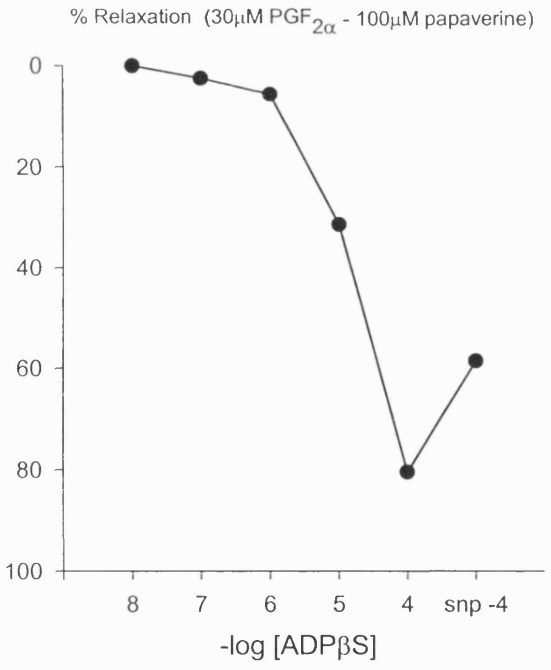
Fig.7. Response of IPA from normal 5 year old child, to cumulative additions of ADPβS.

A.



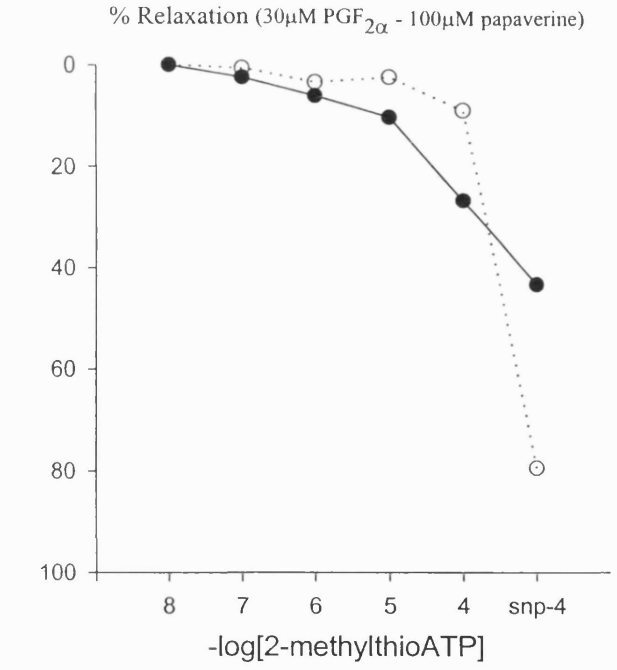
Trace of response to cumulative ADPβS of IPA from an eight year old normal child. A potent relaxation response was produced, which reversed after each addition of ADPβS. Snp, sodium nitroprusside 100μM ; papav, papaverine 100μM.

B.



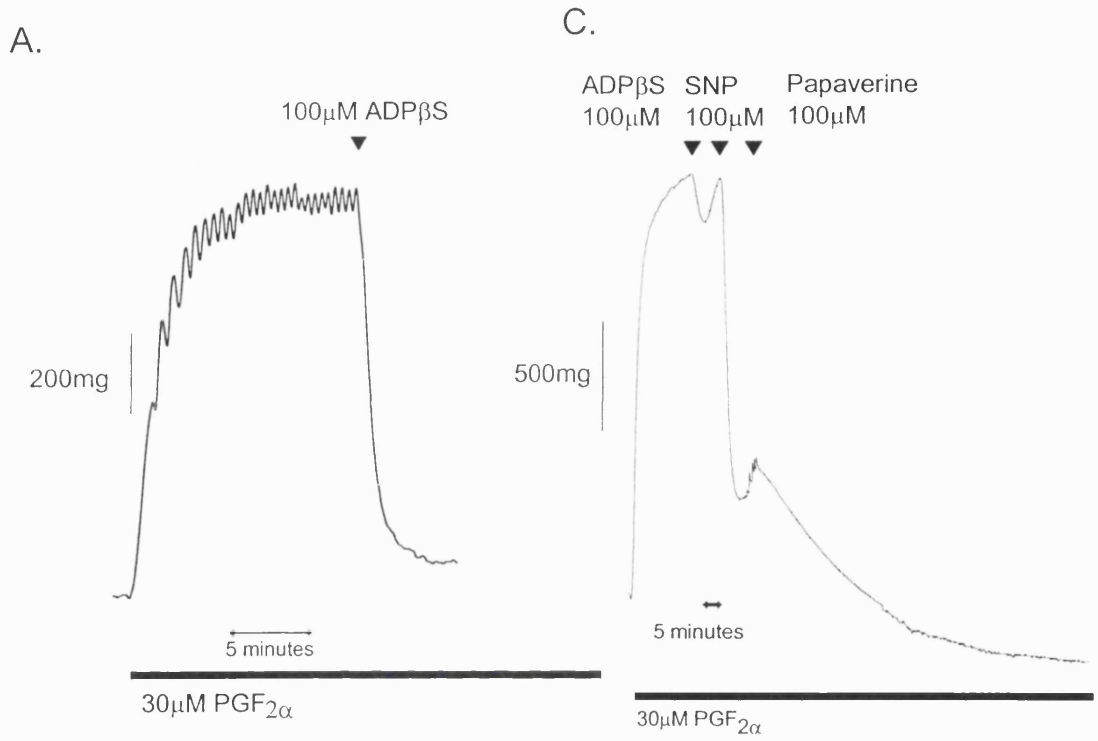
(B) Dose-response to ADPβS in IPA with endothelium precontracted with 30μM $PGF_{2\alpha}$ .The ring was isolated from an 8 year old normal child.

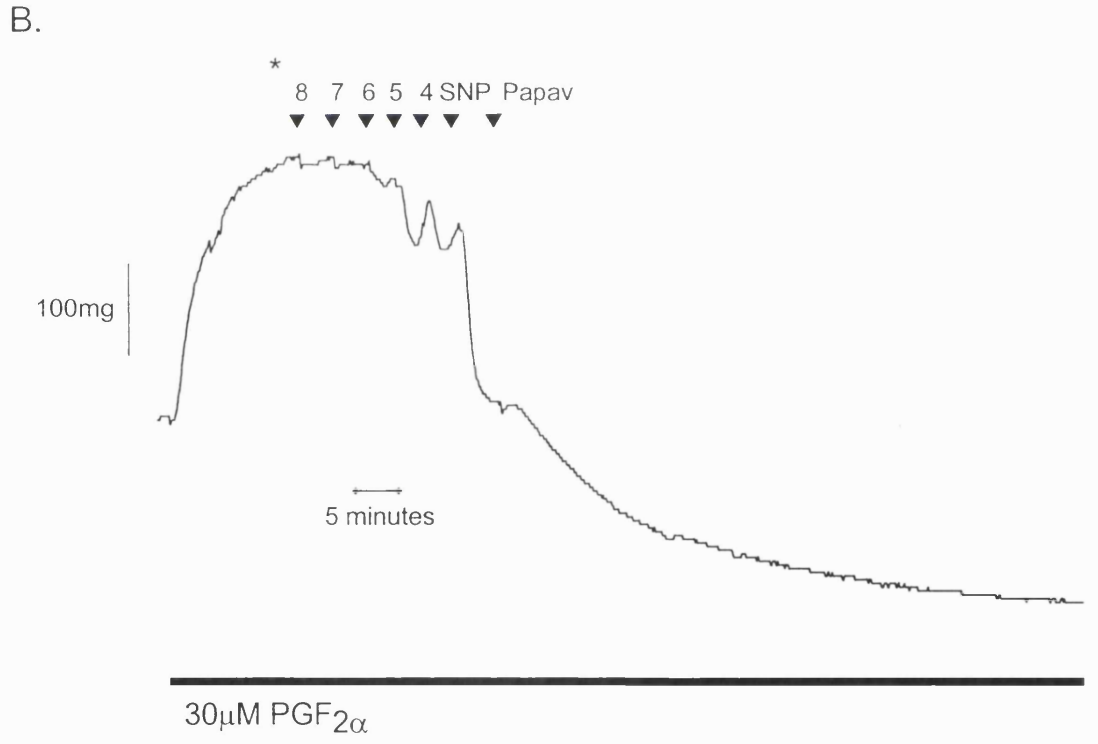
C.



Dose-response to 2-meSATP of IPA precontracted with 30μM $PGF_{2\alpha}$ from an 8 year old child without pulmonary hypertension. Gradual decline of $PGF_{2\alpha}$ tension accounts for a portion of the relaxation by both IPA rings. With (solid symbols) without (empty symbols) endothelium.

Fig.8. Response of IPA from two normal children, to P2Y-receptor agonists.

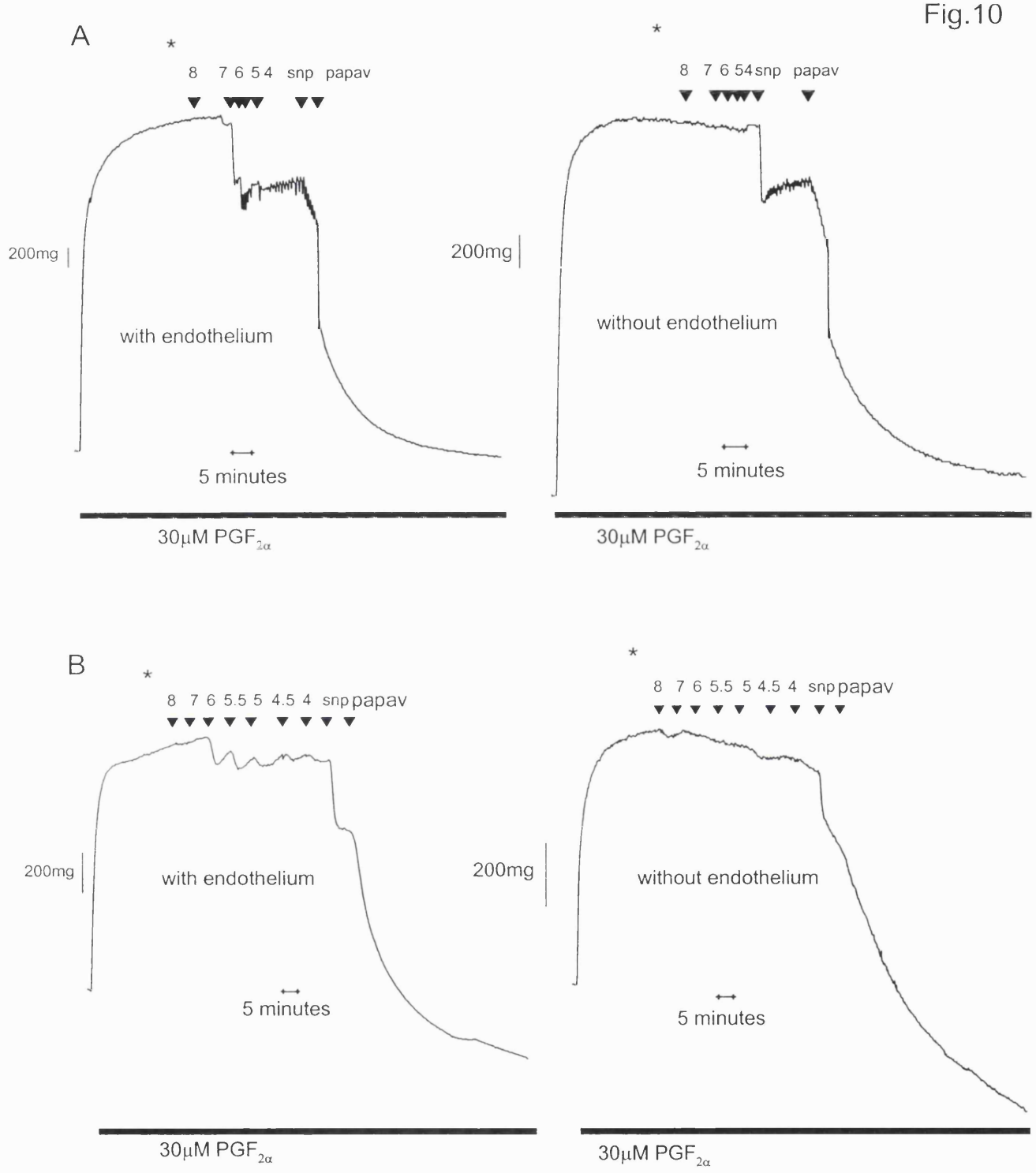




Traces of the reponse to ADPβS of IPA precontracted with 30μM $PGF_{2\alpha}$, (A) from a 36 hr. old child and (B) a 10 day old child, both with pulmonary hypertension. The bolus addition in panel A was more effective than the cumulative series in panel B.

(C) Trace of the response to100μM ADPβS in IPA precontracted with 30μM $PGF_{2\alpha}$,isolated from a 4 month old child with pulmonary hypertension. ADPβS induced a marked transient relaxation, but response was less than in the 36 hour old case. 100μM SNP was more effective than ADPβS.
SNP, sodium nitroprusside. * indicates - log numbers.

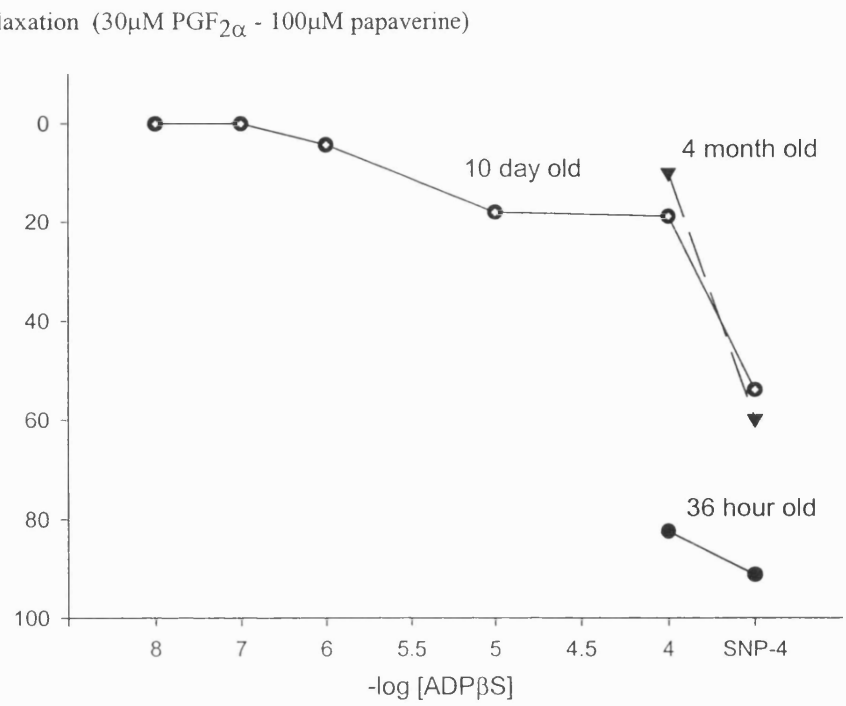
Fig.10



Traces of the responses induced by ADPβS in IPA precontraced with 30μM $PGF_{2\alpha}$. The vessels were isolated from two 15 year old children (A and B) with pulmonary hypertension. The relaxation response was greater with (left hand traces) than without (right hand traces) endothelium. snp, sodium nitroprusside 100μM; papav, papaverine 100μM. * indicates -log values.

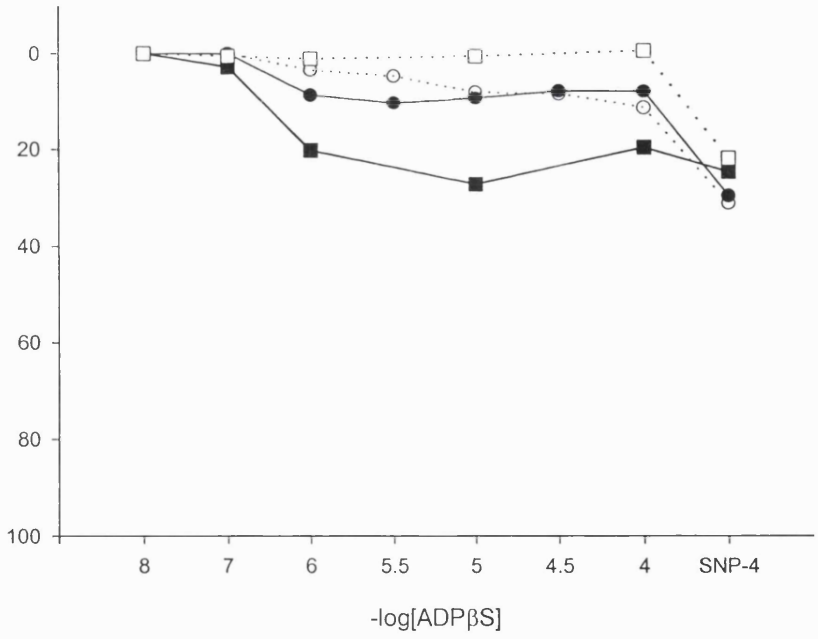
A.

Fig.11



Dose-responses to ADPβS of precontracted (30μM $PGF_{2\alpha}$) IPA with endothelium, from children with pulmonary hypertension. (36 hr. old e+ ●;10 days old e+ ✪ ; 4 month old e+▼).

B.



Dose-response curves to ADPβS of IPA from two 15 year old children with pulmonary hypertension. IPA with (solid symbols) and without (empty symbols) endothelium, precontracted with 30μM $PGF_{2\alpha}$. Note that the relaxation response was endothelium-independent in one case.

Chapter 6. ATP and P2-nucleotide receptor agonists induced contractile responses at resting tone in intrapulmonary arteries from normal and pulmonary hypertensive pigs and children.

Summary.

1.Intrapulmonary arteries (IPA) from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 14-17 days and adults were isolated and mounted with or without endothelium for *in vitro* isometric force recording at resting tension. Pulmonary hypertension was produced in newborn piglets by exposing them to chronic hypobaric hypoxia (CHH) (50.8kPa) for 3 days from birth. IPA from a 36 hour old baby with PPHN, a pulmonary hypertensive 16 year old child and from two children, aged 4 months old, one with and one without pulmonary hypertension were studied.

2. At resting tone IPA from normal fetal and newborn piglets relaxed in response to cumulative additions of ATP, in a concentration-dependent manner. By 3 days of age the IPA studied relaxed to ATP but the response was less marked than in the newborn and was followed by a series of transient contractions at high concentrations of ATP. By 2 weeks of age the relaxation response to ATP was reduced but the contractile response had increased. The response was similar to that seen in IPA from adult pigs.

3. Cumulative application of UTP at resting tone induced a relaxation in IPA from one of two fetal pigs. In the newborn, UTP induced concentration-dependent contractions which increased in sensitivity and magnitude up to 2 weeks of age when the response reached was similar to that seen in adult vessels.

4.At resting tone α,β-meATP produced small transient contractions in adult IPA only. $P2X_{1\ or\ 3}$ -receptor desensitisation by pre-incubation with α,β-meATP abolished the adult α,β-meATP-induced contractile response but increased the UTP-induced contraction of IPA from newborn and 2 week old piglets. Pre-incubation with α,β-meATP reduced the relaxant response to ATP of the IPA from newborn and 2 week old piglets.

5. Exposure to CHH abolished the high-concentration contractile response to ATP and reduced the contractile response to UTP. After exposure to CHH α,β-meATP failed to elicit a contractile response.

6. Removing the endothelium increased the contractile response to UTP at all ages, but the response to ATP or α.β-meATP did not show any endothelial-dependence.

7. At resting tone, the response of IPA from a baby with PPHN to α.β-meATP and ATP was similar to that seen in the 3 day old piglet. The contractile response to bolus additions of ATP in the IPA of a 16 year old PH child was similar to that found in IPA of older normal pigs. The IPA from a 4 month old normal child relaxed to ATP, while α.β-meATP had a greater contractile effect than UTP, at resting tone. In a 4 month old child with pulmonary hypertension by contrast, ATP contracted the IPA at resting tone. The rank order of contractile potency was UTP > ATP >> α.β-meATP . Repeated application of α.β-meATP caused a greater desensitisation of the α.β-meATP contraction response in the IPA from the normal 4 month old child than in those from the pulmonary hypertensive child of the same age. α.β-meATP increased the response to UTP in the normal, and to ATP in the IPA from the 4 month old pulmonary hypertensive infant.

8. In summary, the change in vascular tone of the IPA inherent in the adapting from a high fetal pulmonary resistance to the lower postnatal level revealed a tone-dependent dual-response to ATP. As the vascular tone decreased with age the emphasis of the ATP response altered from relaxation to a dominantly contractile response. The adult porcine IPA possessed an α,β-meATP-sensitive P2X-purine receptor population, but the IPA from younger animals possessed a population of contractile receptors with an affinity for pyrimidine nucleotides, which was influenced by the presence of the endothelium. It would seem that ATP acts at a UTP-sensitive contractile receptor but not at an α,β-meATP-sensitive P2X-contractile receptor. The porcine and human findings were similar.

Our *in vitro* observations of age-related ATP responses support an *in vivo* role for regulating intrapulmonary artery tone. Neonatal pulmonary hypertension caused by chronic hypoxia was not associated with an increase in reactivity of either the α,β-meATP-sensitive P2X-purine or the pyrimidine-receptor population.

Introduction.

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. Previously in the present study, the response to P2-agonists has been studied in precontracted IPA, from pigs during normal development and the effect of pulmonary hypertension. Comparable experiments were also carried out in IPA from normal and pulmonary hypertensive children (see chapters 4 and 5).

When the P2 receptors were subdivided into P2X and P2Y families, the ATP analogue α,β-meATP was found to stimulate a contractile response and then desensitise most of the P2X-receptors, but to have little effect on the responses mediated via the P2Y-receptor {211}. For these reasons α,β-meATP has been used as a classification agonist / antagonist for the purine nucleotide contractile receptor activated by ATP{65}. At raised tone vasodilatation usually occurs through P2Y- G-protein linked receptors either on the endothelium or the smooth muscle cells {19,236,411,245}. At low vascular tone, vasoconstriction to ATP is the dominant response, mediated by P2X-purine receptor(s) in the adult cat {292} . P2X-receptors mediating vasoconstriction are ligand gated ion channels located on the smooth muscle of blood vessels and are permeable mainly to Ca^{2+} ions {25,50,21,401}. Currently there are six recognised P2X-receptors as well as a P2Z ($P2X_7$) non-selective ion pore {305}. The PX_1- purine receptor mRNA has been associated with vascular smooth muscle {6}. ATP has been found to induce a contraction of adult human and rat IPA but not adult rabbit main PA {238, 326 }. P2X-mediated pulmonary vasoconstriction has been shown *in vivo* in the adult cat and α,β-meATP was shown to block ATP-stimulated contractions in adult human and rat pulmonary arteries {287,291,77,238,237}.

There are many examples of UTP contracting different systemic vessels from many species {111,397,313,405,124, 162, 229}. Pyrimidines can be released from the vascular endothelium of rabbit thoracic aorta, and from platelets, establishing physiological sources for the agonist {357,147}. A specific pyrimidine nucleotide contractile receptor has been suggested following observations made in a number of reports {354,406,334, 208}. There is

uncertainty as to whether UTP acts at a purine P2X-receptor through recognition of the nucleotide moiety or at a receptor specifically recognising the pyrimidine moiety, because ATP tends to be as effective as UTP in contracting vessels. Some UTP-induced contractions are not desensitised by either ATP or α,β-meATP, which would not indicate activity at $PX_{1\ or\ 3}$ -receptors {207,355}. UTP induced contractions may have a sustained component, while ATP-induces transient responses suggesting different transduction pathways {400, 355,356}. UTP contractile responses have been associated with the mobilisation of IP_3-sensitive calcium stores and activation of voltage operated calcium channels {358,375,388,207}. However, in rabbit saphenous artery α,β-meATP has also been shown to stimulate accumulation of phosphoinositides, indicative of IP_3 generation {283}. UTP contractions of cerebral arteries have been shown to involve inhibition of K^+-channels{364}. cDNA for the $P2X_4$-purinoceptor has been found in the adult rat lung, but neither α,β-meATP or UTP evoked a significant response when the receptor was expressed in oocytes indicating that this is not the receptor mediating the contractile responses in the present study {37}.

There have been few studies concerning the contractile effects of purine and pyrimidines in the pulmonary circulation and they have not been studied previously in the pulmonary arteries of newborn animals. Intravenous ADP was found to be much more potent than UDP in producing a rise in normal calves and those exposed to hypobaric hypoxia {338}. ATP and UTP have been shown to cause vasoconstriction in isolated adult rat lungs, which was not completely inhibited by α,β-meATP {351,349}. However, the adult rabbit isolated main PA did not contract to either UTP or ATP {326}. ATP induces a rise in pulmonary arterial pressure in the intact cat, partially through P2X-receptors but also by metabolism to adenosine acting at A1-receptors stimulating thromboxane synthesis, making interpretation of data more difficult {289}.

Persistent pulmonary hypertension (PPH) of the newborn is potentially fatal condition often caused by hypoxia {161}. PPH results in abnormal vascular remodelling of the intrapulmonary arteries of the developing lung during the first few days of life {176,8,173,7}. Hypertrophy of the IPA smooth muscle cells is most evident at the respiratory bronchiolar

arteriolar level, decreasing the lumen diameter and increasing resistance to flow. This increase in smooth muscle has been taken to indicate an increase in the contractile reactivity of the IPA to different stimuli, such as hypoxia and other agonists {399}. But in the mature experimental animal P2X-receptor mediated vasoconstriction has not been shown to be responsible for the vasoconstriction seen in response to acute alveolar hypoxia in rat IPA {262}.

In the present study, the response of IPA to cumulative doses of ATP, α,β-meATP and UTP was investigated in the IPA of normal newborn animals and compared to the responses seen in mature vessels. P2X-receptor desensitisation by α,β-meATP of the response to ATP and UTP was also assessed. The effect of exposing newborn piglets to chronic hypobaric hypoxia for 3 days, during adaptation to extrauterine life, was assessed by studying the responses to ATP, α,β-meATP and UTP in isolated IPA. The responses of IPA from one normal and one pulmonary hypertensive child to these P2-agonists was also investigated.

Methods.

Material: intrapulmonary arteries from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 14-17 days and adult pigs. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (CHH) (50.8kPa) from birth for 3 a day period. Human tissue was studied when possible (Table 1).
Methods : vessels were isolated and mounted with or without endothelium for *in vitro* isometric force recording. The effect of P2-agonists were studied in IPA at resting tone and related to potassium chloride contractions and relaxations to papaverine. For further details of the protocols used please refer to sections 1and 3 of Chapter 2.

Table 1. Clinical details of children from whom tissue was studied.

Specimen number	Details of child
Normal	
4183	4 month old. TGA/VSD, pulmonary arterial banding, switch followed by myocardial infarct. ECMO. Normal pulmonary arterial pressure.
Pulmonary hypertensive	
3508	16 year old. Primary PHN, lung removed during transplantation.
4087	4 month old. PHN, congenital lung dysplasia and hypoplasia.
3954	36 hours old. PHN, congenital lung dysplasia and hypoplasia.

Results.

Responses of intrapulmonary arteries from normal pigs.

The cumulative addition of ATP induced a concentration-dependent relaxation which was independent of the endothelium and was similar in vessels from fetal and newborn animals (Fig.1 A,B,C, Fig.2 A). By 3 days of age the response had become biphasic (Fig.1 D). A relaxation response was first induced at concentrations less than 3mM, followed by transient contractions as the concentration increased. Removing the endothelium did not influence either response. The contractile response was present more consistently in 14 day old and adult animals (Fig.1 E,F, Fig.2 B) . It became dominant with age, the relaxation response decreasing. In response to UTP, IPA from one fetal pig which had a high resting tone relaxed at high concentrations, whilst the IPA from a second fetal pig with a lower tone showed no response (Fig.3 A,B). UTP-induced a high concentration-dependent contraction in newborn porcine IPA, which was greater without the endothelium (Fig.3 C). By 3 days of age however, a transient contraction was induced at 0.01mM, though the response was greater in rings without endothelium (Fig.3 D). The magnitude of the response increased in IPA

isolated from normal animals aged 14 days when the response was not significantly different form that in the adult (Fig.3 E,F). The response was significantly greater in rings without endothelium after 2 weeks of age ($p < 0.05$) (Fig.4 E,F).

Cumulative-addition of α,β-meATP induced no significant response in IPA isolated from animals of any age, except the adults (Fig.5 A-E, Fig.6). A small contraction was induced in adult at concentrations ≥ 1μM and 100μM produced only a loss of developed tone. Removing the endothelium had no significant effect on the response at any age. A 20 minute pre-incubation with α.β-meATP, followed by a repeated dose to confirm P2X-receptor desensitisation [total of 2 x 100μM] was found to desensitise the contraction response in IPA from adult pigs, with or without the endothelium (Fig.7 A). The same protocol reduced the low concentration relaxation response to ATP in newborn and 2 week old piglet IPA and removed the endothelium augmented the effect of α,β-meATP in the IPA from normal newborn piglets (Fig.7 B,C). Removing the endothelium had no significant effect on the relaxation response. In newborn IPA UTP-induced an increase in contractile response after preincubation with α.β-meATP and removing the endothelium did not alter the effect (Fig. 8 A). The contractile potency of UTP was also increased in the IPA from 2 week old piglets, and was more evident in the rings with endothelium which at this age displayed responses responses to those in rings without endothelium. However, the stability of the UTP-induced contraction was reduced in the presence of α,β-meATP (Fig. 8 B.).

Responses of intrapulmonary arteries from piglets with PHN.

IPA from only 3 of 4 CHH piglets contracted in response to ATP at high-concentrations, compared to 4 of 4 normal age-matched 3 day old animals (Fig. 9 A). One of 4 did contract but less than in normal IPA. Removing the endothelium from IPA taken from PH neonates produced a significant increase in the tone developed at baseline and induced by the greatest ATP concentration ($p<0.05$). Exposure to CHH from birth for 3 days had no significant affect on the relaxation response at resting tone to low millimolar ATP of IPA, when compared to normal age-matched animals. IPA from newborn piglets exposed to CHH contracted to UTP, but less than in the normal age-matched animals (Fig.9 B). A period of

CHH did not increase the reactivity to α,β-meATP (Fig.9 C). The same lack of response was found as in normal age-matched animals.

Responses of intrapulmonary arteries from normal and PH children.

Bolus addition of α.β-meATP failed to evoke a response in IPA from a 36 hour old baby with PPHN (Fig.10). Repeated application of α.β-meATP did not abolish subsequent responses to ATP. Cumulative doses of ATP produced the same biphasic response seen in neonatal piglets, composed of an initial relaxation followed by contractions at higher concentrations. Bolus addition of ATP-induced a dose-dependent vasoconstriction of IPA from a 16 year old pulmonary hypertensive child over the same concentration range previously found for IPA from a mature pig, despite a maximum response not being reached (Fig.11 A).

The result from this experiment was used to decide which concentration of ATP or UTP should be used to study human vessels. At resting tone ATP relaxed the IPA from a 4 month old normal child, although a degree of spontaneous tone developed prior to addition of the agonist (Fig. 11 B). α.β-meATP induced a greater contractile response than UTP in the normal child. In the IPA isolated from a 4 month old child with pulmonary hypertension the contraction response to UTP was greater than that to ATP at equal concentrations, and both were greater than the response to α,β-meATP (Fig. 11C). Repeated application of α.β-meATP caused a desensitisation of the response to α.β-meATP, reversing this to a vasodilator response. In the presence of α.β-meATP the relaxation response to ATP became a contraction and the contractile response to UTP increased in the normal child. In the IPA from the 4 month old pulmonary hypertensive child the contractile responses to both ATP and UTP were increased in the presence of α.β-meATP.

Discussion.

ATP has been shown by other workers to vasoconstrict intrapulmonary arteries from adult animals *in vitro* and *in vivo*, at resting tone {238,290,237,351}. However, the present study has demonstrated that the reactivity of an intrapulmonary artery at resting tone changes with

age, from birth until adulthood. This may reflects changes in the contractile state of the vascular smooth muscle cells, associated with the changing environment in which they function. The high pulmonary arterial pressure *in utero* and in the newborn is due to vasoconstriction of the intrapulmonary arteries. It is not known whether the contractile mechanism of the hypoxemic fetal intrapulmonary artery smooth muscle cells are in an agonist stimulated active latch state or have developed in a passive "constricted" configuration . However, the present study has shown that the relaxation response to ATP of the IPA of the normal newborn at resting tone was similar to that of precontracted IPA from a normal animal which has adapted to extrauterine life (Chapter 4), suggesting a component of active tension in the blood vessel wall at birth. A fall in the pulmonary arterial pressure must occur at birth to allow blood flow through the respiratory system, therefore our findings of a dominant vasodilatation of the newborn and young animals were not teleologically, surprising. In contrast, a mature pulmonary circulation has an established low arterial pressure and resting tone which may be attributable to a vessel with "passive" smooth muscle cells, where vasoconstriction to an agonist may be more likely to occur.

The contractile response to ATP was poor in newborn porcine IPA . The contractile response of IPA to KCl has been shown to increase with increase in age (see Chapter 3) and therefore the response to ATP may not only be due to the physiological requirement of the vessel reactivity at a given stage in adaptation but also to the immaturity of smooth muscle contractile machinery {233}. The poor contractile response to ATP of newborn IPA might also indicate that the smooth muscle contractile system was only partially activated. The need for a high ATP concentration to induce a contraction could reflect the low sensitivity or number of the P2X-receptor(s) present. The contractions were transient which suggests either receptor desensitisation or metabolism of ATP. Desensitisation seems unlikely as a full cumulative concentration-response , although difficult, could be constructed. If ATP metabolism was occurring by the action of ectoATPase enzymes, then a high -sensitivity receptor may be responding to low concentrations of ATP in the vicinity of the receptor. The relaxation response at low concentration may be mediated by adenosine following metabolism

acting at P1-purine receptors, as postulated for the relaxation response in Chapter 4. Adenosine has been found to mediate the relaxation response when ATP was applied to the rabbit basilar artery at resting tone {374}. This possibility has not been investigated in the present study but considering the rapid degradation of purines in the lung , the action of metabolites should be acknowledged as part of the true physiological response.

The present work is only the second study to demonstrate that a contraction to UTP can be more potent and induce responses of greater magnitude than ATP in a blood vessel {259}. The contractile response to UTP was evident in the normal newborn animals, much earlier than for significant ATP induced contractions. Vasomotor tone results from the many mechanisms interacting with one another and the release of vasodilating factors from the endothelium has generally been found to dampen the contractile responses to physiological agonists. The contractile response to UTP was increased by the removing the endothelium which has been reported for the mesenteric artery of the adult rat {334}. Removing the endothelium had no effect on the ATP-induced response indicating a greater interaction between UTP than ATP in regulating vascular tone. UTP was the dominant contractile agonist compared to ATP and α,β-meATP at all ages studied and the response increased with age. This could be due the number of pyrimidine receptors increasing with age and / or the efficacy of the receptor-coupling mechanism increasing with age. The lack of UTP-induced relaxation in the concentration range at which ATP did induce a relaxation at resting tone, would suggest an absence of pyrimidine sensitive receptors mediating the relaxation responses. Therefore, any concentration of ATP applied to the IPA at any age would be distributed between contractile and relaxant receptors reducing the effect at both. However, UTP only acts at high affinity contractile receptor population at resting tone, producing a greater contractile response than that achieved by the same concentration of ATP. This would be true even if the contractile receptor in question had an equal affinity for ATP and UTP. Only the $P2X_3$- expressed on sensory nerve endings have been shown to respond to UTP and this response was poor when compared to other P2-receptor agonists {83}. The $P2X_4$ receptor has been located in the lung but UTP did not elicit a response {37}. UTP may be acting at one of the metabotropic P2Y-receptors which have been shown to have be

pyrimidine-preferring, such as $P2Y_{4 \text{ and } 6}$. The accepted dogma that contractile responses to P2-agonists are mediated by ionotropic receptors (P2X) has recently been questioned. By following the classification system of agonists rank order, a pyrimidine-preferring P2Y receptor is thought to mediate the contractile response of the adult rabbit coronary artery induced by UDP and UTP {259}. The mRNA for the $P2Y_6$ has been located in the lung and this receptor has a preference for UDP over UTP {74,81}. Having not tested UDP in the present study it would be premature to attribute the results from the present study to a specific subtype of pyrimidine-preferring receptor.

The transient contractile response to α.β-meATP increased with age, but was found to occur in the micromolar rather than the low millimolar UTP and high millimolar ATP responses. The contractile response to, and desensitisation by, α.β-meATP would indicate a $P2X_{1 \text{ and } 3}$-purinergic receptor subtype in the IPA of older pigs. However, the ATP and UTP contractile responses were not blocked by α.β-meATP. Other workers have recently found that the contractile response to ATP was not inhibited by α.β-meATP in systemic vessels {299,391,208,251}. Other investigators have shown that α.β-meATP did not inhibit UTP induced vasoconstriction of adult rat lungs, but reduced the effect of ATP {347}. These findings could suggest that ATP and UTP rather than ATP and α.β-meATP may share a receptor subtype. As stated above, the $P2X_4$-receptor has been located in the lung but neither α.β-meATP or UTP was effective in evoking a response when the receptor was subsequently expressed in xenopus oocytes {37}.

In the present study, α.β-meATP reduced the relaxation response to low concentrations of ATP. This may could be explained by non-specific inhibition of P2Y-receptors {56}. Alternatively, α.β-meATP was shown to be an inihibitor of ectoATPase activity on bovine aortic endothelial cells {86}. EctoATPase inhibition would prevent relaxation if mediated by adenosine at P1-receptor(s) following ATP metabolism {374}. Alternatively, ectoATPase inhibition may increase the levels of ATP and therefore shift the net response towards contractions mediated by P2X-receptors which are stimulated by greater concentrations than are needed to activate P2Y-receptors in the present study.

Chronic hypobaric hypoxia produces vascular remodelling in the newborn piglet and other species including man, contributing to a raised IPA tone. The present study has shown that the pulmonary vasoconstriction associated with pulmonary hypertension in the newborn piglet was not due to an increase in contractile reactivity to ATP. Instead, the study would suggest a dysfunction in the smooth muscle contractile response to ATP. Exposing piglets to CHH from 14-17 days of age abolished contractions to high concentrations of ATP in precontracted IPA (see Chapter 4). If the IPA remodelling associated with PPH involves an increased activation of the "resting " state of smooth muscle cell contractile filaments, this may well be manifested by a reduced contractile reactivity, similar to the descending side of the length-force curve for any blood vessel .

Alternatively, CHH may have remodelled the IPA in such a way as to produce a "precontracted vessel " which would favour a relaxation response to ATP. However, in the present study neither hypothesis would be supported because the resting tension of the IPA after giving papaverine was lower in vessels from animals exposed to CHH than in age-matched normal animals, in the absence of any contractile agonist. Because of the tone-dependent nature of the responses evoked by P2-agonists, a lower tone should favour a contractile response.

An alternative explanation would be that the smooth muscle sensitivity to ATP inducing relaxation was upregulated to balance the vasoconstricting effect of hypoxia *in vivo* . An initial upregulation of IPA vasodilatation to EDRF and activation of K^+-channels can occur in response to acute hypoxia {407}. The increase in medial smooth muscle in chronic hypoxia does not appear to correlate with an increase in contractility, possibly because there is a greater proportion of synthetic smooth muscle cell phenotypes present. However, remodelling does explain the reduction in vessel compliance, which may be involved in maintaining a greater basal tone in hypertensive adult rat arteries {156}. The finding that contraction to ATP does not increase in the remodelled pulmonary artery, but is in fact abolished, would agree with the theory that CHH reduces the proportion of contractile smooth muscle cells and generally increases stiffness of the vessel wall {156}. The finding of a reduced contractility

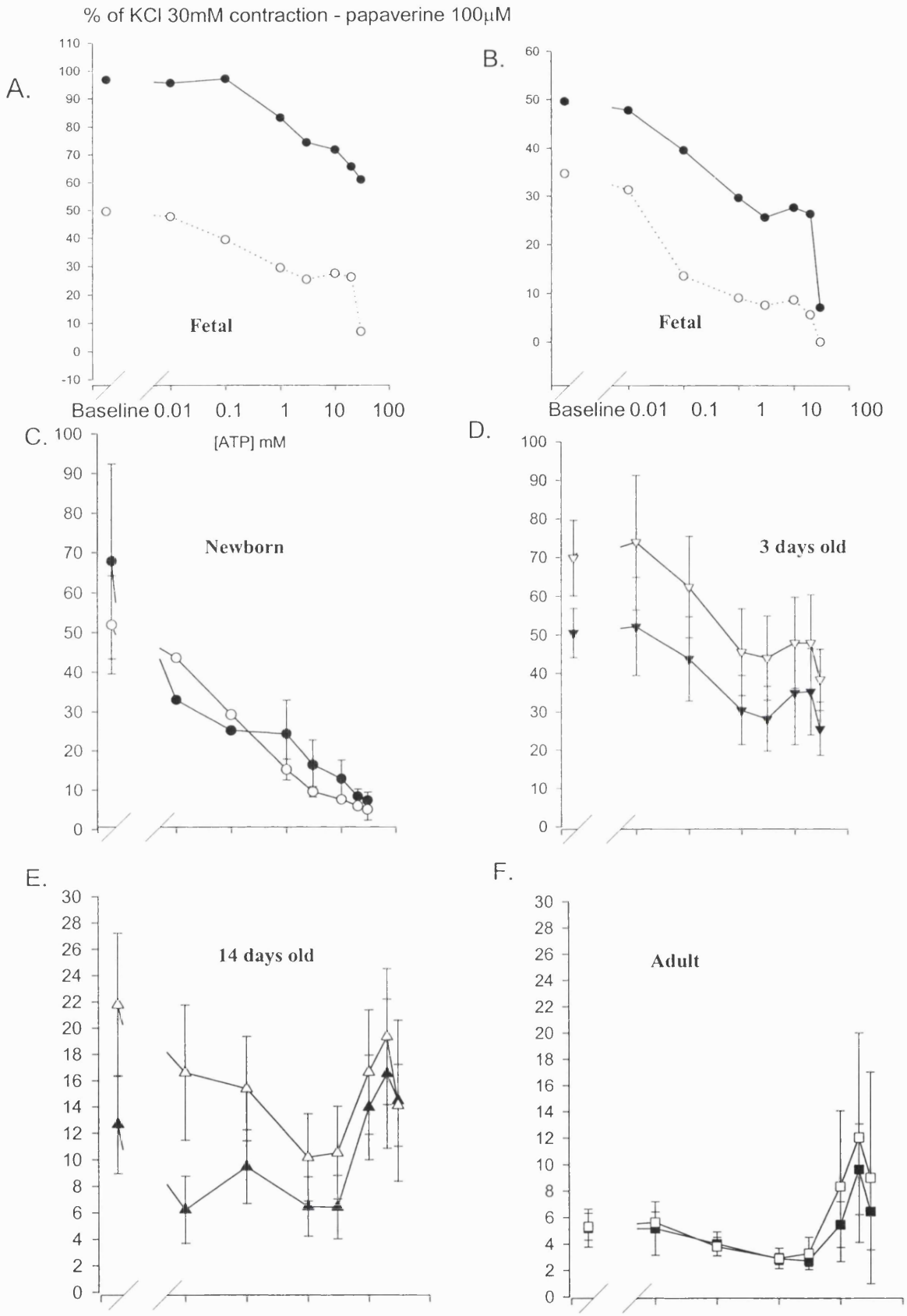
in the vessels from pulmonary hypertensive animals would suggest that the underlying cause for the increased tone *in vivo* is a loss of dilating ability. The present study would suggest that an increase of IPA reactivity to agonists acting at α,β-meATP -sensitive P2X-receptor(s) was not mediating the increased vascular tone produced by CHH. This would agree with work done by McCormack *et al* which demonstrated an absence of P2X-purine receptor mediated vasoconstriction of the adult rat pulmonary circulation subjected to acute hypoxic vasoconstriction {262}.

In the present study it was observed that the IPA from normal neonatal piglets contracted more to UTP following removal of the endothelium. It would seem probable that UTP-induced vasoconstriction may be increased after CHH due to the associated reduction of EDRF {399}. Other workers have shown that CHH reduces EDRF mediated relaxation in the pulmonary circulation, increasing the contractile reactivity of the IPA to certain agonists, including possibly UTP {99}. However, the contractions to UTP were less than normal in the IPA from CHH piglets and removing the endothelium from the IPA of piglets exposed to CHH still increased the contractile response, suggesting that an interaction with released EDRF was still occurring. In summary, increase in the contractile response to purines and pyrimidine with age would suggest that they are not important in mediating the vasoconstriction of the pulmonary vascular bed *in utero*. In addition, CHH causes a general reduction in the capacity of the smooth muscle to contract to P2-agonists.

The findings from human IPA at resting tone indicated that the porcine IPA reactivity mimics the human well. The reactivity of IPA from a 36 hour old baby with PPHN to α,β-meATP and ATP were very similar to that seen in the neonatal piglet of the same age. The conduit IPA from a teenager with pulmonary hypertension contracted in response to ATP over the same concentration range as the porcine vessels. In addition the agonist rank order of contractile response for human IPA was UTP > ATP > α,β-meATP, as found for the mature pig IPA. Another similarity between the species was the ability of α,β-meATP to only desensitise its own response, but not to ATP or UTP.

ATP has been shown to be effective in reducing the high PAP of adult patients with chronic obstructive pulmonary disease and pulmonary hypertension, in which adenosine was ineffective {139,140}. Studies on the mechanism of action indicate inhibition of hypoxic pulmonary vasoconstriction was responsible for the drop in pulmonary resistance {141}. There are also reports of ATP being successfully used in the management of pulmonary hypertensive crises in young children, without the side-effects of more traditional treatments such as tolazoline {54,145}. The theoretical potential for P2-agonists to evoke a pulmonary arterial vasoconstriction in an infant would seem to be unlikely, if low concentrations of drug are administered in the P2Y-receptor vasodilating dose-range. The difficulties associated with attempting to apply results from pigs during development to human infants should be always be considered, despite the apparent similarities in the maturation of the pulmonary circulation in the two species.

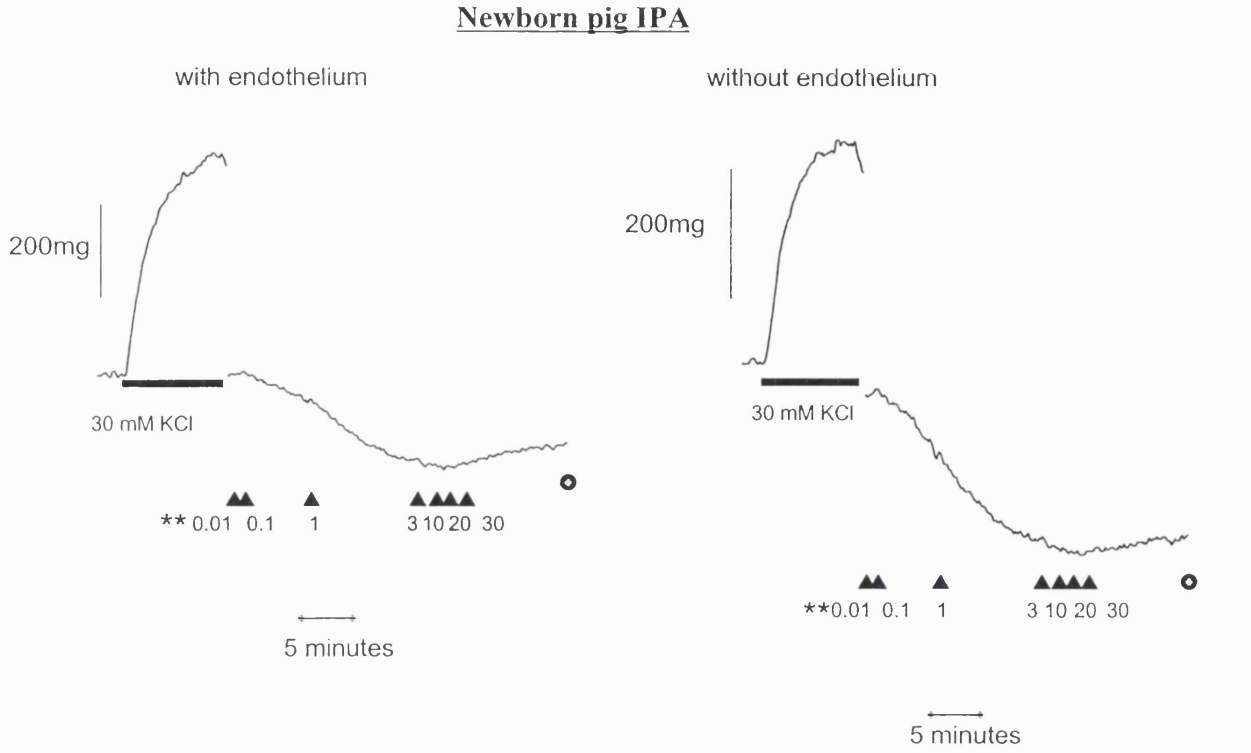
Fig.1



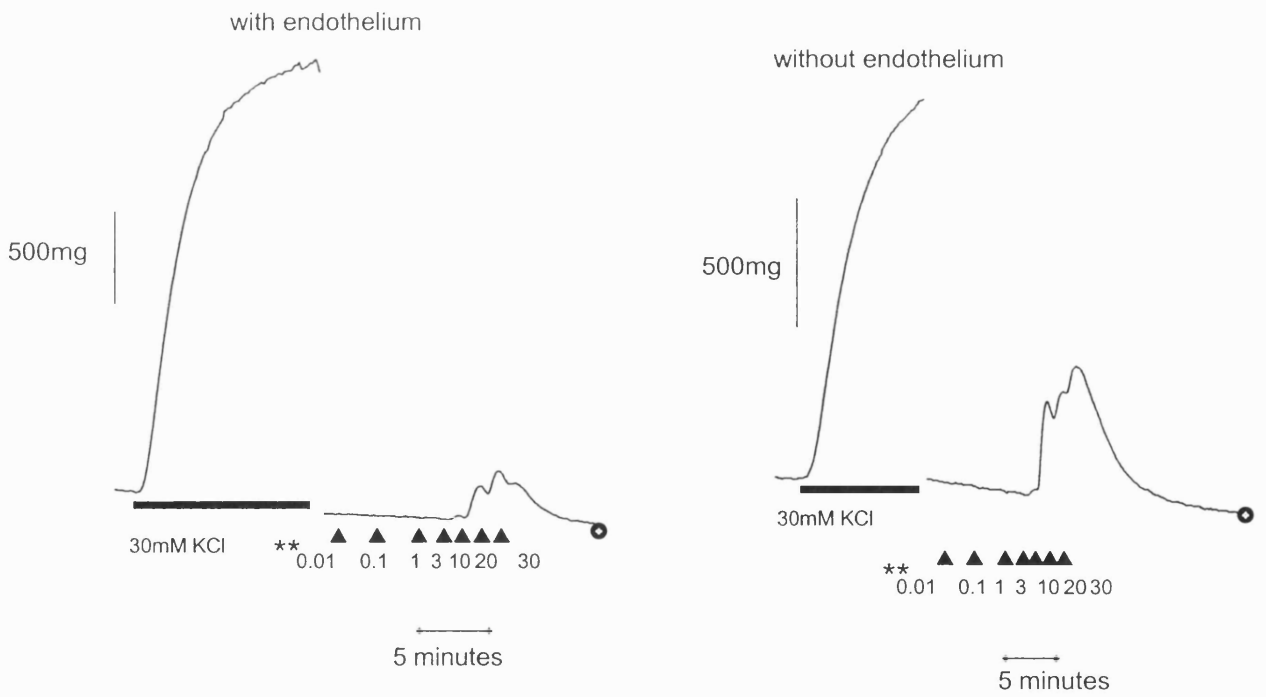
Response of IPA to cumulative doses of ATPat resting tone from (A,B)2 normal fetal piglets ; (C) newborn (n=3/3); (D) 3 day (n=5/3); (E) 14 day (n=6/8) and (F) adult pigs (n=4/4).
Bars = standard error of the mean (sem).

Note: Empty symbols indicates data for IPA with the endothelium removed. The notation "n= 4/4" for example, indicates the number of animals studied with / without endothelium. This applies for all figures in this chapter unless otherwise stated.

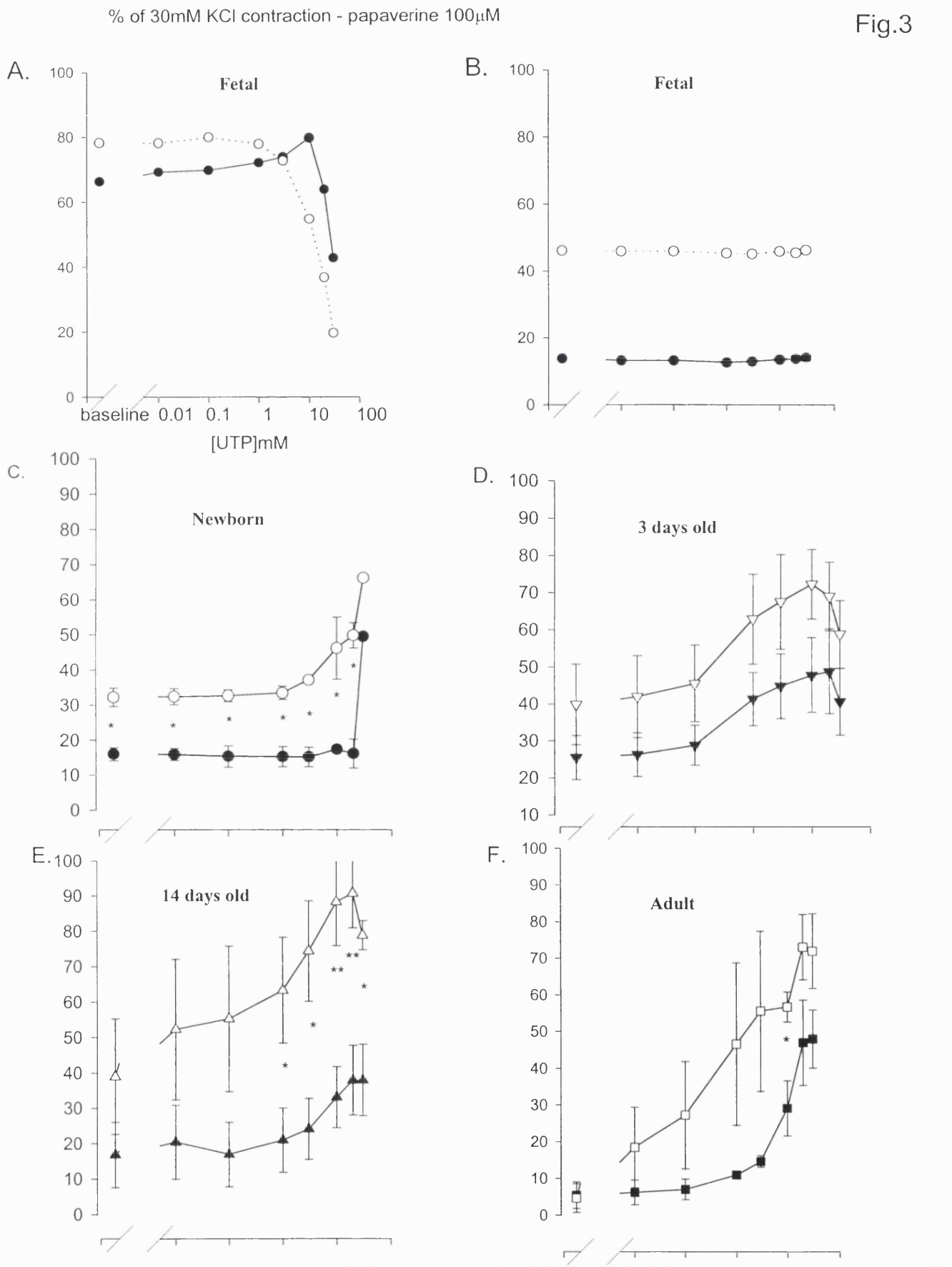
A



B



Representative traces for the response of IPA to cumulative doses of ATP (A) normal newborn and (B) adult pig at resting tone. A low millimolar relaxation was the only response. Bolus of 30mM KCl shown as a reference contractile response. Effect of 100μM papaverine (✪). ** mM values.

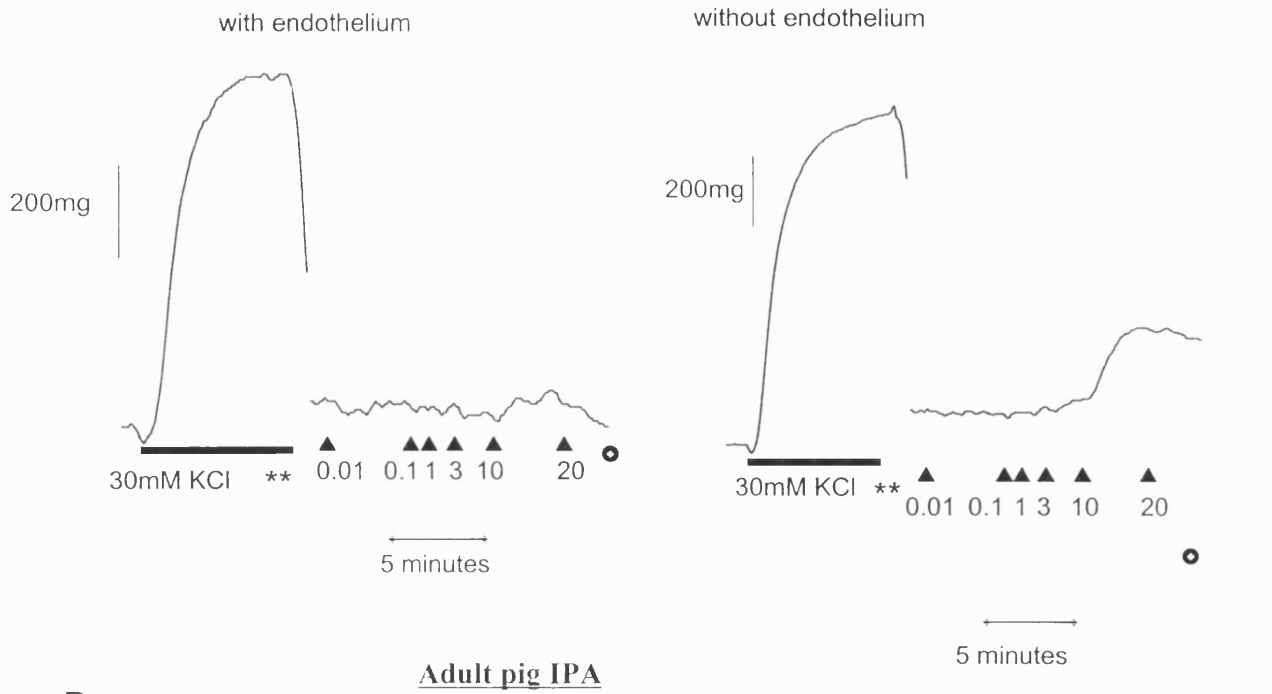


Response of IPA at resting tone to cumulative doses of UTP (A,B) fetal animals; (C) newborn (n=2/2); (D) 3 day (n=4/3); (E) 14 day (n=6/5) and (F) adult pigs (n=3/3). With (solid symbols) and without endothelium (empty symbols). Bars = sem (sd when n=2). * indicates a significant difference at the $p<0.05$ and ** at the $p<0.01$ level.

A

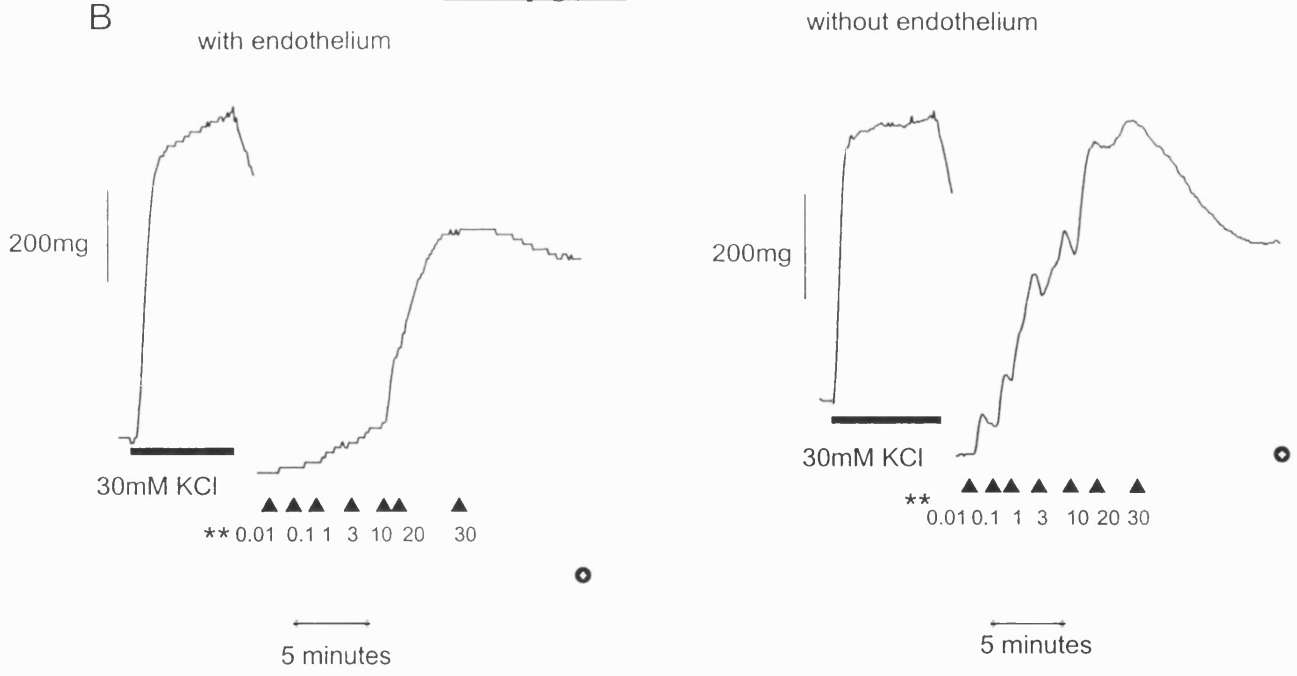
Fig.4

Newborn pig IPA



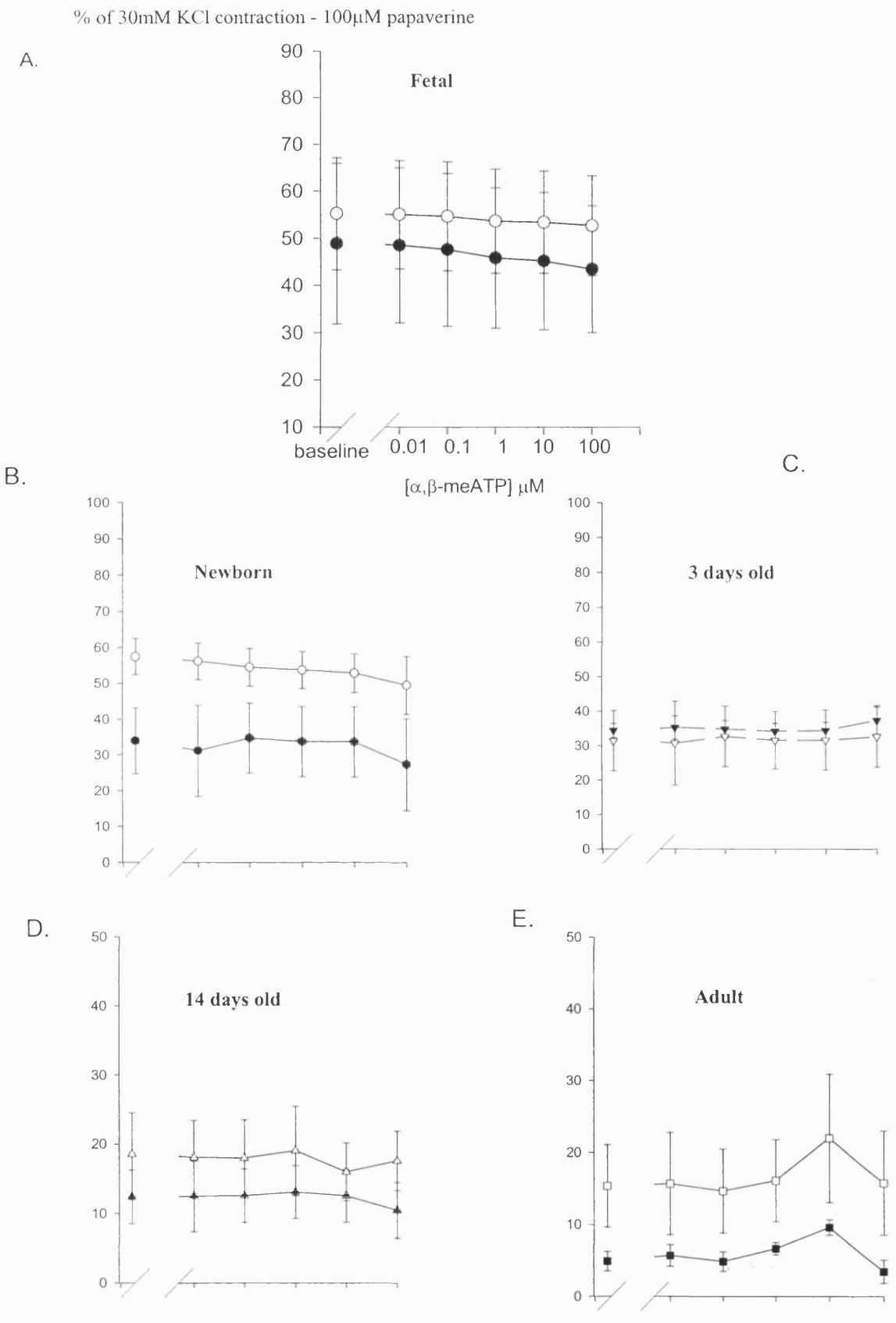
Adult pig IPA

B

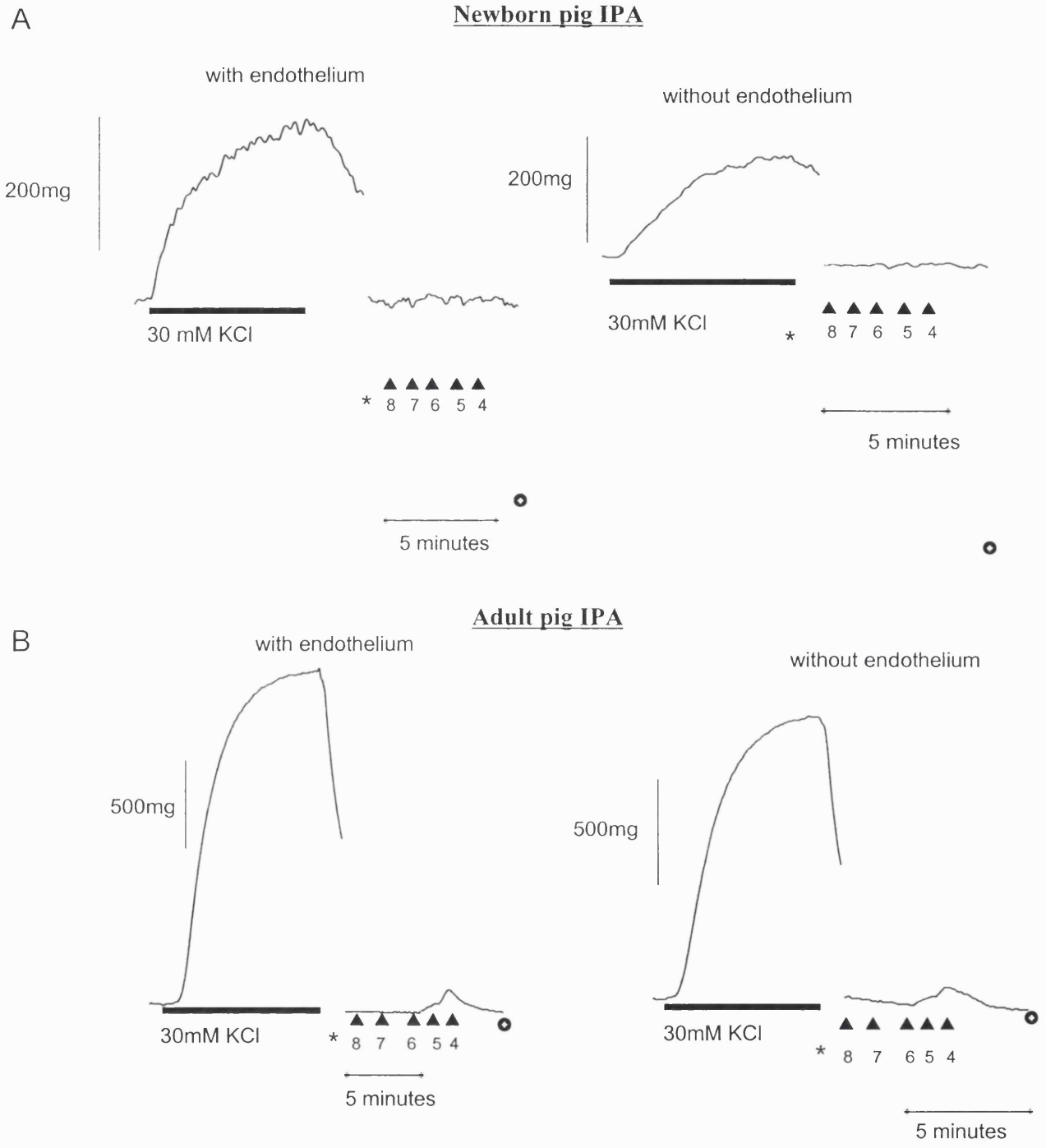


Representative trace of the response of IPA to UTP from (A) a normal newborn and (B) an adult pig. A contraction was induced at high concentrations. Bolus of 30mM KCl as a reference contraction response. Effect of papaverine 100μM (✪). ** indicates millimolar values.

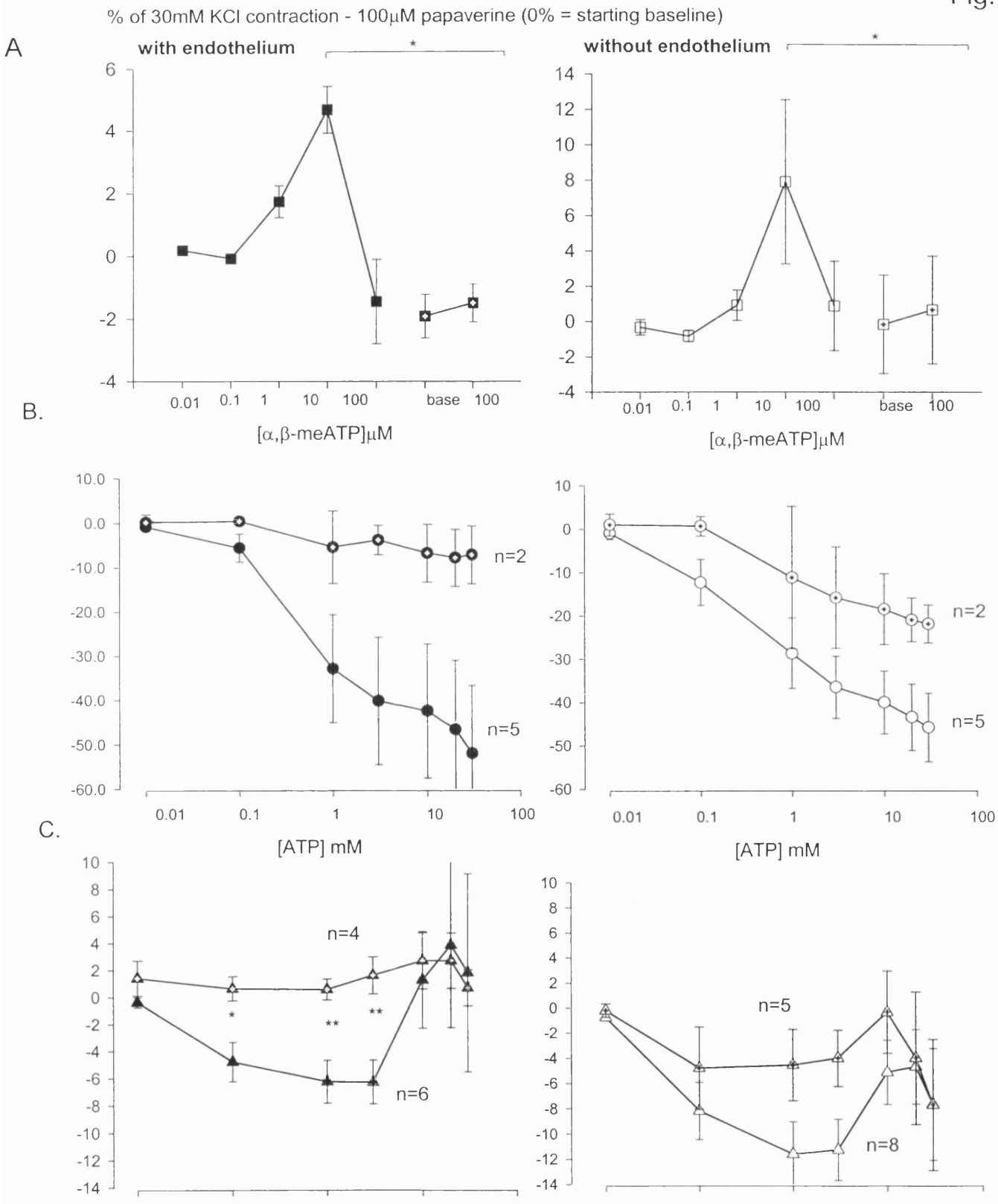
Fig.5



Response of IPA to cumulative doses of α,β-meATP at resting tone from (A) fetal (n=3/3); (B) newborn (n=4/3); (C) 3 day (n=3/3); (D)14 day (n=5/5) and (E) adult pigs (n= 5/6). Bars = standard error of the mean (sem).



Representative traces of response of IPA to α,β-methyleneATP (P2X agonist) of IPA from (A) a normal newborn and (B) an adult pigs. There was no effect on basal tone. Bolus of 30mM KCl for reference contraction. Effect of 100μM papaverine (✪). * indicates -log numbers.

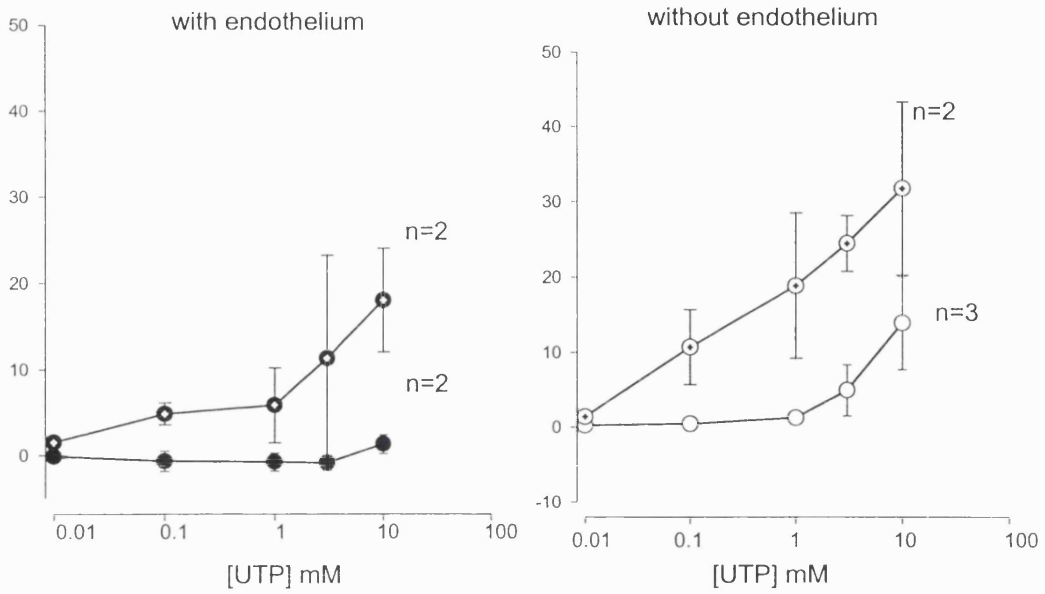


Response of IPA to cumulative doses of α,β-meATP for (A) normal adult pigs at resting tone (n=6) and the crossed symbols indicate the response of IPA following repeated application of α,β-meATP.
The response of IPA to cumulative doses of ATP in (B) normal newborn and (C) normal 14 day old, crossed symbols indicate the response following repeated application of α,β-meATP.
*** indicates a significant difference of P2X-receptor desensitisation $p<0.05$, ** at the $p<0.01$ level.**

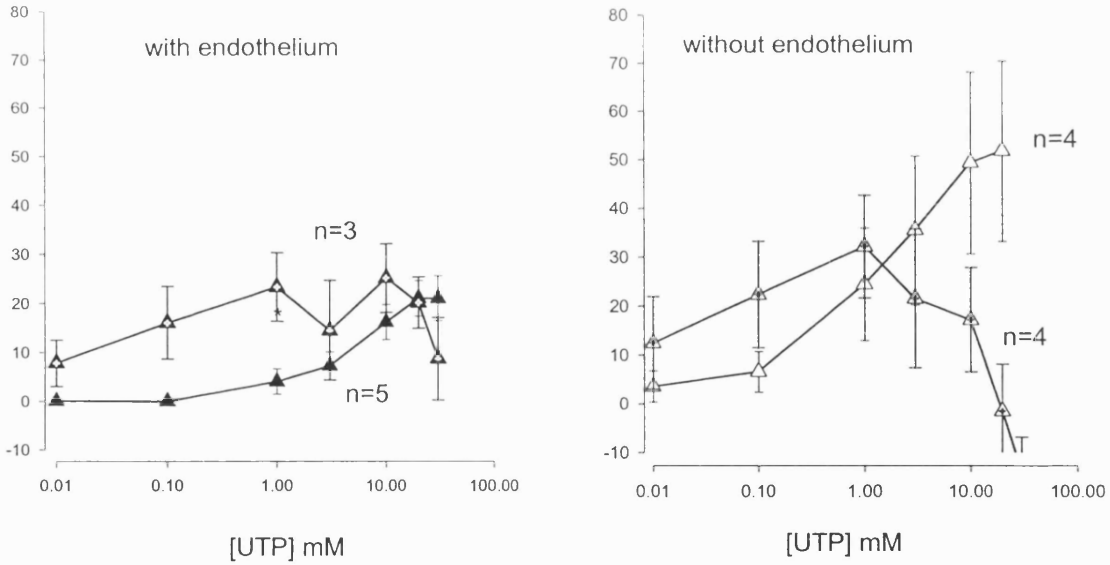
"n" indicates the number of animals used. Error bars represent standard error of the mean (standard deviation when n=2).

Fig.8

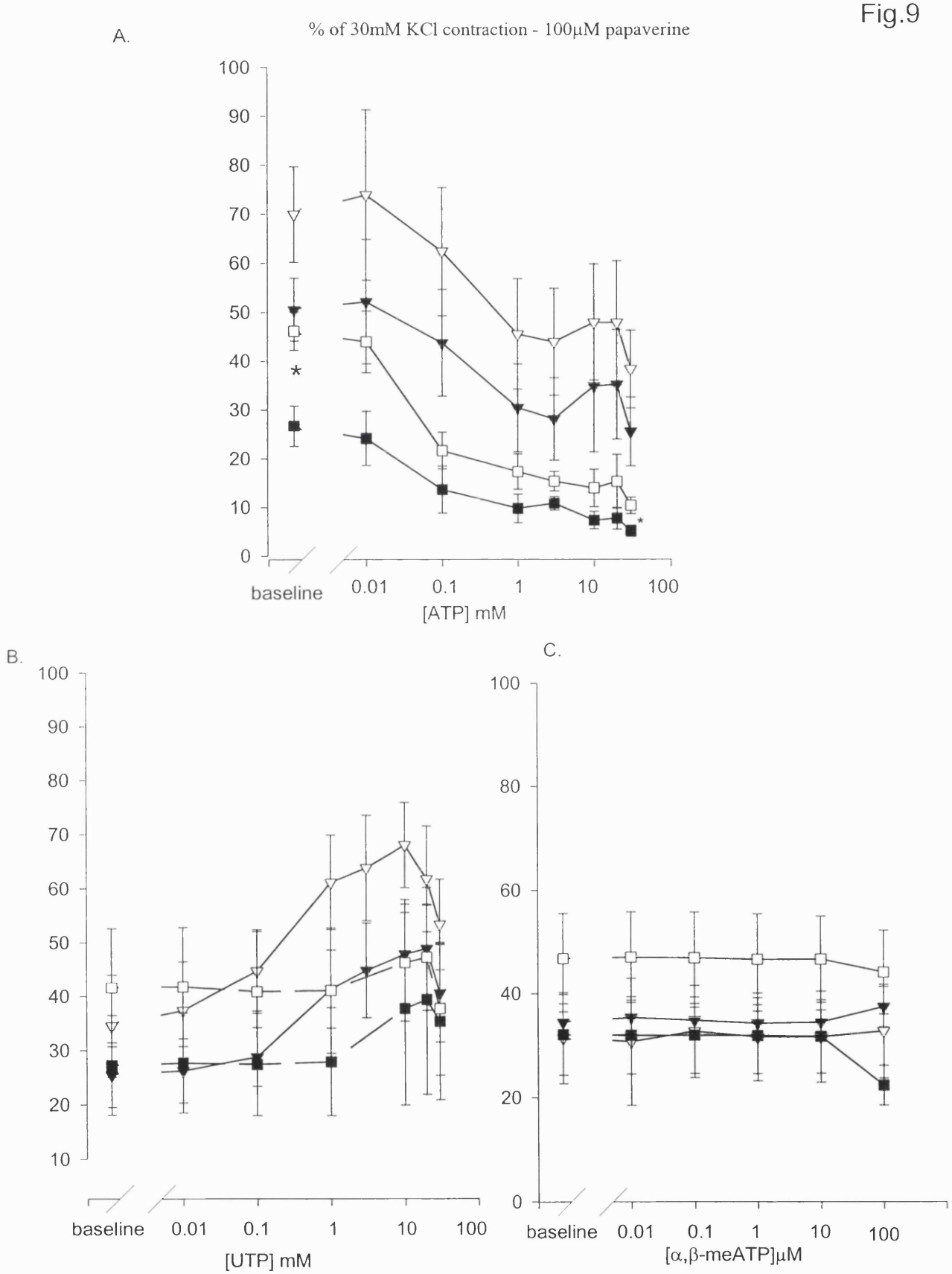
A % of 30mM KCl contraction - 100μM papaverine (baseline= 0%)



B

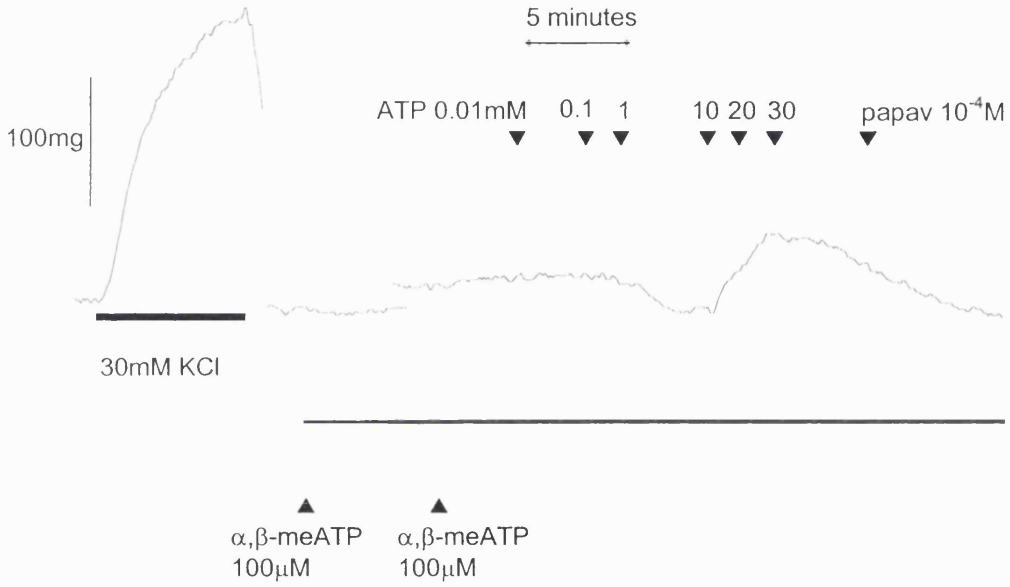


The response of IPA to cumulative doses of UTP in (A) normal newborn and (B) normal 14 day old, **crossed** symbols indicate the response following repeated application of α,β-meATP. Only one pair of IPA with and without α,β-meATP in each age group.
* indicates a significant difference of P2X-receptor desensitisation $p<0.05$ level.



The effect of persistent pulmonary hypertension on the response of IPA at resting tone to cumulative doses of P2-agonists. (A) ATP (normal 3 day old n=5/3▼ , PHN n=4/4■); (B) UTP (normal 3 day old n=4/4 ▼ , PHN n=4/3 ■); (C) α,β-meATP (normal 3 day old n=3/3▼ , PHN n=3/3 ■). Error bars represent standard error of the mean (sem). * indicates a significant difference between response of PPHN IPA with and without endothelium at this concentration, $p<0.05$

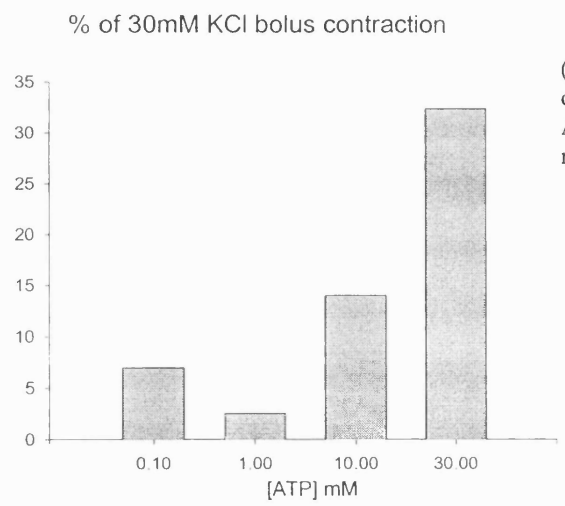
Fig.10



Trace of the response of an IPA with endothelium, isolated from a 36 hour old baby to α,β-meATP and ATP at resting tone. There was no response to repeated application of α,β-meATP, which did not prevent the contractile response to cumulative additions of ATP.
Papav , papaverine.

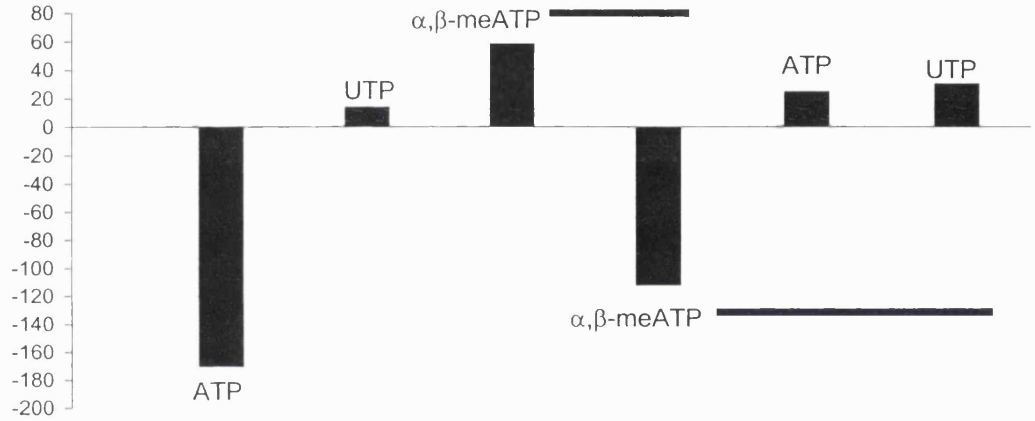
Fig.11

A.

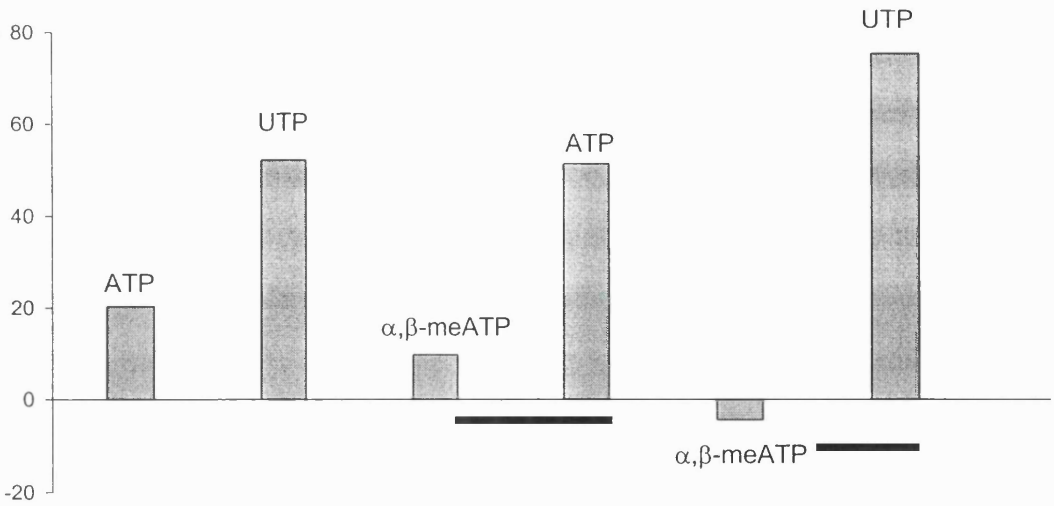


(A) The response of IPA with endothelium from a 15 year old child with pulmonary hypertension, to bolus doses of ATPat resting tone. The response was dose-related but did not reach a maximum.

B.



C.



The responses of IPA with endothelium from (B) a normal 4 month old child and (C) a 4 month old child with pulmonary hypertension, to bolus doses of ATP [10mM], UTP [10mM] and repeated doses of α,β-meATP [100μM] at resting tone. ATP induced relaxation rather than contraction in the "normal" IPA. ATP and UTP remained insensitive to inhibition by α,β-meATP.

Chapter 7. [^{35}S] deoxyATPαS and [^{35}S]UTPαS radioligand binding to intrapulmonary arteries in normal and pulmonary hypertensive lungs.

Summary.

1. 10μM sections of lung tissue were prepared for radioligand binding experiments from normal newborn piglets (aged <5 minutes - 3 hours), animals aged 3 days old and adult pigs. Sections were also prepared from animals with pulmonary hypertension produced by exposing newborn piglets to chronic hypobaric hypoxia (CHH) (50.8kPa) for 3 days from birth.

2. More than 83% of [^{35}S]deoxyATPαS ($P2Y_1$-purine receptor radioligand) binding to the intrapulmonary artery (IPA) media was inhibited by excess 2-meSATP ($P2Y_1$-purine receptor agonist) (100μM) in all groups studied. Greater than 71% of [^{35}S] UTPαS (putative pyrimidine preferring-receptor ligand) binding to the IPA media was inhibited by excess UTP (100μM) (pyrimidine receptor agonist) in all groups studied. This indicates a high level of specific binding for both ligands.

3. The binding for both radioligands was not uniform across the media of the IPA but was particularly associated with the advential border, in all groups studied. The density of specific binding for both radioligands transiently increased at 3 days of age when a high level of binding to the inner media was seen. [^{35}S]deoxyATPαS binding then decreased in the adult to the newborn level, while [^{35}S] UTPαS binding was reduced in the adult but not to the newborn level.

4. Exposure of piglets to CHH from birth for 3 days prevented the appearance of the high binding by [^{35}S] dATPαS and [^{35}S]UTPαS to the inner media.

5. The rank order of binding inhibition by non-radioactive P2-nucleotide agonists was assessed by densitometric quantitative analysis. The findings for porcine IPA in the present study suggested that [^{35}S]dATPαS is binding to $P2Y_1$-**purine** receptors while [^{35}S]UTPαS binds to a P2-**nucleotide**-receptor binding site, on the porcine IPA smooth muscle cells in all groups studied, normal and pulmonary hypertensive.

6. The transient increase of binding by [^{35}S]dATPαS during the period of adaptation to extrauterine life indicates that purines may play a part in this process. Finding that binding may be to a P2Y-purine receptor is in accord with organ chamber pharmacological studies which demonstrate postnatal maturation of the vasodilating response to P2Y-agonists. CHH was found to slightly reduce the P2Y-endothelium-independent relaxation in organ chamber studies, and may be explained by a loss of receptors.

7. Lung tissue from an eight year old normal child was also studied. As found in the pig, both [^{35}S]dATPαS and [^{35}S]UTPαS bound to the media of the IPA. The rank order for inhibition of [^{35}S]dATPαS binding identified a potential P2X-purine receptor sensitive to α,β-meATP, while [^{35}S]UTPαS binding was associated with a non-selective P2-nucleotide receptor, as found in the pig.

Introduction.

Currently the receptors for P2-nucleotides are separated into 2 main groups {65}. The P2Y-receptors are members of the G-protein coupled receptor superfamily and mediate dilatation in blood vessels. The P2X-receptors are members of the ligand-gated ion channel receptor superfamily and mediate constriction in blood vessels. The classification of receptor subtypes is based mainly on the rank order potency determined for a range of P2-receptor agonists with varying degrees of selectivity for the receptor subtypes.

Radioligands have been used by other workers to establish the presence and distribution of purinergic receptors and to support the *in vitro* functional responses. Radioligand binding studies have been carried out on tissue sections, isolated cell receptor expression systems and purified cell membranes {95, 37, 35,413}.

Agonists which display activity in classical pharmacological studies and which may be associated with specific P2-receptor subtypes have been used as radioligands. [^{3}H]ATP was originally used but was susceptible to enzymatic degradation and lacked receptor specificity {232}. The technique has improved following the development of structural analogues suitable for radiolabelling, with greater metabolic stability, such as [^{35}S]ADPβS and

[^{3}H]α,β-meATP {95,211}. Some of the analogues have also been found to exhibit receptor subtype selectivity in functional studies, providing an opportunity to identify receptor subtypes by radioligand binding studies. ADPβS has been used as a P2Y-receptor agonist and recently, following studies involving $P2Y_1$-purine receptors expressed on oocyte cells and binding to purified tissue membranes, [^{35}S]deoxyATPαS has been identified as a ligand with a high specific activity at the $P2Y_1$ -receptor on turkey erythrocytes and oocytes expressing cloned receptors {413, 276}. 2-methylthioATP was used in the original classification of P2Y-receptors and since been associated with $P2Y_1$-receptor agonist *in vitro*. For these reasons, excess 2-meSATP has been routinely used to inhibit ligands targeting P2Y-receptors. α,β-meATP has been used to classify contractile P2X-purine receptors in functional experiments, by virtue of its ability to desensitise the receptor following stimulating and its resistance to metabolism {211,65}. [^{3}H]α,β-meATP has been accepted as the radioligand of choice for investigating the P2X-purine receptors in a range of vascular and non-vascular preparations from many species {32,33,36,276,427}. [^{3}H] α,β-meATP targets $P2X_{1 \text{ and } 3}$, while [^{35}S] ATPγS has been used to locate P2X-purine receptors, in particular $P2X_{1\text{-}4}$ {6}. However, following poor preliminary results with [^{3}H] α,β-meATP in the present study (not included) and a recent report that suggests binding to both P2X- and P2Y-receptors in the adult cat lung, this ligand was not used for the present study {292}.

In the present work, pharmacological studies had shown that UTP, rather than ATP or α,β-meATP, was the most effective constricting agonist of porcine IPA *in vitro*. A novel P2X-receptor activated by UTP has been suggested to mediate vasoconstriction in the adult rat pulmonary circulation {351}. A pyrimidine nucleotide radioligand, [^{35}S]UTPαS, was chosen to investigate binding to pyrimidine-preferring contractile receptors. [^{35}S]UTPαS is used in techniques such as DNA sequencing, however, there are no reports of it being used for radioligand binding to tissue.

Methods.

Material: Lung tissue sections were obtained from normal newborn piglets (aged <5 minutes -3 hours), animals aged 3 days and adult pigs. Sections were also taken from piglets in whom pulmonary hypertension was produced by exposure to hypobaric hypoxia (CHH) (50.8kPa) from birth for 3 days. Lung tissue sections were studied from an 8 year old normal child who had died following a car accident (specimen number 3680).

Methods: Briefly, the inhibition of ligand binding to IPA on lung sections by a range of P2-agonists was determined from densitometric analysis of autoradiographic films. For details of the radioligand binding protocols used please refer to section 5 of Chapter 2.

Results.

Specific binding was seen for both [^{35}S] dATPαS and [^{35}S] UTPαS to lung structures such as parenchyma, airways and veins on sections from all animals, which was not quantified. Binding to conduit IPA, by both ligands, was quantified because conduit IPA were isolated for organ bath pharmacology experiments in the present study, allowing the results from both pieces of work to be combined.

Results from studies confirming the amount of excess non-radioactive ligand to be used.

Binding of 1nM [^{35}S] dATPαS to IPA from normal adult pigs was inhibited in a dose-dependent fashion by a 2-meSATP (Fig.1 A). UTP inhibited the binding by 1nM [^{35}S]UTPαS in a dose-dependent fashion to IPA from normal adult pigs (Fig.1 B).
The binding for both radioligands was distributed across the media of the IPA (Fig.2 and 3, panel D) . From these experiments 100μM was determined to be the excess required to optimise the inhibition of binding (specific binding) recorded.

The effect of age on radioligand binding in neonatal piglets.

The level of specific binding was then determined as the amount of binding inhibited when 1nM of radioligand was co-incubated with 100μM of non-radioactive ligand in Tris buffer at each age. There was less binding by [^{35}S] UTPαS than [^{35}S]dATPαS in each age group (Fig.4 A,B, Table 1).

Table 1 . Specific binding for 1nM [^{35}S] dATPαS and 1nM [^{35}S] UTPαS at different age in normal animals and in animals with PPH.

	[^{35}S] dATPαS		[^{35}S] UTPαS	
Groups	Specific binding density (attomols/mm^2)	% Specific binding	Specific binding density (attomols/mm^2)	% Specific binding
Normal newborn	13± 6.15 (3)	98.66 ± 0.34	2.59 ± 0.05 (2)	75.86 ± 5.54
Normal 3 day old	23.23 ± 14.75 (3)	96.09 ± 2.78	7.46 ± 0.02 (2)	94.46 ± 1.71
Normal adult	11.32 ± 7.64 (3)	83.39 ± 6.77	4.9 ± 2.39 (3)	70.95 ± 4.33
Exposed to 3 days of CHH from birth	18.41 ± 11.96 (3)	89.51 ± 9.5	5.32 ± 5.06 (2)	86.43 ± 9.34

Mean ± standard deviation (where n=2), error of the mean (where n=3). [^{35}S] dATPαS specific binding was taken as that inhibited by excess 2-meSATP (an established P2Y-purine receptor agonist). [^{35}S] UTPαS specific binding was taken as that inhibited by excess UTP (a UTP contractile receptor was under investigation) .

The distribution of binding for both [^{35}S]dATPαS and [^{35}S]UTPαS varied with age (Figs.2,3). There was binding to the media at all ages but greater binding to the outer region of the media. The binding to the outer media was greater at 3 days than in the normal newborn and findings were similar in the 3 day old pulmonary hypertensive and adult animals. Binding towards the inner media was present in the newborn and was obviously greater in the normal 3 day old. Inhibition of binding ranged from 82-99% for [^{35}S]dATPαS (by excess 2-meSATP) and 71-94% for [^{35}S]UTPαS (by excess UTP) in the normal 3 day old and the normal adult, suggesting a high level of specific binding sites in the ages studied

(Table 1). The density of binding increased between birth and 3 days of age for both radioligands, although not significantly (Fig.4 A,B, Table.1). The increase in binding density of [^{35}S]UTPαS was associated with an increase in the % of specific binding ($p< 0.01$) which was not seen for [^{35}S]dATPαS . The specific binding of [^{35}S]dATPαS then declined back to newborn levels while [^{35}S]UTPαS binding was reduced in the adult but remained greater than in the newborn (Fig. 4 A,B).

The rank order for ability to inhibit radioligand binding was determined by ranking the mean binding lost following co-incubation with the non-radioactive agonists (Fig.5 and 6). There was some variation between animals in the same groups but a pattern of inhibition was seen. The rank orders are given in Table 2. By applying the current receptor classification system based on agonist potencies to the findings in Table 2, [^{35}S]dATPαS binds to $P2Y_1$- purine receptor binding site. [^{35}S] UTPαS binds to a receptor(s) with non-selective affinity for P2-nucleotides which makes it impossible to designate any receptor subtype.

The effect of exposure to CHH on radioligand binding

In neonatal piglets exposed to CHH for 3 days from birth, there was less specific binding by [^{35}S] UTPαS than [^{35}S]dATPαS, as was found in all groups of normal animals (Fig.4 A,B, Table 2). The binding across the IPA wall was less in animals exposed to CHH for 3 days than in normal age-matched animals and the increased binding by both radioligands to the inner media seen in 3 day old normal animals was not present (Fig.2,3 panel C). CHH reduced the level of specific [^{35}S]dATPαS binding but not significantly. The rank order of ability to inhibit binding of both radioligand was determined, indicating that [^{35}S]dATPαS bound to a $P2Y_1$- purine receptor and [^{35}S] UTPαS binds to a receptor with non-selective affinity for P2-nucleotides as found in the normal age-matched animals (Figs.5,6 , Table 2).

Table 2 The rank order of displacement activity by non-radioactive agonists of [^{35}S]dATPαS and [^{35}S] UTPαS binding to conduit IPA.

Group	[^{35}S]dATPαS	[^{35}S] UTPαS
Normal newborn	TA = T = TU > AU > A > U	TA = TU ≥ AU > U > T > A
Normal 3 day old	T = TA > TU = AU > A > U	AU = TU = U ≥ TA ≥ T = A
Normal adult	T > TA = AU = TU > A >> U	TU = AU = T = TA > U > A
Exposed to CHH for 3 days from birth.	T = TA = TU > A > AU > U	TA = U = TU > AU = T > A

T, 2-meSATP; A, α,β-meATP; U, UTP; TA, 2-meSATP/α,β-meATP; TU, 2-meSATP/UTP; AU, α,β-meATP/UTP. The order was decided by ranking the mean values of binding inhibition by each of the agonist (s).

Radioligand binding to normal human lung sections.

Frozen sections of lung from an 8 year old normal child were radiolabelled with [^{35}S]dATPαS or [^{35}S]UTPαS . Both ligands bound with uniform density across the media of the conduit IPA but [^{35}S]UTPαS binding to the media of conduit IPA was greater than that for [^{35}S]dATP αS, in contrast to the porcine vessels (Fig.7). The ability of non-radioactive P2-nucleotide agonists to inhibit the binding of [^{35}S]dATPαS or [^{35}S]UTPαS was used to classify the possible receptor subtype associated with the binding sites (Fig. 8 A,B, Table 3). The findings indicated that [^{35}S]dATPαS was binding to a P2X-purine receptor, possibly similar to the human bladder $P2X_1$-subtype , and [^{35}S]UTPαS was binding to a P2-nucleotide receptor.

Table 3 The rank order of inhibition activity by non-radioactive P2-receptor agonists of [^{35}S]dATPαS or [^{35}S]UTPαS binding to the IPA of an 8 year old normal child.

Radioligand	Rank order of binding inhibition
[S^{35}]dATPαS	AU = A > U = TA > T = TU
[S^{35}]UTPαS	TA = T = U > TU = A > AU

T, 2-meSATP; A, α,β-meATP; U, UTP; TA, 2-meSATP/α,β-meATP; TU, 2-meSATP/UTP; AU, α,β-meATP/UTP.

Discussion.

[^{35}S] dATPαS and [^{35}S] UTPαS binding sites were present on the conduit intrapulmonary artery media from normal newborn, 3 day old and adult pigs and from newborns exposed to CHH. [^{35}S] dATPαS was found to bind with a greater density than [^{35}S] UTPαS in all animal groups studied. However, binding to the endothelial layer can not be ruled out because the use of autoradiographic film provides insufficient resolution.

The rank order of activity for agonists to inhibit the binding of was different for [^{35}S]dATPαS and [^{35}S] UTPαS. However, for each ligand a similar rank order was found at each age in the normal animal groups studied. The inhibition of [^{35}S] dATPαS by 2-meSATP was consistently greater than by α,β-meATP in each age group, which would suggest that a $P2Y_1$-purine receptor is present. [^{35}S] dATPαS has been reported to recognise $P2Y_1$-purine receptors in receptor expression systems in oocytes {413}. The binding on the media would support the $P2Y_1$ - endothelium-independent IPA vasodilatation of IPA seen in the present *in vitro* study, and in the small IPA from the normal adult human and the main PA from the adult rabbit {237,326}. The ability of α,β-meATP to partially inhibit [^{35}S] dATPαS binding to a P2Y-receptor might have been predicted. α,β-meATP induced a vasodilatation, though poor, of IPA which may have been due to activity at the P2Y-receptor. In addition, [^{3}H]α,β-meATP has been found to bind P2Y-receptors on the IPA of adult cat lungs {292}.

UTP produced a relatively small inhibition of binding, which would correlate with the poor vasodilatation response seen in the porcine IPA. The ligand would seem to be specific because a high inhibition was seen with 2-meSATP, despite inhibition by the other P2-agonists. However, [^{35}S]dATPαS has recently been found to bind with the characteristics of only a low-potency, partial agonist to $P2Y_1$-receptors expressed in human astrocytomas (1321N1) and monkey kidney cells (Cos-7) {362}. In the same study it was shown that the ligand bound as a full agonist to a $P2Y_2$-purine receptor expressed in the 1321N1 cells. [^{35}S]dATPαS was also shown to involve a large number of high-affinity binding sites which

were not a P2Y-purine receptor with multiple affinity states, bringing into question the selectivity of this ligand {362}. The rank order of binding inhibition from the present binding study would not suggest binding to the $P2Y_2$-purine receptor, where UTP and ATP are equipotent, while 2-meSATP is a much less potent agonist {305,74}.

The transient increase in [^{35}S] dATPαS binding at 3 days of age in the normal animal would indicate a greater number of $P2Y_1$-purine receptors. This could be part of the mechanism underlying the increase in functional endothelium-independent relaxation to $P2Y_1$- purine agonists reported during the neonatal period, in the present organ chamber study. It is possible that normally an increase in the density of dilating receptors on smooth muscle cells close to the source of agonists from the lumen, either blood or endothelium (226), increases during the period when rapid vasodilatation must occur following birth. However, the increase in P2Y-mediated IPA vasodilatation from 3 days of age onwards [see Chapter 5] would not appear to be correlated with an increase in receptor number.

UTP has been found to produce a contractile response in porcine IPA both at rest or after precontraction with $PGF_{2\alpha}$, in the present study (see chapter 5 and 6). The UTP-induced contractions in 3 day old porcine IPA, which increased with age, but UTP-induced endothelium-independent relaxation of precontracted porcine IPA lacked potency and did not increase with age (see chapter 5). This would suggest that the increase in binding may reflect the maturation of a contractile receptor population, but would not explain the increase in contractions from 3 days of age onwards [see Chapter 6]. [^{35}S]UTPαS binding was inhibited equally well by UTP, α,β-meATP and 2-meSATP in each group, which poses a problem for classification of the binding site.

After exposing newborn piglets to CHH, the increased binding of both ligands to the inner media normally seen at 3 days of age did not occur . In the present pharmacological studies *in vitro* ATP-induced relaxation was not affected by CHH, although P2Y-receptor induced (ADPβS) relaxations were slightly reduced (see Chapters 4 and 5). Exposing newborn piglets to CHH has previously been found to abolish IPA endothelium-dependent relaxation to

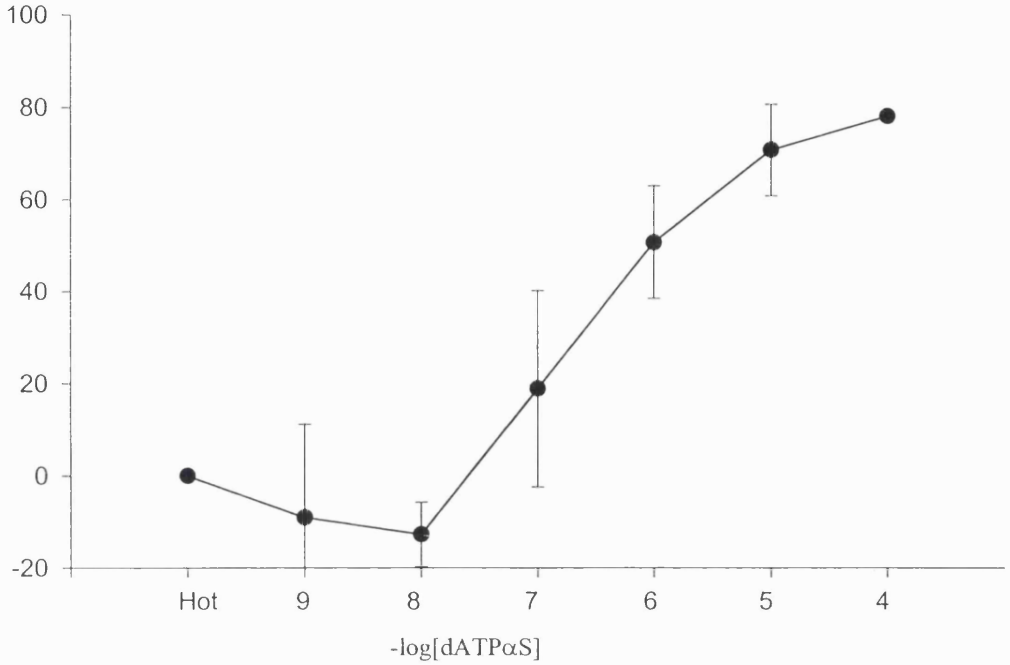
ACh, but did not affect the endothelium-independent relaxation to sodium nitroprusside {399}. The contractile response to UTP was reduced in IPA from piglets after exposure to CHH, and could be explained by the loss of receptor number on the inner media (see Chapter 6).

In the present study, [^{35}S] dATPαS bound to the media of IPA on lung sections taken from a normal 8 year old child. This was unexpected when the P2Y-endothelium-dependent vasodilatation of porcine IPA seen in the present *in vitro* experiments is considered (see chapter 6). P2Y-endothelium-dependent vasodilatation has also been found for the large PA from adult humans and the adult rat {118,152,238}. However, it would agree with the endothelium-independent P2Y-vasodilatation in the small IPA from normal adult human PA and the main PA from the adult rabbit {237,326}. It is still possible that the medial binding may be obscuring an endothelial component, but this could not be resolved using the current technique. The density for [^{35}S] dATPαS was less than for [^{35}S]UTPαS, the opposite to that found from the porcine study. The rank order of binding inhibition for [^{35}S] dATPαS was different from the pig because α,β-meATP was the most active, not 2-meSATP, suggesting a P2X- rather than a P2Y-purine receptor. The absence of rank order of inhibition for [^{35}S]UTPαS binding was as seen in the pig, and therefore supports the conclusion that this ligand recognises a non-selective receptor site in both species.

In the present radioligand binding study, [^{35}S]UTPαS failed to specifically recognise any one P2-receptor on the porcine or human IPA. In contrast, [^{35}S] dATPαS was successfully used to locate $P2Y_1$-purine receptors on the media of IPA from normal and pulmonary hypertensive pigs. It was interesting to find that the same ligand apparently bound to a P2X- receptor on the media of IPA from a normal child.

Fig.1

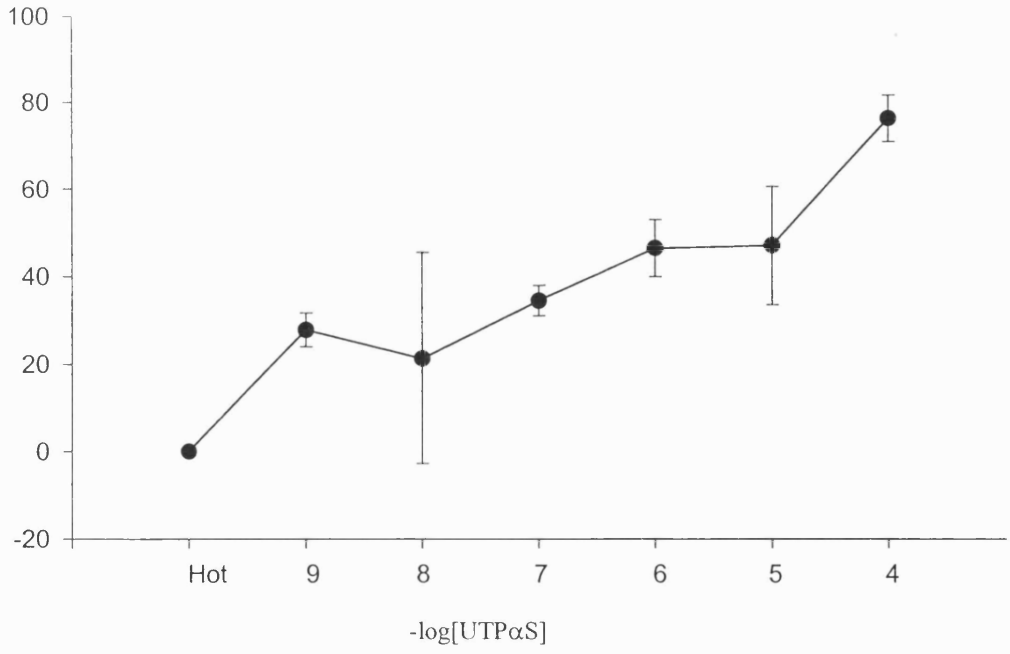
A. % Inhibition of [^{35}S] dATPαS binding to IPA



Binding data from co-incubating 1nM [^{35}S] dATPαS in Tris (=100%), with increasing concentrations of 2-meSATP on IPA from lung sections of 2 adult pigs. These data were used to determine the concentration of non-radioactive ligand required to optimise inhibition of binding. Error bars indicate standard deviation.

B.

% Inhibition of [^{35}S] UTPαS binding to IPA



Binding data from co-incubating 1nM [^{35}S] UTPαS in Tris (=100%), with increasing concentrations of UTP on IPA from lung sections of 2 adult pigs. These data were used to determine the concentration of non-radioactive ligand required to optimise inhibition of binding. Error bars indicate standard deviation.

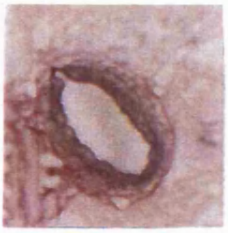
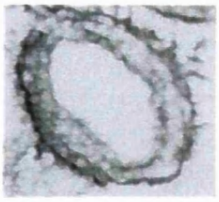
Legend to Fig. 2 Facing page.

[^{35}S] dATPαS

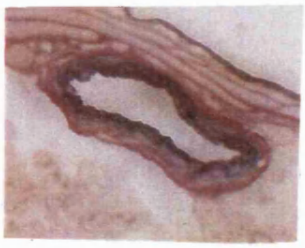
Frozen sections of lung containing conduit IPA were prepared from normal newborn (A), 3 day old (B), newborn exposed to CHH for 3 days (C) and normal adult (D) pigs. Consecutive sections were stained with elastin van Geison stain, shown in the first column. Consecutive sections were used for radioligand binding studies with [^{35}S] dATPαS, and the images on the film were quantified by densitometry (central column of images) *(see section methods 6.)*. To determine the level of specific excess of a non-radioactive ligand, in this case excess 2-meSATP , was co-incubated with the1nM [^{35}S] dATPαS. The non-specific level of binding was analysed from images illustrated in the final column.

The total binding density on the IPA increased from birth to 3 days and was always dense in the outer media region . The normal increase in binding to the luminal region seen at 3 days was absent following exposure to CHH . Binding was inhibited by 2-meSATP in all groups,suggesting a P2Y-receptor site.

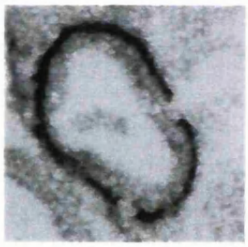
A IPA from normal newborn pig

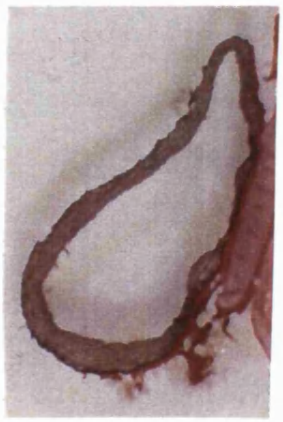
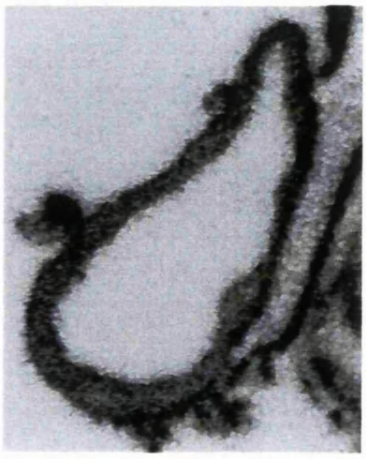
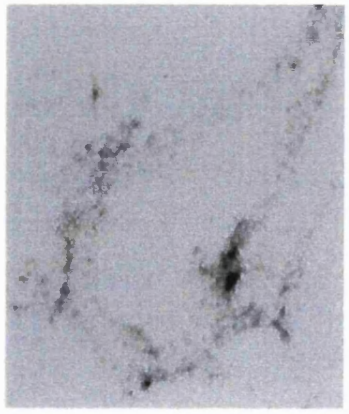

B IPA from normal 3 day old pig



C IPA from piglet exposed to CHH from birth for 3 days


D IPA from normal adult pig

Section stained with van Geisons.

1nM [^{35}S] deoxyATP α S

+ 100 μM 2-meSATP

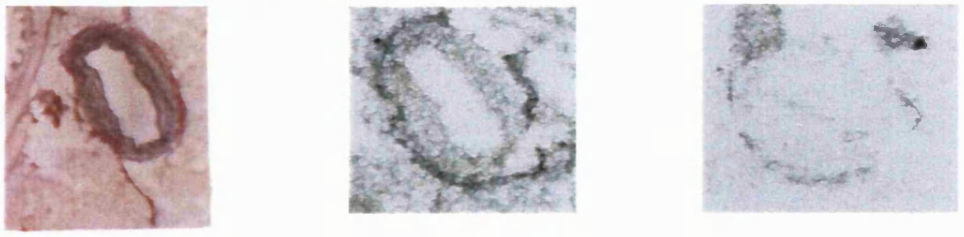
Legend to Fig. 3 Facing page.

[^{35}S] UTPαS

Frozen sections of lung containing conduit IPA were prepared from normal newborn (A), 3 day old (B), newborn exposed to CHH for 3 days (C) and normal adult (D) pigs. Consecutive Sections stained with elastin van Geison stain shown in the first column. Consecutive sections were used for radioligand binding studies with [^{35}S] UTPαS, and the images on the film were quantified by densitometry (central column of images) *(see section methods 6.)*. To determine the level of specific excess of a non-radioactive ligand, in this case excess UTP , was co-incubated with the1nM [^{35}S] UTPαS. The non-specific level of binding was analysed from images illustrated in the final column.

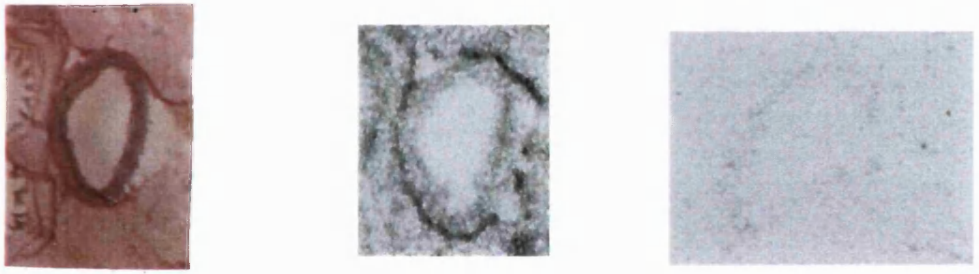
The total binding density on the IPA increased from birth to 3 days and was always dense in the outer media region. The normal increase in binding to the luminal region seen at 3 days was absent following exposure to CHH . Binding was inhibited by all the non-radioactive P2-ligands in all groups, suggesting a non-selective P2-nucleotide receptor.

A IPA from normal newborn pig

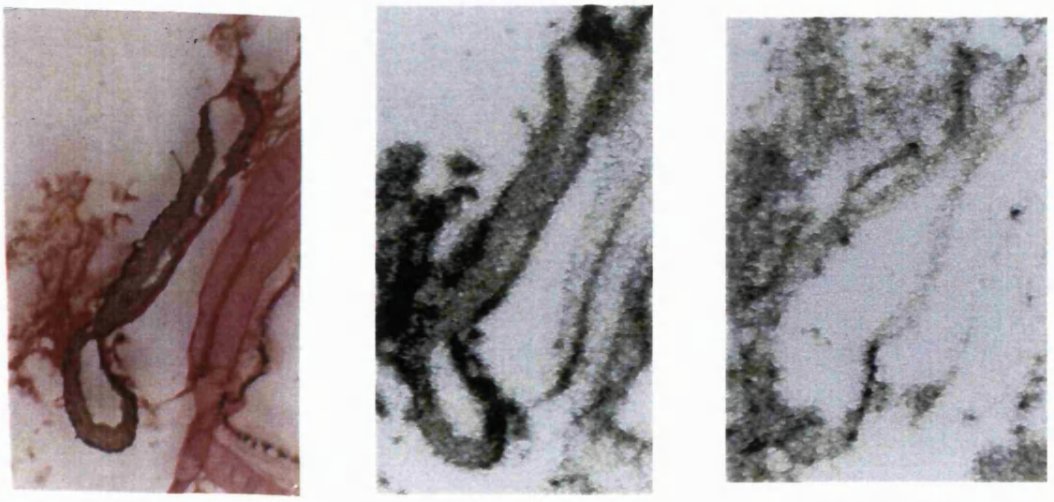


B IPA from normal 3 day old pig

C IPA from a piglet exposed to CHH from birth for 3 days.



D IPA from normal adult pig



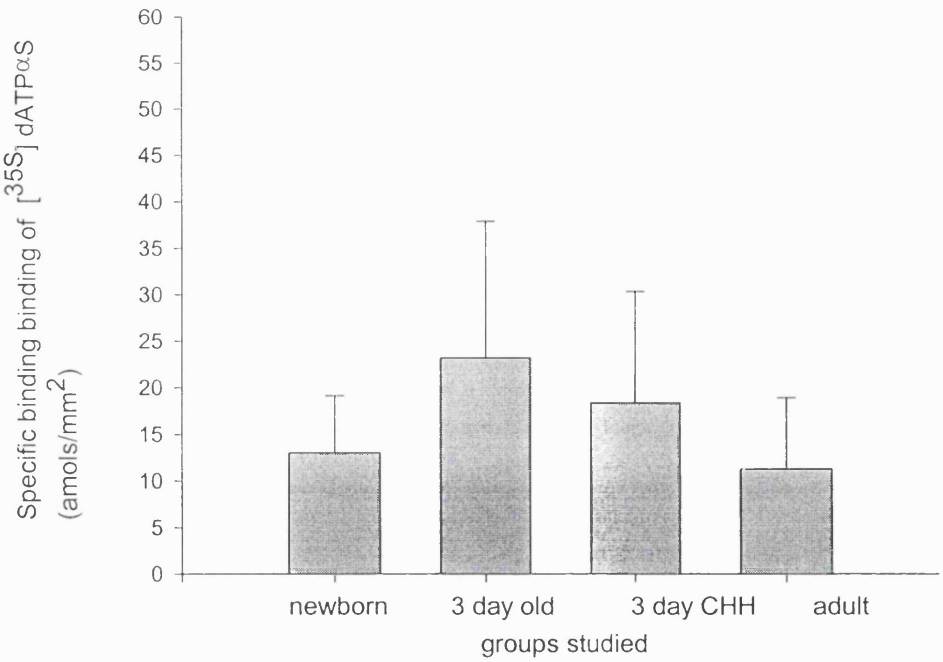
Section stained with van Geisons.

1nM [^{35}S] UTP αS

+ 100 µM UTP

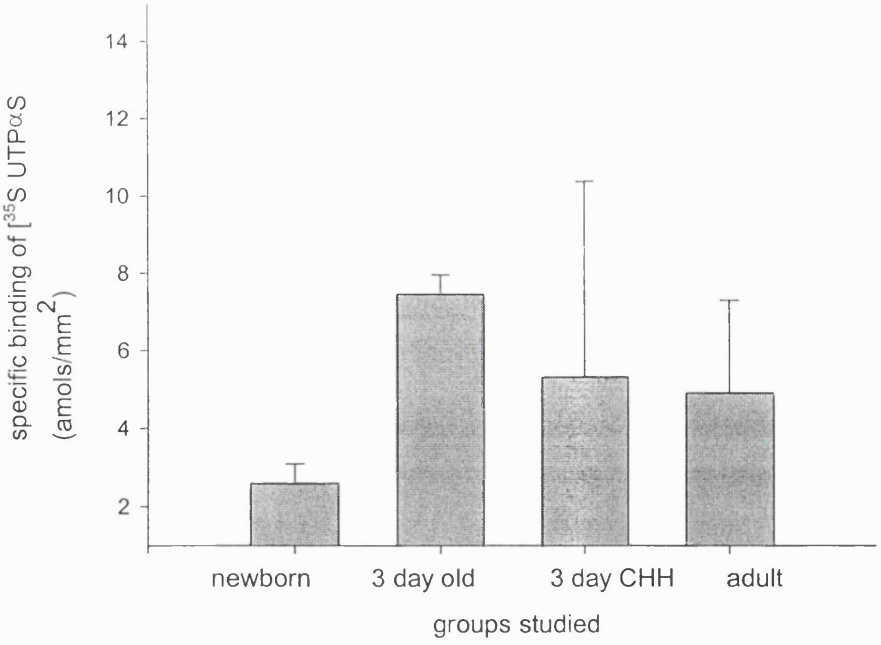
A.

Fig.4



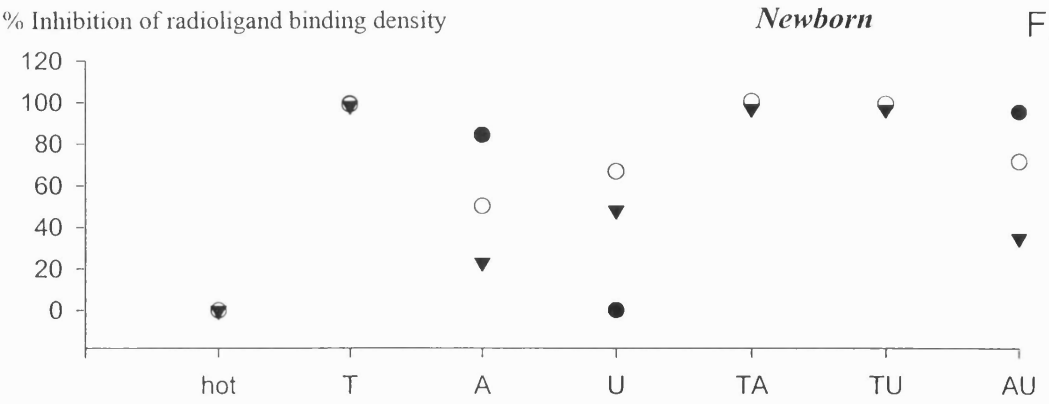
The effect of age and chronic hypobaric hypoxia (50.8kPa CHH) on the specific binding density of 1nM [^{35}S]dATPαS to IPA from porcine lung sections by co-incubating with 100μM 2-meSATP. The binding density increased from birth to 3 days of age and was lower in the adult. Exposure to CHH for 3 days from birth slightly reduced the binding density when compared to age-matched controls.
Error bars indicate standard deviations (2 animals in each group).

B.

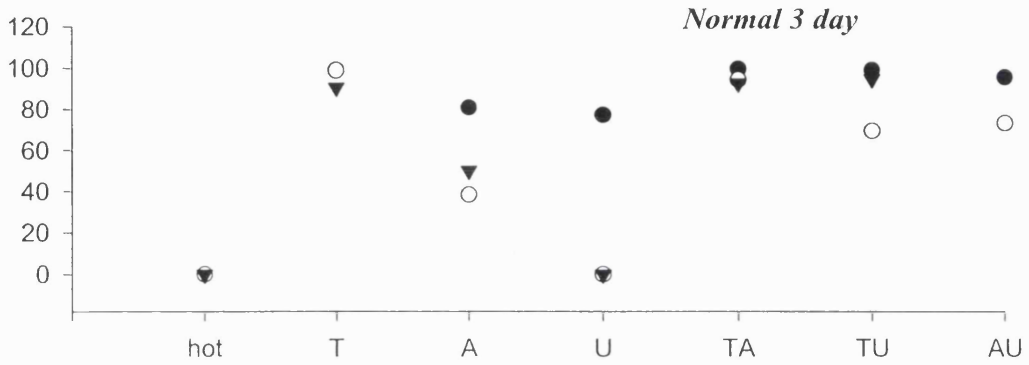


The effect of age and chronic hypobaric hypoxia (50.8kPa CHH) on the specific binding density of 1nM [^{35}S]UTPαSto IPA from porcine lung sections by co-incubating with 100μM UTP.
The binding density increased from birth until 3 days of age. Exposure to CHH for 3 days from birth slightly reduced the binding density when compared to age-matched controls.
Error bars indicate standard deviations (2 animals in each group).

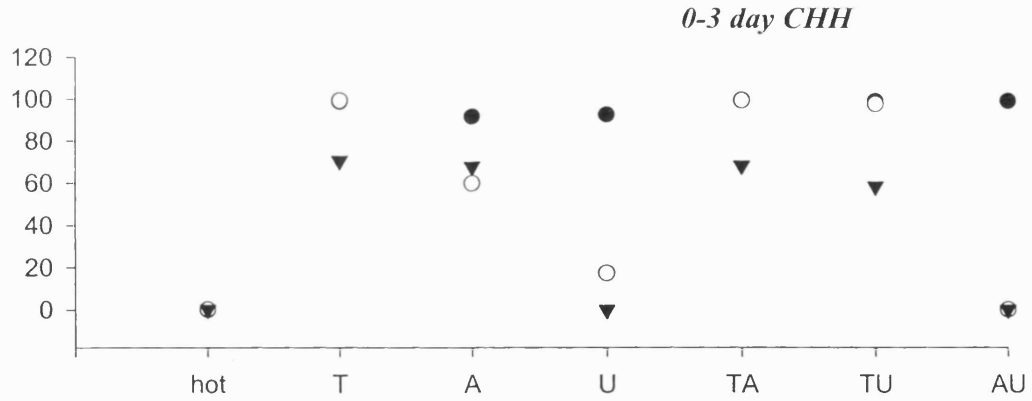
A. % Inhibition of radioligand binding density



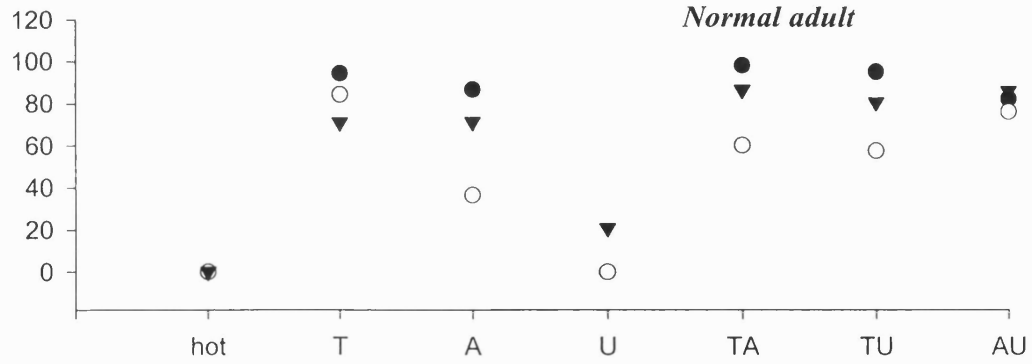
B. % Inhibition of radioligand binding density



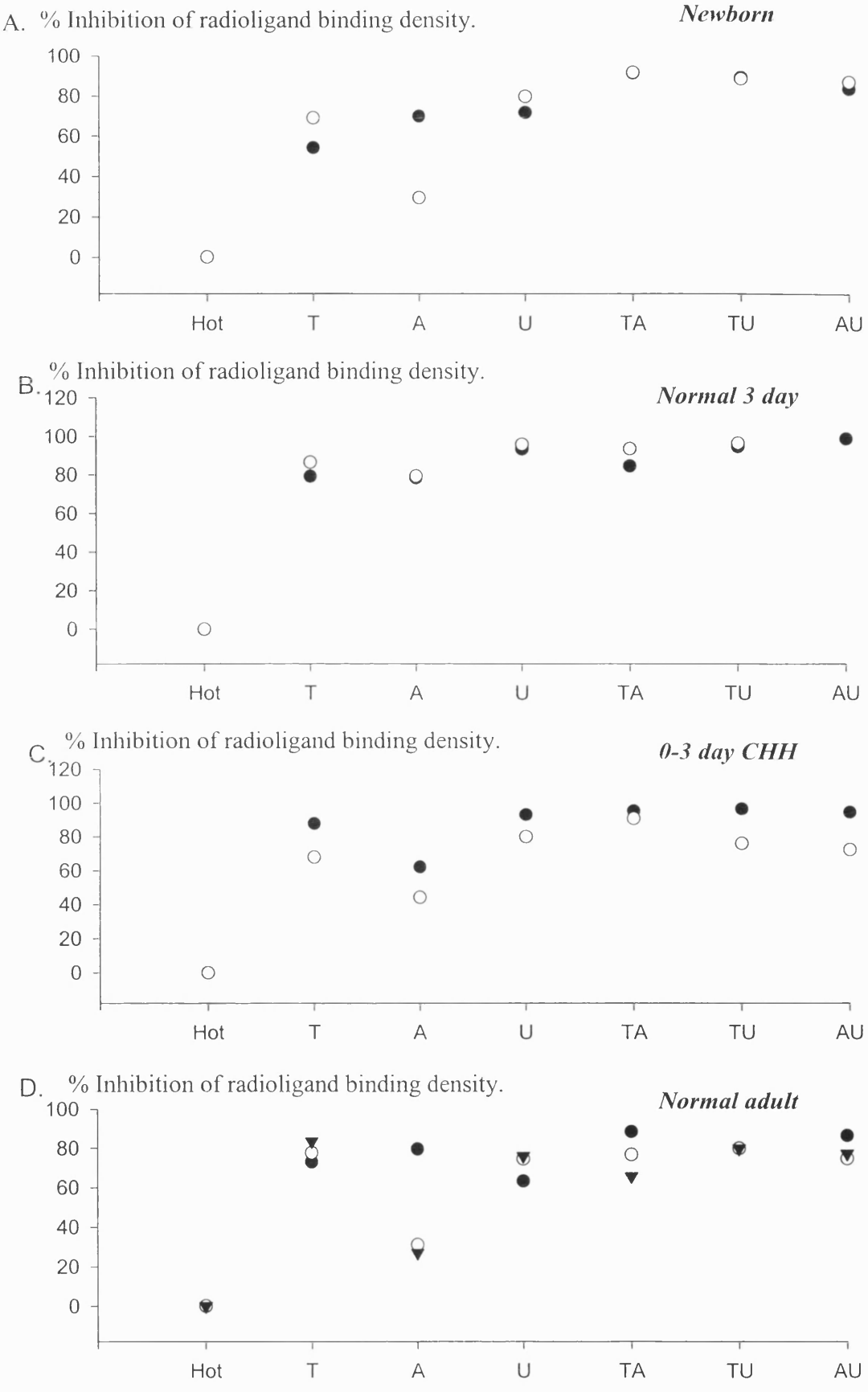
C. % Inhibition of radioligand binding density



D. % Inhibition of radioligand binding density



Vertical scatterplots describing the inhibition of [^{35}S]dATPαS binding by P2-agonists, to porcine conduit IPA, from individual animals in each group. (a) Newborn, (B) normal 3 day, (C) 0-3 day CHH, (D) normal adult. There is some variation within groups but the the rank order of inhibition would indicate binding to a purine P2Y- receptor population in the normal and the pulmonary hypertensive animals.
T, 2-meSATP; A, α,β-meATP; U, UTP.



Vertical scatterplots describing the inhibition of [^{35}S]UTPαS binding by P2-agonists, to porcine conduit IPA, of individual animals within each group. (A) Newborn, (B) Normal 3 days , (C) 0-3 day CHH, (D) Normal adult. There is some variation between animals but the the rank order of inhibition would indicate binding to a non-selective P2-nucleotide receptor population in normal and pulmonary hypertensive animals.
T, 2-meSATP; A, α,β-meATP; U, UTP.

Legend to Fig. 7 Facing page.

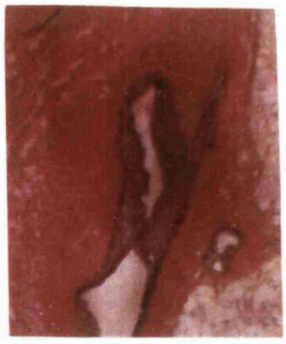
[^{35}S] dATPαS and [^{35}S] UTPαS

Lung tissue from an 8 year old normal child. Consecutive sections were stained with elastin van Geison stain in the first column opposite. Consecutive sections were used for separate radioligand binding studies with either [^{35}S] dATPαS (A) or [^{35}S] UTPαS (B) , and the images on the film were quantified by densitometry (central column of images). To determine the type of binding site binding the radioligand, 1nM of either [^{35}S] dATPαS or [^{35}S] UTPαS were co-incubated with excess [100μM] of non-radioactive P2-ligands and the binding levels analysed from images in the final column.

The binding density was lower with [^{35}S] dATPαS than [^{35}S] UTPαS in contrast to the pig. The binding for both ligands was over the media. [^{35}S]dATPαS was inhibited to the greatest extent by α,β-meATP indicating a P2X-purine receptor binding site. [^{35}S] UTPαS was displaced by all non-radioactive ligands to the same high degree, indicating a P2-nucleotide receptor binding site, as in the pig.

Fig.7

A

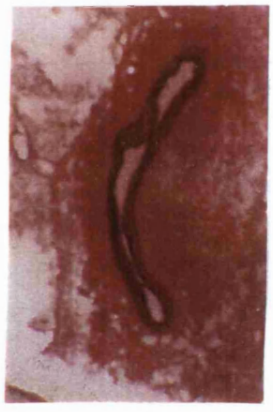


structural photograph

1nM [^{35}S] dcoxyATPαS

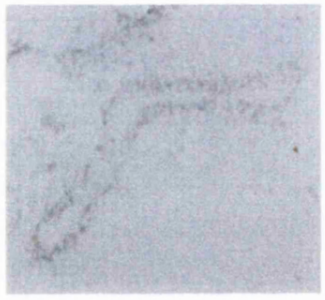
+100 μM α , β -methyleneATP

B



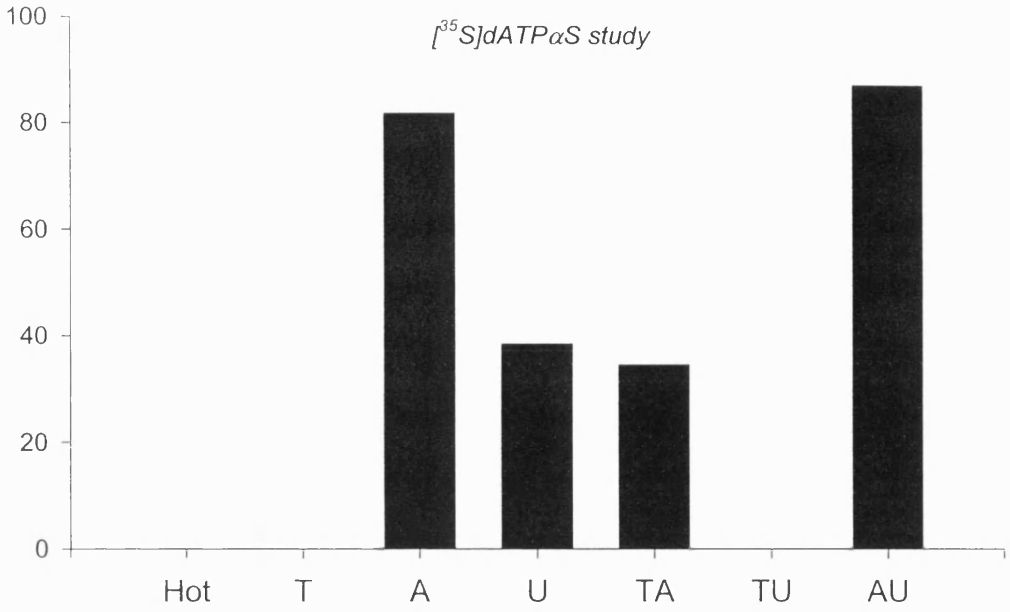
Section stained with van Geisons.

1nM [^{35}S] UTPαS



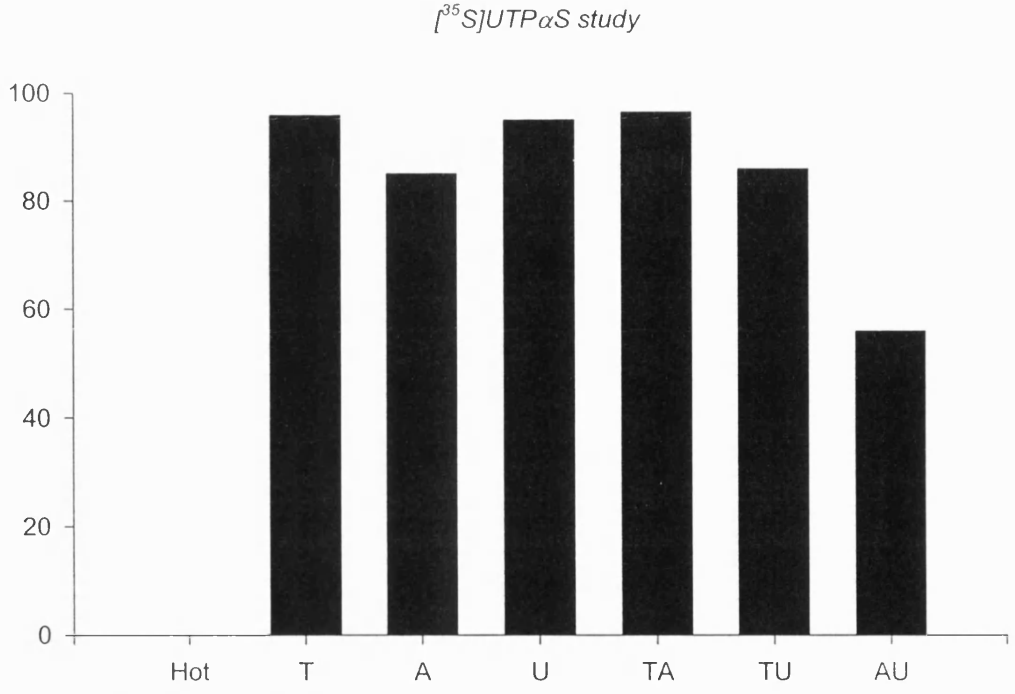
+ 100 μM UTP

A. % Inhibition of radioligand binding density.



A. The inhibition of [^{35}S]dATPαS binding by P2-agonists, in conduit IPA from a normal 8 year old child. The rank order of inhibition suggests a P2X-purine receptor binding site. T, 2-meSATP;A, α,β-meATP; U, UTP.

B. % Inhibition of radioligand binding density.



A. The inhibition of [^{35}S]UTPαS binding by P2-agonists, in conduit IPA from a normal 8 year old child. The rank order of inhibition suggests a non-selective P2-nucleotide receptor binding site. T, 2-meSATP;A, α,β-meATP; U, UTP.

Chapter 8. P2-nucleotide receptor agonist induced responses of intrapulmonary veins from normal and pulmonary hypertensive pigs.

Summary.

This chapter presents preliminary study in the pulmonary venous segment (total of 16 animals and one human baby) which has yielded interesting results and indicates the need for further investigation.

1. Conduit intrapulmonary veins (IPV) from fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs were isolated and mounted with or without endothelium for *in vitro* isometric force recording. Piglets were exposed to chronic hypobaric hypoxia (CHH 50.8kPa) at 14 days of age for 3 days. IPV were isolated from these pulmonary hypertensive animals and mounted for *in vitro* isometric force recording, at resting tone. The IPV from a 36 hour old baby with PPHN were also studied.

2. In younger piglets low concentrations of ATP induced an endothelium-independent relaxation of IPV precontracted by $PGF_{2\alpha}$ [30μM] and transient contractions followed at higher concentrations. The relaxation response did not increase with age. However, removing the endothelium reduced the effect in vessels from older animals.

3. At resting tone cumulative doses of ATP or UTP in produced equipotent contractions in vessels from newborn animals which increased with age, for both agonists. In mature IPV the rank order of contractile potency was α,β-meATP >ATP = UTP. Removing the endothelium did not significantly affect the contractile response induced by any of the agonists at any age.

4. The contractile response to ATP, α,β-meATP and UTP was unchanged after a period of CHH at 14 days of age when compared to responses from normal age-matched animals.

5. The contractile reactivity of the IPV at resting tone, from a baby with PPHN was similar to that seen in the piglets.

6. In summary, the increase in contractile reactivity of IPV with age dominated any changes

in sensitivity of the relaxation response that might be occurring . This would suggest that a reduction in venous resistance does not play a major part in reducing pulmonary arterial pressure in the newborn, but may act to control blood flow through the respiratory units after adaptation to extrauterine life has occurred, and possibly in the presence of pulmonary hypertension. As previously reported for the IPA (see Chapter 6) the contractile response was mediated by separate α,β-meATP and pyrimidine-sensitive receptors, and also appears to be true in the human infant.

Introduction.

Much attention has been focused on the arterial segment of the pulmonary circulation when investigating the fall in PAP during adaptation to extrauterine life. In contrast, the venous segment has received little attention. The established role of the arterial resistance vessels in controlling systemic vascular tone and the passive role by veins would seem to be the basis for this approach {372}. There appears to be a species and age variation regarding the contribution of the different pulmonary vascular segments to total pulmonary vascular resistance. In the adult ferret the IPV contribution has been shown to be greater than in the adult rabbit, but in both species the contribution of IPV was less than that of the IPA and microvessels {332,329}. It has also been shown in the rabbit, but not the ferret, that the IPV contribution was greater in younger animals {331,329}. IPV have a greater basal tone than the IPA, regulated by EDRF, in both the neonatal and adult sheep {379,142}. The relative contribution of each segment of the pulmonary circulation to total pulmonary vascular resistance may change during postnatal life. ACh- induced a greater relaxation of isolated fetal ovine IPV than IPA, although IPV isolated from newborn lambs contracted in response to ACh, relaxing after one week of age {220, 379}. Endothelial cyclooxygenase products partially mediated both the IPV vasodilatation and vasoconstrictor responses. Recent work in our group has shown that porcine IPV display a greater vasodilatation than IPA to ACh {241, personal communication P.J.Boels 1998}.

Little work has been carried out regarding purines in the intrapulmonary veins. ATP has been shown to induce an endothelium-dependent relaxation of precontracted adult rat IPV, classified as a P2Y-purine receptor mediated response due to the high activity of 2-meSATP. In the same study, ATP was found to also induce a contractile response at low tone, acting at P2X-purine receptors, which could be blocked by α,β-meATP {238}. Pyrimidines induced a rise in pressure in the isolated adult rat lung, which was attributed to IPA vasoconstriction despite the technique not allowing assessment of the venous contribution to the effect {351}.

Pulmonary hypertension in the newborn infant is potentially fatal and can result from pulmonary arterial or venous hypertension, depending on the aetiology of the disease {161}. Congenital abnormalities affecting the left side of the heart such as mitral stenosis lead to an increase in pulmonary arterial and venous wall thickness {52}. Thromboxane- and hypoxia-induced pulmonary hypertension of newborn lambs involves venoconstriction {424,330}. Pulmonary veins from adult rat and sheep have been shown to vasoconstrict more than pulmonary arteries to hypoxic stimuli, whilst the response in the adult ferret *in vivo* was shared equally between the IPA and IPV {428,371,333}. In the adult sheep a period of hypoxia was thought to produce an increase in reactive oxygen species and a subsequent increase in vessel reactivity in response to the release of vasoconstricting cyclooxygenase products {371}.

In adult rat IPA, pulmonary vasconstriction induced by acute alveolar hypoxia *in vivo* was not found to be mediated by P2X -receptors {262}. But the reactivity of IPV to P2-nucleotides has not been investigated during adaptation to extrauterine life nor has the effect of chronic hypoxia on IPV been studied in the neonate. Therefore we investigated the venous response to P2-nucleotides during the normal adaptation to extrauterine life in isolated porcine IPV and examined the effect of pulmonary hypertension produced by exposing 14 day old piglets to CHH for 3 days.

Methods.

Materials: intrapulmonary veins from normal fetal piglets (5 days pre-term), newborn piglets (aged <5 minutes - 3 hours), animals aged 3, 6, 14-17 days and adult pigs. Pulmonary hypertension was produced in piglets by exposure to hypobaric hypoxia (CHH) (50.8kPa) from 14 days of age for 3 a day period. Lung tissue was obtained from a 36 hour old baby with PHN , congenital lung dysplasia and hypoplasia (specimen number 3954).

Methods: vessels were isolated and mounted with or without endothelium for *in vitro* isometric force recording. The response to P2-agonists was studied in resting vessels and those precontracted with $PGF_{2\alpha}$ from piglets during normal development and the effect of pulmonary hypertension. For further details of the protocols used please refer to sections 1,2, and 3 of Chapter 2.

Results.

Responses of intrapulmonary veins from normal and PH pigs.

In IPV precontracted with $PGF_{2\alpha}$ [30μM] , ATP induced a relaxation response at low concentrations and at greater concentrations transient contractions followed by a relaxation (Fig.1 A,B). There was no significant difference between the relaxation response of IPV isolated from the young (fetal - 6 days old) or more mature (14 days old - adult) groups (Fig.1 C, Table 1). Removing the endothelium substantially reduced the sensitivity to ATP-induced relaxations in the older animal group (Table 1).

Table 1. Effect of age and removing the endothelium on the relaxation response of IPV precontracted with 30μM $PGF_{2\alpha}$ to ATP.

Group	EC_{50} for Contraction ± sem (n)	% Contraction Emax ± sem (n)
Young : with endothelium	3.5 mM ± 1.4 (5)	100 ± 0 (5)
: without endothelium	1.4 mM ± 0.6 (3)	100 ± 0 (3)
Mature : with endothelium	0.4 mM ± 0.2 (4)	96 ± 4 (4)
: without endothelium	2.3 mM ± 0.5 (2)	100 ± 0 (2)

(n) indicates the number of animals used. Standard deviation given where n=2. Young group = (fetal - 6 days old), mature group = (14 days old - adult).

At resting tone, cumulative doses of ATP induced a series of transient contractile responses in the IPV from normal 3 day old animals (Fig.2). The magnitude and sensitivity of the response in the more mature group (14 days old - adult) of animals was greater than in the younger group (3-6 days old) (Fig.2,5 A) . UTP induced a contractile response in animals as young as 3 days and the magnitude of the response increased with age (Fig.3,5 B). α,β-meATP was not tested in the younger group of animals but it did induce a contractile response at 14 days which increased in the adult (Fig.4). Removing the endothelium had no significant effect upon the contractile response to ATP, UTP or α,β-meATP in a normal adult pig (Fig. 5 C, Table 2). α,β-meATP was 1000 times more potent than UTP and it evoked a greater maximal contraction than ATP. Prior incubation with 100μM α,β-meATP blocked the contractile response to a bolus of α,β-meATP [100μM], but had no effect on ATP or UTP induced contraction, as in the IPA (Fig.6, A,B respectively).

A period of CHH from 14 - 17 days of age had no significant effect on the ATP- or α,β-meATP -induced vasoconstriction, but did reduce the contractile response to UTP (Fig.7 A-C).

Table 2 : The contractile activity of P2-nucleotide receptor agonists in the IPV at resting tone from one normal adult pig.

	EC_{50} Emax With endothelium	EC_{50} Emax Without endothelium
ATP	0.41mM 76.57%	0.51mM 92.66%
UTP	2.37mM 114.96%	1.95mM 143.52%
α,β-meATP	2.71 μM 148.27%	0.724 μM 134.86%

Responses of intrapulmonary veins from a pulmonary hypertensive baby.

Cumulative doses of α,β-meATP, ATP and UTP each-induced a contractile response in IPV with endothelium {Fig.8}. The rank order of potency was α,β-meATP >> ATP = UTP. Preincubation with 100μM α,β-meATP did not abolish the contractile responses to ATP or UTP, but did block further contractions to α,β-meATP.

Discussion.

Several conclusion can be drawn from the limited study carried out to date using conduit porcine intrapulmonary veins. At all ages the sensitivity to ATP for inducing relaxation of the IPV was similar to that seen in neonatal porcine IPA (see Chapter 4). The absence of any increase in the relaxation potency to ATP after birth would suggest that the purine activity in these vessels is not playing a crucial part in reducing the pulmonary pressure immediately after birth.

In the present study, the IPV vasodilatation to ATP at all ages was endothelium-independent , in contrast to the P2Y-mediated relaxation in adult rat IPV which was found to be totally endothelium-dependent {238}.

The large contractile nature of IPV described in the literature was observed here, even in the newborn pig at which age IPA was shown to be unresponsive to ATP (Chapter 6) {220, 371,

379, personal communication P.J.Boels 1998}. It may be that the contractile response at birth, might mask a change in the vasodilatory response at high tone. The contractile response was produced at high concentrations of ATP, but not as high as those required to contract the porcine IPA. This may reflect the small biomass of IPV through which the agonist must diffuse, increasing the efficiency of delivery. The contractions induced by ATP in the IPV appeared to peak and then decline more rapidly than those seen with IPA suggesting that the greater contractile response in the IPV was not caused by a lower degradation activity, and therefore longer agonist availability.

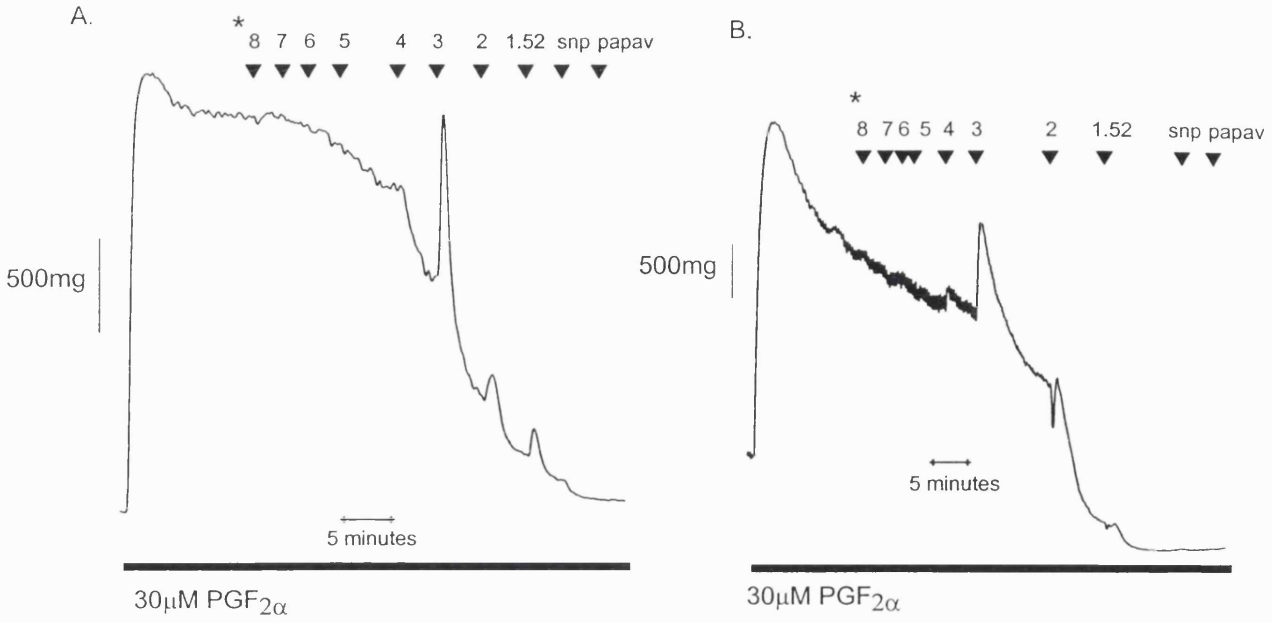
In contrast to the IPA, ATP evoked a contractile response at low tone in the IPV of the newborn. The present data showed that the contractile response of IPV to both ATP and UTP increased with age and that the response to the two agonists was similar at each age. We have not investigated the effect of α,β-meATP in animals younger than 2 weeks of age or the effect of UTP in those younger than 3 days. At low tone α,β-meATP was found to be the most potent contractile agent ($P2X_{1\ and\ 3}$) , and the endothelium had no significant effect upon any P2-agonist tested at any age. The contractile response to α,β-meATP was desensitised by repeated application, indicating $P2X_{1\ and\ 3}$ involvement. Pre-incubation with α,β-meATP did not block the response to ATP or UTP, indicating a different receptor subtype. The $P2X_4$- receptor has been found in the whole lung tissue of the adult rat, responding slightly to α,β-meATP , but not to UTP and probably does not therefore correspond to the receptor mediating UTP-induced vasoconstriction in the perfused adult rat lung{37, 351}.
Some of the findings from the present study differ from those in the only other study regarding the reactivity of adult rat IPA and IPV to P2-agonists {238}. In the rat there was no great difference in the contractile response of IPA and IPV to ATP or α,β-meATP which might suggest a species difference in the vessel contractile structure. In addition, α,β-meATP produced the greatest contractile response in both vessel type, and removing the endothelium increased the IPV response. ATP was equipotent (micromolar) to α,β-meATP and was sensitive to desensitisation by repeated application of α,β-meATP, indicating that a $P2X_{1\ or\ 3}$ - purine receptor mediated the response for both agonists in both vessels. Other than the

species difference, the vessels used in the rat study were main extrapulmonary vessels which may possess different receptor populations than the intrapulmonary segments used in the present study.

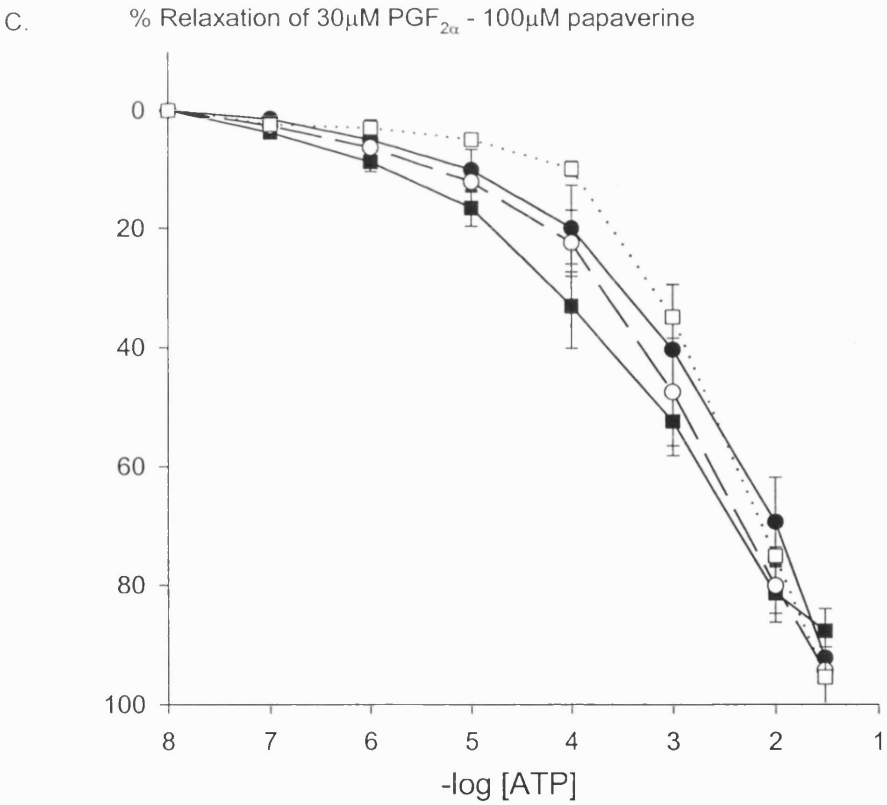
It has been reported that IPV contract vigorously to an hypoxic stimulus, in some studies to a greater extent than the IPA {428,371,330,333}. We investigated the effect of CHH at 14 days of age and found no significant change in contractile response to ATP, UTP or α,β-meATP. Each of these agonists was also effective in evoking a contractile response in the IPV from a 36 hour old baby with PPHN, but without data from the IPV of a normal infant a comment regarding changes in venous reactivity cannot be made.

One could speculate that the venous segment may be involved in regulating pulmonary blood flow in hypoxia because the pulmonary veins are not only passive capacitance vessels in the normal lung, accounting for approximately 20% of the pulmonary pressure. Pulmonary venoconstriction would increase the time spent by the deoxygenated blood in close proximity to the respiratory units, so optimising the gaseous exchange process in an hypoxic environment.

More studies of the IPV, are required to clarify their physiological role in normal adult animals before considering their involvement in neonatal development and pulmonary hypertension.



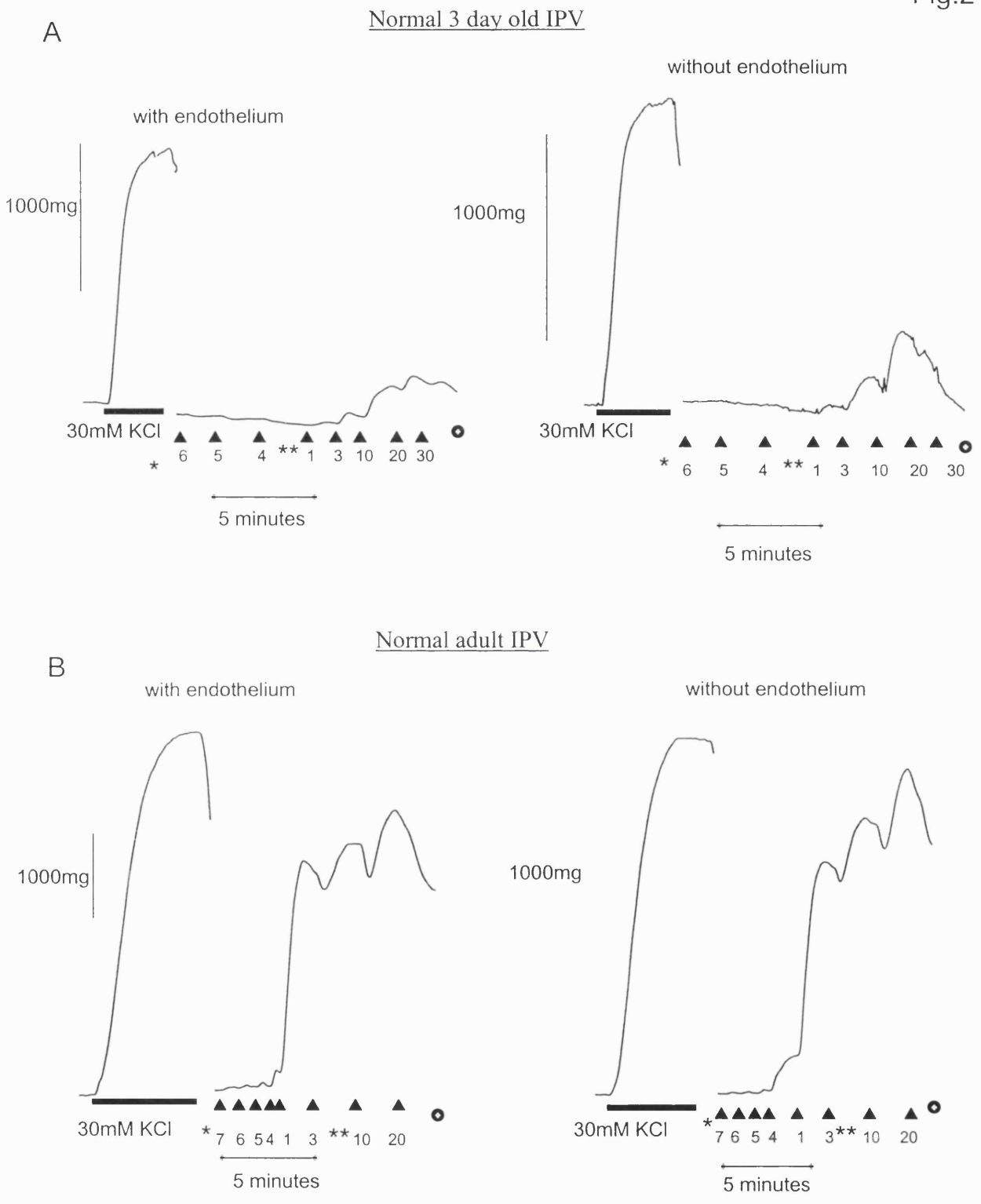
Traces of the response to cumulative ATP added to intrapulmonary veins from (A) normal newborn and (B) 14 day old, mature pig. The vessels have an intact endothelium and have been precontracted with 30μM $PGF_{2\alpha}$. The response showed little change with age.



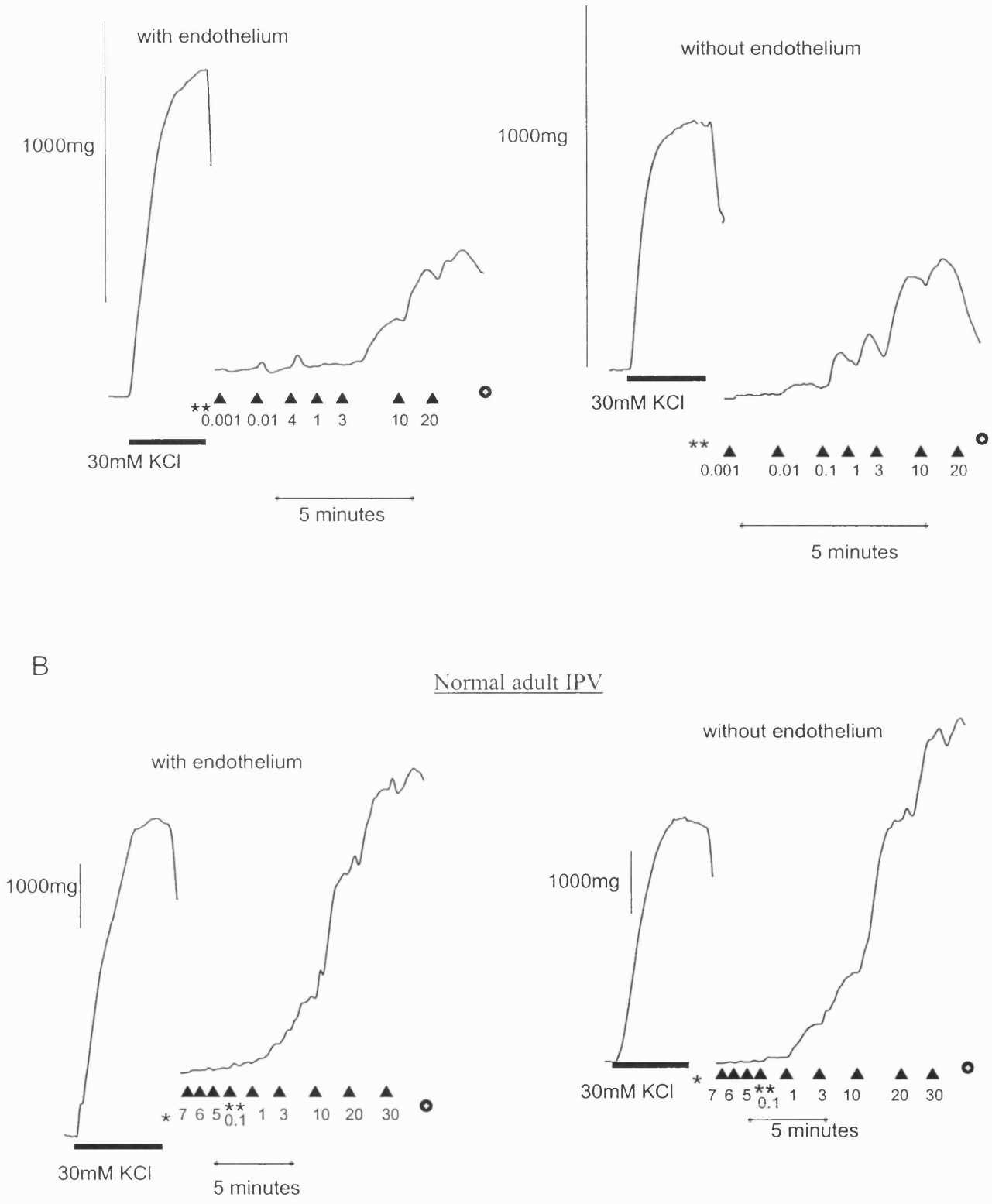
Relaxation response of IPV precontracted with 30μM $PGF_{2\alpha}$, to cumulative doses of ATP. The response was investigated during adaptation in fetuses-6 days old ● (n=5/3) and 14 days old -adult ■ (n=5/2). There was no significant effect of age upon the relaxation response . Removing the endothelium reduced the relaxation of the older age group. Error bars represent standard error of the mean (sem), standard deviation where n=2.

Note : the notation n=4/3 for example indicates that in 4 preparations the endothelium was present (solid symbols) and that for 3 preparations the endothelium had been removed (empty symbols).

Fig.2

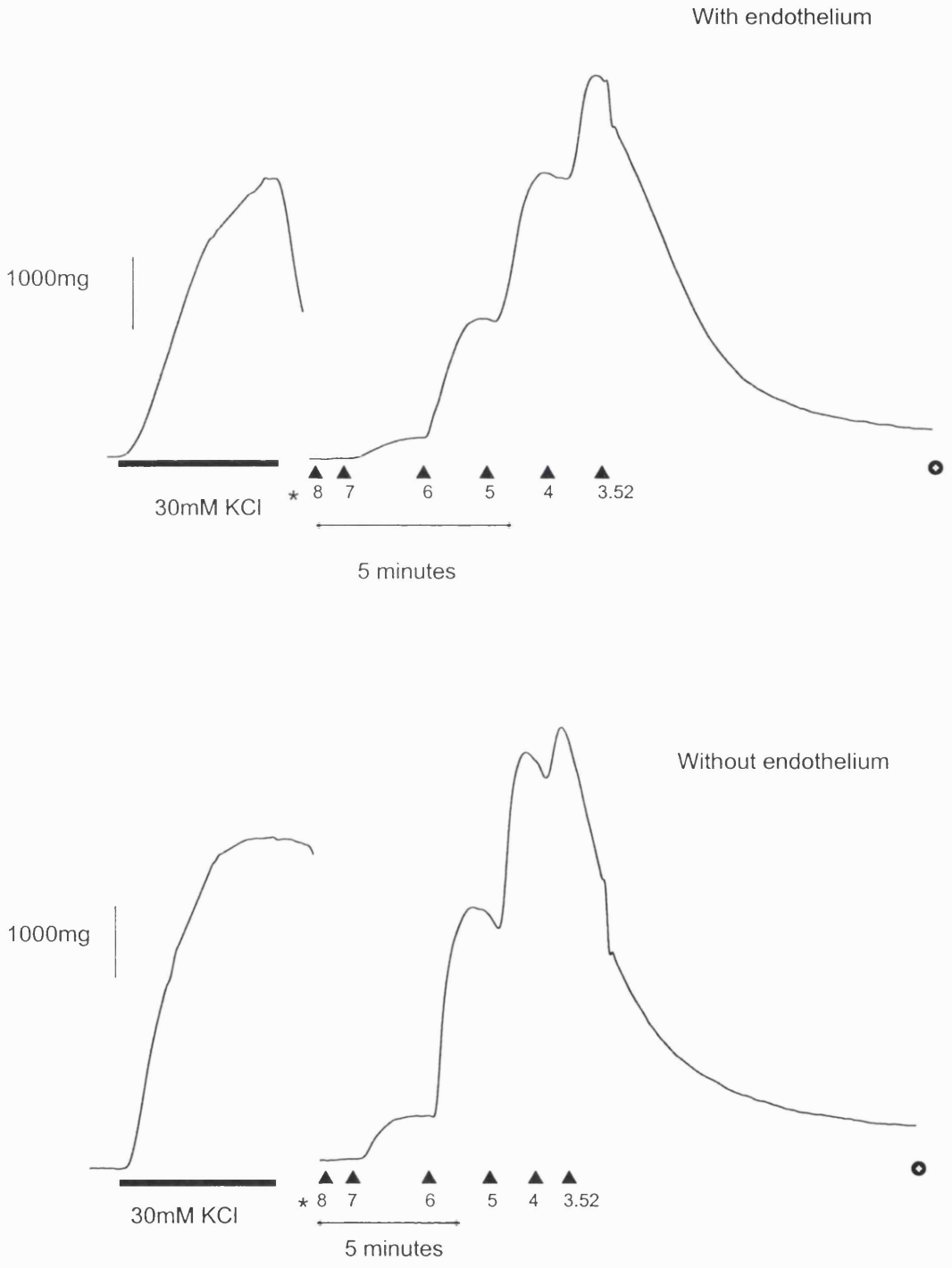


Representative traces of the ATP-induced response of (A) 3 day old normal and (B) adult porcine IPV at resting tone. High concentration transient contractions were recorded which did not differ in IPV with and without endothelium. Tension after 100μM papaverine (✪). * indicates -log values; ** indicates millimolar concentrations.



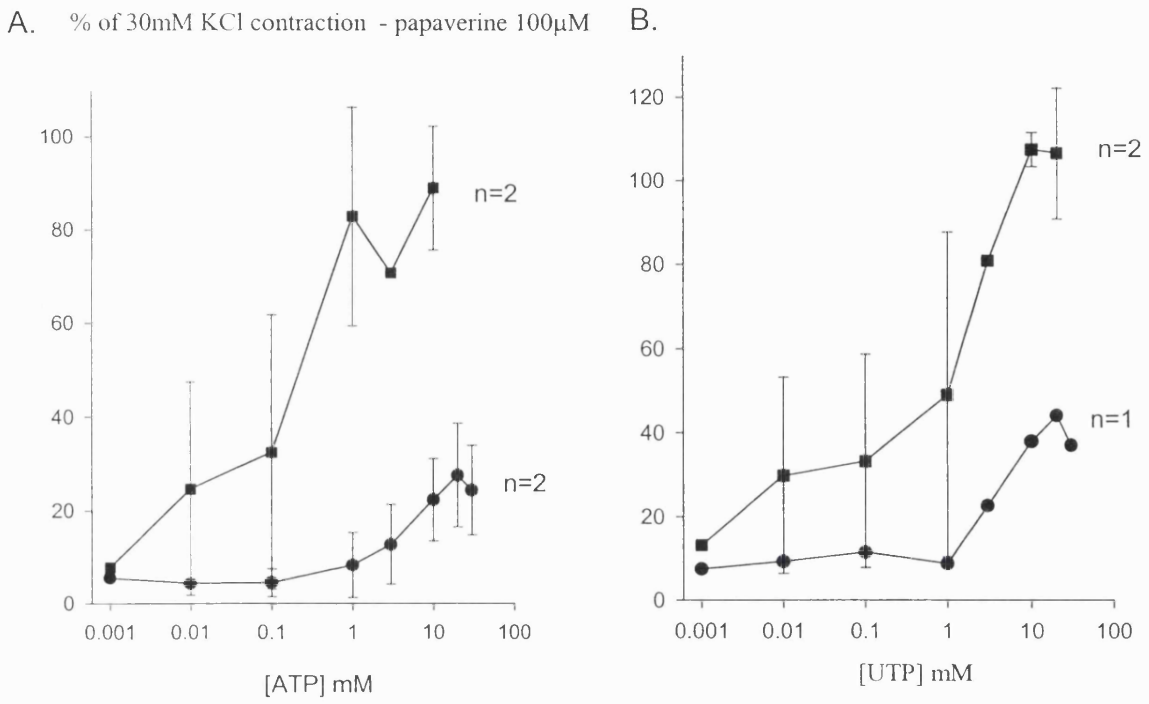
Representative traces of the UTP-induced response of (A) 3 day old normal and (B) adult porcine IPV at resting tone. A set of high concentration transient contractions which were recorded started at a lower concentration in IPV without endothelium. Tension after 100μM papaverine (✪). ** indicates millimolar concentrations.

Normal adult IPV

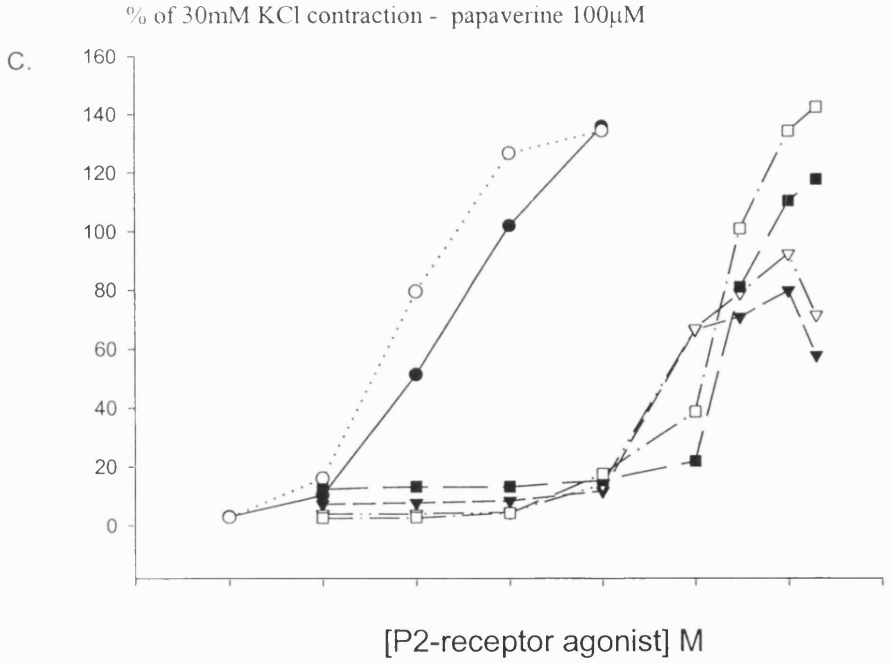


Representative traces of the α,β-meATP-induced response of an adult porcine IPV at resting tone. The contraction response in the IPV was similar in vessels with or without endothelium. Tension after 100μM papaverine (✪).* indicates -log values.

Fig.5



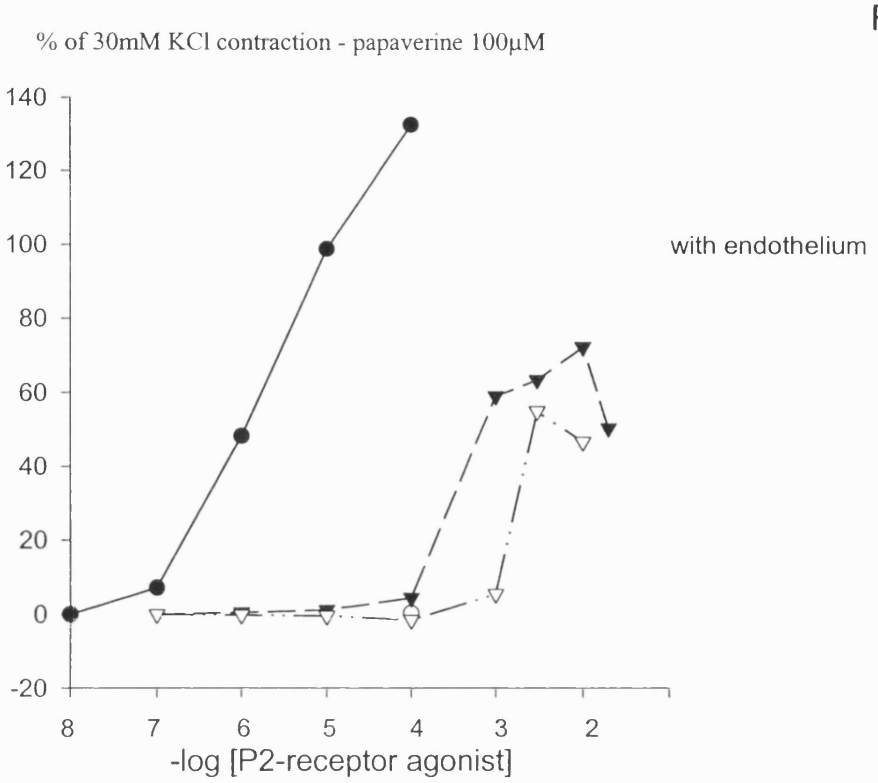
IPV at resting tone showing a contractile response to (A) ATP and (B) UTP of IPV under resting tone, with endothelium. A significant increase in magnitude and sensitivity of response occurred. between the 3-6 days (●) and 14 days -adult (■). Error bars represent the standard deviation .



(C) The contractile response of an IPV with endothelium, to P2-receptor nucleotide agonists from one adult animal under resting tone. α,β-meATP (●) was 1000-fold more potent than UTP (■) which produced a greater maximal contraction than ATP (▼). Removing the endothelium (empty symbols and broken lines) had no significant effect on the responses.

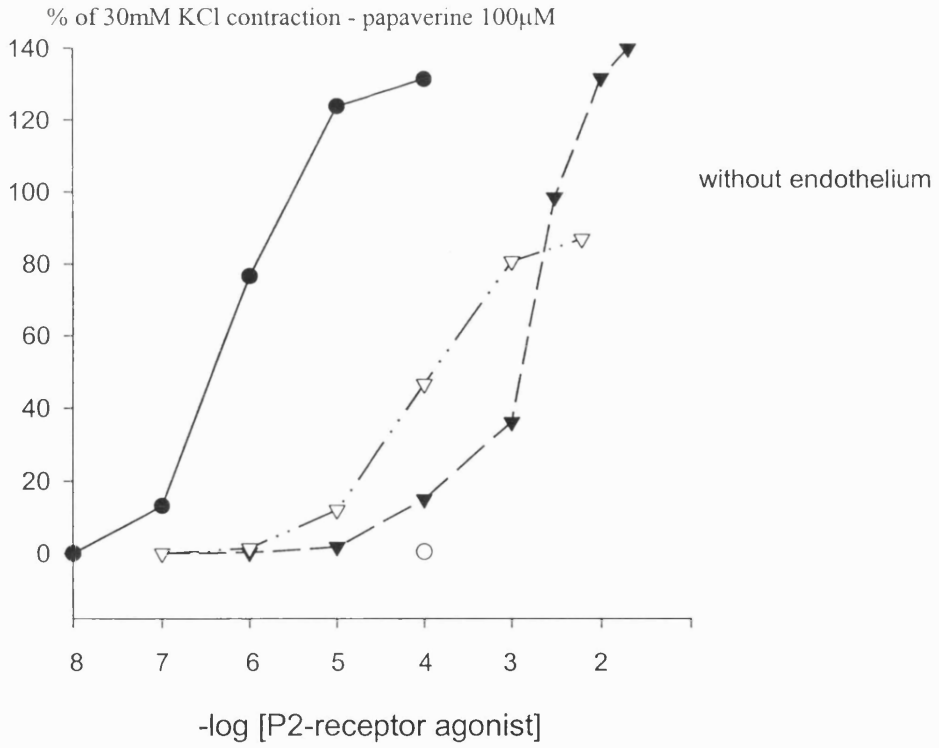
A

Fig.6

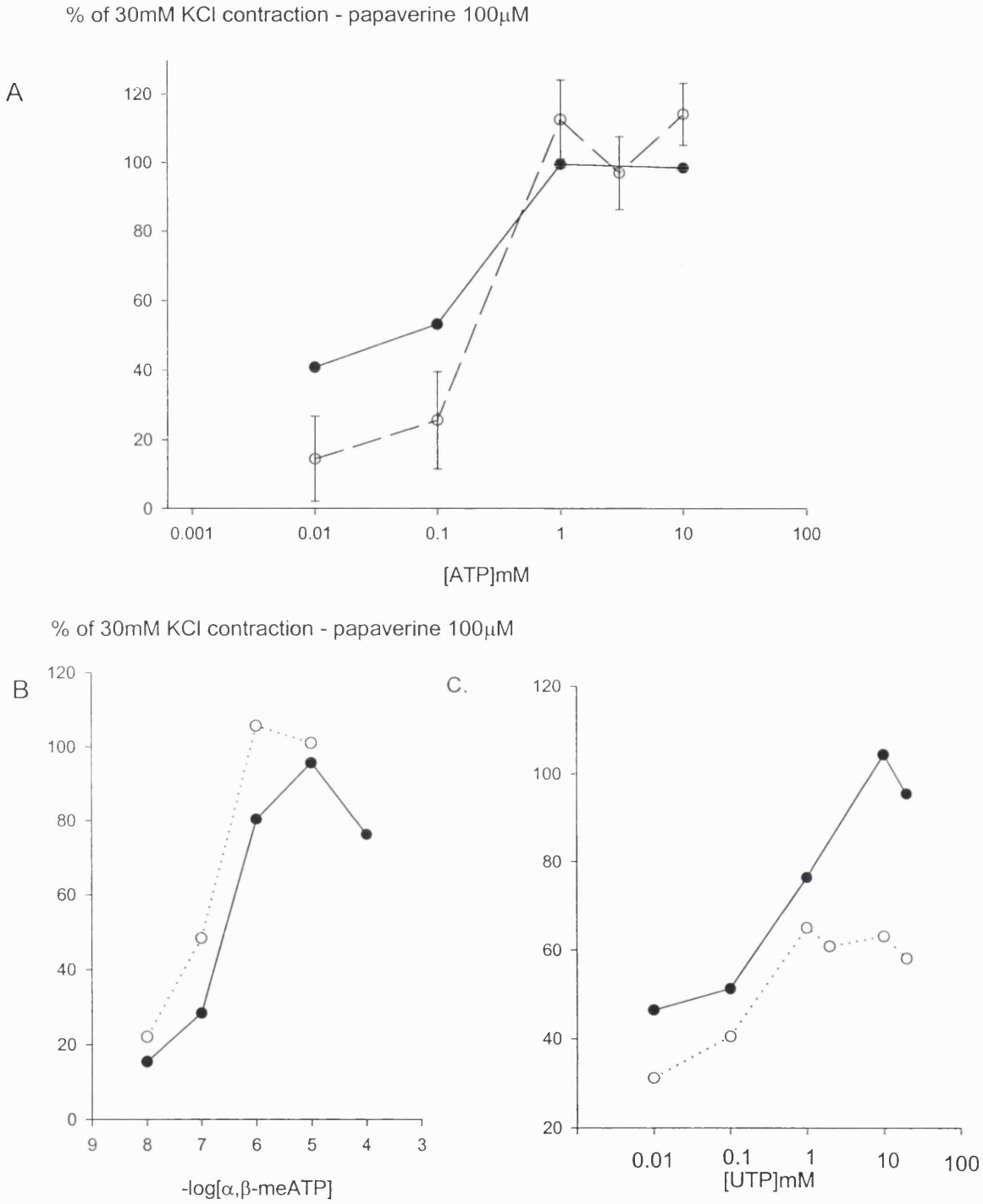


(A) The contractile responses of an IPV with endothelium from one normal adult pig to α,β-meATP (●) and ATP (▼). The effect of repeated application of α,β-meATP on the response to α,β-meATP itself (○) and ATP (▽) is shown.

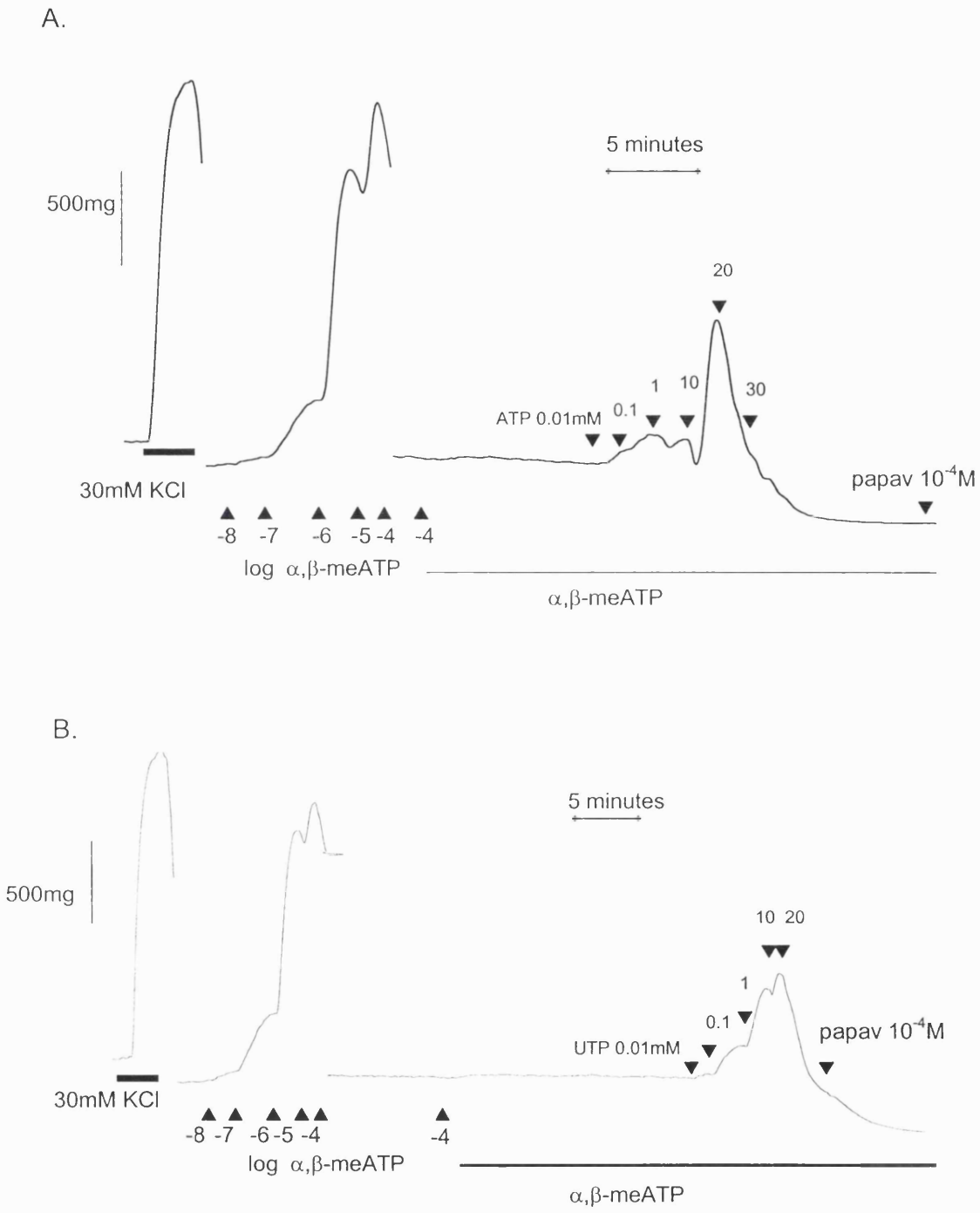
B



(B). The contractile responses of an IPV with endothelium from one normal adult pig to α,β-meATP (●) and UTP (▼). The effect of repeated application of α,β-meATP on the response to α,β-meATP itself (○) and UTP (▽) is shown.



Contractile response of IPVwith endothelium, from one 17 day old piglet which has been exposed to CHH for 3 days at resting tone to cumulative doses of (A) ATP, (B) α,β-meATP and (C) UTP. CHH has reduced themaximal effect of UTP.



Traces of the response of IPV with endothelium isolated from a 36 hour old child with PPHN, to P2-agonists at resting tone. Cumulative additions of α,β-meATP produced the greatest contractile response and blocked the response to a repeated dose after 20 minutes. α,β-meATP did not block the equipotent contractile responses induced by cumulative doses of (A) ATP and (B) UTP.

CHAPTER 9. DISCUSSION

Introduction.

This chapter begins with a brief critical analysis of the methodology used in the present study, before discussing the results of the work. The maturation of the relaxant and contractile responses induced both at rest and at raised tone, the subtype of P2-receptor responsible for these responses assessed by functional and receptor radioligand studies, the effect of chronic hypobaric hypoxia (CHH) and the role of the intrapulmonary veins (IPV) will be addressed. The relation of the observations made concerning the role of purines in the neonatal pulmonary circulation will be discussed in the context of adaptation to extrauterine life and persistent pulmonary hypertension in the human infant.

Critical discussion of the methods used in the present study.

The effects of purines on the pulmonary arterial pressure (PAP) *in vivo* in animals and humans, both immature and mature, have previously been reported {139,352,289,226}. Purine receptors have only been characterised in isolated intrapulmonary vessels from adult animals {238,237}. Therefore only indirect conclusions can be drawn from the literature regarding the site and mechanism of action of purines and pyrimidine within the intact pulmonary circulation.

In the present study, isolated intrapulmonary arteries (IPA) and intrapulmonary veins (IPV) were studied rather than the whole isolated lung preparation or intact animal in order to try and classify the responses in defined segments of the pulmonary vasculature.

Understanding the processes underlying the whole organ response is important when inferring the role of purines and pyrimidines in a physiological process such as adaptation of the newborn circulation to extrauterine life , or in a pathological condition such as pulmonary hypertension. Both of these processes have been traditionally associated with the arterial segment of the pulmonary circulation {159,9,399}. The study of separate vessel segments was a logical starting point in order to understand the reactivity of the whole lung. This

information combined with the knowledge already accumulated about the whole lung responses to various stimuli under different conditions can help determine the possible points of therapeutic intervention likely to have the most beneficial results.

However, there are problems associated with studying isolated vessels. First, the fact that they are *isolated* may alter the reactivity of the blood vessel in such a way as not to represent the *in vivo* situation. Efforts are made to recreate the *in vivo* environment by using pH buffered physiological salt solutions and providing gaseous mixtures, but many interactions cannot be readily reproduced such as those involving the humoral and neural systems, and the signals generated by a whole animal reacting to changes in its environment.

In vivo systemic arteries are exposed to a greater pressure than pulmonary arterial pressure (PAP) {154}. Isolated systemic vessels have been routinely studied for isometric force measurements under a passive tension of 2-5 grams. In order to reflect the lower PAP *in vivo* relative to the systemic arterial pressure, a passive tension of 1 gram was imposed on the porcine IPA. Using the same principle a passive tension of 300mg was imposed on IPV. It is recognised that this process of assigning the resting tone is crude. The process may be improved by determining the passive stretch which reflects the pressure experienced by the vessel *in vivo* . These values have been reported in the literature by this and other groups {154,372}. Therefore in the present study, by imposing the same passive tension on vessels from all groups of animals studied, the results of the study may misrepresent the true *in vivo* situation, which will change with age and in neonates with pulmonary hypertension {41}. However, work by other members of the group has suggested that vessels from both normal and pulmonary hypertensive newborns and normal adult pigs stretched to produce 1000mg of passive tone places the preparations on the plateau of their length-tension curves {personal communication P.J.Boels 1997}. By calculating back from the passive stretch required to obtain a 1000mg tension it has been shown that 1000mgs does represent an *in vivo* PAP of approximately 25mmHg. Taking these findings into consideration, it may be assumed that the conclusions drawn from the results of the present study are valid for looking at changes in vascular reactivity to purines and pyrimidines with age and pulmonary hypertension.

In the present study, the elastic conduit IPA and IPV were studied for two reasons. Firstly, the larger intrapulmonary arteries and veins have both been shown to be involved in adaptation to extrauterine life. The changes in arterial pulmonary vascular tone and remodelling induced by exposure to hypoxia have been shown to occur in the larger vessels, despite having been associated with the pulmonary microvasculature {9,399,349,17,394}. Secondly , on a technical point, isolating small vessels from the neonatal lung is not a simple task and choosing larger vessels allows accurate data to be generated more rapidly.

All vessels were exposed to a 95% O_2 and 5% CO_2 gas mixture in the organ chambers at 37°C. This gas mixture is routinely used in pharmacological laboratories for *in vitro* studies. However, we are very aware that the partial pressure of oxygen *in vivo* may be markedly different from that achieved *in vitro*, particularly for the intrapulmonary vessels. The partial pressure is also different in fetal and postnatal life , and changes on exposure to chronic hypobaric hypoxia.

A bolus of 30mM potassium chloride (KCl) was added to each vessel to assess viability for *in vitro* isometric force measurements. From preliminary work (not shown) the sensitivity of the IPA to KCl did not alter significantly with age, therefore using 30mM KCl as a reference contraction parameter for comparing responses between preparations was thought to be valid. Greater concentrations (≥100mM) of KCl have been used by other workers studying vessels to produce a maximum contraction against which other agonists responses can be compared . However, 30 or 40mM KCl has been routinely used by our group to evoke a significant and reproducible contraction of IPA and IPV from animals of all ages, normal and pulmonary hypertensive {241,399}. 30mM KCl has been used in systemic vessels {66}. In addition it has been found that concentrations greater than 30mM KCl may damage endothelial cell function in some vessels and may release neurotransmitter from nerve endings {45}. But in the present study, a supermaximal concentration of 125mM KCl was used in experiments with human tissue because the first priority was to obtain a response from tissue which we considered might not be of optimal condition.

Repeated application of purines has been shown to desensitise the contractile response mediated by P2X-purines receptor in vascular preparations from the systemic circulation {66}. At the beginning of the present study, non-cumulative additions of purine agonists were used to construct a dose-response at resting tone. However, the errors due to washout artefacts and fatigue of the preparation made this approach inappropriate. Fortunately, transient contractions in the conduit pulmonary vessels were slow to decline compared to those in some systemic vessels, thus cumulative curves could be constructed as previously reported for adult rat and human pulmonary vessels {238,237}. Desensitisation was not a problem in this preparation because only one response curve was produced from each preparation. Bolus additions of agonist were used in experiments with human tissue, again to maximise the likelihood of seeing a response in tissue of uncertain quality.

P2Y-receptor antagonists such as suramin, PPADS, reactive blue, were not used in the present study, although this might be considered theoretically advisable . Firstly, the priniciple aim of the study was to determine the reactivity to P2-receptor agonists of the pulmonary vessels which allowed classification by rank order of potency. Secondly, P2-receptor antagonists are still repeatedly found to have non-specific properties which render conclusions from their use difficult {56} and lastly, the scarcity of tissue would not allow for "screening" of possible candidate antagonists.

Radioligand binding was done to investigate the distribution of receptor mediating the vasodilator reponses recorded in the organ chambers, and the apparent endothelial-independence of the responses. From the literature regarding radioligand binding studies, it seemed sensible to use an analogue of the physiological agent. In addition, an agent which had increased stability to hydrolysis during the incubation period would be advantageous. To investigate the relaxation receptor [^{35}S]ADPβS was considered following the satisfactory outcome of *in vitro* studies, but was suggested to also bind at a P2Y-receptor with pyrimidine-affinity {personal communication from Barnard group}. [^{35}S]deoxyATPαS was

chosen after work published by Webb *et al* showing that the ligand bound to the $P2Y_1$-purine receptor expressed in xenpous oocytes and turkey erythrocytes {413,276}. The specificity of this ligand has recently been brought into question by a report of [^{35}S]deoxyATP αS binding to high affinity, non-P2Y receptor binding sites on 1321N1 and Cos-7 cell membranes{362}. In the present study non-radioactive [^{35}S]deoxyATPαS was not used to inhibit radiobinding, but instead we used agents which encompassed the P2X-, Y- and pyrimidine preferring- receptor subtypes and which had also been used in organ chamber studies. These were used separately and in combination to identify any cross-reactivity of receptor-populations.

[^{35}S]UTPαS was used to investigate the receptors mediating the potent UTP-induced contractions. It was hoped that the chemical derivative would have increased stability compared to UTP, which has been found for the equivalent ATP derivative.

[^{3}H]α,β-methyleneATP was not used in the radioligand binding study because both α,β-methyleneATP and 2-meSATP had been reported to inhibit binding to IPA in lung sections of adult cat and human , indicating a lack of radioligand specificity {34,292}.

Discussion of results from the present study.

Introduction.

In this section the conclusions which have been reached from the each part of the study will be discussed and related to each other, to assimilate all the *in vitro* data and apply it to an *in vivo* environment. The role of purines in normal adaptation of the porcine lung to extrauterine life and in pulmonary hypertension of the newborn pig is discussed. The relevance of the findings in the porcine model to the human infant and the possible clinical implications will be addressed.

Summary of the results from the pharmacological study.

The present study has shown that ATP will dilate both IPA and IPV at increased tone and IPA at low tone in newborn porcine vessels. The relaxation of both types of vessel was endothelium-independent and was shown to be mediated via a $P2Y_1$-purine receptor in the IPA at raised tone, by rank order of P2-agonist potency. The relaxation of the IPA increased with age, but this was not seen in the IPV. The relaxation was not mediated by cyclooxygenase products in the newborn IPA. The number of $P2Y_1$-purine receptors increased during the first 3 days of life as indicated by the increased [^{35}S]deoxyATPαS binding. Therefore an increase receptor number could account for the increase in relaxation after birth, but not that seen after 3 days of age. The increased IPA response with age may be linked to the maturation of a hyperpolarising K^+-channel mechanism. UTP was a poor dilator at high tone in the mature IPA, which does not support the concept of there being of a pyrimidine-preferring receptor, such as $P2Y_{2,4,6}$ -receptor.

UTP was found to be the dominant contractile agonist in the IPA at resting or raised tone. The contractile response to both ATP and UTP gradually increased with age. If it is assumed that [^{35}S]UTPαS bound to a contractile pyrimidine-preferring receptor, the increase in binding seen with age may account for the increase in UTP-induced contraction with age. Only the

UTP-induced contractile response was inhibited by EDRFs in the IPA, suggesting that UTP has a greater involvement in physiological regulation of IPA tone than ATP. The α,β-meATP contractile response was poor and only significant in the adult IPA, indicating the presence of an ineffective P2X $_{1or3}$-purine receptor.

However, the contractile response of IPV to each P2-agonist was much greater than that of the IPA. This would agree with the general observation that IPV contract more than IPA. The IPV contractile responses to α,β-meATP were produced in the same micromolar range as found for the IPA, indicating that the same P2X $_{1or3}$-purine receptors may be responsible. The increased response could be due either to a greater receptor number in the IPV than IPA, or the receptors of the IPV have a greater efficacy. The contractile response to both ATP and UTP did not appear to be mediated by P2X $_{1or3}$-purine receptors in either the IPA or the IPV. It would appear that ATP and UTP operate at a different receptor to α,β-meATP, based on the lack of desensitisation by α,β-meATP and the different concentration ranges of activity in both IPA and IPV.

After exposure to chronic hypobaric hypoxia, the rank order of relaxation response to P2-agonists for porcine IPA was the same as that in the normal animals indicating a $P2Y_1$-mediated response. The endothelium-independent nature of the $P2Y_1$- vasodilatation in the normal pig may confer some resistance to damage by CHH. The contractile response to ATP and UTP was reduced in IPA from newborn animals exposed to CHH. The reduction in density of [^{35}S] UTPαS binding to the inner media could account for the decrease in ATP- and UTP-induced contractions, if both agonists act at the same receptor. The contractile response to ATP, α,β-meATP and UTP was not significantly altered in IPV from animals exposed to CHH at 14 days of age for 3 days.

In IPA from children, both normal and pulmonary hypertensive, a P2Y-mediated endothelium-dependent vasodilatation response was found. The exception to this general finding was the endothelium-independent relaxation to ATP of one normal child. This could indicate metabolism of ATP to adenosine acting on the smooth muscle, while the more

resistant ATP-analogues act at endothelial P2Y-receptors. The relaxation response appeared to be greater in younger children, whether they were normal or pulmonary hypertensive. This may be due to the greater reactivity of immature human pulmonary vessels. In addition, the responses may be greater in children who have been pulmonary hypertensive for a shorter period of time, when the structural remodelling may not be so severe and the vessels can still react normally. The IPA at resting tone from a baby with PPHN displayed the same lack of response to α,β-meATP and biphasic response to ATP as was seen in the neonatal piglets.

Contractile responses were seen with ATP, UTP and α,β-meATP in the IPA from a normal and a pulmonary hypertensive 4 month old child. ATP and UTP appear to operate at a different receptor than α,β-meATP the rationale being the same as for that presented for the porcine IPA.

A rank order of α,β-meATP >> ATP = UTP was demonstrated in the IPV of a baby with PPHN at resting tone. A further similarity with the porcine IPV fndings was that the repeated application of α,β-meATP did not abolish the responses to either ATP or UTP.

Application of the findings to normal adaptation to extrauterine life in porcine pulmonary vessels.

The findings from the present study suggest that ATP could induce a vasodilatation of IPA and IPV under high tone by an endothelium-independent mechanism in the fetal and newborn animal. It did not appear that ATP induced vasodilatation was triggered by birth because the fetal and newborn IPA and IPV responded in a similar way. The trigger is more likely to be an increase in oxygen tension at birth accompanied by mechanical stretch of the vasculature. The PAP falls over the first few days of life as the NO pathway and probably other pathways mature. The present *in vitro* data indicate that ATP could take part in relaxing IPA even at the low, resting tone found in the normally adapted neonate. The absence of an increasing postnatal relaxation response to ATP of the IPV would indicate that changes in IPV reactivity to purines does not play a part in the postnatal adaptation to extrauterine life. The importance of the part played by purines during this time period may be indicated by the parallel increase

in P2Y-purine receptor binding sites on the inner media of the IPA at 3 days and that plasma levels of ATP have been shown to increase during birth in lambs {226}. The dominant vasodilatation response mediated by P2Y-purine receptors may indicate that ATP acts to oppose the maturing constrictive capability of the IPA to maintain a low PAP. This may be linked to the gradual shift from a predominantly synthetic to a contractile phenotype of the IPA smooth muscle cells which occurs for many weeks after birth {8,241,233}. The contractile response to ATP and UTP was found to be relatively poor in the normal newborn when compared to the adult animal response. This would suggest that P2-contractile receptors are not involved in maintaining a high fetal PAP. The maturation pattern of the contractile response would suggest a role for ATP and UTP acting via P2-receptor types, in regulating the IPA tone in mature animals. The contractile reactivity of the IPV could be analogous to the systemic arteriole, regulating blood flow of leaving the repiratory units. The *in vitro* findings would support the view that ATP may be involved in the normal postnatal fall of PAP which appears to occur, as has always been thought, in the arterial segment of the pulmonary circulation.

Application of the present findings to chronic hypoxia-induced pulmonary hypertension of the newborn pig.

The arterial segment of the pulmonary circulation is the site of the structural remodelling by chronic hypoxia which leads to a sustained increase in PAP {172,9}. The *in vitro* findings in the present study have demonstrated that ATP can dilate IPA from animals exposed to CHH. The response was mediated via a $P2Y_1$-receptor through an endothelium-independent mechanism . The response was slightly reduced compared with that in the normal animal. This would appear to be due to a reduced P2Y-purine receptor density on the inner media of the IPA. As the contractile responses to both ATP and UTP were reduced in the IPA following chronic hypoxic exposure, the resultant reactivity shifted towards vasodilatation, suggesting that the vasoconstriction underlying CHH-induced PH is not due to an augmented P2-receptor contractile response. The decrease in contractile response may attributed to a reduction in receptor number if [^{35}S]UTPαS binds to a contractile receptor. Alternatively,

CHH-induced -remodelling may have raised the intrinsic tone in the IPA, creating a vessel with a similar configuration as a vessel precontracted with $PGF_{2\alpha}$. The theory of a tone-dependent dual response to P2-agonists would therefore be predicted in this instance to lead to an increase in relaxation.

The venous, rather than the arterial, segment of the pulmonary circulation is better positioned for monitoring the oxygen levels in the blood stream as it carries blood leaving the respiratory units. The strong hypoxic acute venoconstriction consistently reported in the literature would reduce the rate of blood flow from the site of gaseous exchange and thus optimise the oxygen saturation of the blood {428,371,330,333}. However, at the same time a back pressure would be exerted on the arterial segment which would induce structural vascular remodelling and smooth muscle hypertrophy that is characteristic of the chronic hypoxic disease state. It might be predicted from the literature that CHH-induced PH would not have an effect on the venous segment of the pulmonary circulation, due to the buffering effect of the preceding microvessels. This would be compatible with the findings in the present study, where no change in reactivity was seen in IPV from PH neonatal piglets, suggesting that in this respect they function in a similar manner in acute and chronic hypoxia.

Clinical relevance of the present findings and possible implications.

In the present study ATP dilated the isolated IPA from neonatal and adult pigs by an endothelium-independent mechanism, via P2Y-purine receptors. The relaxation response of IPA was largely resistant to the effects of CHH in pulmonary hypertensive animals. In the present study it is postulated that this may be due to an absence of EDRF involvement, because the response to EDRF has been shown to be reduced in the human pulmonary hypertensive patient {399, 119}. The observation that the P2Y-mediated relaxation in IPA isolated from human infants was endothelium-dependent raises the possibility that the response may also be lost in the pulmonary hypertensive child. However, this was not so. The P2Y-mediated response appeared to operate in IPA from children with pulmonary hypertensive with different aetiologies. However, in some of these children the ACh-induced

endothelium-dependent relaxation was not abolished either. In one normal child the vasodilatation to ATP itself was endothelium-independent, which may indicate metabolism to adenosine. Finding that both endothelium-dependent and -independent P2Y-relaxation responses are resistant to CHH, suggest that the resistance to CHH lies not in the type of cell on which the P2Y-receptor resides but in the pulmonary IPA receptor itself. The current therapies of nitric oxide inhalation and prostacyclin are found wanting in some instances of neonatal pulmonary hypertension and a P2Y-agonist might be more successful.

ATP-infusions have been used in humans with various cardiovascular abnormalities {145,22, 164,109,92,23}, with no ill effect. The concentration of ATP required to induce a fall in PAP has also been shown to be safe in experimental *in vivo* human studies {139,140,141,344,284}. ATP has not been reported to produced any of the unwanted side-effects associated with another vasodilator tolazoline, such as nausea and tachycardia, which reduce clinical use in infants {54}. Also, a large concentration difference between the ATP-induced pulmonary and -systemic hypotensive effects exists in both animal and human studies. The few reports documenting the ability for ATP to reduce the raised pulmonary arterial pressure in infants experiencing a pulmonary hypertensive crises and adults during bypass operations in combination with the present study will hopefully be the starting point for a move for more common clinical use {54,254}. In contrast to vasodilators such as sodium nitroprusside, the effects of ATP have been demonstrated to be rapidly reversed {136,167}. Given acutely, ATP would be a suitable vasodilator drug for assessing the pulmonary reactivity of patients awaiting intracardiac surgery during cardiac catherterisation. For longer term treatment, the aim must be to halt or reverse pulmonary vasoconstriction and structural remodelling and a P2Y-purine agonist with a longer active life in the circulation may have more therapeutic potential, such as ADPβS.

Future experimental work indicated by the present studies.

The present study could be extended :

(a) To further compare interspecies differences between the pig and human pulmonary vasculature. The availablity of normal and diseased human tissue will be a rate limiting factor.

(b) It will be advantageous to investigate porcine and human systemic vessels in order to determine the effect of various P2-agonists of interest in the different vascular beds. Such work could be extended extended to *in vivo* porcine studies to determine the pulmonary selectivity of P2-agonists and the ability of stable analogues to reverse pulmonary hypertension, whilst at the same time being slowly metabolised. This could be essential before clinical use of P2Y-receptor analogues could be contemplated as an alternative to labile ATP as an infusion. ADPβS retains activity (transient P2Y-mediated insulin secretion response) after oral administration in animals which would be an advantage clinically {183}.

(c) The radioligand binding studies should be extended to include lung tissue from more children, normal and with pulmonary hypertensive . It would be advantageous to develop the use of tissue sections dipped in photographic emulsion (*dipped slides*) to allow greater qualitative analysis. The distribution of ligand binding in other lung structures will shortly be analysed, in the airways and veins, to obtain a more complete profile of the binding sites recognised by the radioligands chosen in the present study of lung development.

(d) To further investigate the transduction pathways for both relaxation and contraction of intrapulmonary arteries with development and exposure to chronic hypobaric hypoxia. Precontracting IPA with potassium chloride removed the increase with age of ATP-induced relaxation seen with $PGF_{2\alpha}$ precontraction, suggesting the involvement of K^+-channels in the maturation of the relaxation response. The dependence of the relaxation response to the precontractile agonist may be one avenue of investigation.

(e) The response to electrical nerve stimulation at raised tone should be investigated with respect to purines acting at P2Y receptors on the smooth muscle. Purine nucleotides are released from nerve terminals in the mature pulmonary artery {112,373}.

(f) ATP and UTP are potent mitogens of vascular smooth muscle {126,337,194,252}. They are also released from the perivascular sympathetic nerves of the intrapulmonary artery, but there are few reports of post-junctional purinergic activity in this type of vessel. Neonatal pulmonary hypertension has been found to involve vascular remodelling including smooth muscle proliferation, and accelerated sympathetic innervation {172,10}.

Final statement

The aim of the present study was to investigate whether the *in vitro* responses mediated by P2-nucleotide receptors were relevant to the adaptation of the fetal pulmonary circulation to an extrauterine environment. The pathological role of these receptor in pulmonary hypertension of the newborn was also to be assessed.

In the present study, it has been shown that the arterial-segment of the porcine and human pulmonary circulation favours vasodilatation rather than vasoconstriction, through a P2Y-purinoceptor. The relaxation response was endothelium-independent in the porcine IPA $P2Y_1$-receptors which were identified on the smooth muscle by radioligand binding . The relaxation response was resistant to PHN in both species, despite the endothelium-dependence of the P2Y-relaxation response in human IPA.

The findings indicate that the porcine IPV do not play a part in the fall of PAP at birth, but could be involved in the pulmonary blood flow under normal and hypoxic conditions.

A population of contractile high affinity α,β-methylene ATP-sensitive $P2X_1$- or $_3$- receptors is present on the porcine IPA , IPV and the human IPA. However, the greatest contractile effects in the IPA of both species were induced by UTP and ATP at a greater concentration

range, probably through a different receptor. From current nomenclature a pyrimidine preferring P2Y-receptor, such as the $P2Y_4$, is mediating the UTP-induced contractions {259}. Thus, *in vitro* findings from the present study would support previous *in vivo* reports of purines, that infusion of a P2Y-agonist will tend to cause vasodilatation of a high pressure pulmonary circulation such as that found at birth or in persistent pulmonary hypertension of the neonate.

As ATP infusions are already used for management of various clinical conditions in the USA, Russia and Japan, including successful treatment of neonatal pulmonary hypertension, in light of the present study it may be time to investigate the use of P2Y-agonists in hospitals in the UK.

References:

1. Abbrachio.M.P., Burnstock.G. (1994). Purinoceptors : are there families of P2X and P2Y purinoceptors. *Pharmacol Ther.*, 64, p445-475.
2. Abman.S.H., Chatfield.B.A., Rodman.D.M., Hall.S.L., McMurty.I.F. (1990). Diminished endothelium-derived relaxing factor (EDRF) release by fetal pulmonary arteries in vitro. *Pediatr Res.*, 27 , 351A.
3. Abman.S.H., Chatfield.B.A., Rodman.D.M., Hall.S.L., McMurtry.I.F. (1991). Maturational changes in endothelium-derived relaxing factor activating of ovine pulmonary arteries in vitro. *Am J Physiol.*, 260 (4 pt 1), L280-L285.
4. Adatia.I., Barrow.S.E., Stratton.P.D., Ritter.J.M., Haworth.S.G. (1994). Effect of intracadiac repair on biosynthesis of thromboxane A_2 and prostacyclin in children with left to right shunts. *Br Heart J.*, 72, 452-456.
5. Akbar.G.K.M., Dasari.V.R., Webb.T.E., Ayanathan.K., Pillarisetti.K., Sandhu.A.K., Athwal.R.S., Danile.J.L., Ashby.B., Barnard.E.A., Kunapuli.S.P. (1996). Molecular cloning of a novel P2 purinoceptor from human erythroleukemia cells. *J Biol Chem.*,271(31), 18363-18367.
6. Alexander.S.P.H., Ford.A.P.D.W. (1996). Purines '96. *TIPS.*, V17, No.11, 385-388.
7. Allen.K.M., Haworth.S.G. (1986). Impaired adaptation of pulmonary circulation to extrauterine life in newborn pigs exposed to hypoxia : an ultrastructural study. *J Pathol.*, 150, 205-212.
8. Allen.K., Haworth.S.G. (1988). Human postnatal pulmonary arterial remodelling : ultrastructural studies of smooth muscle cell and connective tissue maturation. *Lab Invest.*,59,702-709.
9. Allen.K.M., Haworth.S.G. (1989). Cytoskeletal features of immature pulmonary vascular smooth muscle cells : the influence of pulmonary hypertension on normal development. *J Pathol.*, 158, 311-317.
10. Allen.K.M., Wharton.J., Polak.J.M., Haworth.S.G. (1989). A study of nerves containing peptides in the pulmonary vasculature of healthy infants and children and those with pulmonary hypertension. *Br Heart J.*, 62, 353-360.

11. Allison.D.J., Stanbrook.(1980). A radiological and physiological investigation into hypoxic vasoconstriction in the dog. *Invest Radiol. 1979 George Simon memorial fellowship award,* 15 (3), 178-190.

12. Altiere.R.J., Olson.J.W., Gillespie.M.N. (1986). Altered pulmonary vascular smooth muscle responsiveness in monocrotaline-induced pulmonary hypertension. *J PET.*,236 (2), 390-395.

13. Axellson.J., Holmberg.B. (1969). The effect of extracellularly applied ATP and related compounds on electrical and mechanical activity of the smooth muscle taenia coli from the guinea-pig. *Acta Physiol Scand.,* 75, 149-156.

14. Ayyanathan.K., Webb.T.E., Sandhu.A.K., Athwal.R.S., Barnard.E.A., Kunapuli.S.P. (1996) . Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. *Biochem Biophys Res Commun.*, 218, 783-788.

15. Badesch.D.B., Orton.E.C., Zapp.L.M., Westcott.J.Y., Voelkel.N.F., Stenmark.K.R. (1989). Decreased arterial wall prostaglandin production in neonatal calves with severe chronic pulmonary hypertension. *Am. J. Respir. Cell. Mol. Biol.*,1 (6), 489-98.

16. Bao.J.X., Eriksson.I.E., Stjarne.L. (1989). Age-related variations in the relative importance of noradrenaline and ATP as mediators of the contractile response of rat tail artery to sympathetic nerve stimulation. *Acta Physiol Scand.,* 136, 287-288.

17. Barer.G., Bee.D., Emmery.C., Wach.R.A. (1985). Changed site of pulmonary vasoconstriction in chronically hypoxic rats. *Proc Physiol Soc.,* Abstract 166P.

18. Barer.G. (1993). Endothelial control of the pulmonary circulation in normal and chronically hypoxic rats. *J Physiol Lond.*, 463, 1-16.

19. Barnard.E.A., Burnstock.G., Webb.T. (1994). G-protein-coupled receptors for ATP and other nucleotides : a new receptor family. *TIPS.*, 15, 67-70.

20. Barst.R.J. (1986). Pharmacologically induced pulmonary vasodilatation in children and young adults with primary pulmonary hypertension. *Chest*, 89 (4), 497-503.

21. Bean.B.P. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *TIPS.*, 13, 87-90.

22. Belhassen.B., Pellag.A. (1984). Acute management of paroxysmal supraventricular

tachycardia: verapamil, adenosine triphosphate or adenosine? *Am J Physiol.*, 54(1) , 225-227.

23. Belhassen.B., Pelleg.A., Shoshani.D., Laniado.S. (1984). Atrial fibrillation induced by adenosine triphosphate. *Am J Cardiol.*, 53 (9), 1405-1406.
24. Belik. J. , Keeley.F.W., Baldwin.., Rabinovitch.M. (1994). Pulmonary hypertension and vascular remodelling in fetal sheep. *Am J Physiol.*, 266 (6 pt2), H2303-9.
25. Benham.C.D., Tsien.R.W. (1987). A novel receptor-operated Ca2+-permeable channel activated by ATP in smooth muscle. *Nature*, 328, 275-278.
26. Berger.J.I., Gibson.R.L., Clarke.W.R., Standaert.T.A., Redding.G.J., Henderson.W.R.Jr., Truog,W.E. (1993). Effects of amirone during group B streptococcus-induced pulmonary hypertension in piglets. *Pediatr Pulmonol.*, 16(5), 303-10.
27. Bernbaum.J.C., Russell.P., Sheridan.H., Gewitz.M.H., Fox.W.W., Peckham.G.J.(1984). Long-term follow-up of newborns with persistent pulmonary hypertension. *Crit Care Medi.*, 12 (7), 579-83.
28. Bertolino.F., Valentin.J., Maffre.M., Jover.B., Bessca.A., John.G.W. (1994). Prevention of thromboxane A2 receptor-mediated pulmonary hypertension by a nonpeptide angiotensin II type 1 receptor antagonist. *JPET.*, 268 no.2 ,747-.
29. Bevan.A.J. 1972. A direct method for recording tension changes in the wall of small blood vessels in vitro. *Agents and Actions*, 2(5), 257-260.
30. Binet and Burstein, (1950). Poumon et action vasculaire de l'adenosine-triphosphate. *Presse Medicale.*, 5, 1201.
31. Bjornsson.O.G., Monck.J.R., Williamson.J.R.(1989). Identification of P2Y purinoceptors associated with voltage-activated cation channels in cardiac ventricular myocytes of the rat. *Eur J Biochem.*, 186 (1-2), 395-404.
32. Bo.X., Burnstock.G. (1989). [^{3}H] -α-β-Methylene ATP, a radioligand labelling P2-purinoceptors. *J A N S.*, 28, 85-88.
33. Bo.X., Burnstock.G. (1992). Species differences in characteristics and distribution of [^{3}H] alpha-beta-methylene ATP binding sites in urinary bladder and urethra of rat, guinea-pig and rabbit. *Eur. J. Pharmacol.*, May 27, 216 (1), 59-66.

34. Bo.X., Neely.C.F. (1992). Autoradiographic localization of P2-purinoceptors in cat and human pulmonary vessels with [^{3}H] α,β-meATP. *FASEB proceedings*. A3586.

35. Bo.X., Simon.J., Burnstock.G., Barnard.E.A. (1992). Solubilization and molecular size determination of the P2X purinoceptor from the rat vas deferens. *J Biol Chem.*, 267 (25), 17581-17587.

36. Bo.X., Burnstock.G. (1993). Heterogenous distribution of [^{3}H] α,β-Methylene ATP binding sites in blood vessels. *J Vasc Res.*, 30, 87-101.

37. Bo.X., Zhang.Y., Nassar.M., Burnstock.G., Schoepfer.R. (1995). A P2X-purinoceptor cDNA conferring a novel pharmacological profile. *FEBS.* , 375 (1-2), 129-133.

38. Bodin.P., Bailey.D., Burnstock.G. (1991). Increased flow-induced ATP from isolated vascular endothelial cells but not smooth muscle cells. *Br J Pharmacol.*, 103, 1203-1205.

39. Bodin.P., Milner.P., Winter.R., Burnstock.G. (1992). Chronic hypoxia changes the ratio of endothelin to ATP release from rat aortic endothelial cells exposed to high flow. *Proc R Soc Lond.*, 247, 131-135.

40. Bodin.P., Burnstock.G. (1995). Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells. *Experentia.*, 51, 256-259.

41. Boels.P.J., Gao.B., Deutsch.J., Haworth.S.G. (1997). K+-(ATP)-channel activation in normal and hypertensive newborn and adult porcine pulmonary vessels. *Pediatr Res.*, 42 (3) 317-326.

42. Boeynaems.J.M., Galand.N. (1983). Stimulation of vascular prostacyclin synthesis by extracellular ADP and ATP. *Biochem Biophysics Res Commun.*, 112 (1), 290-296.

43. Boeynaems.J., Pearson.J.D. (1990). P2 purinoceptors on vascular endothelial cells : physiological significance and transduction mechanisms. *TIPS.*, 11, 34-37.

44. Bogdanov.(1996) (refer to ref. 456).

45. Bolton.T.B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *J Physiol Lond.*, 220 (3), 647-671.

46. Boulanger.C.M., Mombouli.J.V., Vanhoutte.P.M. (1993). Indapamide inhibits endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat. *Fundamental Clnical Pharmacology*, 7(8), 443- 448.

47. Bowler.W.R., Birch.M.A., Gallagher.J.A., Bilbe.G. (1995). Identification and cloning of human P_{2U} purinoceptor present in osteoblastoma, bone and osteoblasts. *J Bone Min Res.*,10, 1137-1145.

48. Boyer.J.L., Downes.C.P., Harden.T.K. (1989). Kinetics of activation of phospholipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J Biol Chem.*, 264 (2), 884-890.

49. Boyer.J.L., Siddiqi.S., Fischer.B., Romero-Avila.T., Jacobson.K.A. (1996). Identification of potent P2Y-purinoceptor agonists that are derivatives of adenosine 5'-monophosphate. *Drug Develop Research*. V37, No.3, p132.

50. Brake.A.J., Wagenbach.M.J., Julius.D. (1994). New structural motif for ligand gated ion channels defined by an ionotopic ATP receptor. *Nature*, 371, 519-523.

51. Brayden.J.E. (1991). Hyperpolarization and relaxation of resistance arteries in response to adenosine diphosphate. Distribution and mechanism of action. *Circ Res.*, 69, 1415-1420.

52. Brenner.O. (1935). Pathology of the vessels of the pulmonary circulation. *Archives of Internal Medicine*, 56, 211.

53. Brizzolara.A.L., Burnstock.G. (1991). Endothelium-dependent and endothelium-independent vasodilatation of the hepatic artery of the rabbit. *Br. J Pharmacol.*, 103, 1206-1212.

54. Brook.M.M., Fineman.J.R., Bolinger.A.M., Wong.A.F., Heymann.M.A., Soifer.S.J. (1994). Use of ATP-$MgCl_2$ in the evaluation and treatment of children with pulmonary hypertension secondary to congenital heart defects. *Circulation*, 90 (3), 1287-1293.

55. Brown.C.M, Burnstock.G. (1981) . The structural conformation of the polyphosphate chain of the ATP molecule is critical for its promotion of prostaglandin biosynthesis. *Euro J Pharmacol.*, 69 (1), 81-6.

56. Brown.C., Tanna.B., Boarder.M.R. (1995). PPADS: an antagonist at endothelial-purinoceptors but not P2U-purinoceptors. *Br J Pharmacol,*, 116 (5), 2413-2416.

57. Buchtal. F., Kahlson.G. (1944). The motor effect of adenosine triphosphate and allied phosphate compounds on mammalian smooth muscle. *Acta. Physiol. Scand.*, 8, 235-334.

58. Buell.G., Lewis.C., Collo.G., North.RT.A., Suprenant.A. (1996). An antagonist-insensitive P_{2X} receptor expressed in epithelia and brain. *EMBO J.*, 15, 55-62.

59. Buga.G.M., Ignarro.L.J.(1992). Electrical field stimulation causes endothelium-dependent and nitric-oxide-mediated relaxation of pulmonary artery. *J Am Physiol.* ,262, H973-H979.

60.Burnstock.G. Campbell.G., Bennett.M., Holman.M.E. (1964). Innervation of guinea-pig taenia coli : Are there intrinsic inhibitory nerves which are distinct from sympathetic nerves. *Int. J. Neuropharmaca.*, 31, 163-66.

61.Burnstock.G., Campbell.G., Satchell.D., Smythe.A. (1970). Evidence that adenosine triphosphate or a related nucleotide is a transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br.J.Pharmacol.*, 40, 668-688.

62. Burnstock.G.(1972). Purinergic nerves. *Pharmacol. Rev.*, 24,509-581.

63. Burnstock.G. (1976). Do some nerve cells release more than one transmitter. *Neuroscience*, 1, 239-248.

64. Burnstock.G., (1978) Straub.R.W., Bolis.L. (eds.) *Cell membrane Receptors for Drugs and Hormones: a multidisciplinary approach.* Raven, New York , p107-118.

65. Burnstock.G., Kennedy.C. (1985). Is there a basis for distinguishing two types of P2-purinoceptor ? *Gen. Pharmacol.*, 16, 433-440.

66. Burnstock.G., Warland.J. (1987). A pharmacological study of the rabbit saphenous artery in vitro : a vessel with a large purinergic contractile response to sympathetic nerve stimulation. *Br J Pharmacol.*, 90, 111-120.

67. Burnstock.G., Warland.J.J.I. (1987). P2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P2y- but not the P2x-purinoceptor. *Br J Pharmacol.*, 90, 383-391.

68. Burnstock.G. (1990). Co-transmission. (The fifth Heymans Memorial Lecture*). Arch Int Pharmacodyn Ther.*, 304, 7-33.

69. Burnstock.G., Fischer.B., Hoyle.C.H.V., Maillard.M., Ziganshin.A.U., Brizzolara.A.L., von Isakovics.A., Boyer.J.L., Harden.T.K., Jacobson.K.A. (1994). Structural activity relationships for derivatives of adenosine-5'-triphosphate as agonists at P2 purinoceptors :

hetreogeneity within P2x and P2y subtypes. *Drug Develop Res.*, 31, 206-219.

70. Burnstock.G. Ralevic.V. (1994). New insights into the local regulation of blood flow by perivascular nerves and endothelium. *Br J Plastic Surgery.*, 47, 527-543.

71. Burnstock.G. (1995). Current state of purinoceptor research. *Pharmaceutica Acta Helvetiae.* 69, 231-242.

72. Burnstock.G. (1995). Receptors for ATP at peripheral neuroeffector junctions. In : Belardinelli L., Pellag.A (eds). *Adenosine and adenine nucleotides: from molecular biology to integrative physiology.* Kluwer Acad., Norwell, MA. P289-295.

73. Burnstock.G. (1996). Purinoceptors : Ontogeny and Phylogeny. *Drug Develop Res.,* 39, 204-242.

74. Burnstock.G., King.B. (1996). The numbering of cloned P_2 purinoceptors. *Drug Develop Res.*, (in press).

75. Bush.A., Busst.C., Booth.K., Knight.W.B., Shinebourne.E.A. (1986). Does prostacyclin enhance the selective pulmonary vasodilator effects of oxygen in children with congenital heart disease. *Circulation*, 74 (1), 135-144.

76. Bush.A., Busst.C.M., Knight.W.B., Shinebourne.E.A. (1988). Comparison of the hemodynamic effects of epoprostenol (prostacyclin) and tolazoline. *Br. Heart Journal*, 60 (2), 141-148.

77. Cahill.B., Lippton.H., Neely.C., Hyman.A., Kadowitz.P. (1988). Evidence for a P2x-purinoceptor in the pulmonary vascular bed. *FASEB J.*, 2 (5), A952, 3815.

78. Carter.T.D., Hallam.T.J., Cusack.N.J., Pearson.J.D. (1988). Regulation of P2y-purinoceptors-mediated prostacyclin from human endothelial cells by cytoplasmic calcium concentration. *Br J Pharmacol.*, 95, 1181-1190.

79. Catravas.J.D. (1984). Removal of adenosine from the rabbit pulmonary circulation, in vivo and in vitro. *Circ Res.,* 54 (5), 603-611.

80. Chang.J., More.P., Fineman.J.R., Soifer.S.J., Heymann. (1992). K^+ channel pulmonary vasodilation in fetal lambs: role of endothelium-derived nitric oxide. *Am J Physiol.*, 73(1),188-194.

81. Chang.K., Hanaoka.K., Kumada.M., Takuwa.Y. (1995). Molecular cloning and functional analysis of a novel nucleotide receptor. *J.Biol.Chem.*, 270 (44), 26152-26158.

82. Chelliah.R., Bakhle.Y.S. (1983). The fate of adenine nucleotides in the pulmonary circulation of isolated lungs. *Q J Exp physiol.*, 68 (3), 289-300.

83. Chen.C., Akopian.A.N., Sivilotti.L., Colquhoun.D., Burnstock.G., Wood.J.N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*, 377, 428-431.

84. Chen.B.C., Lee.C.M., Lee.Y.T., Lin.W.W. (1996). Characteristics of signalling pathways of P2Y and P2U purinoceptors in bovine pulmonary arterial endothelial cells. *J Cardiovasc Pharmacol*, 28(2), 192-199.

85. Chen.B.C., Lee.C., Lin.W. (1996). Inhibition of ecto-ATPase by PPADS, suramin, reactive blue in endothelial cells, C6 glioma cells and RAW 264.7 macrophages. *Br J Pharmacol.*, 119, 1628-1634.

86. Chen.B.C., Lin.W. (1997). Inhibition of ecto-ATPase by the P2-purinoceptor agonists, ATPγS, α,β-methylene-ATP, and AMP-PNP, in endothelial cells. *Biochem PhysicalRes Commun.*, 233, 442-446.

87. Chen.Z.P., Krull.N., Xu.S., Levy.A., Lightman.S.L. (1996). Molecular cloning and functional characterization of a rat pituitary G protein-coupled ATP receptor. *Endocrinology*,137 (5), 1833-1840.

88. Chinellato.A., Pandolfo.L., Regazzi.E., Zambonin.M.R., Froldi.G., De Biasi.M., Caparrotta.L., Fassina.G..(1991). Effect of age on rabbit aortic responses to relaxant endothelium-dependent and endothelium-independent agents. *Blood Vessels*, 28(5), 358-365.

89. Chinellato.A., Ragazzi.E., Pandolfo.L., Froldi.G., Caparrotta.L., Fassina.G. (1992). Pharmacological characterization of a new receptor site in rabbit aorta.. *Gen Pharmacol.*, 23 ,No.6, 1067-1071.

90. Christman.B.W., McPherson.C.D., Newman.J.H., King.G.A., Bernard.G.R., Groves.B.M., Loyd.J.E. (1992). An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Eng J Med.*, 327 (2), 70-75.

91. Collo.G., North.R.A., Kawashima.E., Merlo-Pich.E., Neidhart.S., Suprenant.A., Buell.G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors, and the distribution and properties of an extended family of ATP-gated ion channels. *J Neurosci.*,16(8), 2495-2507.

92. Colson.P., Gaba.S., Saussine.M., Seguin.J., Chaptal.P.A., Roquefeuil.B. (1989). Vasodilating effect of adenosine triphosphate during cardioplumonary bypass. *J Cardiothorac Anesth.*, 3(5 suppl 1), 31.

93. Communi.D., Parmentier.M., Boeynaems.J.M. (1996). Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. *Biochem Biophys Res Comm.*, 222(2), 303-308.

94. Communi.D., Pirotton.S., Parmentier.M., Boeynaems.J.M. (1996). Cloning and functional expression of a human uridine nucleotide receptor. *J Biol Chem.*,270, 30849-30852.

95. Cooper.C.L., Morris.A.J., Harden.T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabelled agonist with a phospholipase C-linked P2Y-purinergic receptor. *J Biol Chem.*, 264 (11), 6202-6206.

96. Cornfield.D.N., McQueston.J.A., McMurtry, Rodman.D.M., Abman.S.H.. (1992). Role of ATP-sensitive potassium channels in ovine pulmonary vascular tone. *Am J Physiol.*,32, H1363-1368.

97. Corr.L., Burnstock.G. (1991). Vasodilator response of coronary smooth muscle to the sympathetic co-transmitters noradrenaline and adenosine 5'-triphosphate. *Br.J.Pharmacol.*, 104, 337-342.

98. Corr.L., Burmnstock.G. (1994). Analysis of P2-purinoceptor subtypes on the smooth muscle and endothelium of rabbit coronary artery. *J Cardiovasc Pharmaco*, 23, 709-715.

99. Cortijo.J., Marti-Cabrera.M., Bernabeu.E., Domenech.T., Bou.J., Fernandez.A.G., Beleta.J., Palacois.J.M., Morcillo.E.J. (1997). Characterization of 5-HT receptors on human pulmonary artery and vein: functional and binding studies. *Br J Pharmacol.*, 122, 1455-1463.

100. Crack.B.E., Beukers.M.W., McKenchnie.K.C., Ijzerman.A.P., Leff.P. (1994). Pharmacological analysis of ectoATPase inhibition : evidence for combined enzyme

inhibition and receptor antagonism in P2x-purinoceptor ligands. *Br J pharmacol.*, 113 (4) , 1432-1438.

101. Crack.B.E., Pollard.C.E., Beukers.M.W., Roberts.S.M., Hunt.S.F., Ingall.A.H., McKechnie.K.C.W., Ijzerman.A.P., Leff.P. (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *Br J Pharmacol.*, 114, 475-481.

102. Cremona.G., Higenbottam.T. (1995). Role of prostacyclin in the treatment of primary pulmonary hypertension. *Am. J. Cardiol.*, 75 (3), 67A-71A.

103. Crouch.E.C., Parks.W.C., Rosenbaum.J.L., Chang.D., Whitehouse.L., Wu.L.J., Stenmark.K.R., Orton.E.C., Mecham.R.P., (1989). Regulation of collagen production by medial smooth muscle cells in hypoxic pulmonary hypertension. *Am Rev Resp Dis.*, 140 (4), 1045-1051.

104. Crutchley.D.J., Eling.T.E., Anderson.M.W. (1978). ADPase activity of isolated perfused rat lungs. *Life Sciences*, 22,1413.

105. Crutchley.D.J., Ryan.U.S., Ryan.J.W. (1980). Effects of aspirin and dipyridamole on the degradation of adenosine diphosphate by cultured cells derived from bovine pulmonary artery. *J Clin Invest.*, 66(1),29-35.

106. Cusack.N.J., Pearson.J.D., Gordon.J. (1983). Stereoselectivity of ectonucleotidases on vascular endothelial cells. *Biochem J.*, 214, 975-981.

107. Dale.H. (1935). Pharmacology and nerve endings. *Proc R Soc Med.*, 28, 319-32.

108. Dalziel.H.H., Takeuchi.T., Bjur.R.A., Shinozuka.K., Sneddon.P., Westfall.D.P. (1990). The effect of purine analogues on neurotransmission in rabbit pulmonary artery. Abstract at *Eur J Pharmacol.*, 183, 134.

109. Darbinian.T.M., Chernikov.V.S., Apasov.K.T., Vlasov.G.P., Garsevanov.G. (1993). Effect of ATP infusion on heart function in the post-perfusion period after aortocoronary bypass surgery in patients with chronic ischemic heart disease. *Anesteziol Reanimatol.*, 3-8.

110. Dawes.G.S. (1968). *Foetal and neonatal physiology. In : Year Book. Chicago: Medical Publishers Inc.* 167.

111. Debdi.M., Seylaz.J., Sercombe.P. (1993). Increased influence of calcium and nicardipine on rabbit basilar artery reactivity after brief subarachnoid hemorrhage. *J Cardiovasc Pharmacol.*, 21(5), 754-759.

112. del Basso-P., Stati.T, Cioci.F., Fabi.F. (1996). Contribution of P2y purinoceptors in the relaxation to electric field stimulation of rabbit femoral artery. *Drug Develop Res.*, 37 (3) (Abstract), 166.

113. De Leyn.P., Lerut.T., Schreinemakers.H., van Belle.H., Lauwerijns.J., van Lommel.F., Verbeken.E., Flameng.W. (1993). Adenine nucleotide degradation in ischemic rabbit lung tissue. *Am J Physiol.*, 264 (4pt2), L329-L337.

114. De Mey.J., Burnstock,G., Vanhoutte.P.M. (1979). Modulation of evoked release of noradrenaline in canine saphenous vein via presynaptic receptors for adenosine but not ATP. *Eur J Pharmacol.*, 55, 401-405.

115. De Mey.J., Vanhoutte.P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J Physiol.*, 316, 347-355.

116. Dieterle.Y., Ody.C., Ehrensberger.A., Stalder.H., Junod.A,F. (1978). Metabolism and uptake of adenine triphosphate and adenosine by porcine aortic and pulmonary endothelial cells and fibroblasts in culture. *Circ Res.*, 42 No.6, 869-876.

117. Dinh-Xuan.A.T. (1990). Acetylcholine and adenosine diphosphate cause endothelium-dependent relaxation of isolated human pulmonary artery. *Eur Respir J.* , 3 , 633.

118. Dinh-Xuan.A.T., Higenbottam.T.W., Clelland.C.A., Pepke-Zaba.J., Cremona.G., Butt.A.Y., Large.S.M. (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N Eng J Med.*, 324 (22), 1539-1547.

119. Dinh-Xuan A.T. (1992). Endothelial modulation of pulmonary tone. *Eur. Respira. J.*, 5 , 757-762.

120. Dormowicz.A.G. (1993). Progressive loss of vasodilator response component of pulmonary hypertension in neonatal calves exposed to 4570m. *Am J Physiol.*,265, H2175.

121. Drury.A.N., Szent-Gyorgi.A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol .Lond.*,68, 213-237.

122. Dubyak.D.R. (1993). Signal transduction by P2-purinergic receptors for extracellular ATP. *Am J Resp Cell Mol Biol.*, 4, 295-300.

123. Dunn.J.A., Lorch.V., Sinha.S.N. (1989). Responses of small intrapulmonary arteries to vasoactive compounds in the fetal and neonatal lamb : norepinephrine, epinephrine, serotonin and potassium chloride. *Pediatr Res.*, 25 (4), 360-363.

124. Ea Kim.L., Sercombe.R., Oudart.N. (1988). Relaxation of rabbit middle cerebral arteries in vitro by H1 histaminergic agonists is inhibited by indomethacin and tranylcypromine. *Fundam Clin Pharmacol.*, 2(6), 463-475.

125. Eddahibi.S., Raffestin.B., Pham.I., Launay.J.M., Aegerter.P., Sitbon.M., Adnot.S. (1997). Treatment with 5-HT potentiates development of pulmonary hypertension in chronically hypoxic rats. *Am J Physiol.,* 272 (3 pt 2), H1173-H1181.

126. Erlinge.D., Yoo.H., Edvinsson.L, Rees.D.J., Wahlestedt.C. (1993). Mitogenic effects of ATP on vascular smooth muscle cells vs. Other growth factors and sympathetic cotransmitters. *Am J Physiol.*, 265, H1089-H1097.

127. Fike.C.D., Lai-Fook.S.J., Bland.R.D. (1988). Microvascular pressures during hypoxia in isolated lungs of newborn rabbits. *J Appl Physiol.*, 65 (1), 283-287.

128. Fike.C.D., Kaplowitz.M.R. (1994). Effect of chronic hypoxia on the pulmonary vascular pressure in isolated lungs from newborn pigs. *J Appl Physiol.,*77 (6), 2853-2862.

129. Filtz.T.M., Li.Q., Boyer.J.L., Nicholas.R.A., Harden.T.K. (1994). Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. *Mol Pharmacol.*, 46, 8-15.

130. Fineman.J.R., Crowley.M.R., Soifer.S.J. (1991). Selective pulmonary vasodilatation with ATP-MgCl2 during pulmonary hypertension in newborn lambs. *Pedatr Res.*, 27 (4) , 30A.

131. Fineman.J.R., Heyman.M.A., Soifer.S.J. (1991). Nw-nitro-l-arginine attenuates endothelium-dependent pulmonary vasodilatation in lambs. *Am J Physiol* .,260 , H1299-H1306.

132. Fischer.B., Yefidof.R., Boyer.J.L., Harden.T.K., Jacobson.K.A. (1996). Novel non-nucleotide P2-purinoceptor agonists. *Drug Develop Research.* V37, No.3, p132.

133. Fox.W.W., Duara.S. (1983). Persistent pulmonary hypertension in the neonate :

diagnosis and management. *J Pediatr.*, 103, 505-514.

134. Fredholm.B.B., Abbracchio.M.P., Burnstock.G., Dubyak.G.R., Harden.T.K., Jacobson.K.A., Schwabe.U., Williams.M. (1997). Towards a revised nomenclature for P1 and P2-receptors. *TIPS.*, 18, 79-81.

135. Fried.R. (1985). The effects of isoproterenol on the development and recovery of hypoxic pulmonary hypertension. *Am J Pathol.*, 121, 102.

136. Fukunaga.A.F., Flacke.W.E., Bloor.B.C. (1982). Hypotensive effects of adenosine and adenosine triphosphate compared with sodium nitroprusside. *Anesth Analg.*, 61 (3), 273-278.

137. Furchgott.R.F. (1981). The requirement for endothelial cells in the relaxation of arteries to acetylcholine and some other nitrovasodilators. *TIPS.*, 2, 173-176.

138. Furnes, Costa (1987). The enteric nervous system. Chuchill Livingston Edinburgh text.

139. Gaba.S.J.M., Bourgouin-Karaouni.D., Dujols.P., Michel.F.B., Prefaut.C.(1986). Effects of adenosine triphosphate on pulmonary circulation in chronic obstructive pulmonary disease. *.Am Rev Resp Dis.*, 134, 1140-1144.

140. Gaba.S., Trigui.F., Dujols.P., Godard.P., Michel.F.B., Prefaut.C. (1986). Compared effects of ATP vs adenosine on pulmonary circulation of COPD. *Eur J Respira Dis.*, 69 (suppl 146), 515-522.

141. Gaba.S.J.M., Prefaut.C. (1990). Comparison of pulmonary and systemic effect of adenosine triphosphate in chronic obstructive pulmonary disease - ATP : a pulmonary controlled vasoregulator. *Eur Resp J.*, 3, 450-455.

142. Gao.Y., Zhou.H., Raj.J.U. (1995). Endothelium-derived nitric oxide plays a larger role in pulmonary veins than in arteries of newborn lambs. *Circ Res.*, 76, 559-565.

143. Garland.C.J., Keatinge.W.R. (1979). Penetration of nerves, and sensitivity to noradrenaline, of inner and outer muscle of sheep pulmonary arteries. *Physiological Soc. Proc.*, c36, 61P.

144. Geggel.R.L., Aronovitz.M.J., Reid.L.M. (1986). Effects of chronic in utero hypoxema on rat neonatal pulmonary arterial structure. *J. Pediatr.*, 108 (5 pt1), 756-759.

145. Gerasimov.N.M., Guliamov.D.S., Karimova.T.Z., Belova.O.A., Ivanova.L.S., Nam.L.N. (1994). Biologically active substances during treatment of pulmonary hypertension with ATP infusions immediately after general anesthesia and surgery of hypervolemic congenital heart defects. *Anesteziol Reanimatol.*, (3), 14-17.

146. Glasgow.R.E., Heymann.M.A. (1990). Endothelium-derived relaxing factor as a mediator of bradykinin-induced pulmonary vasodilatation. *Clin Res.*, 38 , 211A.

147. Goetz.U., Da-Prada.M., Pletscher.A. (1971). Adenine- , guanine- and uridine- 5'-phosphonucleotides in blood platelets and storage organelles of various species. *JPET.* ,178 (1), 210-215.

148. Gordon.J.L., Martin.W. (1983). Endothelium-dependent relaxation of the pig aorta : relationship to stimulation of ^{86} Rb efflux from isolated endothelial cells. *Br J Pharmacol.* 79, 531-541.

149. Gough.G.R. (1973). Three new adenosine triphosphate analogues. Synthesis and effects on isolated gut. *J Medicinal Chem.*, 16 (10), 1188.

150. Graier.W.F., Simiecek.S., Kukovetz.W.R., Kostner.G.M. (1996). High D-glucose-induced changes in endothelial Ca2+/EDRF signaling are due to generation of superoxide anions. *Diabetes*, October, 45 (10), 1386-1395.

151. Green.R., Rojas.J., Sundell.H. (1979). Pulmonary vascular response to prostacyclin in fetal lambs. *Prostaglandins*, 18(6), 927-934.

152. Greenberg.B .(1987). Endothelium-dependent relaxation of human pulmonary arteries. *Am J Physiol.*, 252,H434.

153. Greenberg.S., Diecke.F.P.J., Peevy.K., Tanaka.T.P. (1989). The endothelium modulates adrehergic neurotransmission to canine pulmonary arteries and veins. *Eur J Pharmacol.*, 162, 67-80.

154. Greenwald.S.E., Berry.C.L., Haworth.S.G. (1982). Changes in the distensibility of the intrapulmonary arteries in the normal newborn and growing pig. *Cardiovasc Res.*, 16, 716-725.

155. Greenwald. S.E., Johnson. R.J., Haworth.S.G. (1985). Pulmonary vascular input impedance in the newborn and infant pig. *Cardiovasc Res.*,19 , 44-50.

156. Griffith.S.L., Rhoades.R.A., Packer.C.S. (1994). Pulmonary arterial smooth muscle contractility in hypoxia-induced pulmonary hypertension. *J Appl Physiol.*, 77 (1), 406-414.

157. Guibert.C., Pacaud.P., Loirand.G., Marthan.R.,Savineau.J. (1996). Effect of extracellular ATP on cytosolic Ca2+ concentration in rat pulmonary artery myocytes. *Am J Physiol., 271* ,L450-L458.

158. Hakim.T.S., Michel.R.P., Minami.H., Chang.H.K. (1983). Site of pulmonary hypoxic vasoconstriction studied with arterial and venous occlusion. *J Appl Physiol.*, 54(5), 1298-1302.

159. Hall.S., Haworth.S.G. (1986). Conducting pulmonary arteries : structural adaptation to extrauterine life. *Cardiovasc Res.*, 21, 208-216.

160. Hall S.M., Haworth. S.G. (1986). Normal adaptation of pulmonary arterial intima to extrauterine life in the pig : ultrastructural studies. *J Pathol.*, 153 , 171-76.

161. Hanson.M.A., Spencer.J.A.D., Rodeck.C.H. (1993). *Fetus and the Neonate. Physiology and clinical applications. V1. The circulation.* Chapter by S.G.Haworth, p396.

162. Hardebo.J.E., Kahstrom.J., Owman.C., Salford.L.G. (1987). Endothelium-dependent relaxation to uridine tri- and diphosphate in isolated human pial vessels. *Blood Vessels,* 24 (3), 150-155.

163. Harden.T.K., Lazarowski.E.R., Boucher.R.C. (1997). Release, metabolism and interconversion of adenine and uridine nucleotides : implications for G protein-coupled P2-receptor agonist selectivity. *TIPS.*, 18l, 43-46.

164. Harkema.J.M., Chaudry.I.H. (1992). Magnesium-adenosine triphosphate in the treatment of shock, ischemia and sepsis. *Crit Care Med.*,20(2), 263-275.

165. Hartley.S,A., Kozlowski.R.Z. (1997). Electrophysiological consequences of purinergic receptor stimulation in isolated rat pulmonary arterial myocytes. *Circ Res.*, 80 (2), 170-178.

166. Hasebe.N., Ondera.S., Yamashita.H., Kawamura.Y., Haneda.T., Tobise.K. (1992). Site of hypoxic vasoconstriction in pulsatile perfused canine lung lobes. *Jpn Circ J.*, 56 (8), 837-846.

167. Hashimoto.Y., Rigor.B.M., Moreno.J.A. (1982). Cardiovascular effects of hypotension

induced by adenosine triphosphate and sodium nitroprusside on dogs with denervated hearts. *Tohuku J Exp Med.*, 138 (1), 27-37.

168. Hassessian .H.M. (1993). Old , new and not yet exploited purinergic vasomechanisms of the pulmonary circulation. *Biomedical Reviews.*, 3, 11-25.

169. Hassessian.H., Bodin.P., Burnstock.G. (1993). Blockade by glibenclamide of the flow-evoked endothelial release of ATP that contributes to vasodilatation of the pulmonary vascular bed of the rat. *Br J Pharmacol.*, 190 (2), 466-472.

170. Hassessian.H., Burnstock.G. (1995). Interacting roles of nitric oxide and ATP in the pulmonary circulation of the rat. *Br J Pharmacol.*, 1143, 846-850.

171. Hauge.A. (1968). Role of histamine in hypoxic pulmonary hypertension in the rat. I. Blockade or potentiation of endogenous amines, kinins and ATP. *Circ Res.*, 22(3), p371-383.

172. Haworth.S.G., Reid.L., (1976). Persistent fetal circulation : newly recognised structural features. *J Pediatr.*, 88, 614-620.

173. Haworth.S.G. (1979). Pulmonary vascular structure in persistent fetal circulation. Godman M.J., Marquis.R.M., *eds. Paediatric Cardiology 2 Heart Disease in the Newborn. Edingburgh: Churchill Livingstone.*, 67-78.

174. Haworth.S.G., Hislop.A.A.. (1981). Adaptation of the pulmonary circulation in extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc Research*, 15, 108-119.

175. Haworth.S.G. (1982). Total anomalous pulmonary venous return. Prenatal damage to pulmonary vasculature and extrapulmonary veins. *Br Heart J.*, 48(6), 513-524.

176. Haworth S.G., Hall. S.M., Chew.M., Allen.K. (1987). Thinning of fetal pulmonary arterial wall and postnatal remodelling : ultrastructural studies on the respiration units arteries. *Virchows Arch A.*,411 ,161-171.

177. Heath.D. *et al.* (1981). Small pulmonary arteries in some Natives of La Paz, Bolivia. *Thorax*, v36, 599-604.

178. Heath.D., Williams.D., Rios Dalenz.J., Gosney.J. (1990). Pulmonary vascular disease in a rabbit at high altitude. *Int J Biometerol.*, 34 (1), 20-3.

179. Hellewell.P.G.,Pearson.J.D. (1984). Purinoceptor mediated stimulation of prostacyclin in the porcine pulmonary vasculature. *Br J Pharmacol.*, 83, 457-462.

180. Henderson.D.J., Elliot.D.G., Smith.G.M., Webb.T.E., Dainty.I.A. (1996). Cloning and characterisation of a bovine P2Y receptor. *Biochem Biophys Res Commun.*, 21 (2), 648-656.

181. Heymann.M.A., Rudolph.A.M. (1975). Control of the ductus arteriosus. *Physiol Rev.*, 55 (1), 62-78.

182. Higenbottam.T., Cremona.G. (1993). Acute and chronic hypoxic pulmonary hypertension. *Eur Respirat J.*, 6(8), 1207-12.

183. Hillaire-Buys.D., Bertrand.G., Chapal.J., Puech.R., Ribes.G., Loubatieres-Mariani.M.M. (1993). Stimulation of insulin secretion and improvement of glucose tolerance in rat and dog by the P2y -purinoceptor agonist, adenosine-5'-O-(2-thiodiphosphate). *Br J Pharmacol.*, 109 (1), 183-187.

184. Hillaire-Buys.D., Chapal.J., Petit.P., Loubatieres-Mariani.M.M. (1996). Adenosine-5'-O(2-thiodiphosphate) induce relaxation of rat pancreatic vasculature involves more than NO and PGI_2 release. *Drug Develop Res.*, 37 (3), (Abstract) p165.

185. Hislop.A.A., Reid.L. (1977). Changes in the pulmonary arteries of the rat during recovery from hypoxia-induced pulmonary hypertension. *Br J Exp Pathol.*, 58 (6), 653-62.

186. Hislop.A.A., Buttery.L.K.D., Springall.D.R., Pollock.J., Polak.J.M., Haworth.S.G. (1993). Postnatal changes in localisation of endothelial nitric oxide synthase in the porcine pulmonary vasculature. *Am Rev Resp Dis.*, 147 (4), A224.

187. Hislop.A.A., Zhao.Y.D., Springall.D.R., Polak.J.M., Haworth.S.G. (1995). Postnatal changes in endothelin-1 binding in porcine pulmonary vessels and airways. *Am J Respir Cell Mol Biol.*, 12(5), 557-566.

188. Holden.W.E. (1984). Hypoxia-induced contraction of porcine pulmonary artery strips depends on intact endothelium. *Exp Lung Res.*, 7, 101-112.

189. Holton.P. (1959). The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J Physiol.*, 145, 494-504.

190. Hoshino.Y., Obara.H., Kusunoki.M., Fujii.Y., Iwai.S. (1988). Hypoxic contractile response in isolated human pulmonary artery : role of calcium ion. *J Appl Physiol.*, 65 (6), 2468-2474.

191. Hourani.S.M.O., Chown.J.A. (1989). The effects of some possible inhibitors of ectonucleotidases on the breakdown and pharmacological effects of ATP in the guinea-pig urinary bladder. *Gen Pharmac.*, 20 No.4, 413-416.

192. Housley.G.D., Greenwood.D., Bennett.T., Ryan.A.F. (1995). Identification of a short form of the P2XR1-purinoceptor subunit produced by alternative splicing in the pituitary a and cochlea. *Biochem Biophysica Res Commun.*, 212, 501-508.

193. Hu.Q.H., Wang.D.X., Zhang.Y., Wang.J.X., Chen.R.S. (1994). ATP evokes calcium transients in single pulmonary artery endothelial cells in primary culture. *J Tongji Med Univ,* 14 (1), 42-44.

194. Huang.N., Wang.D., Heppel.L.A. (1994). Role of adenosine 3:5'-monophosphate-dependent protein kinase and cAMP levels in ATP-dependent mitogenesis in swiss 3T3 cells. *J Biol Chem.*, 269 (1), 548-555.

195. Humphries.R.G., Leff.P., Robertson.M.J. (1996). P2T-purinoceptor antagonists : a novel class of antithrombotic agent. *Drug Develop Research*, 36 (No.3), 175.

196. Husted.S.E., Nedergaard.O.A.. (1985). Dual inhibitory action of ATP on adrenergic neuroeffector transmission in rabbit pulmonary artery. *Acta Pharmacol Toxicol Copenh.*, 57 (3), 204-213.

197. Hyman.A.L., Nandiawada.P., Knight.D.S., Kadowitz.P.J. (1981). Pulmonary vasodilator responses to catecholamines and sympathetic nerve stimulation in the cat. *Circ Res.*,48, 407-415.

198. Hyman.A.L., Kadowitz.P.J. (1989). Analysis of response to sympathetic nerve stimulation in the feline pulmonary vascular bed. *J Appl Physiol.*, 67 (1), 371-376.

199. Inoue.T., Kannan.M.S.(1988) . Nonadrenergic and noncholinergic excitatory neurotransmission in rat intrapulmonary artery. *Am J Physiol.,* 23, H1142-1148.

200. Ishii.R., Shinozuka.K., Kunitomo.M., Hashimoto.T., Takeuchi.K. (1996). Regional differences of endogenous ATP release in rabbit arteries. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.*, 113 (3), 387-391.

201. Jahr.C.E., Jessell.T.M., (1983). ATP excites a subpopulation of rat dorsal horn neurons. *Nature*, 304, 730-733.

202. Jannsens.R., Communi.D., Pirotton.S., Samson.M., Parmentier.M., Boeynaems.J.-M. (1996). Cloning and tissue distribution of the human $P2Y_1$ receptor. *Biochem Biophys Res Commun.*, 221, 588-593.

203. Johns.A., Lategan.T.W., Lodge.N.J., Ryan.U.S., Van Breeman.C., Ada,s.D.J. (1987). Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. *Tissue Cell*, 19 (6), 733-745.

204. Johns.R.A., Linden.J.M., Peach.M.J. (1989.). Endothelium-dependent relaxation and cyclic CMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circ res.*, 65 1508-15.

205. Johnson.T.S., Young.J.B., Landsberg.L. (1983). Sympathoadrenal responses to acute and chronic hypoxia in rats. *J Clin Invest.*, 71, 1263-1272.

206. Jones.K., Higenbottam.T., Wallwork.J. (1989). Pulmonary vasodilatation with prostacyclin in primary and secondary pulmonary hypertension. *Chest*, 96 (4), 784-789.

207. Juul.B., Plesner.L., Aalkjaer.C. (1992). Effect of ATP and UTP on [Ca]I, membrane potential and force in isolated rat small arteries. *J Vasc Res.*, 29(5), 385-395.

208. Juul.B., Plesner.L., Aalkjaer.C. (1993). Effects of ATP and related nucleotides on the tone of the isolated rat mesenteric resistance arteries. *JPET*, 264(3), 1234-1240.

209. Kadletz.M., Digan.R.J., Mullen.P.G., Windsor.A.C., Sugerman.H.J., Wechsler.W.R. (1996). Pulmonary artery endothelial cell function in swine pseudomonas sepsis. *J Surg Res.*, January, 60 (1), 186-192.

210. Kanai.Y., Hori.S., Tanaka.T., Yasuko.M., Watanabe.K., Aikawa.N., Hosoda.Y.(1993). Role of 5-hydroxytryptamine in the progression of monocrotaline induced pulmonary hypertension in rats. *Cardiovasc Res.*, 27 ,1619-23.

211. Kasakov.L., Burnstock.G. (1983). The use of the slowly degradable analogue α,β-

methyleneATP, to produce desensitisation of the P2-purinoceptor: effect on non-adrenergic non-cholinergic responses of the guinea-pig urinary bladder. *Eur J Pharmacol.*, 86, 291-294.

212. Katsuragi.T., Su.C. (1980). Purine released from vascular adrenergic nerves by high potassium and calcium ionophore A-23187. *J PET* ., 215 (3), 685-690.

213. Katsuragi.T., Su.C. (1982). Release of purines and noradrenaline by oubain and high potassium from vascular adrenergic nerves. *Br.J.Pharmacol.*, 77, 625-629.

214. Keef.K.D., Pasco.J.S., Ekman.D.M.(1992). Purinergic relaxation and hyperpolarization in guinea-ig and rabbit coronary artery : role of the endothelium. *J PET.*, 260 (2), 592-600.

215. Kennedy.C., Burnstock.G. (1985). Evidence for two types of P2-purinoceptor in longitudinal muscle of the rabbit portal vein. *Eur J Pharmacol.*, 111, 49-56.

216. Kennedy.C., Leff.P. (1995). How should P2x-purinoceptors be classified pharmacologically. *TIPS.*, 16, 168-173.

217. Kinsella.J.P., Abman.S.H. (1994). Efficacy of inhalational nitric oxide therapy in the clinical management of persistent pulmonary hypertension. *Chest*, 105 (3 suppl), 92s-94s.

218. Kinsella.J.P., Truog.W.E., Walsh.W.F., Goldberg.R.N., Bancalari.E., Mayock.D.E., Redding.G.J., de Lemos.R.A., Sardesai.S., McCurnin.D.C., Moreland.S.G., Cutter.G.R., Abman.S.H.. (1997). Randomized, multicentre trial of inhaled nitric oxide and high - frequency oscillatory ventilation in severe, persistent pulmonary hypertension. *J Pediatr.*,131, 55-62.

219. Koga.T., Takata.Y., Kobayashi.K., Fujii.K., Nagao.T., Fujishima.M. (1992) . Age-related changes in P2-purinergic receptors on vascular smooth muscle and endothelium. *Hypertension.* 19, 286-289.

220. Kolab.K., Hibler.S., Raj.J.U. (1993). Vasodilator responses in intrapulmonary arteries and veins of immature fetal lambs. *Am Rev Resp Dis.*, A416.

221. Konduri.G.G., Woodard.L.L. (1991) . Selective pulmonary vasodilation by low-dose infusion of adenosine triphosphate in newborn lambs. *J Pediatr.*, 119, 94-102.

222. Konduri.G.G., Theodorou.A.A., Mukhopadhyay.A., Deshmukh.D.R. (1992).. Adenosine triphosphate and adenosine increase the pulmonary blood flow to postnatal levels in fetal lambs. *Pediatr Res.*,31 No.5, 451-457.

223. Konduri.G.G., Woodard.L.L., Mukhopadyay.A., Deshmukh.D.R. (1992). Adenosine is a pulmonary vasodilator in newborn lambs. *Am Rev Respir Dis.*, 146 (3), 670-676.

224. Konduri.G.G., Gervasio.C.T., Theodorou.A.A..(1993). Role of adenosine triphosphate and adenosine in oxygen-induced pulmonary vasdilation in fetal lambs. *Pediatr Res.*, 33 No.5, 533-539.

225. Konduri.G.G. (1994). Systemic and myocardial effects of ATP and adenosine during hypoxic pulmonary hypertension in lambs. *Pediatr Res.*, 36, 41-48.

226. Konduri.G.G., Mital.S., Gervasio.C.T., Rotta.A.T., Forman.K. (1997). Purine nucleotides contribute to pulmonary vasodilation caused by birth-related stimuli in the ovine fetus. *Am J Physiol.*, 272, H2377-2384.

227. Krishtal.O.A., Marenchenko.S.M., Pidoplichko.V.I., (1983). Receptor for ATP in the membrane of mammalian sensory neurons. *Neurosci Letts.*, 35, 41-45.

228. Kunapuli.S.P., Akbar.G.K.M. , Webb.T.E., Matsumoto.M., Mills.D.C.B., Barnard.E.A. (1996). Cloning and characterization of a novel P2 purinoceptor from human erythroleukemia cells. In: *Structure and Function of P2-purinoceptors*. Satellite meeting of experimental biology, Atlanta, GA, 7-9 April 1995 (Abstract).

229. Laing.R.J., Kakubowski.J., Morice.A.H. (1995). An in vitro study of the pharmacological responses of rat middle cerebral artery : effects of overnight storage. *J Vasc Res.*,32 (4), 230-236.

230. Lazarowski.E.R., Harden.T.K. (1994). Identification of a uridine nucleotide-selective G-protein -linked receptor that activates phospholipase C. *J Biol Chem.*, 269, 11830-11836.

231. Leon.C., Vial.C., Cazenave.J., Gachet.C. (1996). Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. *Gene*,171(2), 295-297.

232. Levin.R.M., Jacoby.R., Wein.A.J. (1983). High-affinity divalent ion-specific binding of 3H-ATP to homogenate derived from rabbit urinary bladder. Comparison with divalent-ion ATPase activity. *Mol Pharmacol.*, January, 23 (1), 1-7.

233. Levy.M., Stuart-Smith.K., Haworth.S.G. (1995). Maturation of the contractile response and its endothelial modulation in newborn porcine intrapulmonary arteries. *PediatrRes.*, 38 No.(1), 25-29.

234. Lewis.C., Neidhart.S., Holy.C., North.R.A., Nuell.G., Suprenant.A. (1995). Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, 377, 432-435.

235. Lipman.F., *et al* (1941). Metabolic generation and utilization of phosphate bond energy. *Adv Enzymol.*, 1, 99-162.

236. Lippton.H.L., Qingzhong.H., Hauth.T., Hyman.A. (1992). Mechanisms of signal transduction for adenosine and ATP in the pulmonary vascular bed. *Am J Physiol., 262*, H926-H929.

237. Liu.S.F., McCormack.D.G., Evans.T.W., Barnes.P.J. (1989). Evidence for two P2-purinoceptor subtypes in human small pulmonary arteries. *Br J Pharmacol.*, 98, 1014-1020.

238. Liu.S.F., McCormack.D.G., Evans.T.W., Barnes.P.J. (1989). Characterization and distribution of P2-purinoceptor subtypes in rat pulmonary vessels. *JPET.*, 251, 1204-1210.

239. Liu.S.F., Crawley.D.E., Evans.T.W., Barnes.P.J. (1991). Endgenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery. *Br J Pharmacol.*, 104, 565-569.

240. Liu.S.F., Crawley.D.E., Evans.T.W., Barnes.P.J. (1992). Endothelium-dependent nonadrenergic, noncholinergic neural relaxation in guinea-pig pulmonary artery. *JPET.*, 260 No.2, 541-548.

241. Liu.S., Hislop.A.A., Haworth.S.G., Barnes P.J. (1992). Developmental changes in endothelium-dependent pulmonary vasodilatation. *Br J Pharmacol.*, 106 , 324-330.

242. Liu.S.F., Crawley.D.E., Rohde.J.A., Evans.T.W., Barnes.P.J. (1992). Role of nitric oxide and guanosine 3',5'-cyclicmonophosphate in mediating nonadrenergic, noncholinergic relaxation in guinea-pig pulmonary arteries. *Br J Pharmacol.*, 107 (3), 861-6.

243. Loesch.A., Bodin.P., Burnstock.G. (1991). Colocalization of endothelin, vasopressin, serotonin in cultured endothelial cells of rabbit aorta. *Peptides*, September - October, 12 (5), 1095-1103.

244. Lukacsko.P., Krell.R.D. (1982). Response of the guinea-pig urinary bladder to purine and pyrimidine nucleotides.*Eur J Pharmacol.*, 80, 401-406.

245 Lustig.K.D., Erb.L., Landis.D.M., Hicks-Taylor.C.S., Zhang.X., Sportiello.M.G., Weisman.G.A. (1992). Mechanisms by which extracellular ATP and UTP stimulate the release of prostacyclin from bovine pulmonary artery endothelial cells. *Biochimica et Biophysica Acta.*, 1134, 61-72.

246. Lustig.K.D., Sporiello.M.G., Erb.L., Weisman.G.A. (1992). A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionised forms of ATP and UTP. *Biochem J.*, 284 (pt), 733-739.

247. Lustig.K.D., Shiau.A.K., Brake.A.J., Julius.D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci USA.*, 90, 5113-5117.

248. MacLean.M.R., McCulloch.K.M., MacMillan.J.B., McGrath.J.C. (1993). Influences of the endothelium and hypoxia on neurogenic transmission in the isolated pulmonary artery of the rabbit. *Br J Pharmacol.*, 108 (1), 150-154.

249. MacLean.M.R., Sweeney.G., Baird.M., McCulloch.K.M., Houslay.M., Morecroft.I. (1996). 5-Hydroxytryptamine receptors mediating vasoconstriction in pulmonary arteries from control and pulmonary hypetensive rats. *Br J Pharmacol.*, 119 (5), 917-30.

250. Maggi.A.A., Patacchini.R., Perretti.F., Tramontana.M., Manzini.S., Geppetti.P., Santicioli.P. (1990). Sensory nerves, vascular endothelium and neurogenic relaxation of the guinea-pig isolated pulmonary artery. *Naunyn Schmeideberg's Arch Pharmacol.*, 342, 78- 84.

251. Maguire.M.H., Dobronyi.I., Hung.K.S., Sartchell.D.G. (1996). Do different receptors mediate ATP- and UTP-elicited contraction of rat pelvic artery? *Drug Develop Res.*, 36(3), 165.

252. Malam.Souley.R., Seye.C., Gadeau.A.P., Loirand.G., Pillois.X., Campan.M., Pacaud.P., Desgranges.C. (1996). Nucleotide receptor P2u partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol.*, 166 (1), 57-65.

253. Mallikharjuna.V.S.N., Karamsetty.R., Kane.K.A., Wadsworth.R.M.. (1995) . The effects of chronic hypoxia on the pharmacological responsiveness of the pulmonary artery. *Pharmac Ther.*, 68 No.2, 233-246.

254. Marro.P.J., Baumgart.S., Delivoria-Papadopoulos.M., Zirim.S., Corcoran.L., McGaurn.S.P., Davis.L.E., Clancy.R.R. (1997). Purine metabolism and inhibition of xanthine oxidase in severe hypoxic neonates going onto extracorporeal membrane oxygenation. *Pediatr Res.*, 41(4), 513-520.

255. Martin.W.,Cusack.N.J.,Carleton.J.S., Gordon.J.L. (1985). Specificity of P_2-purinoceptor that mediates endothelium-dependent relaxation of the pig aorta. *Eur J Pharmacol*,108, 295-299.

256. Martin.T.W., Michaelis.K. (1989). P2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. *J Biol Chem,*, 264 (15), 8847-8856.

257. Mathieson.J.J.I., Burnstock.G.(1985). Purine-mediated relaxation and constriction of isolated rabbit mesenteric artery are not endothelium-dependent. *Eur J Pharmacol.*, 118, 221-229.

258. Matran.R., Alving.K., Lundberg.J.M. (1991). Differential bronchial and pulmonary vascular responses to vagal stimulation in the pig. *Acta Physio Scand.*, 143 (4), 387-393.

259. Matsumoto.T., Nakane.T., Chiba.S. (1997). UTP induces responses in the isolated and perfused canine epicardial coronary artery via UTP-preferring P2Y receptors. *Br J Pharmacol.*,122, 1625-1632.

260. Matsushita.T., Boels.P.J., Haworth.S.G. (1997). Atrial natiuretic peptide induces pulmonary arterial relaxation in normal and hypertensive newborn piglets. *J. Cardiology.* (Abstract).

261. McCormack.A.G., Clarke.B., Barnes.P.J. (1989). Characterization of adenosine receptors in human pulmonary arteries. *Am J Physiol.*, 256, H41-46.

262. McCormack.D.G., Barnes.P.J., Evans.T.W. (1989). Purinoceptors in the pulmonary circulation of the rat and their role in hypoxic vasoconstriction. *Br J Pharmacol.*,98,367-372.

263. McLaren.G.J., Lambrecht.G., Mutchler.E., Baumert.H.G., Sneddon.P., Kennedy.C. (1994). Investigation of the actions of PPADS, a novel P2X-purinoceptor antagonist in the guinea-pig isolated vas deferens. *Br J Pharmacol.*, 111, 913-917.

264. McLean.J.R., Twarog.B.M., Bergofsky.E.H. (1985). The adrenergic innervation of pulmonary vasculature in the normal and pulmonary hypertensive rat. *J A N S.*,14 (2), 111-123.

265. McMahon.T.J., Minkes.R.K., Dewitt.B.J., Osei.S.Y.S., Higuera.T.R., Kadowitz.P.J. (1993). Different mechanisms underlie vasodilator responses to adenosine triphosphate (ATP) in the pulmonary and systemic vascular beds : role of endothelium-derived relaxing factor. *FASEB J.*, 7 (3). Abstract 287, A50.

266. Mentzer.R.M., Rubio.R., Berne.R.M. (1975). Release of adenosine by hypoxic canine lung tissue and its possible role in pulmonary circulation. *Am J Physiol.*, 229 (6), 1625-31.

267. Meyrick.B., Reid.L.,(1980). Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am J Pathol.*,100(1), 151-178.

268. Meyrick.B., Reid.L. (1982). Normal postnatal development of the media of the rat hilar pulmonary artery and its remodelling by chronic hypoxia. *Lab Invest.*, 46(5), 505-514.

269. Meyrick.B., Niedermeyer.M.E., Ogletree.M.L., Brigham.K.L. (1985). Pulmonary hypertension and increased vasoreactivity caused by repeated indomethacin in sheep. *J Appl Physiol.*, 59 (2),443-52.

270. Miller.D.S.(1989). Role of arachidonic acid metabolites in hypoxic contraction of isolated porcine pulmonary artery and vein. *Exp Lung Res.*, 15 (2), 213.

271. Mills. An., Haworth.S.G. (1987). Pattern of connective tissue development in swine pulmonary vasculature by immunolocalisation. *J Pathol* .,153 , 171-176.

272. Milner.P., Bodin.P., Loesch.A., Burnstock.G. (1992). Increased shear stress leads to differential release of endothelin and ATP from isolated endothelial cells from 4 - and 12-month-old male rabbit aorta. *J Vasc Res.*, 29 (6), 420-425.

273. Milner.P., Bodin.P., Loesch.A., Burnstock.G. (1995). Interactions between sensory perivascular nerves and the endothelium in brain microvessels. *Int J Microcirc Clin Res.*, 15 (1), 1-9.

274. Minnette.P.A., Barnes.P.J. (1990). Muscarinic receptor subtypes in lung. Clinical implications. *Am Rev Respir Dis.*, 141 (3 pt 2), s162-s165.

275. Mo.M.. (1990). Flow-induced changes in Ca^{2+} signalling of vascular endothelial cells : effect of shear stress and ATP. *Am J Physiol.*, 260, H1698

276. Mockett.B.G., Bo.X., Housley.G.D., Thorne.P.R., Burnstock.G. (1995). Autoradiographic labelling of P2 purinoceptors in the guinea-pig cochlea. *Heart Res.,* 84 (1-1), 177-193.

277. Mohri.K., Takeuchi.K., Shinozuka.K., Bjur.R.A., Westfall.D.P. (1993). Simultaneous determination of nerve-induced adenine nucleotides and nucleosides released from rabbit pulmonary artery. *Analytical Biochemistry*, 210, 262-267.

278. Molderings.G.J., Collings.E., Likungu.J., Jakschik.J., Gothert.M. (1994). Modulation of noradrenaline release from the sympathetic nerves of the human saphenous vein and pulmonary artery by presynaptic EP3- and DP-receptors. *Br J Pharmacol.*, 111,733-738.

279. Mombouli.J.V., Nephtali.M., Vanhoutte.O.M. (1991). Effects of the converting enzyme inhibitor cilazaprilat on endothelium-dependent responses. *Hypertension*, 18 (4 suppl), I122-I119.

280. Moore.P., Velvis.H., Fineman.J.R., Soifer.S.J., Heymann.M.A.. (1992). EDRF inhibition attenuates the increase in pulmonary blood flow fue to oxygen ventilation in fetal lambs. *J Appl Physiol.*, 73 (5), 2151-2157.

281. Morin III.F.C., Stenmark.K.R.. (1995). Persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care*, 151, 2010-2032.

282. Muller.W., Kachel.W., Lasch.P., Varnholt.V., Konig.S.A. (1996). Inhaled nitric oxide for avoidance of extracorporeal membrane oxygenation in the treatment of severe persistent pulmonary hypertension. *Intensive Care Med.*, 22 (1), 71-76.

283. Nally.J.E., Muir.T.C., Guild.S.B. (1992). The effect of noradrenaline and adenosine 5'-triphosphate on polyphosphoinositide and phosphatidylcholine hydrolysis in arterial smooth muscle. *Br J Pharmacol.*, 106 (4), 865-870.

284. Nanto.S., Masuyama.T., Hori.M., Shimonagata.T., Ohara.T., Kubori.S. (1996). Zero flow pressure in human coronary circulation. *Angiology*, 47(2), 115-122.

285. Needham.L., Cusack.N.J., Pearson.J.D., Gordon.J.L. (1987).Characteristics of the P2 purinoceptor that mediates prostacyclin production by pig aortic endothelial cells. *Eur J Pharmacol.*, 134, 199-209.

286. Needleman.P., Minkes.M.S., Douglas.J.R. Jr. (1974). Stimulation of prostaglandin biosynthesis by adenine nucleotides. *Circ Res.*,34, 455-.

287. Neely.C., Kadowitz.P.J., Lippton.H., Hyman.A.L. (1986). Influence of adenosine 5'-triphosphate and β,γ methylene-adenosine 5'-triphosphate on the pulmonary vascular bed. *FASEB abstract.*, abstract submission form.

288. Neely.C.F., Kadowitz.P.J., Lippton.H., Neiman.M., Hyman.A.L. (1989). Adenosine does not mediate the pulmonary vasodilator response of adenosine 5'-triphosphate in the feline pulmonary vascular bed. *JPET.*, 250 No.1, 170-176.

289. Neely.C., Pellack.D., Beesburg.R., Haile.D. (1989). ATP produces vasoconstriction via P2X receptor and vasodilation via a P2Y receptor in the feline pulmonary vascular bed. D24. *Purine Nucleosides and Nucleotides in cell signalling :targets for new drugs.* abstract submission form.

290. Neely.C.F., Bui.D., Matot.I. (1992). Inhibition of 5'-nucleotidase in vivo enhances adenosine 5'-triphosphate (ATP)- induced pulmonary vasoconstriction. *Purines '92 abstract.* Abstract submission form.

291. Neely.C.F., Haile.D.M., Cahill.B.E., Kadowitz.P.J. (1992). Adenosine and ATP produce vasoconstriction in the feline pulmonary vascular bed by different mechanisms. *JPET.*, v258 No.3, 753-761.

292. Neely.C.F., Matot.I., Batra.V.K., Bo.X., Burnstock.G. (1995). P2X purinoceptors in the feline pulmonary vascular bed : distribution and selective in vivo pharmacological probes. *Am J Physiol.* 270 (6 Pt. 1), L889-897.

293. Neely.C.F., Stein.R., Matot.I., Batra.V., Cheung.A. (1996). Calcium blockage in pulmonary hypertension and hypoxic vasoconstriction. *New Horiz.*, 4 (1), 99-106.

294. Nguyen.T., Erb.L., Weisman.G.A., Marchese.A., Heng.H.H.Q., Garrad.R.C., George.S.R., Turner.J.T., O'Dowd.B.F. (1996). Cloning, expression and chromosmal localization of the uridine nucleotide receptor gene. *J Biol Chem.*, 270, 30845-30848.

295. Nicholas.R.A., Lazarowski.E.R., Watt.W.C., Li.Q., Boyer.J., Harden.T.K. (1996). Pharamacological and second messenger signalling of cloned P2Y receptors. *J A N S.*, 16, 319-323.

296. Noguchi.Y., Hislop.A.A., Haworth.S.G. (1997). Influence of hypoxia on endothelin-1 binding sites in neonatal porcine pulmonary vasculature. *Am. J. Physiol.*, 41, H669-H678.

297. Norel.X., Walch.L., Constantino.M., Labat.C., Gorenne.I., Dulmet.E., Rossi.F., Brink.C. (1996). M1 and M3 muscarinic receptors in human pulmonary arteries. *Br J Pharmacol.*, 119 (1), 149-157.

298. Nuttle.L.C., El-Moatassim.C., Dubyak.G.R. (1993). Expression of the pore forming P2Z purinoceptor in *Xenopus* oocytes injected with poly(A^+) RNA from murine macrophages. *Mol Pharmacol.*,44, 93-101.

299. O'Connor.S.E., Wood.B.E., Leff.P. (1990). Characterization of P2x-receptors in rabbit isolated ear artery. *Br J Pharmacol.*,101(3), 640.

300. O'Connor.S.E., Dainty.I.A., Leff.P.(1991). Further classification of ATP receptors based on agonist studies. *TIPS.*, 12, 137-141.

301. Ohar.J.A., Pyle.J.A., Waler.K.S., Hyers.T.M., Webster.R.O., Lagunoff.D. (1990). A rabbit model of pulmonary hypertension induced by synthetic platelet-activating factor acetylglyceryl ether phosphorylcholine. *Am Rev Respir Dis.*, 141 (1), 104-10.

302. Olivier.K.N., Bennett.W.D., Hohneker.K.W., Zeman.K.L., Edwards.L.J., Boucher.R.C., Knowles.M.R. (1996). Acute safety and effects on mucociliary clearance of aerosolised

uridine 5'-triphosphate +/- amiloride in normal human adults. *Am J Resp Crit Care.Med.*, 154 (1), 217-223.

303. Olson.E.B.Jr., Ghias Ud Din.M., Rankin.J. (1982). Uptake and metabolism of prostaglandin E1 in isolated perfused fetal, newborn and adult rabbit lungs. *Prostaglandins*, 9 (4), 429-436.

304. Orton.E.C., Reeves.J.T., Stenmark.K.R.. (1988). Pulmonary vasodilation with structurally altered pulmonary vessels and pulmonary hypertension. *J Appl Physiol.*, 65 (6), 2459-2467.

305. (1996) *P2 purinoceptors : localization, function and transduction mechanisms*. Ed. D.J.Chadwick, J.A.Goode. Pub. J. Wiley & Sons Ltd.

306. Paidas.C.N., Dudgeon.D.L., Haller.J.A.Jr., Clemens.M.G. (1988). Adenosine triphosphate : a potential therapy for hypoxic pulmonary hypertension. *J of Pediatr Surgery*, 23,No. 12, 1154-1160.

307. Paidas.C.N., Dudgeon.D.L., Haller.J.A.Jr., Clemens.M.G. (1989). The effect of adenosine triphosphate on the functional status of the ductus arteriosus. *J Pediatr Surgery*, 24 No.7, 649-653.

308. Paidas.C.N., Dudgeon.D.L., Haller.J.A.Jr., Clemens.M.G. (1989). Adenosine triphosphate (ATP) treatment of hypoxic pulmonary hypertension (HPH) : a comparison of dose-dependence in pulmonary and renal circulations. *J Surg Res.*, 46 (4), 374-379.

309. Palea.S., Corsi.M., Pietra.C., Artibani.W., Calpista.A., Gaviraghi.G., Trist.D.G. (1994). ADP beta S induces contraction of the human isolated urinary bladder through a purinoceptor subtype different from P2X and P2Y. *J PET.*, 269(1), 193-7.

310. Palmer.R.M.J., Ferrige.A.G., Moncada.S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, 327, 524-526.

311. Parr.C.E., Sullivan.D.M., Paradiso.A.M., *et al.* (1994). Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci USA.*, 91, 3275-3279.

312. Peake.M.D., Harabin.A.L., Brennan.N.J., Sylvester.J.T. (1981). Steady-state vascular responses to graded hypoxia in isolated lungs of five species. *J Appl Physiol.*, 51 (5), 1214-1219.

313. Pearce.W.J., Ashwal.S., Cuevas.J. (1989). Direct effects of graded hypoxia on intact and denuded rabbit cranial arteries. *Am J Physiol.*, 257 (3 pt 2), H824-H833.

314. Pearl.R.G. (1994). Effect of l-glutamine on pulmonary hypertension in the perfused rabbit lung. *Pharmacology*, 48, p260-264.

315. Pearson.J.D., Gordon.J.L. (1979).Vascular endothelial and smooth muscle cells in culture selectively release adenine nucleotides. *Nature*, 281, 384-386.

316. Pearson.J.D., Slakey.L.L., Gordon.J.L. (1983). Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells. *Biochem. J.*, 214, 273-276.

317. Pearson.J.D., Coade.S.B., Cusack.N.J.(1985). Characterization of ectonucleotidases on vascular smooth muscle cells. *Biochem J.*, 230, 503-507.

318. Pepke-Zaba-J., Higenbottam.T.W., Dinh-Xuan.A.T., Stone.D., Wallwork.J. (1991). Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. *Lancet,* 338 (8776), 1173-1174.

319. Perez-Viscaino.F., Villamor.E., Moro.M., Tamargo.J. (1996). Pulmonary versus systemic effects of vasodilator drugs : an in vitro study in isolated intrapulmonary and mesenteric arteries of neonatal piglets. *Eur J Pharmacol.*, 314 (1-2), 91-98.

320. Perkett.E.A., Brigham.K.L., Meyrick.B. (1988). Continuous air embolization into sheep causes sustained pulmonary hypertension and increased pulmonary reactiveness. *Am J Pathol.* ,132 (3), 444-454.

321. Perreault.T., De Marte.J. (1993). Maturational changes in endothelium-derived relaxations in the newborn piglet pulmonary circulation. *Am J Physiol.*, 264 (2pt2), H302-H309.

322. Picher.M., Cote.Y.P., Beliveau.R., Poiter.M., Beaudoin.A.R.(1993). Demonstration of a novel type of ATP-diphosphorylase (EC 3.6.1.5) in the bovine lung. *J Biol Chem.*, 268 (7), 4699-4703.

323. Prielipp.R.C., McLean.R., Rossenthal.M.H., Pearl.R.G. (1991). Hemodynamic profiles

of prostaglandin E1, isoproterenol, prostacyclin, and nifedipine in experimental porcine pulmonary hypertension. *Critical Care Medicine*, 19 no.1, 60-67.

324. Priest.R.M., Hucs.D., Wars.J.P.T. (1997). Noradrenaline, β-adrenoceptor mediated vasorelaxation and nitric oxide in large and small pulmonary arteries of the rat. *Br J Pharmacol.*, 122, 1375-184.

325. Purkiss.J.R., Wilkinson.G.F., Boarder.M.R. (1994). Differential regulation of inositol 1,4,5-triphosphate by co-existing P2Y-purinoceptors and nucleotide receptors on bovine aortic endothelial cells. *Br J Pharmacol.*, 111, 723-728.

326. Qasabian.R.A., Schyven.C., Owe-Uoung.R., Killen.J.P., MacDonald.P.S., Conigrave.A.D., Williamson.D.J. (1997). Characterization of the P2-receptors in rabbit pulmonary artery. *Br J Pharmacol.*,120, 553-558.

327. Rabinovitch.M., Mullen.M., Rosenberg.H.C., Maruyama.K., O'Brodovich.H., Olley.P.M. (1988). Angiotensin II prevents hypoxic pulmonary hypertension and vascular changes in rats. *Am. J. Physiol.*, 254 (3 pt2), H500-H508.

328. Raine.J., Hislop.A.A., Redington.A.N., Haworth.S.G., Shinebourne.E.A. (1991). Persistent pulmonary hypertension of the newborn. *Arch Dis Child.*, 66 , 398-402.

329. Raj.J.U., Bland.R.D., Lai-Fook.S.J. (1986). Microvascular pressures measured by micropipettes in isolated edematous rabbit lungs. *J Appl Physiol.*, 60 (2), 539-545.

330. Raj.J.U., Chen.P. (1986). Micropuncture measurement of microvascular pressures in isolated lamb lungs during hypoxia. *Circ Res.*, 59, 398-404.

331. Raj.J.U., Chen.P., Navazo.L. (1986). Micropuncture measurement of lung microvascular pressure profile in 3- to 4-week-old rabbits. *Pediatr Res.*, 20 (11), 1107-1111.

332. Raj.J.U., Hillyard.R., Kaapa.P., Anderson.J., Gropper.M. (1990). Pulmonary vascular pressure profile in 2-3-week-old, 5-6-week-old and adult ferrets. *Respir Physiol* 82 (3), 307-315.

333. Raj.J.U., Hillyard.R., Kaapa.P., Gropper.M., Anderson. (1990). Pulmonary arterial and venous constrictions during hypoxia in 3- to 5-wk-old and adult ferrets. *J Appl Physiol.*, 69 (6), 2183-2189.

334. Ralevic.V., Burnstock.G. (1991). Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Br J Pharmacol.*, 69 (6), 1583-1590.

335. Ralevic.V., Mathie.R.T., Alexander.B., Burnstock.G. (1991). Characterization of P2X- and P2Y-purinoceptors in the rabbit hepatic arterial vasculature. *Br J Pharmacol.*, 103, 1108-1113.

336. Rapoport.R.M., Draznin.M.B., Murad.F. (1984). Mechanism of adenosine triphosphate-, thrombin- and trypsin-induced relaxation of rat thoracic aorta. *Circ Res.*, 55, 468-479.

337. Rathbone.M.P., Middlemiss.P.J., Gysbers.J.W., DeForge.S., Costello.P., Del Maestro.R.F. (1992). Purine nucleosides and nucleotides stimulate proliferation of a wide range of cell types. *In Vitro Cell Dev Biol.*, 28 A (7-8), 529-536.

338. Reeves.J.T., Joki.P., Merida.J., Leathers.J.E. (1966). Pulmonary vascular obstruction following administration of high-energy nucleotides. *J Appl Physiol.*, 22 (3), 475-479.

339. Reilly.W.M., Saville.V.L., Burnstock.G. (1987). An assessment of the antagonistic activity of reactive blue at P1 and P2 purinoceptors : supporting evidence for purinergic innervation of the rabbit portal vein. *Eur J Pharmacol.*, 140(1), 47-53.

340. Rice.W.R., Burton.F.M., Fiedeldey.D.T. (1995). Cloning and expression of the alveolar type II P_{2U}-purinergic receptor. *Am J Respir Cell Mol Biol.* ,12, 27-32.

341. Robaye.B., Boeynaems.J., Communi.D. (1997). Slow desensitization of the human $P2Y_6$ receptor. *Eur J Pharmacol.*, 329, 231-236.

342. Robertson.B.E., Corry.P.R., Nye.P.C., Kozlowski.R.Z. (1992). Ca2+ and Mg2+ activated potassium channels from rat pulmonary artery. *Pfleugers Arch,* 421(2-3), 94-96.

343. Rodman.D.M.. (1992). Chronic hypoxia selectively augments rat pulmonary artery Ca2+ and K+ channel-mediated relaxation. *Am J Physiol.*, 263 ,1 pt1,L88-94.

344. Rongen.G.A., Smits.P., Thien.T. (1994). Characterization of ATP-induced vasodilation in the human forearm vascular bed. *Circulation*, 90 (4), 1891-1898.

345. Rosenberg.A.A., Kennaugh.J., Koppenhafer.S.L., Loomis.M., Chatfield.B.A., Abman.S.H. (1993). Elevated immunoreactive endothelin-1 levels in newborn infants with persistent pulmonary hypertension. *J Pediatr*, 123, 109-114.

346. Rosenberg.A.A., Kennaugh.J.M., Moreland.S.G., Fashaw.L.M., Hale.K.A., Torielli.F.M., Abman.S.H., Kinsella.J.P. (1997). Longitudinal follow-up of a cohort of newborn infants treated with inhaled nitric oxide for peristent pulmonary hypertension. *J Pediatr.*, 131, 170-175.

347. Rubino.A., Burnstock.G. (1994). A new purinergic receptor may contribute to the control of pulmonary circulation in the rat. Abstract. *5th International Adenosine Symposium* . 1096.

348. Rubino.A., Burnstock.G.. (1994). Contractile actions of pyrimidine and purine nucleotides in the rat pulmonary vascular bed. *Br J Pharmacol.,* 112, 501P.

349. Rubino.A., Burnstock.G. (1995). P2-purinoceptors in the pulmonary circulation. *Res Comm Mol Pathol Pharmacol.*, v87, (1), 53.

350. Rubino.A., Ralevic.V., Burnstock.G. (1995). Contribution of P1-(A2b subtype) and P2-purinoceptors to the control of vascular tone in the rat isolated mesenteric arterial bed. *Br J Pharmacol.,* 115, 648-652.

351. Rubino.A., Burnstock.G.(1996). Evidence foe a P2-purinoceptor mediating vasoconstriction by UTP, ATP and related nucleotides in the isolated pulmonary vascular bed of the rat. *BrJ Pharmacol.,* 1415-1420.

352. Rudolph.A.M., Kurland.M.D., Auld.P.A.M., Paul.M.H. (1959). Effects of vasodilator drugs on normal and serotonin-constricted pulmonary vessels of the dog. *Am J Physiol.*, 197 (3), 617-623.

353. Ryan.J.W., Smith.U. (1971).Metabolism of adenosine 5'-monophosphate during circulation through the lungs. *Transactions of the Association of American Physicians,*84:297.

354. Saiag.B., Milon.D., Guelou.M.C., Van den Driessche.J., Rault.B. (1987). Heterogeneity of purinergic P2 receptors at the level of the caudal artery of the rat and the saphenous vein of the dog. *C R Seances Soc Biol Fil.,* 181 (2), 168-177.

355. Saiag.B., Milon.D., Allain.H., Rault.B., van de Driessche.J. (1990). Constriction of the smooth muscle of rat tail and femoral arteries and dog saphenous vein is induced by uridine triphosphate via 'pyrimidinoceptors', and by adenosine triphosphate via P2X

purinoceptors. *Blood Vessels,* 27(6), 352-364.

356. Saiag.B., Milon.D., Shacoon,V., Allain.H., Rault.B., van den Driessche.J. (1992). Newly evidenced pyrimidinoceptors and the P2-purinoceptors are present on the vascular smooth muscle and respectively mediate the UTP- and ATP- induced contractions of the dog maxillary internal vein. *Res Comm Chem Pathol Pharmacol.,* V76(1), 89-95.

357. Saiag.B., Bodin.P., Shacoori.V., Catherine.M., Rault.B., Burnstock.G. (1995). Uptake and flow-induced release of urindine nucleotides from isolated vascular endothelial cells. *Endothelium*, V2, 279-285.

358. Sanchez-Fernandez-M., Katz.G.M., Suarez-Kurtz.G., Kaczorowski.G.L., ReubenJ.P. (1993). Mobilisation of intracellular calcium in cultured vascular smooth muscle cells by uridine triphosphate and the calcium ionophore A23187. *J Membr Biol.,* 135 (3), 273-287.

359. Sata.T., Kubota.E., Said.S.I., Isra.H.P. (1990). EPR spectroscopic studies of detection of a carbon-centred free radical during acetylcholine-induced and endothelium-dependent relaxation of guinea-pig pulmonary artery. *Free Radic Res Commun.*, 9 (3-6), 213-222.

360. Scamps.F., Rubin.V., Puceat.M., Tkachuk.V., Vassort.G. (1992). A Gs protein couples P2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. *J Gen Physiol.*, 100 (4), 675-701.

361. Schachter.J.B., Li.Q., Boyer.J.L., Nicholas.R.A., Harden.T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human P2Y1 purinoceptor. *Br J Pharmacol.*, 118, 167-173.

362. Schachter.J.B., Harden.T.K. (1997). An examination of deoxyadenosine 5'-(a-thio) triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the $P2Y_1$-receptor. *Br J Pharmacol.*, 121, 338-344.

363. Schaff.A., Bock.M., Dienemann.H., Hesse.U., Nees.S., Gerlach.E. (1986). Degradation of adenine nucleotides (AN) in the circulatory system: relative contributions of the vascular endothelium and whole blood. *Eur J Physiol.*, suppl.1 vol 1. ,44.

364. Schilling.L., Parsons.A.A., Wahl.M. (1995). Effects of potassium channel activators on isolated cerebral arteries of large and small diameter in the cat. *J Neurosurg.*, 83(1), 123-128.

365. Scott.J.A., Craig.I., McCormack.D.G. (1996). Nonadrenergic noncholinergic relaxation of human pulmonary arteries is partially mediated by nitric oxide. *Am J Respir Crit Care Med.*, 154 (3 ptII), 629-632.

366. Seguela.P., Haghihi.A., Sohomonian.J-J., Cooper.E. (1996). A novel P2x ATP receptor ion channel with widespread distribution in the brain. *J Neurosci.*, 16, 448-455.

367. Shaul.P.W., Magness.R.R., Muritz.K.H., DeBeltze.D., Buja.L.M. (1990). α1-adrenergic receptors in pulmonary and systemic vascular smooth muscle. *Circ Res.*, 67, 1193-1200.

368. Shaul.P.W., Kinane.B., Farrar.M.A., Buja.L.M., Magness.R.R. (1991). Prostacyclin production and mediation of adenylate cyclase activity in the pulmonary artery. Alterations after prolonged hypoxia in the rat. *J Clin Invest.*, 88 (2), 447-455.

369. Shaul.P.W., Farrar.M.A., Magness.R.R. (1993). Pulmonary endothelial nitric oxide production is developmentally regulated in the fetus and newborn. *Am J Physiol.*, 34, H1056-H1063.

370. Sheedy.W., Thompson.J.S., Morice.A.H. (1996). A comparison of pathophysiological changes during hypobaric and normobaric hypoxia in rats. *Respiration*, 63 (4), 217-222.

371. Sheenan. D.W., Farhi.L.E., Russell.J.A. (1992). Prolonged lobar hypoxia in vivo enhances the responsitivity of isolated pulmonary veins to hypoxia. *Am Rev Respir Dis.*, 145, 640-645.

372. Shepherd.J.T., Vanhoutte.P.M. (1979). The human cardiovascular System facts and concepts. *Ravens Press*, p8.

373. Shinozuka.K., Kobayashi.Y., Shimoura.K., Hattori.K. (1990). Regional difference of purinergic modulation on adrenergic neurotransmission in isolated rabbit pulmonary artery. *IPVAR Satellite Symposium,* (Abstract). P11.

374. Shirahase.H., Usui.H., Manabe.K., Kurahashi.K., Fujiwara.M. (1988). Endothelium-dependent contraction and independent-relaxation induced by adenine nucleotides and nucleoside in the canine basilar artery. *J P E T.*, 247 (3), 1152-1157.

375. Shirawasa.Y., White.R.P., Robertson.J.T. (1983). Mechanism of the contractile effect induced by uridine 5'-triphosphate in canine cerebral arteries. *Stroke,* 14 (3), 347-355.

376. Soifer.S.J., Kaslow.D., Roman.C., Heymann.M.A.. (1987). Umbilical cord compression induces pulmonary hypertension in newborn lambs : a model to study the pathophysiology of persistent pulmonary hypertension in the newborn. *J Develop Physiol.*, 9 (3), 239-52.

377. Sollevi.A., Lagerkranser.M., Andreen.M., Irestedt.L. (1984). Relationship between arterial and venous adenosine levels and vasodilatation during ATP- and adenosine-infusion in dogs. *Acta Physiol Scand.*, 120 (2), 171-6.

378. Soto.F., Garcia-Guzman.M., Gomez-Hernandez.J.M., Hollman.M., Karschin.C., Stuhmer.W. (1996). P2X0c4: an ATP-activated ionotropic receptor cloned from rat and human brain. *Proc Natl Acad Sci., USA.* 93 (8), 2495-2507.

379. Steinhorn.R.H., Morin III.F.C., Gugino.S.F., Giese.E.C., Russell.J.A. (1993). Developmental differences in endothelium-dependent responses in isolated ovine pulmonary arteries and veins. *Am J Physiol.*, 33, H2162-H2167.

380. Steinhorn.R.H. , Russell.J.A., Morin.F.C 3rd.(1995). Disruption of cGMP production in pulmonary arteries isolated from fetal lambs with pulmonary hypertension. *Am J Physiol.*, 268 (4 pt2), H1483-9.

381. Stenmark.K.R., Orton.E.C., Reeves.J.T., Voelkel.N.F., Crouch.E.C., Mecham.R.P. (1988). Vascular remodelling in neonatal pulmonary hypertension. Role of the smooth muscle. *Chest*, 93 (3 suppl), 127s-1332.

382. Stone.T.W. (1989). Purine receptors and their pharmacological roles. *Advance in Drug research*, 18, 292-429.

383. Su.C. (1975). Neurogenic release of purine compounds in blood vessels. *JPET*, v195 (No.1), 159-166.

384. Su C. (1978). Purinergic inhibition of adrenergic transmission in rabbit blood vessels. *JPET.*,204 (2), 351.

385. Suprenant.A., Rassendren.F., Kawashima.E., North.R.A., Buell.G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science*, 272, 735-738.

386. Susuki.H. (1983). An electrophysiological study of excitatory neuromuscular

transmission in the guinea-pig main pulmonary artery. *J Physiol.*, 336, 47-59.

387. Tada.S., Okajima.F., Mitsui.Y., Kondo.Y., Ui.M. (1992). P2-purinoceptor-mediated cyclic AMP accumulation in bovine vascular smooth muscle cells. *Eur J Pharmacol*, 227, 25-31.

388. Takemura.S., Kawada.N., irohashi.K., Kinoshita.H., Inoue.M. (1994). Nucleotide receptors in hepatic stellate cells of the rat. *FEBS letts.*, 354 (1), 53-56.

389. Takeuchi.K., Shinozuka.K., Akimoto.H., Ishii.R., Hasimoto.T. (1994). Methoxamine-induced release of endogenous ATP from rabbit pulmonary artery. *Eur J Pharmacol.*, 254, 287-290.

390. Takeuchi.K., Shinozuka.K., Ishii.R., Hashimoto.T. (1995). Regional differences of endogenous ATP release in the pulmonary artery of rabbits. *Clin Exp Pharmacol Physiol.*, 22 (9), 675-676.

391. Taylor.E.M., Parsons.M.E., Wright.P.W., Pipkin.M.A., Howson.W. (1989). The effect of adenosine triphosphate and related purines on arterial resistance vessels in vitro and in vivo. *Euro J Pharmacol.*, 161(2-3), 121.

392. Thomas.T., Marshall.J.M.. (1993). The role of adenosine in hypoxic pulmonary vasoconstriction in the anaesthetized rat. *Experimental Physiology*, 78, 541-543.

393. Thompson.B.T., Hassoun.P.M., Kradin.R.L., Hales.C.A.(1989). Acute and chronic hypoxic pulmonary hypertension in guinea pigs. *J Appl Physiol.*, 66 (2), 920-928.

394. Tod.M.L. Yoshimura.K., Rubin.L.J. (1992). Ontogeny of neonatal pulmonary vascular pressure-flow relationships. *Am J Physiol.*, 262 (3 pt 2), H684-H690.

395. Tokuyama.Y., Hara.M., Jones.E.M.C., Fan.Z., Bell.G.I. (1995). Cloning of rat and mouse P_{2Y} purinoceptors. *Biochem Biophys Res Commun.*, 211, 211-218.

396. Torok.T.L., Rubanyi.G., Vizi.E.S., Magyar.K. (1982). Stimulation by vanadate of [3H] noradrenaline release from rabbit pulmonary artery and its inhibition by noradrenaline. *Eur J Pharmacol.*, 84, 93-97.

397. Trezise.D.J., Drew.G.M., Weston.A.H. (1992). Analysis of the depressant effect of the endothelium on contractions of rabbit isolated basilar artery to 5-hydroxytryptamine. *Br J Pharmacol.*, 106 (3), 587-592.

398. Tucker.A., McMurtry.I.F., Reeves.J.T., Alexander.A.F., Will.D.H., Gorver.R.F. (1975). Lung vascular smooth muscle as a determinant of pulmonary hypertension at altitude. *Am J Physiol.*, 228 (3),762-7.

399. Tulloh.R.M.R., Hislop.A.A., Boels.P.J., Deutsch.J., Haworth.S.G. (1997). Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. *Am J Physiol.* 272., H2436-H2445.

400. Urquilla.P.R. (1978). Prolonged contraction in isolated human and canine cerebral arteries induced by uridine 5'-triphosphate. *Stroke*, 9(2), 133-136.

401. Valera.S., Hussy.N., Evans.R.J., Adami.N., North.R.A., Suprenant.A., Buell.G. (1994). A new class of ligand gated ion channel defined by P2x purinoceptor for extracellular ATP. *Nature*,371, 516-519.

402. Valera.S., Talabot.F., Evans.R.J., Gos.A., Antonarakis.S.E., Morris.M.A., Buell.G.N. (1996). Characterization and chromosomal localization of a human P2X receptor from the urinary bladder. *Recept Channels.*, 3, 283-289.

403. Voelkel.N.F. (1986). State of Art. Mechanisms of hypoxic pulmonary vasoconstriction. *American Rev of Resp Disease*, 133, 1186-1195.

404. von Euler.U.S., Liljestrand.G. (1946). Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol Scand.*, 12, 301-320.

405. von Kugelgen.I., Haussinger.D., Starke.K. (1987). Evidence for a vasoconstriction-mediating receptor for UTP, distinct from the P2-purinoceptor, in rabbit ear artery. *Naunyn Schmeideberg's Arch Pharmacol.* 336, 556-560.

406. von Kugelgen., Starke.K. (1990). Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P2X-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn Schmeideberg's Arch Pharmacol.*, 341, 538-546.

407. Wadsworth.R.M. (1994). Vasoconstrictor and vasodilator effects of hypoxia. *TIPS,* 15, 47-53.

408. Wagenvoort.C.A., Wagenvoort.N. (1977). Pathology of pulmonary hypertension. *John Wiley & Sons New York.*

409. Wang.P., Ba.Z.F., Chaudry.I.H. (1995). ATP-$MgCl_2$ restores depressed endothelial cell function after hemorrhagic shock and resuscitation. *Am J Physiol.*, 268 (4pt 2), H1390-6.

410. Wang.C.-Z., Namba.N., Gonoi.T., Inagaki.N., Seino.S. (1996). Cloning and pharmacological characterization of a fourth P2X receptor subtype widely expressed in brain and peripheral tissue including various endocrine tissues. *Biochem Biophys Res Commun.*, 220, 196-202.

411. Webb.T.E., Bateson.A.N., Barnard.E.A. (1993). Isolation and characterization of a novel family of G protein-coupled receptors. *Biochem Soc Trans.*, 21 (2), 199s.

412. Webb.T.E., Simon.J., Krishek.B.J., Bateson.A.N., Smart.T.G., King.B.F., Burnstock.G., Barnard.E.A. (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS letters*, 324 (2), 219-225.

413. Webb.T.E., Simon.J., Batseon.A.N., Barnard.E.A. (1994). Transient expression of the recombinant chick brain purinoceptor and localization of the corresponding mRNA. *Cellular and Molecular Biology*, 40 (3), 437-442.

414. Webb.T.E. (1996) (refer to ref. 456).

415. Webb.T.E., Henderson.D., King.B.F., Wang.S., Simon.J., BatesonA.N., Burnstock.G., Barnard.E.A. (1996). A novel G protein-coupled P_2 purinoceptor (P_{2Y3}) activated preferentially by nucleoside diphosphates. *Mol Pharmacol.*, 50(2), 258-265.

416. Webb.T.E., Kaplan.M.G., Barnard.E.A. (1996). Identification of 6H1 as a P_{2Y} purinoceptor: $P2Y_5$. *Biochem Biophys Res Commun.*, 219, 105-110.

417. Weintraub.W.H., Negulescu.P.A., Machen.T.E. (1992). Calcium signalling in endothelial: cellular heterogeneity and receptor internalization. *Am J Physiol.*, 263 (5pt1), c1029-1039.

418. Weir.E.K., Archer.S.L. (1995). The mechanisms of acute hypoxic pulmonary vasoconstriction, the tale of two channels. *FASEB J.*, 9, 183-189.

419. Wharton.J., Haworth.S.G., Polak.J.M. (1988). Postnatal development of the innervation and paraganglia in the porcine pulmonary arterial bed. *J Pathol.*, 154, 19-27.

420. White.T.D., Chaudry.A., Vohra.M.M., Webb.D., Leslie.R.A.. (1985). Characteristics of

P2 (nucleotide) receptors mediating contraction and relaxation of rat aortic strips : possible physiological relevance. *Euro J Pharmacol.,* 118, 37-44.

421. Wilkinson.G.F., Purikiss.J.R., Boarder.M.R. (1994). Differential heterologous and homologous desensitization of two receptors for ATP (P2y purinoceptors and nucleotide receptors) coexisting on endothelial cells. *Mol Pharmacol.*, 45 (4), 731-736.

422.Windscheif.U., Ralevic.V., Baumert.H.G., Mutschler.E., Lambrecht.G., Burnstock.G. (1994). Vasoconstrictor and dilator response to various agonist in the rat perfused mesenteric arterial bed : selective inhibition by PPADS of contraction mediated via P2x-purinoceptors. *Br J Pharmacol.*, 113, 1015-1021.

423. Woodmansey.P.A., O'Toole.L., Channer.K.S., Morice.A.H. (1996). Acute pulmonary vasodilatory properties of amlodipine in humans with pulmonary hypertension. *Heart*, 75 (2), 171-173.

424. Yoshimura.K., Tod.M.L., Pier.K.G., Rubin.L.J. (1989). Role of venoconstriction in thromboxane-induced hypertension and edema in lambs. *J Appl Physiol.*,66 (2), 929-935.

425. Zellers.T.M., Vanhoutte.P.M. (1991). Endothelium-dependent relaxations of piglet pulmonary arteries augment with maturation. *Pediatr Res.*, 30 , 176-180.

426. Zhang.F., Woodmansey.P.A., Morice.A.H.. (1995). Acute hypoxic vasoconstriction in isolated rat small and large pulmonary arteries. *Physiol Res.*, 44 (1), 7-18.

427. Zhao.M., Bo.X., Burnstock.G. (1996). Distribution of [3H] alpha, beta-methylene ATP binding sites in pulmonary blood vessels of different species. *Pulm Pharmacol.*, 9(3), 167-174.

428. Zhao.Y. (1993). Pulmonary vein contracts in response to hypoxia. *Am J Physiol.*, 265,L87-L92.

429. Zhao.Y.J., Wang.J., Rubin.L.J., Yuan.X.J. (1997). Inhibition of K (V) and K (Ca^{2+}) channels antagonizes NO-induced relaxation in pulmonary artery. *Am J Physiol.*, 272 (2 pt 2), H904-H912.

430. Ziganshin.A.U., Hoyle.C.H., Bo.X., Lambrecht.G., Mutschler.E., Baumert.H.G., Burnstock.G. (1993). PPADS selectively antagonizes P2x-purinoceptor-mediated response in the rabbit urinary bladder. *Br J Pharmacol.*, 110, 1491-1495.

445. Ziganshin.A.U., Hoyle.C.H.V., Burnstock.G. (1994). Ecto-enzymes and metabolism of extracellular ATP. *Drug Develop Res.*, 32, 134-146.

446. Ziganshin.A.U., Hoyle.C.H.V., Lambrecht.G., Mutschler.E., Baumert.H.G., Burnstock.G. (1994). Selective antagonism by PPADS at P2x-purinoceptors in receptor isolated blood vessels. *Br J Pharmacol.*, 111, 923-929.

447. Ziyal.R., Ziganshin.A.U., Nickel.P., Ardunay.U., Mutschler.E., Lambrecht.G., Burnstock.G. (1997). Vasoconstrictor responses via P2X-receptors are selectively antagonized by NF023 in rabbit isolated aorta and saphenous artery. *Br J Pharmacol.,* 120, 954-960.

448. Zubrow.A.B., Imaizumi.S.O., Tulenko.T.N. (1989). Endothelium-mediated relaxation in the fetal pulmonary artery. *Pediatr Res.*, 25 , 75A.