The control of ductus arteriosus smooth muscle contractility.

Studies on the physiological and pharmacological properties of the isolated fetal rabbit ductus arteriosus.

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Abstract.

1. This work studied the smooth muscle of the rabbit ductus arteriosus. The ductus is a shunt blood vessel of the fetus which extends between the main pulmonary artery and aorta and closes after birth.

2. This work had the following aims regarding the isolated fetal rabbit ductus arteriosus (a) to characterise the ideal experimental conditions for study of the vessel; (b) to characterise the dilator prostanoid receptors on the vessel; (c) to characterise the effect of prostaglandin E_2 (PGE_2) on the response of the vessel to noradrenaline; (d) to characterise the effect of indomethacin on the response of the vessel to PGE_2 and other vasodilators; (e) to characterise the effect of oxygen tension on both the response of the vessel to the contractile effect of noradrenaline and the relaxant effect of PGE_2 and other vasodilators; and, (f) to characterise contractile effects of prostanoids on the vessel.

3. The ductus arteriosus was isolated from fetal New Zealand White rabbits obtained at 28 days gestation following Caesarean section of the sacrificed mother. The vessel was mounted *in vitro* as a ring preparation and its responses to contractile and relaxant agonists were quantified. The effects of experimental manipulations on these responses were studied, namely, the effects of receptor antagonists, enzyme inhibitors and varying oxygen tension.

4. The ideal experimental conditions for the study of contractile and relaxant responses were characterised looking at the effects of noradrenaline and PGE_2.

5. The dilator prostanoid receptors (P receptors, e.g. PGE_2 receptor = EP receptor) on the ductus were characterised with the vessel pre-contracted

with 1μM indomethacin and 25mM K^+ in 15% oxygen. Of a range of synthetic prostanoids with selective agonism at EP_1, EP_2 or EP_3 receptors, all were at least 100 times less potent than PGE_2 (EC_{50} [i.e. interpolated molar concentration causing 50% maximum response] = 0.36nM). The rank order of potency was: PGE_2 >> misoprostol (145-fold less potent) > GR63799X (685-fold less potent) >> AH13205 (>100,000-fold less potent) ≥ sulprostone (>10,000-fold less potent) ≥ 0. The EP_1 antagonists, AH6809 (10μM) and SC19220 (30μM), had no significant effect on the sensitivity of the ductus to PGE_2. The EP_4 antagonist, AH23848B, caused concentration-related shifts to the right of the PGE_2 concentration relaxation response curve yielding a pA_2 of 4.91 but with a Schild plot slope of 1.46. The EP_2 agonist, AH13205, while only causing a very small relaxation, acted as a competitive antagonist of PGE_2 with a pA_2 of 4.85. Cicaprost (selective IP agonist) and BW245C (selective DP agonist) both relaxed the ductus but were less potent than PGE_2 (110-fold less potent and 875-fold less potent, respectively). AH23848B and AH13205 (both 30μM) inhibited the sensitivity of the ductus to BW245C to a similar extent as that to PGE_2 but had little or no effect on its sensitivity to cicaprost. The selective DP receptor blocking drug, BW868C (10μM), caused no significant decrease in sensitivity to BW245C.

6. In the absence of indomethacin, PGE_2 (from 1nM to 100nM) increased the EC_{50} to noradrenaline 3 fold. In the presence of 1μM indomethacin, PGE_2 (0.01nM to 100nM) increased the EC_{50} to noradrenaline 245 fold. By comparing the control pEC_{50} (i.e. $-\log_{10}EC_{50}$) to noradrenaline with the relationship between the pEC_{50} to noradrenaline and [PGE_2] in 1μM indomethacin, the effect of endogenous PGE_2 synthesised in the vessel wall was estimated as being equivalent to a bath concentration of approximately 1nM exogenous PGE_2.

7. When the vessel was pre-contracted with 10μM noradrenaline, indomethacin had no effect on the dilator response to PGE_2, but did alter

the response to other vasodilators. The sensitivity of the vessel to cicaprost, cromakalim and forskolin was decreased in 1μM indomethacin compared with control. Forskolin caused complete relaxation in the presence and absence of indomethacin. Indomethacin decreased the maximum response to cromakalim but increased the maximum response to cicaprost. 0.3nM PGE_2 partially reversed the effect of indomethacin on the sensitivity of the vessel to forskolin.

8. Oxygen tension had no effect on the maximum contractile response (MCR) to noradrenaline in 0.01nM PGE_2, but a profound inhibitory effect of fetal oxygen tension on the MCR to noradrenaline was uncovered by PGE_2 in a concentration-dependent manner across the range 0.1-10nM.

9. With the ductus pre-contracted with 1μM indomethacin and 10μM noradrenaline, nifedipine and atrial natriuretic peptide had no effect on ductal tone in either 2% or 15% oxygen (oxygen tension: 19-23mmHg and 100-110mmHg, respectively). In 15% oxygen, the rank order of maximum relaxant response (MRR) to dilator agonists was forskolin > cicaprost > PGE_2 >> cromakalim >> sodium nitroprusside ≈ adenosine ≈ 0. The MRR of all agonists was increased in 2% oxygen except forskolin which caused complete relaxation in 15% oxygen. In 15% oxygen, the rank order of pEC_{50} was PGE_2 >> cicaprost ≈ cromakalim ≈ forskolin. PGE_2 was about 70 times more potent than cicaprost. The pEC_{50} of all four agonists was increased in 2% oxygen. The increase in pEC_{50} could not be explained by a decreased extent of pre-contraction. The MRR to PGE_2 in 15% oxygen and the magnitude of the increase in pEC_{50} to PGE_2 going from 15% to 2% oxygen were the same in 1μM flubriprofen, 1μM indomethacin or in the absence of these drugs. However, in 2% oxygen, the MRR to PGE_2 was increased in 1μM indomethacin or 1μM flubriprofen compared with control.

10. In 1μM indomethacin and 300nM forskolin, both U46619 (TP agonist) and sulprostone (EP_1 and EP_3 agonist) caused concentration-dependent

contractions of the ductus in the nanomolar range (EC_{50}s 33nM and 42nM, respectively). The response to U46619 was shifted to the right by the TP receptor antagonist EP 092. Responses to GR63799X (EP_1 and EP_3 agonist) and $PGF_{2\alpha}$ were complicated by the fact that these agonists caused relaxation at high concentrations (≥30nM). In 10nM PGE_2, U46619, sulprostone and GR63799X elicited similar contractile responses, whereas $PGF_{2\alpha}$ had no effect. Incubation in 10nM U46619 or $PGF_{2\alpha}$ had no effect on the sensitivity of the ductus to the dilator effect of PGE_2. However, 10nM sulprostone increased the EC_{50} to PGE_2 by 119% and 3μM sulprostone increased it by 157%.

11. The main conclusions drawn from this work are as follows:

(a) the dilator EP receptor on the ductus arteriosus is not EP_1, EP_2, or EP_3, but probably the recently described EP_4 sub-type

(b) the ductus arteriosus has a dilator IP receptor, but no DP receptor

(c) PGE_2 inhibits the sensitivity of the ductus arteriosus to noradrenaline

(d) indomethacin alters the response of the ductus to exogenous dilators by the elimination of endogenous PGE_2 and PGI_2

(e) fetal oxygen tension and PGE_2 act synergistically to inhibit the response of the ductus to noradrenaline

(e) raising the oxygen tension in the physiological range which occurs at birth decreases the sensitivity of the ductus to a diverse range of vasodilators

(f) an endogenous cyclo-oxygenase product inhibits the response of the ductus to PGE_2 in fetal oxygen tension

(g) the ductus has a contractile TP receptor and a contractile EP receptor (probably EP_3) and the latter inhibits the vessel's sensitivity to the dilator effect of PGE_2.

(h) pooling the findings with cicaprost (in 5, 7 and 9 above), PGI_2 almost certainly has a physiological role in the control of ductus arteriosus smooth muscle.

List of contents.

List of Tables.

Page

List of Figures.

Page.

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List of full papers, published or in press, from this thesis.

1. Smith GCS, McGrath JC. Prostaglandin E_2 and fetal oxygen tension synergistically inhibit response of isolated fetal rabbit ductus arteriosus to norepinephrine.
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4. Smith GCS, Coleman RA, McGrath JC. Characterization of dilator prostanoid receptors in the fetal rabbit ductus arteriosus.
Journal of Pharmacology and Experimental Therapeutics (in press).

5. Smith GCS, McGrath JC. Contractile effects of prostanoids on the fetal rabbit ductus arteriosus.
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Chapter 1. Introduction.

1.1 The physiology of the ductus arteriosus.

1.1.1 The ductus arteriosus: an overview.

The ductus arteriosus is a fetal shunt blood vessel which extends between the main pulmonary artery and the descending aorta. It is patent in fetal and early neonatal life. The direction of flow is determined by the pressure gradient across the vessel, which is a function, therefore, of the aortic and pulmonary artery pressures. In the fetus, blood flow is from the pulmonary artery to the aorta but the direction of blood flow across the ductus reverses soon after birth. The vessel closes in the first few days of life. In fetal life, the function of the ductus is to divert deoxygenated blood from the pulmonary circulation to the descending aorta where a proportion is diverted to the umbilico-placental circulation, where gaseous exchange takes place. Patency of the ductus in early neonatal life improves pulmonary blood flow and arterial oxygen tension and is one of the features of the transitional circulation of the neonate.

Contraction of ductus arteriosus smooth muscle is inhibited *in utero* by prostaglandins. At birth, elimination of this inhibitory effect and the direct contractile effect of increasing oxygen tension and of humoral vasoconstrictors cause contraction of the ductus resulting in its closure, typically within 48 hours of birth (in the human). The lumen is then obliterated by necrosis. The ability to manipulate ductal patency by controlling ductal smooth muscle is important in a number of clinical situations. This thesis concentrates on the control of contractility of ductus arteriosus smooth muscle.

1.1.2 The fetal circulation.

One of the fundamental differences between the fetal and adult

circulations is that in the fetus the two ventricles of the heart pump in parallel due to the presence of shunts, whereas in the adult the ventricles pump in series. There are three main shunts in the fetal circulation: the ductus arteriosus, the ductus venosus and the foramen ovale (see Mott, 1982 for review). In the fetus, the ductus arteriosus shunts blood from the pulmonary artery to the descending aorta. Right ventricular output in the fetus is 65% of the combined ventricular output[1] (CVO) and, as in the adult, this is deoxygenated blood. Clearly, the lungs are not ventilated *in utero,* and gaseous exchange takes place at the placenta. There is a need, therefore, for the redirecting of this deoxygenated blood to the umbilico-placental circulation. The vast majority of the right ventricular output is diverted from the main pulmonary artery, through the ductus (57% CVO), to the descending aorta, where a proportion (40% CVO) is diverted to the umbilico-placental circulation, where gaseous exchange takes place. There is no left to right shunt across the ductus under normal conditions in the fetus (Teitel *et al,* 1987).

The direction of blood flow across the ductus is determined by the pressure gradient across the vessel. In the fetus, pulmonary vascular resistance is very high due to hypoxic pulmonary vasoconstriction and systemic pressure is low due to the presence of the low resistance umbilico-placental circulation: the shunt across the ductus is, therefore, right to left (Anderson *et al,* 1981). The current understanding of the ductus in the fetus is that it is a relatively passive structure and its role is simply to remain widely patent.

1.1.3 The neonatal transitional circulation.

Following birth, the pressure gradient across the ductus is reversed, which is mediated by ventilation of the lungs, oxygenation of the lungs and the loss of the low resistance umbilico-placental circulation (Teitel *et al,*

[1]As the ventricles pump in parallel, cardiac output in the fetus is expressed as the combined ventricular output. In the adult the output of both ventricles is, necessarily, the same, and the cardiac output refers to that of each ventricle.

1987). In the neonate, flow across the ductus is from the aorta to the pulmonary artery, until the vessel closes in the first 24 to 48 hours of life (Drayton and Skidmore, 1987). Immediately following birth, lung function is sub-optimal and arterial oxygen tension is less than in the healthy adult (Heymann and Rudolph, 1975). Redirection of blood from the aorta to the pulmonary artery through the ductus arteriosus improves arterial oxygen tension at the expense of systemic (but not cardiac or cerebral) blood flow (Clyman *et al,* 1987). Ligation of the ductus in the neonatal lamb decreases pulmonary blood flow and arterial oxygen tension (Dawes *et al,* 1955). Physiological patency of the ductus in the first hours of life is, therefore, an adaptive mechanism which improves arterial oxygen tension at a time of critical changes in cardio-respiratory function.

The left to right shunt in the ductus in the human begins to decline a few hours after birth and falls steadily until closure (Drayton and Skidmore, 1987). Closure of the ductus is effected by contraction of its thick muscular wall. Following this functional closure, fibrosis occurs and ultimately a remnant of the vessel is formed, the *ligamentum arteriosum.* The major focus for research on the ductus arteriosus has been the factors which control contraction of its smooth muscle. This is the primary interest of this thesis and the current state of understanding is reviewed below.

1.2 Control of ductus arteriosus smooth muscle.

1.2.1 Oxygen.

In fetal life, the ductus is exposed to an oxygen tension of about 20-28mmHg and this rises rapidly following birth (see Heymann and Rudolph, 1975). It was first observed over 50 years ago that the ductus arteriosus contracts to increasing oxygen tension (Kennedy and Clark, 1942) and this has been postulated to be important in the post-natal closure of the vessel. The oxygen contraction of the ductus increases towards term (Noel and Cassin, 1976; Clyman *et al,* 1979) and can be enhanced, in the pre-term animal, by antenatal administration of hydrocortisone (Clyman *et al,* 1981c).

There has been a great deal of work directed at elucidating the mechanism of the contractile effect of oxygen. Given the importance of prostaglandins in dilating the vessel *in utero* (see section 1.2.3), a number of authors have speculated that the effect of oxygen might be mediated by a constrictor metabolite of arachidonic acid.

It has been suggested that $PGF_{2\alpha}$ might mediate the oxygen-induced contraction of the ductus (Starling and Eliott, 1974), however, this is unlikely as the response to oxygen persists in the presence of the prostaglandin synthesis inhibitor, indomethacin (Smith and McGrath, 1988). Likewise, lipoxygenase inhibition has no effect on the oxygen contraction (Coceani *et al,* 1982).

Carbon monoxide relaxes the ductus (Coceani *et al,* 1984) which led these authors to hypothesise that a mono-oxygenase cytochrome P_{450} arachidonic acid metabolite may mediate oxygen contraction of the ductus. This hypothesis was supported by two observations: firstly, that monochromatic light reversed the effect of carbon monoxide, an effect which was maximal at a wavelength of 450nM (characteristic of the P_{450}

system) (Coceani *et al,* 1988); and, secondly, that metyrapone, a chemical inhibitor of some cytochrome P_{450} isoenzymes, also relaxed the ductus (Coceani *et al,* 1984). However, the same study demonstrated that a range of other chemical inhibitors of this system had only minor effects on the ductus, which undermined the hypothesis. Furthermore, in the presence of combined cyclo-oxygenase and lipoxygenase inhibition, arachidonic acid had only a dilator effect on the ductus (Coceani *et al,* 1988) which suggests that if a chemical mediator of the oxygen-induced contraction exists, it is not an arachidonic acid metabolite. The fact that carbon monoxide also relaxed the ductus in fetal oxygen tension implies that it may relax the ductus by a mechanism unrelated to the oxygen-induced contraction. It has been demonstrated that carbon monoxide stimulates the synthesis of cyclic guanosine monophosphate (cGMP) and relaxes smooth muscle: this is reversed by monochromatic light with a maximum effect at 422nM (Utz and Ullrich, 1991). While Coceani found that maximal reversal of the effect of carbon monoxide on the ductus occurred at 450nM, other authors have found maximal reversal of the effect of carbon monoxide on the oxygen-contracted ductus to occur at 420-425nM (Fay and Jobsis, 1972). Furthermore, a diverse range of arachidonic acid epoxides has no contractile effect on the ductus (Coceani *et al,* 1988). It seems unlikely, therefore, that the oxygen-induced contraction of the ductus is mediated by a constrictor metabolite of arachidonic acid.

Recently, the same group have proposed that endothelin may mediate the oxygen-induced contraction of the ductus: endothelin contracts the isolated lamb ductus (Coceani *et al,* 1989); it is released both by the endothelium and media of the vessel (Coceani and Kelsey, 1991); the release of endothelin is stimulated by increasing oxygen tension (Coceani *et al,* 1992) and the oxygen-induced contraction of the ductus is inhibited by the endothelin receptor antagonist BQ123 (Coceani *et al,* 1992). However, another study demonstrated that endothelin had no

effect on the lamb ductus *in utero* (Chatfield *et al,* 1991), despite the fact that oxygen can contract the ductus *in utero* (Mentzer *et al,* 1985). Furthermore, another study on human cultured ductal cells demonstrated no stimulatory effect of oxygen on endothelin release and that ductal smooth muscle cells release only 5-10% of the levels of endothelin released by endothelial cells (Day *et al,* 1994). Finally, the oxygen-induced contraction of the ductus is unaffected by removal of the endothelium (Coburn *et al,* 1986). These observations suggest that endothelin does not mediate the oxygen-induced contraction of the ductus.

Fay (1971) demonstrated some fundamental properties of the oxygen-induced contraction of the guinea-pig ductus: oxygen had an effect across the range 0-140mmHg; the effect of oxygen was the same whether the luminal or the adventitial side of the ductus was exposed to elevated oxygen; and local anaesthetics and tetrodotoxin (inhibitors of nerve-mediated effects) had no effect on the response to oxygen. These findings suggest that oxygen acts directly on the smooth muscle cells of the ductus. In addition, Fay observed that the oxygen-induced contraction could be inhibited by a diverse range of inhibitors of oxidative phosphorylation and that the degree of contraction correlated with oxygen consumption. Neither of these facts was true of the acetyl choline-induced contraction of the ductus. Fay postulated that cytochrome a_3 was the oxygen sensor. This was partly based on the effect of carbon monoxide and its reversal by light (Fay and Jobsis, 1972). The effects of these on guanylate cyclase complicate this interpretation.

It has been demonstrated that increasing oxygen tension depolarises the ductus arteriosus (Roulet and Coburn, 1981). More recently it has been shown that the oxygen-induced contraction is dependent on the presence of extracellular calcium and that it is inhibited by calcium channel antagonists to a similar extent to the potassium-induced contraction (Nakanishi *et al,* 1993). Furthermore, glibenclamide, an inhibitor of adenosine triphosphate (ATP)-sensitive potassium channels contracts the

ductus in fetal but not elevated oxygen tension and cromakalim, an ATP-sensitive potassium channel opener, completely reverses the oxygen-induced contraction of the ductus (Nakanishi *et al,* 1993). Taken in conjunction with the work of Fay, a model for oxygen-induced contraction of the ductus has been proposed: in fetal oxygen tension, intracellular levels of ATP are low and ductal smooth muscle is hyperpolarised by ATP-sensitive potassium channels being open; when oxygen is increased, ATP levels rise, closing the potassium channels depolarising the cell membrane and causing contraction (Nakanishi *et al,* 1993). This model is only part of the explanation for the effects of oxygen, however, as oxygen can still cause contraction of the ductus in the presence of 126mM extracellular potassium (Roulet and Coburn, 1981) and calcium channel antagonists do not fully block the oxygen-induced contraction (Nakanishi *et al,* 1993), i.e. oxygen also has a membrane-independent contractile effect on the ductus.

1.2.2 Vasoconstrictors.

The ductus arteriosus of all species studied is innervated by catecholamine containing nerves (Boreus *et al,* 1969; Ikeda, 1970; Ikeda *et al,* 1972; Bodach *et al,* 1980). The catecholamine content of the ductus is similar to peripheral arteries which are known to be under autonomic neural control (Ikeda *et al,* 1972). The vessel contracts both in response to transmural stimulation of these nerves (Ikeda *et al,* 1973a; Bodach *et al,* 1980) and exposure to exogenous noradrenaline (Born *et al,* 1956; Kovalcik, 1963; Aronson *et al,* 1970; Smith and McGrath, 1988). The effect of transmural stimulation increases towards term (Ikeda *et al,* 1973b) and it is also potentiated in raised oxygen tension, as is the response of the vessel to exogenous noradrenaline (Ikeda *et al,* 1973a). The effect of transmural stimulation is blocked in part by α adrenoceptor blockade (Bodach *et al,* 1980) and treatment of the pregnant guinea pig with phenoxybenzamine (an α adrenoceptor antagonist) delays closure of the ductus in the offspring (Hornblad and Larsson, 1972). The ductus also contracts to

acetyl choline (Kovalcik, 1963; McMurphy and Boreus, 1971; Ikeda *et al,* 1973a), and the vessel is innervated with acetyl choline-containing nerves (Silva and Ikeda, 1971). The central control of activity of the pressor nerves of the ductus is unknown.

The adventitia of the guinea pig ductus has many mast cells present, which can release agents such as histamine and 5-hydroxytryptamine (Fay, 1971), both of which contract the isolated ductus (Aronson *et al,* 1970; McMurphy and Boreus, 1971). The factors which control degranulation of ductal mast cells are not known. The endothelium and media of the ductus synthesise the potent peptide vasoconstrictor, endothelin (Coceani and Kelsey, 1991). Endothelin contracts the isolated lamb ductus (Coceani *et al,* 1989) but has no effect on the vessel when infused into the chronically instrumented fetal lamb (Chatfield *et al,* 1991).

The ductus also contracts in response to circulating vasoactive agents, notably adrenaline (Kovalcik, 1963), through α adrenoceptors, and bradykinin (Kovalcik, 1963; Aronson *et al,* 1970). The latter is released following ventilation of the lungs with oxygen (Heymann *et al,* 1969) and levels of bradykinin in human cord blood are higher than the adult (Melmon *et al,* 1968). The ductus also contracts in response to steroid hormones, namely, glucocorticoids (Momma *et al,* 1981) and progesterone (Pulkkinen *et al,* 1986). The effects of the former are probably related to modulating the sensitivity of the ductus to the dilator effect of prostaglandin E_2 (PGE_2) (see section 1.2.3). The mechanism of action of progesterone is unknown but, unlike glucocorticoids (Momma and Takao, 1989a), it does not interact with the effect of indomethacin (Pulkkinen *et al,* 1986).

The response of the ductus to exogenous vasoconstrictors *in utero* seems to be less than the isolated ductus *in vitro* (Friedman *et al,* 1983, and as discussed above for endothelin). This may be due to an inhibitory effect of

prostaglandins, as the response of the ductus to exogenous noradrenaline is potentiated by indomethacin (Smith and McGrath, 1988). Furthermore, fetal oxygen tension inhibits the response of the isolated ductus to transmural stimulation and application of exogenous noradrenaline and acetyl choline (Ikeda *et al,* 1973a).

1.2.3 Prostaglandins.

Prostaglandins are oxygenated derivatives of the 20 carbon chain fatty acid, arachidonic acid, formed from the cyclo-oxygenase pathway. They generally act as local hormones with diverse biological effects. Each of the main naturally occurring prostaglandins acts at its own receptor, named after it (e.g. EP for PGE_2, IP for PGI_2, TP for TxA_2, etc.). The EP receptors have been divided into sub-types. For reviews of prostanoid pharmacology, see Coleman *et al,* (1990 and 1994b) and Smith *et al,* (1991).

In 1973, Coceani and Olley demonstrated that PGE_1 and PGE_2 relaxed the isolated lamb ductus arteriosus. It was then demonstrated that indomethacin contracted the vessel both *in vivo* (Sharpe *et al,* 1974) and *in vitro* (Coceani *et al,*1975). The effect of indomethacin is likely to be due to its inhibitory effect on cyclo-oxygenase, as a range of structurally diverse cyclo-oxygenase inhibitors contracted the vessel (Momma *et al,* 1984). This suggests that the net effect of prostaglandins on the ductus is inhibitory. With one exception (see below), all studies (in diverse species) have demonstrated that prostaglandins relax the ductus (Coceani *et al,* 1975; Sharpe and Larsson, 1975; Starling *et al,* 1976; Clyman *et al,* 1977a, 1978a and 1978c; Coceani *et al,* 1978b; Coceani *et al,* 1980; Momma *et al,* 1980; Friedman *et al,* 1985; Sideris *et al,* 1985).

The ductus is exposed to both locally released and circulating prostaglandins. The isolated ductus synthesises a range of prostaglandins: PGI_2 is the main product of arachidonic acid in the vessel and, indeed, is the only prostaglandin for which there is clear evidence of catalytic

production (Terragno *et al,* 1977; Pace-Asciak and Rangaraj, 1977, 1978 and 1983; Skidgell *et al,* 1984). The ductus also forms small amounts of PGE_2, $PGF_{2\alpha}$ and PGD_2 (all about 10% the level of PGI_2 synthesis). Prostaglandins are formed by both endothelial and smooth muscle cells of the ductus (Rabinovitch *et al,* 1989). It is thought that PGE_2 is formed by degradation of PGH_2 and not through an enzymatic pathway (Needleman *et al,* 1981; Skidgell *et al,* 1984). The ductus does, however, have catabolic enzymes for PGE_2 (Pace-Asciak and Rangaraj, 1978).

The ductus is also exposed to circulating PGE_2 and it has been suggested that circulating PGE_2 is more important in the the control of the ductus than locally released PGE_2 (Clyman, 1987). Circulating concentrations of PGE_2 increase towards term and are approximately 1-2nM in the term fetal lamb (Clyman *et al,* 1980c). These fall dramatically at birth, by 10-fold at 1 hour and by 20-fold at 3 hours (Clyman *et al,* 1980c). Loss of the dilator effect of circulating PGE_2 has been postulated to be fundamental to the closure of the ductus (Clyman, 1987). The fall in circulating concentrations of PGE_2 is thought to be due largely to the increase in lung blood flow that occurs at birth, as the lungs are the major site of prostaglandin catabolism. The main catabolic pathway for PGE_2 is 15 (OH) prostaglandin dehydrogenase: the activity of this enzyme in the lung and kidney increases towards term (Simberg, 1983; Tsai and Einzig, 1989) and activity can be induced in the pre-term animal by the administration of hydrocortisone (Tsai and Brown, 1987).

The contractile effect of indomethacin is assumed to be due primarily to the elimination of PGE_2 (Clyman, 1987; Coceani and Olley, 1988) as it is by far the most potent prostanoid dilator of the ductus and, specifically, is about 1000 times more potent than the main prostanoid synthesised by the vessel, PGI_2 (Coceani *et al,* 1978b, Clyman *et al,* 1978c). 6-keto-PGE_1 is almost as potent as PGE_2 and could, theoretically, be formed from PGI_2, however, the enzymes required for this have not been demonstrated in the

ductus (Coceani *et al,* 1980).

A dose of 10mg/kg indomethacin to the pregnant rat causes 70% constriction of the ductus of its fetuses, and this leads to signs of right sided heart failure in 1-8 hours and right ventricular hypertrophy within 24 hours (Momma and Takao, 1989b). The effect of indomethacin varies with gestational age, but the findings are different comparing *in vitro* and *in vivo* studies. The contractile effect of indomethacin and ibuprofen on the isolated ductus decreased towards term (Clyman *et al,* 1978b; Coceani *et al,* 1979), whereas the contractile effect of indomethacin *in vivo* was either the same comparing term and pre-term, or greater in term animals (Friedman *et al,* 1983; Momma and Takao, 1987; Moise et al, 1988). No explanation has been advanced for this discrepancy.

The sensitivity of the ductus to PGE_2 and PGI_2 decreases towards term (Clyman *et al,* 1980b). The sensitivity of the pre-term lamb to these agents can be decreased by antenatal administration of glucocorticoids to the mother (Clyman *et al,* 1981c). Persistent patency of the ductus in the pre-term neonate is related both to elevated circulating concentrations of PGE_2 and to increased sensitivity of the vessel to its dilator effect (Clyman *et al,* 1983b). Antenatal administration of glucocorticoids decreases the incidence of persistent patency of the ductus in premature human neonates (Clyman *et al,* 1981b). The pre-term ductus has greater PGI_2 synthase activity than term (Clyman *et al,* 1978d), but releases less PGE_2 than the term ductus (Clyman *et al,* 1979). The increased activity of PGI_2 synthase in the pre-term ductus may result in less accumulation of PGH_2 and, since PGE_2 is formed by degradation of PGH_2, may explain the low levels of PGE_2 released. Antenatal administration of hydrocortisone has no effect on PGE_2 release from the isolated pre-term ductus (Clyman *et al,* 1981a).

It has been reported in some studies that increasing oxygen tension decreases the sensitivity of the ductus to PGE_2 (Coceani *et al,* 1975) but

other studies have failed to reproduce this finding (Clyman *et al,* 1977a). Where an effect of oxygen was observed, it was abolished by indomethacin (Coceani *et al,* 1975). The two protagonists in this debate discussed their different findings in some detail and, essentially, the difference remained unexplained (Coceani and Olley, 1977; Clyman *et al,* 1977b). It has been suggested that the discrepancy may be due to the failure of one group to include CO_2 in their gas bubbling system (Smith and McGrath, 1988), a technical difference overlooked by both groups. The release of PGE_2 by the isolated ductus is stimulated by increasing oxygen (Clyman *et al,* 1980a; Coceani *et al,* 1986). Increasing oxygen actually decreases the activity of PGI_2 synthase in the ductus (Needleman *et al,* 1981), possibly by stimulating the release of a hydroperoxy fatty acid with a direct inhibitory effect of the enzyme (Needleman *et al,* 1981; Powell, 1982). This is consistent with the effect of oxygen on PGE_2: a decreased activity of PGI_2 synthase would result in accumulation of PGH_2 which would then degrade to form PGE_2. Oxygen may also act by the stimulation of free radical release, as the hydroxyl radical, but not superoxide anion, stimulates the release of dilator prostaglandins by the ductus (Clyman *et al,* 1989; Saugstad and Sanderud, 1989).

Relaxation of the ductus by PGE_2 is associated with a rise in the intracellular concentration of cyclic adenosine monophosphate (cAMP) (Walsh and Mentzer, 1987). PGE_2 acts, at least in part, by inhibiting the sensitivity of the contractile proteins to calcium (Crichton *et al,* 1994). As well as causing relaxation of the ductus, PGE_2 inhibits myogenic tone (Kriska et al 1990) and may inhibit the response of the isolated ductus to noradrenaline, as the sensitivity of the ductus to noradrenaline is increased following incubation in indomethacin (Smith and McGrath, 1988). The effect of PGE_2 decreases after birth in term animals (Momma *et al,* 1980; Clyman *et al,* 1983a). This decreased sensitivity is probably related to partially irreversible contraction (Clyman *et al,* 1983a) and it is less marked in pre-term neonates (Clyman *et al,* 1985).

Only 2 studies have looked at contractile effects of prostanoids on the ductus. The first looked at the effect of a single very high concentration (about 7μM) of $PGF_{2\alpha}$, which they found caused a small contraction of the ductus (Starling and Eliott, 1974). The other looked at the effect of a biogeneration system for TxA_2 (incubation of PGG_2 or PGH_2 with microsomal fractions of human platelets or guinea pig lungs) on the ductus (Coceani *et al,* 1978a). Whereas PGG_2 or PGH_2 relaxed the ductus (presumably by formation of PGE_2), when PGG_2 or PGH_2 were incubated in platelet or lung microsomes, they had no effect on the ductus arteriosus (lamb). The authors acknowledged the possibility that small quantities of PGE_2 were formed which may have masked effects (Coceani *et al,* 1978a).

1.2.4 Non-prostanoid vasodilators.

Both sodium nitroprusside (SNP) and glyceryl trinitrate dilate the lamb ductus *in vivo* (Walsh *et al,* 1988) and *in vitro* (Walsh and Mentzer, 1987). These agents increase the intra-cellular concentrations of both cAMP and cGMP in the ductus (Walsh and Mentzer, 1987) and, in the absence of indomethacin, their maximum effect on the oxygen-contracted ductus is greater than PGE_1. I have observed that the rabbit ductus pre-contracted with 10μM noradrenaline relaxes in response to acetyl choline in the endothelium-intact but not endothelium-denuded ductus arteriosus (Smith GCS, unpublished observations), which is preliminary evidence for release of nitric oxide by the ductus.

Adenosine reverses the oxygen-induced contraction of the lamb fetus ventilated with oxygen *in utero* (Mentzer *et al,* 1985). Furthermore, the circulating concentrations of endogenous adenosine vary inversely with both the arterial oxygen tension and the degree of contraction of the ductus arteriosus (Mentzer *et al,* 1985). However, adenosine has no effect on the indomethacin-induced contraction of the fetal lamb (Friedman *et al,* 1983).

The ductus also has β adrenoceptors which mediate relaxation (Bodach *et al,* 1980). The contractile effect of adrenaline through α adrenoceptor activation will, therefore, be offset somewhat by its dilator effect through β adrenoceptors. Nevertheless, the net effect of adrenaline in the isolated vessel *in vitro* is contractile (Kovalcik, 1963).

1.3 Closure of the ductus arteriosus.

1.3.1 Control systems in closure.

There is as yet no clear model of control of contraction of the ductus in the neonate. As discussed in section 1.2, there are a number of factors which control ductal smooth muscle and in most cases there is a clear pattern of inhibitory influences decreasing towards term and being eliminated after birth and contractile influences increasing towards term and being promoted following birth. However, the ductus is widely patent immediately prior to birth despite the changes that occur towards term. Furthermore, many of the factors identified as promoting contraction of the ductus at birth change very rapidly following birth which is in contrast to the slower closure of the ductus. A good example of this is the release of bradykinin from the lungs at birth, which had been proposed to be involved in the post-natal closure of the vessel (see section 1.2.2): the release of bradykinin is immediate, whereas the closure of the ductus is (relatively) delayed.

It seems likely that oxygen tension and PGE_2 are central to the control of the ductus, as the two most remarkable features of the control of ductal smooth muscle are its profound contractile response to oxygen and its profound relaxant response to PGE_2. However, it is not clear how these factors interact at birth. As discussed in section 1.2.3, increasing oxygen actually stimulates PGE_2 release by the ductus. Any model of ductal closure will have to explain why the factor which is central to stimulating contraction of the vessel after birth (i.e. oxygen) causes the release of the factor central maintaining the vessel in a patent state *in utero* (i.e. PGE_2).

Finally, in considering the control systems regulating the ductus, the focus has been on the efferent, effector limb of the control model. The nature of the sensor and afferent limb has been somewhat neglected. Interestingly, the ductus has structures in its wall which are similar to the carotid and aortic bodies (Boyd, 1941; Fay, 1971; Macdonald *et al,* 1983) which send afferent fibres to the left vagus nerve (Boyd, 1941). Given the pressor innervation of the ductus (see 1.2.2), there may indeed be a neural control loop. Again, there is no model to link the action of the pressor nerves of the ductus with the effects of oxygen and PGE_2.

1.3.2 Anatomical aspects of ductal closure.

Closure of the ductus in the neonate has been thought of as consisting of two distinct processes: an initial, functional closure by contraction of ductal smooth muscle and a later, irreversible closure of the vessel by microanatomical changes in its structure (Heymann and Rudolph, 1975).

The anatomical changes have been considered in 4 stages (Gittenberger *et al,* 1985): (1) creation of an area of sub-endothelial oedema formed by separation of the endothelium from the internal elastic lamena (2) endothelial cell infolding and ingrowth with migration of undifferentiated smooth muscle cells from the inner media into the sub-endothelial layer, forming "sub-intimal cushions". (3) endothelial cell apposition (4) fibrotic degeneration of the vessel. The anatomical changes are promoted by indomethacin and inhibited by PGE_2 (Mine, 1981), although ductal smooth muscle cell migration is inhibited by indomethacin *in vitro* (Koppel and Rabinovitch, 1993).

The theoretical dissociation of the functional and anatomical contraction of the ductus is a mistake. The ductus begins to close after a few hours of birth, even though the process takes 24 to 48 hours to be completed. Even the initial contraction of the ductus seems to be only partially reversible, which suggests that anatomical changes are taking place before the

functional contraction is complete (Clyman *et al,* 1983a). Interestingly, the initial contraction of the pre-term ductus is fully reversible (Clyman *et al,* 1985), which suggests that the biological processes required for these anatomical changes following closure are not present in the pre-term animal and must develop towards term.

1.4 Clinical significance of the ductus arteriosus.

There are 3 main clinical situations where the therapeutic control of the ductus arteriosus is important. In two of these, useful clinical therapies were developed from basic pharmacological studies on animals, and the ductus arteriosus is a clear example of a situation where animal research has led to novel and important human drug development.

1.4.1 Patent ductus arteriosus.

As the name suggests, patent ductus arteriosus (PDA) is failure of the ductus to close soon after birth. There are two main groups of patients suffering from this: firstly, premature infants and, secondly, older children and adults. Closure of the ductus in the latter case is surgical. There is the option of drug treatment to close the ductus in premature infants which has arisen from an understanding of the vessel's basic pharmacology.

The treatment for PDA in premature infants was originally surgical ligation of the vessel. The disadvantages of this form of treatment were (1) the infants are small and often unwell (2) the required level of surgical expertise was only found in major centres, necessitating transfer of sick premature infants from smaller centres. The observation that indomethacin contracted the ductus arteriosus of lambs and rabbits led directly to its use in human infants and it is currently a widely used technique in closure of PDA (see Gersony, 1986 for review). The success rate of indomethacin is 70%, compared with 35% for placebo. The major adverse effects of indomethacin are similar to the adult: renal impairment, gastro-intestinal

bleeding and bleeding tendency (secondary to its effects on platelets). Recently, more sophisticated 'closed' surgical techniques of ductal closure have been developed, namely the Rashkind occluder (Rashkind *et al,* 1987). The continued need for development of treatments reflects the relatively high failure rate of indomethacin and its adverse effects.

1.4.2 Ductus-dependent circulation.

The term "ductus-dependent circulation" applies to a diverse range of congenital heart defects where, as a consequence of the nature of the defect, continued patency of the ductus arteriosus of the neonate is necessary for its survival (see Freed *et al,* 1981). There are 3 roles that the ductus can have in such circumstances: (1) to maintain adequate pulmonary blood flow, e.g. in pulmonary atresia; (2) to maintain adequate systemic blood flow, e.g. in aortic arch abnormalities; and (3) to improve mixing of the systemic and pulmonary circulations, e.g. in transposition of the great arteries. The natural history of these conditions is that the infant is often healthy at birth, but then deteriorates in early neonatal life as the ductus closes. Formerly, detection of such a defect was an indication for emergency surgery before ductal closure was complete. The observation that PGE_2 was a potent dilator of the lamb ductus led directly to the use of PGE_1 (or PGE_2) to maintain artificially ductal patency until definitive surgery could be performed. This therapy results in often dramatic clinical improvement in infants with these diverse conditions. It is not effective, however, when closure of the ductus is complete and is most effective where the initial degree of closure is smallest (Clyman *et al,* 1983a). With the ability to diagnose congenital heart defects pre-natally, it has been suggested that ductal patency in infants with these conditions could be optimised by pre-natal diagnosis and then commencing therapy immediately following birth in affected infants (Smith, 1992).

Intravenous PGE_1 has major adverse effects, including apnoea (10-15%), pyrexia (10-15%), cardiac arrest, seizures/jitteriness, hypotension, flushing (10%) and diarrhoea (Gersony, 1986). These adverse effects are all the

more serious since the infants are often unwell due to their cardiac defect. There is currently no alternative to the E series prostaglandins in the treatment of these conditions and the importance of the therapy is such that the adverse effects have to be accepted.

1.4.3 Pregnancy.

The use of non-steroidal anti-inflammatory drugs (NSAID) in pregnancy has been recently reviewed (Van den Veyver and Moise, 1993). One of the problems with these drugs in pregnancy is that they cross the placenta and contract the fetal ductus arteriosus. There are 3 main situations where they are used in pregnant women: (1) treatment of pre-existing medical disease, e.g. rheumatoid arthritis- generally, the NSAID would be substituted for a simple analgesic for the duration of pregnancy (2) treatment of premature labour- NSAIDs (indomethacin is most widely used) are uterine tocolytics; and, (3) treatment of polyhydramnios- NSAIDs (again, indomethacin is most widely used) decrease fetal urine output which decreases production of liquor.

Significant contraction of the ductus arteriosus is seen in over half of fetuses in the course of maternal administration of indomethacin for premature labour and the extent of contraction varies directly with the gestational age of the fetus (Moise *et al,* 1988). There are isolated reports of neonatal deaths apparently secondary to NSAID-induced contraction of the fetal ductus, although studies in pre-term labour suggest that constriction of the fetal ductus following maternal administration of indomethacin does not result in significant cardiovascular disease in the neonate (see Van den Veyver and Moise, 1993).

1.5 Potential for drug development.

1.5.1 Patent ductus arteriosus.

The effect of indomethacin on the ductus arteriosus is thought to be mediated entirely by elimination of the dilator effect of PGE_2 (Clyman, 1987; Coceani and Olley, 1988). A selective antagonist of the ductal PGE_2 receptor (EP receptor) would be expected, therefore, to be at least as effective a constrictor of the ductus as indomethacin. Furthermore, it is likely that most of the adverse effects of indomethacin (see above) are mediated by eliminating the effects of prostaglandins on different prostanoid receptors compared with its contractile effect on the ductus. A selective antagonist of the ductal EP receptor would not, therefore, be expected to have many of the adverse effects of indomethacin. Identification of the EP receptor sub-type mediating the effects of PGE_2 on the ductus arteriosus would provide a clear strategy for the development of safer and more effective therapies in PDA.

1.5.2 Ductus-dependent circulation.

PGE_1 is a potent agonist of all the EP receptor sub-types (Coleman *et al,* 1990). Although the EP receptor sub-types mediating the adverse effects of PGE_1 are unknown, it is highly unlikely that the same receptor mediates both the therapeutic effect of PGE_1 on the ductus arteriosus and all its diverse adverse effects. A selective agonist of the ductal EP receptor would be expected to be as effective as PGE_1 in ductus-dependent circulation, but with fewer adverse effects. Again, identification of the EP receptor sub-type mediating the effects of PGE_2 on the ductus arteriosus may lead to the development of safer and more effective drugs in ductus-dependent circulation.

Furthermore, there is some evidence that the effect of PGE_2 on the ductus is inhibited by increasing oxygen tension (Coceani *et al,* 1975). In ductus-dependent cyanotic congenital heart disease, PGE_1 increases

arterial oxygen tension (Freed *et al,* 1981). If oxygen decreases the sensitivity of the ductus to PGE_2, it may be that when PGE_1 is administered in these forms of heart defect, its therapeutic effect (i.e. increasing arterial oxygen tension) may decrease the sensitivity of its target (i.e. the ductus) to its action: i.e. there is potential for this therapy to be self-limiting. If this effect of oxygen is specific to PGE_2, other vasodilators may be more appropriate in these situations.

Finally, it has been demonstrated in some experimental conditions that indomethacin increases the sensitivity of the ductus to PGE_2 (Coceani *et al,* 1975). All the comparisons between PGE_2 and other prostanoids were made in the presence of indomethacin (Clyman *et al,* 1978a and 1978c; Coceani *et al,* 1978b; Friedman *et al,* 1983) and this may have exaggerated the relative potency of PGE_2 as a ductal dilator. There may be other agents which are equally effective dilators of the ductus but have fewer side-effects and could be used in ductus-dependent circulation.

1.5.3 Premature labour.

Indomethacin inhibits uterine contraction by eliminating contractile prostaglandin effects on the myometrium. The prostanoid receptors mediating these contractile effects on human myometrium are EP_1, EP_3, FP and TP (Senior *et al,* 1991, 1992). There are two theoretical approaches to this clinical problem to avoid contraction of the ductus: firstly, one could develop a selective prostanoid receptor antagonist which blocked one or more of the contractile receptors on myometrium but was without activity at the ductal prostanoid receptor(s); or, secondly, one could co-administer a selective agonist of the dilator EP receptor on the ductus with indomethacin to protect the vessel from indomethacin contraction. Both situations require that the dilator EP receptor on the ductus is different from the contractile EP receptors on the uterus, which requires characterisation of the ductal EP receptor.

1.6 Aims of this work.

The aims of this work were directed at improving both the basic understanding of the physiology and pharmacology of closure of the ductus and identifying promising new avenues for the manipulation of the ductus in the clinical conditions discussed above. Clearly, there were areas of overlap between these two areas of study. The specific aims were as follows.

1.6.1 Interactions between PGE_2 and noradrenaline.

Research on the effect of endogenous and exogenous PGE_2 on the ductus has focused on the effect of this drug on the vessel's intrinsic tone. I have demonstrated that indomethacin increases the sensitivity of the ductus to noradrenaline (Smith and McGrath, 1988), implying that PGE_2 decreased the sensitivity of the ductus to noradrenaline. One aim of the study was to characterise the effect of PGE_2 on the vessel's response to noradrenaline.

1.6.2. Interactions between PGE_2 and oxygen.

As discussed in section 1.2.1, the two main factors in the physiological regulation of the ductus are PGE_2 and oxygen tension. A major goal of this study was to characterise the relationship between oxygen tension and the vessel's response to PGE_2, and to establish whether any effect of oxygen on PGE_2 was specific to PGE_2. Furthermore, I sought to establish whether the two factors which dilate the ductus *in utero* (low oxygen and high PGE_2) interact together in controlling the vessel's response to noradrenaline. As well as shedding light on the physiological control of the vessel, these questions are of relevance to the therapeutic control of the ductus as discussed above.

1.6.3 EP receptor sub-type.

Perhaps the clearest avenue for drug development in the therapeutic control of the ductus is the use of selective agonists and antagonists of the EP receptor in the ductus arteriosus. As discussed above such drugs would

be expected to be safer and more potent than existing therapies. One aim was, therefore, to characterise the dilator EP receptor on the fetal rabbit ductus arteriosus as a first step towards that goal.

1.6.4 Characterisation of the effect of other prostanoids.

The ductus synthesises a range of prostaglandins but so far there is only evidence for an effect of PGE_2. This is at least partly due to a lack of good detailed quantitative studies on the effects of other prostanoids on the ductus. It is also explained by the fact that much of the early work on the ductus was carried out prior to the availability of the many synthetic prostanoids developed in recent years. I aimed to screen for the full range of known prostanoid receptors, contractile and relaxant, in the ductus.

1.6.5 Characterisation of the effect of indomethacin.

Indomethacin eliminates the effects of endogenous prostanoids synthesised by the ductus. From its effects, one can deduce the net effect of endogenous prostanoids synthesised by the ductus under any given set of physiological conditions. Thus, the effect of indomethacin may shed light on the physiological effect of prostaglandins synthesised in the wall of the ductus. Furthermore, comparison of the relative potencies of prostaglandins as dilators of the ductus has always been carried out in the presence of indomethacin, despite the fact that indomethacin has been shown to increase the vessel's sensitivity to PGE_2 (Coceani *et al,* 1975). I sought to elucidate the mechanism of this effect of indomethacin on the vessel's response to PGE_2 and to elucidate the effect of indomethacin on the sensitivity of the ductus to other prostanoid and non-prostanoid dilators.

Chapter 2. General Methods.

The basic design of the study was as follows: the ductus arteriosus was isolated from fetal New Zealand White rabbits and mounted *in vitro* for the measurement of isometric tension. Contractile or relaxant responses were elucidated under varying experimental conditions, such as varying oxygen tension, or in the presence and absence of drugs (such as receptor antagonists, enzyme inhibitors etc.).

2.1 Preparation of tissue.

Time-mated pregnant New Zealand White rabbits were killed at 28 days gestation by intra-venous injection of a lethal dose of sodium pentobarbitone into an ear vein and exsanguination by bilateral laceration of the common carotid arteries. The fetuses were delivered by Caesarean section and decapitated prior to the onset of respiration. The thorax was opened and under dissection microscope, one or two rings of ductus arteriosus (1-1.5mm in length) were obtained from each fetus. The investigation was performed in accordance with the Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986,* published by Her Majesty's Stationery Office, London.

2.2 Experimental conditions.

Each ring of ductus arteriosus was suspended between two stainless steel hooks (one stable, the other connected to an isometric tension transducer [Grass, model FT 03C]). Passing the hooks through the narrow vessel lumen denuded it of endothelium, which was confirmed by light microscopy. The mounted vessels were placed in 10ml organ baths containing physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.0, $KHPO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, and glucose 11.0; with 23μM Na_2EDTA when noradrenaline was being

used. The signal from the transducer was relayed to a bridge conditioner (Fylde FE 492 BBS) and thence to a chart recorder (Linseis 7208).

The tension transducer was secured in a Vernier control (Hugo Sachs Electronics, type 850S) which allowed very accurate adjustment of its height relative to the preparation. By elevating or lowering the transducer, the degree of passive tension placed on the vessel could be altered and this could be monitored by the displacement of the pen of the chart recorder.

The vessels were maintained at 36.5-37°C. The baths were bubbled with gas mixtures of variable O_2, 5% CO_2, and remainder N_2. The gas mixtures were made up in a rotameter system (normally used for anaesthetic gas dispension), stored in Douglas bags and bubbled through the system by a small pump. When 2% O_2 was used, the organ bath was partially covered with chemically inert film to reduce diffusion of oxygen from the air. In the experiments where 3% oxygen was used, special organ baths with narrow necks were used which made the film unnecessary. Temperature and oxygen tension were measured continuously in an additional organ bath which was validated by intermittent checks of temperature and oxygen tension in the other baths. The oxygen measuring system (O_2 electrode and O_2 meter, Strathkelvin Instruments, model 781) was zeroed in a 0.01M solution of sodium borate saturated with sodium sulphite. The gain was set at 36.5 to 37°C bubbling with room air, after correction for atmospheric and water vapour pressure and assuming ambient oxygen of 20.93%.

2.3 Contractile and relaxant responses.

After an initial incubation period (typically 90 minutes) the response to agonists was studied. As most responses were in the presence of indomethacin (1μM) and this caused contraction of the vessel as previously described (see section 1.2.3), the vessel was generally relaxed

by some means prior to obtaining a contractile response. When studying relaxations, the vessel was pre-contracted with one of the following: (1) 10μM noradrenaline, (2) 1μM indomethacin plus 10μM noradrenaline or (3) 1μM indomethacin plus 25mM K^+. In all cases, a response (contractile or relaxant) under a given set of experimental conditions was compared with a similar number of concurrent, time-matched controls. The exact experimental protocol used for a given part of the study is detailed in each chapter.

2.4 Quantification of responses.

The potency of agonists was estimated by the pEC_{50}, i.e. $-\log_{10}EC_{50}$ where the EC_{50} is the interpolated molar concentration of the drug causing 50% of its own eventual maximum response, either contractile or relaxant. An EC_{50} was only calculated when the response to a given agonist was clearly maximal. In a small number of cases, the potency was expressed as the concentration required to cause a given decrease in the extent of pre-contraction (Chapter 6 only, see section 6.1.3).

The maximum contractile response (MCR) to vasoconstrictors under a given set of conditions was generally expressed with reference to a control response. The maximum relaxant response (MRR) to a vasodilator was expressed in one of two ways: (1) as a percentage of a preceding control response, either to the same agonist under control conditions or a control response to a different agonist which was being used as a standard, or (2) as a percentage of the maximum relaxation as determined by addition of 100nM PGE_2 and 30μM papaverine at the end of the response, which resulted in loss of all active tone (i.e. below baseline and with no further response to 1μM forskolin).

In each chapter, the exact method used to quantify responses and the reason why a given method was used is made clear.

2.5 Drugs.

The following drugs were used: adenosine, atrial natriuretic peptide (ANP, rat 1-28), forskolin, indomethacin, noradrenaline bitartrate, papaverine hydrochloride, propranolol hydrochloride, sodium nitroprusside (SNP, all Sigma); AH13205, AH23848B, AH6809 and GR63799X (Glaxo); BW245C and BW868C (Wellcome); cicaprost and sulprostone (Schering); misoprostol and SC19220 (Searle); PGE_2 (U-12062), $PGF_{2\alpha}$ tromethamine salt (U-14583E) and U46619 (Upjohn); cromakalim (BRL 38227, Smith Kline Beecham); flubriprofen (Boots); nifedipine (Bayer); and EP 092 (Edinburgh University). The stock solutions of drugs were as follows: PGE_2 was in ethanol (10mg/ml) which was diluted to 100μM in 0.1M phosphate buffer; BW245C was in ethanol (10mg/ml) and the 10mM dilution was in 0.1M phosphate buffer; cromakalim (10mM), nifedipine (10mM), BW868C (10mM), GR63799X (10mg/ml), misoprostol (1mM), SC19220 (5mg/ml) and papaverine (20mM), were in ethanol; indomethacin (10mM) and AH13205 (100mM) were in 10% $NaHCO_3$; AH6809 was in 1% $NaHCO_3$ in 0.9% saline; sulprostone was in 3% v/v ethanol and 0.01% v/v Tween 80 (Sigma); noradrenaline (10mM) was in 23μM Na_2EDTA; forskolin (10mM) was in DMSO; U46619 (10mg/ml) was supplied in methyl acetate and diluted to 1mg/ml in ethanol; adenosine (10mM), ANP (10μM), flubriprofen (1mM), $PGF_{2\alpha}$ (1mM) and SNP (10mM) were in 0.9% saline; cicaprost (0.5mg/ml) and propranolol (10mM) were in distilled water. AH23848B was dissolved in 10% $NaHCO_3$ which was then added to the appropriate volume of HCO_3-free PSS to result in PSS with the desired concentration of the drug and a $[HCO_3]$ of 25mM. Solutions of indomethacin, nifedipine and SNP were protected from light. All subsequent dilutions were made in 0.9% saline, except noradrenaline which was diluted in 23μM EDTA. All drugs were stored on ice for the duration of the experiment.

2.6 Statistics.

All data are expressed as means with either the standard error of the mean (SEM) or 95% confidence intervals (CI) in parenthesis. In graphs, points are means, bars are SEM. Comparison of two means was made by Student's t-test, paired or un-paired as appropriate, when the data was normally distributed. When the data were not normally distributed, or where there were any doubt (especially with small numbers of observations) comparison was made by the Mann-Whitney U test, which is suitable for parametric and non-parametric data. Comparison of three or more means was made by analysis of variance (ANOVA). Multiple comparisons after ANOVA were made using Tukey's method for calculating CI (95%) of pairwise differences. Significance was assumed at the 5% level. Statistical analysis was performed on Minitab Release 8.2 for the Apple Macintosh.

Chapter 3. Characterisation of experimental conditions for study of contractile and relaxant responses of fetal rabbit ductus arteriosus.

The standard animal for studies on the pharmacology of ductus arteriosus smooth muscle has tended to be the fetal lamb (Clyman, 1987). This preparation is limited by several facts: the animals are expensive, they are large and they have a long gestation. Very few studies have used the rabbit. However, the vessel of the fetus and neonate is sufficiently large to use as a standard organ bath preparation and is much cheaper and more convenient to use than the sheep. The studies on the rabbit are so limited, however, that there has been no detailed description of the ideal experimental conditions for the study of the rabbit ductus. I sought to characterise the optimum experimental conditions for the ductus arteriosus of the fetal rabbit at 28 days gestation.

3.1 Methods.

The general methods outlined in Chapter 2 were employed for setting up and maintaining rings of ductus arteriosus. All of the experiments described in this chapter were carried out in 15% oxygen, which resulted in an oxygen tension of 100-110mmHg. Responses to agonists (contractile and relaxant) were obtained cumulatively, with a given response being allowed to attain a maximum level before eliciting the next.

3.1.1 Effect of stretch on responses.

Preparations of the vessel were initially stretched to 0.3g.tension which was re-set at 60 minutes. The PSS was changed at 30 and 60 minutes. At 90 minutes, a standard contraction was obtained by exposing the vessel to a $[K^+]$ of 65mM using PSS where NaCl was isotonically replaced with KCl. This was washed out with three changes of PSS over 15 minutes and, once

the tension had settled, the stretch of the vessel was altered to one of 6 test levels (g.tension): 0.1, 0.2, 0.3, 0.6, 0.9 and 1.2. 30 minutes later, indomethacin was added to the bath (1μM) and after a further 30 minutes, 2M KCl was added to the bath to give a final $[K^+]$ of 25mM. After 10 minutes exposure to the increased $[K^+]$, a cumulative, concentration relaxation response curve (CRRC) to PGE_2 was obtained. Following this, the PSS was changed three times over 15 minutes and the vessel was incubated in 1μM indomethacin for 30 minutes. The vessel was then relaxed with 1nM PGE_2 and a cumulative concentration contraction response curve (CCRC) was obtained to noradrenaline. The last part of that protocol was repeated and a second CCRC to noradrenaline was obtained in 10nM PGE_2.

The magnitude of contractile and relaxant responses at the different degrees of test stretch was related to the control response to 65mM K^+ at the standard degree of stretch of 0.3g.tension. The sensitivity to contractile and relaxant agonists was quantified by the pEC_{50} (see section 2.4 for definition).

3.1.2 Effects of stretch on the time dependence of responses.

The time-dependence of the response to noradrenaline was studied with the vessel set up at initial preparation with 3 different levels of stretch (g.tension): 0.2, 0.6 and 1.2. It was incubated for 90 minutes and the PSS changed at 30 and 60 minutes. Following this, a series of 4 or 5 consecutive CCRC to noradrenaline was obtained. The PSS was changed 3 times after each response and at least 45 minutes allowed between responses. This was carried out in the absence of indomethacin and exogenous PGE_2. In addition, a protocol was used where the vessel was stretched to 0.2g.tension at initial preparation and consecutive responses to noradrenaline were obtained by the same time course described above, but responses were elicited in the presence of 1μM indomethacin (added at least 30 minutes prior to the CCRC) and 1nM PGE_2 (added 10 minutes

prior to the CCRC).

The time-dependence of the CRRC to PGE_2 was studied with the vessel stretched to 0.6g.tension and re-set at 60 minutes. The PSS was changed at 30 and 60 minutes. Indomethacin was added at 90 minutes and 30 minutes later, 2M KCl was added to a bath $[K^+]$ of 25mM. After a further 10 minutes, a CRRC to PGE_2 was obtained. The protocol was repeated to obtain 3 consecutive CRRC to PGE_2. The PSS was changed 3 times after each response and at least 55 minutes allowed between responses.

3.2 Results.

The magnitude of the contractile response to indomethacin increased as the degree of stretch was increased from 0.1g.tension to 0.6g.tension and was steady between 0.6 to 1.2g.tension. (Figure 3.1). The pEC_{50} to PGE_2 was the same across the range of stretch (Figure 3.2). The maximum relaxant response (MRR) to PGE_2 varied with the degree of stretch in a similar pattern to the contractile response to indomethacin (Figure 3.3). The degree of stretch had no effect on the pEC_{50} of the ductus to noradrenaline, both in 1nM and 10nM PGE_2, or on the magnitude of change in pEC_{50} to noradrenaline-induced by increasing PGE_2 from 1nM to 10nM (Figure 3.4). The maximum contractile response (MCR) to noradrenaline, in both 1nM and 10nM PGE_2, varied with the degree of stretch in a similar pattern to the response to indomethacin, but the magnitude of the difference in the MCR comparing 1nM and 10nM PGE_2 did not vary with the degree of stretch (Figure 3.5).

The sensitivity of the vessel to noradrenaline declined over a series of consecutive responses. This was related to the degree of stretch. With an initial stretch of 0.2g.tension, the first response to noradrenaline was more sensitive than subsequent responses, which were all similar (Figure 3.6). A very similar pattern was observed (0.2g.tension) when each CCRC was carried out in the presence of 1μM indomethacin and 1nM PGE_2 (Figure

3.7). With the vessel initially stretched to 0.6 or 1.2g.tension, there was a steady decline in sensitivity to noradrenaline over the course of a series of consecutive responses (Figure 3.8). The maximum response to noradrenaline was the same over the course of the responses in all cases.

The sensitivity of the ductus to PGE_2 was unchanged over the course of 3 responses with a degree of stretch of 0.6g.tension (Figure 3.9). The magnitude of relaxation induced by PGE_2 was also stable.

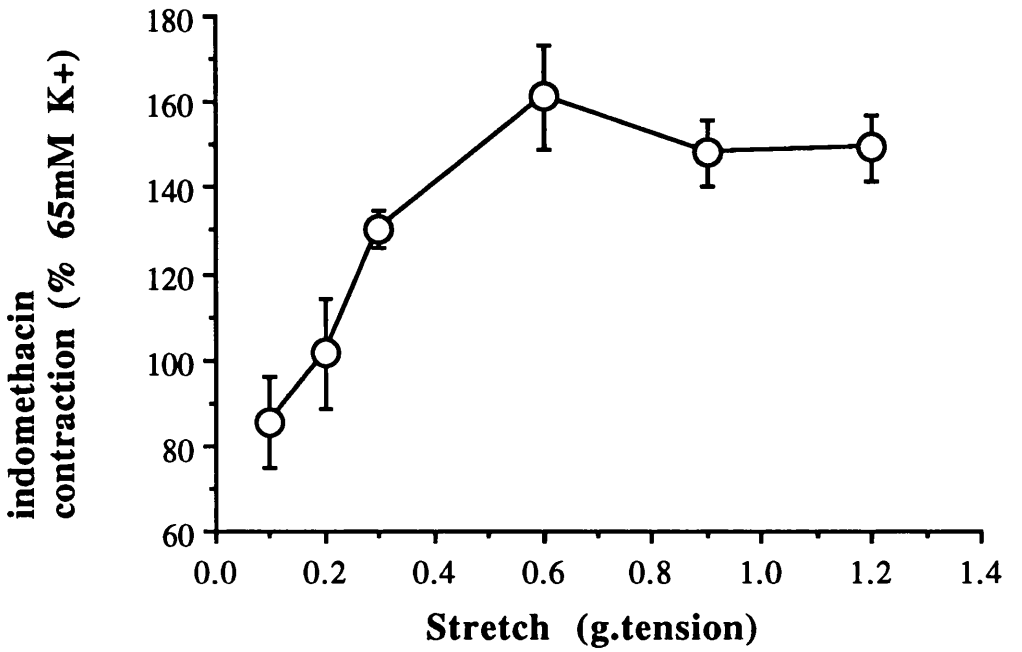

Figure 3.1 The effect of stretch on the magnitude of contraction elicited by indomethacin. The magnitude of the response to indomethacin is expressed as a proportion of the control response to 65mM K^+ at 0.3g.tension. Means are significantly different from each other (ANOVA: F ratio 9.95, $p<0.001$). Multiple comparisons (Tukey's method), all numbers g.tension: 0.1 significantly less than 0.3 to 1.2; 0.2 significantly less than 0.6 to 1.2; no other significant differences. See Appendix (Table A.1) for table of values of 95% CI of pairwise differences. Points are means, bars are SEM. n=7-8.

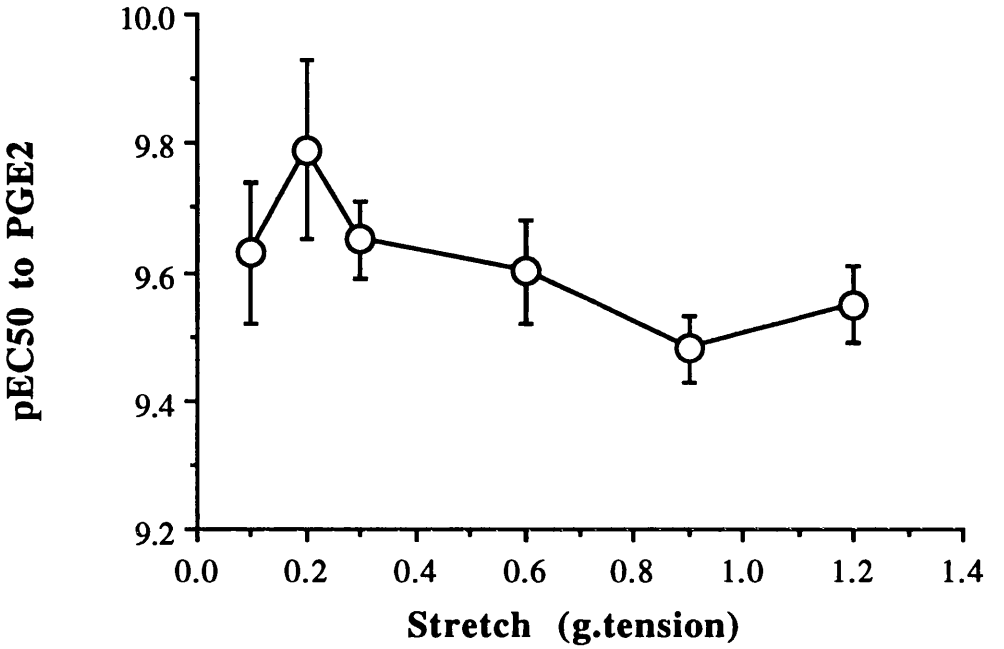

Figure 3.2 The effect of stretch on the pEC_{50} to PGE_2. There is no significant difference between the means (ANOVA: F ratio 1.43, p=0.235). Points are means, bars are SEM. n=7-8.

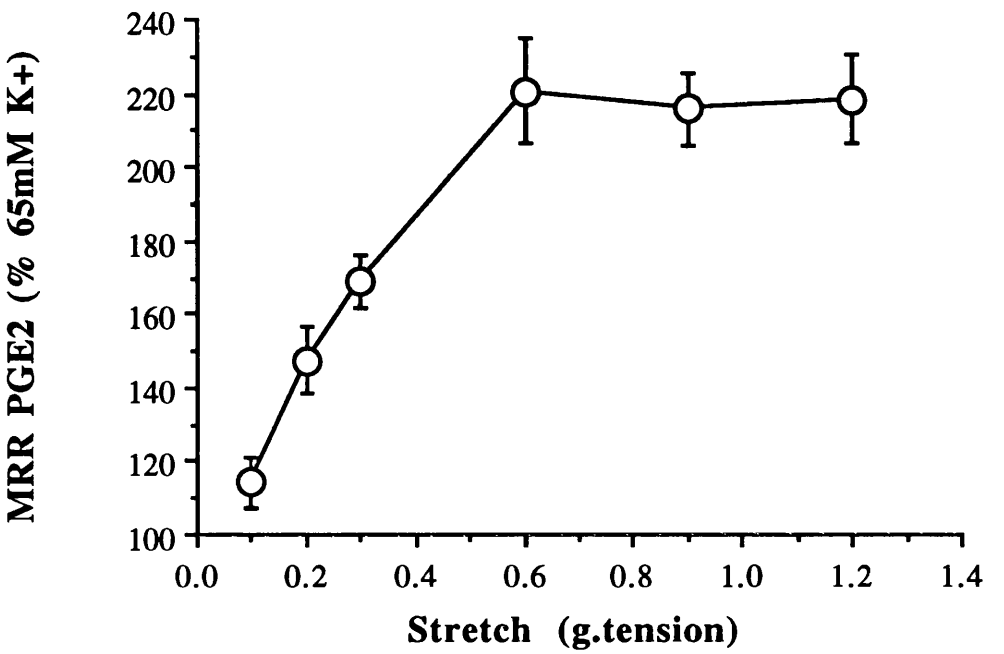

Figure 3.3 The effect of stretch on the maximum relaxant response (MRR) to PGE_2. The magnitude of the MRR to PGE_2 is expressed as a proportion of the control response to 65mM K^+ at 0.3g.tension. Means are significantly different from each other (ANOVA: F ratio 20.01, $p<0.001$). Multiple comparisons (Tukey's method), all numbers g.tension: 0.1 significantly less than 0.3 to 1.2g; 0.2 significantly less than 0.6 to 1.2g; 0.3 significantly less than 0.6 to 1.2; no other significant differences. See Appendix (Table A.2) for table of values of 95% CI of pairwise differences. Points are means, bars are SEM. n=7-8.

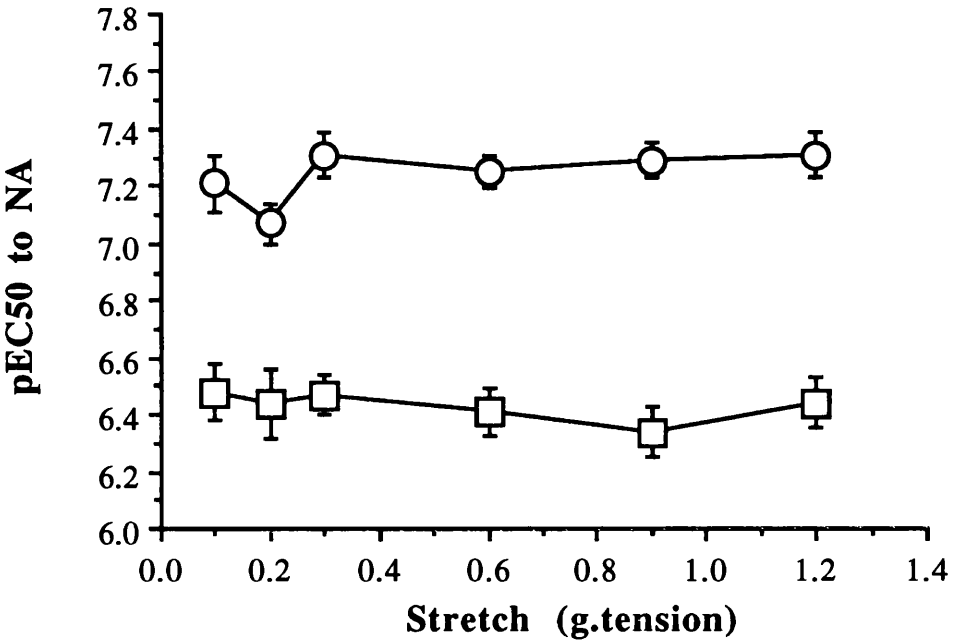

Figure 3.4 The effect of stretch on the pEC_{50} to noradrenaline in 1μM indomethacin and 1 or 10nM PGE_2. (○ 1nM PGE_2 □ 10nM PGE_2) Data for the two concentrations of PGE_2 at a given degree of stretch are from the same vessels, the response in 10nM elicited second (see section 3.1.1 for details of protocol). There was no significant difference in pEC_{50} between the different degrees of stretch in 1nM PGE_2 (ANOVA: F ratio 1.44, p=0.232) and 10nM PGE_2 (F ratio 0.33, p=0.894). The extent of change in pEC_{50} comparing the response in 10nM PGE_2 with that in 1nM PGE_2 was not significantly different comparing the different degrees of initial stretch (F ratio 2.01, p=0.098). Points are means, bars are SEM. n=7-8.

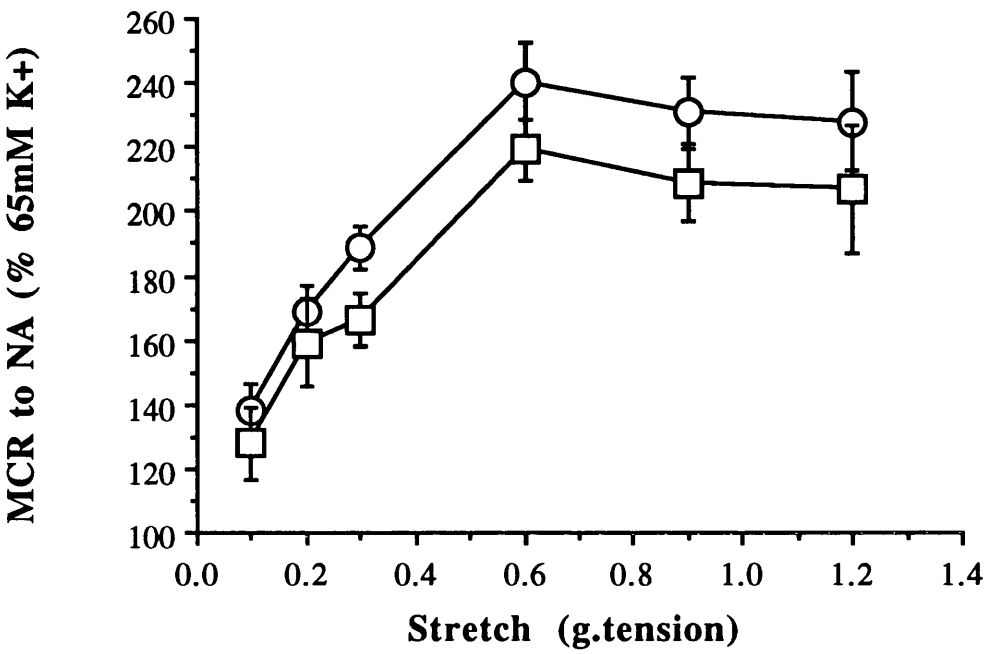

Figure 3.5 The effect of stretch on the maximum contractile response (MCR) to noradrenaline in 1μM indomethacin and 1 or 10nM PGE_2. (○ 1nM PGE_2 □ 10nM PGE_2) The magnitude of the MCR to noradrenaline is expressed as a proportion of the control response to 65mM K^+ at 0.3g.tension. Data for the two concentrations of PGE_2 at a given degree of stretch are from the same vessels, the response in 10nM elicited second (see section 3.1.1 for details of protocol). The MCR to noradrenaline was different with the degree of initial stretch in both 1nM PGE_2 (ANOVA: F ratio 14.02, $p<0.001$) and 10nM PGE_2 (F ratio 7.51, $p<0.001$). Multiple comparisons (Tukey's method), all numbers g.tension: *1nM PGE_2:* 0.1 significantly less than 0.3 to 1.2; 0.2 significantly less than 0.6 to 1.2; 0.3 significantly less than 0.6; no other significant differences. *10nM PGE_2:* 0.1 significantly less than 0.6 to 1.2; 0.2 significantly less than 0.6; no other significant differences. See Appendix (Tables A.3 and A.4) for table of values of 95% CI of pairwise differences. The extent of change in MCR comparing the response in 10nM PGE_2 with that in 1nM PGE_2 was not significantly different comparing the different degrees of stretch (F ratio 0.67, p=0.648). Points are means, bars are SEM. n=7-8.

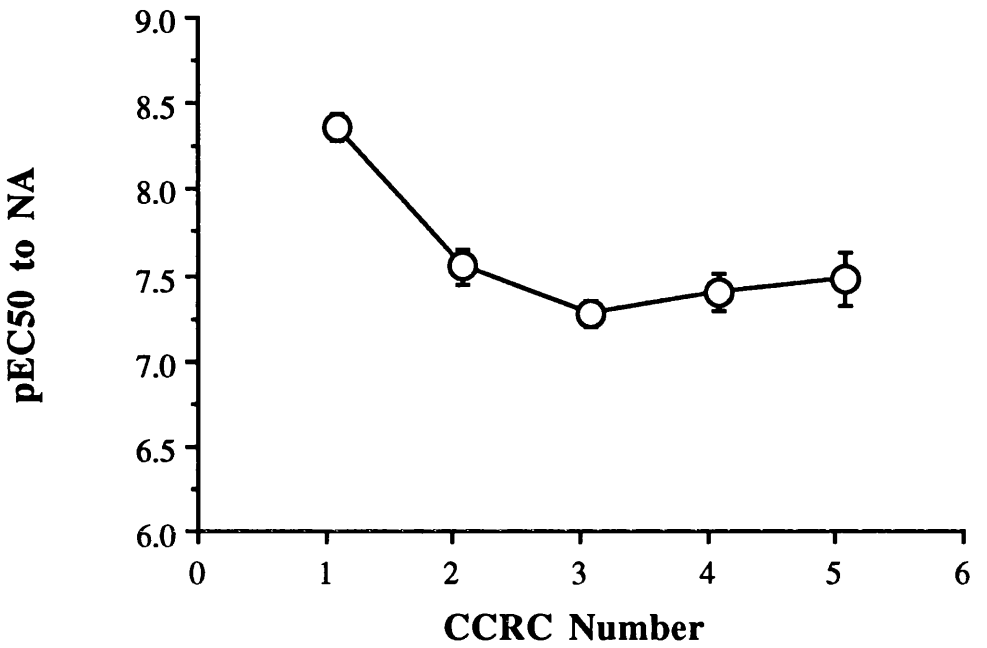

Figure 3.6 The pEC_{50} for 5 consecutive responses to noradrenaline (NA) with an initial degree of stretch of 0.2g.tension and in the absence of indomethacin and exogenous PGE_2. The first pEC_{50} was significantly elevated compared to all the others (all $p<0.0001$). The second pEC_{50} was elevated compared with responses 3 to 5 (mean difference 0.169 [SEM 0.036], $p<0.0001$). There was no significant difference in the pEC_{50} between responses 3-5. Points are means, bars are SEM, n=7-13.

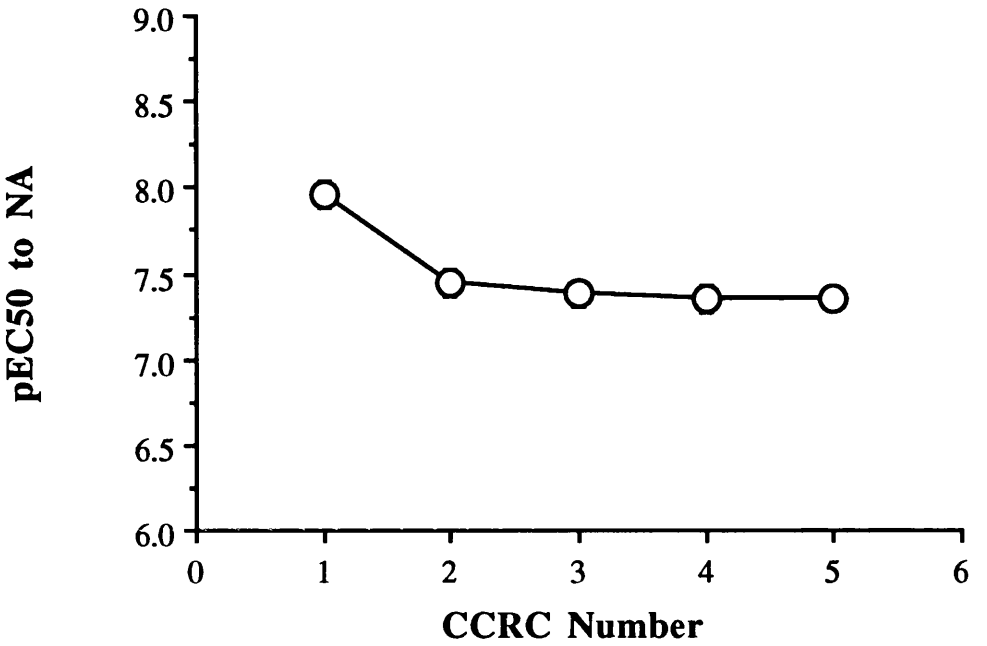

Figure 3.7 The pEC_{50} for 5 consecutive responses to noradrenaline with an initial degree of stretch of 0.2g.tension and in 1μM indomethacin and 1nM PGE_2. The first pEC_{50} was significantly elevated compared with all the others (all $p<0.0001$). There were no significant differences in the pEC_{50} of responses 2 to 5. Points are means, bars are SEM, n=8.

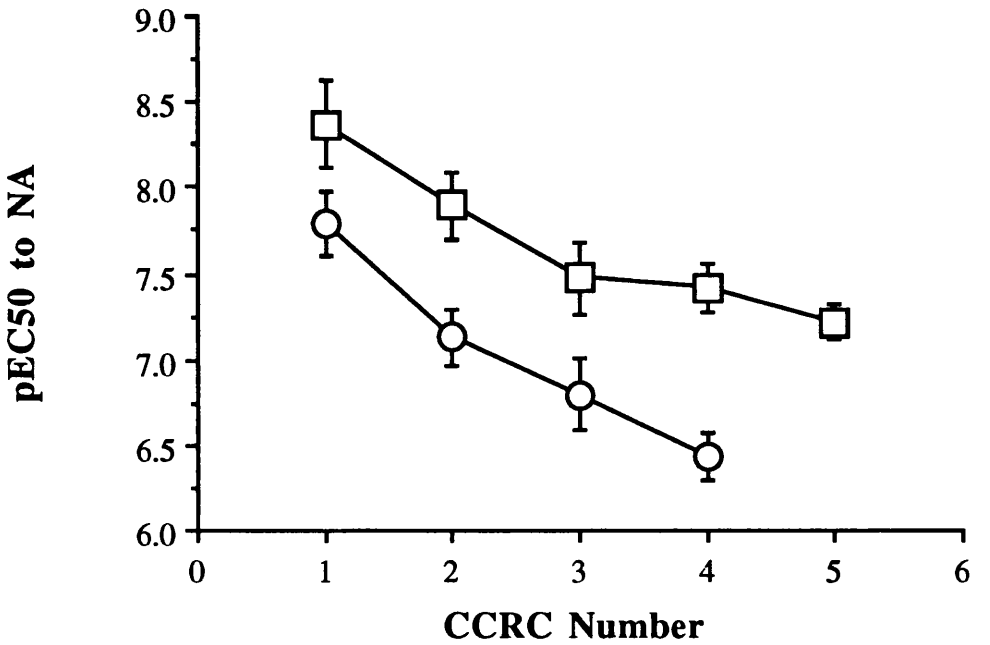

Figure 3.8 The pEC_{50} for consecutive responses to noradrenaline with an initial degrees of stretch of 0.6g.tension or 1.2g.tension and in the absence of indomethacin and exogenous PGE_2. ○ = 0.6g (n=4); □ = 1.2g (n=4). Points are means, bars are SEM.

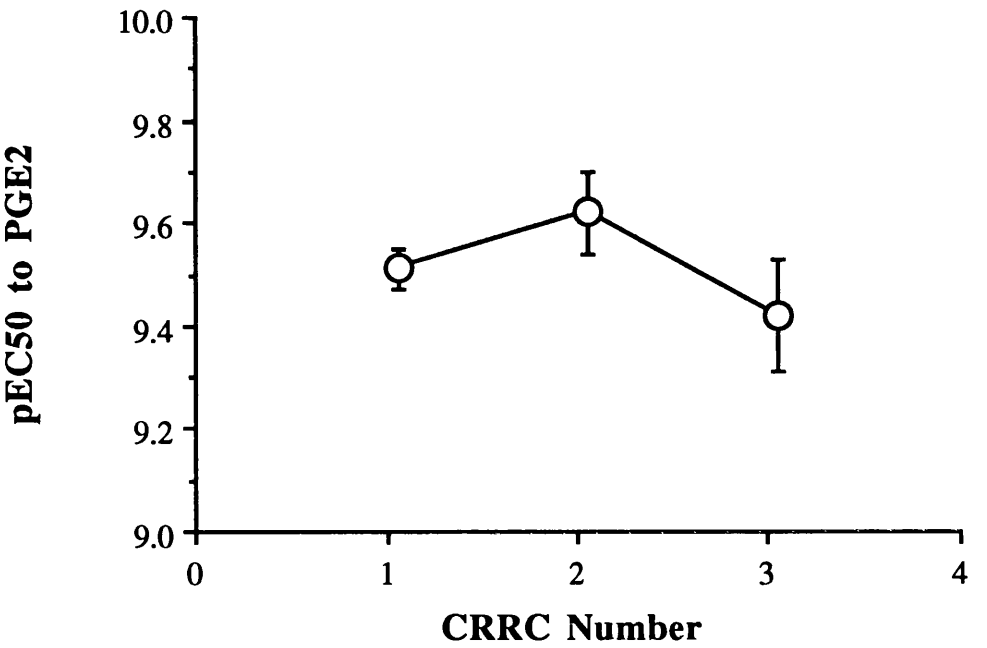

Figure 3.9 pEC_{50} to PGE_2 over 3 consecutive responses with an initial stretch of 0.6g.tension, pre-contracted with 1μM indomethacin and 25mM K^+. There was no significant difference in the pEC_{50} comparing responses 2 and 3 with the initial response (Student's paired t-test, p=0.053 and p=0.33 respectively). Points are means, bars are SEM, n=11.

3.3 Discussion.

The main finding of this series of experiments was that the degree of initial stretch had relatively little effect on the sensitivity of the ductus to agonists (as measured by the pEC_{50}), both contractile (noradrenaline) and relaxant (PGE_2), but that it did alter the magnitude of responses to given agonists. The exception to this was in time-dependent changes in sensitivity. With smaller degrees of initial stretch the first response to noradrenaline was more sensitive than the others (Figures 3.6). This was not due to changes in the vessel's production of prostaglandins, as the same pattern was observed in 1μM indomethacin and 1nM PGE_2 (Figure 3.7). With greater degrees of stretch, the sensitivity of the vessel to noradrenaline continued to change over 5 responses (Figure 3.8). Interestingly, with a degree of stretch (0.6g.tension) which was associated with a consistent decline in sensitivity to noradrenaline over the course of serial responses (Figure 3.3), the response to PGE_2 was relatively stable with respect to time (Figure 3.9). Other than excluding a role for prostaglandins, no light was shed on the mechanism of these changes with time.

In practical terms, this work led to the use of certain fixed protocols:
(1) CRRC: tension was initially set at 0.6g.tension, re-set at 60 minutes and not altered thereafter;
(2) CCRC to noradrenaline: tension was initially set at 0.2g.tension, and not altered thereafter. This degree of stretch resulted in slightly smaller responses, a disadvantage outweighed by the greater time stability of sensitivity of responses compared with greater degrees of stretch.
(3) CCRC to noradrenaline: the second CCRC was used as the control response in view of the data in Figures 3.6 and 3.7.

Furthermore, in all situations where any form of quantitative comparison was being made (e.g. between two agonists or to a given agonist in the

presence and absence of an antagonist) the test response was always compared to a similar number of appropriate concurrent time controls.

As well as the practical implications of these data, they also shed some light on the contractile properties of the isolated ductus arteriosus. The contraction elicited by indomethacin has been described previously in the lamb and is exploited clinically in the treatment of patent ductus arteriosus (see Chapter 1). This contraction is, however, mechanistically quite different from that induced by standard contractile agonists such as noradrenaline. Indomethacin is not an agonist for a receptor coupled to a stimulatory second messenger system. It inhibits cyclo-oxygenase and eliminates the effect of prostaglandins (Smith *et al,* 1991). The net effect of prostaglandins on the ductus are inhibitory and, therefore, indomethacin induces contraction of the vessel by eliminating an inhibitory influence (see Chapter 1). It follows from this, therefore, that the determinants of the magnitude of the indomethacin contraction include (1) the extent of synthesis of prostaglandins by the vessel (2) the sensitivity of the vessel to the inhibitory effects of prostaglandins (3) the degree of spontaneous tone present in the vessel.

This last point is clearly vital. Some authors have concluded in previous studies that the failure of indomethacin to induce a contraction reflects an absence of inhibitory effects of prostaglandins (Clyman *et al,* 1980a). However, if the vessel was producing very high concentrations of prostaglandins and was very sensitive to their inhibitory effects, indomethacin would still only produce a contraction if the vessel had some spontaneous tone present.

Considering the present experiments, the tone present in the vessel prior to the addition of indomethacin would have been from two sources: firstly, myogenic tone; and, secondly, oxygen-induced tone. It has been demonstrated in the isolated ductus arteriosus (both rabbit and guinea pig)

that stretch of the vessel, either as a ring preparation or perfused vessel, induces myogenic tone (Ikeda *et al,* 1973a; Kriska *et al,* 1990). Furthermore, the myogenic response is inhibited by endogenous and exogenous prostaglandins (Kriska *et al,* 1990). It may be that the increasing response of the vessel to indomethacin with stretch reflects increased myogenic tone. Considering oxygen-induced tone, increasing oxygen tension in the range of 0-140mmHg oxygen contracts the ductus (see section 1.2.1). The response to indomethacin in Figure 3.1 was in 100-110mmHg oxygen and would from Fay's paper of 1971 (Figure 8) be associated with over 80% of maximum oxygen-induced tone. Indomethacin removes prostaglandin inhibition of oxygen-induced contraction by the ductus. The extent of the indomethacin contraction may well simply reflect the oxygen-induced contraction. Stretch may well have increased the indomethacin contraction by putting the vessel at the optimal point in the length-tension curve for contractile responses of the vessel. This is supported by the fact that there was a similar relationship between stretch and the MCR to noradrenaline (Figure 3.5). Alternatively, stretch may have altered the response of the ductus to indomethacin by altering its synthesis of or sensitivity to prostaglandins.

Of the various possibilities, I demonstrated that stretch had no effect on the sensitivity of the ductus to PGE_2 (Figure 3.2). The effect of stretch on the MRR to PGE_2 (Figure 3.3) reflects the amount of active tone: PGE_2 caused complete relaxation of the ductus under these circumstances, as indicated by a fall below baseline and no further response to 1μM forskolin (data not shown). With respect to dissecting out the other factors which may mediate the change in contractile responses with stretch, I did not pursue these experiments (e.g. repeating the experiments of Figures 3.1-3.5 in 0 mmHg oxygen): interesting as these questions are, they fell outwith my primary aims and this work fulfilled the limited goal of providing validated experimental conditions for studies on the physiological and pharmacological properties of isolated rings of ductus arteriosus.

Chapter 4. Functional characterisation of dilator prostanoid receptors on the ductus arteriosus.

As discussed in Chapter 1, a major goal of this work was to characterise the dilator prostanoid receptors on the ductus arteriosus. As PGE_2 is the most potent prostaglandin in dilating the vessel, it can be assumed that the ductus has a dilator EP receptor. I wished to identify which of the 4 sub-types of EP receptor was present on the ductus. Secondly, it has been suggested that PGE_2 is the only prostaglandin important in the control of ductus arteriosus smooth muscle (Clyman, 1987; Coceani and Olley, 1988). This hypothesis predicts that the ductus would not have inhibitory receptors for any of the other prostaglandins. I wished to test this hypothesis.

Since much of the early work on the ductus was carried out, a large number of synthetic prostanoids have been developed with selective agonist and antagonist effects on prostanoid receptors (see Coleman *et al,* 1990 and 1994b for reviews). From these, I selected a range of agonists and antagonists which had relatively selective activity at EP receptor sub-types. There is no selective EP_4 agonist, however, the agonists used had activity at one or more of the other sub-types (see section 4.3 for references): misoprostol (EP_2 and EP_3), sulprostone (EP_1 and EP_3), GR63799X (EP_1 and EP_3), and AH13205 (EP_2). Furthermore, three EP antagonists were available: AH6809 (EP_1), SC19220 (EP_1) and AH23848B (EP_4). The non-EP prostanoid receptors which mediate relaxation of smooth muscle are IP and DP (Coleman *et al,* 1990). The selective IP agonist, cicaprost, was used and both a selective agonist and antagonist of the DP receptor were available (BW245C and BW868C, respectively; see section 4.3 for references). Using these pharmacological tools I sought to characterise the inhibitory prostanoid receptors on the ductus arteriosus.

4.1 Methods.

The general methods described in Chapter 2 were employed, which also gives the details of drug solutions.

The rings of ductus arteriosus were mounted *in vitro* and bubbled with 15% oxygen, which resulted in an oxygen tension of 100-110mmHg. The vessels were stretched to 0.6g.tension initially and this was re-set at 60 minutes, and not altered thereafter. The PSS was changed at 30 and 60 minutes following mounting and the vessels were allowed to incubate for 90 minutes prior to addition of indomethacin. The vessel was pre-contracted with 1μM indomethacin and, after a minimum of 30 minutes exposure to indomethacin, by addition of 2M KCl to a bath $[K^+]$ of 25mM. Indomethacin tended to cause contraction with an unstable baseline, making it, on its own, a poor method of pre-contracting the rabbit ductus. The combination of 1μM indomethacin and 25mM K^+ resulted in a sustained, stable elevation of tension. Concentration relaxation response curves (CRRC) were obtained cumulatively, with at least 55 minutes between successive curves. The effect of a given concentration of drug was allowed to reach a maximum before addition of the next. The PSS was changed at least 3 times after each CRRC. The first CRRC was a control response to PGE_2. The potency of agonists from subsequent CRRC was expressed with reference to this initial response to PGE_2.

The potency of agonists was quantified relative to PGE_2 by the equieffective molar ratio (EMR): the EC_{50} of the test agonist / the EC_{50} of PGE_2 from the preceding control response, (see section 2.4 for definition of EC_{50}). The response to a given concentration of agonist and its eventual maximum response were expressed as a proportion of the maximum response to PGE_2 in the initial, control CRRC (Coleman, 1987). All estimates of potency were validated by comparison with concurrent time controls to PGE_2.

Antagonist activity was quantified as a molar concentration ratio (CR), defined as the EC_{50} of the agonist in presence of antagonist/ EC_{50} of the agonist in absence of antagonist. Where a significant effect was observed, data were obtained to construct a Schild plot from which a slope and pA_2 were determined (Arunlakshana and Schild, 1959). All estimates of the effects of antagonists were compared to concurrent time controls to the same agonist in the presence of the appropriate concentration of antagonist vehicle.

4.2 Results.

PGE_2 (0.03–10nM) potently relaxed preparations of rabbit ductus arteriosus in a concentration-dependent fashion (EC_{50} =0.36nM, see Figure 4.1). The highest concentrations of PGE_2 (3–10nM) caused complete inhibition of the vessel's tone. Furthermore, all of the synthetic EP receptor agonists tested, with the exception of sulprostone, relaxed the ductus although none was as potent as PGE_2 and AH13025 produced no more than 20% of the response to PGE_2 at the highest concentration tested (100μM) (Figure 4.2 and Table 4.1). The rank order of potency of the agonists was: PGE_2 >> misoprostol > GR63799X >> AH13205 ≥ sulprostone ≥ 0.

The EP_1 receptor blocking drugs, AH6809 (10μM) and SC19220 (30μM), both caused small apparent leftward shifts of the PGE_2 CRRC compared with the preceding control response to PGE_2 (mean CR [SEM]= AH6809 0.78 [0.12], n=11; SC19220 0.68 [0.12], n=11) but the changes were not significant when compared with concurrent time controls (mean CR [SEM]= AH6809 1.09 [0.19] n=10, p=0.18; SC19220, 1.05 [0.17] n=11, p=0.092).

The selective EP_4 antagonist, AH23848B (10-100μM), caused a concentration-dependent rightward shift of the PGE_2 CRRC (Figure 4.3).

The shift of the curves to PGE_2 did not appear to be parallel and, furthermore, the slope of the Schild plot of these data (1.46, Figure 4.4) was significantly greater than unity. While AH13205 was only a weak dilator of the ductus (Figure 4.2 and Table 4.1) it did antagonise the response of the vessel to PGE_2, causing parallel rightward shifts of the PGE_2 CRRC (Figure 4.5) and the slope of the Schild plot (Figure 4.6) was 1.01.

The selective IP agonist, cicaprost, and DP agonist, BW245C, both caused complete relaxation of the ductus (Figures 4.7 and 4.8, respectively). Cicaprost was approximately 100-fold less potent than PGE_2 and BW245C almost 900-fold less potent than PGE_2 (Table 4.1). The sensitivity of the ductus to both these agonists was reduced by 30μM AH23848B (Figures 4.9 and 4.10, respectively). The decrease in sensitivity to PGE_2 induced by 30μM AH23848B (mean CR [SEM]= 6.53 [2.0], n=12) was significantly greater than that to cicaprost (p=0.04), but not significantly different from that to BW245C (p=0.097).

AH13205 (30μM) had no effect on the sensitivity of the ductus to cicaprost (Figure 4.11) but did cause a rightward shift in the CRRC to BW245C which was significantly greater than concurrent time controls (Figure 4.12). The effect of 30μM AH13205 on the sensitivity of the ductus to PGE_2 (mean CR [SEM]= 4.38 [1.2] was significantly less than on BW245C (p=0.046), but pooling the time controls to BW245C demonstrated a spontaneous 2.0 fold decrease (95% CI 1.3–2.7) in sensitivity to BW245C between two consecutive responses to BW245C.

The selective DP receptor antagonist, BW868C (10μM), appeared to cause a slight shift to the right of the CRRC to BW245C (mean CR [SEM]= 3.76 [0.87], n=6) but this was not significantly greater than seen in concurrent time controls (mean CR [SEM]= 2.27 [0.87], n=5; p=0.26).

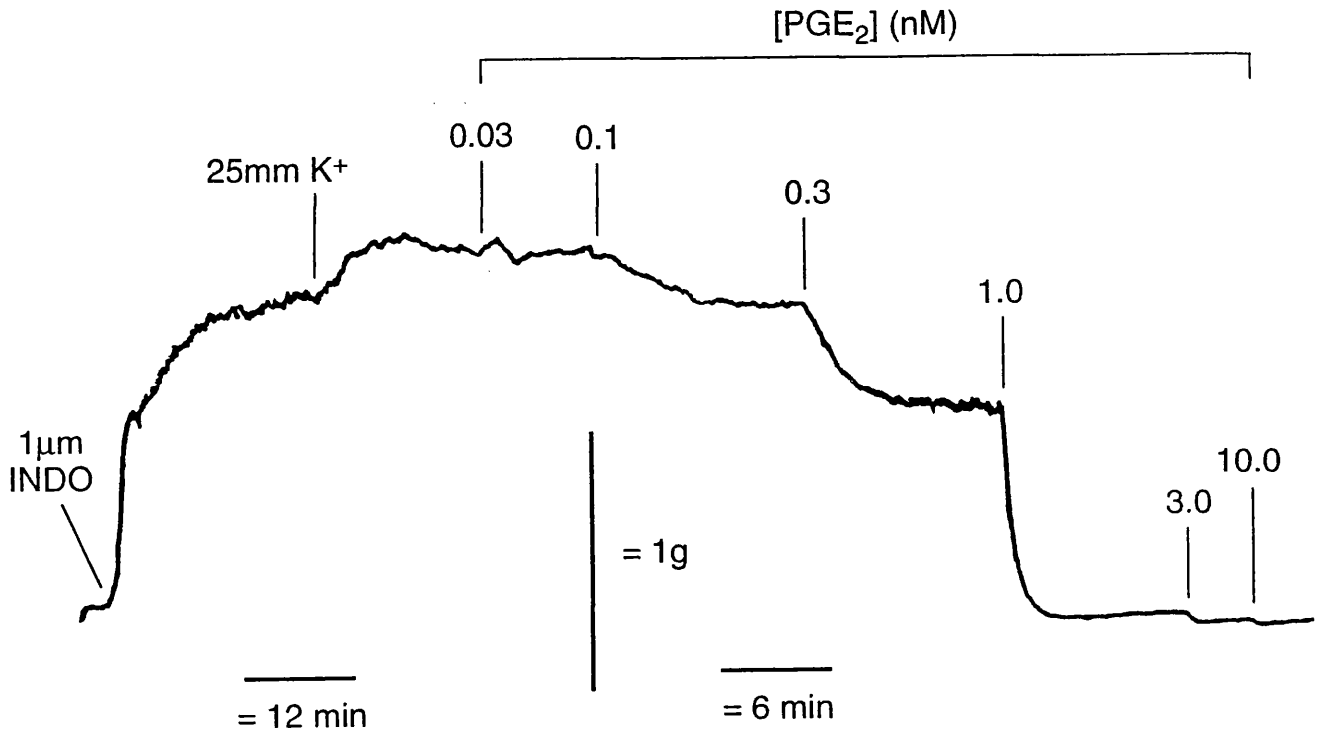

Figure 4.1 Typical cumulative concentration relaxation response curve curve to PGE_2 (0.03nM–10nM) in 1μM indomethacin and 25mM K^+. The 6 minute time bar refers to the PGE_2 CRRC. 1μM INDO = addition of indomethacin to a final bath concentration of 1μM.

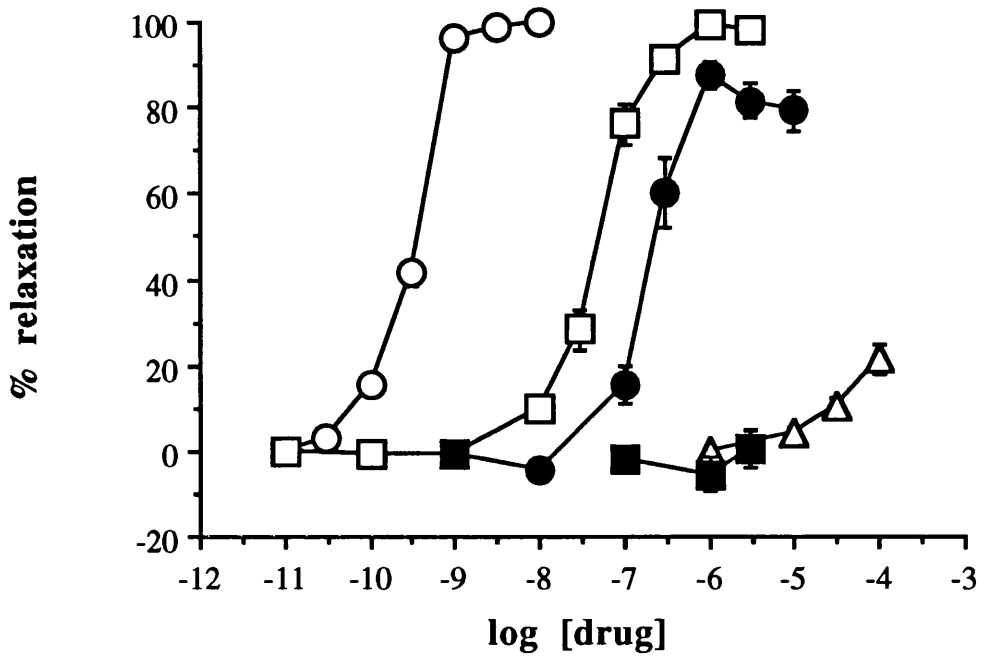

Figure 4.2 The dilator effect of a range of EP agonists in 1μM indomethacin and 25mM K^+. Mean CRRC to PGE_2 (○), misoprostol (□), GR63799X (●), AH13205 (Δ) and sulprostone (■). All n≥8. Relaxation to PGE_2 is expressed as a proportion of its own maximum and relaxation to other agonists is expressed as a proportion of the preceding control response to PGE_2. Points are means, bars are SEM.

Agonist	EC_{50}, nM (95% CI)[a]	EMR (95% CI)[b]	n
PGE_2	0.36 (0.32–0.41)	[1]	44
Misoprostol	54 (37–71)	145 (73–217)	8
GR63799X[c]	239 (164–314)	685 (427–944)	8
Sulprostone[d]	>3,300	>10,000	12
AH13205[d]	>100,000	>100,000	8
Cicaprost	28 (18–38)	110 (80–140)	8
BW245C	312 (180–444)	875 (677–1074)	7

TABLE 4.1. Response to synthetic prostanoids in 1μM indomethacin and 25mM K^+.

[a] EC_{50} = see section 2.4 for definition.

[b]EMR= equieffective molar ratio (see section 4.1 for definition).

[c]Maximum response GR63799X = 87% (95% CI 80-95) of maximum response to PGE_2. All other agonists, where EC_{50} given, maximum response was not significantly less than PGE_2.

[d]Full CRRC not obtained.

Concurrent time controls to PGE_2, obtained for all experiments, showed no spontaneous change with time (mean EMR= 1.07 [95% CI 0.87-1.27], n=37)

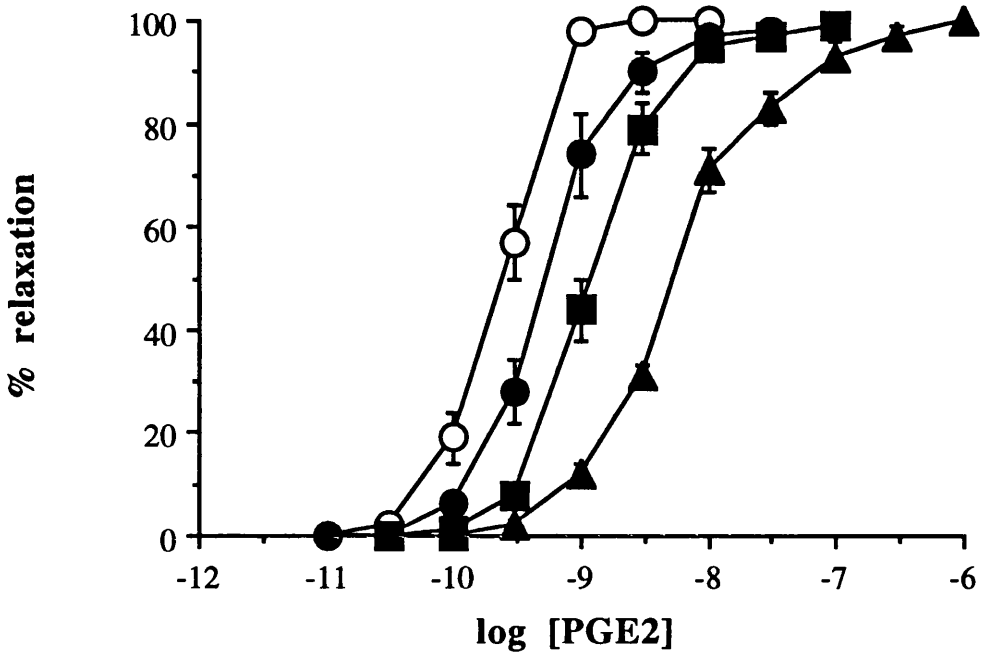

Figure 4.3 The effect of AH23848B on the response to PGE_2. Consecutive CRRC to PGE_2: control (○); in the presence of AH23848B 10μM (●), 30μM (■) and 100μM (▲). n=12. Relaxation to PGE_2 in the absence of AH23848B (control response) is expressed as a proportion of its own maximum and relaxation to PGE_2 in the presence of AH23848B is expressed as a proportion of the preceding control response to PGE_2. Points are means, bars are SEM. Concurrent time controls to PGE_2 (n=8) showed no spontaneous rightward shift (mean CR [SEM]= 10μM 0.95 [0.16]; 30μM 1.10 [0.15]; 100μM 1.09 [0.20], all $p>0.5$ compared with initial control response).

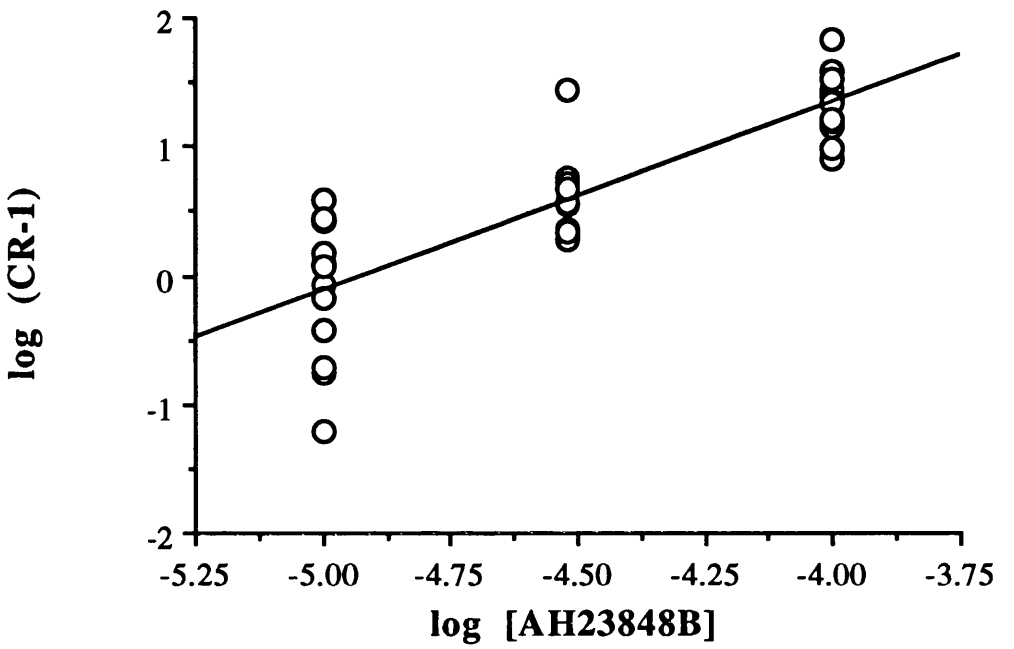

Figure 4.4 Schild plot of the effect of AH23848B on the response to PGE_2. Data obtained from responses displayed in Figure 4.3. Equation of line: log (CR-1) = 7.17 + 1.46 log [AH23848B]. Slope of line 1.46 (95% CI 1.13 to 1.78); pA_2 = 4.91 (95% CI 4.36 to 5.51).

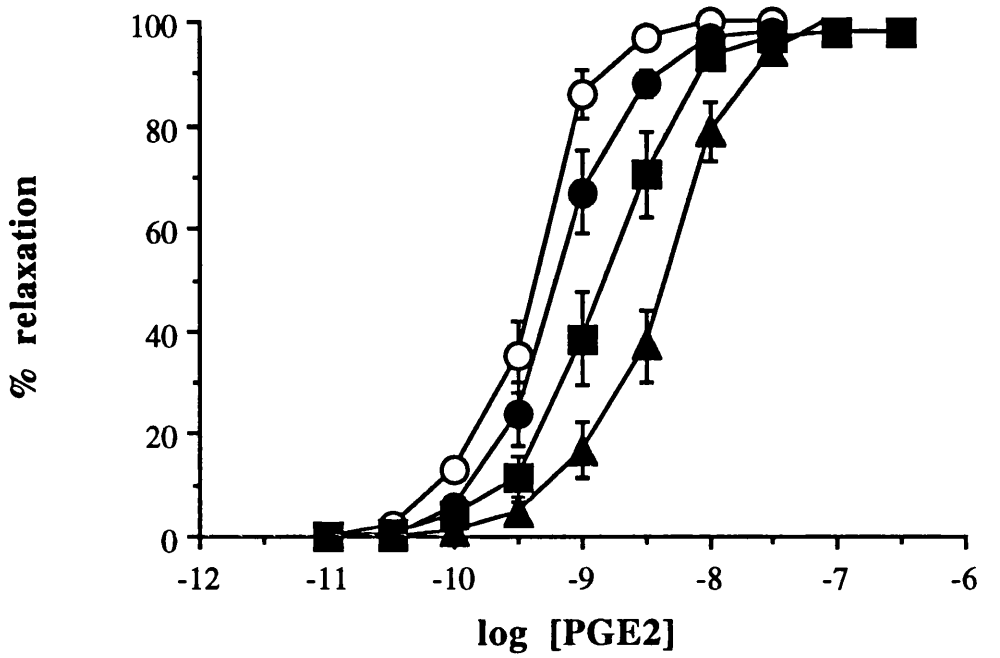

Figure 4.5 The effect of AH13205 on the response to PGE_2. Consecutive CRRC to PGE_2: control (○); in the presence of AH13205 10μM (●) 30μM (■) and 100μM (▲). n=7. Relaxation to PGE_2 in the absence of AH13205 (control response) is expressed as a proportion of its own maximum and relaxation to PGE_2 in the presence of AH13205 is expressed as a proportion of the preceding control response to PGE_2. Points are means, bars are SEM. Concurrent time controls to PGE_2 (n=5) showed no spontaneous change (mean CR [SEM]= 10μM 0.92 [0.12]; 30μM 0.81 [0.11]; 100μM 1.22 [0.19], all $p>0.1$ compared with initial control response).

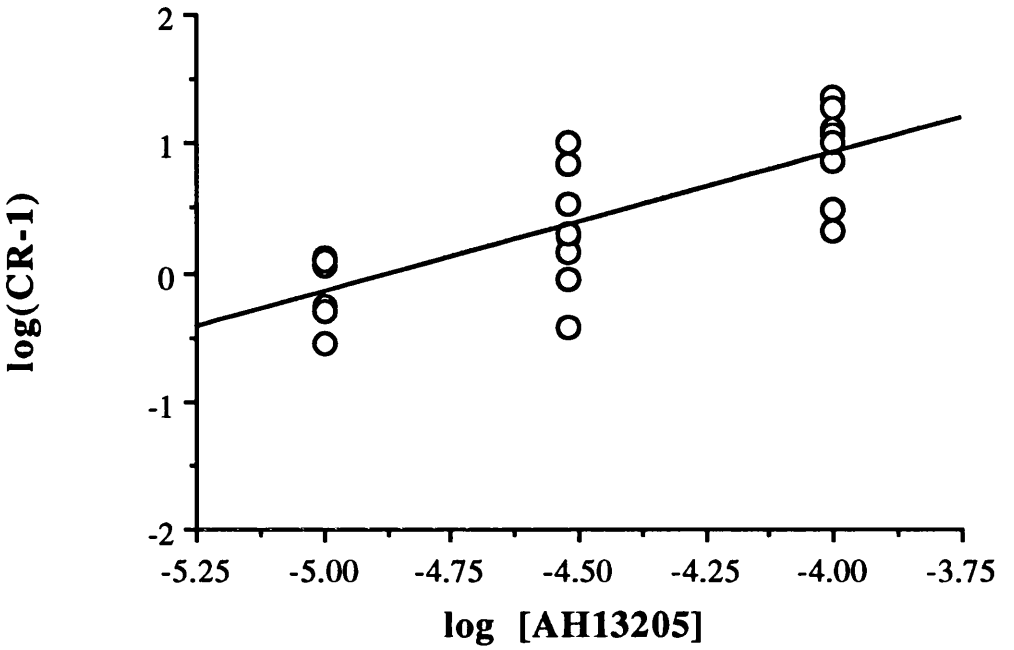

Figure 4.6 Schild plot of the effect of AH13205 on the response to PGE_2. Data obtained from responses displayed in figure 4.5. Equation of line: log (CR-1) = 4.89 + 1.01 log [AH13205]. Slope of line 1.01 (95% CI 0.52 to 1.49); pA_2 = 4.85 (95% CI 3.98 to 5.96).

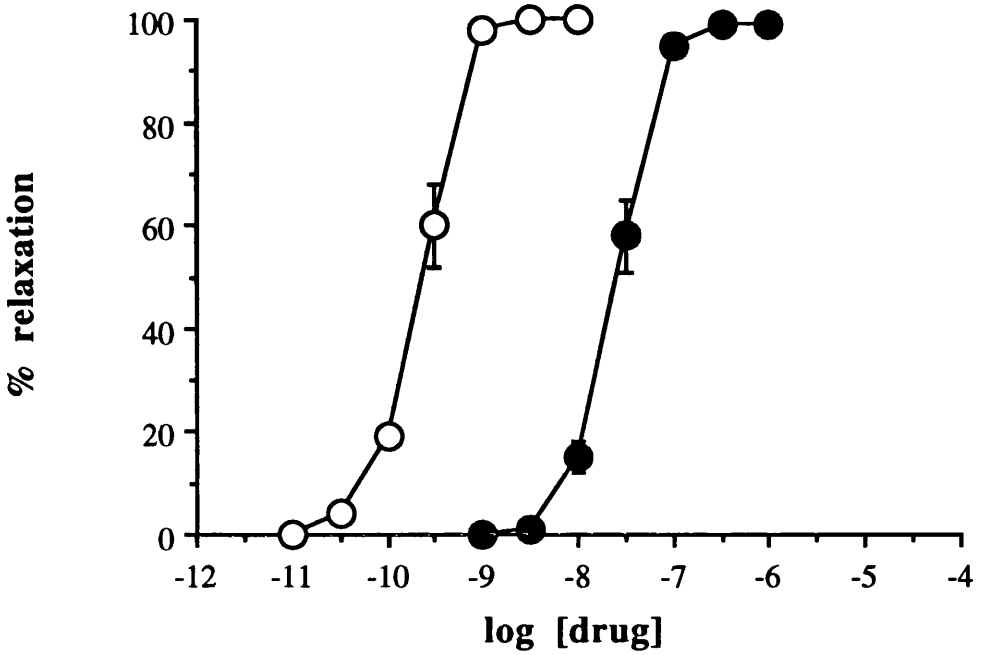

Figure 4.7 The effect of cicaprost in 1μM indomethacin and 25mM K^+. Mean CRRC to PGE_2 (○) and cicaprost (●). n=8. Relaxation to PGE_2 is expressed as a proportion of its own maximum and relaxation to cicaprost is expressed as a proportion of the preceding control response to PGE_2. Points are means, bars are SEM.

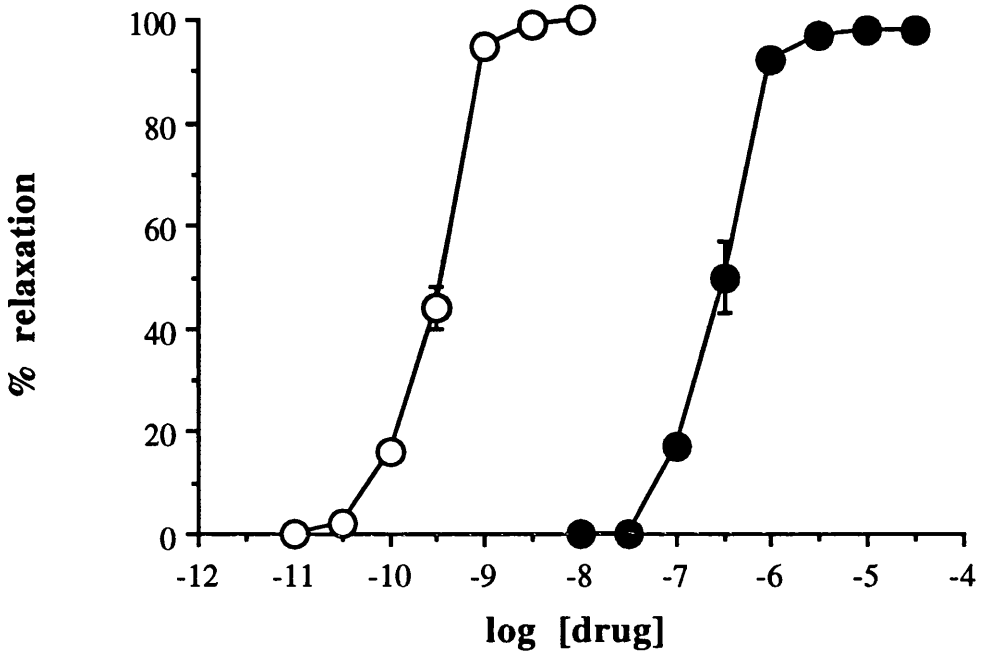

Figure 4.8 The effect of BW245C in 1μM indomethacin and 25mM K^+. Mean CRRC to PGE_2 (○) and BW245C (●). n=7. Relaxation to PGE_2 is expressed as a proportion of its own maximum and relaxation to BW245C is expressed as a proportion of the preceding control response to PGE_2. Points are means, bars are SEM.

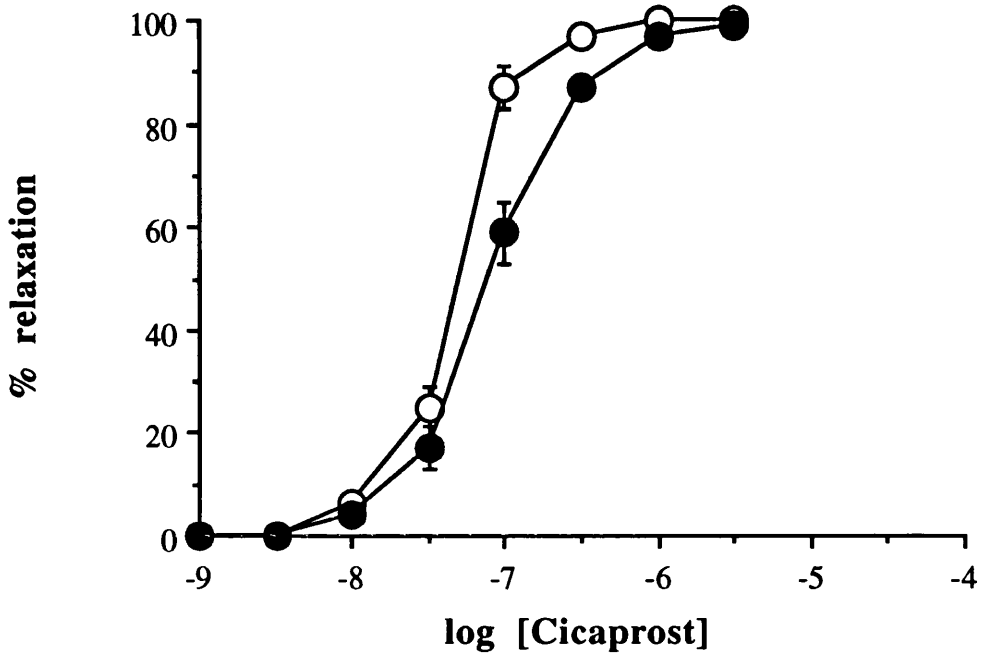

Figure 4.9 The effect of 30μM AH23848B on the response to cicaprost. Mean CRRC to cicaprost in the absence (○) and presence (●) of 30μM AH23848B. Relaxation to cicaprost in the absence of AH23848B (control response) is expressed as a proportion of its own maximum and relaxation to cicaprost in the presence of AH23848B is expressed as a proportion of the preceding control response. Points are means, bars are SEM. Mean CR (SEM) to cicaprost in 30μM AH23848B, 1.72 (0.29), n=7; vs time control, 0.78 (0.05) n=7; p=0.019.

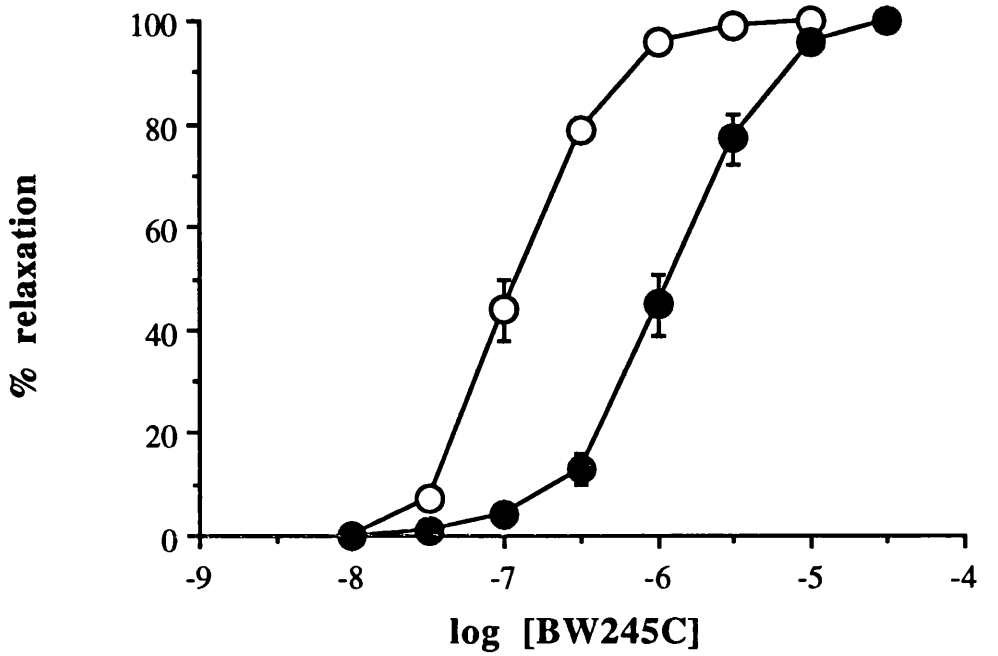

Figure 4.10 The effect of 30μM AH23848B on the response to BW245C. Mean CRRC to BW245C in the absence (○) and presence (●) of 30μM AH23848B. Relaxation to BW245C in the absence of AH23848B (control response) is expressed as a proportion of its own maximum and relaxation to BW245C in the presence of AH23848B is expressed as a proportion of the preceding control response. Points are means, bars are SEM. Mean CR (SEM) to BW245C in 30μM AH23848B 11.6 (2.1), n=8; vs time control 1.98 (0.48) n=6, p=0.0027).

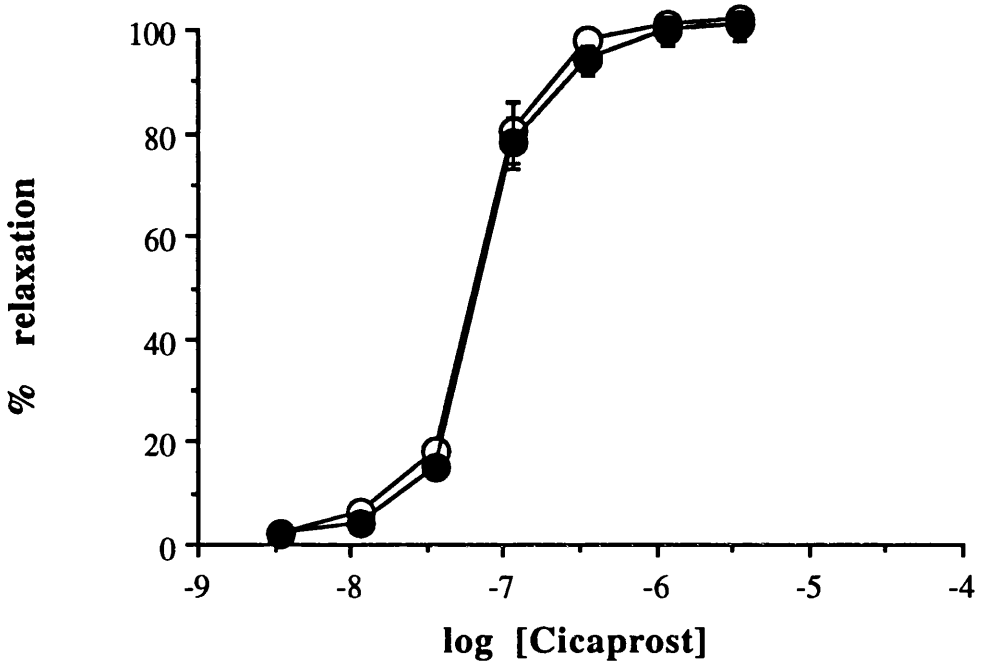

Figure 4.11 The effect of 30μM AH13205 on the response to cicaprost. Mean CRRC to cicaprost in the absence (○) and presence (●) of 30μM AH13205. Relaxation to cicaprost in the absence of AH13205 (control response) is expressed as a proportion of its own maximum and relaxation to cicaprost in the presence of AH13205 is expressed as a proportion of the preceding control response. Points are means, bars are SEM. Mean CR (SEM) to cicaprost in 30μM AH13205, 1.04 (0.08) n=6; vs time control, 0.93 (0.21) n=5; p=0.64.

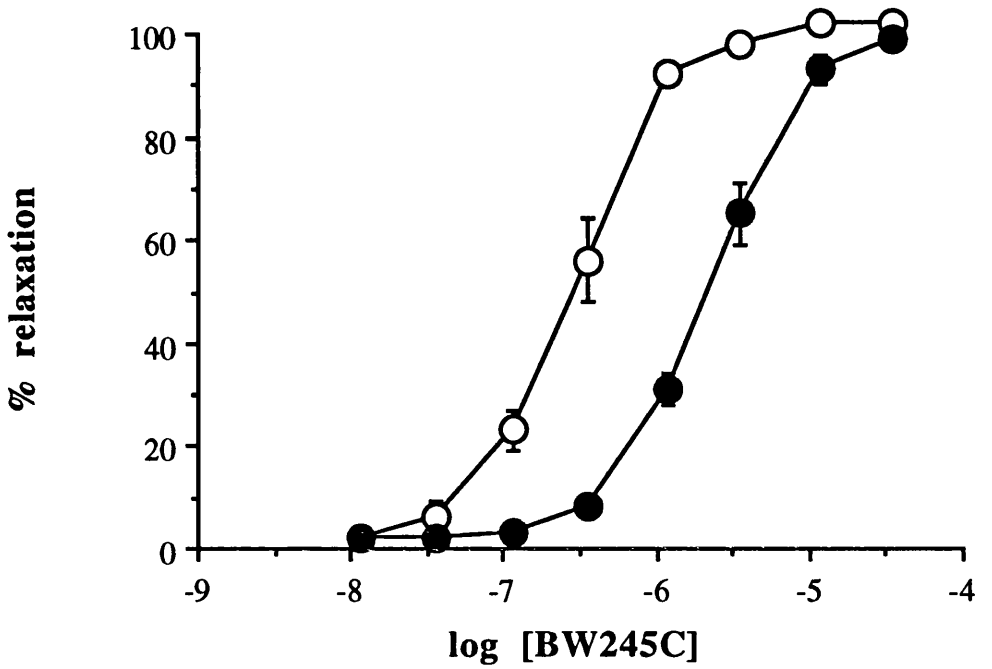

Figure 4.12 The effect of 30μM AH13205 on the response to BW245C. Mean CRRC to BW245C in the absence (○) and presence (●) of 30μM AH13205. Relaxation to BW245C in the absence of AH13205 (control response) is expressed as a proportion of its own maximum and relaxation to BW245C in the presence of AH13205 is expressed as a proportion of the preceding control response. Points are means, bars are SEM. Mean CR (SEM) to BW245C in 30μM AH13205 7.73 (0.87) n=6; vs time control 1.80 (0.36), n=6; p=0.0007.

4.3 Discussion.

It is now well established that receptors for PGE_2 (EP receptors) may be subdivided into at least 3 subtypes, EP_1, EP_2 and EP_3 (Coleman *et al,* 1990). This classification is supported by both selective agonists and the EP_1 receptor blocking drugs, AH6809 and SC19220. More recently, a further type of EP receptor, the EP_4 receptor, has been described which is characterized by a low potency of the existing range of synthetic agonists (Coleman *et al,* 1994a), and weak, but specific antagonist activity by the TP receptor blocking drug, AH23848B, which has no effect on EP_1, EP_2, or EP_3 receptors (Coleman *et al,* 1994a).

The sensitivity of the ductus to PGE_2 was unaffected by the EP_1 receptor antagonists, AH6809 and SC19220, in concentrations 10-fold greater than any previously reported pA_2 (Coleman, 1987), which indicates that PGE_2 does not dilate the vessel through the EP_1 receptor. This is supported by the absence of any dilator effect of the potent EP_1 agonist sulprostone (Coleman *et al,* 1990; Figure 4.2).

The weak dilator action of the selective EP_2 agonist, AH13205 (Nials *et al,* 1993), indicates that PGE_2 does not act through EP_2 receptors on the rabbit ductus. In a range of EP_2 containing tissues, this agonist has been reported to be 10- to 100-fold less potent than PGE_2 (Nials *et al,* 1993), whereas in the rabbit ductus, this difference is at least 100,000-fold (Figure 4.2 and Table 4.1). This interpretation is supported by the fact that misoprostol is more than 100-fold less potent than PGE_2 on the ductus (Figure 4.2 and Table 4.1), whereas it is almost equipotent with PGE_2 at EP_2 receptors in other tissues (Bunce *et al,* 1990).

Both sulprostone and GR63799X are actually more potent than PGE_2 on a range of preparations containing EP_3 receptors (Coleman *et al,* 1987; Bunce *et al,* 1990), but were substantially less potent than PGE_2 on the

ductus (Figure 4.2 and Table 4.1). Thus, it is unlikely that the dilator effect of PGE_2 is mediated by the EP_3 receptor.

AH23848B caused a rightward shift in the PGE_2 CRRC (Figure 4.3) consistent with antagonism of the ductal EP receptor. The drug also had a small, additional non-specific effect, as evidenced by the nature of its effect on PGE_2 (slope of Schild plot >1.0, Figure 4.4) and its small effect on cicaprost (Figure 4.9). AH23848B also blocks the putative EP_4 receptors on pig saphenous vein (Coleman *et al,* 1994a). My findings with selective EP receptor agonists and antagonists suggest, therefore, that the dilator EP receptors on the ductus are of the recently described EP_4 subtype. Alternatively, they may represent a novel subtype of EP receptors. The finding that AH13205 was a selective antagonist of the ductal EP receptor (Figures 4.5, 4.6 and 4.11) was a surprise. This drug is a well characterized EP_2 agonist (Nials *et al,* 1993). However, I am unaware of any previous study that has looked for EP antagonist effects of this drug. A selective EP_4 agonist would help in establishing the nature of the dilator EP receptors in rabbit ductus arteriosus, but no such drug has yet been described.

It may be that the antagonism of PGE_2 by AH13205 points to an involvement of EP_2 receptors, with AH13205 acting as a partial agonist, but this is unlikely. The fact that the absolute potency of PGE_2 on the ductus is high indicates efficient receptor coupling, but AH13205 exhibits little agonist activity, whereas in preparations with substantially poorer coupling e.g. guinea pig trachea, where PGE_2 is approximately 1000-fold weaker than on the ductus, AH13205 exhibits full agonist activity (Nials *et al,* 1993). Secondly, AH23848B has previously been shown to be without antagonist activity at EP_2 receptors (Coleman *et al,* 1994a).

It has been known for some time that the ductus relaxes to PGI_2 in high concentrations (Clyman *et al,* 1978c; Coceani *et al,* 1978b). However,

therehavebeen no data to indicate whether this effect is mediated by an IP receptor, or whether it is a non-specific effect of PGI_2 at the dilator EP receptor (it is characteristic of the native prostaglandins that, at high concentrations, they will act as agonists at receptors for other prostanoids [Coleman *et al,* 1990]). Relaxation of the ductus by the selective IP agonist, cicaprost (Stürzebecher *et al,* 1987; Armstrong *et al,* 1989), indicates that the ductus has IP receptors. This is supported by the fact that the selective antagonists of the ductal EP receptor, AH13205 and AH23848B, had little or no effect on the vessel's response to cicaprost (Figures 4.9 and 4.11). It is concluded that in addition to EP_4 receptors, the fetal rabbit ductus arteriosus also has inhibitory IP receptors.

It has been reported previously that cicaprost is either equipotent with PGI_2 at IP receptors or slightly more potent (Stürzebecher *et al,* 1987). The present finding that cicaprost is about 100-fold less potent than PGE_2 is, therefore, somewhat at odds with studies in the lamb, where the ductus was more than 1000-fold less sensitive to PGI_2 compared with PGE_2 (Clyman *et al,* 1978c; Coceani *et al,* 1978b). While this may simply reflect a species difference, it is relevant that in the lamb ductus the response to PGI_2 is slow, taking up to 12 minutes to plateau (Coceani *et al,* 1978b). Given that these authors noted that leaving PGI_2 at room temperature for 30 minutes eliminated its activity on platelets, it is possible that at 37°C, PGI_2 was broken down over the time course of the response of the ductus. This is not an important consideration with cicaprost which is considerably more stable than PGI_2 (Stürzebecher *et al,* 1987).

Whatever the sensitivity of the ductus to PGI_2 relative to PGE_2, the presence of an IP receptor on the ductus implies a role for PGI_2 in the physiological control of the vessel. This is supported by the fact that PGI_2 is the main prostanoid formed by the ductus (see Chapter 1). Furthermore the absolute sensitivity of the ductus to cicaprost is compatible with other tissues under the physiological control of PGI_2 (the EC_{50} for cicaprost on the rabbit ductus [27.8nM] is consistent with the EC_{50} described for the

rabbit platelet of 22.2nM [Armstrong *et al,* 1989]).

Relaxation of the ductus by the selective DP receptor agonist, BW245C (Town *et al,* 1983; Whittle *et al,* 1983), taken in isolation, suggests that the the ductus has DP receptors (Figure 4.8). However, this is contradicted by a number of other observations. Firstly, BW245C was almost 900-fold weaker than PGE_2 (Table 4.1); secondly, both AH23848B and AH13205 were at least as effective in antagonizing BW245C as they were PGE_2 (Figures 4.10 and 4.12, respectively); and, thirdly, the potent DP receptor antagonist, BW868C, had no significant effect on the sensitivity to BW245C at a concentration more than 1000 times its pA_2 in other tissues (Giles *et al,* 1989). Taken as a whole, these data suggest that the dilator effect of BW245C is due to an action on the ductal EP receptor. This implies that the ductus does not have DP receptors and that the selectivity of BW245C for DP receptors is only relative, being an agonist at the ductal EP receptor albeit almost 900 times less potent than PGE_2 itself.

These data may have real clinical relevance, but clearly, this depends on the similarity in the prostanoid receptor populations in the human and rabbit ductus arteriosus. The use of intra-venous PGE_1 to dilate the ductus in various forms of congenital heart disease in the human neonate is associated with common, serious adverse effects (see Gersony, 1986): seizures, apnoea (10-15%), hyperpyrexia (10-15%), diarrhoea and flushing (10%). Although information as to the prostanoid receptors mediating these effects is not available, it is likely that at least some of these adverse effects are mediated by different EP receptor sub-types from that mediating relaxation of the ductus. Thus, a selective agonist of the ductal EP receptor could have a significantly improved side effect profile compared with PGE_1. The observation that the ductus has an IP receptor suggests the possibility of the use of PGI_2 in treatment of ductus-dependent circulation, but this would probably be associated with other marked cardiovascular activity.

These data may also have relevance to the treatment of PDA. If PGE_2 is the predominant prostaglandin in the maintenance of ductal patency (Clyman, 1987; Coceani and Olley, 1988) and its effects are mediated through EP_4 receptors, as the present work on rabbit ductus suggests, then an EP_4 receptor antagonist should be equally effective as indomethacin but without many (or even any) of its adverse effects. And finally, these data have relevance to the the use of indomethacin as a uterine tocolytic. The contractile receptors for PGE_2 in the human uterus are EP_1 and EP_3 (Senior *et al,* 1991). On the basis of this work, selective antagonists of these receptors would not be expected to contract the ductus.

Chapter 5. Interactions between indomethacin, noradrenaline and vasodilators in the ductus arteriosus.

In my first study on the ductus, I demonstrated that indomethacin increased the sensitivity of the vessel to noradrenaline (Smith and McGrath, 1988), implying that an endogenous cyclo-oxygenase product (probably PGE_2) inhibited the vessel's response to noradrenaline. One aim of the present study was, therefore, to establish whether exogenous PGE_2 inhibited the sensitivity of the vessel to noradrenaline.

I also wished to characterise the effect of indomethacin on the vessel's response to PGE_2 and other vasodilators. Virtually every study on the effects of PGE_2 on the ductus has used indomethacin to pre-contract the vessel and *every* comparison of the relative potency of prostanoids in dilating the ductus that I am aware of has used indomethacin to pre-contract the ductus, both *in vitro* and *in vivo* (see Chapter 1). It has been demonstrated that indomethacin increased the sensitivity of the ductus to the dilator effect of PGE_2 in high oxygen tension, but not low oxygen tension (Coceani et al, 1975). These authors concluded that indomethacin had an effect on the "target-site" for PGE_2 on the ductus (presumably meaning either at the receptor or second-messenger system for PGE_2).

If indomethacin increases the sensitivity of the ductus to PGE_2, but not other prostanoids, the use of indomethacin to pre-contract the ductus in studies looking at the relative potencies of dilators might exaggerate the potency of PGE_2 relative to other vasodilators. Furthermore, there are major flaws in the proposed explanation for the effect of indomethacin on the vessel's response to PGE_2 (see section 5.3). In the following work I set out to characterise the effect of indomethacin on the vessel's response to a number of dilators and constructed experiments to elucidate the mechanism of the effect of indomethacin.

5.1 Methods.

The general methods outlined in Chapter 2 were employed. All experiments described in this chapter were carried out in 15% oxygen, which resulted in an oxygen tension of 100-110mmHg. Different protocols were used for eliciting contraction and relaxation responses, as outlined below.

5.1.1 Effects of PGE_2 on response to noradrenaline.

The vessel was stretched to 0.2g.tension when set up, incubated for 90 minutes before the addition of indomethacin, and the PSS was changed at 30 and 60 minutes. Although the degree of stretch was slightly sub-optimal for contractile responses of the vessel in the presence of indomethacin, higher degrees of stretch resulted in greater variation in the sensitivity to noradrenaline over the course of the experiment (see Chapter 3). Where indomethacin was used, the vessel was incubated in a concentration of 1μM of the drug for at least 30 minutes prior to addition of PGE_2. Where the drug was not used, an identical time protocol was employed. The vessel was exposed to a given concentration of PGE_2 for 10 minutes prior to addition of noradrenaline.

Concentration contraction response curves (CCRC) to noradrenaline were obtained cumulatively. The response at a given concentration was allowed to reach a plateau prior to obtaining the next. The PSS was changed at least 3 times after each CCRC. A period of at least 45 minutes was allowed between CCRC and no more than 5 were obtained from each vessel.

5.1.2 Concentration relaxation response curves.

The vessel was stretched to 0.6g.tension initially and re-set at 60 minutes. The PSS was changed at 30 and 60 minutes and the vessel was incubated for 90 minutes before the addition of any drugs. After the vessel had been incubated in 1μM propranolol (with or without 1μM indomethacin) for 30

minutes, to eliminate any effects of noradrenaline mediated through β-adrenoceptors, it was contracted with 10μM noradrenaline. The contraction was stable (after an initial peak) and was sustained for at least 80 minutes. Concentration relaxation response curves (CRRC) to a given vasodilator were obtained cumulatively and commenced 20 minutes after addition of noradrenaline. The effect of a given concentration of drug was allowed to reach a maximum before addition of the next. The PSS was changed at least 4 times after a CRRC and at least 1 hour was allowed between CRRC. The effect of indomethacin was elucidated by comparing the response in 1μM indomethacin (incubated for at least 30 minutes) with the response in the drug's absence, comparing the responses of different vessels using identical time protocols.

5.1.3 Quantification of responses.

The sensitivity of the vessel to agonists (contractile or relaxant) was quantified by the pEC_{50} (see section 2.4). The magnitude of the relaxation at any given concentration of vasodilator and the maximum relaxant response (MRR) were expressed as a % of the maximum relaxation as determined by addition of 100nM PGE_2 and 30μM papaverine at the end of every CRRC, which resulted in loss of all active tone as indicated by a fall below baseline and no further response to 1μM forskolin. The maximum contractile response (MCR) of noradrenaline was expressed as the increase in tone above changes induced by indomethacin and PGE_2.

Changes in response parameters to noradrenaline in a given concentration of PGE_2 were expressed in relation to a control response. The second CCRC was always used as the control response (in view of the time control data, Figures 3.6 and 3.7), and CCRC 3-5 were obtained in a range of [PGE_2]. In the absence of indomethacin, a control response was obtained and subsequent responses in the presence of exogenous PGE_2 were compared to it. In the presence of indomethacin (1μM) a control response was obtained in 1nM PGE_2 and subsequent responses in a given

concentration of PGE_2 were compared to it. Where the time controls (from Figures 3.6 and 3.7) indicated that the given response parameter was stable comparing CCRC 2 and subsequent CCRC, paired statistical comparison was made between the test and control responses. Where time controls indicated a spontaneous change in response parameter with time, un-paired statistical comparison was made between the extent of change between the test and control response and the extent of change in the time controls.

5.2 Results.

In the presence of 1μM indomethacin, PGE_2 from 0.01nM to 100nM decreased the pEC_{50} to noradrenaline covering a range of 2.39 log units (i.e. a 245 fold increase in EC_{50} [M] to noradrenaline, Figure 5.1). The relationship between [PGE_2] and the MCR (% control maximum) to noradrenaline was bell-shaped. Concentrations of 0.03nM and below and 30nM and above were associated with a decreased MCR compared with the MCR in 1nM PGE_2 (Figure 5.2).

In the absence of indomethacin, PGE_2 of 1nM and above decreased the pEC_{50} to noradrenaline compared with control (Figure 5.3). The maximum decrease in pEC_{50} from control and correcting for the effect of time was between 0.4 to 0.5 log units (i.e an approximately 3 fold increase in the EC_{50} [M]). Addition of PGE_2 between 0.1nM and 10nM increased the MCR (% control maximum) to noradrenaline, whereas 100nM PGE_2 decreased it (Figure 5.4).

By comparing the plot of the raw data for the relationship between [PGE_2] and pEC_{50} to noradrenaline in the presence of indomethacin with the 95% confidence intervals for the control pEC_{50} to noradrenaline in the absence of indomethacin, the effect of endogenous prostaglandins on the response of the vessel to noradrenaline was estimated as being equal to a bath concentration of approximately 1nM PGE_2 with indomethacin present

(Figure 5.5).

With the ductus pre-contracted with 10μM noradrenaline the response to PGE_2 was virtually the same in the presence and absence of 1μM indomethacin (Figure 5.6). A representative trace of the response to PGE_2 with the vessel pre-contracted with 10μM noradrenaline in 1μM indomethacin in 15% oxygen is given in Figure 6.12.A.

The response of the ductus to a range of vasodilators was elucidated in the presence and absence of indomethacin, the vessel pre-contracted with 10μM noradrenaline. Indomethacin decreased the pEC_{50} to cicaprost but increased its MRR (Figure 5.7); decreased the pEC_{50} and MRR to cromakalim (Figure 5.8); and decreased the pEC_{50} to forskolin, but had no effect on the MRR (Figure 5.9). There was no significant difference in the amount of active tone present with 10μM noradrenaline comparing the presence and absence of indomethacin (mean in mN [SEM]: 13.0 [0.52], n=28 vs 13.5 [0.56], n=28, respectively, p=0.55).

Addition of 0.3nM PGE_2 partially reversed the effect of indomethacin on the sensitivity of the ductus to forskolin (Figure 5.10).

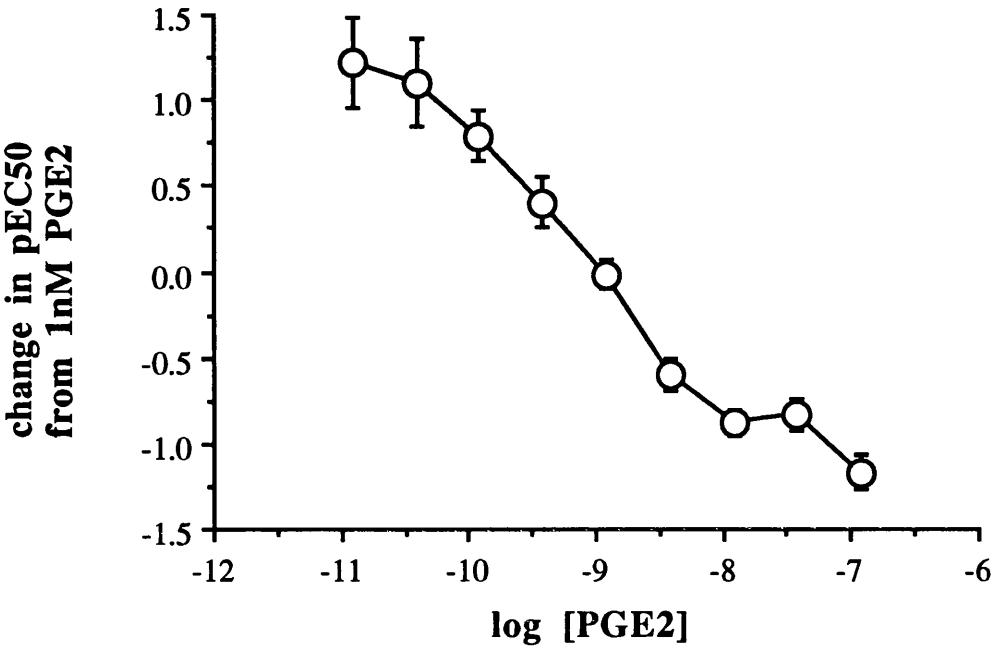

Figure 5.1 The effect of PGE_2 on the pEC_{50} to noradrenaline in the presence of 1μM indomethacin. A control response was obtained in 1nM PGE_2 (CCRC 2, same time protocol as Figure 3.7). The pEC_{50} ($-\log_{10}$ M) of the control response was subtracted from the pEC_{50} of the response in a given $[PGE_2]$ and the difference is plotted on the y axis. The range of change in pEC_{50} across the range of $[PGE_2]$ is 2.39. Points are means, bars are SEM (each point, n=8-14).

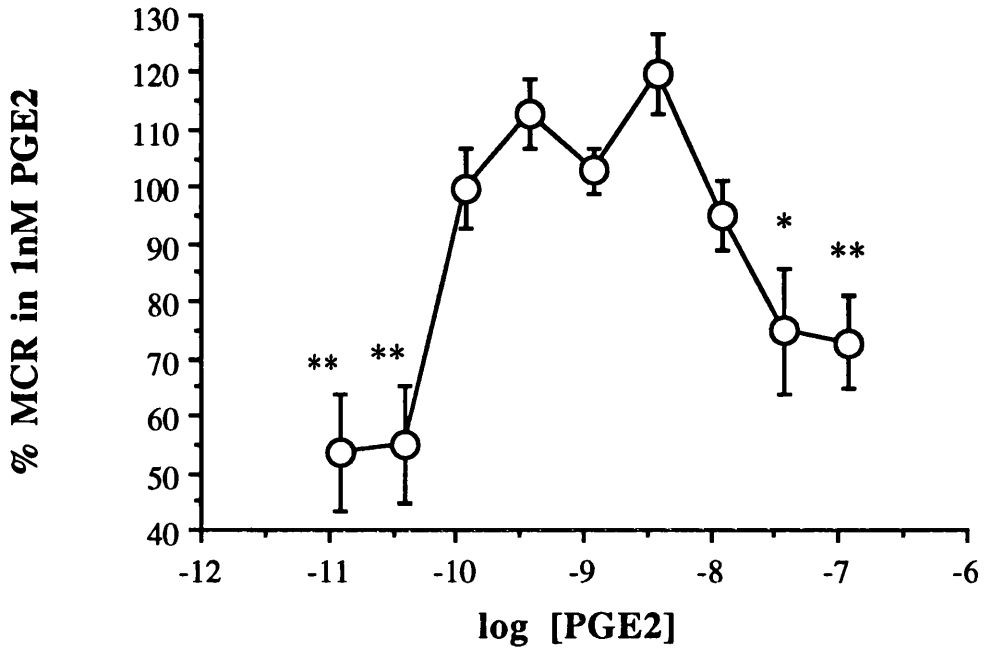

Figure 5.2 The effect of PGE_2 on the maximum contractile response (MCR) to noradrenaline in the presence of 1μM indomethacin. A control response was obtained in 1nM PGE_2 (CCRC 2, same time protocol as Figure 3.7). The MCR in the presence of a given concentration of PGE_2 is expressed as a percentage of the control MCR of CCRC 2. Student's paired t test was used to compare the test and control response *$p<0.05$, **$p<0.01$. Points are means, bars are SEM (each point n=8-14).

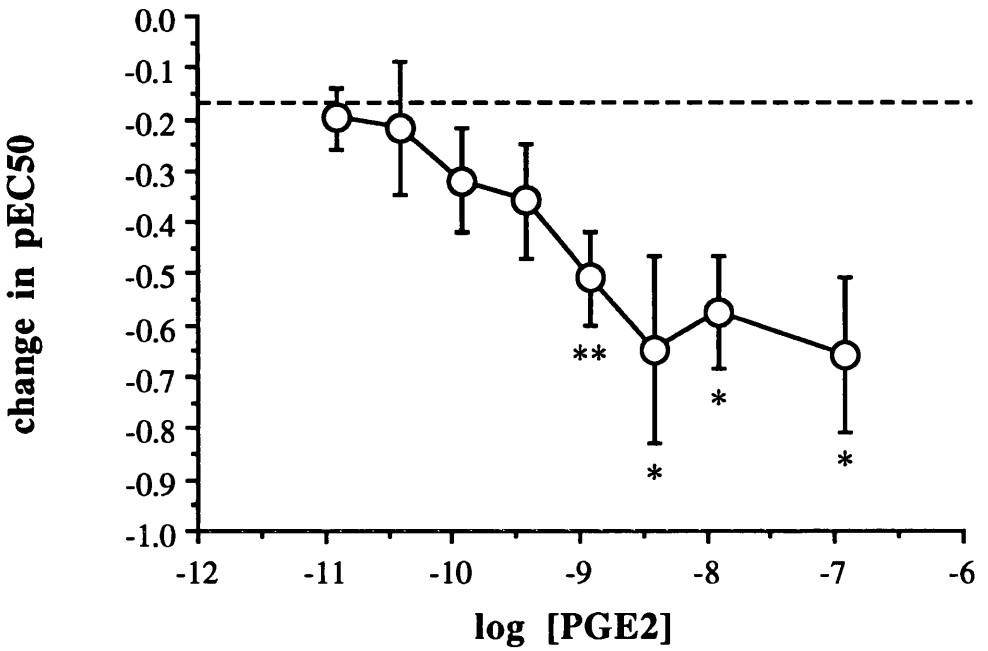

Figure 5.3 The effect of PGE_2 on the pEC_{50} to noradrenaline in the absence of indomethacin. The pEC_{50} ($-\log_{10}$ M) of the control response (CCRC 2, same time protocol as Figure 3.6) was subtracted from the pEC_{50} of the response in a given [PGE_2] and the difference is plotted on the y axis. The difference was compared with the mean difference in pEC_{50} of time controls (-0.169 [SEM 0.036], see Figure 3.6) which is indicated on the graph by the broken line. Student's t-test *$p<0.05$ **$p<0.01$. Points are means, bars are SEM (each point n=7-12).

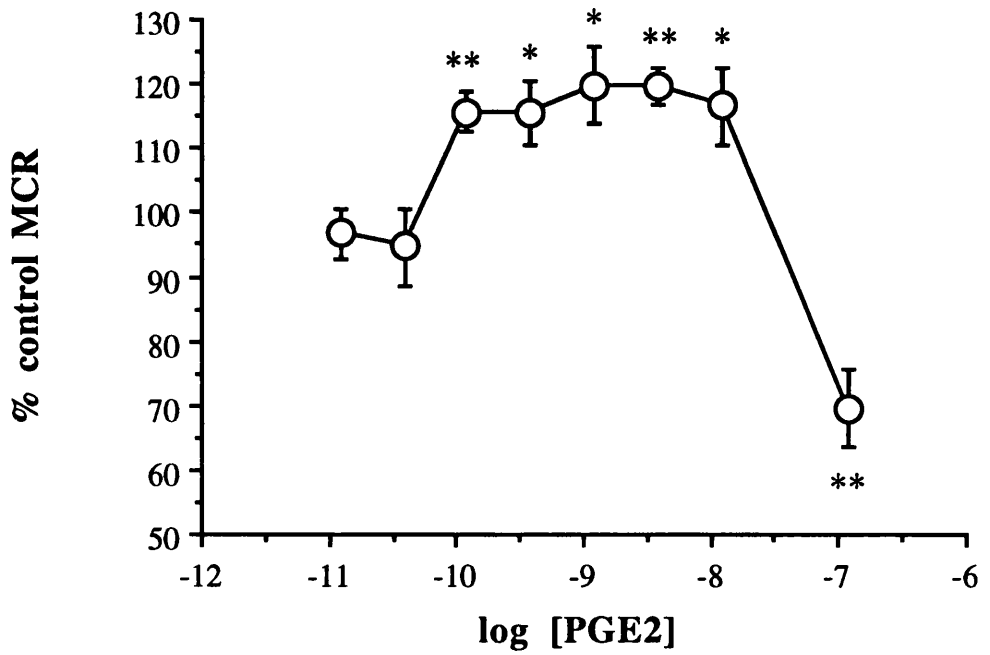

Figure 5.4 The effect of PGE_2 on the maximum contractile response (MCR) to noradrenaline in the absence of indomethacin. The MCR in the presence of a given [PGE_2] is expressed as a % of the MCR of the control response (CCRC 2, same time protocol as Figure 3.6). Student's paired t test was used to compare the test and control response *$p<0.05$, **$p<0.01$. Points are means, bars are SEM (each point n=7-12).

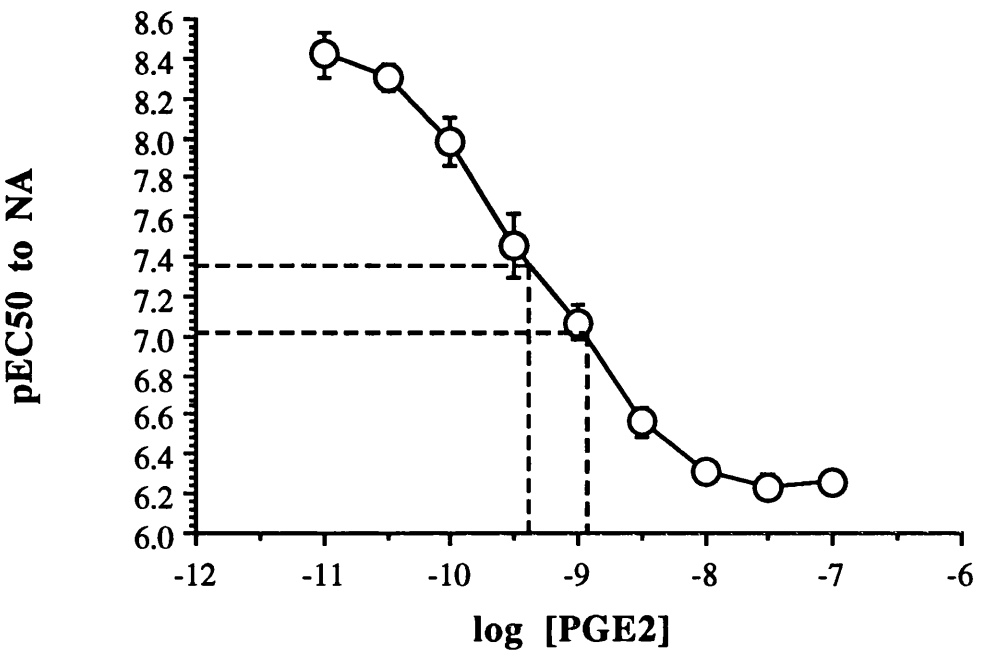

Figure 5.5 The control pEC_{50} ($-log_{10}$ M) to noradrenaline (NA) compared with the pEC_{50} in 1μM indomethacin in the presence of 0.01 to 100nM PGE_2. This graph presents the raw data from Figure 5.1, all responses CCRC 3 to 5. The dotted lines from the y-axis represent the 95% confidence intervals of the pEC_{50} to noradrenaline in the absence of indomethacin and PGE_2 (7.01 to 7.34, n=32: this data is from the control response, CCRC 2, from Figure 5.3). Points are means, bars are SEM (each point, n=8-14).

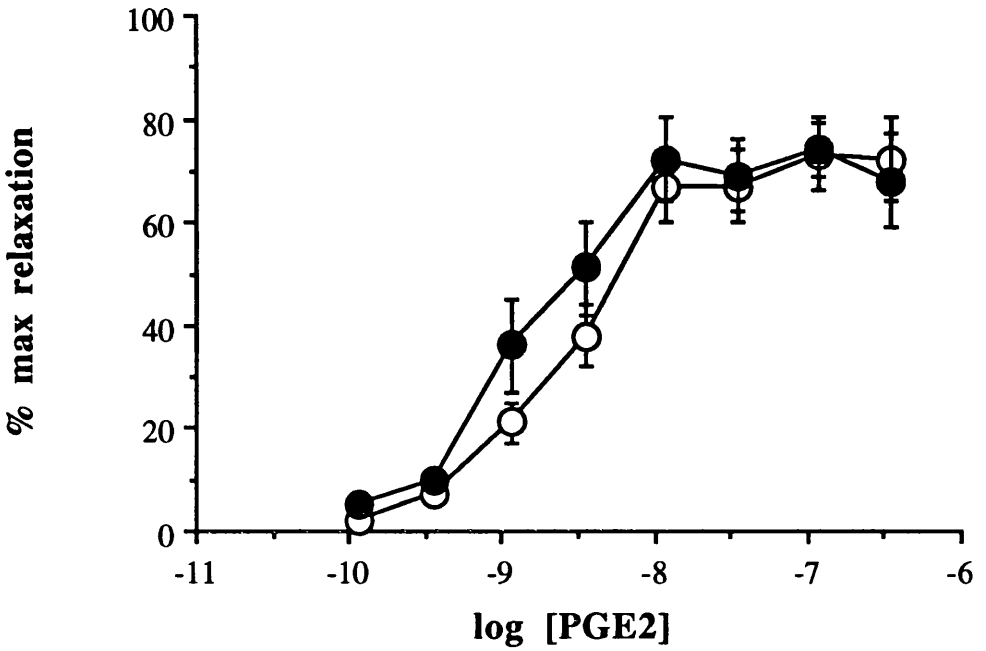

Figure 5.6 The effect of indomethacin (1μM) on the response to PGE_2 in 10μM noradrenaline. 1μM indomethacin (●) and control (○). Comparing the responses to PGE_2, there was no significant difference in either pEC_{50} ($-\log_{10}$ M; mean [SEM]: 8.71 [0.16], n=8, vs 8.50 [0.10], n=8, respectively, p=0.30) or maximum relaxant response (% maximum relaxation, 76.1 [5.6] vs 74.5 [5.5], respectively, p=0.39). Student's t test. Points are means, bars are SEM.

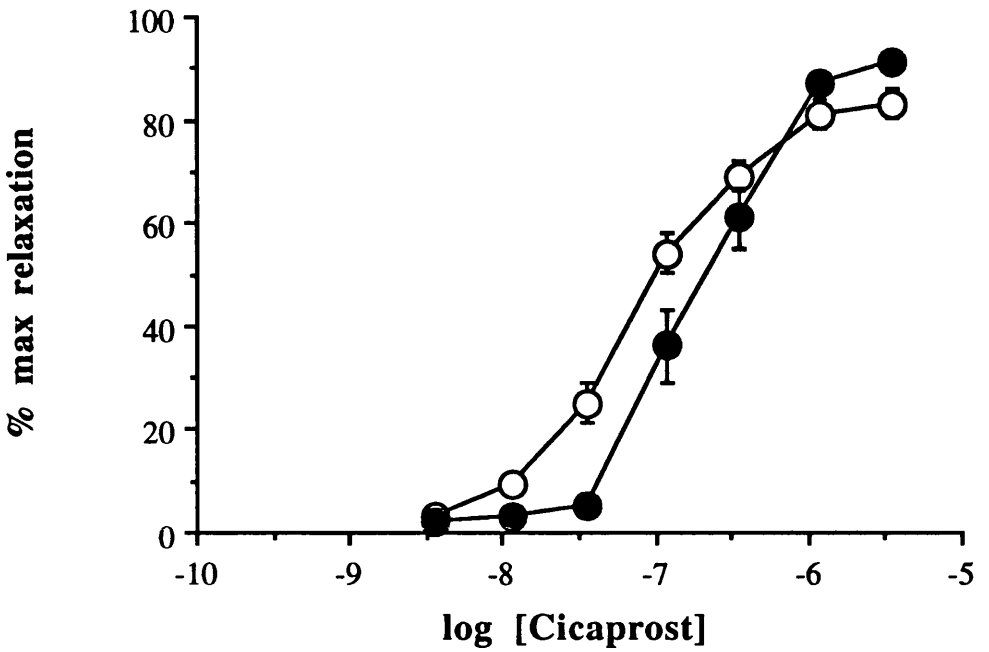

Figure 5.7 The effect of indomethacin (1μM) on the response to cicaprost in 10μM noradrenaline. 1μM indomethacin (●) and control (○). The pEC_{50} ($-\log_{10}$ M) to cicaprost was significantly lower in 1μM indomethacin compared with control (mean [SEM]: 6.78 [0.10], n=8, vs 7.20 [0.07], n=8, respectively, p=0.0053) whereas the maximum relaxant response (% maximum relaxation) was significantly elevated in indomethacin (88.8 [1.6] vs 81.3 [2.5], respectively, p=0.028). Student's t test. Points are means, bars are SEM.

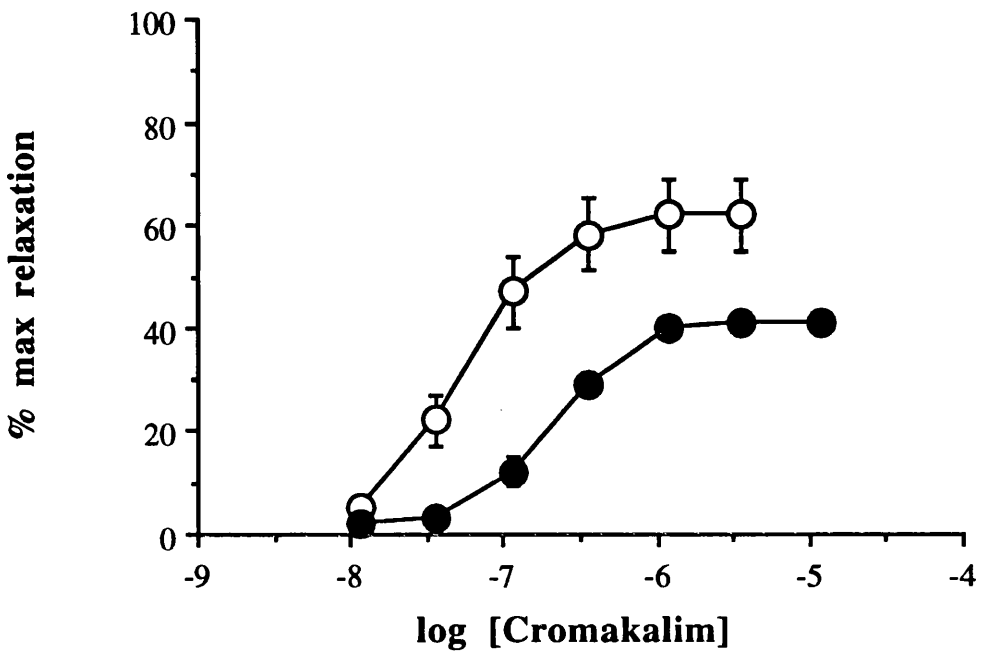

Figure 5.8 The effect of indomethacin (1μM) on the response to cromakalim in 10μM noradrenaline. 1μM indomethacin (●) and control (○). The pEC_{50} ($-\log_{10}$ M) to cromakalim was lower in 1μM indomethacin compared with control (mean [SEM]: 6.75 [0.04], n=4 vs 7.30 [0.03], n=4, respectively, p=0.0001) as was the maximum relaxant response (% maximum relaxation, 39.2 [1.5] vs 59.8 [6.9], respectively, p=0.061). Student's t test. Points are means, bars are SEM.

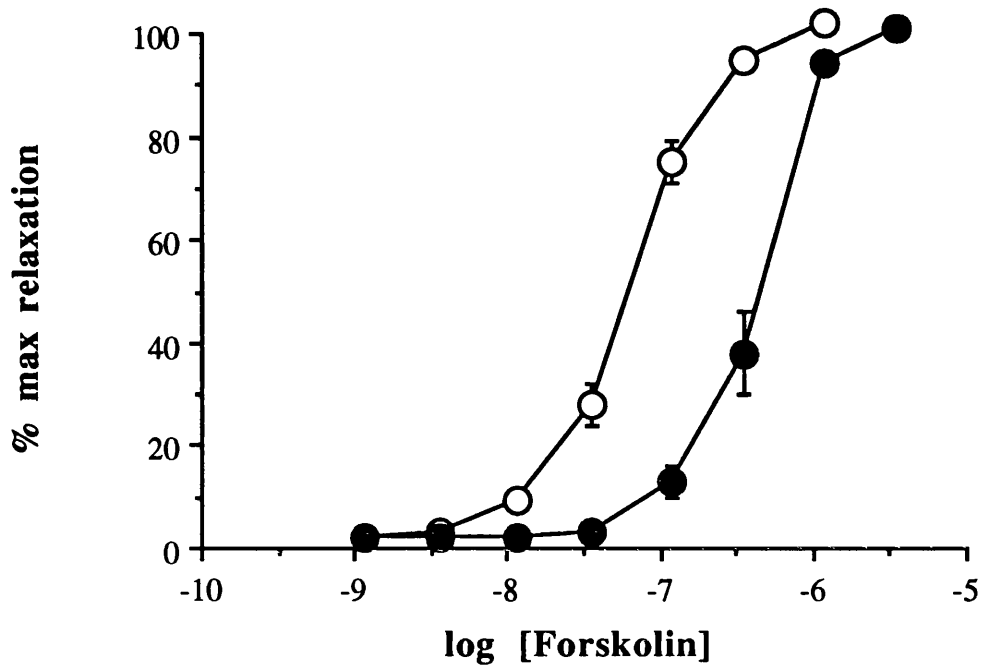

Figure 5.9 The effect of indomethacin (1μM) on the response to forskolin in 10μM noradrenaline. 1μM indomethacin (●) and control (○). The pEC_{50} ($-\log_{10}$ M) to forskolin was significantly lower in 1μM indomethacin compared with control (mean [SEM]: 6.43 [0.07], n=8, vs 7.25 [0.08], n=8, respectively, $p<0.0001$). Student's t test. Points are means, bars are SEM. Note- forskolin caused complete relaxation both in the presence and absence of indomethacin.

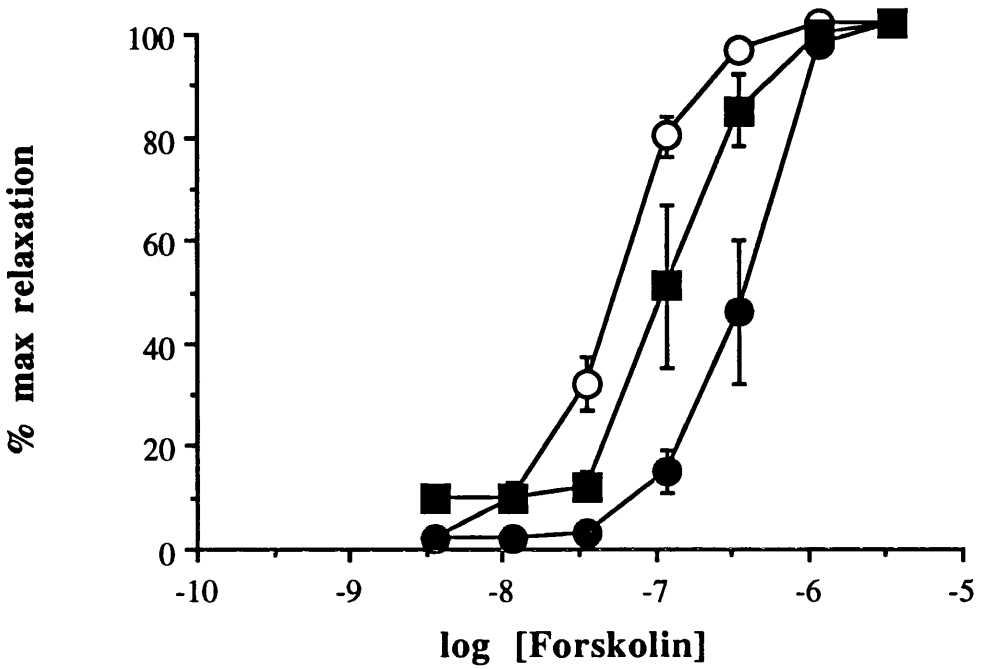

Figure 5.10 Partial reversal of the effect of indomethacin (1μM) on response to forskolin by PGE_2. Control response in absence of indomethacin and PGE_2 (○, n=8). Subsequent response in 1μM indomethacin (●, n=4) and 1μM indomethacin + 0.3nM PGE_2 (■, n=4). The change in pEC_{50} ($-\log_{10}$ M) from the control response was significantly less in the presence of 0.3nM PGE_2 than in its absence (-0.47 [0.06] vs -0.78 [0.09], respectively, p=0.034, Student's t-test). Points are means, bars are SEM.

5.3 Discussion.

In a previous study on the neonatal rabbit ductus arteriosus, I demonstrated increased sensitivity of the vessel to noradrenaline following incubation in indomethacin, implying that prostaglandins synthesised by the ductus inhibit its response to noradrenaline (Smith and McGrath, 1988). The present study demonstrated that PGE_2 is a potent functional antagonist of noradrenaline in the ductus arteriosus. Figure 5.1 reveals that inhibition of ductal sensitivity to noradrenaline is almost maximal by a PGE_2 concentration of 3nM, at which ductal sensitivity to noradrenaline is reduced by about 240 times. Since the concentration of PGE_2 in fetal arterial blood is about 1-2nM (Clyman *et al,* 1980c), it is probable that this action of PGE_2 is of importance in the physiological control of the vessel.

Another aim of this study was to elucidate whether indomethacin has an effect on the sensitivity of the PGE_2 receptor-effector system. The evidence which suggests that it does is work by Coceani *et al,* (1975) which demonstrated an increased response to PGE_2 following incubation in indomethacin in the presence of elevated, but not low, oxygen tension. The ductus synthesises its own prostaglandins (see Chapter 1) which relax the vessel, as evidenced by the contraction induced by indomethacin (Coceani *et al,* 1975). The effects of indomethacin on the ductus are thought to be mediated entirely by elimination of PGE_2 (Clyman, 1987; Coceani and Olley, 1988). In the absence of indomethacin, therefore, the effect of exogenous PGE_2 will be super-imposed on the effects of PGE_2 produced in the vessel wall. It follows that exogenous PGE_2 will only begin to have an effect when it significantly exceeds the concentration of endogenous PGE_2 to which the vessel is exposed. Where a tissue produces a sufficient concentration of prostaglandin to have a biological effect, indomethacin will cause an apparent increase in the tissue's response by removing the contribution of endogenous prostaglandins. Coceani and colleagues (1975) did not consider this explanation for their

results. I wanted to test the hypothesis that the effect of indomethacin on the sensitivity of the ductus arteriosus to PGE_2 could be explained by elimination of the effect of endogenous PGE_2.

I tested the hypothesis in two ways. Firstly I looked at the effect of PGE_2 on the pEC_{50} to noradrenaline, in the presence and absence of indomethacin. Treatment with indomethacin increases the pEC_{50} of the ductus to noradrenaline (Smith and McGrath, 1988) and PGE_2 decreases it (Figure 5.1). It is unaffected by changes in ductal smooth muscle tone *per se* (Smith and McGrath, 1988). It acts as a useful index, therefore, of the effects of endogenous and exogenous PGE_2. The second approach was to look at the dilator effects of PGE_2, with and without indomethacin, in the presence of a very high level of tone which exceeded the inhibitory effects of endogenous PGE_2. For this the ductus was pre-contracted with 10μM noradrenaline.

Looking at the data on the effect of a range of [PGE_2] on the pEC_{50} to noradrenaline in the presence and absence of indomethacin, they appear to confirm that indomethacin increases ductal sensitivity to PGE_2. In the absence of indomethacin, PGE_2 (from 1nM to 100nM) decreased the pEC_{50} to noradrenaline to a maximum of 0.4 to 0.5 log units (i.e. an approximately 3 fold increase in EC_{50} [M], Figure 5.3). In the presence of 1μM indomethacin, PGE_2, across the range 0.01nM to 100nM, decreased the pEC_{50} to noradrenaline by 2.39 log units (i.e. a 245 fold increase in EC_{50} Figure 5.1).

Figure 5.5 illustrates the relationship between [PGE_2] and pEC_{50} to noradrenaline. By superimposing the 95% confidence intervals of the control pEC_{50} to noradrenaline on this graph, it is clear that in the absence of indomethacin endogenous PGE_2 had a profound inhibitory effect on the vessel's pEC_{50} to noradrenaline. Indeed, a rough estimation of the effects of endogenous PGE_2 can be made in terms of equivalence to a bath

[PGE_2] with indomethacin present, which I estimate to be in the region of 1nM. In the absence of indomethacin, PGE_2 had an effect on the pEC_{50} to noradrenaline only at concentrations of 1nM and above. Taking these data together I conclude that the major effect, possibly exclusive effect of indomethacin to increase the sensitivity of the ductus to the effects of PGE_2 is explained by elimination of endogenous PGE_2.

This hypothesis was further tested by raising tone with 10μM noradrenaline and obtaining a CRRC to PGE_2. The absence of a major inhibitory effect of endogenous PGE_2 is indicated by the fact that the extent of pre-contraction was the same in the presence and absence of indomethacin. Under these circumstances, indomethacin had no effect on the sensitivity or MRR to PGE_2 (Figure 5.6), which provided further support for this model of the effect of indomethacin.

This hypothesis predicts that the effects of indomethacin on the sensitivity of the ductus will be increased in situations where endogenous PGE_2 synthesis is stimulated. Elevated oxygen tension massively stimulates ductal synthesis of PGE_2 (Clyman *et al,* 1980b). Coceani *et al,* (1975), looking at similar changes in oxygen tension, found that indomethacin only increased sensitivity to PGE_2 in elevated oxygen tension. I conclude, therefore, that indomethacin potentiates the response to PGE_2 primarily by eliminating the effects of endogenous PGE_2. Any study of the effects of prostaglandin synthesis inhibitors on the response of the ductus to exogenous prostaglandins will have to take this effect into account before postulating effects on the PGE_2 receptor-effector mechanism.

Both in the presence and absence of indomethacin, PGE_2 had complex effects on the MCR (% control maximum) to noradrenaline. With indomethacin present (Figure 5.2), low concentrations of PGE_2 (≤0.03nM) were associated with a decreased MCR to noradrenaline. This was probably due to the fact that the noradrenaline and indomethacin contractions are not additive (as indicated by the fact that the active tone

present in 10μM noradrenaline was the same in the presence and absence of 1μM indomethacin). By eliminating some of the indomethacin-induced tone, moderate concentrations of PGE_2 (0.1 to 10nM) actually increased the MCR to noradrenaline. This effect was overcome by a direct inhibitory effect on the response to noradrenaline of higher concentrations of PGE_2 (≥30nM). The effects of PGE_2 on the MCR to noradrenaline in the absence of indomethacin (Figure 5.2) were more difficult to explain. PGE_2 caused a small, but significant increase in MCR across the range 0.1 to 10nM. This may have been due, again, to elimination of tone. Alternatively, I have some evidence for the existence of a contractile receptor for PGE_2 on the ductus (see Chapter 7) which might explain this stimulatory effect. Whatever the mechanism, this effect was overcome by a direct inhibitory action in high concentrations of PGE_2.

While indomethacin had no effect on the response of the ductus to PGE_2 in the presence of 10μM noradrenaline, it did have effects on the response of the ductus to other vasodilators (Figures 5.7 to 5.9). In the absence of indomethacin, the sensitivity of the ductus to cicaprost (a stable PGI_2 mimetic [Armstrong *et al,* 1989]), forskolin (a direct activator of adenylate cyclase [Seamon and Daly, 1981]) and cromakalim (an activator of ATP-sensitive potassium channels [Bray *et al,* 1991]) was increased. This was probably due to synergy between these exogenous dilators and endogenous PGE_2. I tested this hypothesis by comparing the effect of indomethacin on the response to forskolin in the presence and absence of 0.3nM PGE_2 (Figure 5.10). Addition of this small concentration of PGE_2 reversed somewhat the effect of indomethacin. I conclude, therefore, that the ductus was more sensitive to exogenous vasodilators, other than PGE_2, in the absence of indomethacin and that this was due to a potentiating effect of endogenous PGE_2.

Converting the data in Figures 5.6 and 5.7 into molar concentrations, PGE_2 was about 85 times more potent than cicaprost in the presence of

indomethacin but only 20 times more potent than cicaprost in its absence. The greater the contribution of endogenous PGE_2, the more potent cicaprost will become when compared with PGE_2 since (1) endogenous PGE_2 will increase the vessel's sensitivity to cicaprost; and, (2) endogenous PGE_2 will decrease the vessel's sensitivity to exogenous PGE_2. The above experiments used 10μM noradrenaline to minimise the effects of endogenous PGE_2. The potency of cicaprost compared with PGE_2 in the absence of indomethacin may be increased in situations of lesser constriction of ductal smooth muscle, as the contribution of endogenous PGE_2 will further decrease the vessel's response to exogenous PGE_2. Furthermore, given that the ductus is also exposed to circulating PGE_2 *in vivo* (indeed it has been suggested that this is the major source of PGE_2 to which the vessel is exposed [Clyman *et al,* 1980b]) this suggests that cicaprost may even more potent relative to PGE_2 *in vivo* than in the present study.

Previous comparisons of the relative potency of different prostaglandins as dilators of the ductus, both *in vitro* (Clyman *et al,* 1978a and 1978c; Coceani *et al,* 1978b) and *in vivo* (Friedman *et al,* 1983) have been in the presence of indomethacin and it was on the basis of these studies that PGE_1 was selected to be used to maintain artificially ductal patency in neonates with ductus-dependent circulation. It seems likely, therefore, that comparing the effects of different prostaglandins in the presence of indomethacin as a guide to their potency in the human infant with ductus-dependent circulation has led to two sources of error: (1) the sensitivity of the vessel to PGE_1 was overestimated as the effects of endogenous PGE_2 had been eliminated; and, (2) the sensitivity of the vessel to PGI_2 was underestimated, due to the absence of its potentiation by endogenous PGE_2. The extent of this "error" will depend on the concentration of endogenous PGE_2 to which the smooth muscle of the ductus is exposed *in vivo.* I propose that the relative potencies of ductal vasodilators need to be re-evaluated in better models of the physiological and clinical states.

The effect of indomethacin on the MRR (% maximum relaxation) to dilators was less straightforward. Indomethacin increased the MRR to cicaprost, decreased the MRR to cromakalim and had no effect on the MRR to forskolin (Figures 5.7 to 5.9). The MRR to forskolin was unchanged by indomethacin as it caused complete relaxation whether endogenous PGE_2 was present or not. The decrease in MRR to cromakalim in the presence of indomethacin was probably due to loss of synergy with endogenous PGE_2. Endogenous PGE_2 potentiated the sensitivity to cicaprost (as indicated by the effect of indomethacin). If it had also potentiated its MRR, indomethacin would have decreased rather than increased the MRR to cicaprost. The effect of indomethacin on the MRR to cicaprost cannot, therefore, be explained by eliminating the effects of endogenous PGE_2, but it could be explained by the elimination of endogenous PGI_2. PGI_2 is the main prostanoid formed by the ductus (see Chapter 1). If endogenous PGI_2 inhibited the contraction to 10μM noradrenaline, indomethacin would be expected to increase the maximum relaxation to a PGI_2 mimetic by a similar reasoning as developed above for the effect of indomethacin on the response to PGE_2.

In summary, indomethacin increased the sensitivity of the ductus arteriosus to PGE_2, decreased its sensitivity to cicaprost, cromakalim and forskolin, and decreased its maximum response to cromakalim. All these effects can be explained in terms of eliminating endogenous PGE_2. In addition, indomethacin increased the maximum response to cicaprost, which could be explained by elimination of endogenous PGI_2, but not PGE_2.

Chapter 6. Interactions between oxygen tension, noradrenaline and vasodilators in the ductus arteriosus.

It had been demonstrated that oxygen tension inhibits the sensitivity of the ductus arteriosus to PGE_2 in the absence but not the presence of indomethacin (Coceani *et al,* 1975). As discussed in some detail in the preceding chapter, these authors concluded that indomethacin altered the sensitivity of the "target-site" for PGE_2, and that the drug reversed the inhibitory effect of elevated oxygen tension. In the preceding chapter, it was demonstrated that this was almost certainly a spurious conclusion and that the observed effects of oxygen tension and indomethacin could be explained in terms of their effect on endogenous PGE_2 production which would alter the vessel's response to exogenous PGE_2. Nevertheless, given that PGE_2 is central to keeping the ductus patent in the fetus, and that oxygen is central to closing the vessel in the neonate, it might be expected that some interaction exists between the vessel's sensitivity to PGE_2 and the prevailing oxygen tension. In this part of the work, I set out to test the hypothesis that PGE_2 and oxygen interact.

In this chapter, I describe (1) the effect of oxygen tension on the inhibitory actions of PGE_2 on the response of the ductus to noradrenaline; (2) the effect of oxygen tension on the response of the ductus to PGE_2 and other vasodilators, prostanoid and non-prostanoid, with the vessel pre-contracted with 10μM noradrenaline. These experiments were carried out in the presence of 1μM indomethacin to eliminate misleading effects due to changes in endogenous PGE_2 production. In addition, experiments were carried out where indomethacin was omitted or substituted with a structurally unrelated cyclo-oxygenase inhibitor, flubriprofen.

6.1 Methods.

The general methods outlined in Chapter 2 were employed. In this chapter, the amount of oxygen bubbling the preparations was manipulated to elucidate the effect of oxygen tension on the response of the ductus to contractile and relaxant agonists.

6.1.1 Effects of PGE_2 and oxygen on response to noradrenaline.

These experiments (data in Figures 6.1 to 6.4) were conducted on different equipment from all the rest of the experimental work described in this thesis, although the animals used and general experimental design were identical. The amplification and chart recorder system was a polygraph chart recorder (Grass, model 7PCPA). The organ baths were of a special design, being deep with a narrow top, to reduce diffusion of atmospheric oxygen. The proportion of oxygen used in the gas bubbling system was slightly different, therefore, to that described in section 6.1.2, although the actual oxygen tensions obtained were similar.

When mounted, the vessels were stretched to 0.2g.tension, which was not re-set. Although the degree of stretch was slightly sub-optimal for contractile responses of the vessel in the presence of indomethacin, higher degrees of stretch resulted in greater variation in the sensitivity to noradrenaline over the course of the experiment (see Chapter 3). Following mounting, the vessels were allowed to equilibrate for 90 minutes. The PSS was changed at 30 and 60 minutes. The bath was bubbled with 3, 10, 15, or 95% O_2; 5% CO_2; and remainder N_2. The above procedure yielded a bath PO_2 (in mmHg) of 27.2 (SEM 1.7) with 3% oxygen, 75.4 (SEM 0.8) with 10% oxygen, and 107.0 (SEM 0.8) with 15% oxygen. Vessels were equilibrated in a given PO_2 for a minimum of 45 minutes before a concentration contraction response curve (CCRC) was obtained. All experiments were carried out in the presence of 1μM indomethacin, added at least 30 minutes prior to the addition of PGE_2.

PGE_2 was added to the bath 10 minutes prior to eliciting any response.

CCRC to noradrenaline were obtained cumulatively. The response at a given concentration was allowed to reach a plateau prior to obtaining the next. The PSS was changed at least 3 times after each CCRC. A period of at least 45 minutes was allowed between CCRC and no more than 5 were obtained from each vessel. Contractions due to potassium-induced depolarisation were obtained by addition of molar KCl to the organ bath. When the response to potassium was elicited, the response in 10nM PGE_2 was obtained first and the response in 0.01nM PGE_2 obtained second.

6.1.2 Effects of oxygen on vasodilators

The equipment was used in these experiments was the same as in the rest of the work described in this thesis, with the exception of Figures 6.1 to 6.4.

The vessel was stretched to 0.6g.tension which was re-set 60 minutes later. The PSS was changed at 30 and 60 minutes following mounting and the vessels were allowed to incubate for 90 minutes in 15% oxygen prior to addition of indomethacin. The baths were bubbled with gas mixtures of 2 or 15% O_2, 5% CO_2, and remainder N_2. When 2% O_2 was used, the organ bath was partially covered with chemically inert film to reduce diffusion of oxygen from the air. Bubbling with 2% O_2 resulted in a PO_2 of 19-23mmHg, which is consistent with the fetal oxygen tension to which the ductus is exposed (Heymann and Rudolph, 1975) and bubbling with 15% O_2 resulted in a PO_2 of 100-110mmHg. Vessels were equilibrated in a given PO_2 for a minimum of 45 minutes before a concentration relaxation response curve (CRRC) was obtained.

The vessel was pre-contracted with 10μM noradrenaline. The noradrenaline contraction was stable and time controls demonstrated that it was sustained for at least 80 minutes. Prior to addition of noradrenaline

the vessel was incubated in 1μM propranolol for 30 minutes, to eliminate any effects of noradrenaline mediated through β-adrenoreceptors. Unless otherwise stated, experiments were carried out in the presence of 1μM indomethacin. When it was omitted or substituted by flubriprofen, it is stated in the text and figure legend. Indomethacin (1μM) or flubriprofen (1μM) were added with the propranolol, as appropriate (the two cyclo-oxygenase inhibitors are roughly equipotent in human umbilical artery [MacLennan *et al,* 1988]). In experiments where neither cyclo-oxygenase inhibitor was used, the same time protocol was employed.

The cumulative CRRC to the given agonist was commenced 20 minutes after addition of noradrenaline. The effect of a given concentration of drug was allowed to reach a maximum before addition of the next. In all cases the first CRRC was in 15% oxygen. The effects of oxygen tension were elucidated by obtaining a second response in 2% oxygen. To correct for any effect of time, controls were obtained where both the first and second responses were in 15% oxygen. No separate time controls were obtained for responses to PGE_2 in flubriprofen and in the absence of a cyclo-oxygenase inhibitor. Instead, a third CRRC was obtained in 15% oxygen which, by comparison with the first CRRC in 15% oxygen, confirmed that the response to PGE_2 under these conditions was stable with time. The PSS was changed at least 4 times after an CRRC and at least 1 hour was allowed between CRRC.

6.1.3 Quantification of responses.

The sensitivity of the vessel of agonists (contractile or relaxant) was estimated by the pEC_{50} (see section 2.4 for definition). The sensitivity of the vessel to the dilator effect of cicaprost, forskolin and PGE_2 was also quantified by an index which was relative to the size of pre-contraction: the pEC_{30*}, where the EC_{30*} is the interpolated molar concentration of the agonist which caused a reduction in tone of 30% of the extent of pre-contraction under the given conditions. The asterix is used to emphasize the difference between the method of calculating this and the EC_{50}.

For noradrenaline, only data from CCRC 2-5 were used for comparison of pEC_{50}s, given the time controls of Figure 3.7. However, data from any CCRC were used for comparison of maximum responses to noradrenaline as this was unaffected over the course of time under these conditions (see section 3.2). The maximum contractile response (MCR) to noradrenaline in 10% and 3% oxygen was expressed as a proportion of the same vessel's MCR to noradrenaline in 95% oxygen.

The magnitude of the relaxation at any given concentration and the maximum relaxant response (MRR) were expressed as a % of the maximum relaxation as determined by addition of 100nM PGE_2 and 30μM papaverine at the end of every CRRC, which resulted in loss of all active tone (i.e. below baseline and with no further response to 1μM forskolin).

6.2 Results.

6.2.1 Effects of PGE_2 and oxygen on response to noradrenaline.
In the presence of 1μM indomethacin and 1nM PGE_2, decreasing oxygen decreased the pEC_{50} and maximum response to noradrenaline (Figure 6.1). This effect of oxygen was completely reversible as illustrated by the original tracings in Figure 6.2. The maximum response to noradrenaline in 0.01nM PGE_2 was the same in 95% and 3% oxygen, and the effect of oxygen on the maximum response of the vessel to noradrenaline was dependent on the presence of PGE_2 in a concentration-dependent manner (Figure 6.3)

In the presence of 15% oxygen and 1μM indomethacin, increasing PGE_2 from 0.01nM to 10nM decreased the vessel's sensitivity and maximum response to potassium-induced contraction of the ductus (Figure 6.4).

6.2.2 Effects of oxygen on vasodilators.

With the vessel pre-contracted with 10μM noradrenaline in 1μM indomethacin and 15% oxygen (pooling the first responses in 15% oxygen), the rank order of MRR of agonists was: forskolin > cicaprost > PGE_2 >> cromakalim >> adenosine ≈ SNP > ANP = nifedipine = 0 (Figure 6.5). Only for the first four of these was the response sufficient to calculate a pEC_{50}. The rank order of pEC_{50} ($-\log_{10}M$) of these agonists was (mean [SEM]): PGE_2 (8.65 [0.12] n=15) >> cicaprost (6.80 [0.06] n=15) ≈ cromakalim (6.58 [0.06] n=16) ≈ forskolin (6.42 [0.05] n=12); one way ANOVA of means: F ratio = 156.65, $p<0.0001$. Multiple comparisons revealed that the pEC_{50} for PGE_2 greater than all other agonists, cicaprost was significantly greater than forskolin, but there were no other significant differences (see Appendix [Table A.6] for table of values of 95% CI of pairwise differences). Converting these data into molar concentrations, PGE_2 was about 70 times more potent than cicaprost.

Two agonists, nifedipine (up to 30μM) and atrial natriuretic peptide (up to 100nM) had no significant dilator effect in either 15% or 2% oxygen tension. The MRRs of all the other agonists were potentiated in 2% oxygen (Figures 6.6 to 6.10) except forskolin, which caused complete relaxation in 15% oxygen (Figure 6.11). The effect of oxygen on the response to PGE_2 is illustrated by original tracings (Figure 6.12). With some agonists, the initial response in 15% oxygen was so small that no pEC_{50} value could be obtained. All of the four agonists where a pEC_{50} could be calculated (cicaprost, cromakalim, forskolin and PGE_2) showed a significant increase in pEC_{50} in 2% compared with 15% oxygen (Table 6.1).

The response in 2% oxygen was obtained after the response in 15% oxygen. Time controls for all agonists were obtained for two consecutive responses in 15% oxygen (Table 6.2). Comparing the two responses, there was no change in the response to forskolin, PGE_2 or SNP. However, the

pEC_{50} to cicaprost and cromakalim and the MRR to adenosine, cicaprost, and cromakalim were significantly increased at the second response compared to the first.

For agonists which showed a significant change in time controls, the extent of change with varying oxygen tension was compared with the extent of spontaneous change with time (Table 6.3). In every such case the effect of oxygen was significantly greater than the spontaneous change with time. After correcting the change in pEC_{50} going from 15% to 2% oxygen for the mean change in the time controls (cicaprost and cromakalim only), there was no significant difference in the extent of change in pEC_{50} between the four agonists; one way ANOVA of means: F ratio = 2.30, p=0.100.

The total extent of active tone prior to a CRRC was elucidated by obtaining complete relaxation at the end (see section 6.1.3). The active tone present prior to the first CRRC (i.e 15% oxygen, 1μM indomethacin and 10μM noradrenaline) was 11.6mN (SEM 0.3, n= 86). The amount of active tone prior to the CRRC in 2% oxygen was 99.8% (n=47; 95% CI 94.6 to 105.0) that of the preceding CRRC in 15% oxygen. However, the active tone prior to the second CRRC in 15% oxygen (i.e. the time controls) was 107.8% (n=39; 95% CI 105.5 to 110.1) that of the preceding CRRC in 15% oxygen. The difference between the active tone present in 2% oxygen and its time control in 15% oxygen although small, was statistically significant (p=0.0064, Student's t-test, 95% CI of difference: 2.3 to 13.6).

The amount of tone resulting from the first addition of indomethacin was always greater than later on in the experiment: the active tone prior to addition of 10μM noradrenaline (data from the time controls in 15% oxygen) at CRRC 1 was 12.3mN (SEM 0.4, n= 39) and at CRRC 2 was 5.5mN (SEM 0.6) (p<0.0001). The active tone prior to addition of 10μM noradrenaline in 2% oxygen was 1.5mN (SEM 0.2, n=47) which was

significantly less than the second time control ($p<0.0001$).

For three agonists (cicaprost, forskolin and PGE_2), the response in 15% oxygen was of sufficient magnitude to calculate a pEC_{30*}, which corrects estimation of sensitivity for the magnitude of pre-contraction (see section 6.1.3). In all three cases the pEC_{30*} was increased in 2% oxygen compared with 15% (Table 6.1). There was a spontaneous increase in pEC_{30*} with time to cicaprost, but not the other two agonists (Table 6.2). The extent of change in pEC_{30*} to cicaprost with oxygen was significantly greater than with time (Table 6.3). There was no significant difference in the extent of change in pEC_{30*} with decreased oxygen tension (cicaprost corrected for time controls) between the three agonists; one way ANOVA of means: F ratio 1.15, p=0.337.

Oxygen still altered the PGE_2 CRRC when indomethacin was omitted or when the vessel was incubated in 1μM flubriprofen instead of indomethacin (Figures 6.13 and 6.14, respectively). The magnitude of the effect of oxygen on the pEC_{50} was the same in indomethacin, flubriprofen or in the absence of these drugs (mean change [SEM]: 0.51 [0.12], n=7; 0.56 [0.04], n=4; 0.53 [0.18], n=7, respectively, F ratio = 0.02, p=0.979). The MRR to PGE_2 in 15% oxygen was also the same under the three sets of conditions (Figure 6.15). However, the MRR to PGE_2 in 2% oxygen was decreased in the absence of a cyclo-oxygenase inhibitor compared with both indomethacin and flubriprofen (Figure 6.15).

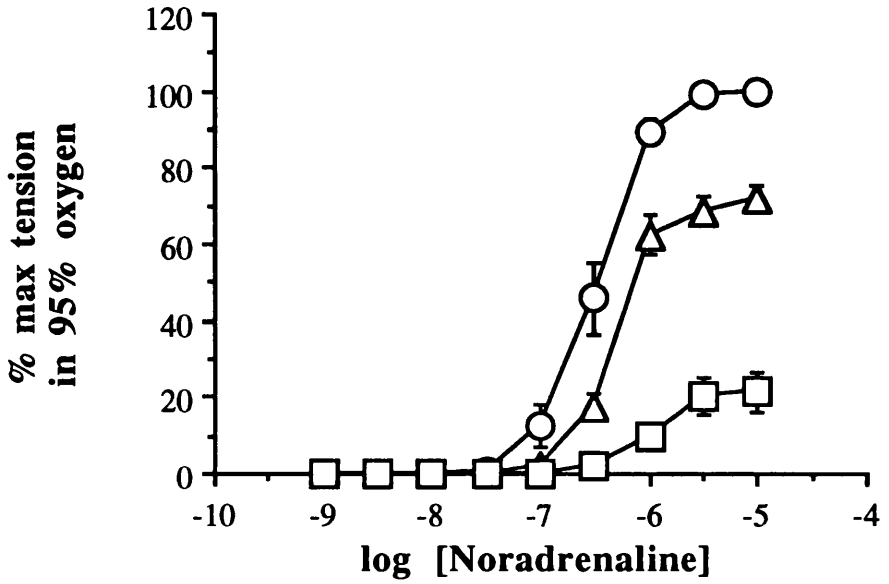

Figure 6.1 Response to noradrenaline in 1nM PGE_2 and 1μM indomethacin 95%, 10% and 3% oxygen. ○ 95% oxygen, n=7 Δ 10% oxygen, n=3; and, □ 3% oxygen, n=7. Responses in 10% and 3% oxygen were paired with responses in 95% oxygen and contractile response in 10% or 3% oxygen were expressed in relation to control response in 95% oxygen. The mean decrease in maximum response from 95% to 10% was 28% (95% CI 12 to 44, p=0.017); from 95% to 3% was 79% (95% CI 68 to 90, p<0.0001); and, from 10% to 3% was 66% (95% CI 30 to 102, p=0.016). The mean change in pEC_{50} ($-\log_{10}M$) from 95% to 10% was 0.30 (95% CI -0.41 to 1.01, p= 0.22), from 95% to 3% was 0.46 (95% CI 0.10 to 0.82, p= 0.02) and from 10% to 3% was 0.40 (95% CI 0.07 to 0.74, p=0.035). All comparisons Student's paired t test. Points are means, bars are SEM.

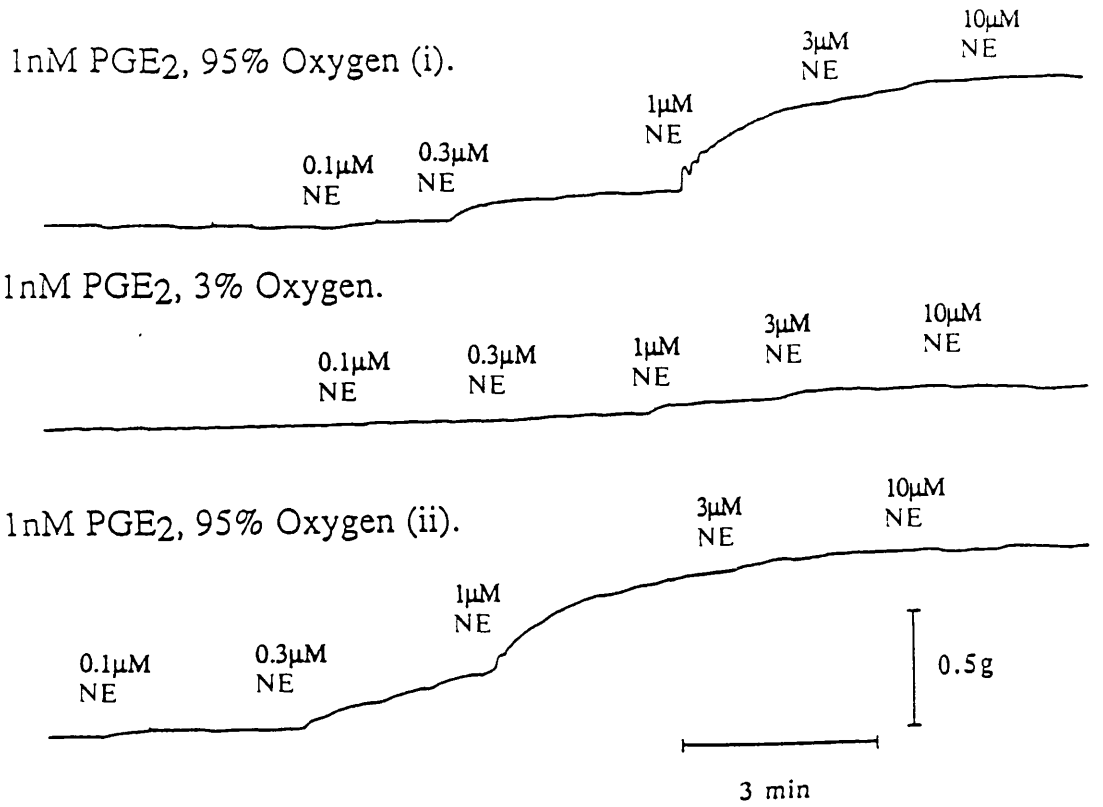

Figure 6.2 Original tracing of consecutive responses to noradrenaline in 1μM indomethacin and 1nM PGE_2 in A. 95%, B. 3% and C. 95% oxygen. Original tracings of 3 consecutive concentration response curves to noradrenaline from a single vessel in 1nM PGE_2 and 1μM indomethacin. **A**-in 95% oxygen, **B**-in 3% oxygen, **C**-in 95% oxygen again. In the four vessel's studied, there was no significant difference between the two CCRC in 95% oxygen in terms of either sensitivity (pEC_{50}: 6.43 [0.11] vs 6.26 [0.12] respectively, n=4, p>0.1) or maximum contractile response (649 mg [85] vs 710 mg [113] respectively, n=4, p>0.1). Student's paired t-test.

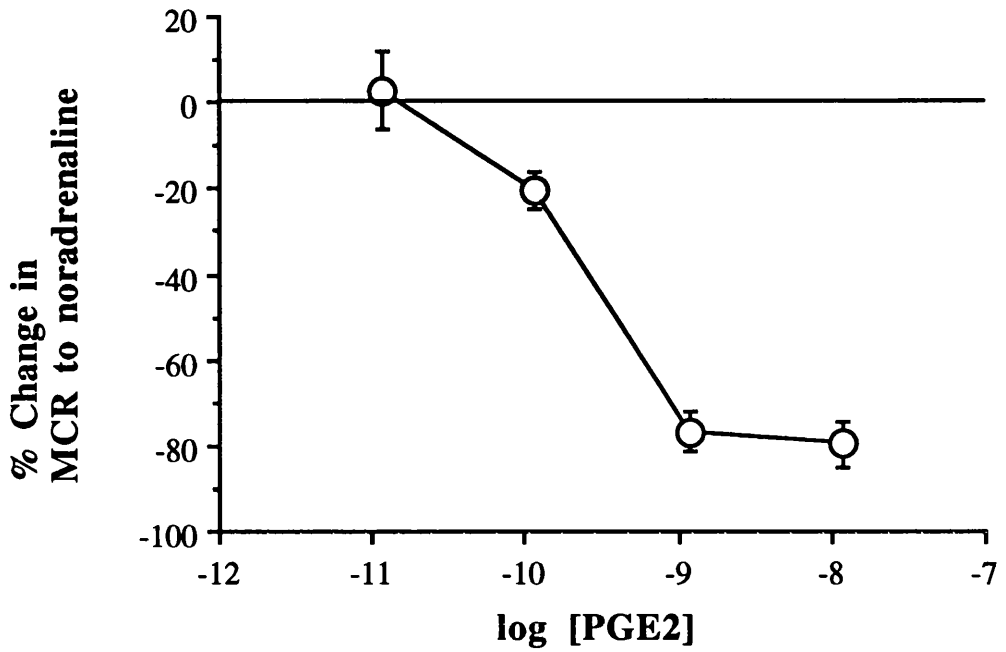

Figure 6.3 Synergy between PGE_2 and oxygen tension to inhibit the maximum contractile response (MCR) to noradrenaline. On the Y axis is plotted the mean change in MCR to noradrenaline from a control response in 95% to a test response in 3% oxygen, each point is the mean of 3-7 different vessels, bars are SEM. The mean decrease from 95% to 3% (95% CI and p value Student's paired t test): 0.01nM PGE_2 -0.2% (-28 to 28, p=0.98); 0.1nM PGE_2 23% (5 to 41, p=0.031); 1nM PGE_2 79% (68 to 90, p<0.0001); 10nM PGE_2 82% (65 to 100, p=0.0007).

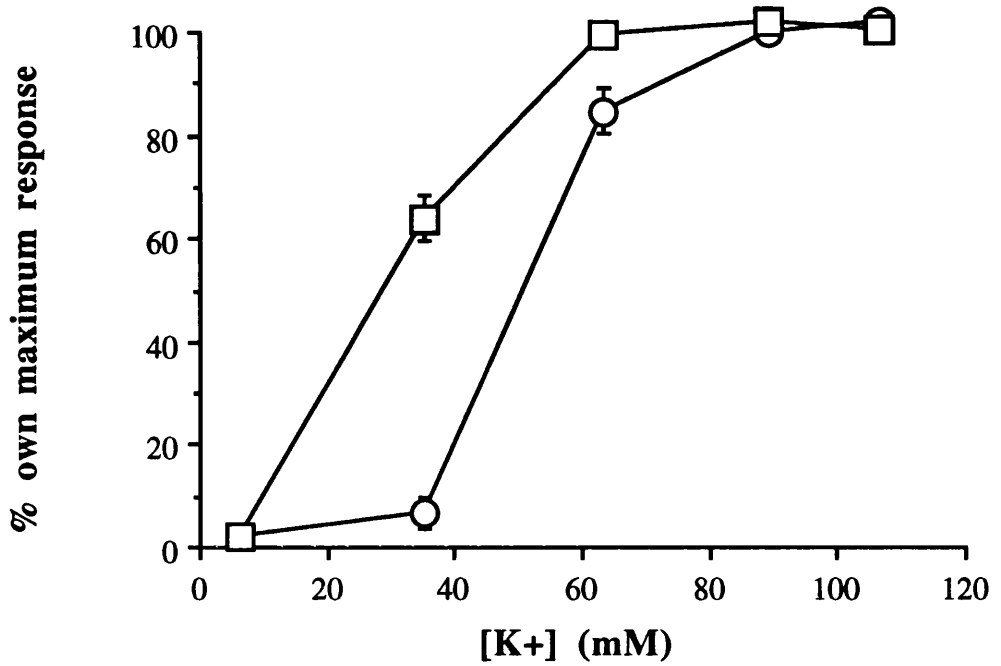

Figure 6.4 The effect of increasing PGE_2 from 0.01nM to 10nM on contractile response to potassium. ○ 10nM PGE_2; □ 0.01nM PGE_2, both responses elicited from same vessels, the response in 10nM was elicited first, n=4. Responses in 15% oxygen and 1μM indomethacin. pEC_{50} ($-\log_{10}M$), mean (SEM), in 10nM 1.32 (0.01); in 0.01nM 1.62 (0.05). Mean change 0.31 (95% CI 0.14 to 0.47), p=0.0099 Student's paired t test. The responses are expressed as a proportion of their own maximum. The response in 10nM PGE_2 was 31% (95% CI 12 to 50, p=0.014) reduced compared to the response in 0.01nM PGE_2.

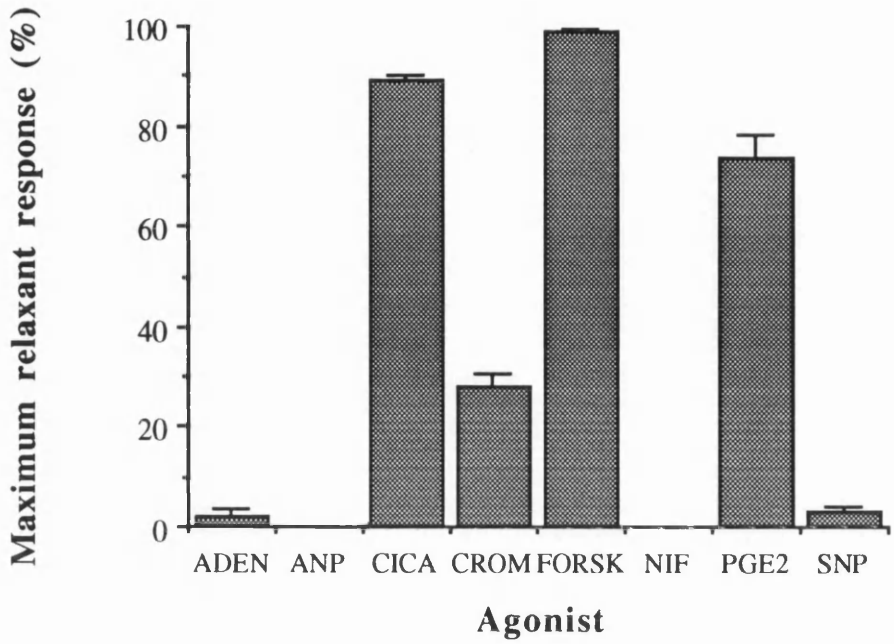

Figure 6.5 Maximum relaxant responses (MRR) of vasodilators in 1μM indomethacin, 10μM noradrenaline and 15% oxygen. MRR expressed as % maximum relaxation. Mean (SEM): Aden = adenosine 2.2% (1.2) n=16, ANP = atrial natriuretic peptide 0.0%, (0.0) n=4, Cica = cicaprost 89.0% (1.0) n=15, Crom = cromakalim 28.2% (2.4) n=16, Forsk = forskolin 98.8% (0.5) n=12, Nif = nifedipine 0.0% (0.0) n=4, PGE_2 73.4% (5.0) n=15, SNP = sodium nitroprusside 2.9% (1.3) n=12. ANOVA of means: F ratio = 282.63, $p<0.0001$. Multiple comparisons: Forskolin vs cicaprost, borderline significant difference, but both significantly greater than all other agonists; PGE_2 significantly greater than cromakalim, which was greater than remaining four agonists (see Appendix [Table A.5] for table of values of 95% CI of pairwise differences).

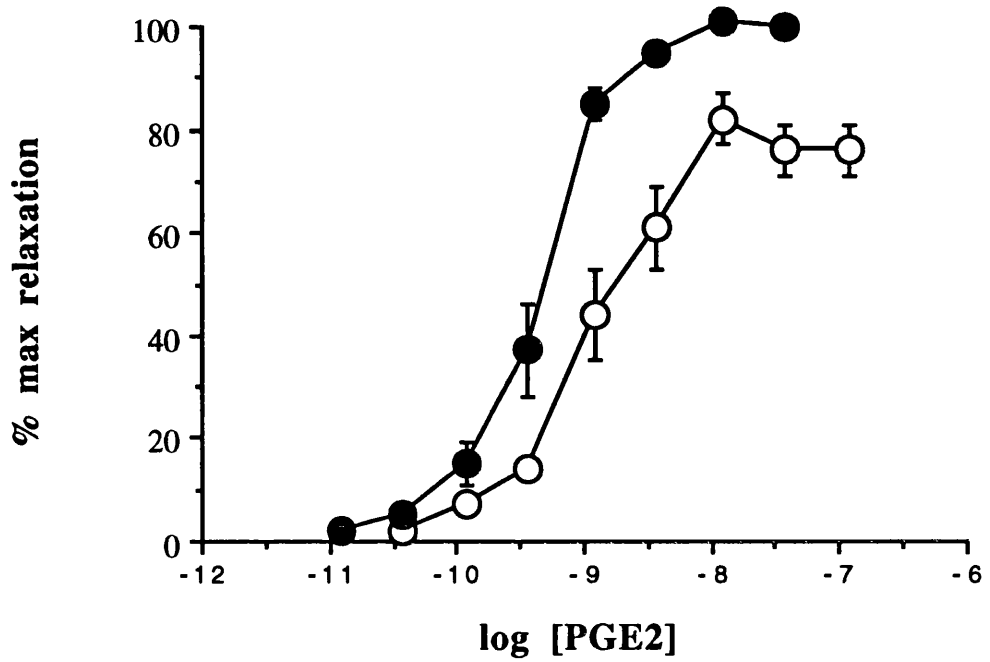

Figure 6.6 The effect of oxygen tension on the response to PGE_2 in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=7. MRR and pEC_{50}s for above and for time controls, see Tables 6.1 and 6.2. Points are means, bars are SEM.

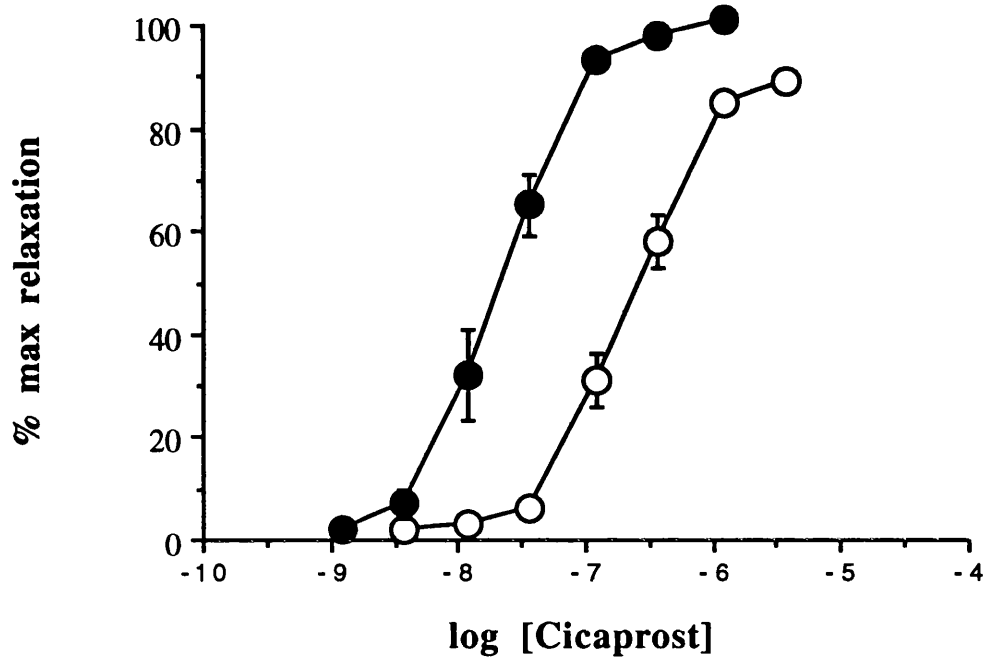

Figure 6.7 The effect of oxygen tension on the response to cicaprost in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=8. MRR and pEC_{50}s for above and for time controls see Tables 6.1 and 6.2. For comparison of extent of change induced by altering oxygen tension with extent of change in time controls, see Table 6.3. Points are means, bars are SEM.

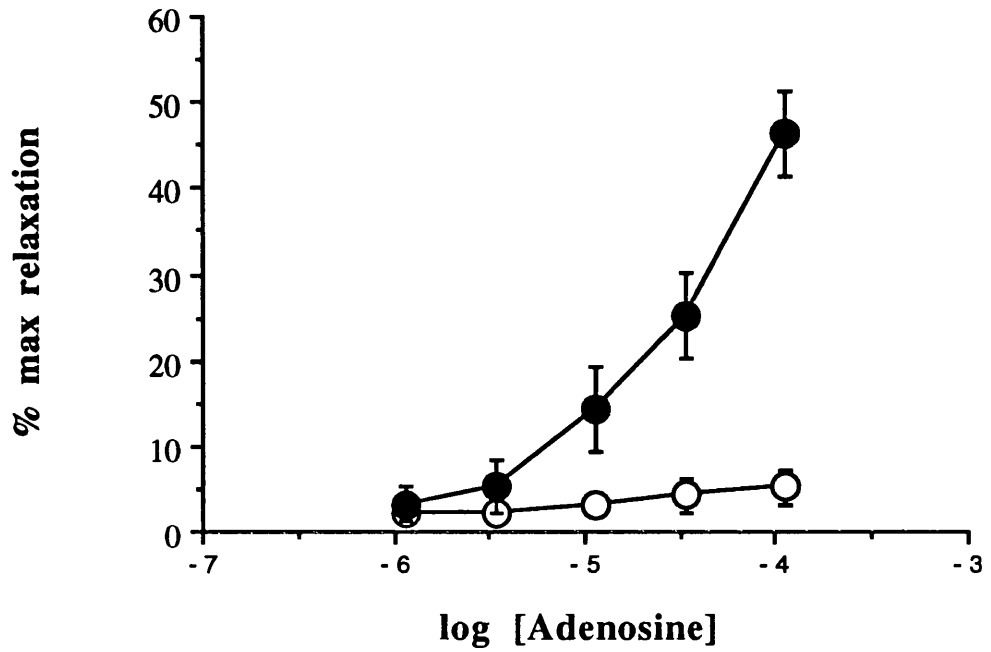

Figure 6.8 The effect of oxygen tension on the response to adenosine in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=8. MRR for above and for time controls see Tables 6.1 and 6.2. For comparison of extent of change induced by altering oxygen tension with extent of change in time controls, see Table 6.3. Points are means, bars are SEM.

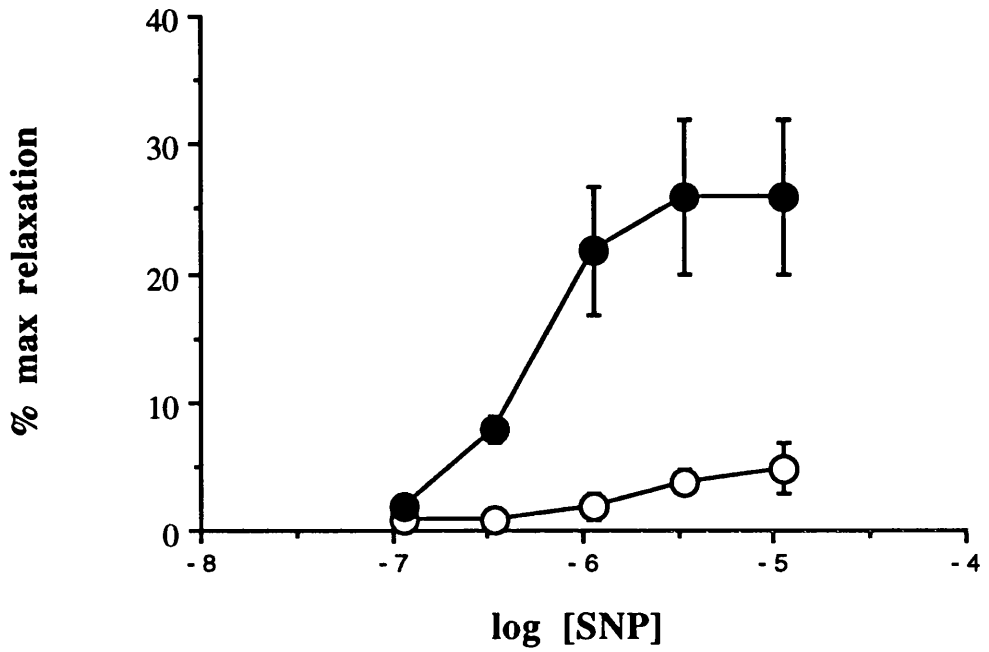

Figure 6.9 The effect of oxygen tension on the response to sodium nitroprusside (SNP) in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=8. MRR for above and for time controls, see Tables 6.1 and 6.2. Points are means, bars are SEM.

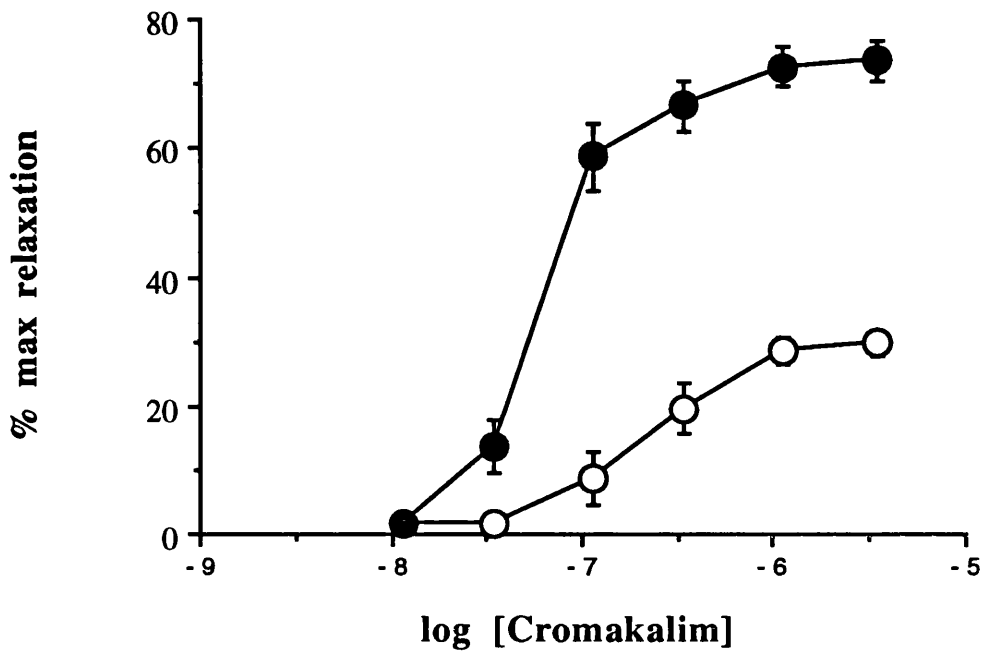

Figure 6.10 The effect of oxygen tension on the response to cromakalim in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=8. MRR and pEC_{50}s for above and for time controls see Tables 6.1 and 6.2. For comparison of extent of change induced by altering oxygen tension with extent of change in time controls, see Table 6.3. Points are means, bars are SEM.

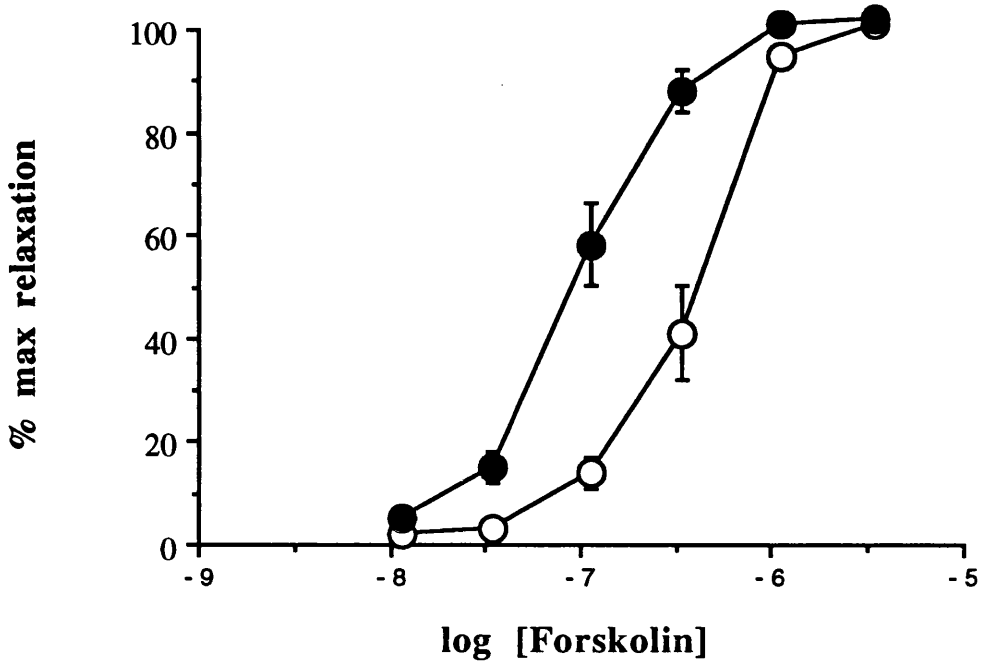

Figure 6.11 The effect of oxygen tension on the response to forskolin in 1μM indomethacin and 10μM noradrenaline. ● 2% oxygen; ○ 15% oxygen; n=8. MRR and pEC_{50}s for above and for time controls, see Tables 6.1 and 6.2. Points are means, bars are SEM.

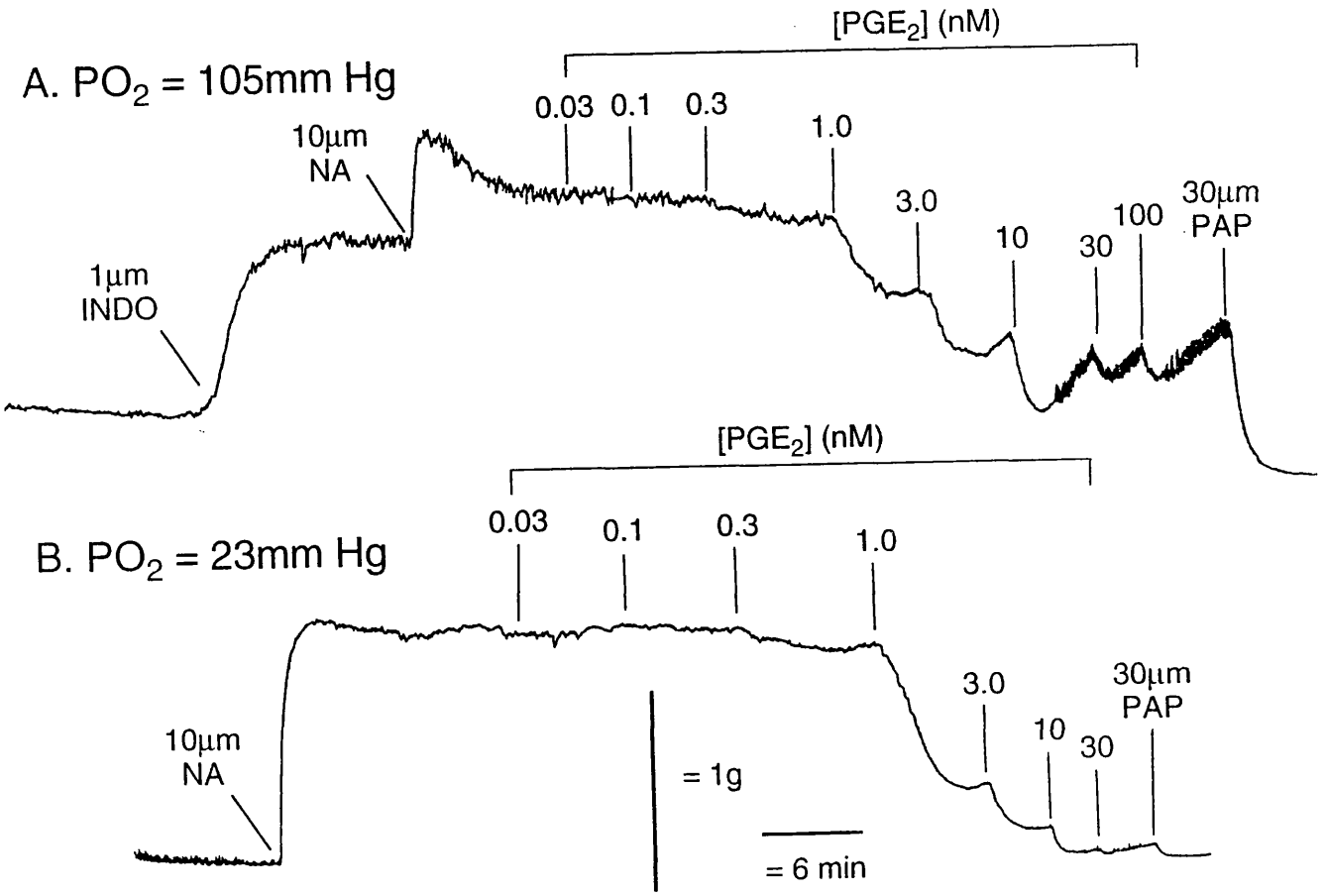

Figure 6.12 Original tracing of CRRC to PGE_2 in 10μM noradrenaline, 1μM indomethacin and in A. 15% oxygen and B. 2% oxygen. Both responses to PGE_2 in the presence of 1μM indomethacin and 10μM noradrenaline. **A** Bubbled with 15% oxygen giving oxygen tension of 105mmHg; **B** Bubbled with 2% oxygen giving oxygen tension of 23mmHg.

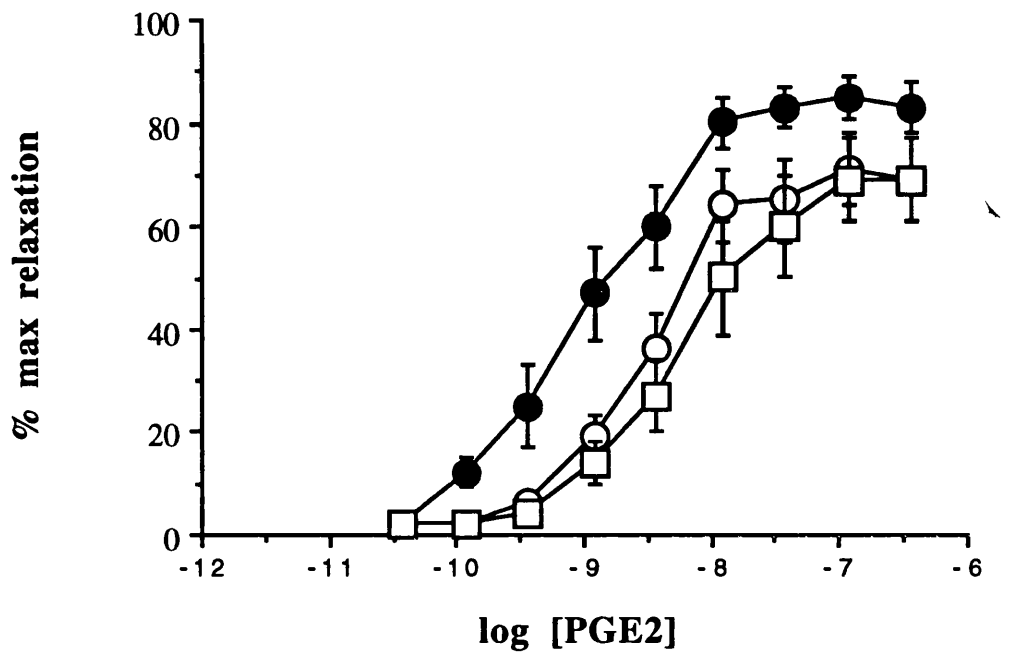

Figure 6.13 The effect of oxygen tension on the response to PGE_2 in 10μM noradrenaline in the absence of indomethacin. ○ CRRC 1, 15% oxygen; ● CRRC 2, 2% oxygen; □ CRRC 3, 15% oxygen; Points are means, bars are SEM, n=7. Three consecutive responses to PGE_2 in absence of cyclo-oxygenase inhibitor in 15% oxygen (CRRC 1 and 3) and 2% oxygen (CRRC 2). Comparing the two responses in 15% oxygen, there was no significant difference in pEC_{50} ($-\log_{10}M$): (mean [SEM] 8.49 [0.11] vs 8.30 [0.16], p=0.24); or MRR (% maximum relaxation): (72.3% [5.8] vs 67.2% [8.4], p=0.26), all Student's paired t-test.

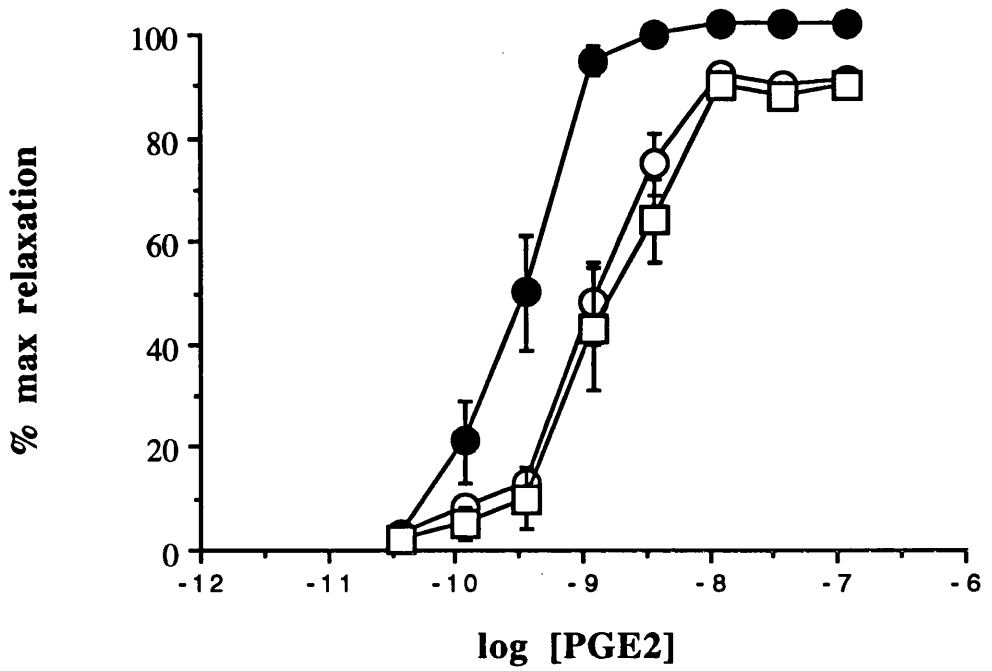

Figure 6.14 The effect of oxygen tension on the response to PGE_2 in 10μM noradrenaline and 1μM flubriprofen. ○ CRRC 1, 15% oxygen; ● CRRC 2, 2% oxygen; □ CRRC 3, 15% oxygen; Points are means, bars are SEM, n=4. Three consecutive responses to PGE_2 in 1μM flubriprofen in 15% oxygen (CRRC 1 and 3) and 2% oxygen (CRRC 2). Comparing the two responses in 15% oxygen, there was no significant change in pEC_{50} ($-\log_{10}M$): (mean [SEM]: 8.99 [0.09] vs 8.86 [0.16], p=0.23) or MRR (% maximum relaxation): (90.1% [1.7] vs 90.0% [1.5], p=0.13), all Student's paired t-test.

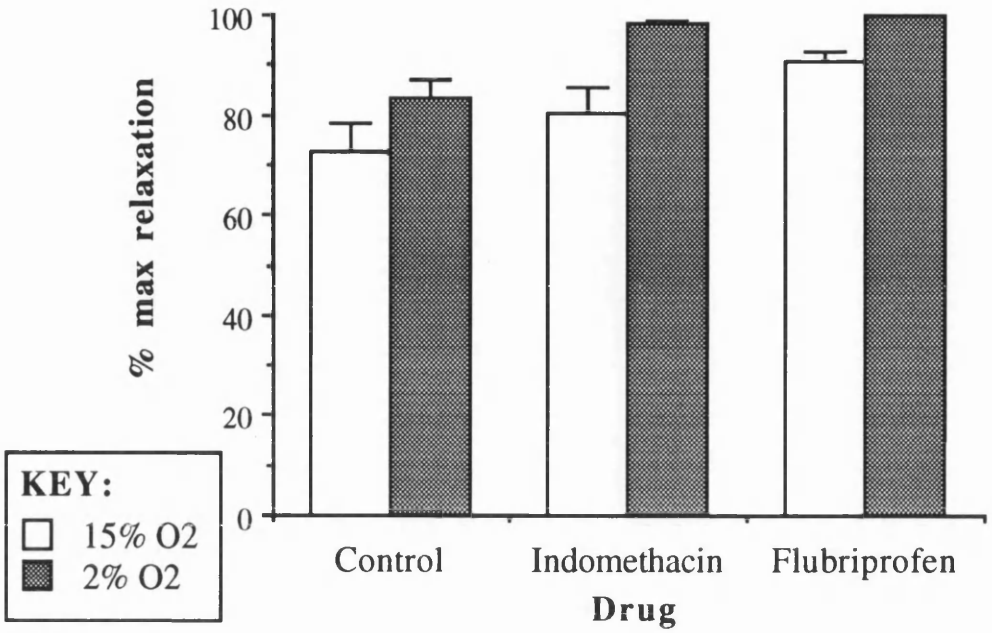

Figure 6.15 Effect of oxygen on the maximum relaxant response (MRR) to PGE_2 in 1μM indomethacin, 1μM flubriprofen and absence of cyclo-oxygenase inhibitor (control). ANOVA of means: *15% oxygen*, F ratio = 2.62, p=0.106; *2% oxygen*, F ratio=12.95, p=0.001- multiple comparisons show no significant difference between MRR to PGE_2 comparing flubriprofen and indomethacin, but both significantly greater than control (see Appendix [Table A.7] for table of values of 95% CI of pairwise differences).

AGONIST		15% oxygen			2% oxygen		
	n=	pEC_{50}	pEC_{30*}	MRR	pEC_{50}	pEC_{30*}	MRR
ADENOSINE	8	–	–	4.4 (2.3)	–	–	44.7*** (5.4)
CICAPROST	8	6.74 (0.08)	6.95 (0.08)	87.4 (1.5)	7.74*** (0.11)	7.98*** (0.10)	99.3*** (0.3)
CROMAKALIM	8	6.62 (0.11)	–	30.6 (2.9)	7.23** (0.04)	–	72.4*** (2.9)
FORSKOLIN	8	6.46 (0.07)	6.64 (0.08)	98.9 (0.8)	7.06** (0.08)	7.28** (0.07)	99.8 (0.2)
PGE_2	7	8.89 (0.14)	9.06 (0.14)	80.4 (5.1)	9.41* (0.09)	9.60* (0.10)	98.5* (0.7)
SNP	8	–	–	4.4 (1.8)	–	–	25.7* (6.2)

Table 6.1 Response to dilators in 15% and 2% oxygen (pEC_{50}, pEC_{30*}, and maximum relaxant response [MRR]). All values are mean (SEM). For definition of pEC_{50} and pEC_{30*} (both $-\log_{10}M$) see sections 2.4 and 6.1.3, respectively; MRR expressed as % maximum relaxation. Statistical comparison between values in 15% oxygen and in 2% oxygen, Student's paired t test, *$p<0.01$, **$p<0.001$, ***$p<0.0001$.

AGONIST	Time Control 1				Time controls 2		
	n=	pEC_{50}	pEC_{30*}	MRR	pEC_{50}	pEC_{30*}	MRR
ADENOSINE	8	–	–	0 (0)	–	–	6.1* (1.2)
CICAPROST	7	6.86 (0.09)	7.04 (0.08)	90.8 (0.8)	7.14* (0.04)	7.33* (0.03)	94.9* (0.9)
CROMAKALIM	8	6.54 (0.05)	–	25.8 (3.5)	6.79* (0.06)	–	43.0*** (4.6)
FORSKOLIN	4	6.34 (0.04)	6.52 (0.06)	98.7 (0.4)	6.47 (0.13)	6.69 ((0.13)	99.2 (0.5)
PGE_2	8	8.44 (0.19)	8.42 (0.29)	67.3 (8.0)	8.26 (0.07)	8.41 (0.10)	77.4 (3.9)
SNP	4	–	–	0 (0)	–	–	6.6 (3.0)

Table 6.2 Response to dilators in time control experiments with both responses in 15% oxygen (pEC_{50}, pEC_{30*}, and maximum relaxant response [MRR]). All values are mean (SEM). For definition of pEC_{50} and pEC_{30*} (both $-\log_{10}M$) see sections 2.4 and 6.1.3, respectively; MRR expressed as % maximum relaxation. Statistical comparison between values from two consecutive responses in 15% oxygen carried out under same time protocol as Table 6.1, Student's paired t test, *$p<0.01$, ***$p<0.0001$.

AGONIST	15% to 2% oxygen CHANGE:				Time controls (15% oxygen) CHANGE:			
	n=	pEC_{50}	pEC_{30*}	MRR	n=	pEC_{50}	pEC_{30*}	MRR
ADENOSINE	8	–	–	40.3 ** (5.2)	8	–	–	6.1 (1.2)
CICAPROST	8	1.00 *** (0.09)	1.02 *** (0.09)	11.9 ** (1.3)	7	0.28 (0.07)	0.28 (0.06)	4.1 (0.9)
CROMAKALIM	8	0.61 * (0.11)	–	41.8 *** (3.3)	8	0.24 (0.06)	–	17.1 (1.7)

Table 6.3. Comparison of changes in pEC_{50}, pEC_{30*} and maximum relaxant response (MRR, %), oxygen tension vs time controls, for adenosine, cicaprost and cromakalim. Values are mean differences (SEM). For definition of pEC_{50} and pEC_{30*} (both $-\log_{10}M$) see sections 2.4 and 6.1.3, respectively; MRR expressed as % maximum relaxation. Statistical comparison: change induced by decreasing oxygen from 15% to 2% vs spontaneous change in time controls (two consecutive responses, same time protocol as oxygen experiments, but both responses in 15% oxygen): *$p<0.05$, **$p<0.001$, ***$p<0.0001$, Student's t-test.

6.3 Discussion.

These data demonstrate that oxygen tension modulates the effect of PGE_2 on the response of the ductus arteriosus to noradrenaline (Figures 6.1 to 6.3). The effect of PGE_2 on both the MCR and the sensitivity of the vessel to noradrenaline was potentiated by fetal oxygen tension, but the potentiating effect of fetal oxygen tension was most pronounced on the MCR (Figure 6.1). However, it required both fetal oxygen tension and physiologically elevated PGE_2 to cause this profound inhibition: oxygen had no effect on the MCR to noradrenaline in 0.01nM PGE_2 but massively inhibited the response of MCR to noradrenaline in 1nM PGE_2 (Figures 6.1 to 6.3). These data point to an interesting synergy between these two fundamental control systems of the ductus in regulating its response to noradrenaline. It can readily be envisaged that following birth with the elimination of circulating PGE_2 (Clyman *et al,* 1980c) and the rise in arterial oxygen tension (Heymann and Rudolph, 1975), that the response of the ductus to both local and circulating catecholamines is uncovered. The interaction between these factors points to the complexity of the control systems which regulate ductal patency at birth.

Interestingly, the effect of PGE_2 on the response of the ductus to noradrenaline is not specific: PGE_2 also inhibits the vessel's response to potassium-induced contraction (Figure 6.4). The effect of PGE_2 on potassium implies that PGE_2 acts at some fundamental cellular level, i.e. it excludes a specific effect on the noradrenaline receptor-effector system (i.e. receptor, G protein, second messengers). This implication has been supported by the observation that PGE_2 inhibits the sensitivity of the contractile proteins to $[Ca^{2+}]_i$, [Crichton et al ,1994]). This effect is probably mediated by stimulation of adenylate cyclase, as PGE_2 only has an effect in the presence of the phosphodiesterase inhibitor, IBMX, and forskolin also inhibits the sensitivity of the contractile proteins of the ductus to $[Ca^{2+}]_i$ (Crichton CA, Smith GCS, Smith GL, paper in

preparation).

The interaction between PGE_2, oxygen and noradrenaline could also be demonstrated by pre-contracting the ductus with 10μM noradrenaline and obtaining CRRC to PGE_2 in low and high oxygen tension (Figures 6.6 and 6.12). I then sought to determine whether this inhibitory effect of neonatal oxygen tension on the response of the ductus to PGE_2 was specific to this agonist or whether it was a general property of vasodilators. As Figures 6.7 to 6.11 illustrate, oxygen tension modulates the sensitivity of the ductus to a range of dilators with different mechanisms of action, including a prostanoid other than PGE_2 (cicaprost, a stable PGI_2 mimetic [Stürzebecher *et al,* 1986]); by activating adenylate cyclase directly (forskolin [Seamon and Daly, 1981]); by stimulating guanylate cyclase (SNP [Walsh and Mentzer, 1987]); by hyperpolarising the membrane through activation of ATP-sensitive potassium channels (cromakalim [Bray *et al,* 1991]); and, by adenosine, which can cause vasodilation in different vessels by different mechanisms (Merkel *et al,* 1992 [its mechanism in the ductus has not been elucidated]).

These data contradict the hypothesis that this effect of oxygen is specific to PGE_2 and suggest that the effect(s) of oxygen also lie at a fundamental cellular event modulating smooth muscle contractility. The model for the oxygen-induced contraction proposed by Nakanishi et al (1993)- see section 1.2.1- namely that oxygen contracts the ductus by depolarising the membrane through the closure of ATP-sensitive potassium channels would predict that cromakalim would not be affected by changes in oxygen tension. As discussed in section 1.2.1, there is evidence that oxygen also contracts the ductus by a mechanism independent of changes in the membrane potential. The effect of oxygen on the response of the ductus to cromakalim (Figure 6.10) is another piece of evidence for a membrane-independent effect of oxygen. Interestingly, our work on the permeabilised ductus indicates that at ambient oxygen (i.e. physiologically

elevated), the intrinsic sensitivity of the contractile proteins of the ductus to $[Ca^{2+}]_i$ is higher than the aorta and pulmonary artery, the vessels it connects and, indeed, any other smooth muscle preparation studied (Crichton *et al,* 1994). We did not have the equipment to study the sensitivity of the permeabilised ductus to $[Ca^{2+}]_i$ in fetal oxygen tension but it would clearly be of interest to elucidate the effect of oxygen on this variable. It may be at this level that oxygen exerts its membrane-independent effect.

These data indicate that in infants with cyanotic congenital heart defects which are ductus dependent, PGE_1 has the potential to be self-limiting: its therapeutic effect (increasing arterial oxygen tension) will decease the sensitivity of the ductus to its effect. The data in Figures 6.7 to 6.11 indicate that this will be a problem with any vasodilator used in this clinical setting.

The ability of adenylate cyclase activation by forskolin to reverse completely the combined contractile effects of indomethacin, elevated oxygen and 10μM noradrenaline (Figure 6.11) suggests a major role for this enzyme in the regulation of ductal tone. The maximal effect of forskolin was significantly greater than PGE_2, which is also believed to dilate the ductus through adenylate cyclase (Walsh and Mentzer, 1987; Crichton *et al,* 1994). The implication of this is that there is adenylate cyclase in ductal smooth muscle which is not coupled to the PGE_2 receptor/effector mechanism.

The effects of PGI_2 on other blood vessels are coupled to adenylate cyclase (Dembinska-Kiec *et al,* 1979; Miller *et al,* 1979). Cicaprost, a stable, selective PGI_2 mimetic (Stürzebecher *et al,* 1986), caused a greater maximal relaxation than PGE_2 (Figure 6.1). If, as is likely, the ductal receptor for PGI_2 is also coupled to adenylate cyclase, this implies that there is actually more of the enzyme coupled to the receptor for PGI_2 than PGE_2, although, clearly, other explanations exist. Despite the fact that PGI_2

is the main prostanoid formed by the ductus, PGE_2 has always been seen as the more important prostaglandin in the regulation of ductal tone as, in the lamb, it is over 1000 times more potent than PGI_2 (see section 1.2.3 for discussion and references). Converting the data in Figure 2 to molar concentrations, PGE_2 was about 70 times more potent than cicaprost. Taken in combination with its greater MRR than PGE_2 these data imply a physiological role for PGI_2 in the regulation of the rabbit ductus. It remains to be seen why studies on the lamb using PGI_2 yielded such different results. It may represent a species difference, it may reflect a difference between the synthetic analogue (cicaprost) and the natural agonist (PGI_2), or it may reflect the nature of the pre-contraction. Given the potential for use of PGI_2 in ductus-dependent circulation, the physiological role of PGI_2 in the regulation of the ductus needs to be clarified.

The contractile response of the vessel to indomethacin declined from CRRC 1 to CRRC 2 (time controls). This was in spite of the fact that the total amount of active tone (i.e. with 10μM noradrenaline as well) actually increased over the same period. This decline in response to indomethacin may be related to the increased response to some, but not all of the dilator agonists with time. It remains to be seen which cellular events are involved in mediating these effects.

Compared with the ability of forskolin to cause complete relaxation in 15% oxygen, 10μM noradrenaline and 1μM indomethacin, SNP induced only a 3% relaxation (Figure 6.1). This suggests that the main second messenger in the control of the ductus arteriosus is adenylate cyclase rather than guanylate cyclase. Indeed, in the lamb ductus, SNP stimulates both adenylate and guanylate cyclase (Walsh and Mentzer, 1987), and the small effect of SNP may be due to an action on adenylate cyclase. Whatever the case, the small effect of the nitric oxide donor, SNP, suggests that while endogenous nitric oxide may have a role in dilating the ductus (see section 1.2.4), its role may be a relatively minor one.

The minor effect of adenosine and SNP on the ductus in 15% oxygen differs from previous studies. It has been suggested that adenosine has a physiological role in maintaining ductal patency *in utero:* micromolar concentrations reverse oxygen-induced contraction of the vessel and its circulating concentrations correlate with oxygen tension (inversely) and ductal blood flow (directly) during ventilation of the fetal lungs with oxygen (Mentzer *et al,* 1985). Similarly, some nitrovasodilators have been demonstrated as having greater efficacy than PGE_1 in reversing the oxygen-induced contraction of the isolated lamb ductus (Walsh and Mentzer, 1987). These data for both agonists seem to be at odds with my results. This may reflect a species difference or the use of noradrenaline to pre-contract the vessel in the present study. Perhaps the most likely explanation, however, is that neither of the other studies used indomethacin. I have found that the ductus is more sensitive to vasodilators other than PGE_2 in the absence of indomethacin (see Chapter 5). This is almost certainly due to synergy between endogenous PGE_2 and the exogenous dilator.

In the absence of cyclo-oxygenase inhibition, elevated oxygen still decreased the pEC_{50} and MRR of the ductus to PGE_2 (Figure 6.13). The extent of the effect of oxygen on the pEC_{50} to PGE_2 was unaffected by cyclo-oxygenase inhibition. This indicates that the effect of oxygen on the pEC_{50} of the ductus to PGE_2 is not mediated by a cyclo-oxygenase product. However, cyclo-oxygenase inhibition did alter the maximum response to PGE_2: the MRR to PGE_2 was increased in the presence of both indomethacin and flubriprofen in 2% but not 15% oxygen, compared with control (Figure 6.15).

As discussed at length in Chapter 5, when comparing the response of tissues to exogenous prostaglandins in the presence and absence of cyclo-oxygenase inhibitors, there can be an apparent decrease in sensitivity to prostaglandins in the absence of a cyclo-oxygenase inhibitor as the effects

of exogenous prostaglandins are being super-imposed on the effects of endogenous prostaglandins. It could be argued that the decrease in MRR to PGE_2 in 2% oxygen in the absence of indomethacin or flubriprofen is such a case. However, in the presence of indomethacin or flubriprofen, exogenous PGE_2 caused complete relaxation in 2% oxygen. In the absence of these drugs it did not. This phenomenon is a change in an absolute response, not a shift in sensitivity and cannot, therefore, be explained in the above terms. These data suggest that an endogenous prostanoid prevents complete relaxation of the vessel to PGE_2, but that its effect is only seen in fetal oxygen tension. Several models of the interaction between the cyclo-oxygenase product and oxygen fit these data: (1) its rate of synthesis may vary inversely with oxygen tension; (2) its receptor/effector mechanism may be modulated by oxygen tension; or, (3) its effect may be masked by elevated oxygen tension e.g. if the cyclo-oxygenase product and raised oxygen tension both decreased the response to PGE_2 by the same mechanism.

By inhibiting the response to PGE_2, this cyclo-oxygenase product will make the ductus contract. These data are, to my knowledge, the first evidence for a constrictor prostanoid released by the ductus. The data suggest that when indomethacin is administered in patent ductus arteriosus, while it will cause contraction by removing the dilator effects of PGE_2 and PGI_2, it will also have a dilator effect itself by removing this endogenous constrictor prostanoid. If this prostanoid has an important physiological role in neonatal closure of the vessel, it may even explain the tendency of the vessel to re-open after initial closure by indomethacin in PDA (Gersony, 1986). Further experimental work is required to identify this prostanoid and to clarify its physiological role in regulating patency of the ductus arteriosus.

Chapter 7. Contractile effects of prostanoids on the ductus arteriosus.

In the preceding chapter, evidence was presented that cyclo-oxygenase inhibition potentiated the dilator effect of PGE_2, implying the presence of a endogenous contractile prostanoid. For this to be the case, there must be one or more types of prostanoid receptor in the ductus coupled to contractile pathways. There have, however, been very few studies of contractile effects of prostaglandins on the ductus. It has been demonstrated that $PGF_{2\alpha}$ contracts the ductus (Starling and Eliott, 1974). However, that finding has been largely discounted due to the fact that these authors studied the effect of a single, very high concentration of $PGF_{2\alpha}$ (about 7μM). Another study failed to demonstrate any contractile effect of native TxA_2 (Coceani *et al,* 1978a). This study was flawed by the fact that the system used for generating TxA_2 also generated PGE_2, picomolar concentrations of which inhibit the response of the ductus to constrictors (see Chapter 5).

The existence of contractile receptors on the ductus arteriosus would be expected from the general properties of other tissues also under the control of prostaglandins, such as other vascular smooth muscle, myometrium and platelets. In these systems, the inhibitory effects of prostanoids are all opposed by stimulatory effects, for instance myometrium has contractile EP_1, EP_3, FP and TP receptors and relaxant EP_2, IP and DP receptors (Senior *et al,* 1991 and 1992); platelets have pro-aggregatory EP_3 and TP receptors and anti-aggregatory DP and IP receptors (Whittle *et al,* 1983; Armstrong *et al,* 1985 and 1989; Matthews and Jones 1993). The current view of prostaglandin pharmacology in the ductus arteriosus is that it is a relatively unique tissue in having purely inhibitory prostaglandin effects (Clyman, 1987; Coceani and Olley, 1988). However, as stated above, the data which have failed to demonstrate contractile effects are sparse, out of

date and methodologically flawed.

The prostanoid receptors mediating contraction of smooth muscle are almost exclusively EP_1, EP_3, TP and FP (Coleman *et al,* 1990). A range of selective agonists and some receptor antagonists are available with activity at these receptors. There has been no study, to my knowledge, of the effects of synthetic contractile prostanoids on the ductus arteriosus of any species. I sought to characterise the effect on the fetal rabbit ductus arteriosus of synthetic and native prostanoids with activity at the prostanoid receptors which mediate contraction in other smooth muscle preparations. The effects on the ductus arteriosus of sulprostone and GR63799X, (potent, selective agonists of the EP_1 and EP_3 receptors [Bunce *et al,* 1990; Coleman *et al,* 1990]) and U46619 (a potent agonist of the TP receptor [Coleman *et al,* 1990]) were studied. The highly selective FP receptor agonist, fluprostenol (Coleman *et al,* 1990), is no longer commercially available and the potency of the native FP receptor agonist, $PGF_{2\alpha}$, had to be compared with the other agonists to make inferences regarding the presence of an FP receptor on the ductus.

7.1 Methods.

The baths were bubbled with a gas mixture of 15% O_2, 5% CO_2, and 80% N_2, which resulted in a PO_2 of 100-110mmHg. When mounted, the vessels were stretched to 0.6g.tension and this was re-set 60 minutes later. The PSS was changed at 30 and 60 minutes following mounting and the vessels were allowed to incubate for 90 minutes prior to addition of indomethacin. All responses, contractile and relaxant, were obtained in the presence of 1μM indomethacin (added at least 30 minutes before eliciting all responses) which contracts the ductus (see section 1.2.3). The PSS was changed at least 3 times after a given response was completed.

7.1.1 Concentration contraction response curves.

Following the indomethacin contraction, the vessel was relaxed with either 300nM forskolin or 10nM PGE_2. When the fall in tension had equilibrated, a standard contraction to 10μM noradrenaline was obtained. The PSS was changed 3 times and indomethacin added. After at least 30 minutes, the vessel was relaxed by the same method, and a cumulative concentration contraction response curve (CCRC) obtained to the given prostanoid. When the effect of an antagonist was studied, a second CCRC was obtained in the presence of the antagonist and the change in sensitivity compared to the same number of concurrent time controls in the presence of the antagonist's vehicle.

7.1.2 Concentration relaxation response curves.

Essentially, the methods were the same as outlined in section 4.1. Following the indomethacin contraction, the vessel was further contracted by addition of 2M KCl to a bath K^+ concentration of 25mM. The combination of 25mM K^+ and 1μM indomethacin caused a sustained, stable elevation of tone which was always obtained within 10 minutes of adding the KCl. After 10 minutes, a cumulative concentration relaxation response curve (CRRC) was obtained. The first CRRC was a control

response to PGE_2. At least 55 minutes was allowed between consecutive CRRC. The second CRRC was obtained 10 minutes after addition of a given prostanoid (itself added 10 minutes after the KCl). The change in response parameters to PGE_2 was compared with the initial control response. The extent of change between the two CRRC was compared with the extent of change in the response parameters of concurrent time controls in the presence of the appropriate vehicle.

7.1.3 Quantification of responses.

The magnitude of the contractile response of prostanoids was expressed as a proportion of the maximum response to noradrenaline (10μM) of the same vessel in the same conditions (i.e. 300nM forskolin or 10nM PGE_2). The magnitude of the relaxation induced by PGE_2 in the presence of a contractile prostanoid and the magnitude of the relaxation induced by PGE_2 in the second time control were expressed as a proportion of an initial control response (Coleman, 1987) The sensitivity of the vessel to both contractile and relaxant responses was expressed as the EC_{50} or the pEC_{50}, see section 2.4 for definition.

7.2 Results.

In 300nM forskolin, both the potent TP receptor agonist, U46619, and the potent EP_1/EP_3 agonist, sulprostone, caused concentration dependent contractions of the ductus in the nanomolar range (Figure 7.1). The agonists were of very similar potency but the maximum response to U46619 was greater than sulprostone. Figure 7.2 illustrates a typical response to U46619. The TP receptor antagonist, EP 092 (Armstrong *et al,* 1985) caused a parallel rightward shift of the U46619 CCRC (Figure 7.3). In 300nM forskolin, $PGF_{2\alpha}$ had no effect at concentrations of 1-10nM but caused relaxation at higher concentrations. GR63799X caused variable contractions at 3-10nM but caused marked relaxation at 30nM. In the presence of 10nM PGE_2, U46619, sulprostone and GR63799X caused similar concentration-dependent contractions of the ductus arteriosus whereas $PGF_{2\alpha}$ had no effect (up to 30μM, Figure 7.4).

There was no significant difference in the response to 10μM noradrenaline in 300nM forskolin (mean 11.8mN [1.1] n=12) and that in 10nM PGE_2 (mean [response prior to U46619, sulprostone and GR63799X only] = 13.4mN [0.5], n=21; p=0.0852). Expressed as a percentage of the response of the same vessel to 10μM noradrenaline under the given conditions, the maximum responses to U46619 and sulprostone in 300nM forskolin (data-see above) were significantly greater than in 10nM PGE_2 (U46619: 29.9% [7.4], n=9, p<0.0001; Sulprostone 17.8% [2.1], n=6, p=0.0142).

Compared with concurrent time and vehicle control responses to PGE_2, there was no significant change in the sensitivity of the ductus to PGE_2 in the presence of 10nM U46619 or 10nM $PGF_{2\alpha}$ (Figure 7.5). There was, however, a significant decrease in the pEC_{50} to PGE_2 in 10nM sulprostone and 3μM sulprostone (Figure 7.5). Calculated from the estimate of the difference from time controls, 10nM sulprostone increased the EC_{50} to PGE_2 by 119% and 3μM sulprostone increased it by 157%.

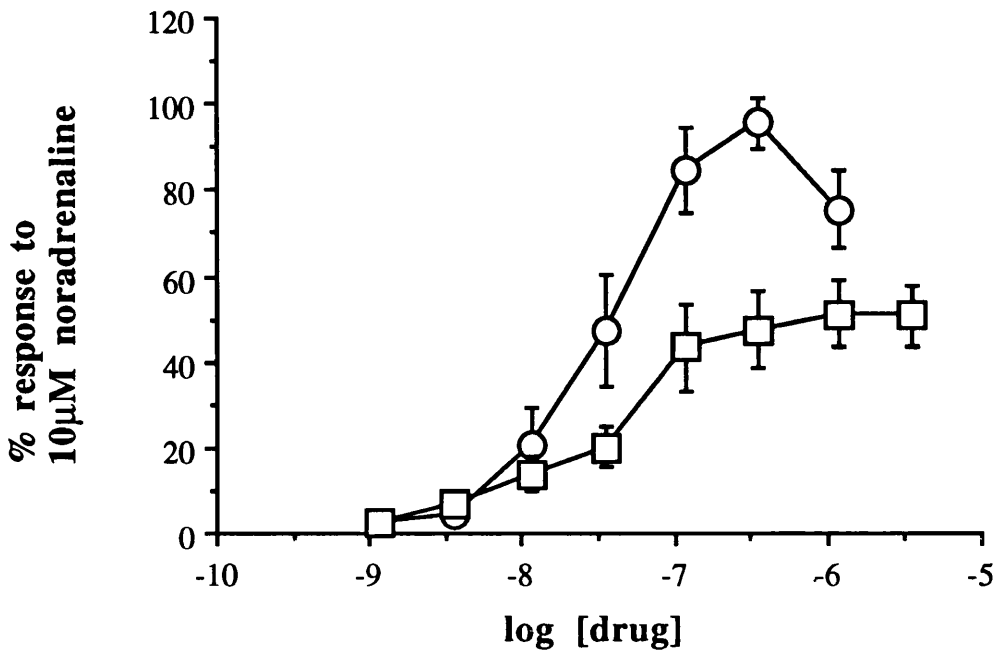

Figure 7.1 Contractile effect of U46619 and sulprostone in 300nM forskolin. U46619 (○ n=8) and sulprostone (□ n=4), in the presence of 1μM indomethacin and 300nM forskolin. Contraction at each concentration is expressed as a proportion of the same vessel's response to 10μM noradrenaline in 1μM indomethacin and 300nM forskolin. U46619: pEC_{50} ($-\log_{10}$ M) = 7.47 (SEM 0.13), n=8, EC_{50} = 33nM; Sulprostone: pEC_{50} = 7.37 (SEM 0.20), n=4, EC_{50} = 42nM. There was no statistically significant difference in the pEC_{50}s (p=0.6711). The maximum response to U46619 was 92.8% (SEM 5.8) that of 10μM noradrenaline, sulprostone was 49.1% (SEM 7.4) that of 10μM noradrenaline, which was significantly less than U46619 (p=0.0085). All comparisons, Mann-Whitney U test. Points are means, vertical bars are SEM.

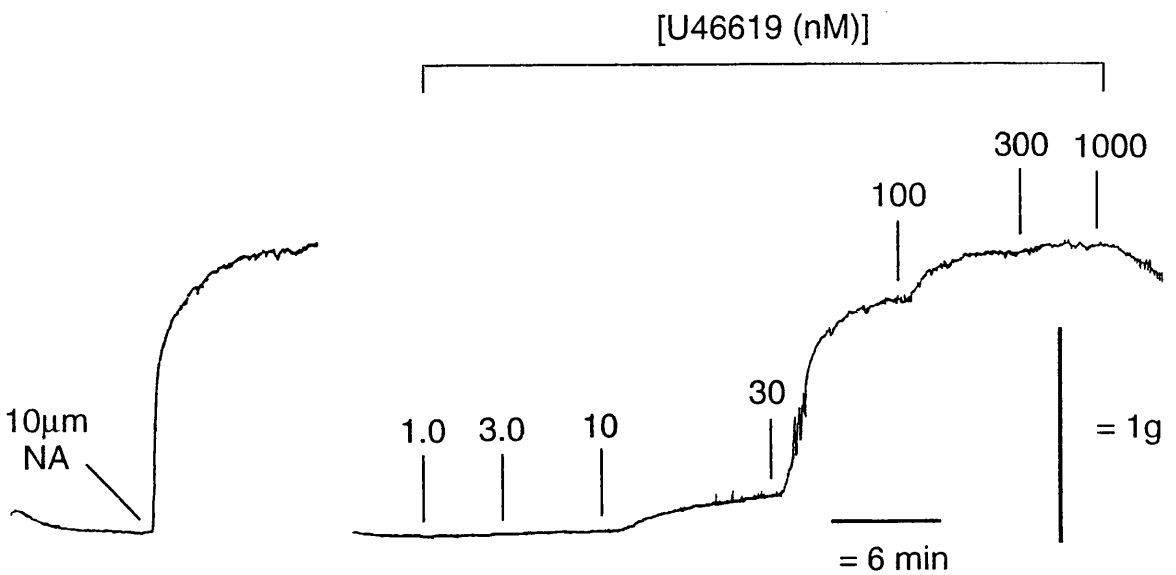

Figure 7.2 Original tracing of response of ductus arteriosus to U46619 (1-1000nM). Preceding control contraction to 10μM noradrenaline (NA), both responses in the presence of 1μM indomethacin and 300nM forskolin.

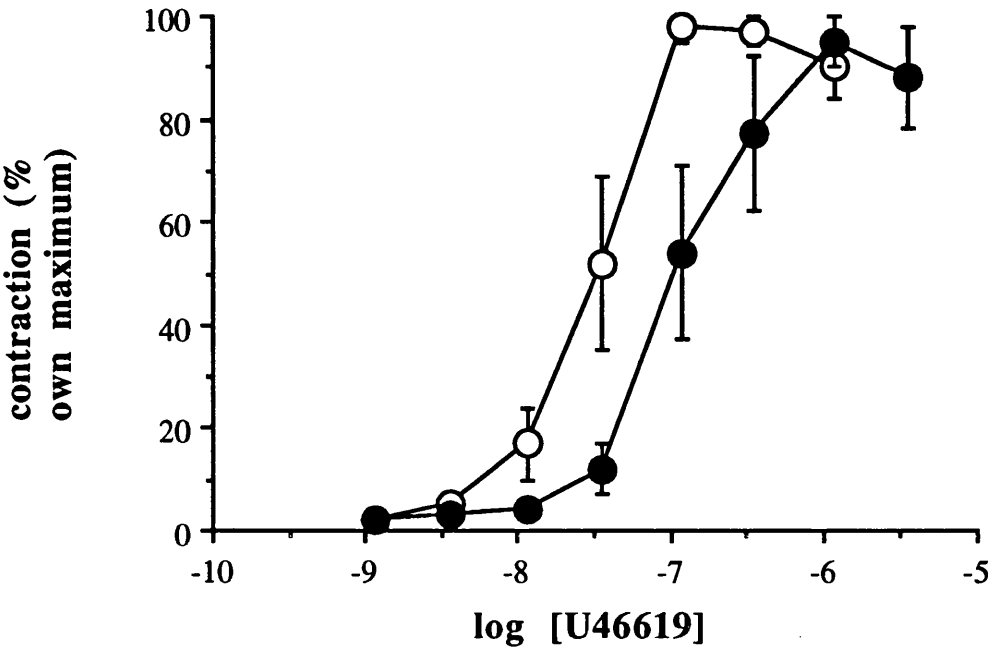

Figure 7.3 Effect of EP 092 on response to U46619 in 300nM forskolin. Response of the ductus arteriosus to U46619 in the presence of 1μM indomethacin and 300nM forskolin, in the presence (●) and absence (○) of 1μM EP 092, both n=4. Contraction of a given vessel is expressed as a % of its own maximum. Points are means, vertical bars are SEM. Compared with the initial control response, the mean change in pEC_{50} [$-\log_{10}$ M] in 1μM EP 092 was -0.48 (0.20), n=4 vs time control 0.03 (0.05), n=4; p=0.0304, Mann-Whitney U test.

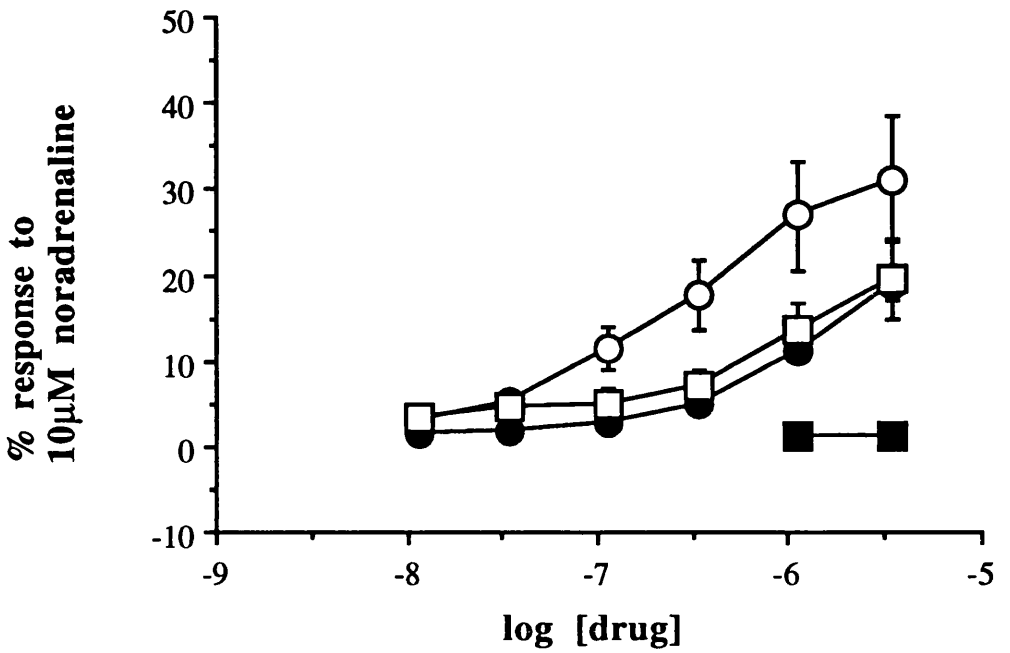

Figure 7.4 Contractile response to U46619, sulprostone, GR63799X and $PGF_{2\alpha}$ in 10nM PGE_2. U46619 (○ n=9), sulprostone (● n=6), GR63799X (□ n=6) and $PGF_{2\alpha}$ (■ n=6) in the presence of 1μM indomethacin and 10nM PGE_2. Contraction at each concentration is expressed as a proportion of the same vessel's response to 10μM noradrenaline in the presence of 1μM indomethacin and 10nM PGE_2. Points are means, vertical bars are SEM. Looking at the response at each concentration by ANOVA, there were no significant differences in the response to U46619 and sulprostone, and sulprostone and GR63799X at any concentrations. The responses to U46619 and GR63799X were similar but the response to GR63799X was significantly less than the response to U46619 at 100nM and 300nM (see Appendix [Tables A.8 and A.9 respectively] for table of values of 95% CI of pairwise differences).

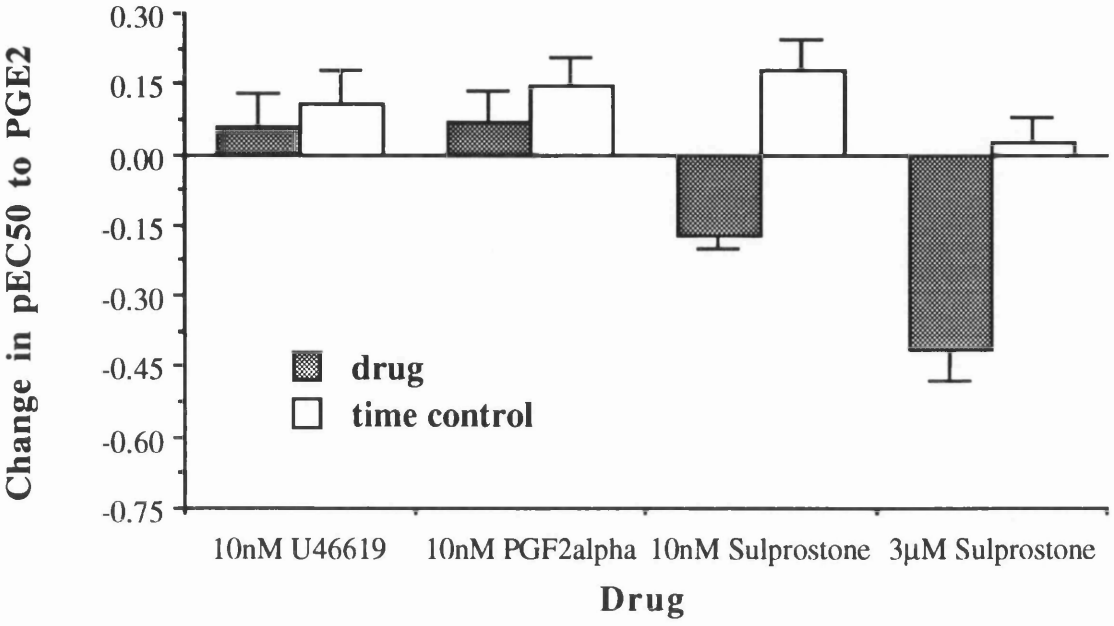

Figure 7.5 Effect of contractile prostanoids on the sensitivity of the ductus arteriosus to PGE_2. Data are the mean change in pEC_{50} to PGE_2 between an initial control response and a subsequent test response, comparing test responses in the presence of a contractile prostanoid with time controls in the presence of the appropriate vehicle. The mean (SEM) change in pEC_{50} ($-\log_{10}$ M) in 10nM U46619 was 0.06 (0.07) n=7 vs time control 0.11 (0.07), n=8; p=0.7273; in 10nM $PGF_{2\alpha}$ was 0.07 (0.07), n=7 vs time control 0.15 (0.06), n=8; p=0.4863; in 10nM sulprostone was -0.17 (0.03), n=4 vs time control 0.18 (0.07), n=4; p=0.0304, point estimate of difference=0.34 (95% CI 0.12–0.58); in 3µM sulprostone was -0.41 (0.07), n=12 vs time control 0.03 (0.05), n=7; p=0.0015, point estimate of difference = 0.42 (95% CI 0.23–0.66). All comparisons Mann-Whitney U test.

7.3 Discussion.

The preceding chapter presented data which suggested contractile effects of prostanoids in the ductus. This hypothesis predicts that the ductus would have prostanoid receptors coupled to contractile pathways. The data presented in this chapter strongly support this hypothesis.

This study provides strong evidence that the fetal rabbit ductus arteriosus has thromboxane receptors (TP receptors): namely, the ductus contracts to nanomolar concentrations of the stable thromboxane mimetic, U46619 (Figures 7.1 to 7.4), none of a range of synthetic prostanoids with activity at other contractile prostanoid receptors were more potent than U46619 (Figure 7.4) and the selective TP receptor antagonist, EP 092 (Armstrong *et al,* 1985), caused a rightward shift in the concentration response curve to U46619 (Figure 7.3). Furthermore, the potency of EP 092 on the ductus was consistent with its potency on the TP receptor of the adult rabbit aorta (Armstrong *et al,* 1985).

Only one other study has, to my knowledge, looked for thromboxane effects on the ductus arteriosus, where native TxA_2 was prepared through incubation of cyclic endoperoxides (PGG_2 and PGH_2) with microsomal fractions of human platelets or guinea pig lungs. This mixture did not contract the lamb ductus arteriosus (Coceani *et al,* 1978a) The discrepancy between that work and the current study may be due to one or more of the following: species difference, failure of the bio-generation system to produce sustained, high concentrations of TxA_2 or, perhaps most likely, due to generation of PGE_2 by the reaction mixture. Whether the last of these is the explanation depends on the relative potency of PGE_2 in relaxing the vessel and TxA_2 in contracting it plus the relative proportions of TxA_2 and PGE_2 produced by the reaction mixture in the lamb study. I have found that picomolar concentrations of PGE_2 inhibit the response of the ductus to vasoconstrictors (see Chapter 5) whereas in the present

study I have described an EC_{50} for U46619 of 33nM. There is clearly potential, therefore, for relatively small concentrations of contaminant PGE_2 to mask the effect of TxA_2. Quantifying the response of the lamb ductus to U46619 would resolve this question.

The present study also provides strong evidence for the existence of a contractile EP receptor on the ductus arteriosus, as the vessel contracted to nanomolar concentrations of sulprostone (Figure 7.1), which is a potent agonist of contractile EP receptors (see below) in many tissues (Coleman *et al,* 1990) and is ineffective at the dilator EP receptor on the ductus (see Chapter 4). It is possible that this effect of sulprostone was due to agonism of non-EP contractile prostanoid receptors, namely TP or FP receptors. This is highly unlikely, however, as sulprostone was equipotent with U46619 and more potent than $PGF_{2\alpha}$. In other smooth muscle preparations, sulprostone is almost 200-fold less potent than $PGF_{2\alpha}$ at FP receptors and 500-fold less potent than U46619 at TP receptors (Coleman *et al,* 1988). Furthermore, 10nM sulprostone decreased the sensitivity of the ductus to the dilator effect of PGE_2, whereas U46619 and $PGF_{2\alpha}$ did not (Figure 7.5). Even if the TP receptor of the ductus were unique in that sulprostone and U46619 were equipotent agonists, it would not explain why 10nM sulprostone had this effect whereas 10nM U46619 did not.

The advantage of looking at contractile effects of prostanoids in 10nM PGE_2 was that this eliminated the effect of agonism of the dilator EP receptor of the ductus by prostanoids. For instance GR63799X is a potent agonist of contractile EP receptors in a number of tissues (Bunce *et al,* 1990), but it is also a partial agonist of the ductal EP receptor about 700 times less potent than PGE_2 itself (see Chapter 4). This means that the effect of 1μM GR63799X on the EP_4 receptor would be equivalent to about 1.5nM PGE_2. In the presence of 10nM PGE_2, therefore, even very high concentrations of GR63799X cause only very small additional relaxation and a more reliable CCRC is obtained. In 10nM PGE_2,

GR63799X elicited contraction of the ductus with a similar potency to sulprostone and only slightly less potent than U46619 (Figure 7.4), which supports my conclusion that the ductus has a contractile receptor for PGE_2. In other smooth muscle preparations, GR63799X is more than 200-fold less potent than $PGF_{2\alpha}$ at FP receptors and about 50-fold less potent than U46619 at TP receptors (Bunce *et al,* 1990).

Contractile effects of PGE_2 are mediated through the EP_1 and EP_3 receptors (Coleman *et al,* 1990). Sulprostone and GR63799X are both more potent than PGE_2 at the EP_3 receptor and roughly equipotent with PGE_2 at the EP_1 receptor (Bunce *et al,* 1990; Coleman *et al,* 1990). The data presented above, therefore, do not resolve whether the contractile EP receptor on the ductus is EP_1 or EP_3. I constructed experiments to elucidate the effect of the selective EP_1 receptor antagonist, AH6809 (Coleman *et al,* 1990), on the sensitivity of the ductus to sulprostone and GR63799X. However, I found that AH6809 caused direct relaxation of the vessel even in the absence of PGE_2 (data not shown) which would interfere with analysis of any effect due to receptor blockade. A similar finding has been observed in other tissues and may be due to an action of AH6809 on phosphodiesterase (Coleman, 1987).

The EP_3 receptor induces contraction by decreasing intracellular concentrations of cAMP (Coleman *et al,* 1990). As discussed in Chapters 1 and 6, PGE_2 dilates the ductus by increasing cAMP. Stimulation of the EP_1 and TP receptors induce contraction independently of effects on cAMP (Coleman *et al,* 1994b). The fact that sulprostone decreased the sensitivity of the ductus to PGE_2, whereas U46619 did not, suggests that sulprostone has a specific inhibitory effect on cAMP and, therefore, that its effect on the ductus is likely to be on an EP_3 receptor. This needs to be confirmed by other techniques.

Recent studies have been described studying EP receptors at the molecular level (Namba *et al,* 1993; Sugimoto *et al,* 1993). The EP

receptor subtypes present in the ductus could be described definitively by analysis of mRNA sequences in ductal smooth muscle, either by Northern blot or *in situ* hybridization. The physiological balance between contractile and dilator EP effects in the ductus could be better clarified with more potent, selective agonists and antagonists, which are not yet available.

No data were found to support the existence of an FP receptor on the ductus.

Applying these data to the physiological control of the ductus, it would be anticipated that the dilator EP receptor is active in fetal and early neonatal life and that closure of the vessel would be associated in a shift in the relative activation of the contractile EP receptor over the dilator EP receptor in the face of a given concentration of PGE_2. It would also be expected that the ductus would develop contractile EP and TP receptors with advancing gestational age.

Increasing arterial oxygen tension is a feature of the neonatal circulation and oxygen causes direct contraction of the ductus (see section 1.2.1). In view of this, it is paradoxical that increasing oxygen tension increases the release of PGE_2 by the isolated ductus arteriosus (Coceani *et al,* 1986) given that it is a potent dilator of the vessel (see section 1.2.3). These data make sense of this fact, particularly as increasing oxygen decreases the sensitivity of the ductus to the dilator effect of PGE_2 (see Chapter 6). It may be that oxygen is one factor which regulates the balance of contractile and relaxant EP effects on the ductus.

It might be argued that the presence of TP receptors on the ductus is of no physiological significance, as the ductus does not synthesise TxA_2 (Pace-Asciak and Rangaraj, 1977). However, the vessel may be exposed to TxA_2 as it closes when blood clots in the lumen as TxA_2 is released following

platelet aggregation. Alternatively, the TP receptor is also stimulated by the cyclic endoperoxides, PGG_2 and PGH_2 (Coleman *et al,* 1990), and 8-epi-$PGF_{2\alpha}$ (Banerjee *et al,* 1992). As oxygen stimulates the synthesis of all prostaglandins by the ductus (Coceani *et al,* 1986) it may be that the TP receptor is involved in ductal closure by mediating contractile effects of PGG_2 and PGH_2, although this would be distinct from the direct contractile effect of oxygen which is not blocked by cyclo-oxygenase inhibitors (Smith and McGrath, 1988). Alternatively, increasing oxygen can stimulate the release of 8-epi-$PGF_{2\alpha}$ (Morrow *et al,* 1990), a potent TP receptor agonist (Banerjee *et al,* 1992), the synthesis of which is cyclo-oxygenase independent (Morrow *et al,* 1990). Finally, TP receptors can be stimulated by 15-hydroperoxy metabolites of arachidonic acid (Van Diest *et al,* 1991). There is no study to my knowledge which has examined whether the oxygen-induced contraction of the ductus is altered by TP receptor blockade.

These data also have relevance to the therapeutic use of indomethacin to close the ductus in premature infants where the vessel fails to close spontaneously (see Gersony, 1986, for review). Indomethacin contracts the ductus because the net effect of all prostaglandins synthesised by the ductus is dilator. However, since the ductus has contractile prostanoid receptors, part of the contractile effect of indomethacin (mediated by eliminating dilator effects of PGE_2 and PGI_2) may be offset by eliminating contractile effects of PGE_2 and TP receptor stimulation. Given that the dilator effect of PGI_2 appears to be somewhat dependent on the presence of PGE_2 (see Chapter 5) it may be that a selective EP_4 receptor antagonist (see Chapter 4) would be a more effective constrictor of the ductus than indomethacin.

Considering the use of intravenous PGE_1 or PGE_2 to dilate the ductus in a range of congenital heart defects (Gersony, 1986) the current data suggest that the dilator effect of EP_4 stimulation may be offset somewhat by

activation of the contractile EP receptor. Indeed, the present data may explain the failure of this therapy in some infants. A selective EP_4 agonist would be expected to be a more effective dilator of the ductus than PGE_1 or PGE_2, as well as having fewer adverse effects (see Chapter 4).

Chapter 8. General discussion.

The specific aims outlined in section 1.6 have been largely achieved, and are discussed in detail in each of the chapter discussions. Furthermore, the general aims, of improving the understanding of the control of ductal closure in the neonate and identifying promising new avenues for the development of better drugs in the therapeutic control of the ductus, have also been achieved. In this chapter, I will try briefly to bring together some of the main findings of individual chapters into more general control models of ductal closure and clinical hypothesis for drug development.

8.1 A model for ductal closure in the neonate.

A major finding of this work was that PGE_2 and oxygen tension interact in the regulation of ductal smooth muscle tone (Chapter 6). As discussed in Chapter 1, the main factors identified which stimulate contraction of the ductus after birth are decreasing circulating concentrations of PGE_2 and increasing arterial oxygen tension and, as discussed in Chapter 6, these factors act synergistically to promote contraction of the ductus. The factors which initiate the elimination of circulating PGE_2 and the rise in oxygen tension at birth are ventilation of the lungs, the reversal of hypoxic pulmonary vasoconstriction and the development of normal pulmonary function. As discussed in Chapter 1, the patent ductus in the transitional circulation of the neonate improves pulmonary blood flow and increases arterial oxygen tension. A feedback loop is proposed between the patency of the ductus, its effects on lung function, and the effect of lung function on circulating PGE_2 and oxygen tension. This control loop and the wider relationship between lung function and ductal closure are presented in a model, Figure 8.1.

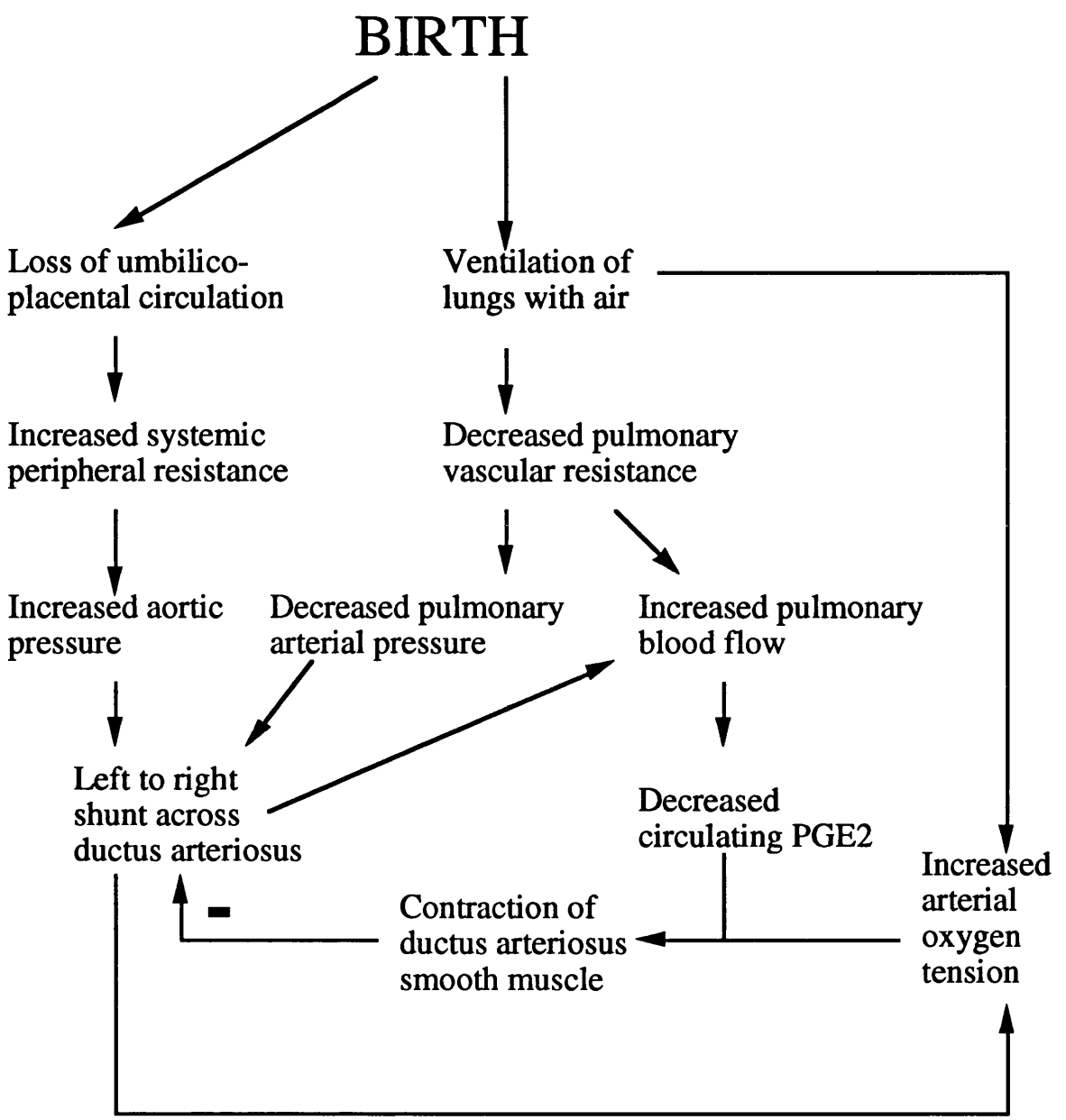

Figure 8.1 A model for the control of the ductus arteriosus in the transitional circulation. Arrows indicate a stimulatory effect unless there is a minus sign next to the point, in which case the effect is inhibitory.

8.2 The effects of oxygen on the ductus arteriosus.

As discussed above, the effect of oxygen is central to the closure of the ductus in the neonate. Understanding of the effects of oxygen had been incomplete. It was clear that the rise in oxygen that occurred at birth stimulated direct contraction of the vessel. However, it had been demonstrated that increasing oxygen tension also stimulated the release of PGE_2 (Clyman *et al,* 1980a; Coceani *et al,* 1986). No explanation had ever been proposed why the factor which is central to stimulating the vessel to contract (oxygen) stimulates the release of the agent which is central to making the vessel relax (PGE_2). Furthermore, following birth there is a massive decrease in the circulating concentrations of PGE_2 (Clyman *et al,* 1980c) but an apparent stimulation of its local release.

My work advances a series of explanations for this apparent anomaly. Firstly, the ductus has a TP receptor (see Chapter 7) which can be stimulated by PGH_2 (Coleman *et al,* 1990a). PGE_2 is formed by degradation of PGH_2 in the ductus (see Chapter 1) and it follows, therefore, that if PGE_2 is increased, PGH_2 must also be increased. Any inhibitory effect of the PGE_2 formed may, therefore, be offset by the contractile effect of increased PGH_2. Secondly, the increase in formation of PGH_2 is at least in part secondary to decreased PGI_2 (see Needleman *et al,* 1981). The role of PGI_2 has been discounted hitherto (Clyman, 1987; Coceani and Olley, 1988). My work indicates that a decrease in the release of PGI_2 will stimulate contraction as PGI_2 almost certainly has a physiological dilator effect (see below). Thirdly, as oxygen tension rises, the sensitivity of the ductus to the dilator effect of PGE_2 is inhibited (see Chapter 6). The net inhibitory effect of PGE_2 may actually be reduced in raised oxygen tension, despite increased local concentrations. Finally, the PGE_2 released will also stimulate the contractile EP receptor (see Chapter 7). It would be anticipated that responses mediated by this receptor will be increased following birth or in raised oxygen tension.

A model for the effect of oxygen to promote contraction of the ductus is outlined in Figure 8.2. This model incorporates both the direct contractile effect of oxygen by depolarising the vessel, the membrane-independent effect and its effects on prostaglandin function.

One of the areas of study of the ductus which warrants further investigation is the mechanism of the membrane-independent effect of oxygen on the ductus. A particularly promising line of investigation would follow up the work on the factors controlling the sensitivity of the contractile proteins of the ductus to calcium. In elevated oxygen tension, the selectively permeabilised ductus is more sensitive to calcium than any other smooth muscle studied (Crichton *et al,* 1994). It would be interesting to establish whether the membrane-independent effect of oxygen tension acts by modulating the sensitivity of the contractile proteins of the ductus to calcium.

8.3 PGI_2 and the ductus arteriosus.

The current orthodoxy is that PGI_2 has little or no role in the control of ductus arteriosus smooth muscle (Clyman, 1987; Coceani and Olley, 1988). One of the major findings of the work described in this thesis is that PGI_2 almost certainly does have a role in the control of ductus arteriosus smooth muscle. The evidence for this is as follows: firstly, PGI_2 is the by far main product of arachidonic acid in the ductus (see Chapter 1); secondly, the maximal dilator effect of PGI_2 is greater than PGE_2 (see Chapter 6); thirdly, the sensitivity of the ductus to PGI_2 under certain conditions is only an order of magnitude less than PGE_2 (see Chapter 5) as opposed to three orders of magnitude as generally supposed (Clyman *et al,* 1978c; Coceani *et al,* 1978b); and, forthly, the ductus has a receptor for PGI_2 (IP receptor, see Chapter 4). It will require the availability of potent selective antagonists of the EP_4 and IP receptor to clarify more fully the relative roles of PGE_2 and PGI_2 in the regulation of ductal patency.

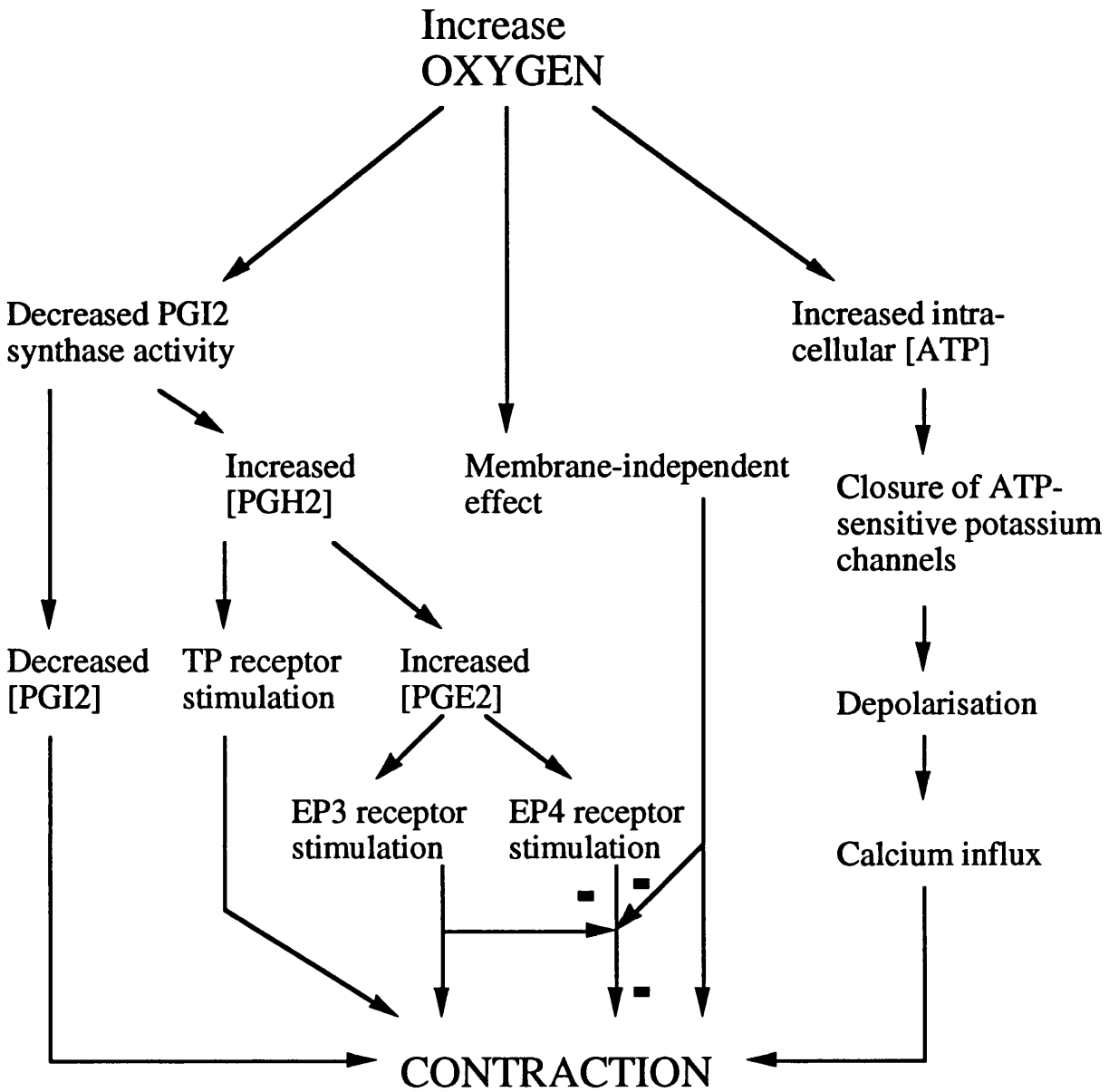

Figure 8.2 A model for the effects of oxygen on the ductus arteriosus. Arrows indicate a stimulatory effect unless there is a minus sign next to the point, in which case the effect is inhibitory. The model assumes that the contractile EP receptor on the ductus is EP3. This is based on an understanding of its pharmacology, but remains to be established (see Chapter 7).

8.4 Vasoconstrictors and the ductus arteriosus.

Recent studies on the ductus have tended to concentrate on the effects of oxygen and PGE_2, whereas the effect on the ductus of humoral vasoconstrictors has been somewhat neglected. However, as outlined in Chapter 1, there is evidence that neurally released, locally released and circulating vasoconstrictors have effects on the ductus arteriosus. As outlined in Chapters 5 and 6, oxygen tension and PGE_2 modulate the response of the ductus to noradrenaline-induced contraction, and the two factors act synergistically to control profoundly the vessel's response to noradrenaline. The effect of PGE_2 is not specific to noradrenaline (Chapter 6) and it seems likely that fetal oxygen tension and high concentrations of PGE_2 are responsible for the relative lack of sensitivity of the ductus *in utero* to vasoconstrictors (Friedman *et al,* 1983). Conversely, the rise in oxygen tension and elimination of the dilator effects of PGE_2 after birth will uncover the response of the vessel to vasoconstrictors.

The control of the activity of pressor nerves and the control of locally released and circulating vasoconstrictors in the regulation of patency of the ductus arteriosus deserve further attention.

8.5 The role of ductus arteriosus endothelium.

In contrast to many other blood vessels, the endothelium of the ductus arteriosus is not thought to have a major role in the regulation of its smooth muscle (Fay, 1971; Coburn *et al,* 1986; Walsh and Mentzer, 1987). The relatively small maximal effect of the nitric oxide donor, SNP, compared with forskolin and prostaglandins (Chapter 6), suggests that endogenous nitric oxide by the ductus is likely only to have a minor role in the control of the ductus. Nevertheless, I have evidence for release of nitric oxide by the ductus (data not shown) and the physiological role of this should be investigated. In all the experiments described in this thesis, I

used the endothelium-denuded ductus arteriosus to eliminate any complicating factors of the endothelium e.g. changes in endothelial function over the course of experiments and the release of nitric oxide complicating the effects of contractile agonists.

The role of endothelin in the control of the ductus also needs to be addressed, given the conflicting data on its effects (see section 1.2.2).

8.6 Horizons for the treatment of ductus-dependent circulation.

This work indicated a number of areas for drug development for this spectrum of conditions.

Firstly, assuming that the EP receptors on human and rabbit ductus are the same, a selective EP_4 agonist would be expected to have fewer side effects than PGE_1 and be more potent, as it would not stimulate excitatory EP receptors on the ductus. There are several pharmaceutical companies working on such drugs at present and there is the very real possibility that such a drug may be available in the near future.

Another approach would be the use of a non-EP_4 dilator of the ductus. The E series prostaglandins were selected for use in ductus-dependent circulation in experiments conducted in the presence of indomethacin (see Chapter 1). As discussed in Chapter 5, the use of indomethacin will have distorted the comparison between E series prostaglandins and other vasodilators. The extent of this distortion will depend on the amount of endogenous PGE_2 to which the ductus is exposed (see Chapter 5). I propose that the relative potencies of prostanoid and non-prostanoid ductal dilators need to be established in animal models of ductus-dependent circulation, in the absence of indomethacin, as this may identify other equally effective dilators of the ductus with less severe adverse effects than PGE_2.

Of the various alternative dilators one might consider, I propose that an ATP-sensitive potassium channel activator such as cromakalim might be the most promising candidate: firstly, cromakalim will specifically inhibit the membrane-dependent effect of raised oxygen tension (Nakanishi *et al,* 1983); secondly, the sensitivity of the ductus to cromakalim will be potentiated by endogenous PGE_2 (see Chapter 5); and, thirdly, the ductus is very sensitive to cromakalim compared to other vessels- with the ductus pre-contracted with 10μM noradrenaline, 100nM cromakalim caused about 50% relaxation (Figure 5.8), whereas in adult rabbit aorta pre-contracted with only 0.1μM noradrenaline, 100nM cromakalim was sub-threshold (Bray *et al,* 1991). This suggests that low concentrations of cromakalim may be relatively selective for the ductus.

8.6 Horizons for the treatment of patent ductus arteriosus.

It is proposed that an EP_4 receptor antagonist would be more effective and less toxic than indomethacin (see Chapter 4). Although this drug would not eliminate the effect of PGI_2 directly, it may well do so indirectly by removing potentiation of the effect of PGI_2 by PGE_2 (see Chapter 5) Furthermore, it would preserve the contractile effects of TP and EP_3 stimulation, which will be eliminated by indomethacin (see Chapter 7). Again, such drugs are being developed commercially for other applications and there is the real possibility of their being available for evaluation in PDA.

Appendix.

Tables of 95% confidence intervals of pairwise differences.

Tables of 95% confidence intervals (CI) of pairwise differences calculated using Tukey's method, following ANOVA where $p<0.05$.

Values are the 95% CI of the difference between the mean from the column and the mean from the row. Clearly, where the 95% CI do not include zero, a p value of at least less than 0.05 can be inferred.

In each case see text or figure legend for details of units and calculation of values.

Table A.1. Data from Figure 3.1, The effect of stretch on the magnitude of contraction elicited by indomethacin.

	0.1	**0.2**	**0.3**	**0.6**	**0.9**
0.2	-56.5				
	24.4				
0.3	-83.7	-69.0			
	-5.6	11.8			
0.6	-116.0	-101.3	-71.4		
	-35.2	-17.8	9.5		
0.9	-101.7	-87.0	-57.1	-27.5	
	-23.6	-6.2	21.0	53.4	
1.2	-103.0	-88.3	-58.3	-28.7	-40.3
	-24.9	-7.4	19.8	52.1	37.8

Table A.2 Data from Figure 3.3, The effect of stretch on the maximum relaxant response (MRR) to PGE_2.

	0.1	**0.2**	**0.3**	**0.6**	**0.9**
0.2	-76.0				
	9.7				
0.3	-95.7	-64.0			
	-12.9	21.7			
0.6	-149.2	-117.5	-94.9		
	-63.5	-28.9	-9.2		
0.9	-142.9	-111.1	-88.6	-37.9	
	-60.1	-25.4	-5.8	47.8	
1.2	-145.7	-113.9	-91.4	-40.7	-44.2
	-62.9	-28.2	-8.6	45.0	38.6

Table A.3 Data from Figure 3.4, The effect of stretch on the pEC_{50} to noradrenaline in 1μM indomethacin and 1nM PGE_2.

	0.1	**0.2**	**0.3**	**0.6**	**0.9**
0.2	-77.5 15.9				
0.3	-96.1 -5.8	-66.8 26.6			
0.6	-149.2 -55.8	-120.0 -23.5	-98.3 -4.9		
0.9	-137.9 -47.6	-108.6 -15.2	-86.9 3.3	-36.9 56.5	
1.2	-135.2 -45.0	-106.0 -12.6	-84.3 6.0	-34.3 59.1	-42.5 47.8

Table A.4 Data from Figure 3.4, The effect of stretch on the pEC_{50} to noradrenaline in 1μM indomethacin and 10nM PGE_2.

	0.1	**0.2**	**0.3**	**0.6**	**0.9**
0.2	-87.7 24.9				
0.3	-93.0 15.8	-63.6 49.1			
0.6	-147.8 -35.2	-118.3 -2.0	-109.2 3.4		
0.9	-135.6 -26.8	-106.2 6.4	-97.0 11.8	-46.0 66.6	
1.2	-133.6 -24.8	-104.1 8.5	-95.0 13.8	-44.0 68.6	-52.4 56.4

Table A.5 Data from Figure 6.5, Maximum relaxant responses (MRR) of vasodilators in 1μM indomethacin, 10μM noradrenaline and 15% oxygen.

	Adenosine	Cicaprost	Cromakalim	Forskolin	PGE_2
Cicaprost	-96.84				
	-76.70				
Cromakalim	-35.95	50.66			
	-16.14	70.80			
Forskolin	-107.35	-20.73	-81.31		
	-85.95	0.97	-59.91		
PGE_2	-81.27	5.34	-55.23	14.60	
	-61.13	25.80	-35.09	36.31	
SNP	-11.43	75.20	14.62	84.49	59.62
	9.97	96.90	36.02	107.37	81.33

Table A.6, Data from section 6.2.2, pEC_{50} to vasodilators in 1μM indomethacin, 10μM noradrenaline and 15% oxygen.

	Cicaprost	Cromakalim	Forskolin
Cromakalim	-0.0868		
	0.5175		
Forskolin	0.0506	-0.1602	
	0.7018	0.4819	
PGE_2	-2.1616	-2.3721	-2.5564
	-1.5477	-1.7679	-1.9052

Table A.7 Data from Figure 6.15, Effect of oxygen on the maximum relaxant response (MRR) to PGE_2 in 1μM indomethacin, 1μM flubriprofen and absence of cyclo-oxygenase inhibitor (control): MRR in 2% oxygen.

	Control	Indomethacin
Indomethacin	-24.029	
	-6.291	
Flubriprofen	-27.026	-11.866
	-6.226	8.934

Table A.8 Data from Figure 7.4, Contractile response to U46619, sulprostone, GR63799X and $PGF_{2\alpha}$ in 10nM PGE_2 at 100nM.

	Sulprostone	GR63799X
GR63799X	-6.000	
	10.400	
U46619	-13.908	-16.108
	1.063	-1.137

Table A.9 Data from Figure 7.4, Contractile response to U46619, sulprostone, GR63799X and $PGF_{2\alpha}$ in 10nM PGE_2 at 300nM.

	Sulprostone	GR63799X
GR63799X	-10.57 14.67	
U46619	-22.27 0.78	-24.32 -1.27

Abbreviations.

AH13205	(+/-)- trans -2- [4- (1-hyroxyphexyl) phenyl] -5-oxocylopentaneheptanoic acid
AH23848B	(1a(z),2b5a]-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid
AH6809	6-isopropoxy-9-oxoxanthine-2-carboxylic acid
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BW245C	5- (6-carbohexyl) -1- (3- cyclohexyl -3-hydroxypropylamino) hydantoin, one racemic diastereomer
BW868C	3-Benzyl -5- (6- carbohexyl) - 1 - (2- cyclohexyl - 2-hydroxyethylamino) hydantoin
cAMP	cyclic adenosine monophosphate
CCRC	concentration contraction response curve
CI	confidence interval
cGMP	cyclic guanosine monophosphate
CR	concentration ratio
CRRC	concentration relaxation response curve
CVO	combined ventricular output
DMSO	dimethyl sulphoxide
EC_{50}	see section 2.4
EMR	equieffective molar ratio
EP 092	(*rac* 5-*endo*(6'-carboxyhex-2'Z-enyl)-6-*exo*{1"-[N-(phenylthiocarbamoyl)hydrazono]ethyl}-bicyclo[2.2.1]heptane

GR63799X	[1R-[1a(Z),2b(R*), 3a]]-4-(benzoylamino) phenyl 7-[3-hydroxy- 2 (2-hydroxy- 3-phenoxypropoxy)- 5- oxocyclopentyl]- 4-heptenoate, single enantiomer
MCR	maximum contractile response
MRR	maximum relaxant response
NA	noradrenaline
NSAID	non-steroidal anti-inflammatory drug
PDA	patent ductus arteriosus
pEC_{30*}	see section 6.1.3
pEC_{50}	see section 2.4
PG	prostaglandin
PSS	physiological salt solution
SC19220	8-Clorodibenz [b,f] [1,4] oxazepine-10 (11H) - carboxylic acid, 2-acetylhydrazide
SEM	standard error of the mean
SNP	sodium nitroprusside

Note: less commonly used abbreviations are defined on their first use in each chapter; common abbreviations are defined once only, on their first use in the thesis (eg PG).

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