Study of the actions of vasoactive substances in the rat isolated perfused lung

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Study of the Actions of Vasoactive Substances in the Rat Isolated Perfused Lung

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To my parents,

wife, and son

Summary

A ventilated perfused rat lung model is described enabling simultaneous measurement of pulmonary perfusion pressure (PPP), lung weight (LW) and pulmonary inflation pressure (PIP). This model was stable for 2 h. Phenylephrine selectively increased PPP, carbachol selectively increased PIP whereas bradykinin (BK) caused increased PPP, LW and PIP. Low doses of BK caused reversible increases in LW. Higher doses caused irreversible increases in LW which were associated with albumin accumulation. Manipulation of venous outflow pressure, retrograde perfusion and the vasodilator papaverine revealed that the changes in LW were explicable as sequelae of vascular hydrostatic pressure changes.

Endothelin (ET) receptors were also characterised in orthogradely and retrogradely perfused lungs. Determination of agonist potency profiles and use of ET receptor antagonists showed that ET_{B2} receptors are located arterially whereas ET_A receptors are predominantly venous in distribution. The bronchoconstrictor actions of ETs and SX6C suggest the presence of ET_B receptors in the bronchial smooth muscles. Results also suggest the possibility of heterogeneity of ET_A and ET_B receptor subtypes.

Pulmonary actions of ET-1 and SX6C were augmented by L-NOARG suggesting that these substances stimulated the release of nitric oxide (NO) which subsequently attenuated their bronchoconstrictor effects. When the vascular tone was elevated, ET-1, ET-3 and SX6C produced ET_{B1} receptor-mediated pulmonary vasodilation as indicated by the potency profile, and the effects of ET antagonists. The vasodilator responses to ET-1 are mediated by the release of NO. In addition to NO, prostaglandins also contributed to the vasodilator actions of ET-1 but not to the actions of SX6C.

Contents

An understanding of biological processes at different levels will be needed to provide answers to many questions concerning diseases of humans. An early molecular biologist, Albert Szent-Gyorgyi (1963) wrote in his article entitled " The Promise of Medical Science", which appeared in Man and His Future:

> *One important point I overlooked was "organization". Organization means if nature puts two things together in a meaningful way, something new is generated, when I take two things apart, I have thrown away something, something which has been the very essence of that system, of that level of organization.*

SECTION 1

Introduction

1 1 The structure of the lung

1.1.2 Airways

The lungs are situated bilaterally within the thorax, and separated from each other by the heart. The structure of the lung has been reviewed by many investigators (Levitzky, 1982; Carola *et al.*, 1990; Vander *et al.*, 1990a; Tyler and Julian, 1992). Briefly, each lung has an apex that lies in the plane of the thoracic inlet and a base which rests upon the convex surface of the diaphragm. The left lung is divided into two lobes, superior and inferior and the right lung in humans is divided into three lobes, superior, middle and inferior. However, the right lungs from laboratory mammals have four lobes: cranial, middle, caudal and accessory (the terms cranial and caudal are given for superior and inferior lobes respectively in all common animals). The conducting zones of the lung include the trachea and extra pulmonary bronchi and various orders of small intrapulmonary bronchi (Figure 1). The bronchi continue to branch into smaller and smaller tubes called bronchioles and then terminal bronchioles which after repeated branching finally end in a number of respiratory bronchioles consisting of alveolar ducts that lead into microscopic air sacs called alveoli. The whole systems look so much like an inverted tree that it is commonly called the "respiratory tree".

The alveolar surface of the lung is lined with a continuous sheet of epithelial cells referred to as type 1 and type 2 epithelial cells. Type 1 epithelial cells cover > 90 % of the alveolar surface which provide a large surface for gas exchange and also serve as a permeability barrier. Type 2 cells secrete a surfactant which helps to keep alveoli inflated. In addition resident mast cells and macrophages are also present in the lung (Levitzky, 1982; Simon, 1992).

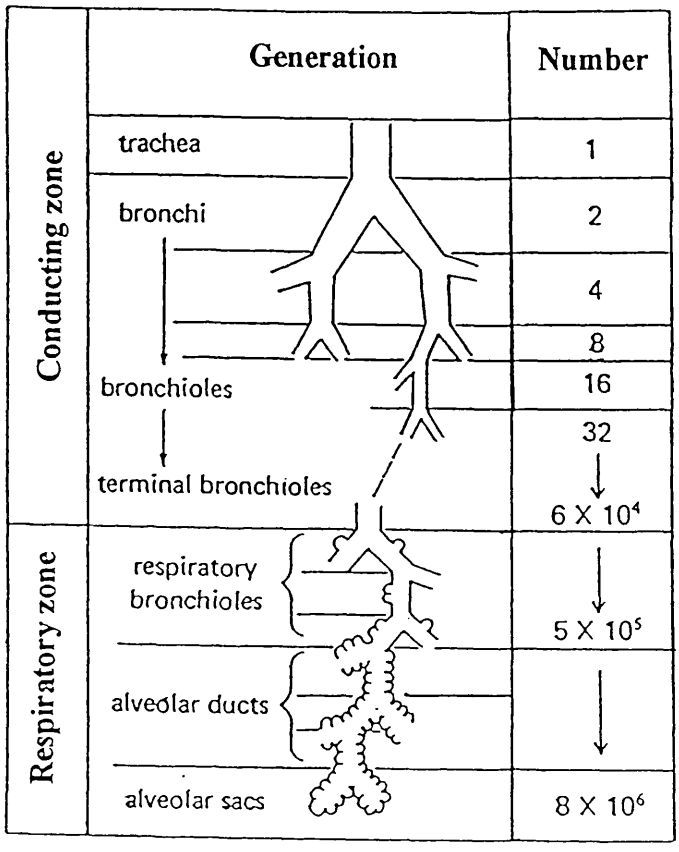


Figure 1 Schematic representation of airway branching and approximate numbers of tubes in each branch. Modified from Levitzky, (1982); Vander *et al.*, (1990a).

1.1.2 *Pulmonary vasculature*

The lung has two separate blood supplies the pulmonary and bronchial circulations respectively. These circulations are different in size, origin and function and are described as follows:

(a) Pulmonary circulation

The main pulmonary artery divides into two branches which supply the right and left lung. The pulmonary arteries and arterioles run along the bronchi, bronchioles, and alveolar ducts. Arteries less than 100 μm in diameter lose their muscular coat and become capillary in structure, 60% of vessels 32-48 μm in diameter are non-muscular (see Snashall and Hughes, 1981). The capillaries arise as side branches or terminations of the arterioles, lying in the alveolar septa and separated from the alveolar space by a small amount of interstitial tissue and the alveolar lining cells. The capillaries are suspended in air which encourages gas exchange. However, this can predispose the lung to oedema formation when capillary pressures are increased (Effros, 1984). The alveolar capillaries drain into vessels which are histologically identical to the arterioles, and then into pulmonary veins which tend to be situated away from the bronchial tree. Finally, the large pulmonary veins pursue an independent course towards the hilum of the lung. The pulmonary veins drain into the left atrium.

(b) The Bronchial circulation

The bronchial circulation is also known as the "pulmonary collateral circulation", or the "systemic blood supply to the lungs" (Deffebach *et al.,* 1987). This circulation is unique in that the arteries arise from the systemic circulation i.e. from the aorta, subclavian, internal mammary, and intercostal vessels, whilst the venous components drain into the pulmonary system.The bronchial artery is considered the nutrient artery supplying the bronchi, hilar lymph nodes, and *vasa vasorum* to the pulmonary arteries and pulmonary veins (Kay, 1992). Both the bronchial and pulmonary circulations communicate freely with each other on the arterial and venous sides. An examination of the distribution of bronchial arteries in 11 mammalian species including humans and rat found anastomoses

between the bronchial and pulmonary arteries in all species, except the pig, dog, cat, and rhesus monkey (McLaughlin *et al.,* 1966; McLaughlin 1983). There are two distinct types of bronchial veins; the deep or true veins which are intrapulmonary vessels related to the bronchi and which drain into the pulmonary veins or direct into the left atria. The second group of bronchial veins drain the subpleural and hilar structures into the azygos veins (Marchand *et al.,* 1950). The relationships between the bronchial and pulmonary circulations are shown in Figure 2.

1.1.3 Lymphatic drainage of the lung

Lymphatic channels are present in the interstitial space. The first group of lymphatics originates in the visceral pleura or in the interlobular septa, draining the periphery of the lung lobules by channels that follow the course of the pulmonary veins to the hilum of the lung. The second group of lymphatics begin as blind-ended tubes in the acini around the alveolar ducts, draining to the hilum via the peribronchial lymphatics. Normally, the lymphatics of the upper part of the left lung drain into the thoracic duct while those from the remainder of the lung and from the whole of the right lung drain into the right lymphatic duct (Cole and Mackay, 1990).

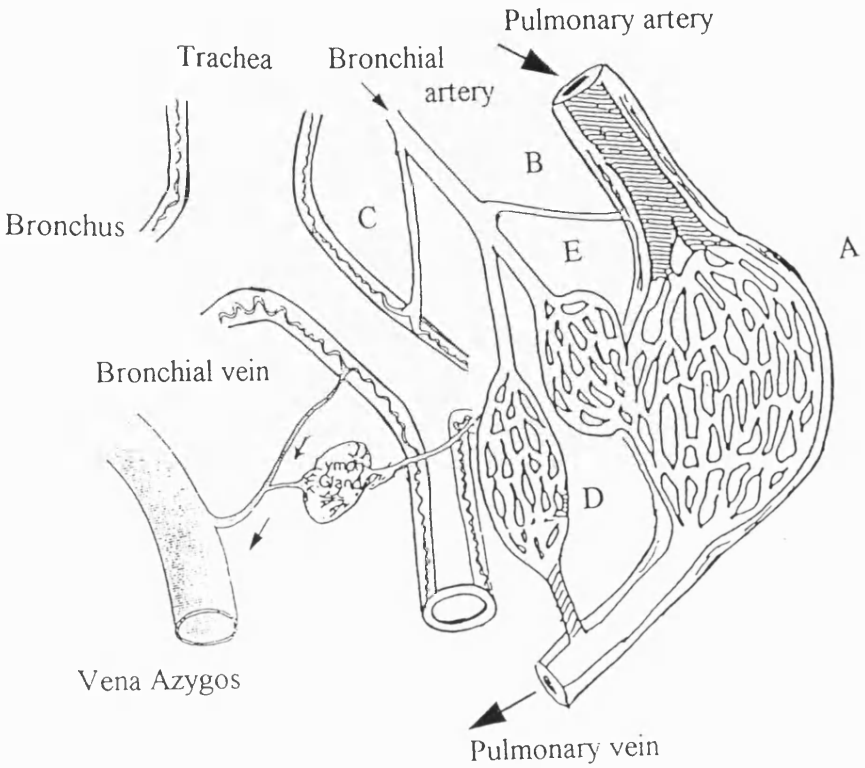


Figure 2 Schematic of the blood supply to the lung, showing the relationship between the bronchial and pulmonary circulations. The pulmonary artery supplies the pulmonary capillary network (A). B represents the bronchial arterial supply to the walls of the pulmonary artery (*vaso vasorum*). C is the bronchial artery supply to the airways. Network D represents the bronchial capillary supply to most of the bronchi; these vessels drain into the pulmonary veins. Network E represents the bronchial capillary supply that anastomoses with the pulmonary capillaries and drains into the pulmonary veins. The bronchial veins drain into the vena azygos, or intercostal veins. Shaded areas represent blood of low O_2 content. Arrows indicate the direction of the blood flow.

1.2 Regulation of pulmonary vascular tone

The pulmonary blood vessels are particularly suited to maintain a delicate balance of pressure and distribution of flow that optimizes gas exchange. In normal mature mammals, the pulmonary circulation is a low pressure, high flow, low-resistance system and pulmonary arterial pressure is normally about one-sixth that of the systemic arterial pressure (Kay, 1992). The pulmonary blood vessels are capable of being distended, narrowed, or collapsed, as well as actively constricting and dilating. Pulmonary vascular smooth muscle is responsive to both neural and humoral influences. These influences produce active alterations in pulmonary vascular resistance, as opposed to passive factors such as gravity. The factors which can affect pulmonary vascular tone are given below.

1.2.1 Neural control

The autonomic nerve fibres are found in close association with pulmonary blood vessels (Zorychta and Richardson, 1992). The elastic pulmonary artery has the highest density of innervation whereas it diminishes toward the periphery. However, the pulmonary veins are devoid of any autonomic control. Sympathetic activation causes an increase in pulmonary vascular resistance which is primarily mediated by α_1-adrenoceptors whereas β-adrenergic receptor activation produces pulmonary vasodilation (Hyman *et al.*, 1981; Hyman *et al.*, 1990). Retrograde and orthograde perfusion of dog lungs has shown that arteries are the major site of the vasoconstrictor response to sympathetic nerve stimulation (Daly *et al.*, 1970).

The pre-ganglionic cholinergic efferent nerve fibres pass down the vagus nerve into the lungs. The extra-pulmonary arteries and large pulmonary veins of rat contain acetylcholinestrase positive nerve fibres, whereas they are absent in intra-pulmonary arteries of the rat (El-Bermani, *et al.*, 1970). Investigators have reported that vagal

stimulation produces variable responses in different species depending upon the pulmonary vascular tone; that is, under low basal tone it caused vasoconstriction, but when the tone was raised, vagal stimulation caused vasodilation. Heterogeneous muscarinic receptors have been identified. Muscarinic receptors mediating vasodilation in the pulmonary vascular bed are of the M_3 sub-type (Post *et al.,* 1991).

The neural mechanisms that are not inhibited by adrenergic or cholinergic blockade are attributed to transmitter release from non-adrenergic non-cholinergic (NANC) fibres. NANC mediated relaxation in pulmonary arteries is reduced by nitric oxide synthase inhibitors, providing evidence that nitric oxide is a NANC neurotransmitter (Liu *et al.,* 1992). This neural mechanism may play a role in the regulation of pulmonary vascular tone (for review see Barnes and Liu, 1995).

1.2.2 Humoral control and role of the endothelium

The pulmonary vasculature responds actively to many humoral and autocoid factors. Many vasoactive substances circulating in the blood or which are synthesised by the lung can affect pulmonary vascular resistance. For instance, endogenous angiotensin-2 produces vasoconstriction, bradykinin is potent in releasing nitric oxide (Palmer *et al.,* 1987) and also stimulates prostacyclin release (Peers and Hoult, 1986; Ignarro *et al.,* 1987). Bradykinin play an important role in the regulation of vascular tone and two types of bradykinin receptors BK_1, BK_2 have been found in pulmonary vascular smooth muscle (Regoli *et al.,* 1986). Other vasoactive substances which can influence pulmonary vascular tone are substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), atrial natriuretic peptides (ANP), 5-hydroxytryptamine, eicosanoids, histamine and platelet activating factor (for review see Barnes and Liu, 1995).

Endothelial cell-derived mediators also play an important role in the regulation of pulmonary vascular tone by synthesizing and releasing endothelin (Yanagisawa *et al.,* 1988), prostacyclin (Moncada *et al.,* 1976) and endothelium -derived relaxant factor (EDRF), now widely accepted to be nitric oxide (Palmer, *et al.,* 1987). Basal and

stimulated nitric oxide both participate in the maintenance of low pulmonary vascular tone (Liu and Barnes, 1994; Barnes and Liu, 1995) and the localisation of nitric oxide synthase has been reported in the pulmonary arterial and bronchial vessels in rat (Xue *et al.*, 1994). Endothelium-derived hyperpolarizing factor (EDHF) has also been shown to be a determinant of vascular calibre under normal conditions, and may therefore be important in the regulation of vascular resistance (Garland *et al.*, 1995).

1.3 Regulation of airway tone:

1.3.1 Neural control

The airways are supplied by nerves, derived from the sympathetic and parasympathetic nervous systems, and by sensory nerves that originate from the sensory ganglia of the vagus nerve. The sensory fibres terminate on the bronchi and are responsible for carrying the impulses from irritant and stretch receptors in the lung to the central nervous system (Said, 1989; Matthew and Altura, 1990; Barnes, 1992). The vagal parasympathetic fibres produce bronchoconstriction via activation of the muscarinic receptor. The nerve fibres from thoracic sympathetic ganglia produce bronchodilation. Both α - and β - adrenoceptor subtypes have been shown to be present on the bronchial smooth muscles (Kneussl and Richardson, 1978; Rugg *et al.*, 1978).

Neuropeptides that may influence airway smooth muscle function include; ANP, bombesin, CGRP, VIP, substance P, neurokinins A and B (reviewed by Said, 1989). The majority of peptides with activity on airway smooth muscle are bronchoconstrictor such as bombesin, CGRP, neurokinins. The peptides that relax airway smooth muscle make up a shorter list i.e. VIP, ANP etc. (see Said, 1989). In addition, a third component in the neural control of respiratory smooth muscle is NANC transmission. Linden *et al.*, (1992) showed that NANC responses can counteract changes in airway smooth muscle tone via a contraction or a relaxation depending on the tone prior to activation. Tucker *et al.*, (1990) have shown that nitric oxide is invoved in mediating NANC relaxations produced by electrical field stimulation in the guinea pig trachea.

NANC relaxations have been observed in airways isolated from a large number of species, including human (for review see Ellis and Undem, 1994).

1.3.2 Humoral regulation and role of airway epithelium

The epithelium plays an important role in modulating the responsiveness of the underlying smooth muscle. The mediators released from the epithelial cells include leukotrienes B_4, C_4, and prostaglandins D_2, E_2, and $F_{2\alpha}$ (see review by Davies and Devalia,1992; Delamere *et al.,* 1994). There has also been considerable interest in the possibility that relaxant factors can be released from airway epithelial cells. Barnes *et al.,* (1985) reported that the tracheobronchial epithelium may release one or more factors promoting airway relaxation. Pharmacological evidence has shown that one of the epithelium-derived relaxing factors might be nitric oxide, for example in guinea pig, the epithelium-dependent tracheal relaxation has been shown to be blocked in the presence of nitric oxide synthase inhibitors (Folkerts *et al.,* 1995). The epithelial cells appear to have both constitutive and inducible forms of nitric oxide synthase (Hamid, et al., 1993; Xue, et al., 1994; Guo, *et al.*, 1995) and the localisation of nitric oxide synthase enzyme has been reported in airway epithelium in rat lung (Xue *et al.,* 1994). Nitric oxide synthases are found in a variety of other cell types in the lung such as macrophages, neutrophils, mast cells, fibroblasts and smooth muscle cells (see review by Drazen *et al.,* 1995).

1.4 Lung functions

1.4.1 Respiratory

The most well known function of the lungs is that of respiration. Gas exchange between air and blood is the joint function of the alveoli and the networks of blood capillaries that envelop them. The capillaries provide an enormous surface area, making possible an extremely rapid diffusion of gasses between alveolar air and pulmonary capillary blood. The calculated number of alveoli in human lung (body weight 74 kg) are 950 million (Volberg and Blanchard, 1992) There may be as many as 280 billion pulmonary

capillaries, or nearly 1000 pulmonary capillaries per alveolus (Levitzky, 1982). It has been estimated that if the alveoli could be opened up flat, they would form a surface area about the size of a tennis court; that is about 85 square meters or well over 40 times the surface area of the entire body (Thibodean and Patton, 1993).

1.4.2 Non -respiratory

The other functions of the lung are non-respiratory functions. The pulmonary circulation receives the entire cardiac output and within the pulmonary vasculature, blood is exposed to a huge capillary endothelial surface. The vascular endothelial cells synthesise an apparently ever-growing number of physiologically important molecules. In part these substances are secreted into the blood or directed towards the subendothelial matrix. These characteristics make the pulmonary microcirculation uniquely suited for exercising a controlling influence on blood-borne vasoactive hormones. Vane, (1969) proposed that the lung should be considered as an endocrine organ owing to its capability to regulate the amounts of vasoactive materials reaching the arterial circulation. The lung can activate, inactivate, synthesize, and release a variety of biologically active compounds which are briefly summarised in table 1.

Table 1 Mediators which are metabolised or released by the lung:

(a) Activation

Angiotensin-I to angiotensin-II (Ng and Vane, 1967)

Big endothelin-1 to endothelin-1 (Hisaki et al., 1994b)

(b) Inactivation

Serotonin (Alabaster and Bakhle, 1970)

Bradykinin (Alabaster and Bakhle, 1972; Bakhle, 1977; Pesquero *et al.*, 1992; Baker *et al.*, 1992)

Prostaglandins E_1, E_2, $F_{2\alpha}$ (Piper *et al.*, 1970)

Endothelin-1 (Westcott *et al.*, 1990; Sirvio *et al.*, 1990)

Norepinephrine (Bryan-Lluka *et al.*, 1992)

(d) Synthesis or release of vasoactive agents

Histamine (Martins *et al.*, 1991)

Substance P (Martins *et al.*, 1991)

Prostaglandins, I_2, $F_{2\alpha}$ (Palmer *et al.*, 1973; Robinson and Hoult, 1980; Korbut *et al.*, 1981)

Endothelin-1 (Vemulapalli *et al.*, 1992)

The processes outlined in the table above are designed to regulate the concentrations of agents such as prostaglandins, amines and peptides in the blood before they reach the systemic circulation. In addition to their actions at non-pulmonary sites, these hormones can also change intrapulmonary blood flow and airway tone through their effects on vascular and bronchial smooth muscle; therefore, mechanisms for terminating such effects in the lung assume considerable interest.

1.5 Lung diseases

1.5.1 Pulmonary vascular diseases

(1) Pulmonary hypertension: Pulmonary hypertension is defined as an increase in pulmonary vascular pressure and this often leads to right ventricular hypertrophy and ultimately heart failure. Chronic pulmonary hypertension is a progressive disease of the pulmonary vascular wall. The histological changes that occur in the pulmonary vasculature are swelling, hypertrophy and hyperplasia of the cells in the intima and the medial coat of the pulmonary arteries leading to narrowing of the lumen. There is also an extension of the smooth muscle cells into non-muscular pulmonary arteries (see Matthew and Altura, 1990). Some of the important features of pulmonary hypertension are hypoxaemia, pulmonary oedema and bronchoconstriction (Matthew and Altura, 1990; Weitzenblum, 1994). The other clinically distinct syndromes of pulmonary hypertension are:

(a) Primary pulmonary hypertension; This is characterised by elevated pulmonary arterial pressure in the absence of primary cardiac or parenchymal pulmonary disease. It affects small pulmonary arteries, in which proliferative lesions involving endothelial and smooth muscle cells, obstruct flow. This damage leads to alterations in pulmonary vascular smooth muscle content resulting in exaggerated pulmonary vasoconstriction. Deranged vasodilating mechanisms appear inadequate to oppose vasoconstriction caused by lung injury and associated hypoxia. Recent evidence suggests that endothelial dysfunction plays a key role in causing chronic hypoxic pulmonary hypertension observed in humans and in animal models of pulmonary hypertension (Liu and Barnes, 1994; Barnes and Liu, 1995). Right ventricular dysfunction and pulmonary vascular fluid leakage with resultant interstitial oedema are common clinical sequelae as summarised by Wilson *et al.*, (1992).

(b) Secondary pulmonary hypertension; Elevated arterial pressure in the pulmonary circulation may be secondary to chronic lung diseases, including chronic bronchitis, emphysema, bronchiectasis, cystic fibrosis, etc (Wilson *et al.,* 1992). Pulmonary hypertension associated with an increase in pulmonary vascular resistance is frequently observed in patients with adult respiratory distress syndrome (ARDS).

(2) Pulmonary oedema

Oedema in the pulmonary circulation continues to be a major cause of morbidity and mortality in critically ill patients suffering from pulmonary hypertension (Cope *et al.,* 1992). Pulmonary oedema is a pathologic state in which there is abnormal extravascular water storage in the lung (Visscher *et al.,* 1956). Normally, a small amount of fluid containing plasma proteins filters out of the blood stream into the pulmonary interstitial space. The lymphatic system then drains this fluid into the systemic veins. When capillary filtration increases, the flow of fluid and proteins into the interstium can eventually exceed the maximal removal rate by the lymphatics, and the intersitium begins to swell. When the interstitial water exceeds the interstitial space capacity, fluid seeps into the alveoli (Cope *et al.,* 1992). The factors affecting liquid movement from the pulmonary capillaries is shown in Figure 3.

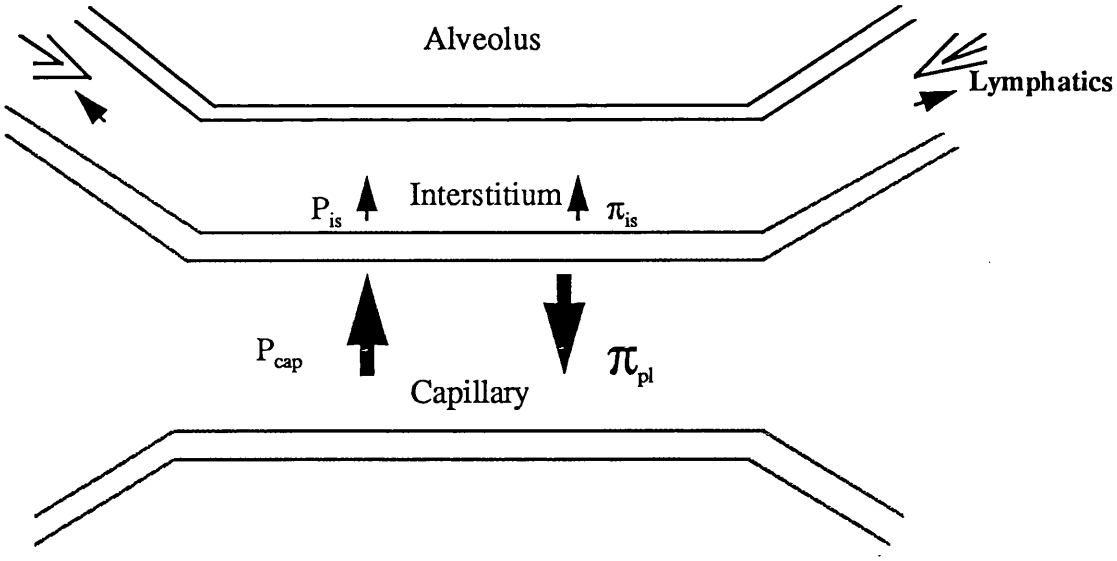


Figure 3 Schematic illustration of the factors affecting liquid movement from the pulmonary capillary. P_{cap} = capillary hydrostatic pressure; P_{is} = interstitial hydrostatic pressure; π_{pl} = plasma colloid osmotic pressure; π_{is} = interstitial colloid osmotic pressure. (P_{is} is assumed to be negative).

The conditions that can produce pulmonary oedema may be summarised as: (a) an increase in permeability of capillary endothelium, (b) an increase in capillary hydrostatic pressure or decrease in colloid osmotic pressure, (c) decrease in interstitial hydrostatic pressure or increased interstitial colloid osmotic pressure, (d) lymphatic insufficiency (Levitzky, 1982). In the pulmonary circulation plasma colloid osmotic pressure is usually greater than capillary hydrostatic pressure, therefore there is very little bulk fluid movement out of the capillary (see Vander *et al.*, 1990b).

In addition, Staub (1974) suggested three pathways that can be taken by water and solute molecules to cross membrane: (a) across cell membranes, (b) through intracellular junctions, and (c) by non selective leak. As shown in Figure 4 the pores crossing cell membranes (arrow 1) are water-filled channels of 4-5 A° radius and only allow water

diffusion and flow but are highly restrictive to the movement of solute molecules due to size. The chief site of ultrafiltration and protein flow is probably via the intracellular junctions. Arrow 2 shows the intracellular junctional pores which have a pore radius too small for plasma protein molecules to penetrate. Arrow 3 indicates the intracellular junctions of larger size (> 1000 A°) which allow plasma protein molecules to pass through (Staub, 1974).

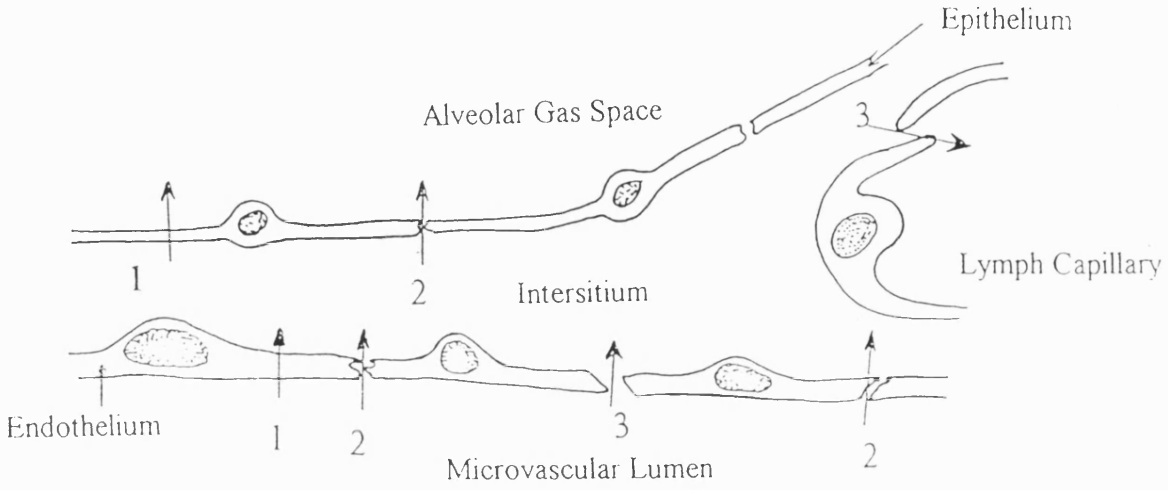


Figure 4 Schematic representation of the fluid and protein exchange features of the lung. There are three extracellular compartments; microvascular lumen, interstitium including lymph capillaries, and alveolar gas space. The three routes of passive transport are: (1) small pores in the cell membrane, (2) intercellular junctions (large pores), (3) intercellular gaps.

1.5.2 Airway diseases

Asthma was first described by Hippocrates in the fourth century BC and its name derives from the Greek word for panting (meaning; swift and shallow breathing with a fast respiratory frequency and small tidal volume) described by Wardlaw, (1993). Although, medical advances of the nineteenth and twentieth centuries have established asthma as a diagnostic entity, it is recognised that this disease is heterogeneous and difficult to classify. Jacobs and Kaliner, (1989) stated that asthma can be defined clinically as a recurrent disease causing intermittent wheezing, breathlessness, and cough with sputum production. Barnes *et al.,* (1992) described asthma as a clinical syndrome characterised by increased responsiveness of the tracheo- bronchial tree to a variety of stimuli and variable airflow obstruction caused by a combination of oedema, smooth muscle spasm and mucous plugging, which in turn are due to airway inflammation. Allergy is the most frequent cause of asthma, accounting for 90 % of asthmatics and the mediators derived from the mast cells play a pivotal role (Jacobs and Kaliner, 1989). The other causes of asthma include respiratory tract infections and enironmental pollution.

1.6 Methods used to investigate lung function

1.6.1 Isolated pulmonary tissues

Lung function can be studied by using isolated pulmonary tissues in a conventional organ bath and pulmonary vascular smooth muscle reactivity to many vasoactive agents has been studied both in human and animal tissues by using rings or strips of pulmonary blood vessels (McKay *et al.,* 1991; La Douceur *et al.,* 1993; Buchan *et al.,* 1994; MacLean *et al.,* 1994; Maguire and Davenport, 1995). Despite the fact that vasoactive agents can produce selective actions on venous pulmonary smooth muscle, limited data is available from isolated pulmonary veins (Sudjarwo *et al.,* 1993; Zellers *et al.,* 1994; Gao *et al.,* 1995a). Isolated tracheal or bronchial preparations have also been used (Maggi *et al.,* 1990; McKay *et al.,* 1991; Hulsmann and de Jongste, 1993; Nally *et al.,* 1994; Yoneyama *et al.,* 1995).

Alternatively, investigations of the actions of agents which produce contractions of pulmonary vascular and airway smooth muscles have been carried out using lung parenchymal strips (Lulich *et al.*, 1976; Lach *et al.*, 1994). In this preparation thin strips are dissected from a lung lobe and studied in a conventional organ bath. Parenchymal strips have also been used to investigate the activation or degradation of blood-borne polypeptides such as endothelins, the plasma concentrations of which are regulated in the pulmonary circulation and are known to exert extremely potent vascular and bronchial actions (Battistini *et al.*, 1995).

The practical advantage of using isolated pulmonary tissues is that, they are easy to prepare and only small pieces of tissue are needed. However, when using pulmonary blood vessels no information can be gained on the responses of the studied agents on the bronchial smooth muscles. When only tracheal or bronchial tissues are used no information can be collected for the actions of substances on pulmonary vascular smooth muscle. When lung parenchymal strips are used, it is assumed that the drug-induced effects in parenchymal strips reflect the responses of smooth muscle of small airways present in the bronchioles and alveolar ducts. However, the involvement of non-airway components such as vascular smooth muscle complicates interpretation of the results (Mirbahar and Eyre, 1980). Moreover, the parenchymal strip preparations isolated from different regions of lung can respond in a quantitatively distinct manner to various pharmacological agents (Wong *et al.*, 1992). Therefore, the anatomical complexity of the parenchymal strip restricts its value as a method to study small airway pharmacology.

1.6.2 Whole lung models

Lung function has been studied by perfusing lungs with homologous whole blood or physiological salt solution (Czartolomna *et al.*, 1991; Rodman *et al.*, 1992). Depending on the experimental design, the lung can be perfused with whole blood, plasma or a blood -and plasma-free physiological salt solution (Chang and Voelkel, 1991). Blood-perfused lungs closely resemble the *in vivo* state and are often used in the studies of hypoxic pulmonary vasoconstriction. However, this is costly, for example using rats

about 8 to 10 ml of whole blood may be removed from each donor rat and for each perfused lung experiment approximately 30 ml of whole blood is required (Chang and Voelkel, 1991). In studies assessing the vascular actions of lipid mediators or measuring lung metabolism of eicosanoids, binding of the mediators or metabolites to plasma proteins and metabolism by blood cells may complicate the interpretation of results. This can be improved by using a single pass perfusion model, however, a very large volume of perfusate is required, making the use of whole blood impractical.

The single-pass isolated lung model perfused with a physiological salt solution offers several advantages to study lung function as;

(a) It lacks blood components and the associated complications mentioned above.

(b) The components of the pulmonary perfusate can be precisely regulated. Additionally, the lung can be easily lavaged or studied microscopically.

(c) There is strict control over perfusate flow, and the pulmonary arterial, venous , and microvascular pressures can be accurately measured. These data allow calculation of arterial and venous resistance changes in response to the experimental conditions.

(d) Lung oedema formation can be continuously monitored.

A variety of methods have been developed to perfuse lungs either *in situ* or *in vitro* (Levey and Gast, 1966; Kroll *et al.,* 1986; Niemeier, 1984; Ryrfeldt *et al.,* 1990; Uhlig and Wollin, 1994). Isolated perfused lungs have been used extensively to assess the metabolism of vasoactive agents and vascular reactivity and several reviews of isolated perfused lung preparations have been published in past years. Most of these have focused on metabolic and toxicological studies (Bakhle and Vane, 1974; Gill and Roth, 1976; Said, 1979; Gillis and Pitt, 1982; Chang and Voelkel, 1991).

Lungs perfused via the pulmonary artery have been utilised to study the release of vasoactive substances in response to infusions of a variety of substances (Bakhle and Vane, 1974; Said, 1979; Robinson and Hoult, 1980; Bakhle *et al.,* 1985; Peers and

Hoult, 1986). In another study the lungs were inflated via the trachea, which was then clamped off and lung perfused via the pulmonary artery for studying the release of substances (Conroy *et al.*, 1992).

Lungs perfused via the pulmonary artery are widely used to study the pulmonary vasoconstrictor activity of injected agents, however, the actions of such agents on lung weight and respiratory smooth muscles have not been studied (Okpako, 1972; Raj *et al.*, 1992; Perreault and Baribeau, 1995).

Lungs are extensively utilised to investigate the actions of agents on bronchial resistance i.e. the bronchoconstrictor effects of bradykinin have been studied without monitoring its vascular actions (Lou, 1993). Alternatively, effects of bradykinin on pulmonary vascular and bronchial resistance have been monitored, while the effects on vascular permeability were ignored (Kroll *et al.*, 1986; Ryrfeldt *et al.*, 1990).

Some permeability investigations have been carried out using cultured pulmonary arterial endothelial cell monolayers (Horgan *et al.*, 1991; Rodman *et al.*, 1992). However, an endothelial monolayer system derived from one circumscribed locus cannot represent the complete lung circulation which is an important aspect of vascular reactivity. Therefore, studies of mechanisms of injury and loss of membrane integrity have also employed the isolated perfused lung preparation to investigate permeability in a complete alveolar capillary membrane in a physiologically intact system (Czartolomna *et al.*, 1991; Horgan *et al.*, 1991). Studies on isolated lungs have also been performed to investigate actions of substances on pulmonary vascular resistance and microvascular permeability by initially inflating the lung via the trachea which was then clamped (Seale *et al.*, 1991).

Ventilated perfused lung models have been utilised to investigate the effects of perfusate composition on oedema formation. In these studies development of gross pulmonary oedema was indicated by swelling and translucency of the pulmonary parenchyma and by the appearance of tracheal fluid (Fisher *et al.*, 1980).

In recent years isolated lung models have been modified to allow simultaneous measurements of the actions of agents on pulmonary vascular and bronchial smooth muscles (Selig *et al.*, 1993; Uhlig and Wollin, 1994). However, in many of these studies the perfusate was recirculated and no indication with regard to the effect of perfusion time on lung viability was given.

In summary, the models used which range from simple pulmonary blood vessels and bronchial strip preparations to complex ventilated and perfused lung preparations have contributed substantially to progress in understanding the pathophysiology of pulmonary diseases such as pulmonary hypertension, adult respiratory distress syndrome, asthma. However, the precise relationship between pulmonary vasoconstriction, oedema and increase in bronchial resistance are still not clear. In order to gain more insight into the mechanisms of lung disease and the development of potential beneficial therapeutic agents, adequate models are needed. The study of isolated perfused and ventilated lung tissue in which the normal relationship between the ventilatory and circulatory unit is preserved *in vitro* would be an important step in this direction.

Many of the studies mentioned above have shown that the lung can metabolise and produce numerous vasoactive agents including endothelins. These recently discovered mediators may have an important role to play in pulmonary and other disease states, and their actions have been one of the major subjects examined in this thesis. A brief introduction on the history of endothelins, endothelin receptors / antagonists and their pathophysiological roles is given below.

1.7 Endothelins

1.7.1 Discovery of endothelins

Investigations have confirmed that vasoactive products produced from endothelial cells are potent modulators of vascular smooth muscle contractility. Initially two unstable vasodilators were described, prostacyclin (Moncada *et al.*, 1976) and endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980) later identified as nitric oxide (Palmer, *et al.*, 1987). Hickey *et al.*, (1985) reported the characterisation of a potent and stable peptidergic vasoconstrictor produced by cultured endothelial cells, originally termed endotensin or endothelium-derived constricting factor (EDCF). After confirmation of these findings EDCF was isolated, purified, sequenced, cloned and the molecule renamed as endothelin (ET) by Yanagisawa *et al.*, (1988).

1.7.2 ET isopeptides

Analysis of human genomic sequences revealed the existence of three distinct genes for ET; these were named as ET-1, ET-2, and ET-3 (Figure 5). All family members contain 21 amino acid residues and show structural similarities, including four cysteine residues (positions 1, 3, 11, and 15), as well as similar amino acids in position 8, 10, 20, 21. In the ETs, all four cysteine residues participate in disulfide bonding (Cys^{1}-Cys^{15}; Cys^{3}-Cys^{11}) (Inoue *et al.*, 1989). In addition, a fourth peptide, known as vasoactive intestinal contracting factor (VIC) or endothelin-ß was discovered by genomic cloning in mice (Saida *et al.*, 1989).

ETs are widely distributed in mammals, including humans and non-human primates. ETs are similar in structure to VIC and a peptide sarafotoxin (SX) found in the venom of a rare snake, *Atractaspis engaddensis* (Kloog and Sokolovsky, 1989). In fact several sarafotoxins, SX6-A, -B, -C, -D and -E all derived from the Israeli snake have been characterised (Bdolah *et al.*, 1989; Sokolovsky, 1992). The identification of these peptides and the characterisation of their binding sites has

generated intense and widespread study of the physiology and pathophysiology of ETs.

1.7.3 Structural similarities between ET and SXs

As shown in Figure 5 the sequence of the carboxy-terminal tail of ET/SX is conserved and the amino-terminal residue is always Cys, which in SX6A, SX6B, ET-1 and ET-2 is followed by Ser and in SX6C, SX6D and ET-3 by Thr. The most important differences between the various peptides of the ET/SX family are to be found within the sequence of the inner loop Cys^{3}-Cys^{11}. All of these peptides possess Glu^{10}, and except for SX6C all have Asp^{8}-Lys^{9} (Sokolovsky, 1992).

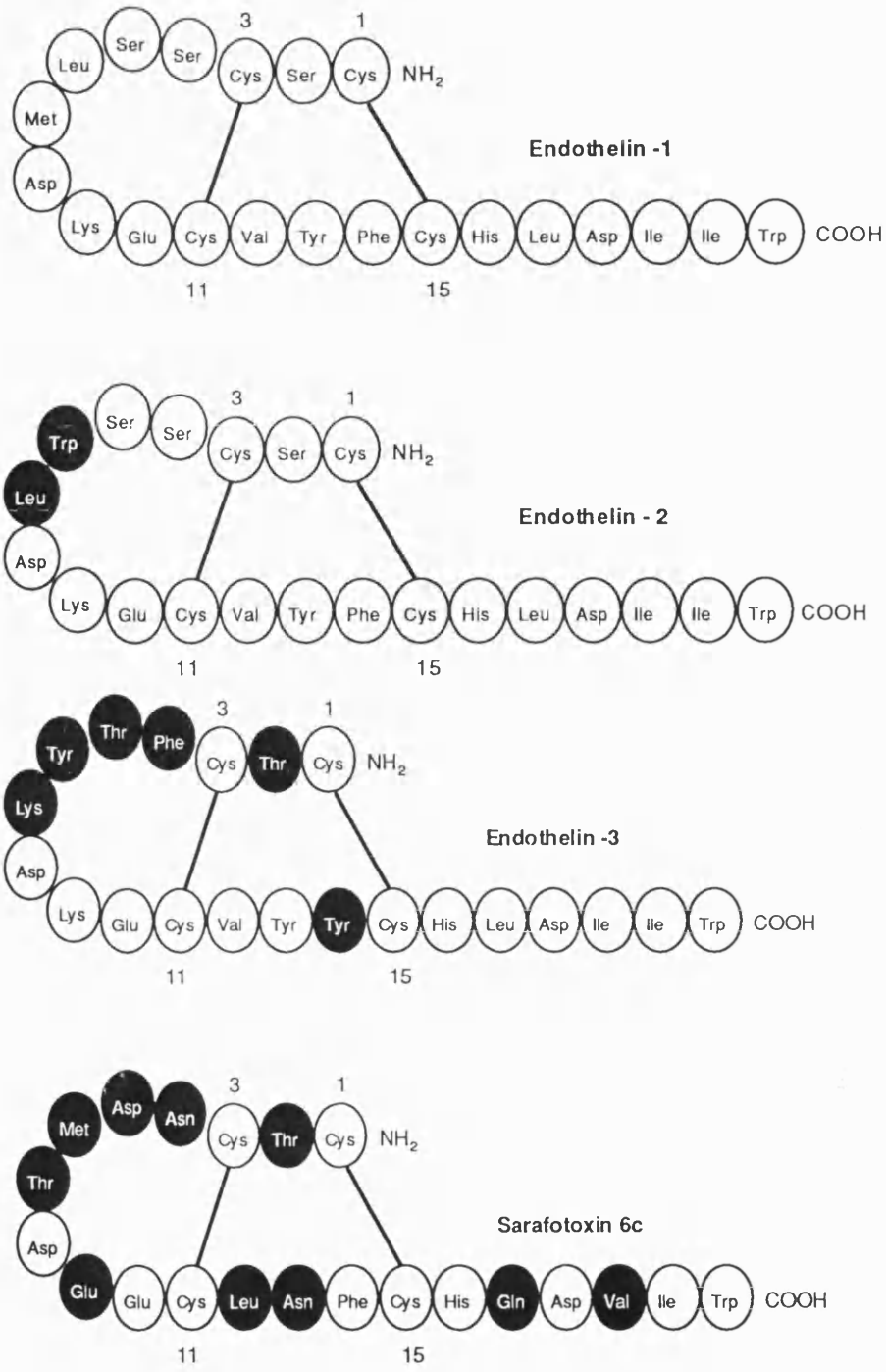


Figure 5 The primary sequences of endothelin (ET)-1, -2 and -3 showing the positions of the disulphide bonds and residues that differ from those of endothelin-1 (shaded). The snake venom sarafotoxin 6c is shown for comparison (Webb, 1991; Huggins *et al.,* 1993).

1.7.4 Endothelin biosynthesis

The biosynthesis of ETs occurs in a manner much like that of other peptide hormones and neurotransmitters. ET isopeptides arise through proteolytic processing of isopeptide-specific prohormones. Their precursors, preproendothelins-1, -2, -3 are polypeptides consisting of approximately 200 aminoacids that undergo proteolytic cleavage to form a 38 to 39 amino acid peptides designated as big endothelins (Inoue *et al.,* 1989; Rubanyi and Polokoff, 1994). The mature 21 amino acid peptides ET-1, ET-2 and ET-3 arise through the proteolytic processing of the "big" ETs by an endopeptidase called endothelin converting enzyme (ECE). The important intermediate steps which are involved in the biosynthesis of mature ETs are shown in Figure 6. The ECE is a membrane-bound neutral metalloprotease and has structural homology to neutral endopeptidase (Shimada *et al.*, 1994). More recent studies have demonstrated the cloning and functional expression of human ECE (Shimada *et al.,* 1995), its amino acid sequence has 91 % and 94 % homology to those of rat ECE (Shimada *et al.,* 1994) and bovine ECE (Ikura *et al.,* 1994), respectively. The conversion of big ET-1 to ET-1 by ECE in rat and rabbit lung is blocked by phosphoramidon, a metalloproteinase inhibitor (Hisaki *et al.,* 1994b). In rat lung there appear to be two isoforms of ECE both of which show preference for big ET-1, or big ET-2 over big ET-3. One is sensitive to phosphoramidon and exhibits optimal activity at pH range 5.3-8.1. Investigations have shown that the ECE activity found in endothelial cells which specifically cleaves big ET-1 was inhibited by phosphoramidon, whereas other neutral endopeptidase inhibitors such as thiorphan or the angiotensin converting enzyme inhibitor, captopril were inactive (Opgenorth *et al.,* 1992). In contrast the second ECE is only sensitive to pepstatin-A and exhibits ET-converting activity over a pH range of 5.5 - 9.7 (Chiou *et al.,* 1994). Recent investigations in guinea pig lung parenchymal strips have also shown the presence of two ECEs which selectively convert big ET-1 and big ET-2 but not big ET-3. A similar substrate-selective ECE showing preference for big ET-1: big

ET-2: big ET-3 in a ratio of 40: 2.5: 1 has also been reported in a human endothelial cell line (Ahn *et al.*, 1995).

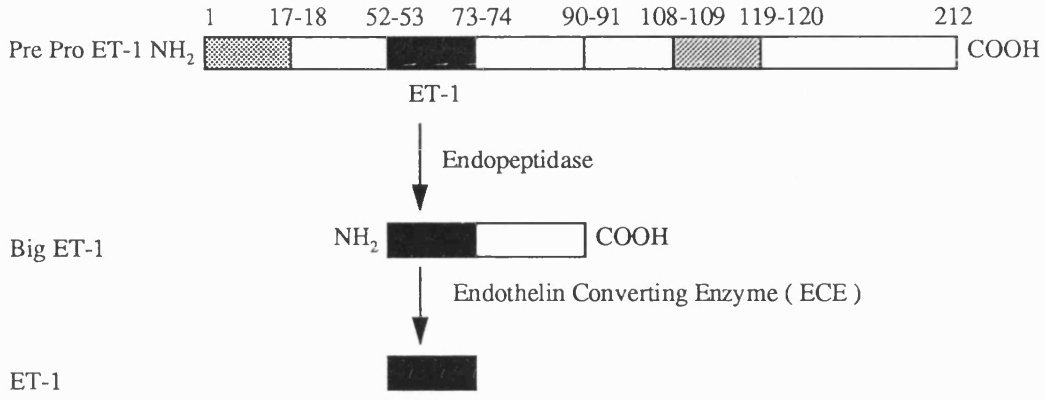


Figure 6 Schematic representation of biosynthesis of ET-1 (Yanagisawa *et al.*, 1988; Rubanyi and Polokoff, 1994).

The physiological importance of the conversion of big ET to ET was demonstrated by the observation that ET-1 was 140-fold more potent as a vasoconstrictor compared to the precursor peptide (Kimura *et al.*, 1989).The prepro- form of ET-1 is devoid of any vasoconstrictor action (Cade *et al.*, 1990).

1.7.5 Endothelin receptors

1.7.5.1 Molecular cloning and characterisation of receptors

The ET family exerts diverse biological effects which are thought to be mediated via the interaction with specific ET membrane receptors. Cloning of two endothelin receptors was reported at the end of 1990. One of these clones was isolated from a bovine cDNA library and, when expressed in *Xenopus oocytes*;was named as the ET_A receptor and has a very high selectivity for ET-1, ET-2 and SX6B over ET-3 and SX6C (ET-1 > ET-2 > SX6B >> ET-3 > SX6C).The binding affinity of ET-1 was 100 times greater than that of ET-3 (Arai *et al.*, 1990). The second clone, identified in a rat lung cDNA library was named as ET_B and had equal affinity for ET-1, ET-2 and ET-3 (Sakurai *et al.*, 1990). Human forms of both receptors were soon reported and the potency difference of ET-1 relative to ET-3 for the ET_A receptor was confirmed as approximately 1000-fold (Hosoda *et al.*, 1991). The lack of selectivity of the ETs for the human ET_B receptors has also been reported (Nakamuta *et al.*, 1991; Sakamoto *et al.*, 1991).

Despite the cloning of only two mammalian receptors, functional evidence suggests the presence of additional subtypes. The most widely proposed of these is the ET_C receptor (Karne *et al.*, 1993) which was cloned from *Xenopus laevis* in dermal melanophores. This is classified as being selective for ET-3 over ET-1 (ET-3 > ET-1). This receptor had approximately 50 % amino acid homology with ET_A and ET_B receptors (Karne *et al.*, 1993). In addition to the cloning of ET_A, ET_B and ET_C receptors a fourth endothelin receptor named as ET_{AX} was cloned recently in *Xenopus* heart and shares 74%, 60% and 51% of identities with human ET_A ET_B and *Xenopus* ET_C receptor respectively (Kumar *et al.*, 1994).

In rat lung membranes the existence of two ET receptors (44 kD and 32 kD molecular weight) which have different affinities for ETs was proposed. The 44kD receptor had a higher affinity for ET-1 or ET-2 compared with ET-3, whereas the 32 kD receptor exhibited selectivity for ET-3 (Masuda *et al.*, 1989). Cloning of

ET_A receptor subtypes from rat alveolar epithelial cells has recently been reported (Markewitz *et al.*, 1995).

ET_A receptors have often been found to mediate contractile responses in isolated smooth muscle preparations. However, recent studies have shown that a subtype of ET_A receptor is also present on endothelial cells (Nishimura *et al.*, 1995). In addition , the existence of ET_{A1} and ET_{A2} receptors is also proposed, where the former are BQ-123-sensitive and the latter BQ-123-insensitive (Sudjarwo *et al.*, 1994; Yoneyama *et al.*, 1995). No selective agonist at ET_A receptors has yet been reported. Thus, identification of this receptor subtype has to rely on the use of selective antagonism of responses to ET-1, either where it is significantly more potent than ET-3 or where selective ET_B receptor agonists such as SX6C, BQ-3020 or IRL 1620 are inactive (see Bax and Saxena, 1994).

ET_B receptors were originally considered as receptors mediating vasorelaxation. However, later it was shown that ET_B receptors were also involved in smooth muscle contraction i.e. rabbit saphenous vein (Moreland *et al.*, 1992). Thus ET_B receptors have been subdivided into ET_{B1} (located on the endothelium and mediating vasorelaxation) and ET_{B2} (located on the vascular smooth muscle and inducing contraction) (Sudjarwo *et al.*, 1993; Warner *et al.*, 1993a; Warner *et al.*, 1993b). Sokolovsky *et al.*, (1992) also reported that there appeared to be two subtypes of ET_B receptors i.e. the 'super high affinity', ET_{B1}- receptors and the ' high affinity', ET_{B2} receptors. It was suggested that the super high sites showing binding affinity in the picomolar range are related to the vasodilator property of endothelins whereas the high affinity sites with binding affinity in the nanomolar range participate in the vasoconstrictor actions.

1.7.5.2 Receptor antagonists

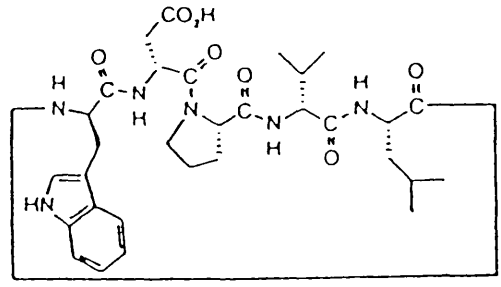
The best known of the ET_A receptor selective antagonists are BQ-123 and BQ-153. These were initially isolated from the fermentation broth of *Streptomyces misakiensis* (Ihara *et al.*, 1992). Most recently a sulphonamide derivative BMS-

182874, has been reported to be a potent orally active selective ET_A receptor antagonist (Webb *et al.*, 1995).

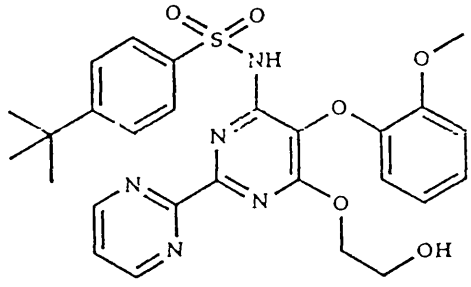
The non-selective agents PD-142893 and PD-145065 were developed by a rational approach starting with ET(16-21), which is known to interact with ET receptors (Doherty *et al.*, 1993). PD-145065 has 4.0 and 15 nM binding affinity (IC_{50} values) for the ET_A and (rabbit renal artery vascular smooth muscle cells) and ET_B receptors (rat cerebellum), respectively. The compound is also an antagonist of ET-1-and SX6C stimulated vasoconstrictor activity, with pA_2 values of 6.9 (rabbit femoral artery, ET_A assay) and 7.1 (rabbit pulmonary artery, ET_B assay), (Doherty *et al.*, 1993).

The orally active, mixed ET_A / ET_B endothelin receptor antagonist bosentan, (Ro 47-0203), shows 20-50 fold selectivity for ET_A receptors. The binding affinities for ET_{B1} and ET_{B2} receptors was 35- and 11- fold lower respectively, than for ET_A receptors in functional studies with pA_2 values of 7.28 , 6.72 and 5.94 respectively, against ET-1- induced contractions in de-endothelialized rat aorta (ET_A), SX6C-induced relaxation of rabbit mesenteric artery (ET_{B1}) and SX6C-induced contractions of rat trachea (ET_{B2}) (Clozel *et al.*, 1994).

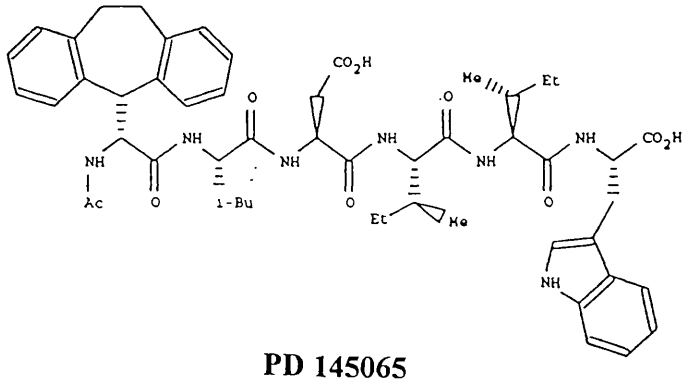
Selective antagonists at ET_B receptors which have been described include, IR1038 (Urade et al., 1992; Karaki, et al., 1993) RES-701-1 (Karaki, *et* al., 1994) and BQ 788 (Ishikawa *et al.*, 1994); for review see Huggins *et al.*, 1993; Warner, 1994. Figure 7 shows the structures of some endothelin receptors antagonists. The anatomical location of the putative endothelin receptor subtypes and their agonists / antagonists is shown in Figure 8.



BQ123



Bosentan (RO 47-0203)



PD 145065

Figure 7 The structures of some endothelin receptor antagonists.

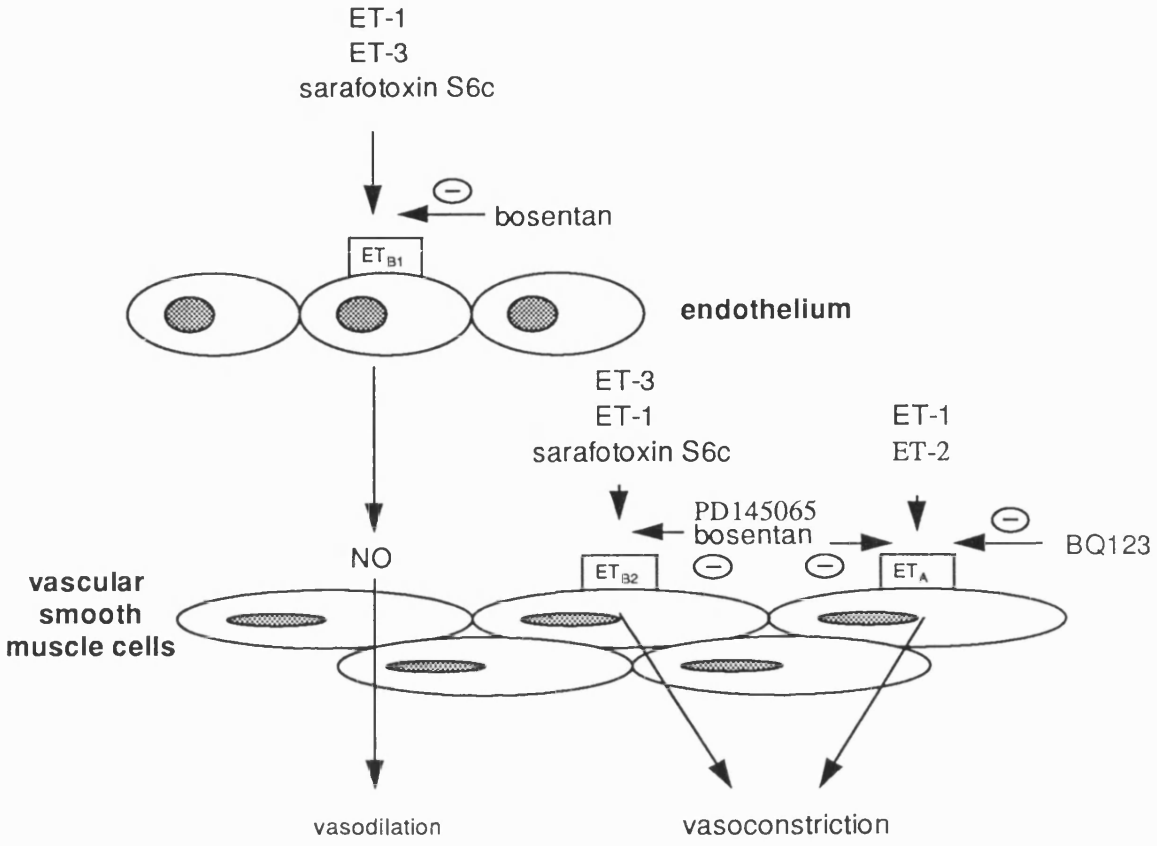


Figure 8 The vascular location of putative subclasses of endothelin receptor subtypes and their agonists / antagonists. Modified from Douglas *et al.*, (1994). NO is nitric oxide.

1.7.6 *Pharmacological actions*

ETs / SXs produce several actions in vascular and non-vascular smooth muscles (for review see Rubanyi and Polokoff, 1994). The contraction of isolated vascular smooth muscle in response to ET-1 generally begins at a threshold dose of about 100 pM (Highsmith *et al.*, 1992). The contractions in vascular smooth muscle are mediated via ET_A and ET_B receptors in animals and humans (Seo *et al.*, 1994; White *et al.*, 1994). ET-1-induced contractions in isolated human blood vessels were blocked with a mixed ET_A / ET_B receptor antagonist bosentan (Seo *et al.*, 1994). In conscious rats ET-1 produces a sustained increase in mean arterial blood pressure which is preceded by a transient depressor response (Bird *et al.*, 1993; Filep *et al.*, 1993; McMurdo *et al.*, 1993). ETs also produce relaxation of pre-contracted isolated blood vessels (Namiki *et al.*, 1992; Karaki *et al.*, 1993; Shetty *et al.*, 1993; Zellers *et al.*, 1994). Among the three ET isopeptides, ET-3 exerted the most potent initial depressor response, but the least vasoconstrictor response. Warner *et al.*, (1989) have reported that ET-3 is a vasodepressor rather than a vasoconstrictor in the isolated perfused rat mesentery acting via the release of EDRF. Recent investigations of the distribution of nitric oxide synthase and endothelin in coronary and pulmonary arteries of new-born rats have shown that in the endothelial cells, nitric oxide synthase and endothelin are co-localised and may be substantially involved in the vasomotor control of the cardiac and pulmonary circulations (Loesch and Burnstock, 1995). ET-1 is a potent constrictor of smooth muscle in isolated trachea and bronchus (see Rubanyi and Polokoff, 1994). ET-1 also stimulates the proliferation of the vascular smooth muscle cells and is a potent stimulus of DNA synthesis and proliferation of pulmonary vascular smooth muscle cells (see Barnes, 1994).

ETs also increase vascular permeability in many isolated vascular beds (Filep *et al.*, 1992; Kurose *et al.*, 1993). *In vivo* studies have also demonstrated the oedemagenic effects of ETs as ET-1 enhanced albumin extravasation in the stomach and duodenum of anaesthetised rats (Filep *et al.*, 1993; Filep *et al.*, 1994).

1.7.7 Physiological and pathophysiological role of ETs

Endothelins are a family of a novel regulatory peptides. Knockout of the ET-1 gene in mice cause severe craniofacial and thoracic blood vessel malformations (Kurihara, 1994) suggesting an important role for ET-1 in normal development. However, clinical and experimental evidence has shown that an increase in the production of endothelin may be a promoting factor in a number of disease states (see review Huggins *et al.*, 1993; Rubanyi and Polokoff, 1994). ETs may play an important role in the regulation of pulmonary vascular tone (Barnes and Liu, 1995) and are also implicated in pulmonary pathophysiology (Filep, 1993). There is an increase in the plasma concentration of ET-1 in patients with pulmonary hypertension (Stewart *et al.*, 1991) which could be a reflection of reduced uptake of ET-1 from the pulmonary vascular bed resulting from endothelial dysfunction (DeNucci *et al.*, 1988) or there may be increased production of ET-1 in the lungs of patients with primary pulmonary hypertension. Increased expression of ET-1 mRNA in an animal model of primary pulmonary hypertension (Stelzner *et al.*, 1992) and in patients with pulmonary hypertension (Giaid *et al.*, 1993) seems to support this hypothesis (Barnes, 1994; Rubanyi and Polokoff, 1994).

Immunoreactive ET-1 has been detected in normal lungs but only in 10 % of healthy volunteers. In contrast, ET-1 expression was evident in airway epithelium in more than 60 % of asthmatic patients (Springall *et al.*, 1991; Oparil *et al.*, 1995). ET-1 circulates in normal animals and humans (Ando *et al.*, 1989), and its levels have been reported to be increased in several experimental situations i.e. acute pulmonary alveolar hypoxia increases lung ET-1 levels in conscious rats (Shirakami *et al.*, 1991), and in several clinical conditions i.e. human patients with status asthmaticus (Nomura *et al.*, 1989), adult respiratory distress syndrome (Druml *et al.*, 1993).

Under physiological conditions the lungs are by far the most important sites of endothelin disposal. Recent evidence has shown that in isolated perfused rat lungs

about 80 % of a bolus injection of $^{[125]}$ET-1 was retained by the lungs during single passage (Westcott *et al.*, 1990; Fukuroda *et al.*, 1994). In lung disease such uptake of ET-1 may be impaired whereas if lung ET release remained constant or increased, the pulmonary circulation would be exposed to elevated ET concentrations which would adversely affect the microcirculation.

1.8 Objectives:

I. To develop a rat isolated perfused lung model which would allow simultaneous monitoring of pulmonary perfusion pressure, lung weight and pulmonary inflation pressure.

II. To validate the model by using selective agonists for the measured parameters.

III. To determine whether increases in lung weight are associated with increased filtration and / or permeability changes.

IV. To characterise and localise the actions of ETs and SX6C in the perfused lungs.

V. To determine the ET receptor subtypes involved by the use of ET receptor antagonists.

SECTION 2

Materials and Methods

2.1 Animals

Male Wistar rats (300-350 g) were supplied by the University of Bath Animal House.

2.2 Drugs and Chemicals

Drugs and Chemicals were obtained from the following sources:

British Drug Houses (BDH), Poole:

Atropine sulphate

Evans blue dye

Novabiochem (UK) Ltd, Nottingham:

[D-Arg^{0},Hyp^{3},Thi^{5,8},D-Phe^{7}]-bradykinin

Endothelin-1

Endothelin-2

Endothelin-3

Peninsula Laboratories, Merseyside.

Sarafotoxin 6c

Sigma Chemical Company, Poole, Dorset:

Bovine serum albumin

Bradykinin acetate

Carbamylcholine chloride (carbachol)

Formamide

Heparin sulphate

Indomethacin

L-phenylephrine hydrochloride

Prazosin hydrochloride

Phosphoramidon

9,11-Dideoxy-11$_\alpha$, 9$_\alpha$-Epoxy-Methanoprostaglandin F$_{2\alpha}$ (U46619)

Sodium nitroprusside

Nitro-L-Arginine

Nitro-D-arginine

Papaverine hydrochloride

Rhone Merieux Ltd, Harlow, Essex

Pentobarbitone hydrochloride (Sagatal)

General laboratory grade chemicals were purchased from British Drug Houses (BDH), Poole and Sigma Chemical Company, Poole.

The following were gifts:

BQ123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) was supplied by Dr. K. Clark, Glaxo Group Research (Ware, Herts)

Bosentan (Ro 47-0203; 4-tert-Butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide sodium salt) was provided by Hoffmann-La Roche Ltd (Basel, Switzerland).

PD145065; Ac-(D-2-(10, 11 -dihydro- 5H - dibenzo [a, d] cyclohepten-5-yl) Gly-LLeu-LAsp-L1le-L1le-LTrp. Na (1 : 2) was a kind gift from Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan (USA).

2.2.1 Physiological salt solutions

Krebs` solution was prepared with the following composition (mM): potassium chloride (4.7); potassium dihydrogen phosphate (1.2); calcium chloride (1.25); magnesium sulphate (1.2); sodium chloride (118); sodium bicarbonate (25); glucose (11.1).

2.2.2 ***Heparin solution:*** Heparin was dissolved in normal saline and stored in ampoules (1000 i.u. ml^{-1}) at 4 °C.

2.2.3 Indomethacin solution: prepared just before use by dissolving in an equimolar amount of sodium carbonate in normal saline.

2.2.4 U46619 solution: U46619 was dissolved in 95 % ethanol, dilutions were made in 0.9 % normal saline (10^{-3} M) this stock was stored at -80 °C.

Stock solutions of all drugs were prepared in normal saline and aliquots were stored at -20 °C.

2.3 *Isolated ventilated perfused lung preparation*

Male Wistar rats were anaesthetized with an intraperitoneal injection of Sagatal (60 mg kg^{-1} body weight). Heparin (500 i.u.) was injected via the tail vein to prevent intravascular clotting and 5 min later the chest wall was opened by a midline sternotomy. The thymus and fatty tissue were carefully excised and a suture was placed around the pulmonary artery and secured with a loose knot. At this time the perfusion circuit was checked to ensure that it was filled with perfusate and that no air bubbles remained in the tubing. A small cut was made in the right ventricular wall and the pulmonary artery cannulated with a stainless steel cannula (external diameter = 1.5mm, internal diameter = 1mm) via the right ventricle. The cannula was secured by tightening the suture. The left atrium was cut and the major part of the ventricles removed to allow free efflux of the perfusate. The lungs were flushed with Krebs' solution via the pulmonary artery cannula.

Following pulmonary artery cannulation, a medial incision was made in the ventral surface of the neck and a section of the trachea exposed by blunt dissection. The trachea was partially transected, and a metallic cannula inserted and secured with a cotton suture. Thereafter, the trachea and the lungs were isolated from the chest cavity and immediately transferred into a warming jacket maintained at 37°C. Lungs were perfused via the pulmonary artery at constant flow of 5ml min^{-1} with Krebs' solution (gassed with 95% O_2 & 5% CO_2) using a roller pump. Pulmonary artery perfusion pressure (PPP) was recorded via a side arm of the inflow perfusion tubing connected to a pressure transducer. The tracheal cannula was attached to a ventilator and the lungs were ventilated with room air, stroke volume (1 ml) using 28 strokes min^{-1} (no positive end expiratory pressure). A side arm was attached to a pressure transducer for recording the pulmonary inflation pressure (PIP). Lungs were attached to an isometric transducer by a wire from the tracheal cannula for recording changes in lung weight (LW). The experimental set up is shown in Figure 9. All parameters were recorded on a multichannel recorder (Grass model 7D polygraph). Lungs were allowed to stabilize for 30 min before drug administration. Drugs were injected as bolus doses (10-100 µl) via the pulmonary artery.

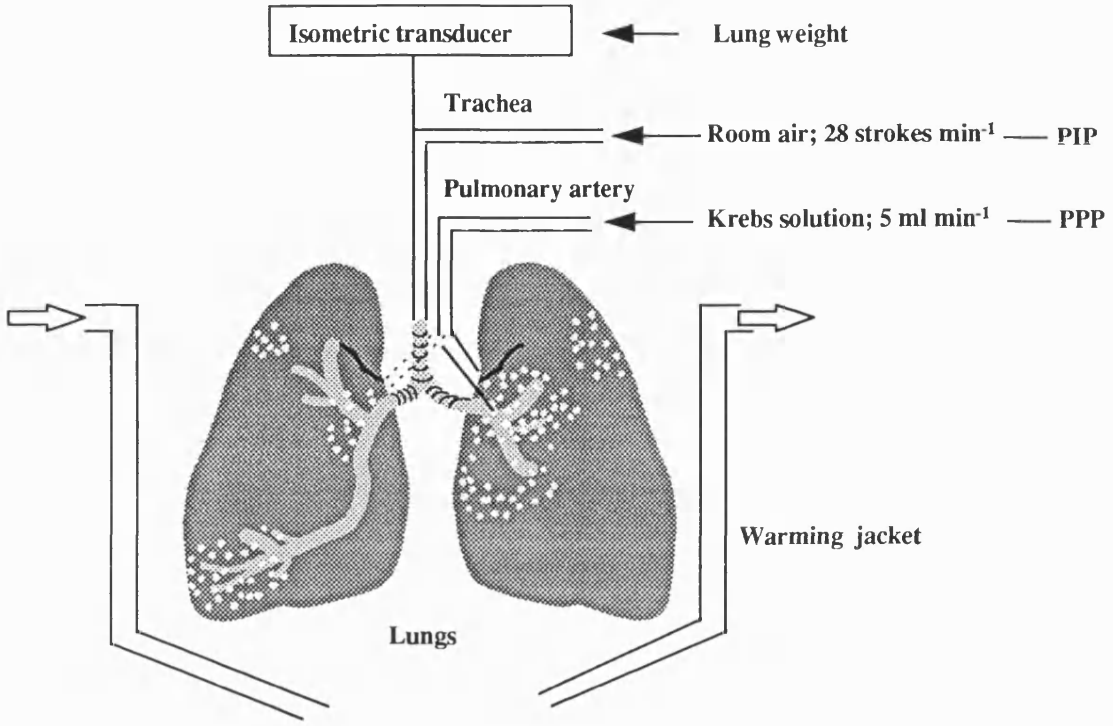


Figure 9 Diagram of the rat isolated perfused lung. PPP- pulmonary perfusion pressure, PIP- pulmonary inflation pressure.

2.3.1 Alteration of venous outflow pressure (VOP)

In a different series of experiments, a plastic cannula (external diameter = 2mm, internal diameter = 1.5mm) was also introduced into the left atrium via the left ventricle. This cannula was attached to a constant-head reservoir which was connected to a pressure transducer and VOP was monitored along with PPP, lung weight and PIP (Figure 10). By raising the level of the reservoir VOP could be manipulated over the range 5-25 mmHg. These increases in pressure were maintained for 1 min before returning outflow pressure to 0 mmHg.

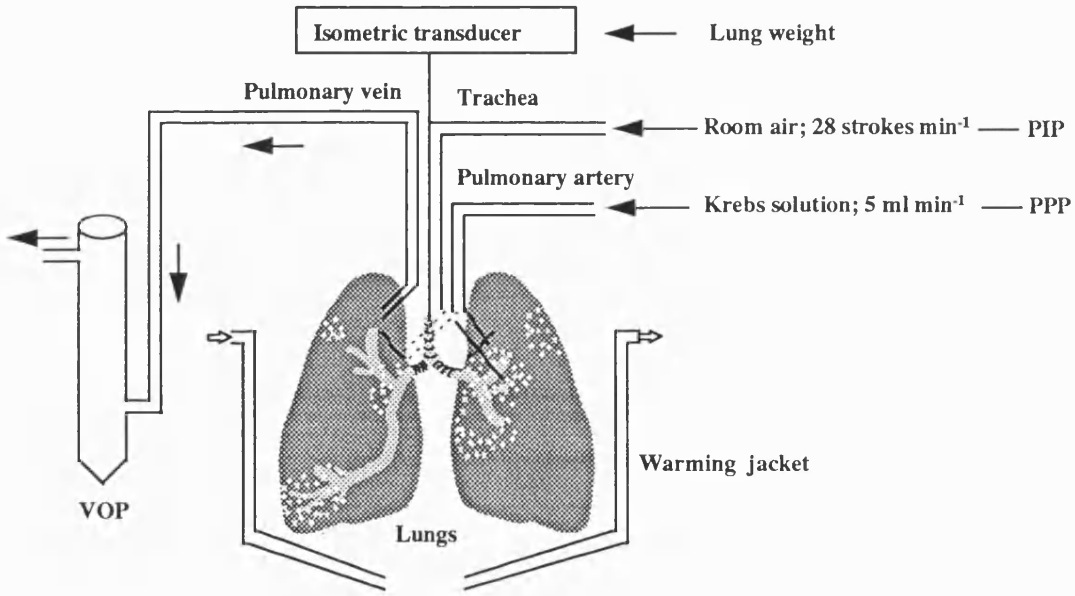


Figure 10 Manipulation of venous outflow pressure (VOP) in the rat isolated perfused lung. PPP- pulmonary perfusion pressure, PIP- pulmonary inflation pressure.

2.3.2 Retrograde perfusion

In another set of experiments a cannula was introduced into the left atrium via the left ventricle and lungs were perfused via the pulmonary vein and pulmonary venous pressure monitored together with LW and PIP. Drugs were injected as bolus doses (10-100 µl) via the pulmonary venous cannula.

2.4 Bioassay methods

For bioassay, the effluent from the lungs was superfused over the assay tissue in the cascade below the lung chamber. The assay tissues were (a) rat fundic stomach strips, (b) rat ascending colon (Vane, 1969). The bioassay tissue preparations were allowed to equilibrate for 30-45 min before the lungs were included in the circuit. The amount of substances activated or inactivated by the lungs was determined by comparing the contractions of assay tissues to bolus injections of agents made directly into the fluid superfusing the assay tissues with responses to injections of agents made into the pulmonary arterial cannula. When used, inhibitors were added into the perfusate 30 min before giving the agonist injections.

2.5 Determination of albumin-bound dye extravasation

Evans blue dye was added to a 10 % (w v^{-1}) solution of bovine serum albumin (BSA) in saline to give a final concentration of 1 mg ml^{-1}. The mixture was dialysed overnight in Visking tubing (22/32) against an excess of distilled water. Aliquots of the solution were infused into the pulmonary artery (0.1 ml min^{-1}) for a 5 min period. Drugs were injected one min after the start of dye infusion. In separate experiments VOP was raised to 20 mmHg for 1 min during dye infusion. Lungs were perfused for a further 3 min to remove unbound dye from the vasculature. Lungs were removed and after removal of cardiac tissue and trachea the lungs were oven dried (50-60 °C) overnight and dry weight noted. Lungs were then digested with formamide (2-4 ml / 0.3 g dry tissue weight) at 40 °C in a water bath for 20 h and the contents were centrifuged (Eppendorf centrifuge 5414) at 9880g for 30 min. The absorbance of standard concentrations of Evans blue (0.25- 20

μg ml^{-1}) or lung extracts were read at 622 nm in a spectrophotometer (Pye Unicam) The concentration of dye in lung extracts was calculated by reference to a standard curve range of Evans blue dye. Using this value fluid accumulation in the lung could be calculated by reference to the concentration of dye in the fluid perfusing the lung.

In a different protocol albumin-bound Evans blue dye solution was infused into the pulmonary artery (0.1 ml min-1) for 5 min, 1 min after agonist injection. Lungs were perfused for a further 10 min to remove unbound dye from the vasculature and retained dye was extracted as explained above.

2.6 Effects of inhibitors and receptor antagonists

2.6.1 Inhibitors

In a series of experiments effects of the inhibitors of: cyclooxygenase (indomethacin 10μM), angiotensin converting enzyme (captopril 10 μM), neutral endopeptidase (phosphoramidon1 μM), nitric oxide synthase (nitro-L-arginine 100 μM)were investigated. Each inhibitor was studied in individual experiments and was added into the perfusate 30 min before giving the agonist injections and was present for the remainder of the experiment.

In some experiments to differentiate the increases in PPP from changes in lung weight, the vasodilator papaverine (20 μM) was added 30 min before giving the bolus injections of agonists.

2.6.2 Receptor antagonists

Antagonists were perfused 30 min before giving the bolus injections of the appropriate agonists in to the pulmonary artery. Dose-response curves for each agonist were completed in the presence of the antagonist.

2.7 Elevation of basal PPP to investigate the pulmonary vasodilator actions of agonists

In a series of experiments basal PPP was elevated using the thromboxane mimetic U46619. This was infused with an infusion pump (Harvard Apparatus 22) into the pulmonary artery. Infusion of U46619 was started after 20 min of an initial stabilisation period and a further 5-10 min were given to stablise the perfusion pressure after which bolus injections of agonists were given into the pulmonary artery.

2.8 Statistical analyses

Increases in PPP, PIP and lung weight induced by bolus injections of phenylephrine, carbachol, bradykinin, endothelins and sarafotoxin 6c are expressed relative to the basal values prior to the addition of each dose. Pulmonary vasodilator responses to ETs, sarafotoxin 6c and sodium nitroprusside are expressed as % decreases with respect to the raised PPP induced by U46619 infusion. When lung weight measurement was needed for comparative purposes, weight gain was calculated 7 min after giving the maximum dose of agonist. Data were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Dunnett's (Dunnett, 1964) or Student's (unpaired) t-tests were used to test the level of significance as appropriate. Probability values of $P < 0.05$ were considered significant.

SECTION 3

Results

3.1 Effects of perfusion time on lung weight

To determine the effects of perfusion time on lung integrity, an initial series of experiments monitored lung weight after differing periods of perfusion at 5 ml min^{-1} (Figure 11). Initial lung weight (2.8 ± 0.2 g ; mean ± SEM, n =5) had increased by 0.22 ± 0.11g after 2h (NS); 0.7 ± 0.2g after 2.5h of perfusion ; by 1.15 ± 0.28g after 3h and 2.9 ± 0.98g after 4h perfusion. These later changes were significant when compared with initial lung weight ($P< 0.05$, ANOVA). When perfusion rate was increased to 10 ml min^{-1} in 2 experiments lung weight was increased markedly after one hour which was too short to carry out the experiments. Therefore in all subsequent experiments lungs were perfused at 5 ml min^{-1} and a maximum perfusion time of 2h was employed.

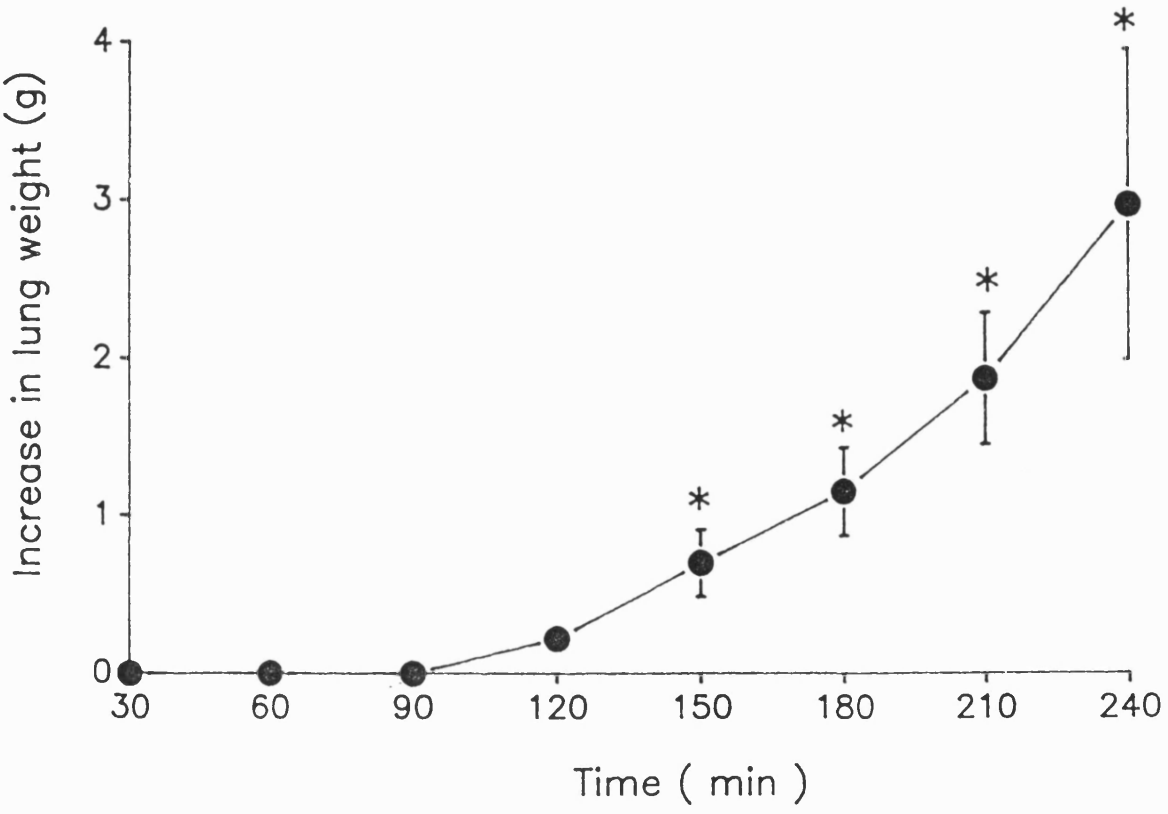


Figure 11 Effect of perfusion time on lung weight. Each point represents mean ± SEM, n= 5. *p< 0.05; 1 way ANOVA.

3.2 Differential pulmonary vascular and bronchial actions of vasoactive substances

The effects of three agonists, phenylephrine (PHE), carbachol (CCh) and bradykinin (BK) on three lung parameters (PPP, lung weight & PIP) are shown in Figure12. As shown PHE (3.12-50 nmol) dose-dependently increased PPP. However PHE was without effect on lung weight or PIP. CCh (1.5-48 nmol) selectively increased PIP without affecting PPP or lung weight. BK (6.25-200 nmol) dose-dependently increased PPP, PIP and lung weight. Low doses of BK (12.5 - 50 nmol) produced reversible increases in lung weight and PIP, but higher doses of BK (100 - 200 nmol) produced increases in lung weight and PIP which were only partially reversible.

Accumulated results for the actions of all three substances on the three monitored parameters are shown in Figure 13. From Figure13a, it can be seen that PHE was the most potent vasoconstrictor agent having an ED_{50} of 4.0 ± 0.7 nmol (n =5). BK had a lower potency (ED_{50} 22 ± 3.0 nmol, n =8) while CCh did not increase PPP in any experiment (n =7).

Figure 13b illustrates that CCh was the most potent bronchoconstrictor with an ED_{50} of 4.25 ± 0.4 nmol (n =7). BK was less potent (ED_{50} of 35.6 ± 3.6 nmol, n =8) while PHE was devoid of bronchoconstrictor activity.

Figure 13c illustrates that BK was the only substance which affected lung weight. Increases in weight were linear over the dose range studied. Such a curve disguises the fact that BK caused a reversible increase in lung weight at low doses (12.5-50 nmol) as illustrated in Figure 12c. However at higher doses (100-200 nmol) the gain in lung weight was sustained.

Figure 12 The differential actions in the perfused lung of (a) phenylephrine, (b) carbachol and (c) BK on lung weight (LW), pulmonary perfusion pressure (PPP) and pulmonary inflation pressure (PIP). Each trace represents one of 5-8 such experiments. Note: different scale for LW parameter in BK.

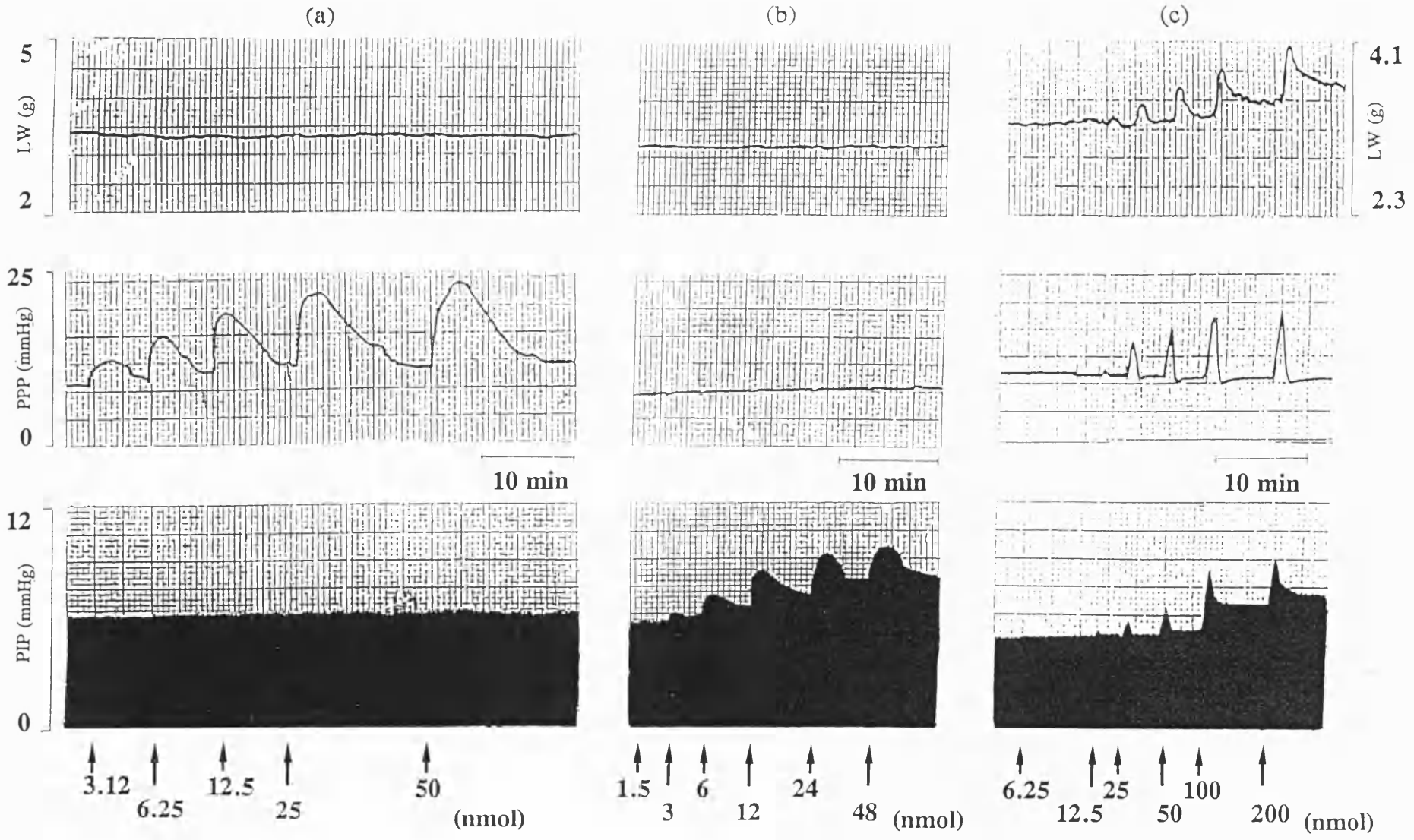
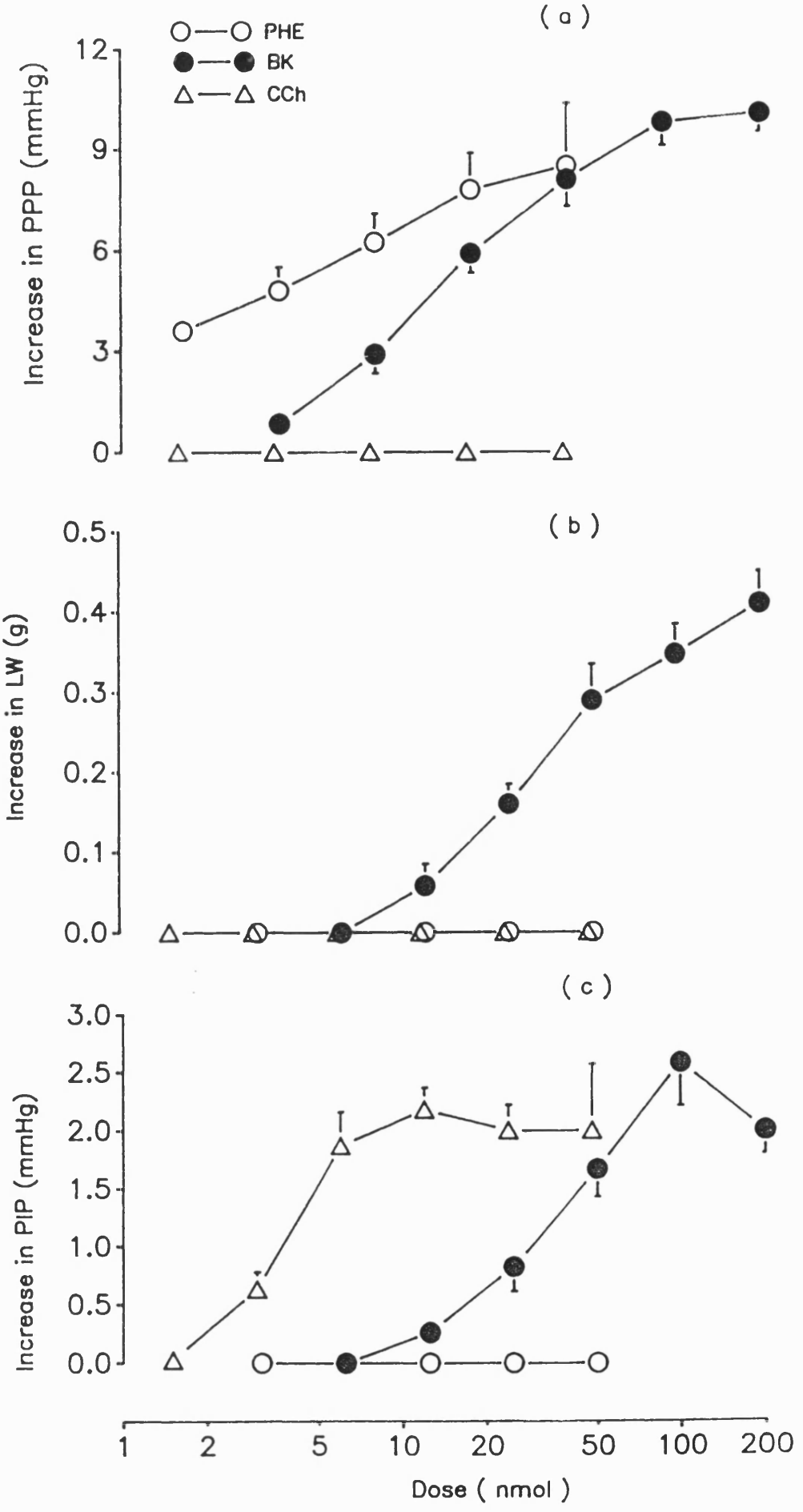

(a)
(b)
(c)
5
LW (g)
2
4.1
LW (g)
2.3
25
PPP (mmHg)
0
10 min
10 min
10 min
12
PIP (mmHg)
0
3.12
6.25
12.5
25
50
(nmol)
1.5
3
6
12
24
48
(nmol)
6.25
12.5
25
50
100
200
(nmol)

Figure 13 Actions on (a) pulmonary arterial perfusion pressure (PPP), (b) pulmonary inflation pressure (PIP) and (c) lung weight (LW) of phenylephrine (PHE) (○), carbachol (CCh) (Δ) and bradykinin (BK) (●) Each point represents mean ± SEM, n= 5-8 experiments.


(a)
PHE
BK
CCh
Increase in PPP (mmHg)
12
9
6
3
0
(b)
Increase in LW (g)
0.5
0.4
0.3
0.2
0.1
0.0
(c)
Increase in PIP (mmHg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
5
10
20
50
100
200
Dose (nmol)

3.3 *Effects of inhibitors and receptor antagonists*

3.3.1 Indomethacin

Effects of the cyclooxygenase enzyme inhibitor indomethacin were examined on the pulmonary vascular and bronchial actions of PHE, CCh and BK. Inclusion of indomethacin (10 μM) in the perfusate did not influence any of the basal parameters. There was a small but significant ($p < 0.05$) increase in the maximal PPP response (from 10.1 ± 0.56 mmHg, n =8 to 12.9 ± 0.9 mmHg, n =6) following 200 nmol BK but not to PHE. Furthermore ED_{50} values for BK (ED_{50} 31.2 ± 2.0 nmol, n =6) and PHE (ED_{50} 4.5 ± 0.9 nmol, n =4) were not significantly different to controls. Indomethacin had no effect on the bronchoconstrictor action of BK (ED_{50} 47.1 ± 4.6 nmol, n =6) or carbachol (ED_{50} 3.0 ± 0.13 nmol, n =4) compared to control values quoted above.

3.3.2 Captopril

Effects of the angiotensin converting enzyme inhibitor captopril on BK- mediated PPP, lung weight and PIP increases were investigated.

Perfusion of captopril (10 μM), a concentration which completely attenuated the pulmonary vascular and bronchial responses to angiotensin-I (62.5-1000 pmol), significantly potentiated the effects of BK on PPP (control ED_{50} value reduced from 22 ± 3 nmol, n = 8 to 0.34 ± 0.04 nmol, n =5 $p< 0.001$), lung weight and PIP (control ED_{50} value reduced from 35.6 ± 3.6 nmol, n =8 to 0.8 ± 0.2 nmol, $p< 0.001$, n =5).

In addition the superfusion bioassay method was used to study the role of angiotensin converting enzyme in the pulmonary circulation to inactivate BK. Experiments have shown

that in the absence of angiotensin converting enzyme inhibitor captopril, angiotensin-I when given into the pulmonary arterial cannula produced large contractions of the rat colon, whereas captopril (10 μM) completely attenuated angiotensin-I induced contractions of rat colon. Furthermore BK given direct over the assay tissue rat stomach fundal strip, induced large contractions. However in the absence of captopril, when BK was given via the pulmonary arterial cannula its responses were reduced, whereas in presence of captopril (10 μM) BK induced responses of the stomach strip were markedly potentiated (data not shown).

3.3.4 Receptor antagonists

Effects of receptor antagonists on pulmonary vascular and bronchial actions of agonists were studied. In four experiments perfusion of the selective kinin B_2 receptor antagonist [D-Arg^0,Hyp^3,$Thi^{5,8}$, D-Phe^7]-bradykinin (500 nM) markedly attenuated the PPP, PIP and lung weight responses to BK (6.25-200 nmol). However it had no effect on the vasoconstrictor responses to PHE or bronchoconstrictor responses to CCh (data not shown).

In a different set of experiments the vasoconstrictor effects of PHE (3.12-50 nmol, n=4) were selectively antagonised in the presence of prazosin (1μM) and the bronchoconstrictor effects of carbachol (1.5-48 nmol, n=4) were also completely blocked with atropine (100 nM) without effecting BK responses (data not shown).

3.3.5 Papaverine

Effects of the vasodilator papaverine on BK-mediated responses were studied to see if lung weight changes produced by BK are secondary to increases in vascular resistance. Papaverine (20 μM) had no effect on the basal PPP, lung weight and PIP (n =3). However perfusion of papaverine markedly attenuated BK- mediated(6.25-200 nmol) increases in

PPP, lung weight and PIP. Accumulated results from 3 different experiments have shown that the maximum increase in PPP (10 ± 0.6 mmHg, n =8) in response to 200 nmol of BK in control lungs was significantly attenuated to 5.3 ± 1.5 mmHg, n =3; $p< 0.01$) in the presence of papaverine. In control lungs 200 nmol of BK produced 0.41 ± 0.04 g increase in lung weight which was only partially reversible. However, in the presence of papaverine (20 μM) the increase in lung weight produced with 200 nmol of BK was reversible and markedly reduced to 0.12 ± 0.06 g, n =3 ($p<0.01$). BK- mediated maximum PIP increase in the absence of papaverine was 2.6 ± 0.4 mmHg, n=8. However in the presence of papaverine maximum PIP was reduced to only 1 ± 0.2 mmHg, n=3 ($p< 0.001$).

3.4 Effects of raised VOP

To investigate if increases in VOP could account for lung weight changes associated with BK, VOP was increased in increments of 5 mmHg. As illustrated in Figure 14 such increases produced corresponding increases in PPP. Increases in VOP from 5-15 mmHg also produced reversible increases in lung weight and PIP. However rises from 20-25 mmHg in VOP produced gains in lung weight and PIP which were only partially reversible. Furthermore when VOP was increased to 25 mmHg the increase in lung weight was accompanied by the appearance of fluid in the tracheal cannula.

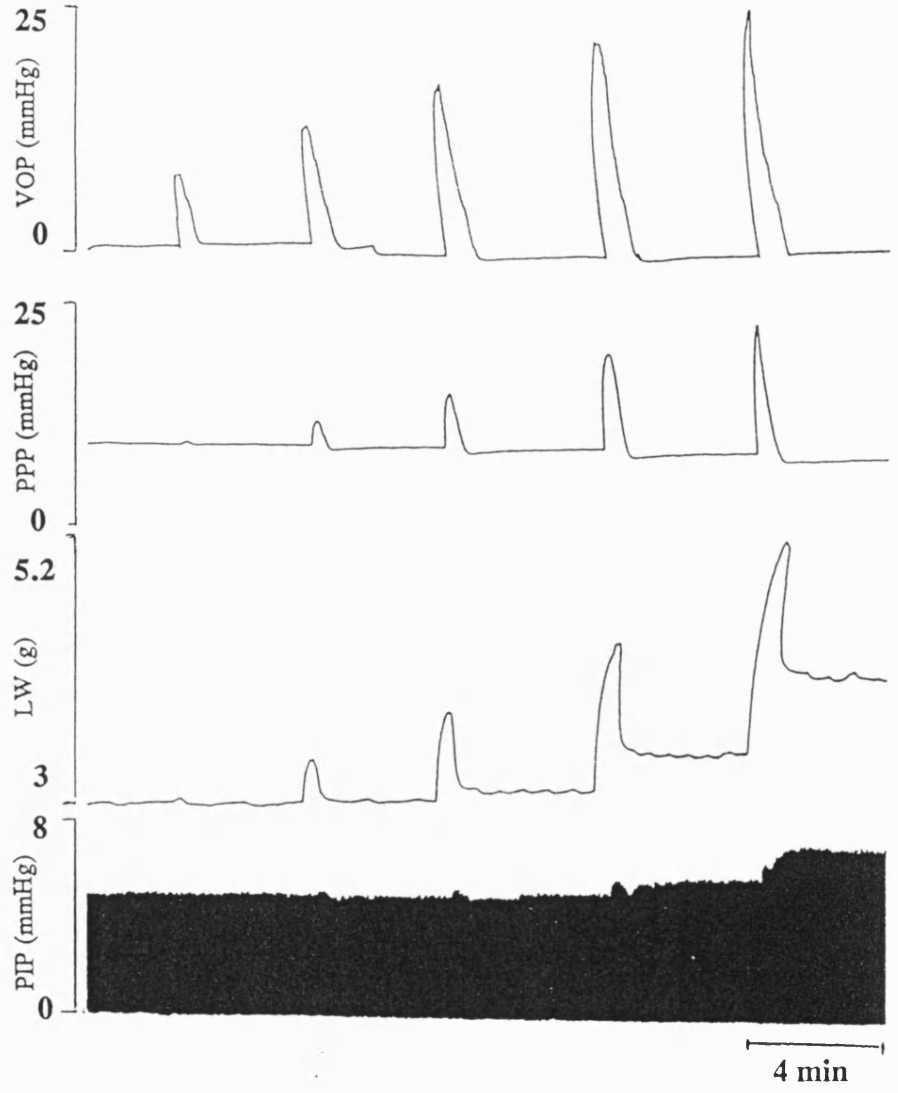


Figure 14 The effects of increasing venous outflow pressure (VOP) for 1 min periods on pulmonary perfusion pressure (PPP), lung weight (LW) and pulmonary inflation pressure (PIP). This figure represents one of 6 similar experiments.

3.5 Determination of albumin-bound dye extravasation

To investigate whether both reversible and irreversible changes in lung weight induced by BK or VOP were due to changes in vascular permeability, experiments utilizing Evans blue dye linked to BSA were carried out. The results are shown in Figure 15. Control lungs perfused with dye did not retain any detectable activity. Similarly lungs which received a low dose of BK (25 nmol) did not retain dye despite registering a small reversible increase in lung weight (Figure 12c). At a higher dose of 200 nmol (which caused a large sustained increase in lung weight) marked retention of dye by the lung was noted (23.3 ± 0.4 ng mg^{-1} of dry tissue, n =6). Back-calculation of fluid retention from retained dye indicated that 200 nmol of BK caused an accumulation of 0.32 ± 0.05 ml (n =6) of fluid. Assuming a specific gravity of 1 for this fluid a comparison of this calculated weight gain with weight gain recorded experimentally (0.38 ± 0.05g, n =6) illustrates that the values are in good agreement.

In a different set of experiments a rise in VOP of 20 mmHg increased PPP by 11.1 ± 0.9 mmHg (n = 6), which was associated with an irreversible lung weight increase of 0.61 ± 0.21 g, n =3). There was a large concomitant accumulation of albumin-bound dye (30.5 ± 8.4 ng mg^{-1} of dry tissue, n=3, Figure 15). In comparison 200 nmol of BK caused a similar increase in PPP of 10 ± 0.6 mmHg (n=6) which was associated with an irreversible weight increase of 0.38 ± 0.05 g.

Furthermore using retrograde perfusion 200 nmol of BK produced an increase in PPP (9.75 ± 0.5 mmHg, n=4) similar to that produced by BK when using orthograde perfusion (10 ± 0.6 mmHg, n=6). However with retrograde perfusion 200 nmol of BK produced significantly less increase in lung weight (0.15 ± 0.02 g, $p< 0.05$) and dye retention (12 ± 4.5 ng mg^{-1} $p< 0.05$, n=4) compared to responses of BK (200 nmol) when using orthograde perfusion (Figure 15).

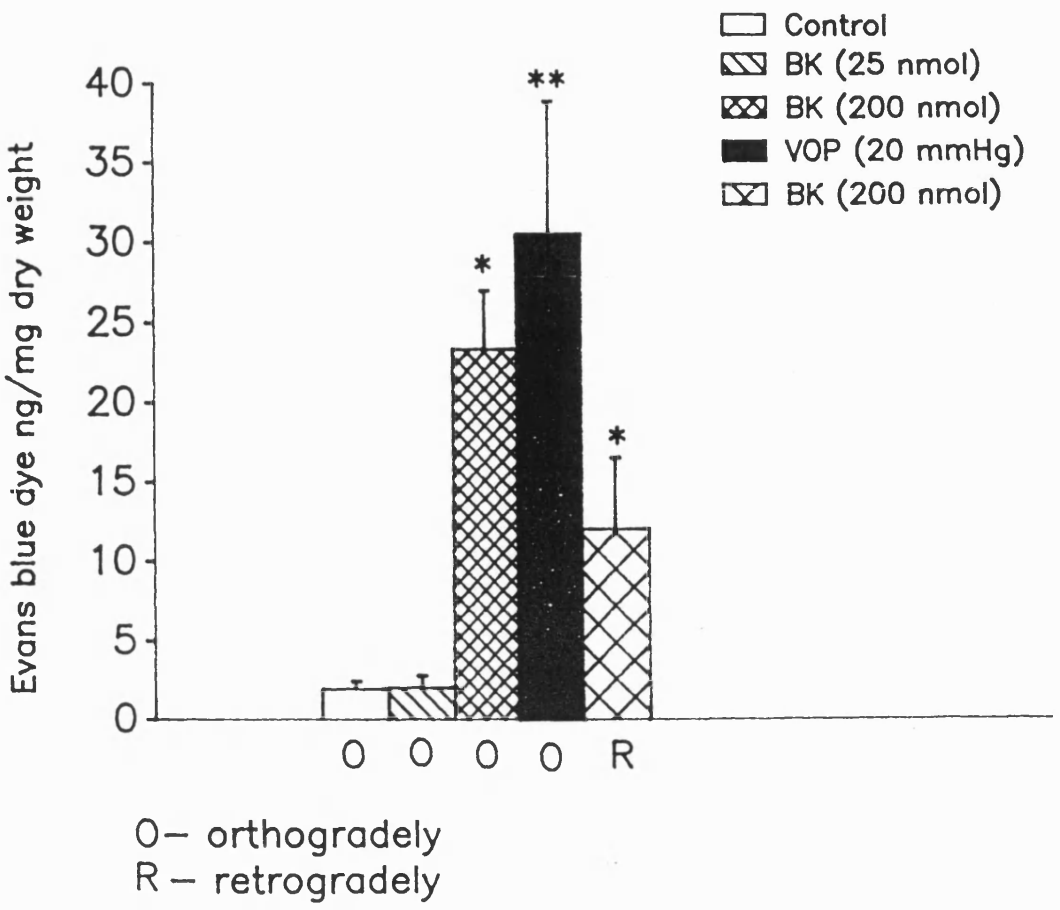


Figure 15 Effects of bradykinin (BK) on vascular permeability in the perfused lung. BK 25 nmol (hatched column) did not cause dye retention when compared to control lungs (open column). BK 200 nmol (cross hatched column) orthogradely and manipulation of (VOP) (solid column) both produced significant extravasation and dye retention (*$p<0.05$; **$p<0.01$ (1 way ANOVA). In retrogradely perfused lungs BK (200 nmol) produced significantly less dye retention compared with orthogradely perfused lungs ($p< 0.05$; Students t-test). Results are mean ± SEM, n= 4-6 experiments.

3.6 Vascular and bronchial actions of endothelins (ETs) and Sarafotoxin 6c (SX6C)

3.6.1 Effects on pulmonary perfusion pressure (PPP)

The effects of endothelin-1 (ET-1), endothelin-3 (ET-3) and sarafotoxin 6c (SX6C) on PPP in 3 different experiments are illustrated in Figure 16. As shown over the dose range used (50-800 pmol) all three agonists dose-dependently increased PPP (basal PPP was 6.7 ± 0.5 mmHg, n =27). The increases in PPP produced by ET-1 (50 - 200 pmol) were not completely reversible at low doses, whereas higher doses (400-800 pmol) produced reversible increases in PPP. In contrast ET-3 and SX6C produced reversible increases in PPP at all doses. The accumulated results for ETs (ET-1, ET-2 & ET-3) and SX6C on PPP are shown in Figure 17. From Figure 17a, it can be seen that over the dose range used the order of potency of the ETs was ET-1 > ET-2 > ET-3. SX6C was of similar potency to ET-1 with ED_{50} values of 165 ± 19 pmol, n = 6 and 130 ± 17 pmol respectively (n = 10).

3.6.2 Effects on lung weight

Figure 16 shows that ET-1 produced a very steep and irreversible increases in lung weight (basal lung weight 2.8 ± 0.06 g, n =27) which was associated with the appearance of fluid in the tracheal cannula. ET-3 produced an increase in lung weight at higher doses (800 pmol) when compared to ET-1. Interestingly SX6C (50-800 pmol) produced very little increase in lung weight. Figure 17b shows the accumulated results for the effects of ETs and SX6C on lung weight. The order of potency of the ETs was ET-1 > ET-2 > ET-3. In contrast, SX6C which was equipotent with ET-1 on PPP caused no significant change in lung weight over the dose range used.

3.6.3 Effects on pulmonary inflation pressure (PIP)

As shown in Figure 16 ETs (ET-1 and ET-3) were equipotent on PIP (basal PIP 5.0 ± 0.01 mmHg, n=27) but SX6C was the most potent agent. Figure 17c illustrates SX6C was the most potent bronchoconstrictor agent (ED_{50} 80 ± 6 pmol, n=6) when compared with ET-1, ET-2 and ET-3. The three ETs were equipotent on PIP. The ED_{50} values for the ETs (ET-1 216 ± 2 pmol, n= 10; ET-2 277 ± 7 pmol, n=4 and ET-3 268 ± 4 pmol, n= 8). In addition the slope of the PIP response to SX6C was significantly steeper than that for any of the ETs ($p< 0.001$).

Figure 16 Experimental traces showing the actions of ET-1, ET-3 and SX6C on pulmonary perfusion pressure (PPP), lung weight (LW) and pulmonary inflation pressure (PIP). Each trace is representative of 4-10 similar experiments..

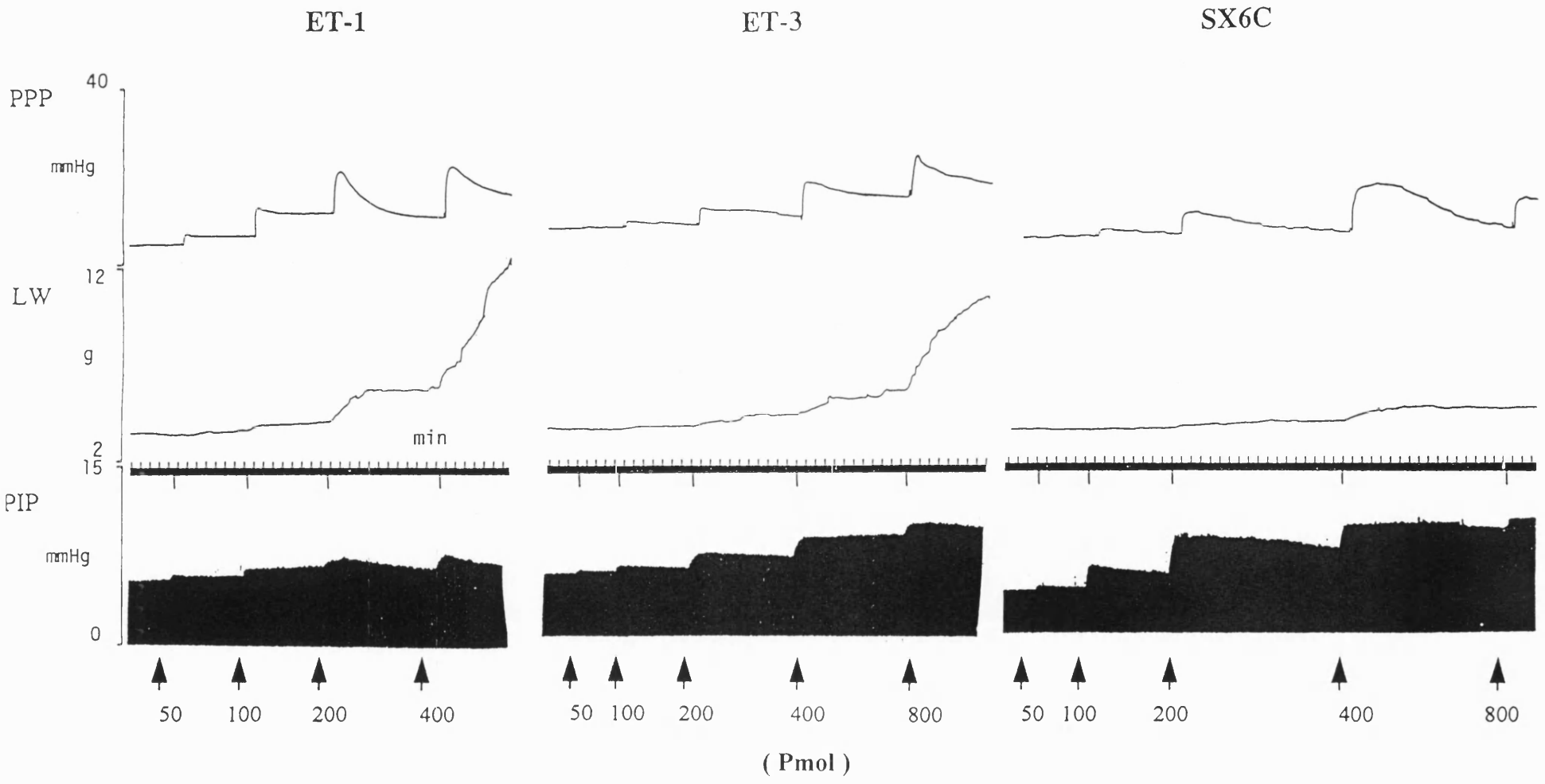
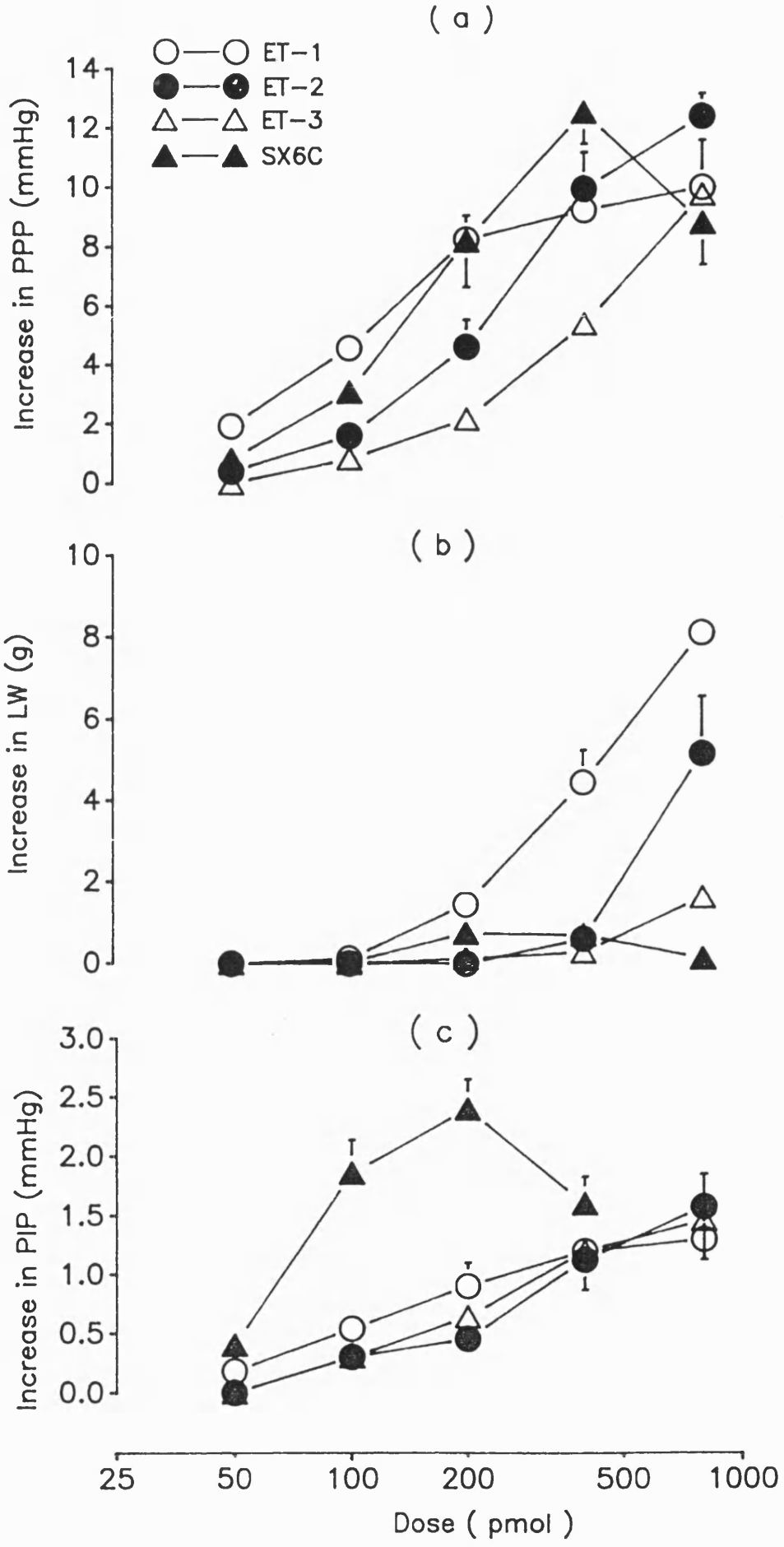

ET-1
ET-3
SX6C
PPP
40
mmHg
LW
12
g
2
min
15
PIP
mmHg
0
50
100
200
400
50
100
200
400
800
50
100
200
400
800
(Pmol)

Figure 17 Effects of ETs and SX6C on (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP). ET-1 (○), ET-2 (●), ET-3 (Δ) and SX6C (▲). Each point represents mean ± SEM, n = 4-10 experiments.


(a)
ET-1
ET-2
ET-3
SX6C
Increase in PPP (mmHg)
14
12
10
8
6
4
2
0
(b)
Increase in LW (g)
10
8
6
4
2
0
(c)
Increase in PIP (mmHg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

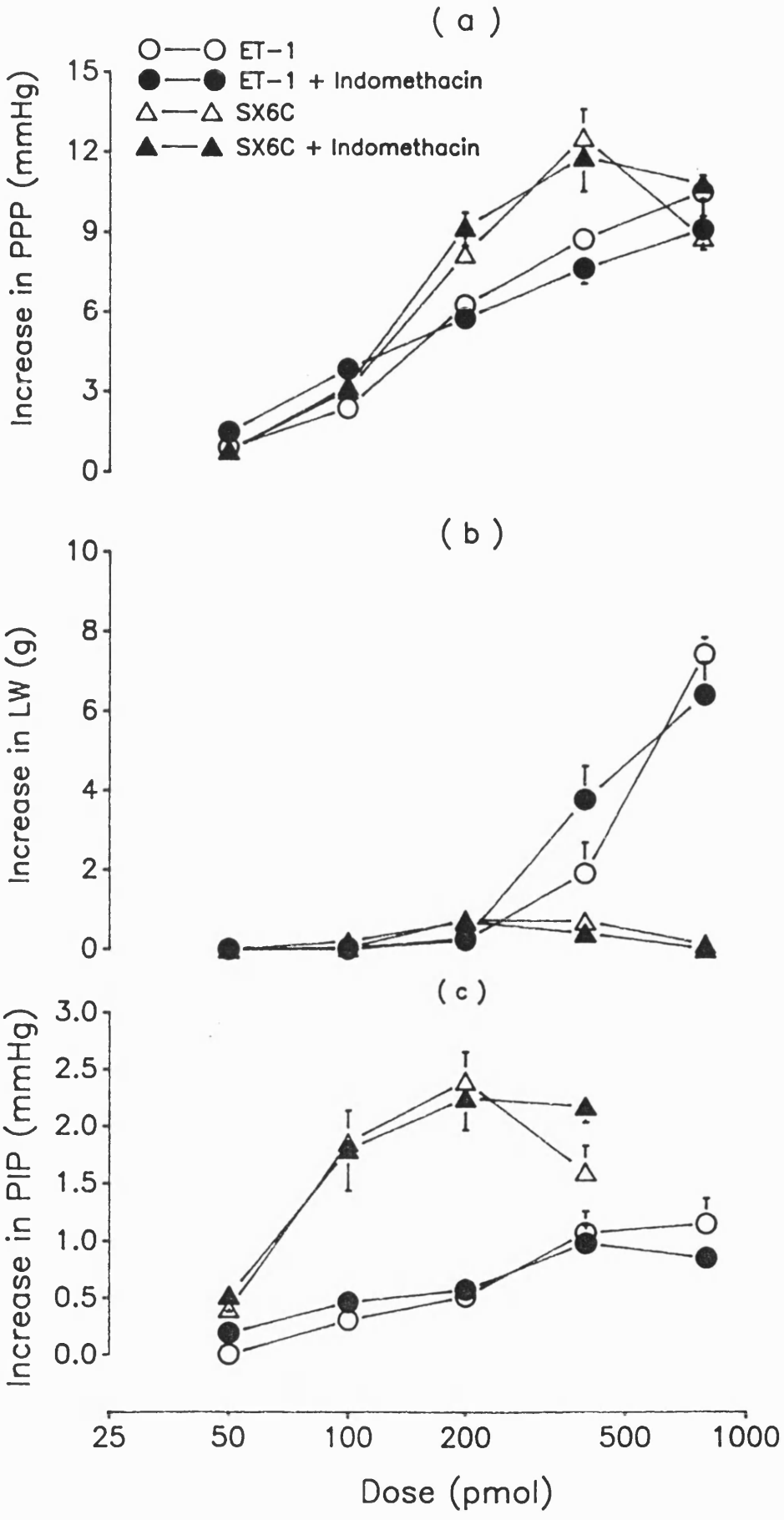
3.7 Effects of inhibitors and receptor antagonists on responses to ETs and SX6C

3.7.1 Indomethacin

The cyclooxygenase inhibitor indomethacin (10μM) had no effect on basal PPP (5.4 ± 0.43 mmHg), PIP (5 ± 0.3 mmHg) or lung weight (2.8 ± 0.1 g) (n= 14), and did not affect the actions of ET-1 on PPP (ED_{50} value of 132 ± 14 pmol was not different to control ED_{50} of 130 ± 17 pmol, n= 10), PIP (ED_{50} of 200 ± 2 pmol compared to control ED_{50} of 216 ± 2 pmol, n= 10) or lung weight (maximum increase produced by 400 pmol ET-1 in the presence and absence of indomethacin was 4 ± 0.9 g and 4.5 ± 0.8 g, n= 10 respectively) Figure 18.

SX6C-mediated responses were also unaffected by indomethacin (10μM) Figure 18a. PPP ED_{50} values for SX6C in the presence and absence of indomethacin were 142 ± 11 pmol (n= 4) and 165 ± 19 pmol (n= 6) respectively, while the corresponding PIP ED_{50} values in the presence and absence of indomethacin were 81 ± 8 pmol (n= 4) and 80 ± 6 pmol (n= 6). SX6C had no significant effect on lung weight with or without indomethacin.

Figure 18 Effects on ET-1 and SX6C on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the presence (filled symbols) and absence (open symbols) of indomethacin (10 μM). ET-1 (○, ●) and SX6C (Δ, ▲). Each point represents mean ± SEM, n= 10-14 experiments.


(a)
ET-1
ET-1 + Indomethacin
SX6C
SX6C + Indomethacin
Increase in PPP (mmHg)
15
12
9
6
3
0
(b)
Increase in LW (g)
10
8
6
4
2
0
(c)
Increase in PIP (mmHg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

3.7.2 Phosphoramidon

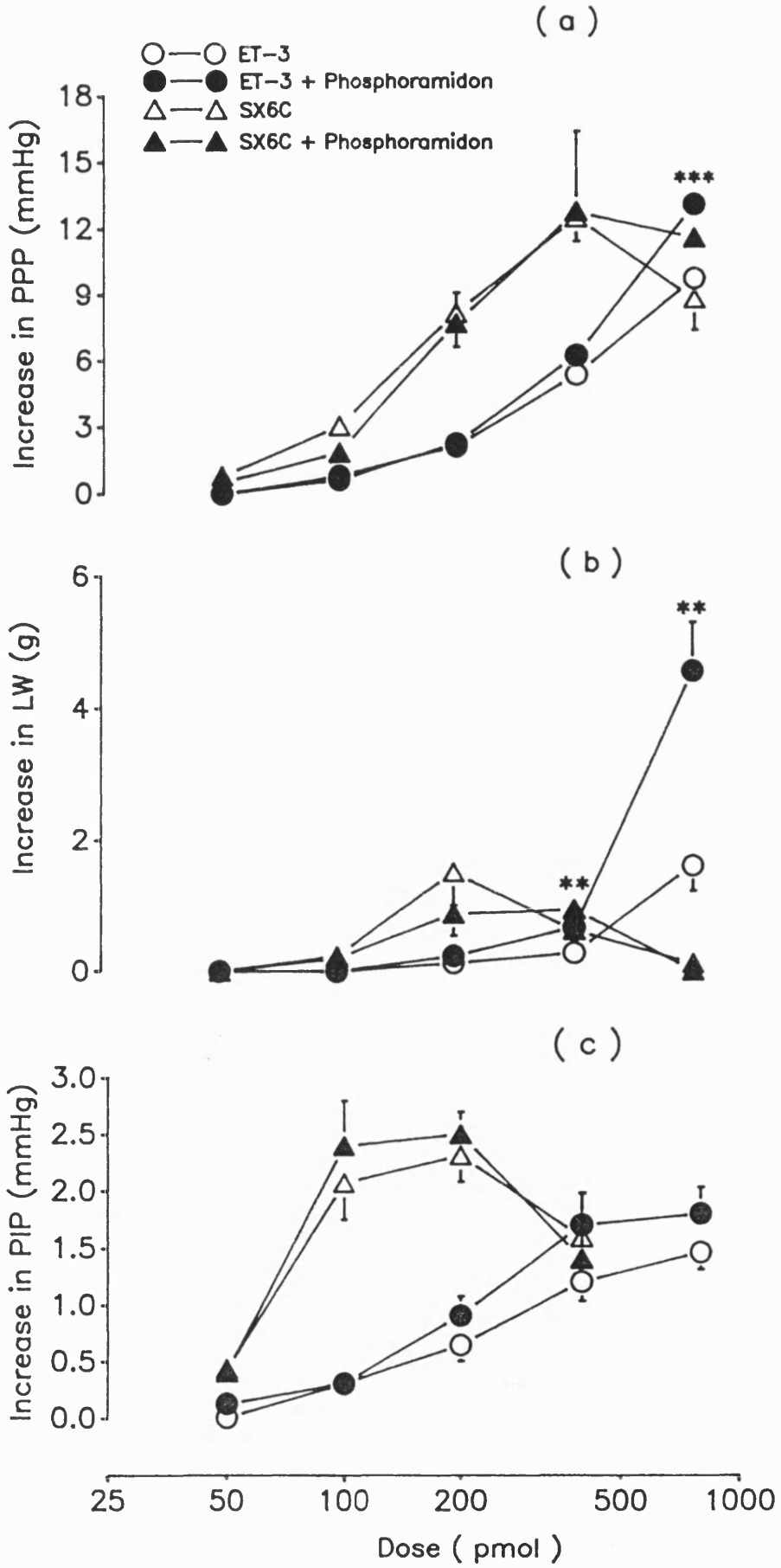
The effect of the neutral endopeptidase inhibitor phosphoramidon was studied, to determine if breakdown of ETs by neutral endopeptidases reduced their bronchoconstrictor potencies. Phosphoramidon (1 μM) had no effect on the basal PPP (6 ± 0.5 mmHg), lung weight (2.7 ± 0.06 g) and PIP (4.8 ± 0.17 mmHg) (n= 9). However perfusion of phosphoramidon (1 μM) significantly potentiated the effect of ET-3 (800 pmol) on PPP (800 pmol increase PPP in control 9.75 ± 0.57 mmHg, n=8 was potentiated to 13 ± 0.4 mmHg, n= 6; $p< 0.01$). Whereas it had no effect on PPP responses to lower doses of ET-3 (50- 400 pmol) Figure 19a.

Lung weight increases to ET-3 were significantly potentiated in the presence of phosphoramidon (1 μM). ET-3 (800 pmol) lung weight increase in control lungs was 1.6 ± 0.4 g, n=8, this was potentiated to 4.56 ± 0.74 g, n=6 ($P< 0.001$) in the presence of phosphoramidon (Figure 19b).

Figure 19c illustrates that phosphoramidon (1 μM) had no marked effect on the PIP responses to ET-3, the ED_{50} value 264 ± 45 pmol(n=6) was not different to the control value quoted above.

The selectivity of action of phosphoramidon is illustrated by its lack of effects on SX6C mediated responses. Figure 19 shows that phosphoramidon (1 μM) had no effect on the PPP (ED_{50} 168 ± 17 pmol, n=3) lung weight and PIP (ED_{50} 76 ± 8 pmol, n=3) responses to SX6C when compared to control responses.

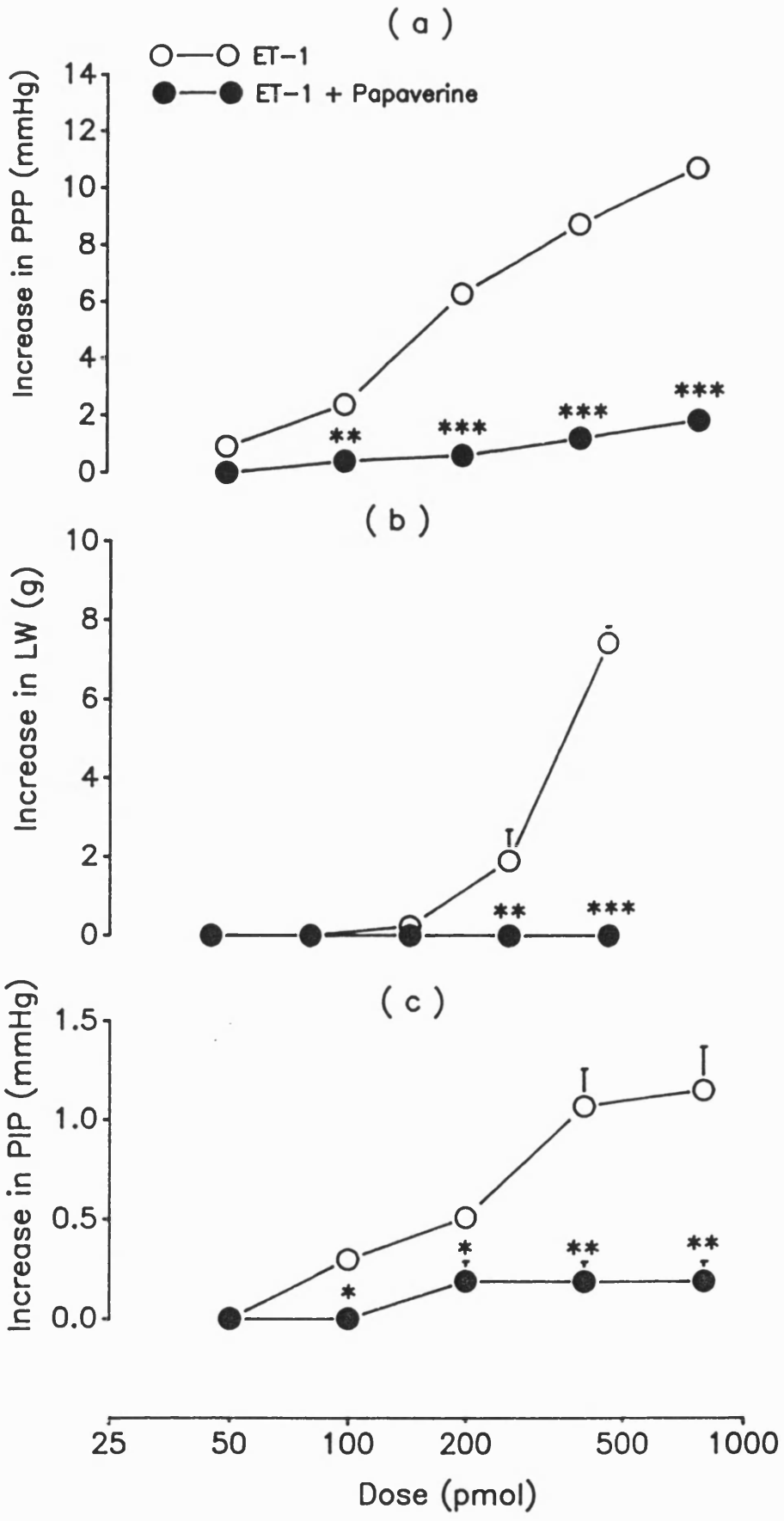
Figure 19 Effects of ET-3 and SX6C on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the presence (filled symbols) or absence (open symbols) of phosphoramidon (1μM). ET-3 (○, ●) and SX6C (Δ, ▲) Each point represents mean ± SEM, n= 3-8 experiments. **p< 0.01; ***p< 0.001. Student's t-test.


(a)
ET-3
ET-3 + Phosphoramidon
SX6C
SX6C + Phosphoramidon
Increase in PPP (mmHg)
18
15
12
9
6
3
0
***
(b)
Increase in LW (g)
6
4
2
0
**
**
(c)
Increase in PIP (mmHg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

3.7.3 Papaverine

Pulmonary actions of ET-1 were investigated in the presence of the vasodilator papaverine, to see if increases in vascular resistance accounted for lung weight changes. Papaverine (20 μM) had no effect on basal PPP (6.8 ± 0.5 mmHg), lung weight (2.9 ± 0.06 g) and PIP (5 ± 0.12 mmHg). However, it abolished ($P< 0.001$, n=4-7) the changes in PPP, lung weight and PIP in response to ET-1 (50- 800 pmol). Results are shown in Figure 20.

Figure 20 ET-1-induced increases in (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (○) and presence (●)of papaverine (20 μM). Each point represents mean ± SEM, n= 4 - 7 experiments. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$: significantly different from control. (Student's t-test).


(a)
ET-1
ET-1 + Papaverine
Increase in PPP (mmHg)
14
12
10
8
6
4
2
0
**
***
***
***
(b)
Increase in LW (g)
10
8
6
4
2
0
**
***
(c)
Increase in PIP (mmHg)
1.5
1.0
0.5
0.0
*
*
**
**
25
50
100
200
500
1000
Dose (pmol)

3.8 Endothelin receptor antagonists

3.8.1 Selective ET_A receptor antagonist

BQ123

BQ123 a selective ET_A receptor antagonist (Ihara et al., 1992) in the concentration range 1 μM to 10 μM had no effect on basal PPP (6.3 ± 0.4 mmHg), PIP (4.7 ± 0.15 mmHg) and lung weight (2.6 ± 0.08 g) (n= 18). Figures 21 and 22 illustrate the effects of BQ123 on ET-1 mediated changes in PPP, lung weight and PIP. The effects of BQ123 on ET-1 mediated increases in PPP are shown in Figure 22a. In the presence of BQ123 (1μM) PPP responses to low doses of ET-1 (50-100 pmol) were significantly inhibited ($p< 0.05$) whereas the response to the highest dose of ET-1 (800 pmol) was significantly augmented (ET-1 800 pmol PPP increase in control 10.4 ± 1.6 mmHg, n= 4 was potentiated to 21 ± 3.6 mmHg, n= 5; $p< 0.01$). However, perfusion of 2μM BQ123 significantly attenuated the effects of ET-1 on PPP and the dose-response curve showed a rightward parallel shift with a significant increase in ED_{50} value (control ED_{50} 130 ± 17 pmol (n= 10) to 311 ± 3 pmol (n = 4; $p< 0.01$) in the presence of BQ123. However, BQ123 at 2 μM had no effect on the maximal increase in PPP induced by ET-1. Furthermore use of the higher concentration of BQ123 (10 μM) markedly attenuated PPP responses to ET-1 (50- 800 pmol). The increase in PPP was reduced to 5.3 ± 1.3 mmHg, n= 3 ($p < 0.01$) in the presence of 10 μM BQ123, when compared to the control value quoted above. Interestingly, in the presence of BQ123, ET-1 caused transient increases in PPP (Figure 21).

Figure 22b illustrates, that BQ123 produced a concentration-dependent reduction of the lung weight increase in response to ET-1 (50- 800 pmol). The maximum increase in lung weight produced in response to 800 pmol ET-1 (8.1 ± 0.1 g, n =4) was reduced in the presence of 1 μM BQ123 to 4.5 ± 0.8 g, n = 4 ($p < 0.001$). BQ123 (2 μM and 10 μM)

further attenuated the ET-1 (800 pmol)-induced increase in lung weight to 0.8 ± 0.2 g, n = 4 and 0.4 ± 0 g, n =3 ($p< 0.001$) respectively.

Interestingly, BQ123 (1-10 μM) augmented the effects of ET-1 on PIP (Figure 22c). The control ED_{50} (216 ± 2 pmol, n =10) for ET-1 was reduced to 78 ± 12.3 pmol, n =5 ($p< 0.001$) in the presence of 1 μM BQ123, 107 ± 17 pmol, n= 4 ($p<0.05$) in the presence of 2 μM BQ123, and 87 ± 20 pmol, n =3 ($p< 0.001$) in the presence of 10 μM BQ123. In addition the dose-response curves to ET-1 over the dose range 50-200 pmol in the presence of BQ123 were significantly steeper than the controls ($p<0.001$).

The selectivity of BQ123 for ET_A receptors was studied with its effects on SX6C - mediated responses. BQ123 (2μM) did not affect the PPP response to SX6C; control ED_{50} was 165 ± 19 pmol (n = 6) and in the presence of BQ123 was 184 ± 20 pmol (n = 3). Use of the higher concentration of BQ123 (10 μM) also had no effect on PPP responses to SX6C (50- 400 pmol). Similarly, SX6C-induced changes in PIP were not altered by the higher concentration of BQ123; control ED_{50} was 80 ± 6 pmol (n = 6) and in presence of 2 μM and 10 μM BQ123 was 68 ± 2 pmol and 71 ± 4 pmol (n = 3) respectively. These results are shown in Figure 23.

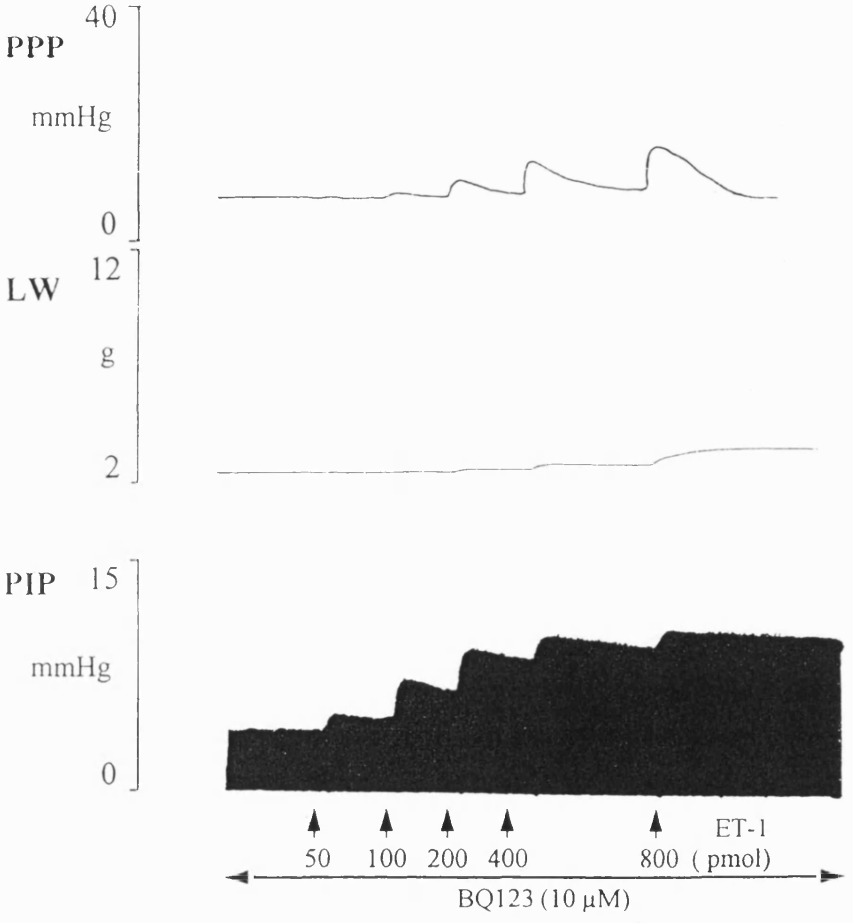


Figure 21 Experimental trace showing the effects of ET-1 on PPP, lung weight and PIP in the presence of BQ123 (10 μM). This represents one of 3 such experiments.

Figure 22 ET-1-induced increases in (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open circles) or presence of BQ123 (1 μM) (filled circles), (2 μM) (open triangles) and (10 μM) (filled triangles). Each point represents mean ± SEM, n= 4-10 experiments. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$: significantly different from control (1 way ANOVA).

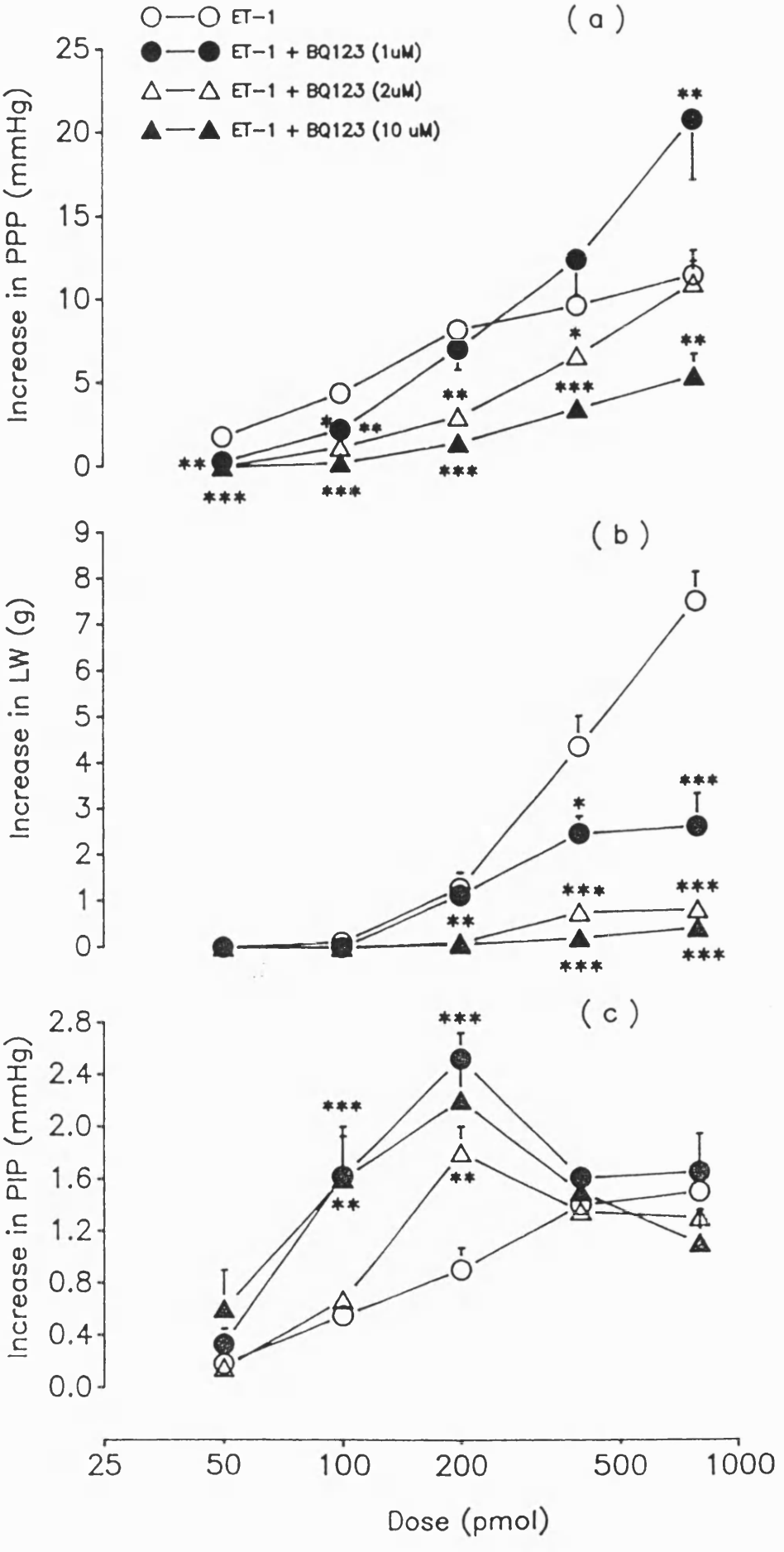
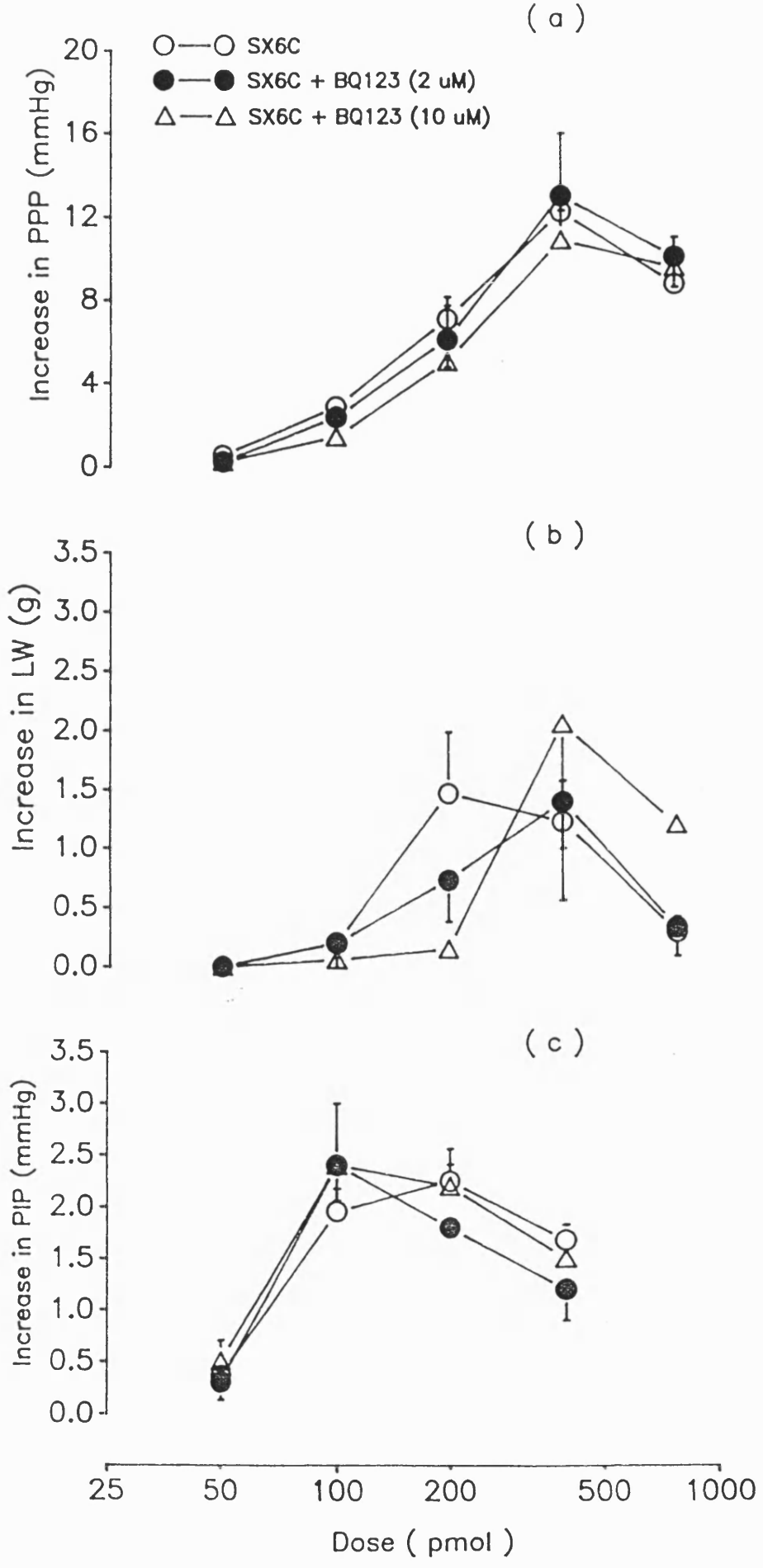

ET-1
ET-1 + BQ123 (1uM)
ET-1 + BQ123 (2uM)
ET-1 + BQ123 (10 uM)
(a)
Increase in PPP (mmHg)
(b)
Increase in LW (g)
(c)
Increase in PIP (mmHg)
Dose (pmol)
25
50
100
200
500
1000

Figure 23 SX6C-induced increases in (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open circles) or presence of BQ123 (2 μM) (filled circles), (10 μM) (open triangles). Each point represents mean ± SEM, n = 3 - 6 experiments.


(a)
SX6C
SX6C + BQ123 (2 uM)
SX6C + BQ123 (10 uM)
Increase in PPP (mmHg)
20
16
12
8
4
0
(b)
Increase in LW (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(c)
Increase in PIP (mmHg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

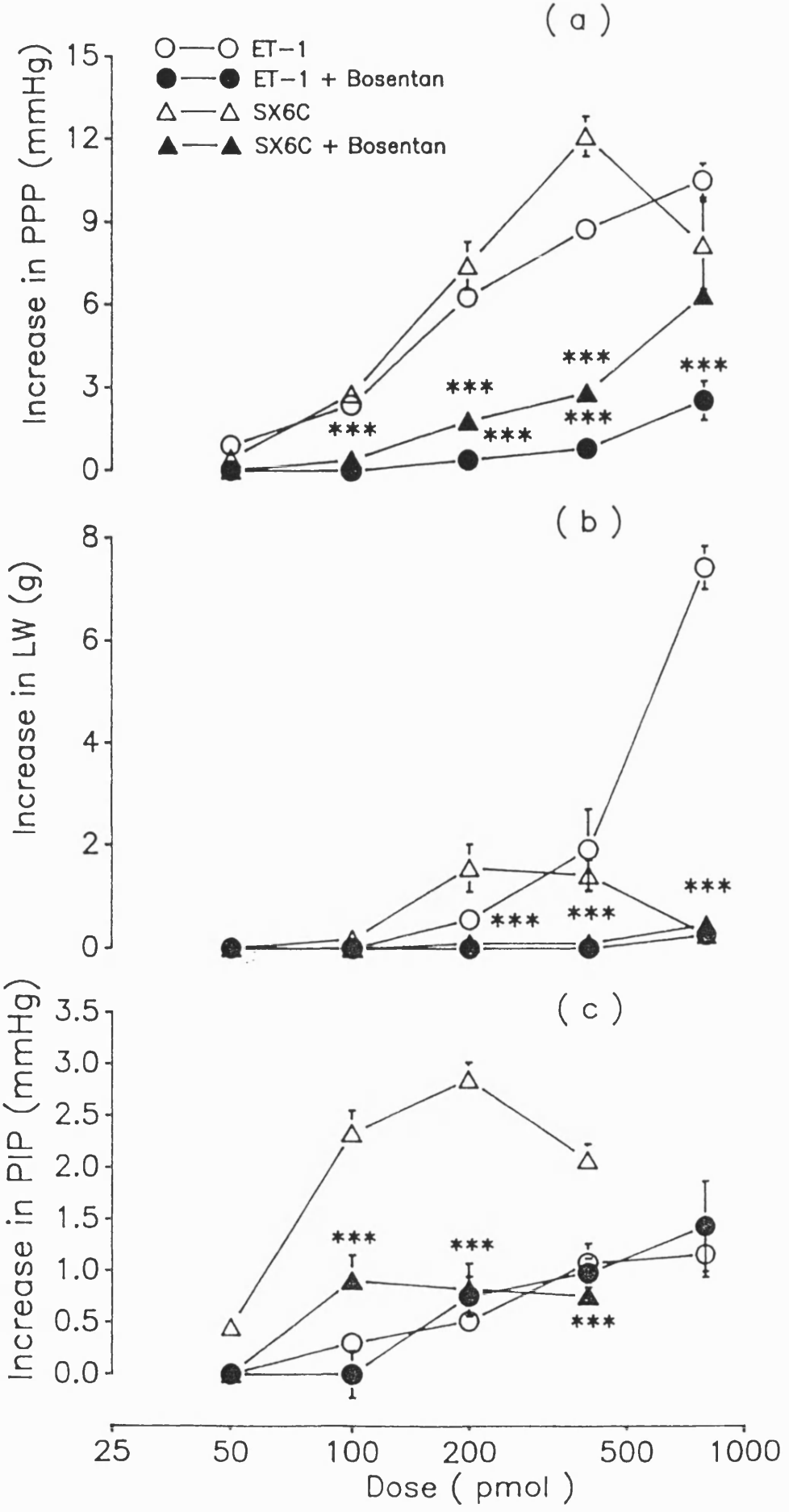
3.8.2 Mixed (ET_A / ET_B) endothelin receptor antagonists

3.8.2.1 Bosentan (Ro 47-0203)

Bosentan (5 μM), a non-peptide mixed endothelin receptor antagonist (Clozel *et al.*, 1994), had no effect on the basal PPP (5 ± 1 mmHg), PIP (5 ± 0.2 mmHg) and lung weight (2.7 ± 0.14 g) (n= 8). However, it reduced ($p< 0.001$, n=4) the ET-1 (50-800 pmol)-induced PPP and lung weight responses as shown in Figure 24a and 24b, respectively. In contrast, bosentan did not affect ET-1-induced PIP responses (control ED_{50} 242 ± 34 pmol, n=7; presence of bosentan ED_{50} 291 ± 90, n=4) (Figure 24c). Responses to SX6C on PPP, PIP and lung weight were attenuated ($p< 0.001$, n=4) by bosentan (Figure 24).

The selectivity of bosentan for endothelin receptors was illustrated by its lack of effects on the PHE mediated responses. In the presence of 5 μM bosentan PPP responses to PHE were not altered (control ED_{50} 4 ± 0.7, n=5; ED_{50} in the presence of bosentan 3 ± 0.2 nmol, n=3).

Figure 24 Effects of ET-1 and SX6C on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open symbols) or presence of bosentan (5 μM) (filled symbols). ET-1 (○, ●) and SX6C (Δ, ▲) Each point represents mean ± SEM, n= 4-13 experiments. *** $p< 0.001$: significantly different from ET-1 or SX6C control (Student's t-test).


(a)
ET−1
ET−1 + Bosentan
SX6C
SX6C + Bosentan
Increase in PPP (mmHg)
15
12
9
6
3
0
***
(b)
Increase in LW (g)
8
6
4
2
0
(c)
Increase in PIP (mmHg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

3.8.2.2 PD145065

PD145065 (0.3-1 μM) another mixed endothelin ET_A / ET_B receptor antagonist (Doherty et al., 1993) had no effect on the basal PPP (5.7 ± 0.5 mmHg), lung weight (2.8 ± 0.13 g) and PIP (5 ± 0.2 mmHg) (n= 13). However, 0.3 μM PD145065 attenuated ($p< 0.001$) the PPP responses to ET-1 (50 - 400 pmol). Perfusion of 1 μM of PD145065 further attenuated ($P< 0.001$, n=3) the PPP responses to ET-1 (50-800 pmol) (Figure 25a).

Figure 25b illustrates that both concentrations of PD145065 (0.3 μM and 1 μM) abolished ($p< 0.001$) the increases in lung weight produced by ET-1 (50-800 pmol).

Interestingly, PD145065 (0.3 - 1 μM) potentiated the PIP responses to ET-1 (Figure 25c). ED_{50} values in the presence of 0.3 μM (134 ± 33 pmol, n=4) and 1 μM (112 ± 2.5 pmol, n=3; $p< 0.01$) were significantly reduced when compared with control value.

PD145065 (0.3 - 1 μM) attenuated ($p< 0.001$, n=3) the PPP, lung weight and PIP responses to SX6C (50- 800 pmol) Figure 26.

The selectivity of PD145065 for endothelin receptors,was studied by its effects on the vasoconstrictor responses to PHE. Use of 1 μM PD145065 had no significant effect on PPP responses to PHE (control ED_{50} 4 ± 0.7 nmol, n= 5; ED_{50} in the presence of PD145065, 3 ± 1 nmol, n=3).

Figure 25 ET-1-induced increases in (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open circles) or in the presence of PD1450656 (0.3 μM, filled circles), (1 μM) (open triangles). Each point represents mean ± SEM, n= 4 -13 experiments. *** $p< 0.001$: significantly different from ET-1 control (1 way ANOVA).

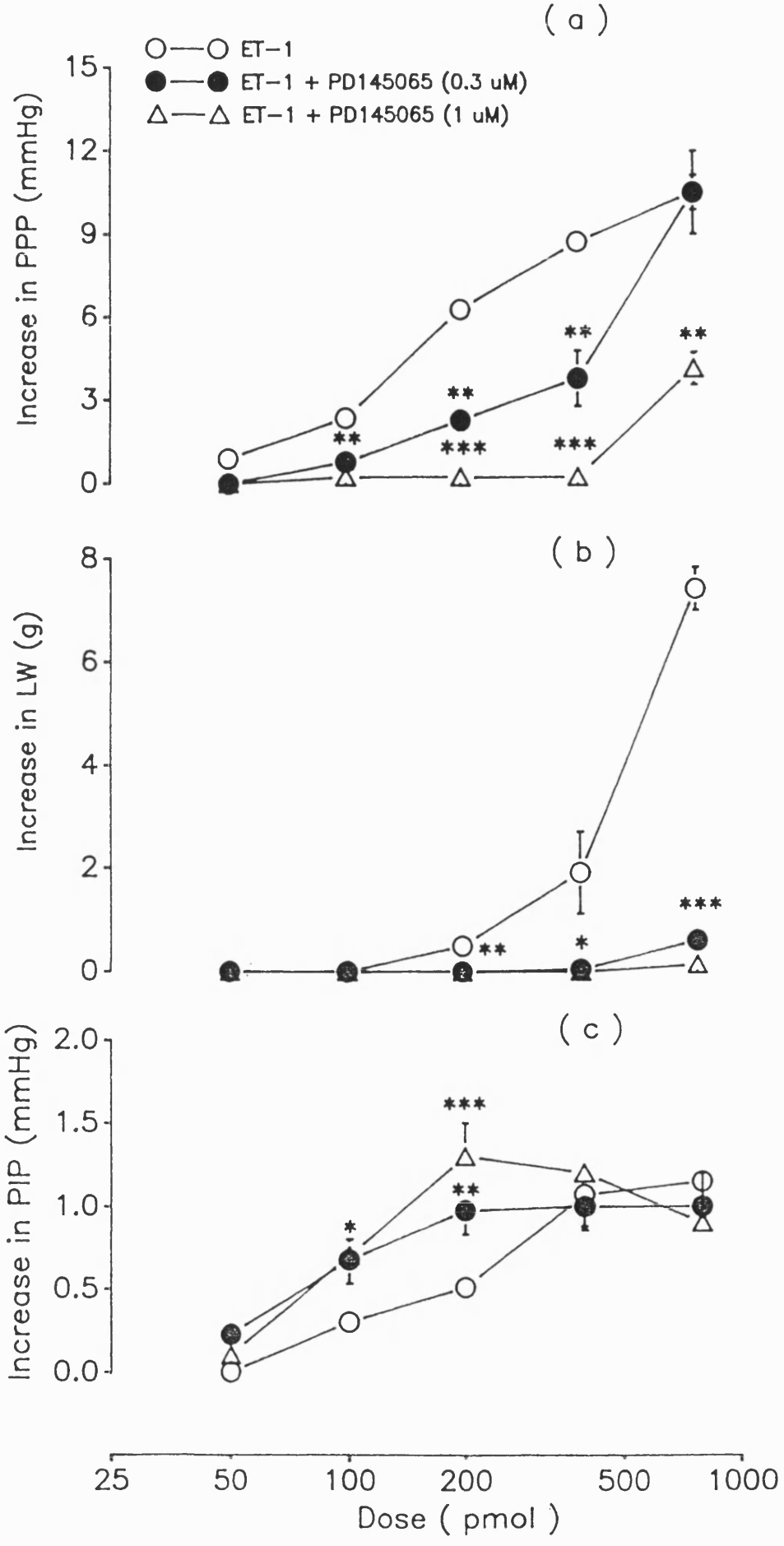
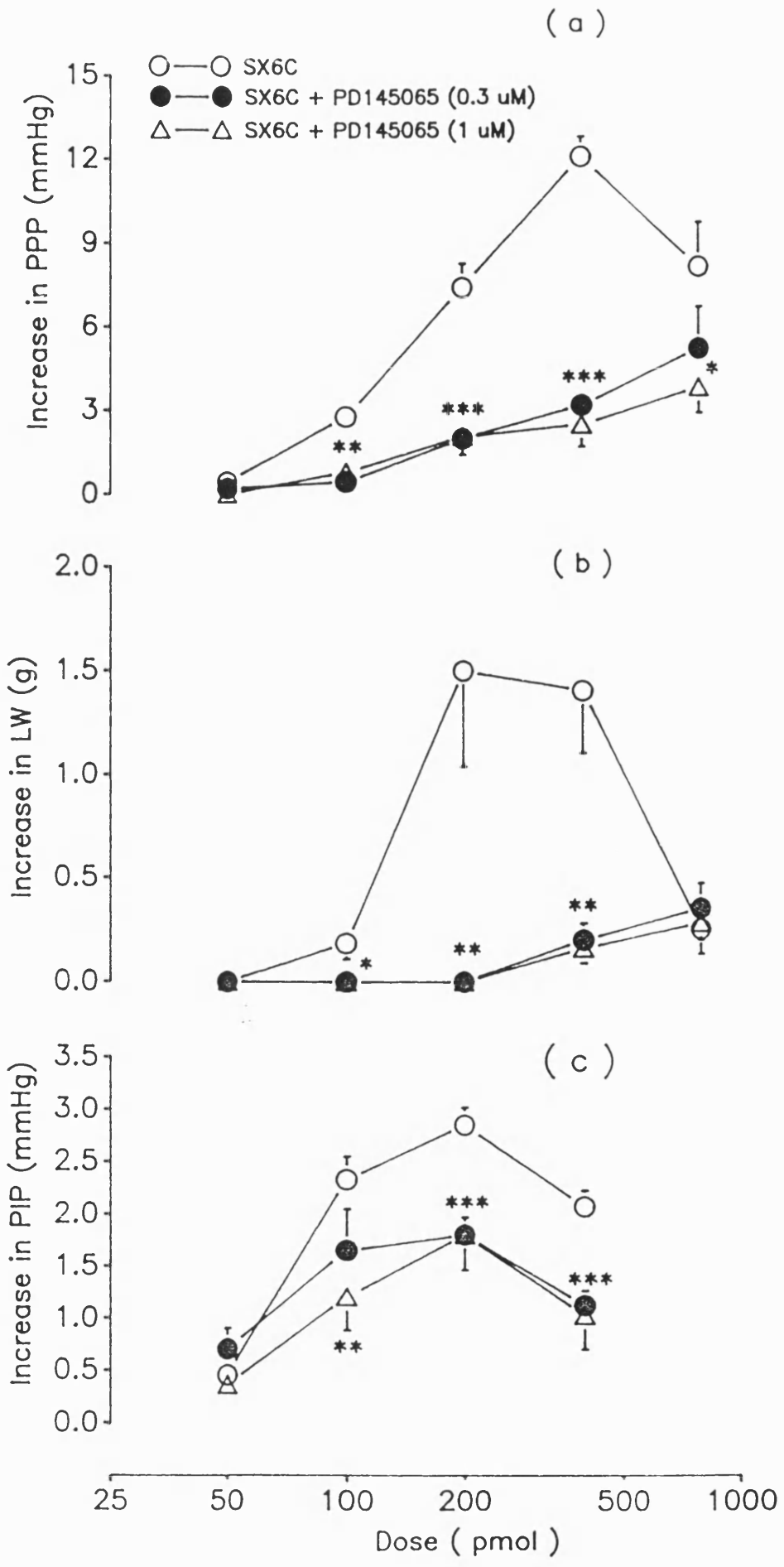

(a)
ET-1
ET-1 + PD145065 (0.3 uM)
ET-1 + PD145065 (1 uM)
Increase in PPP (mmHg)
15
12
9
6
3
0
(b)
Increase in LW (g)
8
6
4
2
0
(c)
Increase in PIP (mmHg)
2.0
1.5
1.0
0.5
0.0
25
50
100
200
500
1000
Dose (pmol)

Figure 26 SX6C-induced increases in pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP); control (open circles) or in the presence of PD1450656 (0.3 μM, filled circles), (1 μM, open triangles). Each point represents mean ± SEM, n= 3- 13 experiments. *** $p < 0.001$: significantly different from SX6C control (1 way ANOVA).

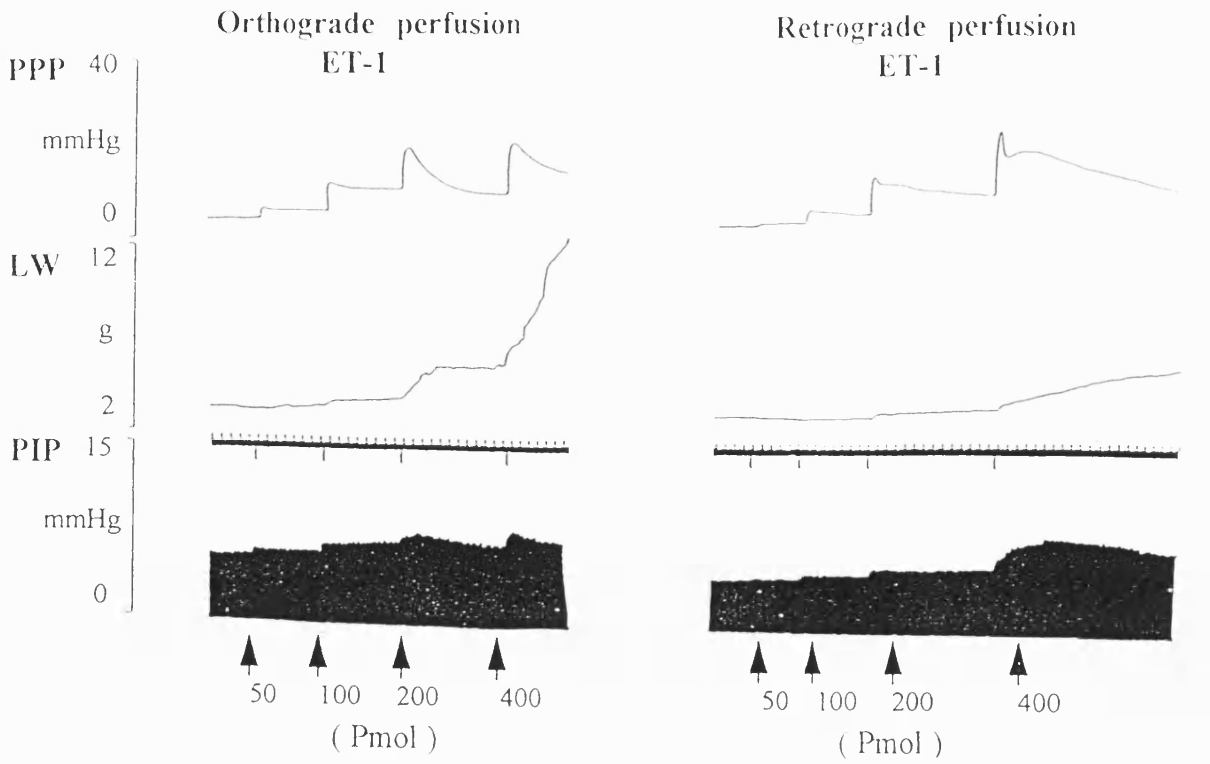

(a)
SX6C
SX6C + PD145065 (0.3 uM)
SX6C + PD145065 (1 uM)
Increase in PPP (mmHg)
15
12
9
6
3
0
**
***
***
*
(b)
Increase in LW (g)
2.0
1.5
1.0
0.5
0.0
*
**
**
**
(c)
Increase in PIP (mmHg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
25
50
100
200
500
1000
Dose (pmol)

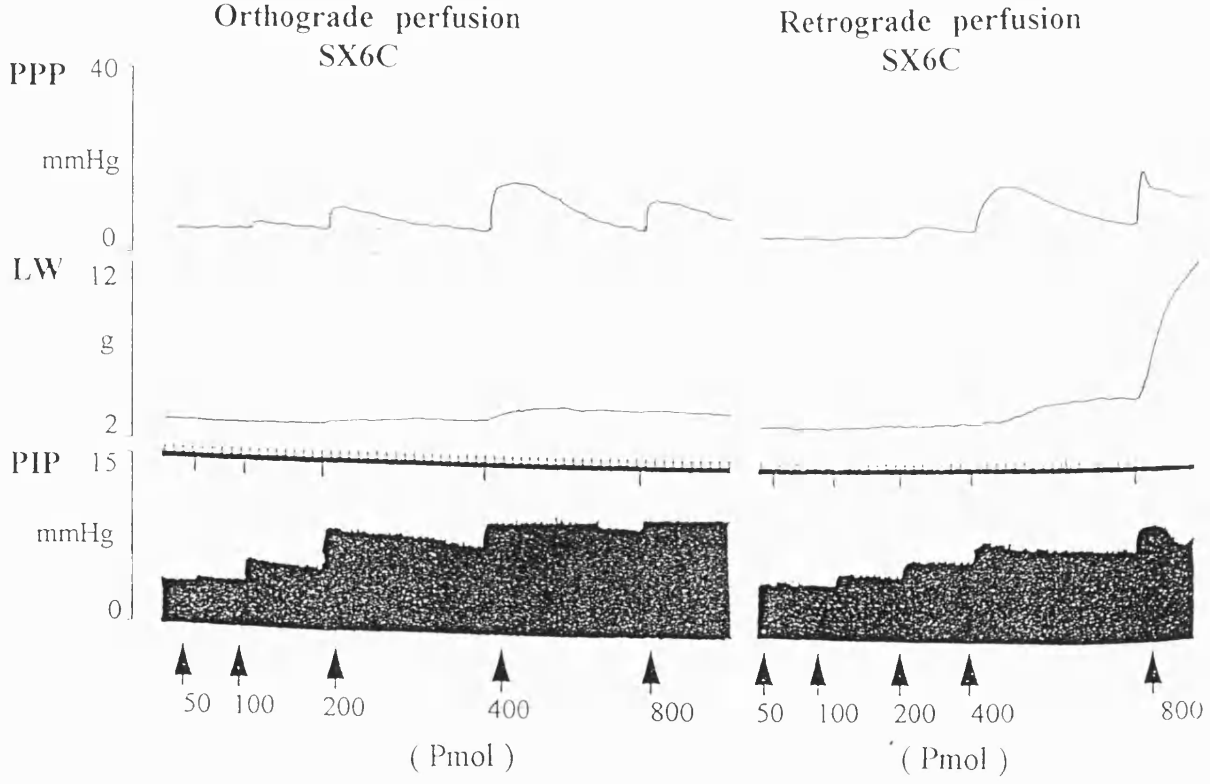
3.9 Effects of ET-1 and SX6C in retrogradely perfused lungs

Retrograde perfusion of lungs was used to study the sites of actions of ET-1 and SX6C in the pulmonary circulation. Comparative actions of ET-1 and SX6C on recorded parameters in orthogradely and retrogradely perfused lungs are shown in Figure 27. ET-1 (50-400 pmol) and SX6C (50-800 pmol) produced dose-dependent increases in PPP, PIP and lung weight. However, a comparison of the upper panels shows that the actions of ET-1 on lung weight were markedly reduced by retrograde perfusion. Using orthograde perfusion, 400 pmol of ET-1 produced an increase in lung weight of 6.9 ± 0.21 g (n=4) whereas 400 pmol of ET-1 in retrograde perfusion produced only a 1.8 ± 0.36 g (n= 4, $p< 0.01$) increase.

Comparison of the lower panels in Figure 27 shows that the actions of SX6C on lung weight were markedly increased by retrograde perfusion. Using orthograde perfusion, 800 pmol of SX6C produced a 0.11 ± 0.06 g (n= 4) increase in lung weight whereas the same dose given via retrograde perfusion produced a 7.5 ± 0.29 g (n= 4, $p< 0.001$) increase in lung weight (Student's t-test).

Figure 27 Experimental traces comparing the effects of ET-1 (upper section) and SX6C (lower section) on pulmonary perfusion pressure (PPP), lung weight (LW) and pulmonary inflation pressure (PIP) in rat lungs perfused orthogradely (right hand panels) or retrogradely (left hand panels). This Figure represents one of 4 similar experiments.


Orthograde perfusion
ET-1
Retrograde perfusion
ET-1
PPP 40
mmHg
0
LW 12
g
2
PIP 15
mmHg
0
50 100 200 400
(Pmol)
50 100 200 400
(Pmol)


Orthograde perfusion
SX6C
Retrograde perfusion
SX6C
PPP 40
mmHg
0
LW 12
g
2
PIP 15
mmHg
0
50 100 200 400 800
(Pmol)
50 100 200 400 800
(Pmol)

3.10 Albumin-bound dye extravasation

To investigate whether increases in lung weight induced by ET-1 or SX6C were due to changes in vascular permeability, experiments utilizing Evans blue dye linked to BSA were carried out. In a series of experiments the increase in lung weight caused by ET-1 (800 pmol) was shown to be associated with a large accumulation of albumin-bound dye (97.5 ± 22 ng mg^{-1} dry weight of tissue compared to 1.32 ± 0.54 ng mg^{-1} in control lungs without ET-1; $p < 0.01$, n= 4). Back-calculation of fluid retention from retained dye indicated that 800 pmol of ET-1 caused an accumulation of 1.62 ± 0.28 ml of fluid (n=4). Assuming a specific gravity of 1 for this fluid a comparison of this calculated weight gain with weight gain recorded experimentally (4.25 ± 0.25 g, n=4) illustrates that the values are significantly different ($p < 0.01$), i.e. fluid accumulation without albumin. Furthermore, albumin bound dye accumulation (1.48 ± 0.85 ng mg^{-1} of dry wt, n= 4) produced by a higher dose of ET-1 (800 pmol) with retrograde perfusion was still significantly lower ($p< 0.01$) than the accumulation seen with 400 pmol ET-1 (25 ± 9.5 ng mg^{-1} of dry wt., n=4) given via orthograde perfusion. Results are shown in Figure 28.

Furthermore, accumulation of albumin bound dye (216 ± 2.6 ng mg^{-1}dry tissue, n=4) caused by SX6C (800 pmol) in retrograde perfusion was significantly greater ($p< 0.01$) than albumin bound dye accumulation (70 ± 4 ng mg^{-1} dry wt, n=4) caused by SX6C in orthograde perfusion (Figure 28). Back calculation of fluid retention from retained dye indicated that 800 pmol of SX6C caused an accumulation of 1.44 ± 0.06 ml and 6.6 ± 0.39 ml fluid in orthogradely and retrogradely perfused lungs, respectively (n=4). Assuming a specific gravity of 1 for this fluid a comparison of this calculated weight gain with weight gain recorded experimentally (1.46 ± 0.24 g and 7.3 ± 0.15 g, n=4, in orthogradely and retrogradely perfused lung, respectively) illustrates that the values are in good agreement.

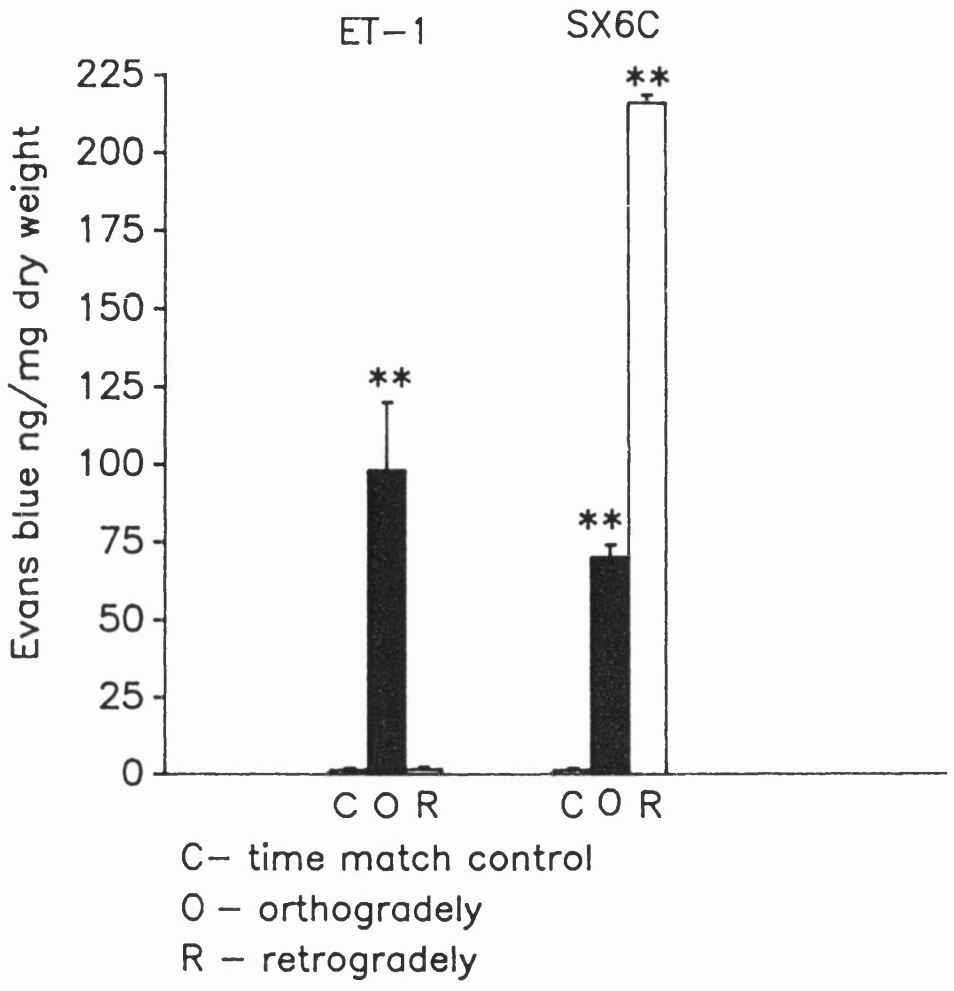


Figure 28 Effects of ET-1 or SX6C on vascular permeability in orthogradely (filled column) or retrogradely (open column) perfused lungs. Each point represents mean ± SEM, n= 4 experiments, **p< 0.01 compared with time matched control (hatched column).1 way ANOVA.

3.11 Effects of nitro-L-arginine (L-NOARG)

Inclusion of L-NOARG (100 μM) in the perfusate had no effect on the basal parameters recorded; basal PPP 5.4 ± 0.5, n=9 vs. 5.4 ± 0.6 mmHg, n=7 in control; basal lung weight (2.84 ± 0.12 g, n=9 vs. 2.85 ± 0.14g, n= 7 in control); basal PIP (5.24 ± 0.3 mmHg, n=9 vs.4.15 ± 0.2 mmHg, n=7 in control).

3.11.1 Effects of L-NOARG on responses to ETs, SX6C, and BK

3.11.1.1 PPP

From Figure 29a it can be seen that 100 μM L-NOARG potentiated PPP increases in response to ET-1 50 - 400 pmol ($p< 0.001$, n=4). Similarly, SX6C (25-200 pmol) mediated increases in PPP were also significantly augmented ($p< 0.001$, n=5) in the presence of L-NOARG (Figure 30a). The maximum constrictor response to SX6C was also increased in the presence of L-NOARG.

The vasoconstrictor effects of BK (6.25- 200 nmol) were potentiated by 100 μM L-NOARG. In the presence of L-NOARG the BK ED_{50} was reduced from control value of 24 ± 4 nmol, n=7 to 16 ± 1.0 nmol, n=9. The maximum response to BK was also significantly increased from 10 ± 0.8 mmHg, n=7 to 14 ± 0.65 mmHg, n=9 ($p< 0.01$) (Figure 31a).

In contrast to the potentation of responses to ET-1, SX6C and BK it was of interest to note that L-NOARG did not affect the increases in PPP induced by PHE (3.12- 50 nmol) (ED_{50} 5.4 ± 1.7, n=3 vs. 4 ± 0.7, n=5) in controls (Figure 32a).

3.11.1.2 Lung weight

In the presence of L-NOARG the lung weight increases caused by ET-1 were markedly potentiated (Figure 29b). Similarly SX6C-induced increases in lung weight were also potentiated in the presence of L-NOARG (Figure 30b).

In contrast to the potentiation of ET-1 and SX6C -induced increases in lung weight L-NOARG had no effect on BK-mediated increases in lung weight (Figure 31b).

3.11.1.3 PIP

Figure 29c illustrates, that the increases in PIP in response to ET-I were significantly augmented in the presence of L-NOARG (ED_{50} 132 ± 5 pmol, n=4; $p< 0.05$ vs. 242 ± 33 pmol, n=7 in control). Similarly, the SX6C-induced increases in PIP were also significantly potentiated (ED_{50} 48 ± 5 pmol, n=5; $p< 0.01$ vs. 85 ± 6 pmol, n=13 in controls) Figure 30c.

The presence of L-NOARG did not alter the bronchoconstrictor responses to CCh (ED_{50} 4 ± 0.3 nmol, n=4 vs. 4.25 ± 0.4 nmol, n=7 in controls, Figure 32b) or BK (ED_{50} 54 ± 12 nmol, n=7 vs. 40 ± 8 nmol, n=9 in controls (Figure 31c).

Figure 29 The effects of ET-1 on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open circles) or presence (filled circles) of L-NOARG (100 μM) Each point represents mean ± SEM, n= 4-7 experiments **p <0.01; ***p < 0.001 compared with control ET-1 (Student's t-test).

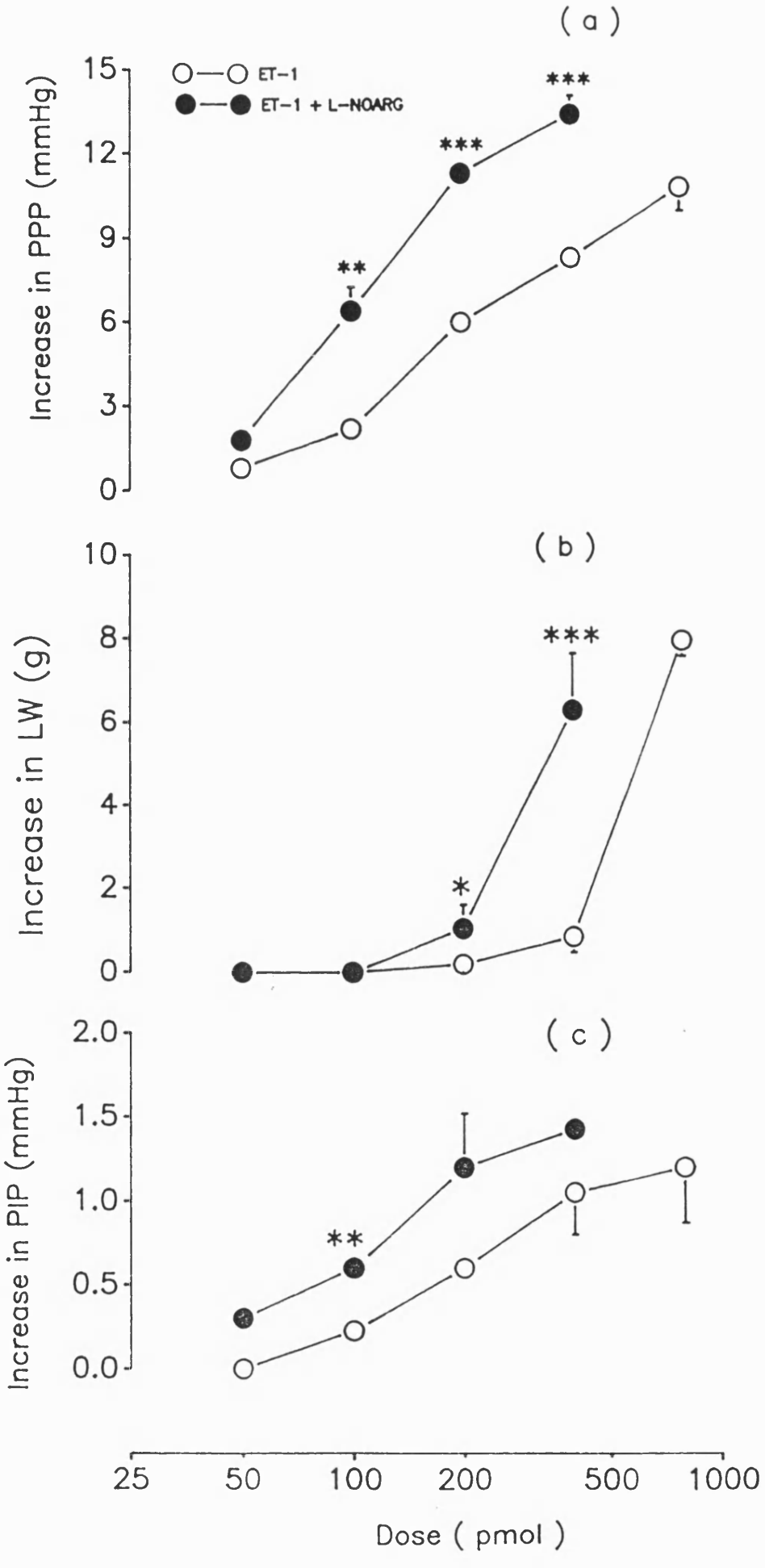

(a)
ET-1
ET-1 + L-NOARG
Increase in PPP (mmHg)
15
12
9
6
3
0
**
***
***
(b)
Increase in LW (g)
10
8
6
4
2
0
*
***
(c)
Increase in PIP (mmHg)
2.0
1.5
1.0
0.5
0.0
**
25
50
100
200
500
1000
Dose (pmol)

Figure 30 The effects of SX6C on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (open circles) or presence (filled circles) of L-NOARG (100 μM) Each point represents mean ± SEM, n= 5-7 experiments. ***$p < 0.001$ compared with control SX6C (Student's t-test).

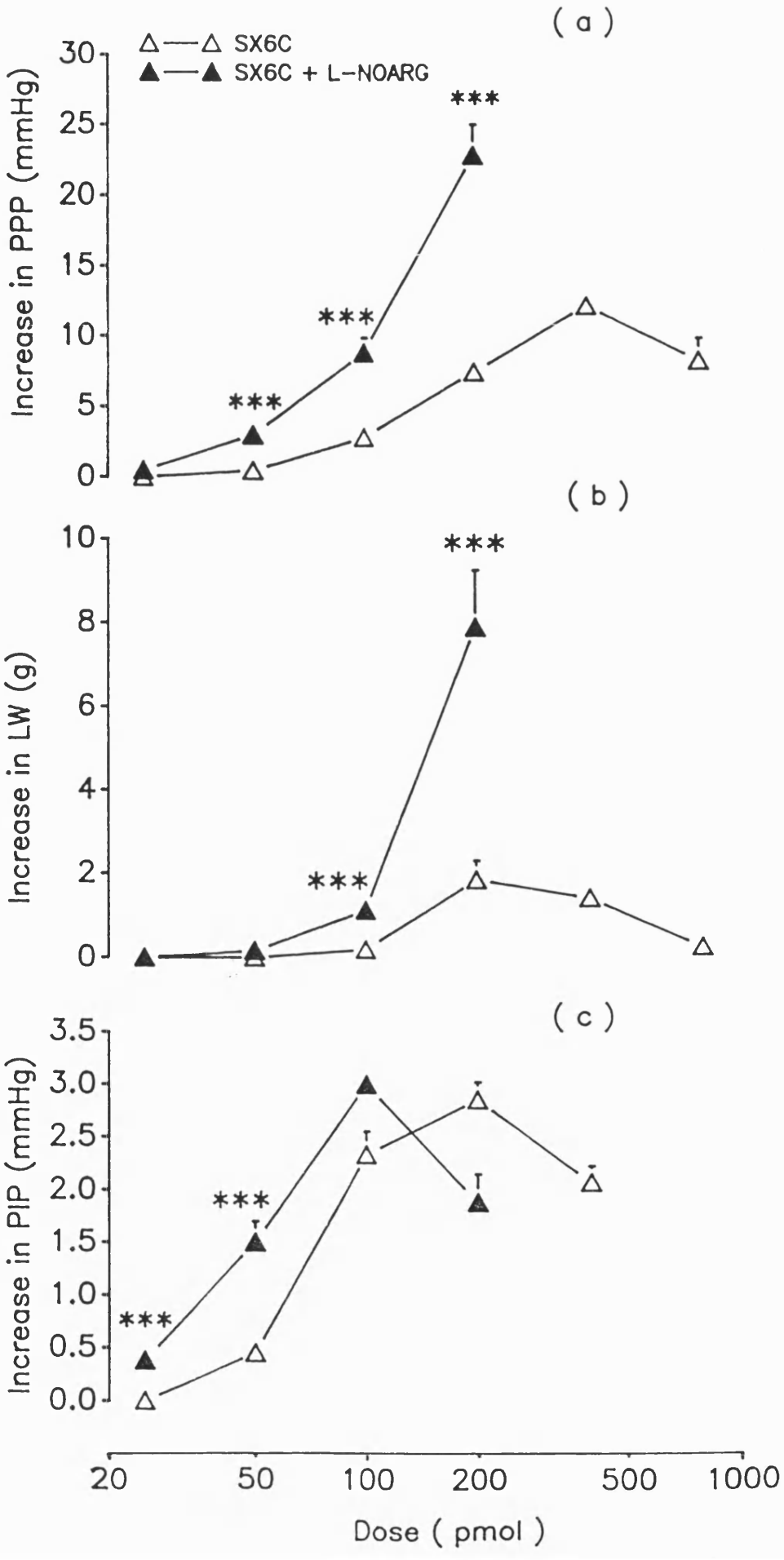

(a)
△—△ SX6C
▲—▲ SX6C + L-NOARG
Increase in PPP (mmHg)
30
25
20
15
10
5
0
***
***
***
(b)
Increase in LW (g)
10
8
6
4
2
0
***
***
(c)
Increase in PIP (mmHg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
20
50
100
200
500
1000
Dose (pmol)

Figure 31 The actions of bradykinin (BK) on (a) pulmonary perfusion pressure (PPP), (b) lung weight (LW) and (c) pulmonary inflation pressure (PIP) in the absence (open circles) and presence (closed circles) of L-NOARG (100 μM)). Each point represents mean ± SEM, n= 7-9 experiments. * $p< 0.05$; ** $p< 0.01$ compared with control (Student's t-test).

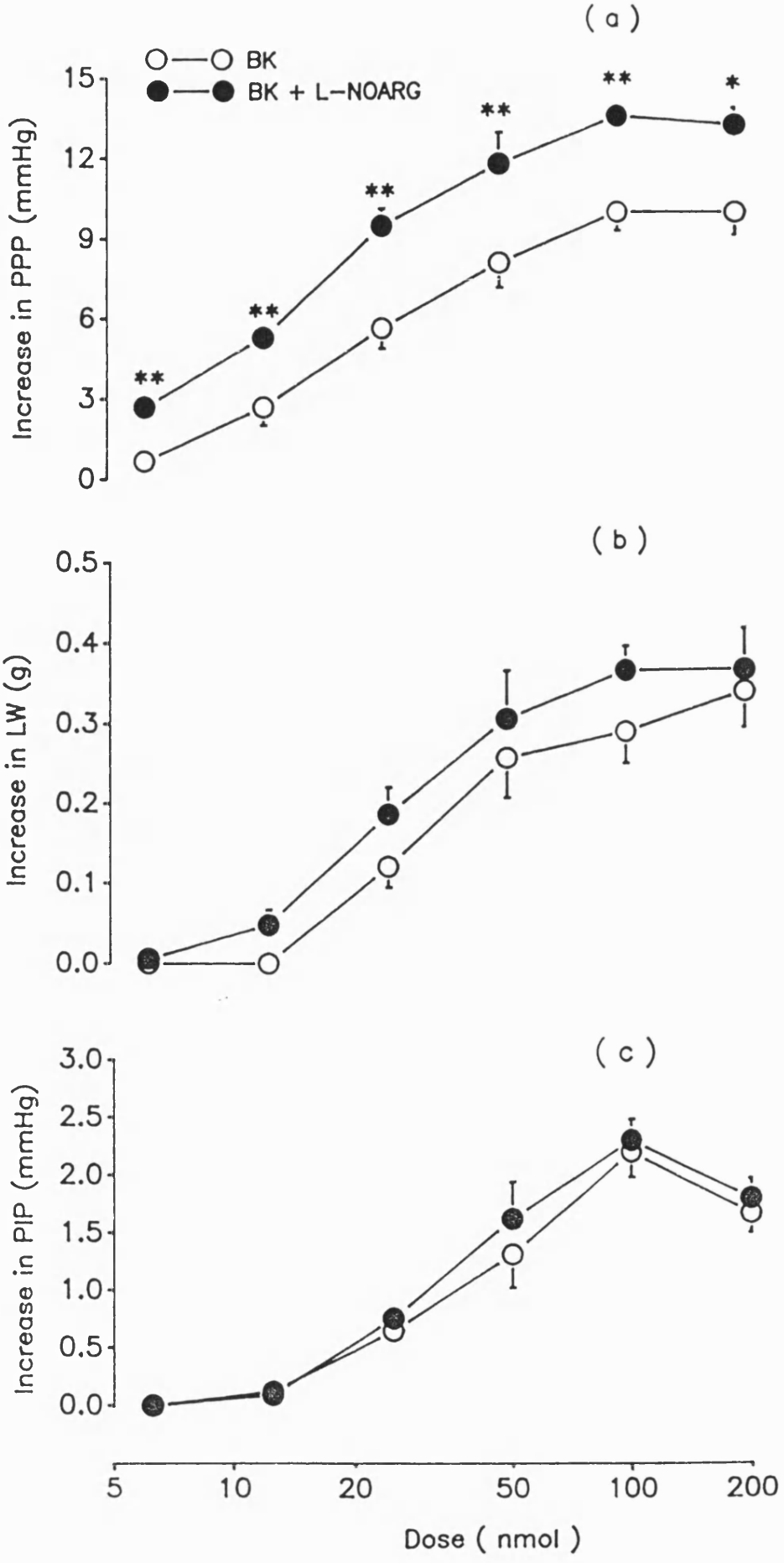
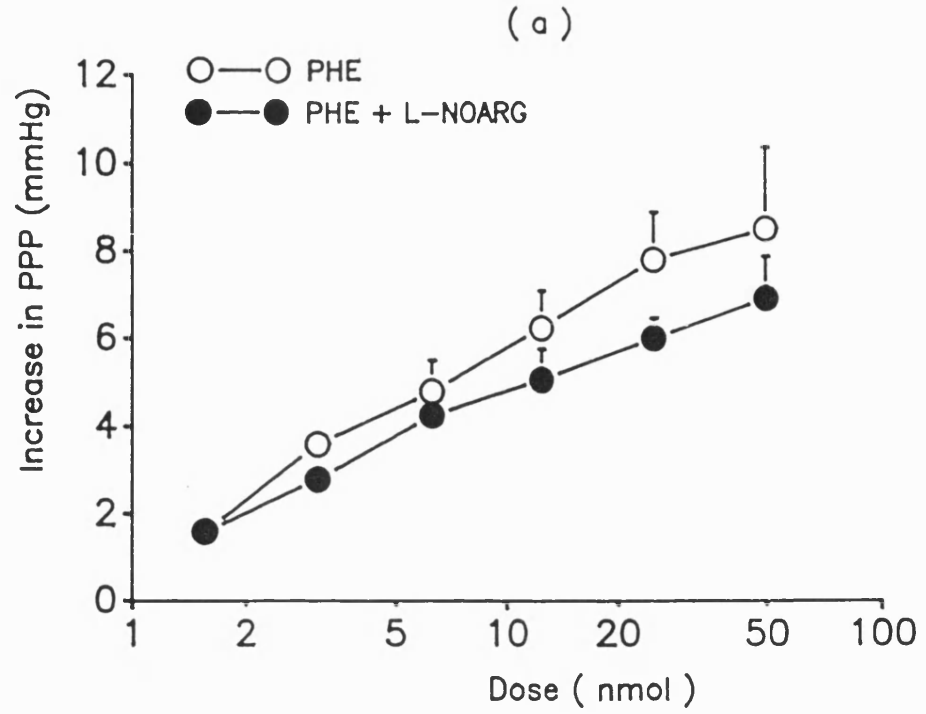
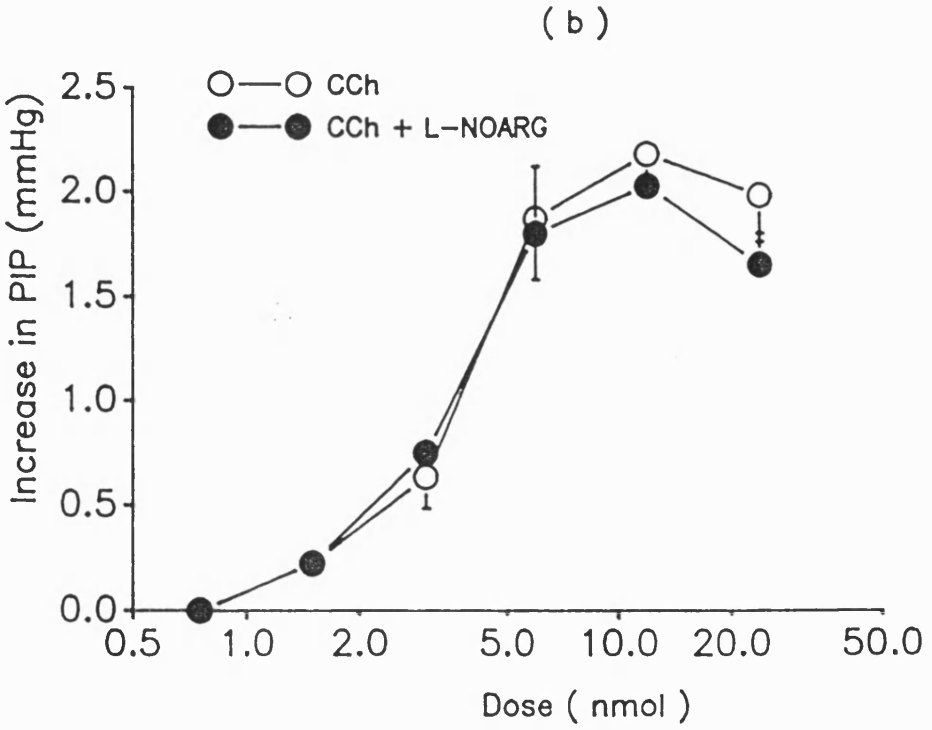

(a)
BK
BK + L-NOARG
Increase in PPP (mmHg)
15
12
9
6
3
0
**
**
**
**
**
*
(b)
Increase in LW (g)
0.5
0.4
0.3
0.2
0.1
0.0
(c)
Increase in PIP (mmHg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
20
50
100
200
Dose (nmol)

Figure 32 The effects of (a) phenylephrine (PHE) on pulmonary perfusion pressure (PPP) and (b) carbachol (CCh) on pulmonary inflation pressure (PIP) in the absence (open circles) and presence of (closed circles) of L-NOARG (100 μM). Each point represents mean ± SEM, n= 3-5 experiments.


(a)
PHE
PHE + L-NOARG
Increase in PPP (mmHg)
12
10
8
6
4
2
0
1
2
5
10
20
50
100
Dose (nmol)


(b)
CCh
CCh + L-NOARG
Increase in PIP (mmHg)
2.5
2.0
1.5
1.0
0.5
0.0
0.5
1.0
2.0
5.0
10.0
20.0
50.0
Dose (nmol)

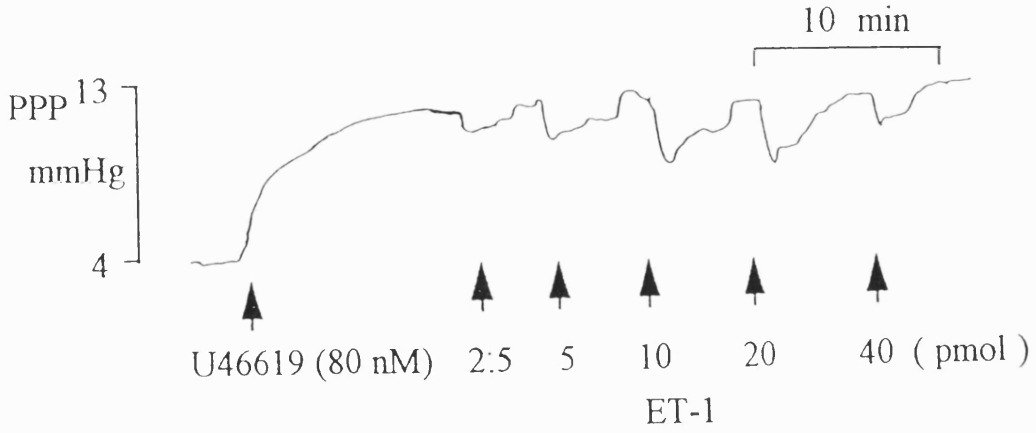
3.12 Effects of ET-1 and SX6C in lungs where vascular tone is increased by infusion of the thromboxane A_2 analogue U46619

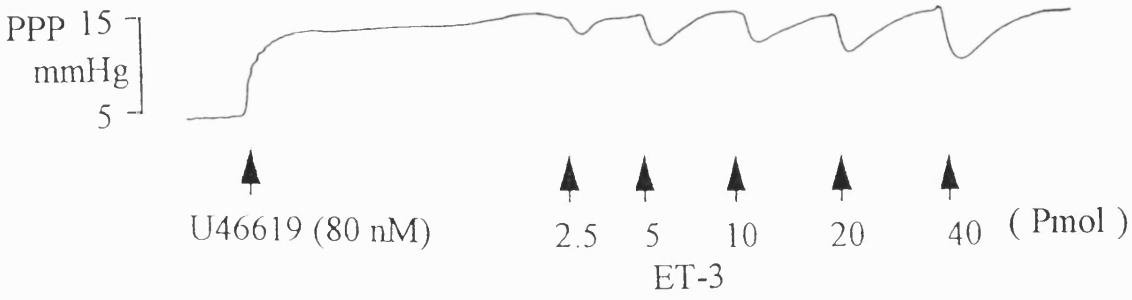
U46619 produced concentration dependent increases in PPP (EC_{50} 31 ± 6 nM, n =3), lung weight and PIP (EC_{50} 37 ± 9 nM, n =3). In subsequent experiments a concentration was selected to produce approximately 80 % of the maximum increase in PPP.

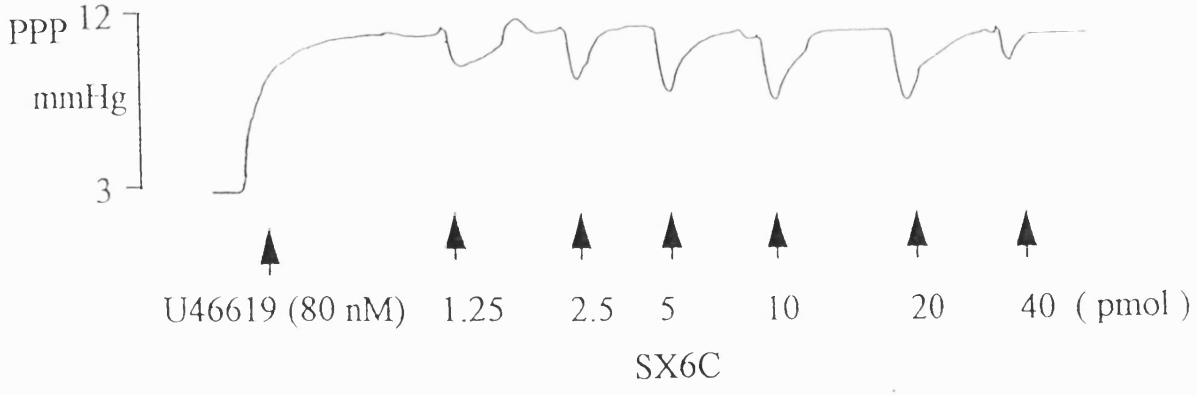
The infusion of U46619 (80 nM) into the pulmonary artery produced an increase in PPP of 9 ± 0.5 mmHg over the basal PPP of 5 ± 0.5 mmHg (n =16). Under these conditions with an elevated PPP bolus injections of ET-1 (2.5-40 pmol), ET-3 (2.5-80 pmol) and SX6C (1.25-40 pmol) all produced dose-dependent falls in PPP (Figure 33, 34).

The vasodilator potencies of ET-1 (ED_{50} 3.6 ± 0.6 pmol, n=5) and ET-3 (ED_{50} 3.9 ± 1.05 pmol, n =4) were similar, however SX6C (ED_{50} 2.2 ± 0.14 pmol, n =7; $p< 0.05$) was significantly more potent as a pulmonary vasodilator than either of the ETs.

Figure 33 Experimental traces showing the elevation of basal pulmonary perfusion pressure (PPP) with U46619 (80 nM) and the fall in PPP in response to (a) ET-1, (b) ET-3 and (c) SX6C. Each trace represents one of 4-7 such experiments.


10 min
PPP 13
mmHg
4
U46619 (80 nM)
2.5
5
10
20
40 (pmol)
ET-1


PPP 15
mmHg
5
U46619 (80 nM)
2.5
5
10
20
40
(Pmol)
ET-3


PPP 12
mmHg
3
U46619 (80 nM)
1.25
2.5
5
10
20
40 (pmol)
SX6C

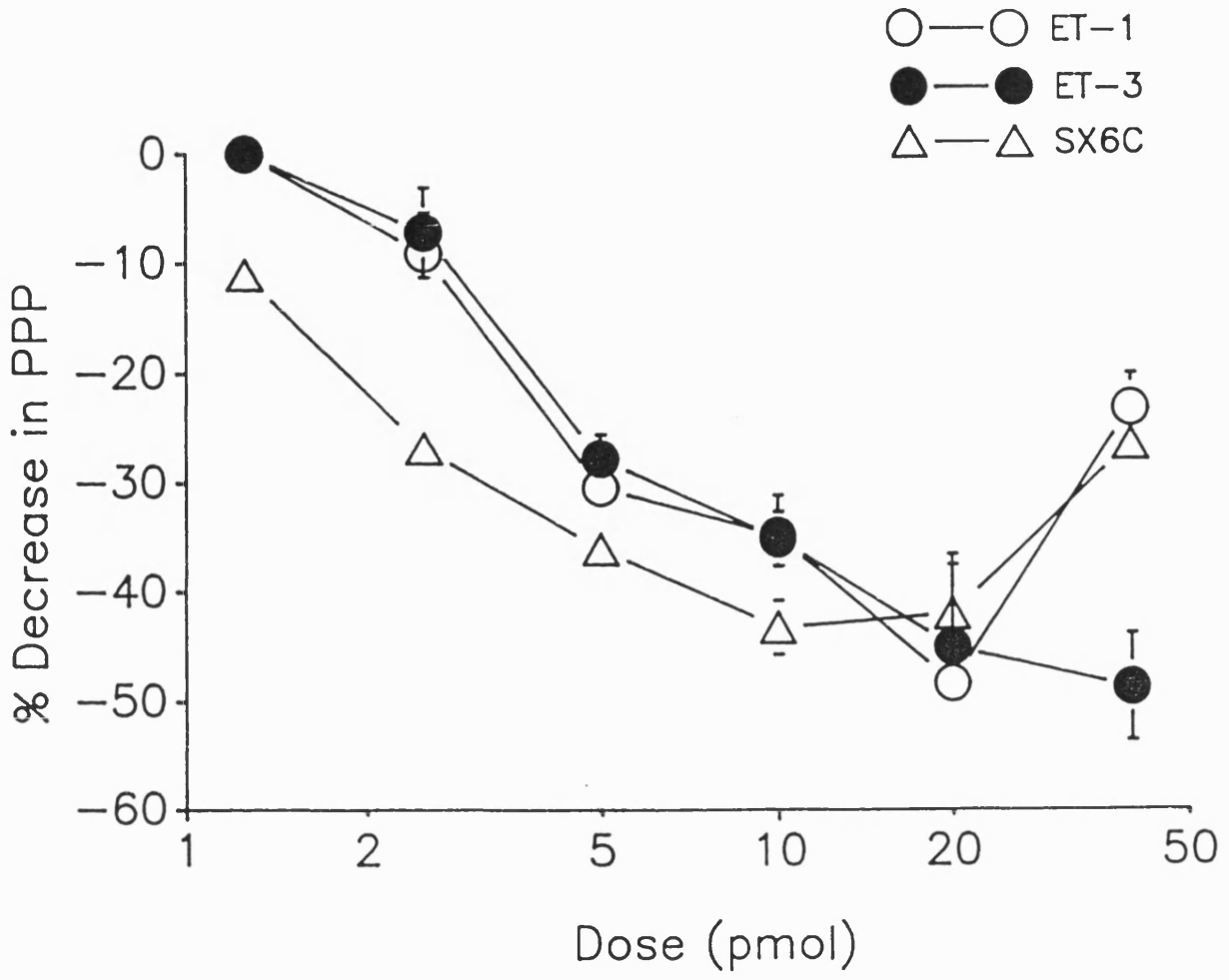


Figure 34 Graph showing the pulmonary vasodilator responses to ET-1 (open circles), ET-3 (filled circles) and SX6C (open triangles) in lungs in which perfusion pressure had been increased withU46619. Each point represents mean ± SEM, n= 4-7 experiments.

3.12.1 Effect of cyclooxygenase inhibitor and ET receptor antagonist on vasodilator responses to ET-1 and SX6C

In many other vascular preparations the vasodilator responses to ETs and SX6C has been reported to be partially mediated by cyclooxygenase and nitric oxide synthase metabolites (Warner et al., 1989; Ekelund et al., 1994). Therefore, experiments were carried out to examine this in lungs.

3.12.1.1 Indomethacin

Inclusion of the cyclooxygenase inhibitor indomethacin (10 μM) did not alter basal PPP (5.6 ± 0.6 mmHg, n=7) or the increase in PPP produced by U46619 (80 nM) (PPP of 10 ± 0.7 mmHg, (n=7) vs. 9 ± 0.5 mmHg, (n= 16) in controls). However, indomethacin did reduce the vasodilator response to low doses of ET-1 (1.25-5 pmol), whereas responses to higher doses of ET-1 (5- 20 pmol) were not affected (Figure 35b). Accumulated results are shown in Figure 37a. ET-1 ED_{50} vasodilator value was significantly increased to 9 ± 3.4 pmol, n=3 ($p< 0.05$) vs. 3.6 ± 0.6 pmol, n=5 in controls. In contrast, indomethacin (10 μM) augmented the decrease in PPP in response to low doses of SX6C (1.25-2.5 pmol). However, responses to higher doses (5-20 pmol) of SX6C were not altered Figure 36b. SX6C ED_{50} (1.7 ± 0.3 pmol, n=4) vasodilator value was not different to the control value quoted above. Accumulated results are shown in Figure 37b.

3.12.1.2 L-NOARG

The nitric oxide synthase inhibitor L-NOARG (100 μM) had no effect on basal PPP (5 ± 0.4 mmHg, n=8). However it augmented PPP increases in response to U46619. Therefore a lower concentration of U46619 was used in order to elevate the PPP by a similar amount seen earlier approximately 9 mmHg.

In a series of experiments 40 nM of U46619 produced an increase in PPP of 8.5 ± 0.8 mmHg, n=8. Figure 35c illustrates that in the presence of L-NOARG a low dose of ET-1 (1.25 pmol) produced vasodilation, whereas, at higher doses (5-20 pmol) vasoconstrictor responses were seen. Accumulated results from 4 different experiments are shown in Figure 38.

In a different set of experiments perfusion of indomethacin (10 μM) and L-NOARG (100 μM) in combination had no effect on basal PPP (5 ±0.5 mmHg, n=3) or the increase in PPP in response to U46619 40 nM (10.6 ± 1.3 mmHg, n=4). However, this combination of inhibitors completely abolished the vasodilator response to ET-1, whereas 5- 20 pmol of ET-1 now produced marked rises in PPP. Accumulated results from 3 different experiments are shown in Figure 38.

In contrast to ET-1-induced vasodilation the vasodilator responses to SX6C (1.25-20 pmol) were completely abolished in the presence of L-NOARG (100 μM). The highest dose of SX6C (20 pmol) now produced an increase in PPP (Figure 36c). Accumulated results are shown in Figure 39.

Perfusion of L-NOARG (100 μM) had no effect on vasodilation produced by the nitric oxide donor sodium nitroprusside (SNP) 10-1000 pmol (Figure 40). This shows that the effects of L-NOARG on ET-1 and SX6C responses were not due to non-specific action.

3.12.1.3 Nitro-D-arginine (D-NOARG)

To check the stereoselectivity of L-NOARG, experiments were carried out in the presence of the inactive isomer D-NOARG. Inclusion of D-NOARG (100 μM) into the perfusate had no effect on basal PPP (5 ± 0.6 mmHg, n=8) or the rise in PPP (9.2 ± 0.9 mmHg, n= 8 vs. control value quoted above) in response to U46619 (80 nM). Similarly, D-NOARG did not alter the vasodilation produced by ET-1 (2.5- 40 pmol) (Figure 35e) or SX6C (1.25- 40 pmol) (Figure 36d).

Accumulated results are shown in Figure 38 and 39. ED_{50} values for ET-1 and SX6C in the presence and absence of D-NOARG were ET-1, 3.2 ± 1.2 pmol, n=4 vs. 3.6 ± 0.6 pmol, n=5 in controls and SX6C, 2.3 ± 0.6 pmol, n=4 vs. 2.2 ± 0.14 pmol, n=7 in controls.

Figure 35 Experimental traces showing the elevation of basal pulmonary perfusion pressure (PPP) with U46619 (40-80 nM) and the subsequent changes in PPP in response to ET-1 in (a) control, (b) in the presence of indomethacin (10 μM), (c) in the presence of L-NOARG (100 μM), (d) in the presence of indomethacin (10μM) plus L-NOARG (100 μM) and (e) in the presence of D-NOARG (100 μM). Each trace is representative of 3-5 similar experiments.

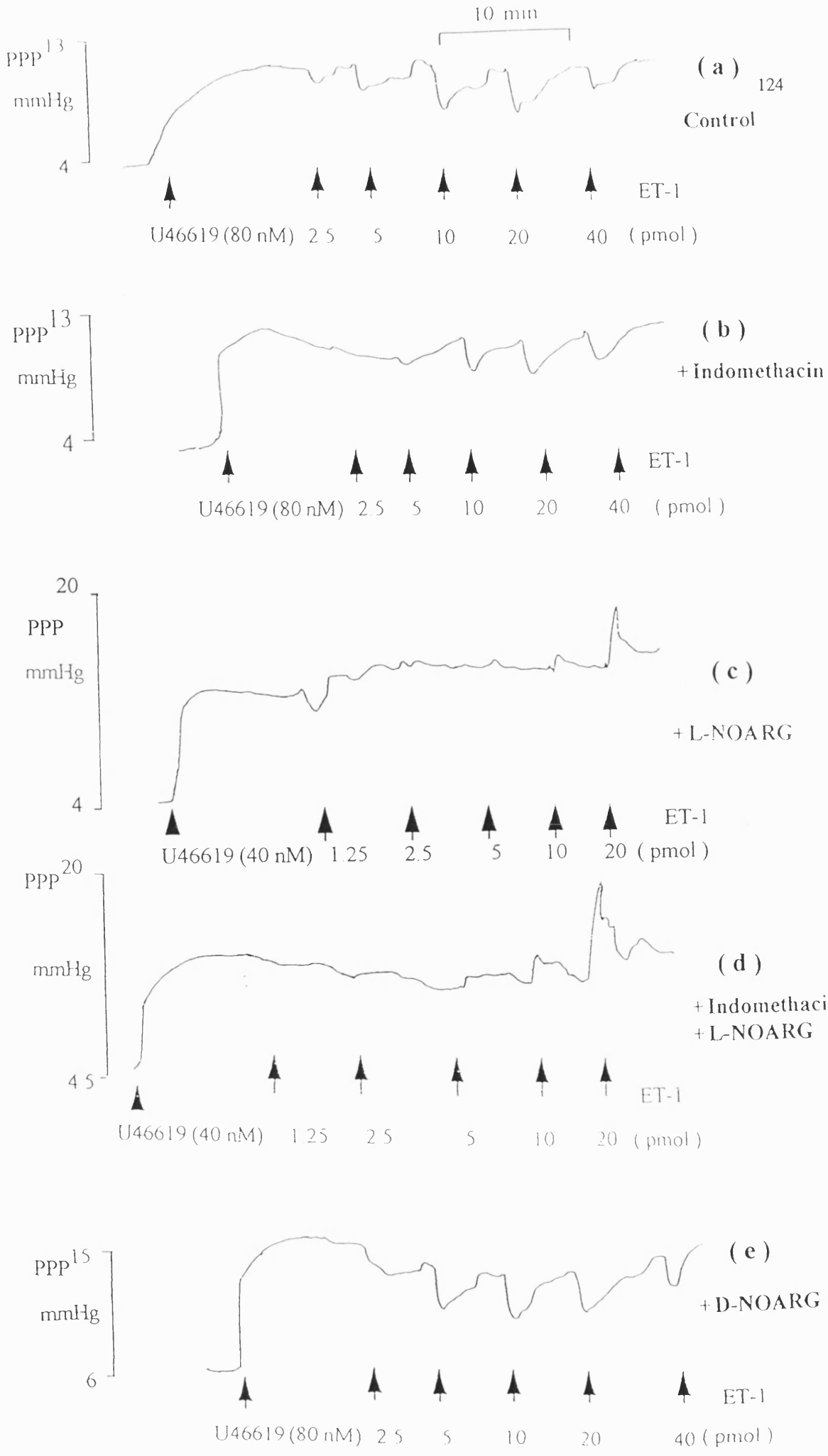

10 min
PPP 13
mmHg
4
(a)
124
Control
ET-1
U46619 (80 nM) 2.5 5 10 20 40 (pmol)
PPP 13
mmHg
4
(b)
+ Indomethacin
ET-1
U46619 (80 nM) 2.5 5 10 20 40 (pmol)
20
PPP
mmHg
4
(c)
+ L-NOARG
ET-1
U46619 (40 nM) 1.25 2.5 5 10 20 (pmol)
PPP 20
mmHg
4.5
(d)
+ Indomethacin
+ L-NOARG
ET-1
U46619 (40 nM) 1.25 2.5 5 10 20 (pmol)
PPP 15
mmHg
6
(e)
+ D-NOARG
ET-1
U46619 (80 nM) 2.5 5 10 20 40 (pmol)

Figure 36 Experimental traces showing the elevation of basal pulmonary perfusion pressure (PPP) with U46619 (40-80 nM) and the subsequent changes in PPP in responses to SX6C (a) control (b) in presence of indomethacin (10 μM) (c)in the presence of L-NOARG (100 μM) and (d) plus D-NOARG (100 μM). Each trace is one of 4-7 similar experiments.

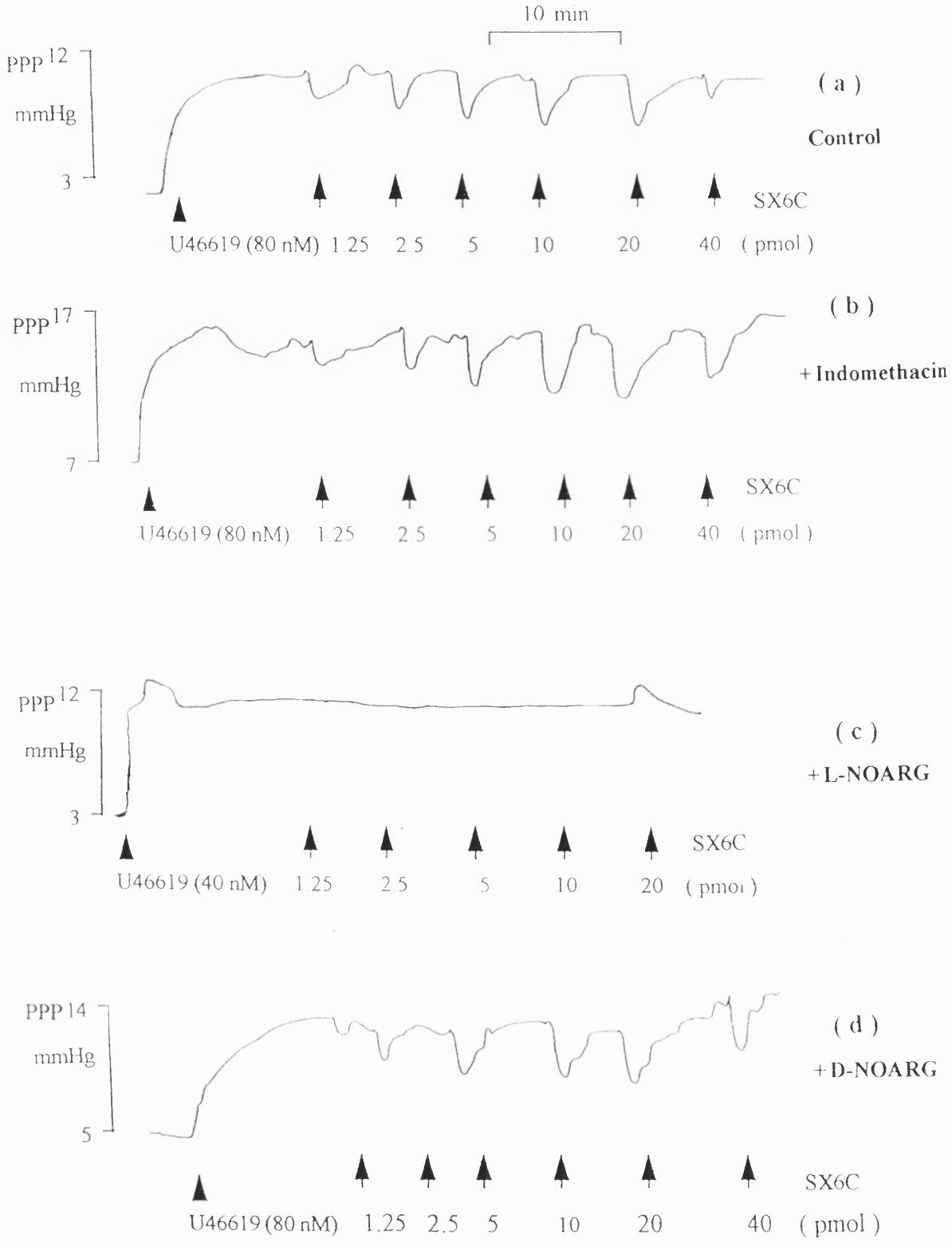
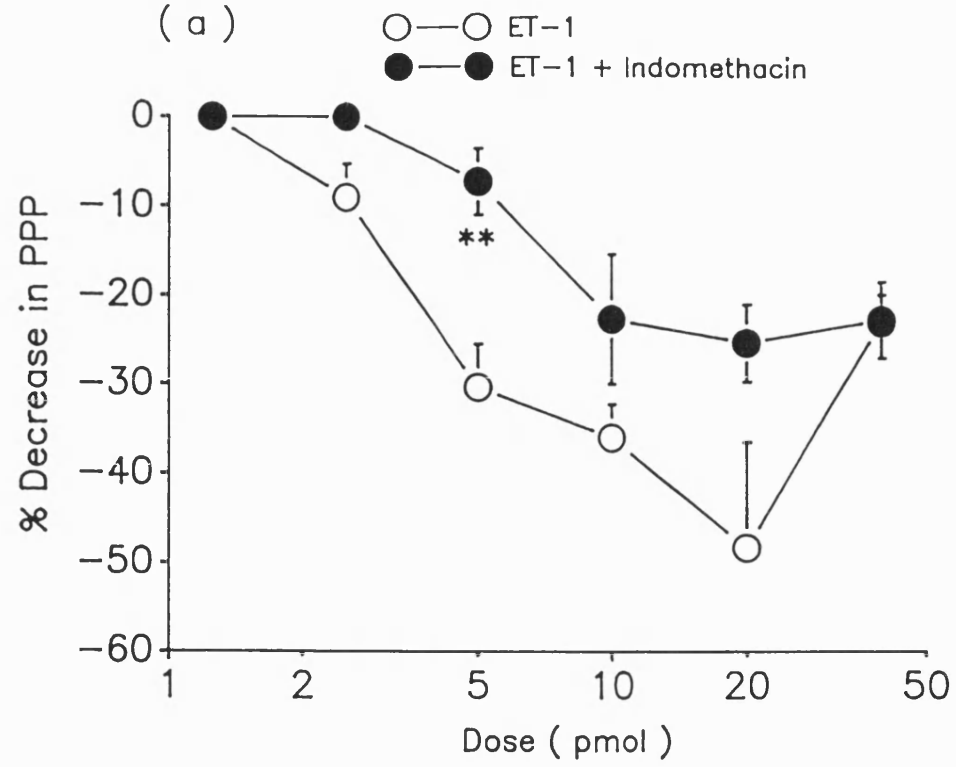
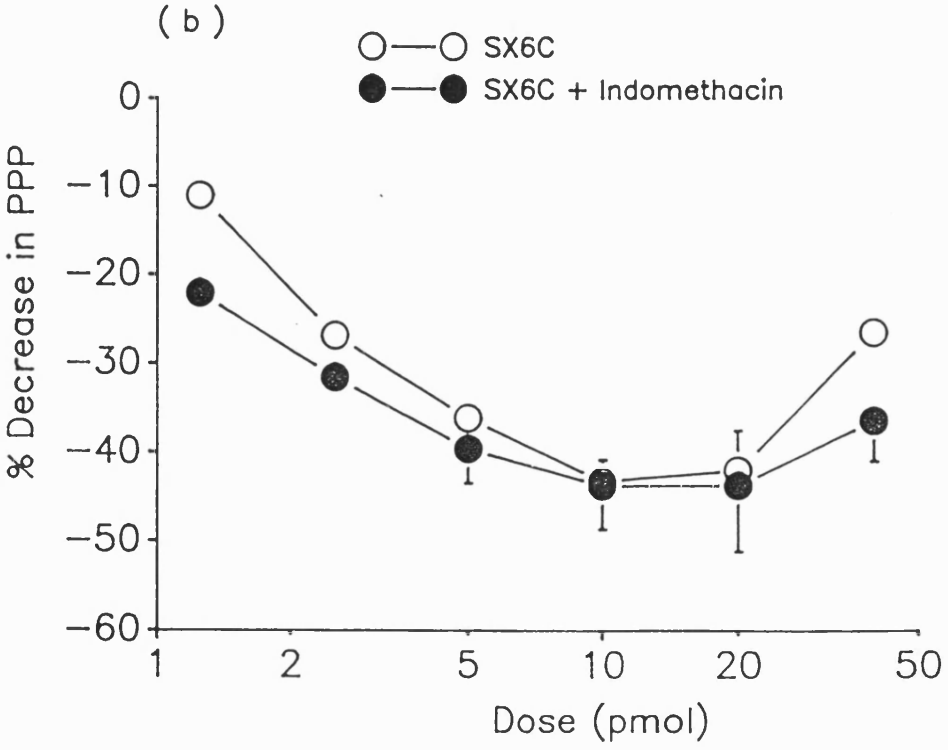

10 min
PPP 12
mmHg
3
(a)
Control
SX6C
U46619 (80 nM) 1.25 2.5 5 10 20 40 (pmol)
PPP 17
mmHg
7
(b)
+ Indomethacin
SX6C
U46619 (80 nM) 1.25 2.5 5 10 20 40 (pmol)
PPP 12
mmHg
3
(c)
+ L-NOARG
SX6C
U46619 (40 nM) 1.25 2.5 5 10 20 (pmol)
PPP 14
mmHg
5
(d)
+ D-NOARG
SX6C
U46619 (80 nM) 1.25 2.5 5 10 20 40 (pmol)

Figure 37 Graphs showing the pulmonary vasodilator response to (a) ET-1 and (b) SX6C in the absence (open circles) and presence (filled circles) of indomethacin (10 μM) in U46619 pre-treated preparations. Each point represents mean ± SEM, n= 3-7 experiments. *$p < 0.05$ (Student's t-test).


(a)
ET-1
ET-1 + Indomethacin
0
−10
−20
−30
−40
−50
−60
% Decrease in PPP
**
1
2
5
10
20
50
Dose (pmol)


(b)
SX6C
SX6C + Indomethacin
0
−10
−20
−30
−40
−50
−60
% Decrease in PPP
1
2
5
10
20
50
Dose (pmol)

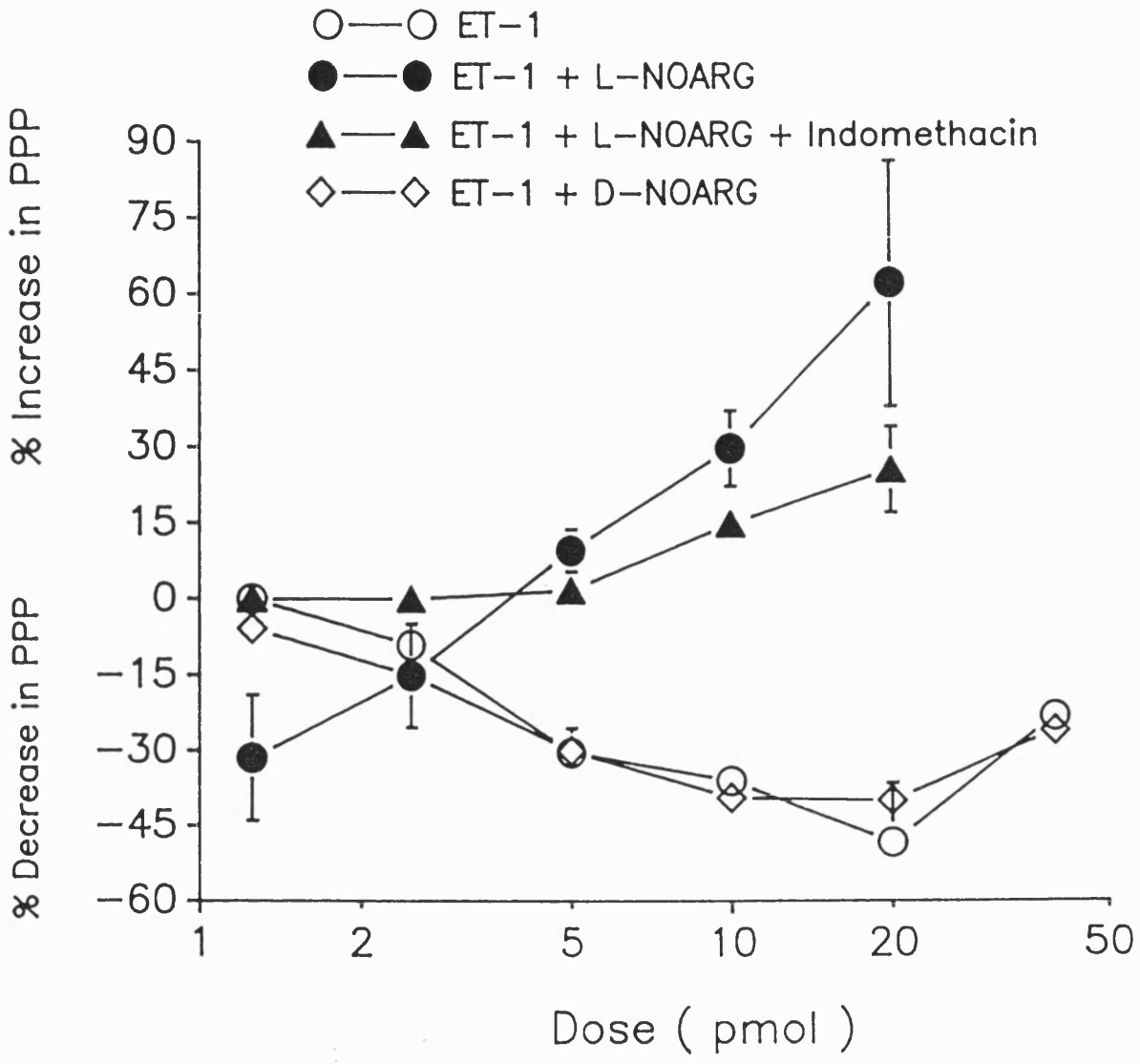


Figure 38 Graph showing the pulmonary vascular responses to ET-1: controls (open circles), in the presence of D-NOARG (100 μM) (open squares), L-NOARG 100μM (filled circles)and indomethacin (10 μM) plus L-NOARG (100 μM) (filled triangles) in U46619 pre-treated preparations. Each point represents mean ± SEM, n=4-5.

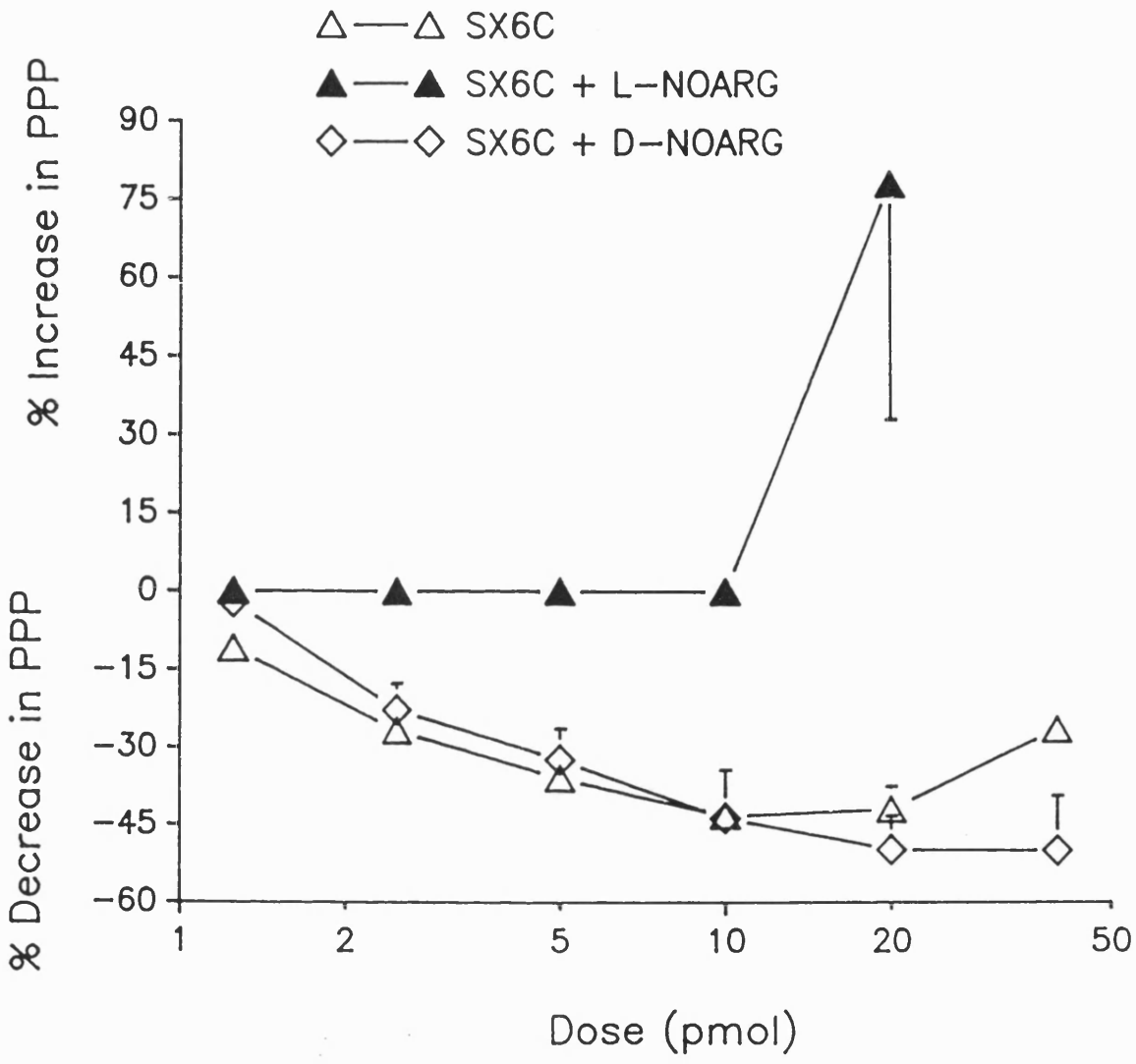


Figure 39 Graph showing the pulmonary vascular responses to SX6C controls, n= 7 (open triangles), and in the presence of; D-NOARG (100 μM), n= 4 (open squares), L-NOARG 100μM, n= 4 (filled triangles), in U46619 pre-treated preparations. Each point represents mean ± SEM.

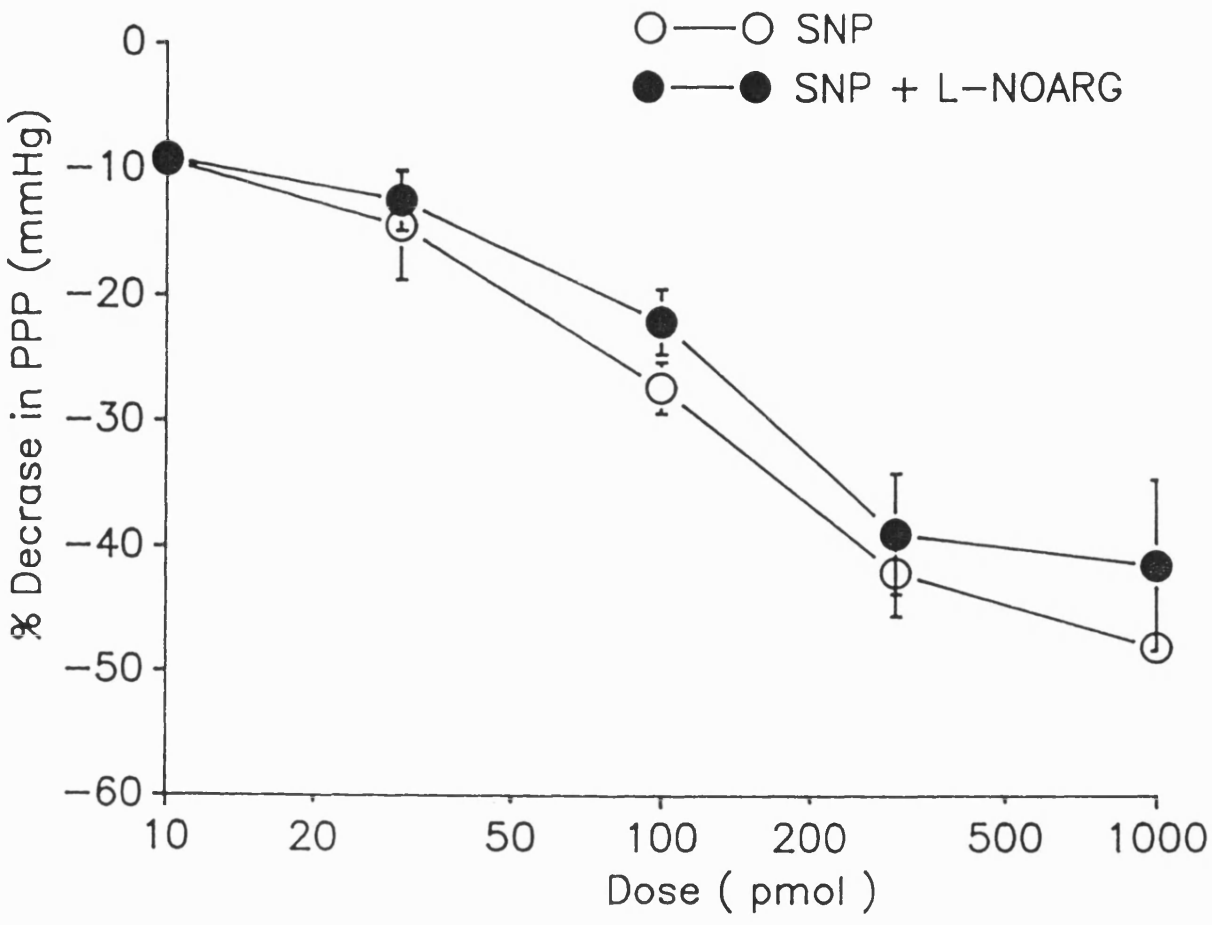


Figure 40 Graph showing the pulmonary vasodilator responses to sodium nitroprusside (SNP) in the absence (open circles) or presence (filled circles) of L-NOARG (100 μM) in U46619 pre-treated preparations. Each point represents mean ± SEM, n= 4

3.12.1.4 BQ123 (selective ET_A receptor antagonist)

Inclusion of BQ123 (10 μM) in the perfusate did not alter basal PPP or the increase in PPP in response to U46619 (80 nM), n=3.

Figure 41b illustrates, that in U46619 preconstricted preparations BQ123 was without any marked effect on the vasodilator responses to ET-1 (2.5-40 pmol). Accumulated results are shown in Figure 42a (ET-1 ED_{50} 5.6 ± 1 pmol, n=3 vs. 3.6 ± 0.6 mmHg, n= 5 in controls).

3.12.1.5 Bosentan (Ro47-0203, mixed endothelin ET_A / ET_B receptor antagonist)

The effects of the mixed ET_A/ET_B receptor antagonist bosentan were studied on pulmonary vasodilator responses to ET-1 and SX6C. Perfusion of bosentan (5 μM) had no effect on basal PPP (5.1 ± 0.5 mmHg, n=8). The infusion of U46619 (80 nM) produced an increase in PPP of 9.25 ± 0.6 mmHg, n=8. Figure 41c and 41e illustrate that bosentan completely abolished ($p< 0.001$) the decreases in PPP produced in response to ET-1 (1.25- 40 pmol) or SX6C (1.25 - 40 pmol). Accumulated results are shown in Figure 42.

To check the selectivity of actions, the effects of bosentan on SNP-induced pulmonary vasodilator responses were examined. Bosentan (5 μM) had no effect on SNP-induced decreases in PPP (100 pmol of SNP in the presence of bosentan produced fall in PPP of 28 % ± 4, n=3 vs. 27 % ± 2 in control lungs).

Figure 41 Experimental traces showing the falls in PPP in response to ET-1; (a) control, (b) in the presence of BQ123 (10 μM), (c) in the presence of bosentan (5 μM). SX6C ; (d) control or (e) in the presence of bosentan (5 μM). Each trace is representative of 3-7 similar experiments.

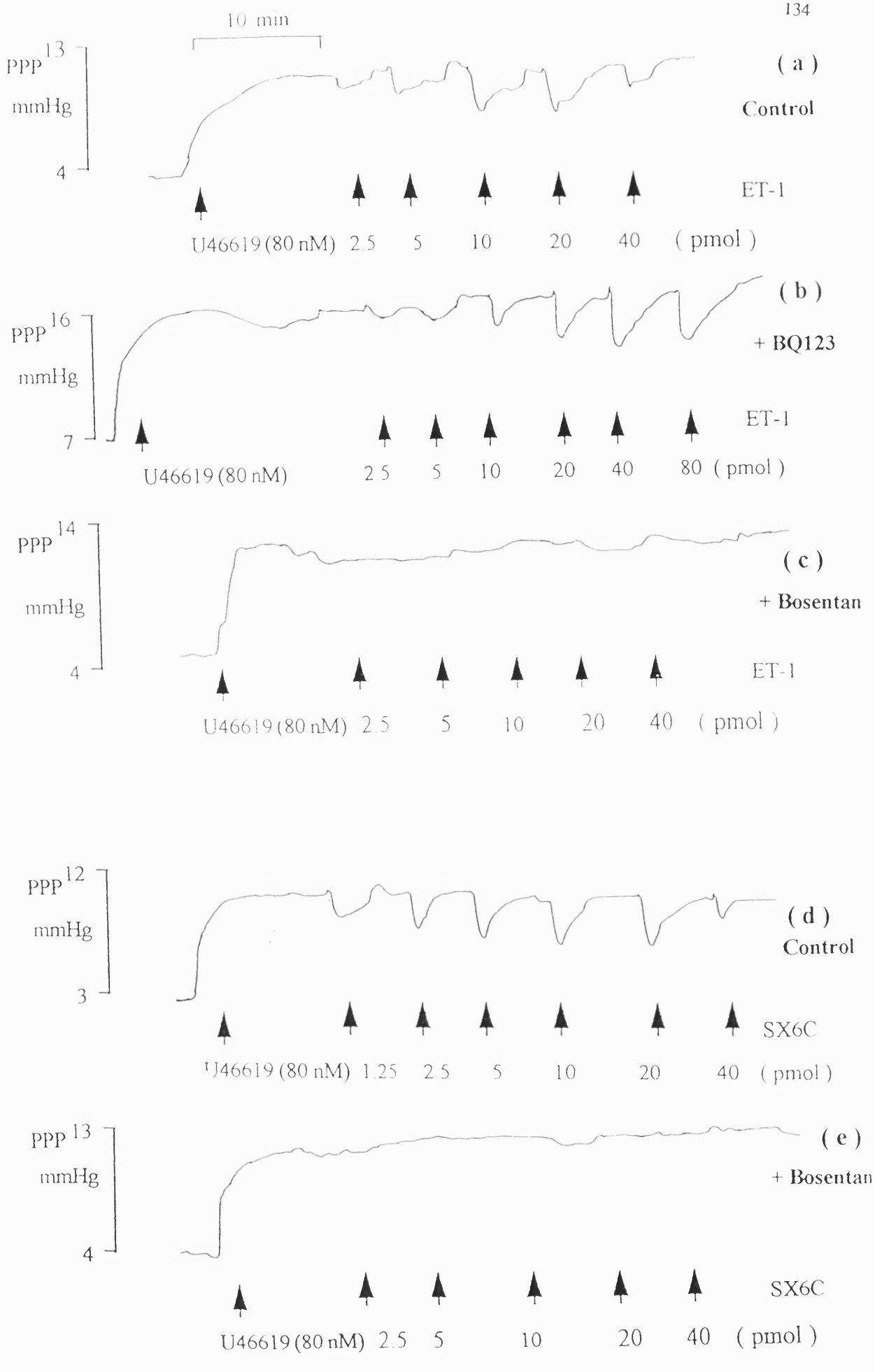
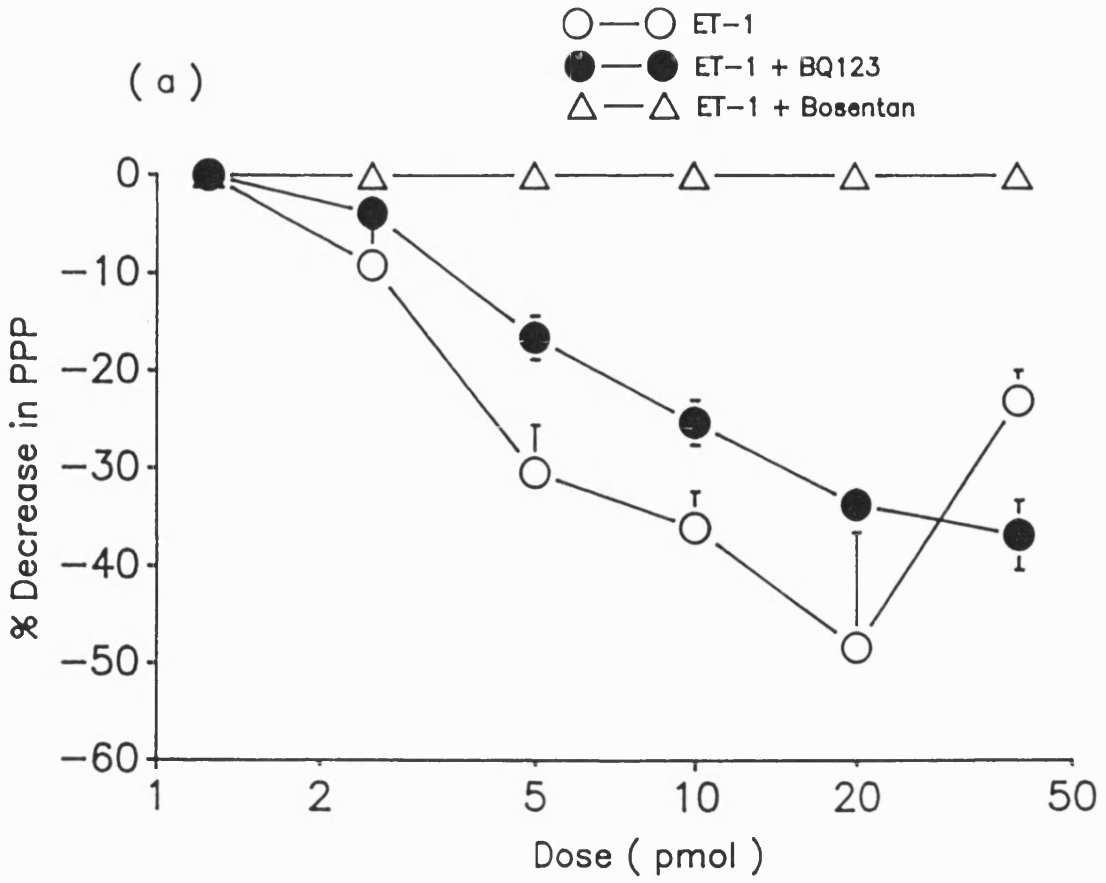
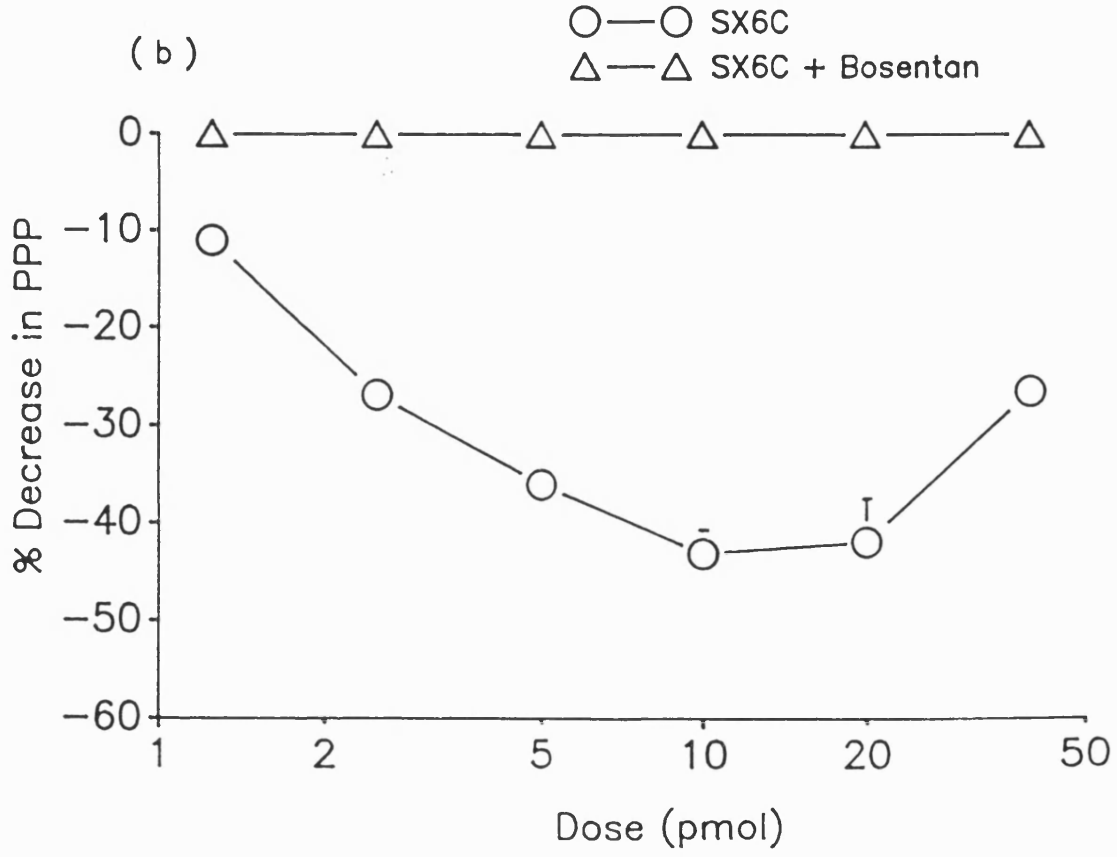

10 min
PPP 13
mmHg
4
(a)
Control
ET-1
U46619 (80 nM) 2.5 5 10 20 40 (pmol)
PPP 16
mmHg
7
(b)
+ BQ123
ET-1
U46619 (80 nM) 2.5 5 10 20 40 80 (pmol)
PPP 14
mmHg
4
(c)
+ Bosentan
ET-1
U46619 (80 nM) 2.5 5 10 20 40 (pmol)
PPP 12
mmHg
3
(d)
Control
SX6C
U46619 (80 nM) 1.25 2.5 5 10 20 40 (pmol)
PPP 13
mmHg
4
(e)
+ Bosentan
SX6C
U46619 (80 nM) 2.5 5 10 20 40 (pmol)

Figure 42 Graphs showing the pulmonary vasodilator responses to (a) ET-1) (b) SX6C alone (open circles)or in the presence of BQ123 (10 μM) (filled circles) or bosentan (5 μM) (open triangles) in U46619 pre-treated preparations. Each point represents mean ± SEM, n= 3-7 experiments.


(a)
ET-1
ET-1 + BQ123
ET-1 + Bosentan
% Decrease in PPP
0
−10
−20
−30
−40
−50
−60
1
2
5
10
20
50
Dose (pmol)


(b)
SX6C
SX6C + Bosentan
% Decrease in PPP
0
−10
−20
−30
−40
−50
−60
1
2
5
10
20
50
Dose (pmol)

3.13 Tachyphylaxis to ETs and SX6C

Although dose-response curves to the vasodilator effects of ET-1 and SX6C could be constructed, it was noted that repeated injections of the dose which produced a maximum vasodilation, caused rapid loss of response. Therefore, a series of experiments were carried out to study this phenomena in more detail. In these experiments the infusion of U46619 (80 nM) produced an increase in PPP of 9 ± 0.6 mmHg, n=3.

The effects of several repeated injections of ET-1 or SX6C (20 pmol each) are shown in Figure 43. As shown the dilator responses to ET-1 (Figure 43a) and SX6C (Figure 43b) given repeatedly at 5-7 min intervals showed a marked tachyphylaxis. As can be seen, the fifth dose of ET-1 (panel A) actually produced vasoconstriction rather than a vasodilator response. The final portion of the recording shows that injection of a series of doses of SX6C after the ET-1 injections also produced dose-dependent increases in PPP. This contrasts markedly with the results shown in panel B of Figure 43 where SX6C injected before ET-1 caused marked depressor responses which decreased in magnitude with repeated injections. As can be seen, the sixth injection actually produced a vasoconstrictor as opposed to a vasodilator response. Subsequent injections of ET-1 now caused dose-dependent increases in PPP. Accumulated data is shown in Figure 44.

Figure 43 Experimental traces showing the tachyphylaxis / cross tachyphylaxis of the vasodilator response to (a) ET-1, (b) SX6C. Each trace represents one of 3 separate experiments.

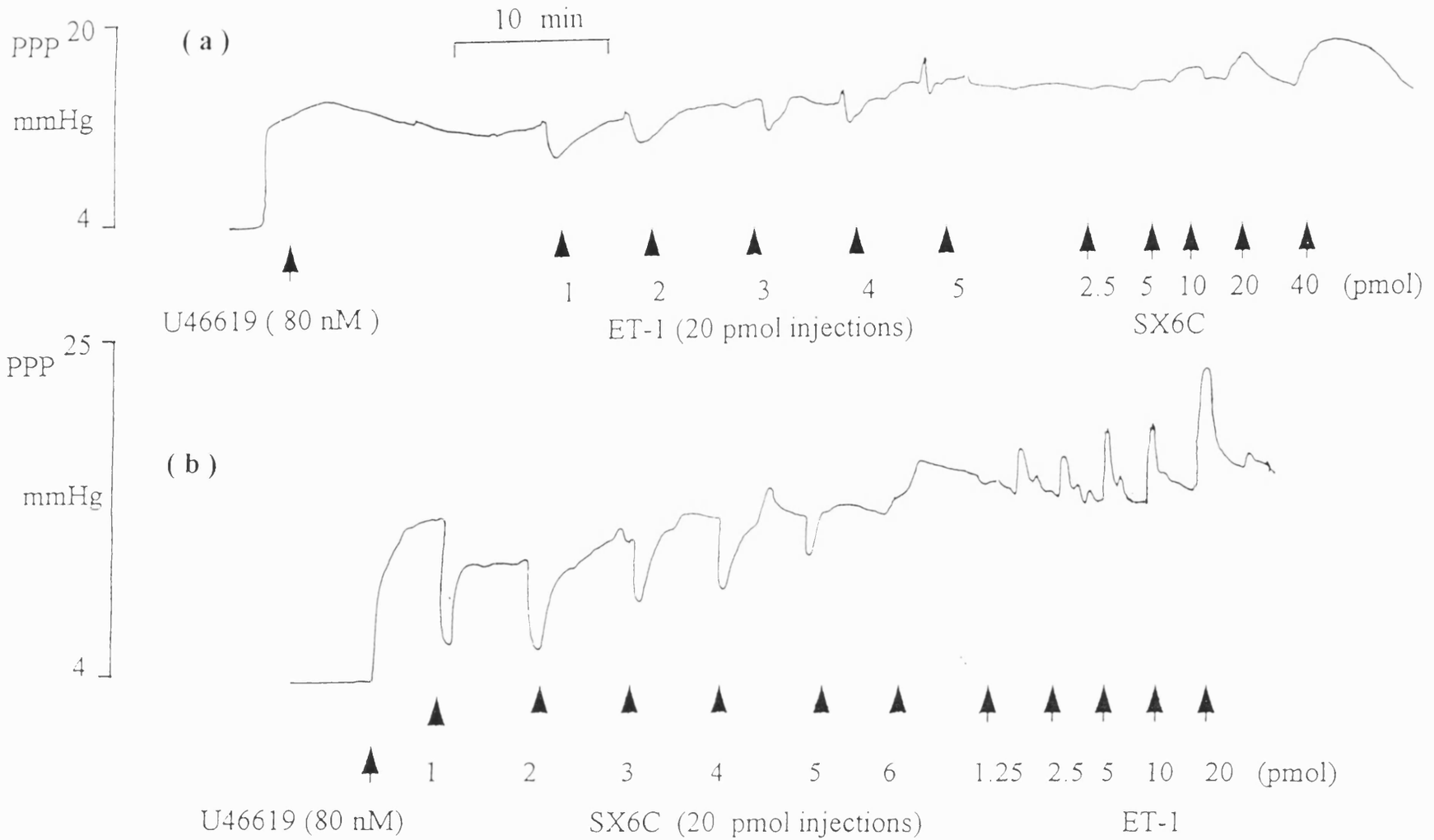
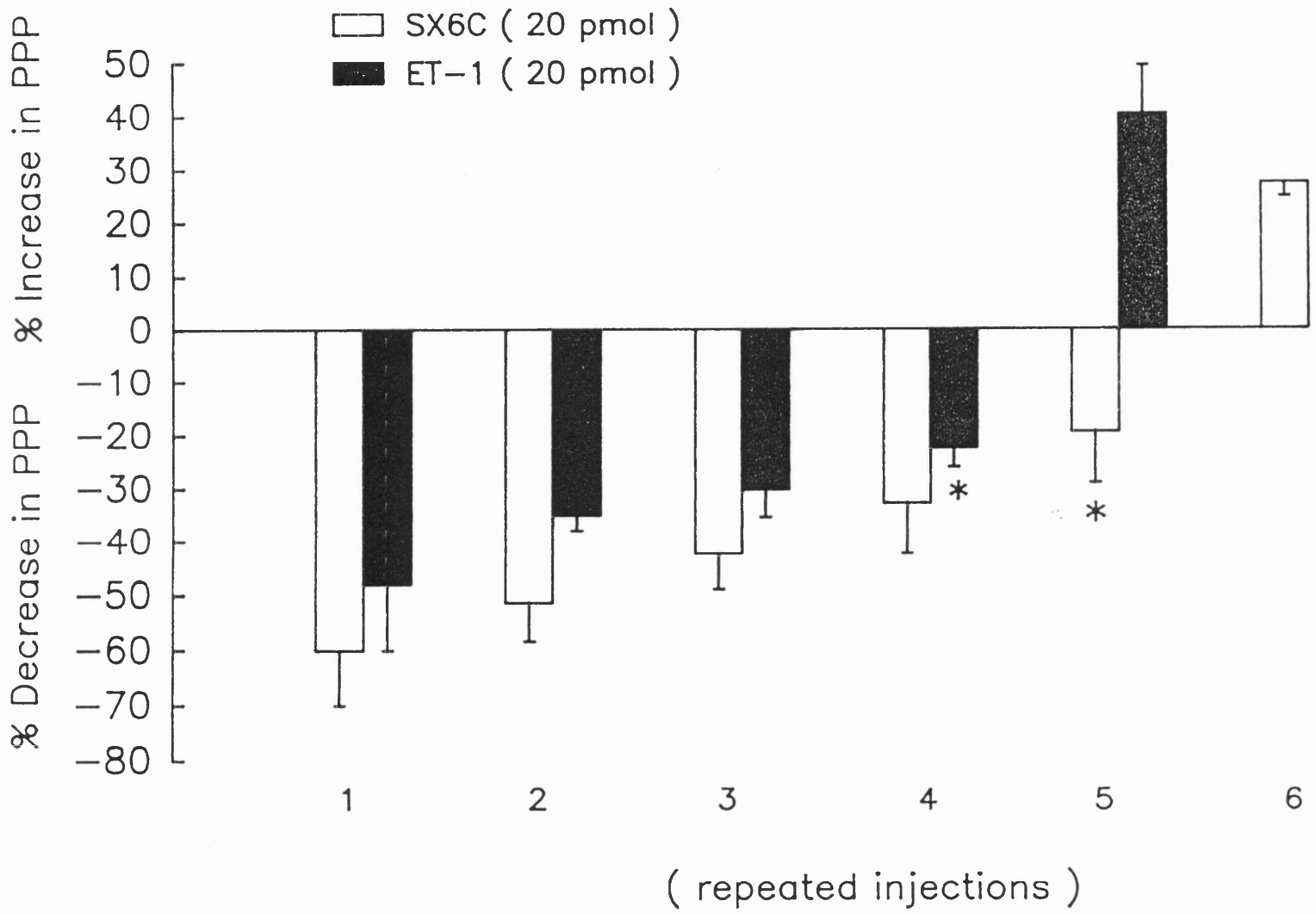

PPP 20
mmHg
4
(a)
10 min
U46619 (80 nM)
1 2 3 4 5
ET-1 (20 pmol injections)
2.5 5 10 20 40 (pmol)
SX6C
PPP 25
mmHg
4
(b)
U46619 (80 nM)
1 2 3 4 5 6
SX6C (20 pmol injections)
1.25 2.5 5 10 20 (pmol)
ET-1

Figure 44 Histogram showing the tachyphylaxis to the pulmonary vasodilator responses to ET-1 (filled columns) and SX6C (open columns) in U46619 pre-treated preparations. Each histogram represents mean ± SEM, n= 3 for each agonist. *$p < 0.05$; 1 way ANOVA.


SX6C (20 pmol)
ET−1 (20 pmol)
% Increase in PPP
% Decrease in PPP
50
40
30
20
10
0
−10
−20
−30
−40
−50
−60
−70
−80
1
2
3
4
5
6
*
*
(repeated injections)

SECTION 4

Discussion

Isolated perfused lungs have been used for a long time to study aspects of pulmonary function. However, no generally accepted criteria for the viability of such preparations have yet been defined. The present experiments have showed that under the conditions described this model is viable for 2 h as; (a) there was no increase in lung weight noticed within this period indicating that lung integrity was not altered, moreover PPP and PIP remained stable, (b) the responses to PHE, CCh, BK and angiotensin-I were reproducible indicating that reactivity to various agonists was not changed within this 2 h of perfusion time, (c) the metabolic capability of the endothelial cells was preserved, as the responses to angiotensin-I reappeared after the angiotensin converting enzyme inhibitor captopril was washed out.

A single-pass perfusion model was utilised rather than a recirculating one as any substance robust enough to survive passage through the pulmonary circulation would continue to act in the latter, making quantitation of effects complex. Using single-pass perfusion also avoids accumulation of any products released by the lungs into the perfusate which could influence lung function. In this lung preparation plasma expanders were not included into the perfusate because of the cost. However, in many previously described lung preparations recirculation procedures have been used in the presence of plasma expander such as dextran, albumin, ficoll, etc. (Selig *et al.,* 1988; Drazen *et al.,* 1989; Westcott *et al.,* 1990; Czartolomna *et al.,* 1991; Seale *et al.,* 1991; Bonvallet *et al.,* 1993; Pino *et al.,* 1992; Uhlig and Wollin, 1994) to offset oedema in lung models.

Rate of perfusion is easily varied and is usually a "trade-off" between maintaining tissue viability yet avoiding higher rates which increase the rate of oedema formation through hydrostatic damage. Results from the present study indicate that even at low rates of perfusion (5 ml min^{-1}) lung integrity is relatively short-lived as increases in lung weight started to occur after 2h of perfusion. Use of higher flow rate (10 ml min^{-1}) in this model produced quick oedema formation. Fisher *et al.,* (1980) have also shown that rat lungs perfused at 12-25 ml min-1 with Krebs' solution alone developed gross alveolar oedema

after 1 h and the addition of 3 % bovine serum albumin at these higher flow rates increased the lung viability to 2-3 hours.

In the present model lungs were ventilated at a volume and rate which did not cause any significant increase in lung weight over 2 h, as even moderate overinflation is known to cause lung injury (Cilley *et al.*, 1993). In addition, investigators have suggested that overinflation of the lung can lead to stress failure, a cause of increased capillary permeability and pulmonary oedema (West and Mathieu-Costello, 1992).

4.1 Differential effects of PHE, CCh, and BK

In order to evaluate the actions of vasoactive agents on the three monitored lung parameters initial experiments were carried out by using PHE, CCh and BK. Experiments showed that under the conditions described these three substances exerted differential pulmonary vascular and bronchial effects. PHE-produced selective dose-related increases in PPP, CCh-produced dose-dependent increases in PIP without having any effect on PPP and lung weight, whereas BK-produced dose-related increases in PPP, lung weight and PIP.

Indomethacin did not alter any of the basal parameters recorded suggesting that cyclooxygenase products do not play a major role in regulating basal pulmonary vascular or bronchial smooth muscle tone. Indomethacin also had no effect on the pulmonary vascular and bronchial responses to PHE and CCH or BK respectively. However, it significantly potentiated the increase in the PPP response to the highest dose of BK but responses to lower doses, and the ED_{50} values were not affected by indomethacin. This suggests that the release of cyclooxygenase products is not a major factor in lung responses to PHE, CCH and low doses of BK. However, the higher doses of BK may produce the release of vasodilator prostanoids which would inhibit the vasoconstrictor effects of BK. In another report the cyclooxygenase inhibitor aspirin potentiated the BK-induced responses *in vivo* (Bhoola *et al.*, 1962). BK has been reported to release

prostacyclin from the perfused lung preparation (Bakhle *et al.*, 1985), which can produce relaxations of porcine isolated pulmonary blood vessels (Zellers *et al.*, 1994). Therefore, inhibition of PGI_2 production by indomethacin could explain why it potentiated the constrictor actions of higher doses of BK.

In the present experiments the pulmonary vasoconstrictor action of PHE was antagonized by a selective α_1-adrenoceptor antagonist prazosin. Previous reports have demonstrated the presence of α_1- adrenoceptors on the isolated pulmonary blood vessels and in the pulmonary vascular bed of rabbit, cat, and dog (Barnes and Liu, 1995). The bronchoconstrictor action of CCh was markedly attenuated with atropine suggesting that the action of CCh is mediated via activation of muscarinic receptors (Post *et al.*, 1991).

The selective BK_2 receptor antagonist D-Arg-[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin (Regoli *et al.*, 1986; Schachter *et al.*, 1987; Steranka *et al.*, 1989) inhibited BK-induced increases in PPP and lung weight and PIP suggesting that the responses to BK are mediated via activation of BK_2 receptors. These findings are in agreement with a recently reported BK receptor binding investigation in rat lung membranes *in vivo* and *in vitro* (Tsukagoshi *et al.*, 1995). Studies in other vascular beds have shown that BK_2 receptors are located on the venous smooth muscles which mediate the permeability effects of BK (Murray *et al.*, 1991). D-Arg-[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin also blocks BK- induced vascular permeability in rabbit skin *in vitro* (Schachter *et al.*, 1987). Similarly, BK_2 receptors have been reported in human isolated bronchi (Molimard *et al.*, 1995) and guinea pig lung parenchyma (Lach *et al.*, 1994).

4.1.2 Pulmonary perfusion pressure and lung weight

PHE was more potent in producing increases in PPP than BK. This is possibly because of extensive inactivation of BK which occurs in the pulmonary circulation (Alabaster and Bakhle, 1972; Baker *et al.*, 1992; Pesquero *et al.*, 1992;). The potency of BK was significantly increased in the presence of captopril.

The increases in PPP produced by PHE were not associated with changes in lung weight Although BK also increased PPP such a precapillary action of BK is unlikely, as BK is known to produce potent arteriolar vasodilation in many vascular beds (Bonner *et al.,* 1990; Feletou *et al.,* 1995). Toda (1977) showed that BK tends to relax arterial smooth muscle and constrict venous smooth muscle both in human and animals. In the present model the increases in PPP seen with low doses of BK were associated with reversible increases in lung weight whereas, at higher doses, BK produced irreversible increases in lung weight. Experiments have shown that albumin-bound dye was not retained with the low dose of BK but higher doses did increase permeability as shown by accumulation of albumin-bound dye.

A clue to the site of vasoconstrictor action of PHE and BK comes from examining the changes in lung weight. Arteriolar constriction would not increase capillary pressure and would not be expected to cause hydrostatic oedema. In contrast, venular constriction would increase capillary pressure and this would be expected to produce hydrostatic oedema. The fact that PHE did not increase lung weight would therefore suggest an arteriolar site of action, while the BK-induced increase in lung weight would suggest a venular constrictor action.

The major questions which arise from the vascular actions of BK are whether BK-induced oedema results from; (1) a direct action on capillary membrane permeability, or (2) indirectly from actions on vascular smooth muscle causing hydrostatic changes due to opening of previously unperfused vessels, or (3) a combination of both such effects. In order to determine which of these factors were responsible, the effects of BK on lung weight were examined; (a) in retrogradely perfused lungs and (b) in the presence of a vasodilator papaverine.

The results showed that in retrogradely perfused lungs the increases in PPP produced by BK were not different to those produced by a similar dose of BK given via the orthograde route. However, there was ≈50% reduction in weight gain compared to

weight gain produced by BK when given in the orthogradely perfused lungs. This suggests that when given via the orthograde route BK-induced increases in lung weight were partially mediated via its constrictor action on the venous side of the pulmonary circulation leading to a hydrostatic increase in lung weight. Other reports have also suggested that the venoconstrictor effects of BK contribute to oedema formation in many vascular beds (Rowley, 1964; Bonner *et al.*, 1990). In the present experiments such venoconstriction could explain the reversible increase in lung weight seen with low doses of BK, the reversibility being a response of the compliance of the capillary bed. This would reverse as the effect of BK at its receptor sites declined due to rapid inactivation of BK in the pulmonary circulation.At higher doses the compliance of the microcirculation would be exceeded and fluid would be forced into the interstitial space leading to oedema formation. Furthermore, at high doses BK would evoke more powerful venoconstriction which, aside from mere increased hydrostatic sieving, might accelerate egress of liquid from the vasculature via an increase in permeability or in permeable surface area. Powerful venoconstriction, might lead to a hydrostatically-mediated permeability increase (Rowley, 1964). Alternatively, higher doses of BK may cause contraction of the endothelial cells of capillary and post-capillary venules and open up junctions between cells as has been reported to occur in the bronchial circulation (Ichinose and Barnes, 1990; Sakamoto *et al.*, 1992) and this would also increase vascular permeability. BK is reported to contract actomyosin elements located within the endothelial cells which results in the formation of large venular gaps which form at cell junctions in venules subsequent to cell separation owing to a "rounding up" of the ends of adjacent endothelial cells (see Grega *et al.*, 1982). Furthermore, reversible modulation may occur within seconds in response to specific agents and involve activation of the contractile apparatus i.e. activation of actin and myosin system in the endothelial cells (Crone, 1986). Another possibility is that high intravascular pressures could also increase total perfused surface area through recruitment of poorly-perfused or unperfused vessels (Hyman et al., 1967), this may enhance egress of liquid and albumin from the vasculature in the presence or absence of increased permeability per se.

In a different approach, the effects of BK on lung weight were investigated in the presence of the vasodilator papaverine, in order to see whether lung weight changes associated with BK were secondary to increases in PPP. Results showed that, papaverine reduced the increases in PPP and lung weight in response to BK. This supports the hypothesis that the increase in lung weight in response to BK is linked to its vasoconstrictor actions.

In addition, VOP was raised in order to investigate if increases in VOP would produce similar changes in lung weight to those seen with BK. It was shown that when VOP was raised (5-15 mmHg) for 1 min it produced increases in PPP which were associated with reversible increases in lung weight. At higher levels of VOP (20-25 mmHg) increases in PPP were associated with irreversible increases in lung weight suggesting that there may be a threshold pressure which when exceeded produces irreversible changes in lung weight. Guyton and Lindsey, (1959) have reported that increments of left atrial pressure had little effect on the fluid content of dog lung tissue until the pressure exceeded 25 mmHg. They have also suggested the that the pressure above which fluid accumulation in the lung becomes sensitive to left atrial pressure should be called the "critical pressure". When VOP was raised to 20 mmHg the irreversible increase in lung weight was also associated with a marked retention of albumin-bound dye. This suggests that an increase in VOP alone can increase vascular permeability increases to albumin. This is in agreement with the findings of Sakai *et al.*, (1989) who also reported increased albumin escape when outflow pressure was elevated. In rabbit lung preparations the limit of VOP elevation not leading to outward filtration was 5 to 10 mmHg (Hauge and Nicolaysen, 1971). Retention of albumin bound dye caused with elevated VOP could be explained due to the fact that the increase in pulmonary capillary pressure stretches the pores through which fluid and protein traverse the capillary wall, thereby accounting for high rates of fluid and protein accumulation at high capillary pressure (Michel, 1994). The fact that increases in VOP to levels which induce changes in PPP similar to those produced by 200 nmol of BK caused a significant albumin-bound dye accumulation suggests that the weight increase induced by VOP is not due to hydrostatic sieving across a normally

permeable vasculature but that VOP like BK, may increase permeability (Sakai *et al.*, 1989) and / or permeable surface area.

4.1.3 Pulmonary inflation pressure

The present experiments have shown that both CCH and BK when administered into the pulmonary artery produced increases in PIP, indicative of bronchoconstriction. However, the bronchial region normally receives its main blood supply from the tracheo-bronchial arteries which arise from the systemic arterial circulation (Marchand *et al.*, 1950; Deffebach *et al.*, 1987) which is not perfused in this model. This suggests that either a proportion of the bronchial smooth muscle receives perfusate via the pulmonary artery or that there may be anastomoses between the pulmonary and the tracheobronchial microvessels (Kroll *et al.*, 1987). Indeed, pulmonary artery forming anastomoses have been shown in the rat pulmonary circulation (McLaughlin *et al.*, 1966; McLaughlin *et al.*, 1983). Perfusion of the pulmonary artery with fluorescein isothiocyanate-dextran (FITC-D) (Kroll *et al.*, 1987) led to the presence of FITC-D in the tracheobronchial tissue. Therefore, it is possible that in the present study the flow from the pulmonary to the airway circulation is via these microvascular anastomoses.

The maximal bronchoconstrictor effects achievable with CCh and BK were similar but CCh was more potent than BK. Results have shown that the bronchoconstrictor potency of BK was markedly increased in the presence of captopril which again indicates that the activity of BK is being attenuated by angiotensin converting enzyme. Increases in PIP in response to CCh were fully reversible at all doses but this was only true with smaller doses of BK. In addition at higher doses BK also produced vascular leakage as indicated by the Evans blue study and the appearance of fluid in the tracheal cannula. Therefore it is possible that the residual irreversible increase in PIP induced by BK is secondary to increased vascular permeability resulting in fluid accumulation in the interstitial space, the airways or the alveoli (Bachofen *et al.*, 1993). BK-induced airway exudation has also been reported by other investigators.(Arakawa *et al.*, 1992; Hui *et al.*, 1992; Yokoyama

et al., 1993). The increased basal PIP caused by BK may therefore result from mechanical resistance caused by fluid in the airway. The fact that increases in VOP which caused irreversible increases in lung weight also increased PIP with associated fluid accumulation supports this hypothesis.

4.2 Endothelins and sarafotoxin 6c

Having validated the perfused lung model utilising the agonists discussed above experiments were then undertaken to investigate the effects of ETs and SX6C in this preparation.

4.2.1 ET receptors- mediating pulmonary vasoconstriction

In this perfused lung preparation ET-1, ET-2 and ET-3 produced dose-dependent increases in PPP suggesting ETs induced pulmonary vasoconstriction. These findings are in agreement with those reported in the other rat lung preparations (Rodman *et al.*, 1992; Bonvallet *et al.*, 1993; Hisaki *et al.*, 1994a; Hisaki *et al.*, 1994b) and rat isolated pulmonary blood vessels (Wanstall and O'Donnell, 1990; Rodman *et al.*, 1992; Bonvallet *et al.*, 1993; MacLean *et al.*, 1994). The present data also supports previous studies in human isolated pulmonary blood vessels (McKay *et al.*, 1991; Hay *et al.*, 1993; Buchan *et al.*, 1994; Maguire and Davenport, 1995). The fact that the pulmonary vasoconstrictor potencies of Ets are ET-1> ET-2 >ET-3 suggests an action mediated via ET_A receptors (Webb, 1991). This was further supported by the findings that a selective ET_A receptor antagonist BQ123 (Ihara *et al.*, 1992) caused a concentration-dependent inhibition of the pulmonary vasoconstrictor responses to ET-1. Previous studies using isolated pulmonary arteries have shown that ETs- induced contractions were inhibited in the presence of the selective ET_A receptor antagonists BQ123 or FR139123 (Bonvallet *et al.*, 1993; Sogabe *et al.*, 1993). The fact that higher concentrations of BQ123 (10μM) significantly reduced the maximum increase in PPP induced by ET-1 indicates that

BQ123 may act as a non-competitive antagonist at ET_A receptors. A similar observation has been reported by Hiley *et al.,* (1992).

The finding that a low concentration of BQ123 (1 μM) potentiated the increase in PPP to the highest dose of ET-1 may indicate the presence of different subtypes of ET_A receptor in the pulmonary circulation, one situated on smooth muscle mediating contraction, the second situated on the endothelium which on activation releases a vasodilator substance (s) in response to ET-1. The blockade of this component of the response in the presence of low concentration of BQ123 could account for the potentiation of the vasoconstrictor response to ET-1. Previous findings have shown that ET-1-induced release of the vasodilator prostanoid PGI_2 in isolated perfused rat lung is blocked in the presence of 1 μM BQ123 (D'Orléans-Juste *et al.,* 1992; D'Orleans-Juste *et al.,* 1993a). However, in the present experiments indomethacin did not alter ET-1-induced vasoconstriction. Interestingly, other studies have shown that ET-1 dose-dependently stimulates cAMP formation in vascular smooth muscle cells and this was blocked by BQ123 (Eguchi *et al.,* 1993). Therefore, it is possible that activation of such an ET_A receptor subtype increases cAMP levels which could then oppose ET-1-induced vasoconstriction. Potentiation of constrictor responses to ET-1 in the presence of BQ123 has also been reported in other vascular preparations (Cornet *et al.,* 1993).

The fact that higher concentrations of BQ123 (2- 10 μM) markedly blocked the ET-1-induced increase in PPP suggests that at higher concentrations BQ123 is blocking the ET_A receptor subtype which is responsible for the vasoconstrictor actions. The present data also shows that BQ123 had no effect on the vasoconstriction produced by SX6C indicating that at the concentrations used BQ123 is not producing non-selective blockade of ET receptors in this model.

SX6C, a highly selective ET_B receptor agonist (Williams *et al.,* 1991) was also found to be a pulmonary vasoconstrictor with a potency similar to ET-1. Thus stimulation of ET_{B2} receptors can also produce vasoconstriction in the lung, a similar finding has been

reported in guinea-pigs (Noguchi *et al.*, 1993). In addition ET_{B2} receptors have been shown to be present on isolated rings of rat pulmonary arteries (MacLean *et al.*, 1994).

In the present model ET-1 at low doses produced sustained vasoconstriction, whereas, at higher doses it caused a biphasic effect, a transient increase followed by a sustained increase in PPP. In contrast ET-3 and SX6C produced only a transient increases in PPP. This may indicate that the biphasic vasoconstriction produced by ET-1 could be due to the activation of different ET receptors sub-types in the pulmonary vascular bed. The fact that ET-1 has a high binding affinity for the ET_A receptor could explain the sustained rise in PPP (Noguchi *et al.*, 1993). This was further supported by the finding that in the presence of BQ123 ET-1 caused only a transient vasoconstriction which is probably due to the activation of ET_{B2} receptors. Interestingly, it has been shown in receptor binding studies that ET-1 remains bound to ET_A receptors for a prolonged time and continues to activate a signal-transducing G protein, which accounts for the sustained contractions induced in smooth muscle (Chun *et al.*, 1995).

In the present experiments perfusion of the mixed ET_A / ET_B receptor antagonists bosentan, or PD145065, (Doherty *et al.*, 1993; Clozel *et al.*, 1994), blocked the responses to ET-1 and SX6C on PPP confirming that pulmonary vasoconstriction is mediated via both ET_A and ET_{B2} receptors. More recently a similar finding has been reported by Eddahibi *et al.*, (*1*995). Furthermore, *in vivo* experiments have shown that bosentan, or PD145065, decreased the pressor responses to ET-1 and SX6C in rat and guinea pig (Clozel *et al.*, 1994; Filep *et al.*, 1994; Teerlink *et al.*1995; Warner *et al.*, 1995). The fact that bosentan, or PD145065, were both more potent in blocking PPP increases induced by ET-1 compared to SX6C suggests that both of these antagonists are more potent in blocking ET_A receptor mediated vasoconstriction as compared to ET_{B2} mediated responses. This is in agreement with the reported potency profile which shows that bosentan is 20-50 fold selective for the ET_A receptors compared with ET_B receptors (Clozel *et al.*, 1994). In addition, previous reports have shown a lower IC_{50} value of PD145065 for ET_A receptors compared to ET_B receptors (Doherty *et al.*,

1993). The fact that bosentan, or PD145065, had no effect on the PHE-induced vasoconstrictor responses suggests that these antagonists are acting selectively at ET receptors.

The neutral endopeptidase inhibitor phosphoramidon (1 μM) had no effect on basal monitored parameters. However, phosphoramidon significantly potentiated ET-3-induced increases in PPP which suggests that ET-3 is inactivated by a phosphoramindon sensitive enzyme (probably neutral endopeptidase) present in the lung tissue (Battistini *et al.*, 1995). Similar findings have been reported in isolated airways of rabbit and human (McKay *et al.*, 1992), and in guinea pig lung parenchyma (Battistini *et al.*, 1995). The fact that SX6C-induced responses were not altered in the presence of phosphoramindon indicates that ETs are more susceptible to breakdown by neutral endopeptidase present in the lung than SX6C (Sokolovsky *et al.*, 1990).

4.2.2 ET-induced pulmonary oedema

Results indicated that ETs are also very potent in producing increases in lung weight, which supports the findings of Ercan *et al.*, (1993). The potency profile of ETs in causing increases in lung weight paralleled their vasoconstrictor potencies (ET-1> ET-2 > ET-3) which suggests that changes in lung weight are secondary. This is further supported by the fact that in the presence of the vasodilator papaverine, ET-1-induced vasoconstriction and lung weight increases were abolished. Interestingly SX6C produced similar increases in PPP to that of ETs but caused very little increase in lung weight.This raises the possibility of a differential distribution of ET receptor subtypes within the pulmonary vascular bed.

As shown earlier PHE selectively increased PPP whereas BK increased both PPP and lung weight. These different effects were explained by PHE producing arteriolar vasoconstriction whereas BK-caused venoconstriction. Thus SX6C, like PHE could be acting on the arteriolar side of the pulmonary circulation whereas ETs, acting like BK-

cause venoconstriction, an action ETs are known to exert (Horgan *et al.,* 1991; Rodman *et al.,* 1992), and oedema formation.

Previous studies using rat isolated pulmonary blood vessels have shown that veins are more sensitive than arteries to ET-1 (Rodman *et al.,* 1992). In another report Raj *et al.,* (1992) showed that ET-1 predominantly constricted pulmonary veins. In order to investigate this possibility, retrograde perfusion was used. Under these conditions arterial vasoconstriction will now cause an increase in hydrostatic pressure within the microcirculation, and increase lung weight via hydrostatic means, whereas venous constriction would be expected to cause little effect on lung weight because the microvascular pressure would not be significantly altered. This is exactly what was found. With retrograde perfusion SX6C now produced significant increases in lung weight, whilst ET-1 had a reduced effect on lung weight, suggesting that the ET_{B2} receptors stimulated by SX6C are predominantly located on the arterial side of the pulmonary circulation whereas the majority of ET_A receptors appear to be located on the venous vessels. These results agree with findings in other vascular beds (D'Orléans-Juste *et al.,* 1993b; White *et al.,* 1993; MacLean *et al.,* 1994).

Results in the present experiments have also shown that increases in lung weight produced by ET-1 are associated with a large accumulation of albumin bound dye. When ET-1 was given via orthograde perfusion it produced a marked accumulation of albumin bound dye, whereas when given retrogradely, significantly less dye retention was seen. These findings further support the suggestion that increases in lung weight and retention of dye produced with ET-1 are due to potent venoconstriction. Furthermore, increases in lung weight resulting from fluid accumulation were significantly higher than the expected lung weight increases calculated from the retention of dye. This suggests that accumulation of fluid in the lung is primarily due to hydrostatic oedema rather than to a direct increase in vascular permeability to albumin. When SX6C was given using orthograde perfusion it also caused retention of albumin bound dye which indicates the presence of some ET_{B2} receptors mediating constriction of pulmonary venous smooth

muscle which would contribute to the pulmonary oedema via a hydrostatic mechanism. However, when SX6C was given retrogradely it produced a significantly higher retention of albumin bound dye compared to orthograde perfusion. This provides further evidence that the majority of ET_{B2} receptors are located at the arteriolar level.

The potency profile of ETs in increasing lung weight suggests a role for ET_A receptors. This is supported by the findings that a selective ET_A receptor antagonist, BQ123, produced a concentration -dependent blockade of ET-1-induced lung weight responses. *In vivo* investigations in many organs have also shown that ET-1-mediated albumin extravasation is attenuated by another selective ET_A receptor antagonist, FR139317, (Filep *et al.*, 1993; Filep *et al.*, 1994). However these findings are not in agreement with those of Bonvallet *et al.*, (1993), who showed that in the rat isolated perfused lung, ET-1 associated hydrostatic oedema was unaffected by BQ123. This may be due to different experimental conditions as the latter study used a recirculating model which was complicated by periods of hypoxia and angiotensin-II addition. The fact that SX6C-induced increases in lung weight were not blocked by BQ123 also suggests that a small population of ET_{B2} receptors activated by SX6C are also present on the venous smooth muscle.

In the present model the mixed ET_A / ET_B receptor antagonists, bosentan (Clozel *et al.*, 1994) and PD145065, both blocked the lung weight increases produced by ET-1 or SX6C. This again supports the suggestion that both ET_A and ET_{B2} receptors play a role in mediating ET-1 and SX6C -induced lung weight responses. In another study it has been reported that both ET-1, or IRL1620 (another ET_B receptor agonist), *in vivo,* caused albumin extravasation in many vascular organs by the activation of both ET_A and ET_B receptors (Filep *et al.*, 1994).

The finding that phosphoramidon markedly potentiated the increase in lung weight produced by ET-3, while having no significant effect on SX6C -induced lung weight changes, suggests that degradation of ET-3 in the lung by endopeptidase may reduce the

effects of ET-3, whereas SX6C is resistant to breakdown by endopeptidases (Sokolovsky *et al.*, 1990). Phosphoramidon -sensitive degradation of ET-3 has also been reported in guinea pig lung parenchymal strips (Battistini *et al.*, 1995). The cyclooxygenase inhibitor indomethacin had no effect on the lung weight increases produced in response to ET-1 or SX6C. This suggests that prostanoids do not play any role in the effects of ET-1 or SX6C.

4.2.3 Receptors mediating bronchial actions of ETs and SX6C

The bronchoconstrictor potency profile of ETs (ET-1≈ ET-2 ≈ ET-3) and SX6C suggests an ET_{B2} mediated response in bronchial smooth muscle. Previous evidence in guinea pig lung parenchymal strips has shown that ETs -induce contractions via ET_{B2} receptors (Hay *et al.*, 1993). It has also been reported that ET-1-induced guinea pig tracheal contractions are not affected in the presence of the ET_A receptor antagonist FR139317 (Cardell *et al.*, 1993).

The fact that BQ123, at concentrations which reduced ET-1-induced increases in PPP and oedema, did not produce any reduction in the bronchoconstrictor effects of ET-1 or SX6C shows that bronchoconstriction was unlikely to be mediated by ET_A receptors. Similar findings have been reported in the guinea pig (Hay *et al.*, 1993; Battistini *et al.*, 1994) and in human bronchus (Hay *et al.*, 1993). SX6C was more potent than the ETs in causing bronchoconstriction, and the slope of the SX6C dose-response-curve was significantly steeper, and achieved a higher maximum than that of ETs . This could be due to a number of factors. Firstly, ETs are known to be more susceptible than SX6C to breakdown by neutral endopeptidase present in the lung, this would reduce the effects of ETs compared with SX6C (Sokolovsky *et al.*, 1990). However, this was discounted as the bronchoconstrictor actions of ETs were not influenced by the addition of the neutral endopeptidase inhibitor phosphoramidon. A recent report by Hisaki *et al.*, (1994b) has also shown that in rat isolated lung, pressor responses to ET-1 were not influenced by phosphoramidon. Secondly, ETs could be releasing some bronchodilator substance resulting in a physiological antagonism, as reported by Uchida *et al.*, (1991). In the

present study, use of the selective ET_A receptor antagonist BQ123, or a mixed ET_A/ ET_B receptor antagonist PD145065, significantly augmented the bronchoconstrictor response of ET-1. This could indicate that activation of ET_A receptors may release a bronchodilator substance which antagonizes the bronchoconstrictor response of ET-1. Interestingly, Battistini *et al.*, (1994) also reported that BQ123 potentiated the ET-1 induced contractions of isolated guinea pig trachea. It has recently been shown that ET-1 increased the levels of cAMP in cloned rat alveolar epithelial cells and these effects were blocked by the ET_A receptor antagonist BQ123 (Markewitz *et al.*, 1995).

Perfusion with the mixed ET_A / ET_B receptor antagonists bosentan, or PD145065, produced a significant reduction in the bronchoconstrictor responses of SX6C without having any effect on ET-1-induced responses. This could indicate that in bronchial smooth muscles the ET_{B2} receptors activated by ET-1 are different to the ET_{B2} receptors activated by SX6C. The existence of different ET_B receptor sub-types in the isolated guinea-pig trachea has also been suggested by Battistini *et al.*, (1993) and Yoneyama *et al.*, (1995). Alternatively, the strong binding affinity of ET-1 for ET_B receptors may explain this discrepancy (Takasuka *et al.*, 1992; Wu-Wong *et al.*, 1994; Wu-Wong, *et al.*, 1995).

The cyclooxygenase inhibitor indomethacin did not alter the pulmonary vascular or bronchial actions of ET-1 or SX6C. This suggests that the effects of ETs or SX6C were not mediated or modulated by eicosanoids. Similar findings have been reported in other rat lung preparations (O'Donnell *et al.*, 1990; Raffestin *et al.*, 1991; Barnard *et al.*, 1991; Rodman *et al.*, 1992). The present results also support the findings in human pulmonary vascular and bronchial tissues (McKay *et al.*, 1991).

4.3 Modulation of lung responses to ETs in the presence of the nitric oxide synthase (NOS) inhibitor nitro-L-arginine (L-NOARG) under basal conditions

4.3.1 Pulmonary vascular actions

The pulmonary vasculature has a low basal tone. In order to determine if constant release of nitric oxide is responsible for this, the effect of the nitric oxide synthase inhibitor L-NOARG was studied at a concentration reported to block constitutive NOS (Moore *et al.*, 1990). The results showed that L-NOARG had no effect on basal PPP, lung weight, or PIP suggesting that NO is not a major factor contributing to the low basal tone. These findings are in agreement with reports in other rat perfused lung preparations (Barer *et al.*, 1993; Hampl *et al.*, 1993; Hassessian and Burnstock, 1995).

ET-1 and SX6C -induced pulmonary vascular and bronchial actions were also studied in the presence of L-NOARG. The results show that L-NOARG markedly augmented the vasoconstrictor responses to ET-1 and SX6C. In contrast L-NOARG did not alter the responses to PHE suggesting that it is not producing non-selective effects. Potentiation of ET-1-induced vasoconstriction in the presence of NOS inhibition has also been reported in other perfused rat lung preparations (Raffestin *et al.*, 1991; Eddahibi *et al.*, 1991). In rat isolated pulmonary arteries potentiation of ET-1-induced contractions in the presence of a NOS inhibitor was reversed after the removal of the endothelium (MacLean *et al.*, 1994).

A possible explanation of this potentiation is that ET-1 and SX6C release endogenous nitric oxide via activation of the endothelial ET_{B1} receptors (Douglas *et al.*, 1994). Inhibition of this process with L-NOARG would result in potentiation of vasoconstriction mediated via ET_A and ET_{B2} receptors located on the vascular smooth muscle. Another possible explanation is that under normal circumstances basally released endogenous nitric oxide suppresses the vasoconstrictor responses to these agonists. However, in this model results suggests that NO is not a major factor contributing to the

low basal vascular tone because (a) L-NOARG had no effect on basal pulmonary vascular tone, (b) L-NOARG did not potentiate PHE responses.

In the presence of L-NOARG the increases in PPP induced by SX6C were far greater than those induced by ET-1. This may be due to the fact SX6C being a selective ET_B receptor agonist (Williams *et al.*, 1991) is more potent in releasing nitric oxide, therefore in presence of NOS inhibitor its effects would be potentiated more as compared to the non-selective ET receptor agonist such as ET-1 (Arai *et al.*, 1990; Sakurai *et al.*, 1990).

L-NOARG also potentiated BK-induced increases in PPP suggesting that BK, like ET-1 and SX6C, also stimulates the endogenous production of nitric oxide. This is in agreement with reports showing that BK-induces endothelium-dependent relaxation of pulmonary blood vessels (Feletou *et al.*, 1995; Gao *et al.*, 1995b).

4.3.2 Lung weight

L-NOARG also potentiated lung weight increases in response to ET-1 or SX6C suggesting that evoked release of nitric oxide inhibits pulmonary oedema in response to these agonists. Responses to ET-1 and SX6C in the absence of L-NOARG suggested that the increases in lung weight were due to their venoconstrictor actions leading to hydrostatic oedema. Therefore one possibility which could explain lung weight increases in the presence of L-NOARG is an augmented vasoconstrictor response which would cause a hydrostatic increase in lung weight. The second possibility is that nitric oxide causes preservation of endothelial barrier functions to prevent extravasation (Zi-Qiang *et al.*, 1994). The fact that L-NOARG potentiated the vasoconstrictor responses to BK without altering its effects on lung weight suggests that the second possibility may be unlikely. However, in the presence of L-NOARG, albumin-bound dye extravasation in response to ET-1, SX6C and BK was not examined and this would need further investigation.

In the presence of L-NOARG the potency of SX6C to increase lung weight was greater than ET-1, whereas it produced little increase in lung weight in the absence of L-NOARG. This further supports the suggestion that SX6C is more potent in releasing nitric oxide than ET-1. The fact that L-NOARG potentiated lung weight responses to SX6C may indicate the presence of small population of ET_{B2} receptors on the venous smooth muscle and that venoconstriction mediated via these receptors was masked by the release of nitric oxide. Zellers *et al.,* (1994) have reported that the ETs and SX6C - induced release of vasodilators is greater in porcine pulmonary veins than arteries. Gao *et al.,* (1995a) have also shown that endothelium-derived nitric oxide plays a larger role in neonatal lamb pulmonary veins than pulmonary arteries, which they suggested may be due to differences in the activity of guanylate cyclase in venous smooth muscle. The present studies do not provide any evidence for the site of release or action of nitric oxide in this model. Further experiments using retrograde perfusion in the presence of L-NOARG would be needed to understand the mechanisms involved in the vascular actions of ET-1, SX6C and BK.

4.3.3 Bronchial actions

ET-1- and SX6C- induced bronchoconstriction was also potentiated by L-NOARG suggesting that evoked release of nitric oxide was inhibiting their bronchial constrictor actions. Because nitric oxide is a diffusible gas (Butler *et al.,* 1995) it is possible that induced release of nitric oxide from the vascular endothelium could pass to the bronchial smooth muscle to reduce the ET-1 or SX6C induced responses. However, this is unlikely, because L-NOARG augmented the vasoconstriction caused by BK but had no significant effect on its bronchoconstrictor responses. Alternatively, ET-1 or SX6C could release nitric oxide from the bronchial and alveolar epithelium via activation of ET_B receptors which in turn would reduce the bronchoconstrictor effects of these agonists. In support of this, investigators, have reported the presence of NOS in the airway epithelium in rat lungs (Xue *et al.,* 1994). Previous studies have also shown that ET-1 can produce relaxation of precontracted guinea pig trachea (Uchida *et al.,* 1991;

Battistini *et al.*, 1994). In addition the contractile effects of ETs on the isolated guinea pig bronchus were also potentiated after the removal of epithelium (Maggi *et al.*, 1990). The bronchoconstrictor effects of SX6C in the presence of L-NOARG were potentiated to a greater extent compared to effects of ET-1. This may be due to the fact that SX6C is more potent in releasing nitric oxide. Alternatively ET-1 may be releasing some weaker bronchodilator component(s) which attenuate its constrictor effects on the airway smooth muscle. Recently, Markewitz *et al.*, (1995) reported that rat alveolar epithelial cells posses a novel ET_A receptor subtype which on stimulation with ET-1 increases cAMP levels in these cells. Therefore it is possible that in this model such a mechanisms would suppress the bronchoconstrictor effects of ET-1 but not SX6C. The fact that L-NOARG had no effect on the bronchoconstrictor responses to BK or CCh suggests that neither of these agonists stimulates NO production by the respiratory epithelium.

Effects of ETs and SX6C were also examined when the basal vascular tone was elevated by infusion of U46619. The infusion of very low amounts produced concentration-dependent increases in PPP, lung weight and PIP. Interestingly, when the infusion of U46619 was stopped, the increased PPP and lung weight were rapidly reversible. This may indicate that U46619 predominantly produced venoconstriction which resulted in hydrostatic pulmonary oedema. However, further experiments utilising retrograde perfusion in the presence of U46619 would be needed to study this phenomenon.

4.4 Vasodilation and ET receptor antagonists

In the U46619 preconstricted preparations very low doses of ET-1, ET-3 and SX6C produced dose-dependent falls in PPP. The results support the findings in other rat perfused lung preparations (Eddahibi *et al.*, 1993; Eddahibi *et al.*, 1995). The fact that ET-1 was equipotent with ET-3 in producing vasodilation, whereas, the highly selective ET_B receptor agonist SX6C (Williams *et al.*, 1991) was significantly more potent suggests a role for ET_{B1} receptors in these responses. This is supported by the

observations that the ET_A receptor antagonist BQ123 did not block the vasodilator response to ET-1, whereas, a mixed ET_A / ET_B receptor antagonist bosentan completely abolished the dilator responses to ET-1 and SX6C. Similar findings have been reported in other rat lung preparations (Eddahibi *et al.,* 1993; Eddahibi *et al.,* 1995). The present results are also in agreement with *in vivo* reports in rat (Clozel *et al.,* 1992; Clozel *et al.,* 1994). BQ123 augmented the fall in PPP produced at the highest (40 pmol) dose of ET-1, however, responses to lower doses were not altered. This suggests that at higher doses of ET-1 ET_A receptors are activated and this causes a physiological antagonism of the ET_{B1}-mediated vasodilation. Potentiation of ET-1-induced pulmonary vasodilation in the presence of BQ123 has also been reported in lungs from new born piglet (Perreault and Baribeau, 1995) and in isolated porcine pulmonary vessels (Zellers *et al.,* 1994). The fact that bosentan had no effect on the vasodilatation caused by sodium nitroprusside, suggests that it is not having a non-selective action on cyclic GMP production.

4.5 Tachyphylaxis and cross-tachyphylaxis

Repeated injections of a single dose of ET-1 or SX6C (20 pmol) caused a rapid loss of pulmonary vasodilator responses indicating desensitization. Investigators have reported a similar phenomenon *in vivo* in cats (Le Monnier De Gouville *et al.,* 1990; Lippton *et al.,* 1993; Ekelund *et al.,* 1994) and in rat basilar artery (Kitazono *et al.,* 1995). Results have shown that repeated administration of these agonists desensitized only the vasodilator responses, whereas vasoconstriction to both the agonists was still seen. Furthermore, under basal conditions activation of ET_B receptors resulted in vasoconstriction. This provides evidence of a functionally distinct ET_B receptor populations in the rat pulmonary vascular bed i.e. ET_{B1} receptors located on the endothelium which mediate vasodilatation whereas ET_{B2} receptors located on the smooth muscle are responsible for the vasoconstrictor action (Shetty *et al.,* 1993). These findings are in agreement with the recently reported ET_B receptor subtypes in new born piglet lungs (Perreault and Baribeau, 1995). In ET-1 desensitized lungs further injections of very low doses of SX6C also failed to cause vasodilatation, instead it produced a dose-dependent

vasoconstriction. Similarly in lungs desensitized with SX6C further injections of low doses of ET-1 failed to produce any vasodilatation, and a dose- dependent vasoconstriction was seen. This suggests the development of cross-tachyphylaxis to the vasodilator actions of ET-1 and SX6C.

Several possibilities could explain the cross-tachyphylaxis phenomenon seen in this model. First, ET-1 and SX6C may stimulate a single type of receptor that becomes rapidly refractory to activation by these peptides. Alternatively, ET-1 and SX6C could bind to distinct receptor sites which share at least one common point in the second messenger system. This shared portion of the reaction chain may then becomes impaired or refractory, after repeated stimulation of the receptors. Consequently, all receptors using this common pathway to transform their membrane signal into a response will fail to evoke functional responses when activated by their agonists. Thus, for instance cross tachyphylaxis to ET-1 and SX6C may reflect the depletion of an endogenous vasodilator such as nitric oxide. Another possibility which could explain the tachyphylaxis phenomenon is that ET_{B1} receptors may get internalised thus reducing the receptor population available for mediating vasodilation.

4.6 Vasodilation and inhibitors

In the U46619 precontracted preparations indomethacin did not affect SX6C-induced vasodilation but it did reduce the vasodilator effects of low doses of ET-1. However, responses to the higher doses were not significantly altered . This suggests that ET-1 induced vasodilatation is partially mediated by the release of vasodilator prostanoids. Vasodilator actions of ETs have been shown to be partially blocked by indomethacin *in vivo* in cats (Ekelund *et al.*, 1994), while DeNucci *et al.*, (1988) have also shown ET-1 stimulated prostacyclin synthesis in rat lungs.

The possible role of nitric oxide in the ET-1- and SX6C-induced pulmonary vasodilation was evaluated using L-NOARG. The fact that L-NOARG potentiated the pressor

responses to U46619 suggesting that U46619 also evoked endogenous production of nitric oxide in the rat pulmonary circulation. Similar findings have been reported in lungs from neonatal pigs (Pinheiro and Malik, 1993). Therefore, in lungs treated with L-NOARG a lower concentration of U46619 had to be used in order to elevate the PPP (≈ 9mmHg) to a similar level seen in the absence of L-NOARG.

L-NOARG blocked the vasodilatation induced by the higher doses of ET-1, whereas, responses to lower doses of ET-1 were not altered. D-NOARG was without effect. Vasodilator responses to ET-1 were completely abolished by the presence of both indomethacin and L-NOARG. This suggests that the vasodilator actions of ET-1 in rat pulmonary circulation are mediated by the release of both nitric oxide and prostanoids. Similar findings have been reported in porcine pulmonary blood vessels (Zellers *et al.*, 1994) and perfused lungs (Pinheiro and Malik, 1993). The finding that L-NOARG alone completely abolished the vasodilatation caused by SX6C, whereas D-NOARG did not alter these responses indicates that the dilator effects of SX6C are mediated exclusively by nitric oxide production. The present findings are in agreement with studies using isolated pulmonary arteries (Namiki *et al.*, 1992). However, these finding are not in agreement with the observations of Eddahibi *et al.*, (1993) who also used perfused rat lung. This may be due to different experimental conditions, or the use of a different NOS inhibitor, L- N^G- monomethyl arginine (L-NMMA) which has been reported to selectively inhibit basal release of nitric oxide without affecting the agonist-evoked release (Frew *et al.*, 1993). These workers also used the guanylate cyclase inhibitor methylene blue to show that ETs vasodilation is not due to cyclic GMP generation caused by nitric oxide, however, it has been shown that nitric oxide can cause smooth muscle relaxation independent of guanylate cyclase activation (Bolotina *et al.*, 1994). The fact that L-NOARG had no effect on the vasodilator actions of the nitric oxide donor sodium nitroprusside suggests that L-NOARG is not having a non-selective action on cGMP generation.

In summary the present experiments have shown that activation of both ET_A and ET_{B2} receptors can lead to constriction in the pulmonary vascular bed. In addition, evidence is provided to show that ET_A receptors are predominantly present on the venous side of the pulmonary circulation whereas ET_{B2} receptors are located arterially. The changes in lung weight in responses to ETs probably reflects hydrostatic oedema resulting from intense venoconstriction. Results also suggest the possibility of heterogeneity of ET_A and ET_{B2} receptor subtypes. Furthermore, it has been shown that under basal conditions endogenous release of nitric oxide modulates the pulmonary vascular and bronchial actions of ETs and SX6C. The pulmonary vasodilator actions of ETs are mediated by the activation of ET_{B1} receptors and involve endogenous release of nitric oxide. Results have also shown the tachyphylaxis to the pulmonary vasodilator actions of ET-1 and SX6C.

A simple ventilated perfused rat lung model has been used to study the actions of variety of substances which exert selective actions on the pulmonary vascular or bronchial smooth muscle. Furthermore, by studying dye accumulation associated with manipulation of VOP, or injections of vasoactive agents with orthograde or retrograde perfusion, valuable information can be gathered regarding the actions of agents which influence vascular permeability.

SECTION 5

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SECTION 6

Appendix

Publications

Lal, H., Woodward, B., & Williams, K.I. (1994). Differential effects of agents on bronchial and vascular tone and lung weight in the rat isolated perfused lung. *Pulmonary Pharmacol.*, **7**, 271-278.

Lal, H., Woodward, B. & Williams, K.I. (1995). Actions of endothelins and sarafotoxin 6c in the rat isolated perfused lung. *Br. J. Pharmacol.*, **115**, 653-659.

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Appendix-II

Abbreviations

ANOVA	analysis of variance
ANP	atrial natriuretic peptide
Arg	arginine
ARDS	adult respiratory distress syndrome
Asp	aspartic acid
BK	bradykinin
BSA	bovine serum albumin
CCh	carbachol
cAMP	cyclic adenosine 3′, 5′ -monophosphate
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
Cys	cystine
DNA	deoxyribonucleic acid
D-NOARG	nitro-D-arginine
ET	endothelin
EDCF	endothelium-derived contracting factor
ECE	endothelin converting enzyme
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
FITC-D	flourescein isothiocyanate-dextran
Glu	glutamic acid
Hyp	4-hydroxyproline
i.u.	international unit
Leu	leucine
L-NMMA	L-N^G-monomethyl arginine
L-NOARG	nitro-L-arginine

LW	lung weight
Lys	lysine
mRNA	messenger ribonucleic acid
NANC	non-adrenergic non-cholinergic
NO	nitric oxide
NOS	nitric oxide synthase
NS	not significant ($p > 0.05$)
p	probability
PGI_2	prostacyclin
PHE	phenylephrine
Phe	phenylalanine
PIP	pulmonary inflation pressure
PPP	pulmonary perfusion pressure
Pro	proline
SEM	standard error of mean
SNP	sodium nitroprusside
SX	sarafotoxin
Trp	tryptophan
U46619	9,11- Dideoxy-11_{α}, 9_{α}- epoxy-methanoprostaglandin $F_{2\alpha}$
Val	valine
VIP	vasoactive intestinal peptide
VIC	vasoactive intestinal contracting factor
VOP	venous outflow pressure