LOCAL CONTROL OF CORONARY VASCULAR RESISTANCE

by

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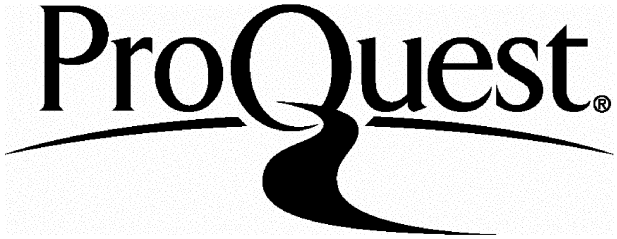

ABSTRACT

This thesis presents a study of the responses to vasodilators in the guinea-pig and rat heart, carried out by perfusing the coronary circulation using the method of Langendorff. In the guinea-pig coronary vasculature it was found that the vasodilator response to substance P and low doses of 5-hydroxytryptamine acted almost exclusively via nitric oxide. The vasodilator responses to adenosine 5'-triphosphate and bradykinin appeared to involve other mechanisms in addition to nitric oxide.

While a major part of the response to adenosine was mediated directly via A_2-receptors on the smooth muscle, activation of a subpopulation of A_2-receptors on the endothelial cells by adenosine and its analogues induced relaxation via production of nitric oxide. 2-MethylthioATP induced relaxation almost exclusively via a subpopulation of P_{2Y}-purinoceptors on the endothelium, which when activated induced release of nitric oxide. In addition to adenosine 5'-triphosphate acting at this population of P_{2Y}-purinoceptors adenosine 5'-triphosphate appears to induce relaxation via release of a prostanoid. Pyrimidines also induced relaxation via nitric oxide, possibly via a subclass of the P_{2Y}-purinoceptor superfamily. Prostanoids do not play any role in the relaxation induced by pyrimidines.

During periods of increased flow, adenosine 5'-triphoshate release from the guinea-pig heart was rapidly and significantly increased. The pressure/flow ratio was reduced in high flow conditions suggesting that coronary vasodilatation had occurred.

It is suggested that the release of adenosine 5'-triphosphate induced coronary vasodilatation.

In the rat coronary vasculature the vasodilator response to 2-methylthioATP was inhibited by the P_2-purinoceptor antagonist suramin. However, the response to adenosine 5'-triphosphate was not inhibited by suramin or the P_1-purinoceptor antagonist 8-(*p*-sulfophenyl)theophylline. It is suggested that adenosine 5'-triphosphate may be acting at another receptor site to elicit vasodilatation, which is neither a known P_{2Y}- or P_1-purinoceptor.

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ABBREVIATIONS

Acetylcholine	ACh
Acetylcholine esterase	AChE
Adenosine 5'-diphosphate	ADP
Adenosine 5'-monophosphate	AMP
Adenosine 5'-triphosphate	ATP
Arachidonic acid	AA
Arylazidoaminopropionyl ATP	$ANAPP_3$
Calcitonin gene-related peptide	CGRP
2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine	CGS 21680
Central nervous system	CNS
2-Chloroadenosine	2-CA
3-[(3-cholamidopropyl)-dimethylammino]-1-propanesulfonate	CHAPS
Choline acetyl transferase	ChAT
Cyclic 3',5'-adenosine monophosphate	cAMP
Cyclic guanosine monophosphate	cGMP
N^6-Cyclopentyladenosine	CPA
Cytidine 5'-triphosphate	CTP
Dimethylsulfoxide	DMSO
1,3-Dipropyl-8-cyclopentylxanthine	DPCPX
Endothelium-derived contracting factor	EDCF
Endothelium-derived hyperpolarizing factor	EDHF
Endothelium-derived relaxing factor	EDRF
5'-N-Ethyl-carboxamido adenosine	NECA
Guanosine 5'-triphosphate	GTP
5-Hydroxytryptamine	5-HT

N-Iminoethyl-L-ornithine	L-NIO
Inosine 5'-triphosphate	ITP
Lactate dehydrogenase	LDH
α,β-Methylene ATP	α,β-meATP
β,γ-methylene ATP	β,γ-meATP
2-Methylthio ATP	2-meSATP
N^G-Monomethyl-L-arginine	L-NMMA
Neuropeptide Y	NPY
Nicotinamide adenine dinucleotide phosphate	NADPH
Nitric oxide	NO
N^G-Nitro-L-arginine	L-NA
N^G-Nitro-L-arginine methyl ester	L-NAME
Non-adrenergic, Non-cholinergic	NANC
Noradrenaline	NA
N^6-phenylisopropyl-adenosine	PIA
Prostaglandin I_2	PGI_2
Sodium nitroprusside	SNP
8-(*p*-Sulphophenyl)theophylline	8-PSPT
Thymidine 5'-triphosphate	TTP
Uridine 5'-triphosphate	UTP
Vasoactive intestinal peptide	VIP
Xanthine amine congener	XAC

PREFACE

The cardiovascular system is a transport system and it links the external environment to the tissues and distributes substances essential for metabolism. These are oxygen from the lungs and nutrients from the gastrointestinal tract. At the same time, the cardiovascular system removes from the tissues carbon dioxide and other by-products of metabolism, carrying them to the lung, kidney and liver. Such actions of the cardiovascular system are essential for homeostasis of the plasma component of the blood and the interstitial fluid, which both comprise the extracellular fluid or internal environment, and hence ensure the even distribution of available water and electrolytes to all parts of the body. Blood pressure and vascular tone are maintained in accordance with the metabolic demands of the tissue by regulation from the autonomic nervous system and from locally released and circulating humoral agents. While neuronal control of vascular tone appears to be physiologically relevant in most components of the vascular system, regulation of vascular tone by locally released and circulating humoral agents appears to be more important in terminal arterioles.

Many studies on control of the vasculature have been dominated by consideration of the role of catecholamines released from sympathetic perivascular nerves and from the adrenal medulla into the bloodstream. New and improved techniques in immunohistochemistry, pharmacology, electron microscopy and electrophysiology have led to many discoveries that have reshaped understanding of the way in which vascular tone is regulated. The autonomic nervous system has seem a dramatic rise in the number of

putative neurotransmitters existing in perivascular nerves, almost invariably along with other neurotransmitters, and this has led to the formulation of the concepts of co-transmission and neuromodulation. Since the discovery in 1981 by Furchgott and Zawadski, of the essential role played by the endothelium in mediating vasomotor relaxant responses to acetylcholine (ACh), an increasing number of agents have been shown to exert their effects on vascular tone via the endothelium. More recently the demonstration that some of these agents may arise from the endothelium itself, has highlighted the importance of endothelial cells as a source of vasoactive agents in addition to being a target, and introduced a new dimension to the concept of regulation of vascular tone.

Some vasoactive agents, such as the purine nucleotides participate in the regulation of vascular tone both by having a role in the autonomic nervous system and by being effectors of the vascular endothelial system. One example is adenosine 5'-triphosphate (ATP). In the autonomic nervous system ATP may be released from perivascular nerves to mediate vasoconstriction, or from non-sympathetic inhibitory nerves to cause relaxation. In addition, ATP may exert modulatory effects both pre- and postjunctionally following ectoenzymatic breakdown to adenosine. Also an effector of the vascular endothelium, ATP can act on its receptors on the endothelial cell surface to cause relaxation, and in this case the source of its release is more likely to be non-neuronal, for example from endothelial cells or from circulating blood.

The aim of this thesis was to examine aspects of local regulation of vascular tone with particular emphasis on the influence of endothelial cells as mediators of vasodilatation in response to purines, pyrimidines, bradykinin, 5-hydroxytryptamine (5-HT) and substance P, in the coronary vasculature. A background to the regulation of vascular tone, including milestones in the history together with more recent observations is given in the introduction (Chapter 1). After a description of the general methods employed (Chapter 2) the experimental results are presented in three sections.

Section A (Chapter 3) deals with endothelial cell mediated relaxation in response to ATP, bradykinin, substance P and 5-HT in the guinea-pig coronary vasculature. Particular emphasis is paid to the role nitric oxide (NO) played in the relaxant response to these agents, and two closely related inhibitors of the synthesis of NO are compared.

Section B (Chapters 4,5,6 and 7) examines the vasodilator effects of purines and pyrimidines in the coronary vasculature. In chapter 4 possible mechanisms of action of the endothelial response to adenosine and some of its analogues are investigated by using inhibitors of the synthesis of NO and prostaglandins. The same inhibitors are used to investigate (Chapter 5) the possible mechanisms of action of the endothelial response to ATP and 2-methylthio ATP (2-meSATP). The information obtained is used to examine whether ATP acts on the same receptors as the classical P_{2Y}-purinoceptor agonist 2-meSATP to initiate relaxation. In the rat coronary vasculature (Chapter 6) the P_2-purinoceptor

antagonist suramin is used to determine whether ATP acts at the same receptors as 2-meSATP to induce relaxation. In chapter 7 the vasodilator effects of pyrimidines are examined and compared with those of purines to establish whether there is a common mechanism of action.

In section C (Chapter 8) the release of ATP from the isolated perfused guinea-pig heart submitted to increased flow is investigated.

The results of the experimental work are discussed at the end of each chapter and then assessed in a wider context in the General Discussion (Chapter 9).

Chapter 1

INTRODUCTION

This chapter is not intended to be a comprehensive review of the literature but rather to give perspective on topics relevant to this thesis, with particular emphasis on neural and endothelial control of the cardiovascular system. With this in mind, this chapter is divided into four main parts: 1.1 Structure and function of the coronary circulation; 1.2 Perivascular nerves; 1.3 Vascular endothelium; 1.4 Control of blood vessel tone by purines.

1.1 STRUCTURE AND FUNCTION OF THE CORONARY CIRCULATION

1.1.1 The coronary circulation

The basic anatomic pattern of the major coronary arteries is comparable from the smallest to the largest mammals (Gregg & Fisher, 1963). The three major vessels located on the epicardial surface of the heart are known as the left anterior descending, left circumflex and right coronary artery, which originate from the aorta at the sinuses of Valsalva. The two left coronary arteries arise from a single coronary ostium behind the posterior cusp of the aortic valve and in general supply the anterior wall of the left ventricle (anterior descending) and the left atrium and lateral and posterior portions of the left ventricle (circumflex). The right coronary artery supplies the right atrium, right ventricle and variable proportions of the posterior portion of the left ventricle. These large epicardial vessels give rise to smaller intramural arteries which arise at sharp

angles to penetrate the myocardium. In some species these eventually form a plexus of interconnecting arteriolar anastomoses and supply a dense network of capillaries. There are two venous drainage systems: a superficial system and a deep system. The superficial system ends in the coronary sinus and anterior cardiac veins and drains the left ventricle, whereas, the deep system drains the rest of the heart. The deep system is made up in large part of the arteriosinusoidal vessels, sinusoidal capillary-like vessels that empty directly into the heart chambers. There are also direct connections to the atria and ventricles from the coronary arterioles (arterioluminal vessels) and the veins (thebesian veins).

1.1.2 Factors that control coronary blood flow

The heart has only a short lived capacity for anaerobic metabolism and the metabolic needs of the myocardium can be considered almost solely in terms of oxidative metabolism. Thus, there is a close relationship between myocardial oxygen supply and demand (Shipley & Gregg, 1945; Eckenhoff *et al.*, 1947a; Lichtlen *et al.*, 1971; Berne & Rubio, 1979) and since 65 - 85 % of the coronary arterial oxygen is extracted by the myocardium in a single passage through the coronary bed, there is very little capacity for the myocardium to adjust to increased demand by increased oxygen extraction. The primary mechanism by which the heart adjusts is therefore by changes in coronary blood flow.

Coronary blood flow is affected by the simple fact that

the heart is a mechanically active pump. The pressure inside the left ventricle is slightly greater than in the aorta during systole. Consequently, flow occurs in the arteries supplying the heart subendocardial portion of the left ventricle only during diastole, although the force is sufficiently dispersed in the more superficial portions of the left ventricle myocardium to permit some flow in this region throughout the cardiac cycle. Since diastole is shorter when the heart rate is rapid, left ventricular coronary flow is reduced during tachycardia. On the other hand, the pressure differential between the aorta and the right ventricle and the differential between the aorta and the atria, are somewhat greater during systole than during diastole. Therefore, coronary flow in those parts of the heart is not appreciably reduced during systole. Due to the fact that there is no blood flow during systole in the subendocardial portion of the left ventricle, this region is prone to ischemic damage and is the most common site of myocardial infarction.

The heart has a remarkable ability to autoregulate coronary blood flow over a wide range of pressures (Bayliss, 1902) and to match blood supply closely to myocardial demands (Cross, 1964; Berne & Rubio, 1979). The contribution of myogenic and metabolic factors to this autoregulation has been reviewed. Substances produced by metabolising myocardial tissue such as ATP, prostaglandins, lactic acid, histamine and potassium can directly influence the responses of the coronary arteriolar smooth muscle and appear to be of importance in altering total myocardial blood flow to match myocardial metabolic demands (Berne, 1964).

1.1.3 The structure of blood vessels

The walls of most blood vessels in mammals are composed of three layers (see Rhodin, 1980) known as the tunica intima, the tunica media and the tunica adventitia (Cox, 1982). The tunica intima or inner layer consists of a lining of endothelium with its basal lamina (Majno, 1965). These endothelial cells are in direct contact with the circulating blood. Endothelial cells were traditionally considered as a non-thrombogenic surface which provided a permeability barrier between the blood-stream and the vascular wall, but it is now well established that the endothelium plays an important role in the local control of vascular smooth muscle tone (Vanhoutte & Rimele, 1983). The internal elastic lamina is a variable subendothelial layer of collagen, elastin fibrils and some smooth muscle cells and fibroblasts. It separates the intima from the tunica media.

The tunica media contains the vascular smooth muscle cells, which appear to be circularly arranged, but which are actually arranged in a low pitched spiral, along with some connective tissue, largely elastin and collagen. It is the smooth muscle cells in this layer that are responsible for the development of active tone. They also have important synthetic properties as well as the ability to migrate and proliferate (Wissler, 1968). They are also responsible for the production of the collagenous and elastic components of this layer. The thickness of the smooth muscle layer varies according to the nature and site of the blood vessel. In the muscular arteries the

smooth muscle cells may be several layers deep, while arterioles have only a single layer of cells and capillaries have none. The proportion of connective tissue in the tunica media also varies greatly. In large 'shock-absorbing' arteries such as the aorta the elastic connective tissue predominates, while the epicardial coronary arteries are 'muscular' arteries and have only a poorly defined elastic laminae. In fact, the coronary artery has the lowest elastin to collagen ratio of any vessel of similar size (Cox, 1982). The tunica media is bounded by the internal elastic lamina on the luminal side and by the external elastic lamina on the adventitial side.

The tunica adventitia is a connective tissue layer consisting mainly of collagen and elastin fibres. It contains the vasa vasorum as well as a plexus of autonomic nerves which do not penetrate into the media but run along the adventitial-medial border outside the external elastic lamina, relatively remote from effector smooth muscle cells (Dahlstrom *et al.*, 1965; Lever *et al.*, 1965a; Malor *et al.*, 1973; Denn & Stone, 1976). Branches of the vasa vasorum enter the tunica media to supply the blood vessel wall. The blood vessels only supply the outer portion of the arterial wall; the inner portion is supplied from the lumen of the vessel. Cells of the tunica adventitia are fibroblasts and macrophages.

1.2 PERIVASCULAR NERVES

1.2.1 The vascular neuroeffector junction

In the majority of blood vessels perivascular autonomic nerves are confined to the adventitial side of the media and are characterised by extensive branching such that a two-dimensional plexus is formed (Hillarp, 1946; Burnstock, 1975a). Electron microscope and fluorescent histochemical studies (Hillarp, 1946; Burnstock, 1970) of the relationship of individual nerves to smooth muscle cells along with studies on the electrophysiology of transmission (see Bennett & Burnstock, 1968) led Burnstock and Iwayama (1971) to propose a general model of the autonomic neuroeffector junction. Typically, autonomic nerves have extensive terminal varicose regions (1-2 μm in diameter) which are free of Schwann cell envelopments and are separated by narrow (0.1-0.3 μm in diameter) intervaricose regions (Gabella, 1981; Burnstock, 1986a). The varicosities contain high levels of neurotransmitters which are released 'en-passage' during the conduction of nerve impulses to interact with receptors on the outer smooth muscle cells of the tunica media. The subsequent change in membrane potential of these outer smooth muscle cells is spread between neighbouring cells via specialised low resistance junctions known as 'nexuses' or 'gap-junctions' (Burnstock, 1986b). Therefore, neurogenic input to a single smooth muscle cell is propagated to adjacent cells to allow control of tone of the muscle bundle. The separation between nerve varicosities and

muscle cells varies between different vessels. The minimal junction cleft may be as small as 20 nm in vessels with dense innervation (e.g. small muscular arteries or large arterioles) and as much as 2000 nm in large elastic arteries. In the epicardial coronary artery, Gerova (1982) has estimated that the distance between the nerve terminals and the smooth muscle cells may be greater than 1 μm. This wide gap would allow both pre- and postjunctional modulation of neurotransmission by locally released factors (Burnstock, 1990). In small arteries the gap falls to about 500 nm and in arterioles it is said to be approximately 55-140 nm (Malor *et al.*, 1973). However, Lever *et al.* (1965a) have reported denuded nerve terminals approaching the smooth muscle of coronary arterioles in cats, guinea-pigs, rats and rabbits in the order of 300-700 nm, with the smallest distance as much as 200 nm. This is far greater than in the arterioles of other organs (Appenzeller, 1964; Lever *et al.*, 1965b). In precapillaries and capillaries, the denuded end of the nerve terminals run only 70-80 nm from the endothelial cell and face the endothelial cells rather than the smooth muscle cells of the precapillaries. There are even reports of fusion of the basement membranes of the nerve terminals and endothelium (Thaemert, 1966; Malor *et al.*, 1973). In several studies, true terminals have been located sandwiched between the vascular cells and the cardiac cells, implying that neurotransmitter release may influence both elements (Gerova, 1982).

The vascular neuroeffector junction between varicosities and smooth muscle cells is not a true synapse with well defined

pre- and postjunctional specialization as exemplified by the classical synapses of the skeletal neuromuscular junction and of those present in the ganglia. The varicosity membrane sometimes has thickening associated with vesicle grouping suggesting possible site of neurotransmitter release, but postjunctional specialization is rarely seen.

1.2.2 Sympathetic nerves

Sympathetic outflow from the central nervous system (CNS) is said to be thoracolumbar. This is due to the fact that the preganglionic neurones emerge from the CNS in the ventral roots of the spinal nerves of the first thoracic (T1-T12) to third lumbar (L1-L3) segment of the spinal cord. The axons of these preganglionic neurones, which are typically short and myelinated, run to the autonomic ganglia situated outside the CNS, where most synapse with the cell bodies of postganglionic neurones. Two chains composed of these ganglia and their nerve fibres run along each side of the spinal cord and these make up the paravertebral sympathetic trunks/chains. Not all preganglionic neurones synapse in the paravertebral sympathetic trunk. Instead, they pass through and make synaptic connections with the cell bodies of postganglionic neurones in the coeliac, superior mesenteric and inferior mesenteric prevertebral ganglia and plexuses in the abdomen. Fibres from these then innervate all the abdominal viscera. The axons of the postganglionic neurones, which are long and mostly unmyelinated, emerge from the ganglia to innervate

effectors of the sympathetic system.

The sympathetic nerve supply to the heart is diffuse and derives branches from each of the cervical ganglia and (usually) the upper four thoracic ganglia, forming a superficial and deep cardiac plexus. Branches from these pass with the coronary arteries to supply the myocardium, coronary arteries and conducting systems of the heart (Gerova, 1982).

Sympathetic nerves are the most prolific nerve type and represent the main neurogenic element in the homeostatic regulation of vascular tone. The principal transmitter released from the sympathetic nerve endings is noradrenaline (NA), which is synthesized in the nerve varicosity and stored in small dense-core granular vesicles (35-60 nm). The release of NA is evoked by an action potential which invades the terminal axon causing a change in membrane permeability. Na^+, Cl^- and Ca^{2+} enter the cell and K^+ emerges (Hodgkin & Huxley, 1952). The influx of Ca^{2+} triggers the release of NA from the storage vesicles into the junctional cleft (Cunnane, 1984). When NA is released from the nerve terminal it diffuses down its concentration gradient and initiates a response in the postjunctional tissue by reversibly combining with one of two main types of receptor known as α- and β-adrenoceptors (Ahlquist, 1948). These receptors have been further subdivided into α_1 and α_2 (Langer, 1977; Starke, 1977; Drew, 1981) and β_1 and β_2 (Furchgott, 1967; Lands *et al.*, 1967a, 1967b) on the basis of pharmacological studies.

Postjunctionally, α_1- and α_2-adrenoceptors are found on

the smooth muscle where they mediate vasoconstriction (Vanhoutte *et al.*, 1981). Action at β-adrenoceptors initiates relaxation of the vascular smooth muscle (Vanhoutte, 1978). Unless the β-adrenoceptor is the dominant receptor present, existence of β-adrenoceptors on the smooth muscle may only be revealed after pharmacological blockade of α-adrenoceptors. α_2-Adrenoceptors are also found prejunctionally where they inhibit transmitter release (Langer, 1979; Drew, 1981). β-Adrenoceptors may also be located prejunctionally and action at these receptors may mediate a positive feedback mechanism to enhance transmitter release (Osswald & Guimaraes, 1983). In addition, α_2- and β-adrenoceptors have also been located on the endothelium of some blood vessels (Miller & Vanhoutte, 1985a; Rubanyi & Vanhoutte, 1985a).

Co-transmission and Neuromodulation

Dale's hypothesis states that a single neuron releases similar substances at all of its terminals (Dale,1935) and for many years it was believed that each nerve could synthesis and release only one neurotransmitter (Dale's principle). Inconsistencies in this principle were addressed in a review by Burnstock in 1976 (Burnstock, 1976) and the existence of nerves that can synthesis, store and release more than one pharmacologically active neurotransmitter is now widely accepted (Cuello, 1982; Chan-Palay & Palay, 1984; Burnstock, 1985a; Bartfai *et al.*, 1988).

It has long been known that ATP coexists with NA in

sympathetic nerves (Stjarne & Lishajko, 1966; Geffen & Livett, 1971) and subsequent studies have demonstrated co-storage and co-release of these two substances (Su *et al.*, 1971; Langer & Pinto, 1976). There is now a large body of evidence to show that NA and ATP act as cotransmitters, being released from sympathetic nerves in variable proportions depending on the tissue and species (Burnstock, 1986c, 1988a, 1988b).

It is now clear that ATP is not the only cotransmitter with NA. Advances, particularly in immunohistochemical techniques have identified approximately 16 putative transmitters, including peptides and monoamines as well as purines. One such transmitter is a 36 amino acid peptide known as neuropeptide Y (NPY), which is widely distributed throughout the sympathetic nervous system (Lundberg *et al.*, 1982a; Hokfelt *et al.*, 1983; Lundberg *et al.*, 1983; Ekblad *et al.*, 1984). It co-exists with NA in many sympathetic nerves supplying the cardiovascular system and is released upon sympathetic stimulation (for reviews see Grey & Morley, 1986; Edvinsson *et al.*, 1987; Lundberg *et al.*, 1987). The action of NPY on blood vessels varies markedly. Its primary role appears to be as a neuromodulator, however, in some arteries NPY has direct vasoconstrictor actions. For example, in the cerebral arteries (Edvinsson, 1985; Hanko *et al.*, 1986), skeletal muscle arteries (Pernow *et al.*, 1987) and the coronary vascular bed (Allen *et al.*, 1983; Clarke *et al.*, 1987) NPY appears to have a pronounced direct vasoconstrictor effect.

A neuromodulator modifies the process of neurotransmission (Burnstock, 1985a; 1986b). For example,

neuromodulation is said to occur when a substance acts prejunctionally to increase or decrease the amount of transmitter released, or alternatively acts postjunctionally to alter the time course or extent of action of the neurotransmitter (Burnstock, 1987a). Acting prejunctionally, NPY has been shown to exert an inhibitory effect on the release of NA (Dahlof *et al.*, 1985; Pernow *et al.*, 1986) while postjunctionally, it acts in many vascular beds to enhance NA-induced vasoconstriction (Edvinsson *et al.*, 1984; Lundberg *et al.*, 1985). Postjunctional inhibitory effects have also been demonstrated in canine cerebral arteries (Suzuki *et al.*, 1988).

1.2.3 Parasympathetic nerves

Efferent fibres of the parasympathetic nervous system arise from both a cranial outflow, passing along cranial nerves III (oculomotor), VII (facial), IX (glossopharyngeal) and X (vagus), and a sacral outflow, which is carried in the spinal nerves of sacral segments 2, 3 and 4 of the spinal cord. Preganglionic fibres are generally long in comparison to preganglionic sympathetic fibres since the parasympathetic ganglia are located in the walls of, or in the vicinity of, the effector organ. The postganglionic parasympathetic neurones are therefore relatively short. The most important component of the cranial parasympathetic system is conveyed with the Xth cranial nerve; the vagus and the fibres from this supply the heart via the nodose

ganglion as well as the lungs and gastrointestinal system. The vagal fibres to the heart converge on the paratracheal plexus and then pass with the sympathetic nerves to the cardiac plexuses (Mizeres, 1957). The sacral outflow passes in the pelvic splanchnic nerves to supply the pelvic organs.

Early observations of vagal innervation of the coronary arteries of dog, based on nerve profiles remaining after sympathetic denervation (Woollard, 1926) have been extended to other species, including man (Nonidiez, 1939; Hirch & Borghard-Erdle, 1961). Substantial amounts of acetylcholinesterase (AChE) were found in the coronary arteries of rats, cattle and rabbits (Navaratnam & Palkama, 1965). The innervation was reported to be so dense in humans that Hirch and Borghard-Erdle (1961) mistakenly concluded that the coronary arterioles were only supplied by the vagal fibres. However, nondegenerated fibres were found within the coronary wall after sectioning of the vagus (Denn & Stone, 1976), and although cholinergic fibres were seen in the wall of the coronary artery, stimulation of the peripheral portion of the cut vagus nerve did not induce any change in the diameter of the conduit coronary artery (Gerova, 1982). One possible source of these fibres is intracardiac cholinergic ganglia. These have been detected in the atria (Tcheng, 1951; Jacobowitz, 1967; Yamauchi *et al.*, 1975; Ellison & Hibbs, 1976) where postganglionic fibres from these ganglia pass across the atrioventricular groove (Blomquist *et al.*, 1987) to supply the myocardium and the intramural coronary arteries (Jacobowitz, 1967; Osborne & Silva, 1970).

The principal transmitter of most parasympathetic nerves

is ACh. For many years histochemical localisation of cholinergic nerves was assumed from the localisation of the degradatory enzyme of ACh, AChE (Koelle, 1951; Karnovsky & Roots, 1964), however, AChE does not act exclusively on ACh (Chubb *et al.*, 1980). Since then a more specific method, relying on the immunocytochemical localisation of the ACh synthesizing enzyme choline acetyltransferase (ChAT), has been developed (Eckenstein *et al.*, 1981). It appears that perivascular parasympathetic nerves do innervate the coronary arteries (Navaratnam & Palkama, 1965; Schenk & Badawi, 1968; Denn & Stone, 1976; Pillay & Reid, 1982) but the supply is sparser than that of sympathetic nerves (Burnstock, 1975b).

Cholinergic nerves have been shown to produce vasodilator effects by a direct action on muscarinic receptors on vascular smooth muscle cells of the feline posterior auricular artery (Brayden & Bevan, 1985) and ACh had long been known to dilate the coronary artery of many species via an atropine-sensitive mechanism (Wiggers, 1909; Smith *et al.*, 1926; Katz *et al.*, 1938; Eckenhoff *et al.*, 1947b; Herxheimer, 1960). Relaxant effects mediated by prejunctional inhibition of NA release have also been demonstrated in the dog coronary artery (Cohen *et al.*, 1984). However with the recent advances in understanding of the functions of vascular endothelium and the interaction of neural and humoral factors on vascular smooth muscle, the role of parasympathetic cholinergic neurotransmission is now being reconsidered. The demonstration that in most blood vessels denuded of the endothelium; ACh either produces contraction or has little effect

in many vessels (Furchgott & Zawadzki, 1980a), demands a reappraisal of the effects of ACh released from parasympathetic nerves.

Co-transmission and neuromodulation

The classical example of co-transmission in parasympathetic nerves is ACh released with vasoactive intestinal polypeptide (VIP). VIP is a 28 amino acid polypeptide which is widely distributed in peripheral and central neurones (Said, 1982). It acts as a neurotransmitter causing direct relaxation of the vascular smooth muscle (Larsson *et al.*, 1976; Duckles & Said, 1982; Unverferth *et al.*, 1985; Brum *et al.*, 1986) through action at specific VIP binding sites (Barnes *et al.* 1986). In addition to the demonstration that VIP is co-localised with AChE (Lundberg & Hokfelt, 1983) and ChAT (Leblanc *et al.*, 1987), it has been suggested that vasodilatation in many exocrine glands is mediated by co-release of VIP and ACh from postganglionic neurones in both parasympathetic and sympathetic pathways (Lundberg *et al.*, 1980; Lundberg, 1981; Lundberg *et al.*, 1982b). In the cat salivary gland ACh and VIP are co-stored in parasympathetic nerves and co-released on nerve stimulation. Low frequency stimulation evokes the release of ACh, causing salivary secretion and some vasodilatation (Bloom & Edwards, 1980; Lundberg, 1981). At higher frequencies the nerves release both ACh and VIP to produce a marked vasodilatation. VIP may also neuromodulate the action of ACh, increasing both the postjunctional effect on acinar cell

secretion and the release of ACh from nerve varicosities via a prejunctional mechanism (see Burnstock, 1987b).

1.2.4 Sensory-motor nervous system

In addition to the efferent fibres, there is also an afferent component to the autonomic nervous system. Afferent fibres containing sensory information may pass from the viscera along both sympathetic and parasympathetic pathways and reach the CNS without synapsing. However, it has been known for many years that stimulation of sensory nerves in the dorsal roots of the spinal column, or stimulation of the distal end of cut sensory nerves, could give rise to a motor response producing vasodilatation in the skin (Bayliss, 1901). This response was called 'antidromic' vasodilatation. In 1927, Lewis described the 'axon reflex' in which antidromic impulses in primary afferent sensory neurones pass down collateral branches and cause vasodilatation via distal release of the sensory neurotransmitter (Lewis, 1927; see Dale, 1935; Burnstock, 1977). More recently, evidence has accumulated to support a 'sensory-efferent' role of sensory nerve endings whereby capsaicin-sensitive neurones have the ability to release the stored transmitter from the same terminal which is excited by the environmental stimulus (Maggi & Meli, 1988).

Substance P, the oldest-known neuropeptide, was discovered by von Euler and Gaddum in 1931 (von Euler & Gaddum, 1931). The immunohistochemical localization of substance P in

cell bodies of the sensory ganglia as well as in nerve fibres in the periphery and in the dorsal horn of the spinal cord (Jessell *et al.*, 1978; Furness *et al.*, 1982; Wharton & Gulbenkia, 1987) forms the neurochemical basis for the role of substance P as a neurotransmitter in primary sensory nerves, as originally suggested by Lembeck (1953). This hypothesis has been substantiated in subsequent studies in which it has been demonstrated that there is a loss of substance P-immunoreactivity following treatment with capsaicin (Duckles & Buck, 1982; Furness *et al.*, 1982; Saria *et al.*, 1985; Duckles, 1986). Specific receptor sites for substance P have been identified on vascular smooth muscle and endothelial cells (Regoli *et al.*, 1984a, 1984b, 1989).

Substance P is not the only peptide present in capsaicin-sensory neurones. Calcitonin gene-related peptide (CGRP), a 37 amino acid neuropeptide, has also been localised in sensory neurones (Fischer *et al.*, 1983; Rosenfeld *et al.*, 1983). In some species it appears to be extensively co-stored with substance P (Gibson *et al.*, 1984; Gibbins *et al.*, 1985; Lee *et al.*, 1985; Lundberg *et al.*, 1985; Gulbenkian *et al.*, 1986). Like substance P, CGRP is a potent vasodilator (Brain *et al.*, 1985). CGRP may also modulate the effects of substance P, potentiating release of substance P and inhibiting its breakdown (Le Greves *et al.*, 1985; Oku *et al.*, 1987).

It has also been demonstrated that ATP was released on antidromic stimulation of sensory nerves (Holton, 1959). It is now known that ATP co-exists with substance P in sensory nerves

(Burnstock, 1977). It is possible that ATP may be found to play a role in mediating cardiac pain as has previously been suggested for adenosine (Crea *et al.*, 1990) and to mediate antidromic coronary vasodilatation described by Lewis (1947).

1.3 THE VASCULAR ENDOTHELIUM

The vascular endothelium is a simple squamous epithelium which is the layer of the intima in direct contact with the circulating blood. Endothelial cells play a major role in capillary transport, regulation of plasma lipids and the control of homeostasis. For many years it was considered to be a simple diffusion barrier moderating vascular permeability. Due to the tight interconnections between adjoining endothelial cell membranes the entry of large macromolecules, such as plasma proteins, into the vessel wall was prevented. The movement of these proteins into the vessel wall occurred only by pinocytosis and was an actively regulated process (Goldberg *et al.*, 1982). Endothelial cells were also found to possess important metabolic functions. For example, they contain lipoprotein lipase which regulates the binding of lipoprotein to the vessel wall and maintains homeostasis of vascular lipids (Scow *et al.*, 1976). They can also synthesis prostacyclin which inhibits adhesion of platelets to the vessel wall (Moncada *et al.*, 1977a). The role of the endothelium as a non-thrombogenic surface was supported by the finding of proteoglycans (Gimbrone, 1976), heparin sulphate, antithrombin III (Chan & Chan, 1979) and plasminogen activator

(Loskutoff & Edgington, 1977) within endothelial cells. Substances produced by cultured endothelial cells resulted in the identification of many factors which promote thrombosis or activation of the coagulation system, such as von Willebrand factor (Jaffe *et al.*, 1974), platelet activating factor (Zimmerman *et al.*, 1985), thromboxane A_2 (Mehta & Roberts, 1983), thromboplastin and fibronectin (Jaffe & Mosher, 1978). This suggests that the endothelium plays a more subtle role in maintaining a well regulated balance between coagulation and fibrinolysis. The endothelium has also been shown to metabolise several vasoactive agents, including NA (Hughes *et al.*, 1969; Gillis & Pitt, 1982; Rorie & Tyce, 1985), 5-HT (Strum & Junod, 1972; Small *et al.*, 1977), adenine nucleotides (Pearson & Gordon, 1985), bradykinin and angiotensin I (Ryan & Ryan, 1977). The endothelium can also synthesis and release endothelium-derived relaxing factors (e.g. prostacyclin and NO) and endothelium-derived contracting factors (e.g. endothelin, prostanoids, angiotensin and histamine) (see Gerlach *et al.*, 1985; Vane *et al.*, 1987; Lüscher, 1988).

This section will concentrate on the role of the vascular endothelium in the control of vascular smooth muscle tone.

1.3.1 Endothelial prostacyclin

Arachidonic acid (AA) is the principal precursor of prostaglandins in mammalian tissue. The main metabolic product of

AA in vascular tissue is prostacyclin (Moncada *et al.*, 1976). The ability of medium sized vessels to synthesize prostacyclin is greatest at the intima surface and decreases progressively towards the adventitia (Moncada *et al.*, 1977b). Removal of the endothelium from the rabbit aorta virtually abolishes the ability of the luminal surface of the artery to produce prostacyclin from exogenous AA (Eldor *et al.*, 1981). These observations have been supported by studies with cells from the vessel wall in culture which show that endothelial cells are the most active producers of prostacyclin (Weksler *et al.*, 1977). The biosynthesis of prostacyclin from endothelial cells is initiated by intracellular generation from AA, which is liberated from endothelial phospholipids by an active phospholipase. For example, release of prostacyclin from porcine endothelial cells by bradykinin is accompanied by activation of both phospholipase A_2 and phospholipase C (Hong & Deykin, 1982; Lambert *et al.*, 1986). Biosynthesis of prostacyclin by endothelial cells can also be initiated through a transmembrane transferance of prostaglandin endoperoxides from platelets (Bunting *et al.*, 1976; Shafer *et al.*, 1984). Prostacyclin is chemically unstable, with a half-life of 2-3 minutes, breaking down to 6-Keto-prostaglandin $F_{1\alpha}$ (see Gryglewski *et al.*, 1988). The vasodilatation and inhibition of platelet aggregation induced by prostacyclin are correlated with an activation of the adenylate cyclase system, leading to a rise in intracellular cyclic 3', 5'-adenosine monophosphate (cAMP) (Gorman *et al.*, 1977; Ito *et al.*, 1980).

Several drugs as well as exogenous mediators, stimulate

the generation of prostacyclin. Human endothelial monolayers in culture produce prostacyclin in response to stimulation by AA, prostaglandin H_2, thrombin and the calcium ionophore A23187 (Weksler *et al.*, 1978a). Prostacyclin release can be activated by receptor operated mechanisms, for example, bradykinin in cat and rabbit coeliac and mesenteric arteries induces relaxations that are blocked by indomethacin suggesting that in these cases prostacyclin mediates the response (Cherry *et al.*, 1982; Förstermann *et al.*, 1986). Many substances evoking endothelium-dependent relaxations, including ACh, ATP and adenosine 5'-diphosphate (ADP), may also release prostacyclin and other prostanoids from endothelial cells in some vessels (Beetens *et al.*, 1983; Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984; Förstermann *et al.*, 1986; Forsberg *et al.*, 1987; Needham *et al.*, 1987).

Monolayers of bovine aortic endothelial cells have also been shown to produce bursts of prostacyclin in response to step increases in shear stress up to levels found in arteries (Grabowski *et al.*, 1985). It has also been demonstrated that prostacyclin release from endothelial cells occurs in response to shear stress in cultured human umbilical vein endothelial cells (Frangos *et al.*, 1985; Bhagyalakshmi & Frangos, 1989) and in response to hypoxia in the rat tail artery (Busse *et al.*, 1983).

1.3.2. Endothelium-derived relaxing factor (EDRF)

One of the first reports of endothelium-dependent

vasodilatation was published in 1980 (Furchgott & Zawadzki, 1980a) and addressed the vascular responses to ACh. In preparations with intact endothelium, low concentrations of ACh were found to relax precontracted rabbit aorta, while higher concentrations ($>10^{-6}$ M) exerted direct contractile effects independent of the presence of the endothelium. It was postulated that the endothelium released a factor that acted on arterial smooth muscle to promote relaxation of muscle tone. Demonstration of a diffusible factor released from endothelial cells has come from bioassay perfusion systems utilizing vessel strips (Rubanyi *et al.*, 1987) and cultured endothelial cells (Angus & Cocks, 1984; Loeb *et al.*, 1987) as donors of this relaxing factor. Prostacyclin was an immediate candidate for the factor; however, endothelium-dependent vasodilatation was obtained in arteries (i.e. rabbit thoracic aorta) that do not respond to prostacyclin and the response was not altered by treatment with cyclooxygenase inhibitors (Furchgott & Zawadzki, 1980). The term *"endothelium-derived relaxing factor"* was proposed for the substance that induces smooth muscle relaxation (Furchgott, 1983). Investigations into the nature and mechanisms of action of EDRF have not been facilitated by its short half-life: 6-49 seconds (see Lüscher, 1988). Based on the similarities in the pharmacological behaviour of EDRF and NO Furchgott in 1986 suggested that EDRF may be NO (see Furchgott, 1988). At the same time Ignarro *et al.* (1988) also speculated that it may be NO or a closely related species. The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF released from vascular endothelial cells

was detected by the chemical means used to identify NO. NO may be measured as the chemiluminescent product of its reaction with ozone (Downes *et al.*, 1976). It was shown using this method that the concentrations of bradykinin that induce the release of EDRF from porcine aortic endothelial cells in culture also caused concentration-dependent release of NO. Also the amounts of NO released by the cells were sufficient to account for the relaxation of vascular strips (Palmer *et al.*, 1987). Furthermore, the levels of NO released by these cells also accounted for the inhibition of platelet aggregation and adhesion induced by EDRF (Radomski *et al.*, 1987a, 1987b). A detailed comparison of the biological actions of EDRF and NO on vascular strips (Hutchinson *et al.*, 1987; Palmer *et al.*, 1987) also showed that the two compounds were indistinguishable. The release of NO from isolated perfused rabbit (Amezcua *et al.*, 1988) or guinea-pig (Kelm & Schrader, 1988) hearts has been shown to account for the vasodilator actions of ACh and bradykinin in these preparations.

In 1988, the amino-acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells, cultured in the absence of L-arginine for 24 hours prior to the experiments, showed a decreased in the release of EDRF induced by bradykinin and A23187 which could be restored by L-but not D-arginine (Palmer *et al.*, 1988a). The formation of NO_2^- from L-arginine by endothelial cells was also reported (Schmidt *et al.*, 1988). It was found that the release of NO from endothelial cells could be inhibited in an enantiomerically specific manner by N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et*

al., 1988b), an inhibitor of the generation of NO_2^- and NO_3^- and citrulline from L-arginine in macrophages (Hibbs *et al.*, 1987). Endothelial homogenates form citrulline from L-arginine by a mechanism that is nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) dependent and inhibited by L-NMMA (Palmer & Moncada, 1989). In endothelial cell cytosol, depleted of L-arginine, there was an L-arginine-dependent increase in cyclic guanosine monophosphate (cGMP) which was also concentration-dependent, required NADPH and was accompanied by the formation of [^{3}H] citrulline from [^{3}H] arginine (see Moncada & Palmer, 1990). Both the production of [^{3}H] citrulline and the increases in cGMP were inhibited by L-NMMA. This data is consistent with NO and citrulline being co-products of the same enzymatic process. The formation of [^{3}H] citrulline and the increase in cGMP were inhibited by Ca^{2+} chelators, indicating that this enzyme, which is commonly known as *NO synthase*, is Ca^{2+}-dependent (see Moncada & Palmer. 1990). The production and/or release of EDRF appears to be dependent on intracellular (Defeudis, 1985; Hallam & Pearson, 1986) and extracellular Ca^{2+} (Long & Stone, 1985; Winquist *et al.*, 1985; Collins *et al.*, 1986; Lückhoff *et al.*, 1988). Accordingly, an increase in extracellular Mg^{2+} has been shown to inhibit the release of EDRF in canine coronary arteries (Ann & Ku, 1986). In addition, it has been suggested that Ca^{2+}-dependent stimulation of NO synthase in endothelial cells is mediated by calmodulin (Busse & Mulsch, 1990). In contrast to prostacyclin the relaxation of smooth muscle cells by EDRF is usually coupled to cGMP in a time and concentration-dependent manner (Holzmann, 1982; Rapoport &

Murad, 1983; Griffith *et al.*, 1985) and is antagonised by the inhibitors of guanylate cyclase, haemoglobin and methylene blue (Holzmann, 1982; Martin *et al.*, 1985).

L-NMMA is a competitive inhibitor of NO synthase (Palmer & Moncada, 1989; Mayer *et al.*, 1989). It also inhibits the release of NO from endothelial cells (Palmer *et al.*, 1988b) and vascular tissues (Amezcua *et al.*, 1989; Rees *et al.*, 1989). The importance of NO, formed from L-arginine, in regulating basal tone and the response to endothelium-dependent vasodilators in resistant vessels has been demonstrated in the isolated perfused rabbit heart (Amezcua *et al.*, 1989). In this preparation, L-NMMA caused an increase in coronary perfusion pressure and an inhibition of the decrease in coronary perfusion pressure induced by ACh, accompanied by inhibition of the release of NO into the coronary effluent. L-NMMA also inhibited vasodilator responses to ACh, A23187, substance P and L-arginine in the rabbit aorta (Rees *et al.*, 1989). Other L-arginine analogues have been described as inhibitors of NO generation in vascular tissue. For example, N^G-nitro-L-arginine (L-NA) is an inhibitor of NO synthase (Mulsch & Busse, 1990) and has effects on vasculature tissue similar to those described for L-NMMA (Moore *et al.*, 1989; Ishii *et al.*, 1990; Mulsch & Busse, 1990). L-NA has been shown to inhibit vasodilator responses to ACh and 5-HT in the isolated perfused rabbit heart (Lamontagne *et al.*, 1991) and to attenuate the duration of the vasodilator response to bradykinin in the isolated rat heart (Baydoun & Woodward, 1991). It has also been shown to be more potent than L-NMMA at inhibiting the generation of NO

(Moore *et al.*, 1990). Further more, N-iminoethyl-L-ornithine (L-NIO), L-NA and its methyl ester N^G-nitro-L-arginine methyl ester (L-NAME) induced enantiomerically specific effects on vascular tissue *in vivo* and *in vitro* which were qualitatively similar to those described for L-NMMA (Rees *et al.*, 1990a, 1990b).

Endothelium-dependent vasodilatation has been shown to occur in response to many different vasodilators. ATP (Furchgott, 1981; Kennedy *et al.*, 1985; Martin *et al.*, 1985; Houston *et al.*, 1987; Dudel & Förstermann, 1988; Williams *et al.*, 1988), ADP (DeMey & Vanhoutte, 1981; Houston *et al.*, 1987), substance P (Angus *et al.*, 1983; Furchgott, 1983; D'Orleans-Juste *et al.*, 1985; Beny *et al.*, 1986; Dudel & Förstermann, 1988; Griffith *et al.*, 1988; Malomvögyi *et al.*, 1988), 5-HT (Cocks & Angus, 1983; Stewart *et al.*, 1987), histamine (Toda, 1984) and bradykinin (Cherry *et al.*, 1982; Gordon & Martin, 1983; Ignarro *et al.*, 1987a) have been shown to act at least partially through this mechanism, although there is considerable variation between species and even between different vessels in the same species (Vanhoutte & Miller, 1985; Nyborg & Mikkelsen, 1990). It follows that damage to the endothelium may quantitatively and qualitatively alter the response of blood vessels to locally released or circulating agents (Busse *et al.*, 1985). Diseases, such as atherosclerosis, which affect the intima *in vivo* may also change responses to these vasoactive agents (Ginsberg *et al.*, 1984, Kalsner & Richards, 1984).

The endocardial cells are ontogenetically similar to the endothelium. Removal of the endocardium has been shown to induce

a negative inotropic effect in isolated papillary muscle preparations (Smith *et al.*, 1991). This, together with the finding that cultured porcine endocardial cells release NO and possess an NO synthase (Schulz *et al.*, 1991), suggests that the L-arginine: NO pathway plays a role in myocardial contractility (Lewis *et al.*, 1990; Smith *et al.*, 1991).

In addition to EDRF, endothelial cells have been shown to release a hyperpolarizing factor (endothelium-derived hyperpolarising factor; EDHF) for example, in the rat aorta and pulmonary artery response to ACh (Chen *et al.*, 1988).

The similarities between the properties and effect of EDRF and the mediator of inhibitory non-adrenergic, non-cholinergic (NANC) transmission were first pointed out for the rat anococcygeus and the bovine retractor penis muscles by Gillespie (1987) and Furchgott (1988). Since then blockade of neurogenic vasodilatation in vascular beds by NO synthase inhibitors has also been reported. For example, vasodilator responses of arterioles evoked by stimulation of the nerve to the tenuissimus muscle in the hindleg of the rabbit, in the presence of pancuronium to block skeletal muscle contraction and guanethidine to block vasoconstriction, were blocked by L-NAME and restored by L-arginine, as were vasodilator responses to ACh (Persson *et al.*, 1991). In perfused segments of the bovine penile artery, stimulation-induced NANC relaxations were abolished by L-NA (Martin *et al.*, 1991). Both L-NMMA and L-NA increased the tone and blocked ACh-induced relaxations but L-NMMA differed from L-NA in that it did not block NANC relaxations, indicating a difference

between endothelial and neuronal NO synthase (Liu *et al.*, 1991). In rat cerebral blood vessels, NO synthase was detected by immunohistochemistry in adventitial nerve fibres as well as in endothelial cells, whereas in the aorta and coronary arteries it was detected only in endothelial cells (Bredt *et al.*, 1990).

1.3.3 Endothelium-derived constricting factors

Besides EDRF's, the endothelium can release substances which induce contraction of the underlying smooth muscle. This suggestion was first made on the basis of experiments in canine femoral arteries, where removal of the endothelium reduced the contractions evoked by increasing concentrations of potassium ions (DeMey & Vanhoutte, 1981). Later work in isolated arteries and veins demonstrated that rapid endothelium-dependent increases in tension can be obtained which are explained best by the production of mediators that activate the underlying smooth muscle. These substances have been termed *"endothelium-derived contracting factors"* (EDCF) (Vanhoutte, 1987; Lüscher & Vanhoutte, 1990).

Exogenous AA induces endothelium-dependent contractions in canine vessels (DeMey & Vanhoutte, 1982; Miller & Vanhoutte, 1985b). In the rabbit aorta, AA evoked more pronounced increases in tension in rings with endothelium than in those without endothelium (Singer & Peach, 1983a, 1983b) and the endothelium-dependent component of the contractions induced by AA were inhibited by indomethacin. In the aorta of the spontaneously hypertensive rat, in the renal artery of the normal rat and in the

basilar artery of the dog and rabbit, ACh induces endothelium-dependent contractions which can be blocked by indomethacin (Altiere *et al.*, 1986; Lüscher & Vanhoutte, 1986; Shirahasa *et al.*, 1987a, 1987b; Katusic *et al.*, 1988; Toda *et al.*, 1988). Likewise, in the basilar artery of the dog, NA, the calcium ionophore A23187 and nicotine cause endothelium-dependent contractions which are prevented by inhibitors of cyclooxygenase (Usui *et al.*, 1983, 1987; see Katusic & Shepherd, 1988). Thromboxane A_2 is the major vasoconstrictor product of cyclooxygenase; its formation from endoperoxides is catalysed by the enzyme thromboxane synthase, and this may occur in endothelial cells (Ellis *et al.*, 1976; Moncada & Vane, 1979). This may be the case in the isolated pulmonary artery of the rabbit where thromboxane synthetase inhibitors reduced endothelium-dependent contractions evoked by ACh (Altiere *et al.*, 1986). However, the production of thromboxane A_2 cannot explain the endothelium-dependent contractions evoked by AA in the canine veins or by ACh and endoperoxides in canine cerebral arteries (Miller & Vanhoutte, 1985b; Katusic *et al.*, 1988; Toda *et al.*, 1988). In the canine basilar artery, superoxide anion appears to be the cyclooxygenase-dependent EDCF (Vanhoutte & Katusic, 1985; Katusic & Vanhoutte, 1989).

Cultured bovine aortic endothelial cells release a potent vasoconstrictor peptide(s) (Hickey *et al.*, 1985; Gillespie *et al.*, 1986; Highsmith *et al.*, 1988). It is a small peptide that has been characterized as endothelin (Yanagisawa *et al.*, 1988a; Masaki, 1989). Endothelin may contribute to the long-term

regulation of basal tone of the blood vessels in response to stimuli such as thrombin, vasopressin and angiotensin II, which cause delayed but long-lasting stimulation of the production of the peptide (Yanagisawa *et al.*, 1988b; Schini *et al.*, 1989; Boulanger & Lüscher, 1990). Receptors for endothelin have been localised on cultured rat aortic smooth muscle cells (Hirata *et al.*, 1988), rat kidney (Kohzuki *et al.*, 1989) and human and porcine coronary arteries (Power *et al.*, 1989). Besides its ability to cause a vasoconstrictor response, endothelin-1 releases prostacyclin and EDRF (De Nucci *et al.*, 1988) and elicits vasodilatation *in vivo* (Wright & Fozard, 1988).

Anoxia augments the contractions of isolated canine peripheral, coronary and cerebral arteries (DeMey & Vanhoutte, 1982, 1983; Rubanyi & Vanhoutte, 1985b; Katusic & Vanhoutte, 1986). This anoxic facilitated contraction is abolished or reduced by the removal of the endothelium (Rubanyi & Vanhoutte, 1985b; Iqbal & Vanhoutte, 1988). The anoxic endothelium-dependent contractions are not blocked by inhibitors of phospholipase A_2 or lipoxygenase, which rules out a product of the metabolism of AA as a mediator (Rubanyi & Vanhoutte, 1985b). The mediator of the hypoxic response is unlikely to be endothelin since: (1) anoxic endothelium-dependent facilitation is not observed in canine peripheral veins, although their endothelium release EDCF and their smooth muscle is sensitive to endothelin (DeMey & Vanhoutte, 1982; Rosen & Freeman, 1984; Miller *et al.*, 1989); (2) Ca^{2+} antagonists prevent endothelium-dependent contractions in response to anoxia in canine vessels, but not contractions evoked by

endothelin (Katusic & Vanhoutte, 1986; Iqbal & Vanhoutte, 1988; Miller *et al.*, 1989) and (3) the anoxic EDCF cannot be bioassayed under conditions where the bioassay tissue contracts to exogenous endothelin (Vanhoutte *et al.*, 1989).

1.3.4 Endothelium as a source of vasoactive agents

Obviously, the blood is a source of vasoactive substances which may act on the endothelium, either because the substances are present in the circulation (e.g. catecholamines, angiotensin, vasopressin) or because they are released from aggregating platelets (e.g. 5-HT, ATP, ADP). Transmitters released from perivascular nerves are unlikely to diffuse across the media and basal lamina without degradation to act on endothelial receptors. ACh and ATP in particular would be subject to degradation by AChE and 5'-nucleotidase respectively, located at the myo-endothelial border (see Burnstock, 1985b, 1987b). The blood cannot account for the entire source of vasoactive substances as certain vasoactive substances which are known to produce an endothelium-dependent response, such as ACh and substance P, do not persist in the circulation in appreciable amounts but are rapidly broken down.

The first study to specifically investigate the endothelium as a source of vasoactive agents was carried out by Parnavelas and colleagues (Parnavelas *et al.*, 1985). They showed that the enzyme ChAT, which is responsible for the synthesis of ACh, could be localised inside the endothelial cells lining the

capillaries and small vessels in the rat cortex. Immunohistochemical staining methods at the electron microscopic level were used to localise ChAT. Since then, this technique has been used to show ChAT, substance P, 5-HT, vasopressin and angiotensin II inside endothelial cells of several blood vessels including the rat femoral, mesenteric and coronary vessels (Burnstock *et al.*, 1988; Loesch & Burnstock, 1988; Milner *et al.*, 1989).

ATP has been found in the effluent of organs perfused with blood-free medium (see Forrester, 1981; Pearson & Gordon, 1984, 1985; Gordon, 1986). It has been shown that 5-HT, substance P, ATP and ACh can be released from the Langendorff heart in response to hypoxia (Paddle & Burnstock, 1974; Burnstock *et al.*, 1988; Milner *et al.*, 1989). Since ATP has been shown to be contained within and may be released from cultured coronary endothelial cells (Nees & Gerlach, 1983), a source of the ATP released during hypoxia may be from endothelial cells. Substance P has been shown to be released from the endothelial cells of the rat hindlimb (Ralevic *et al.*, 1989) and from cultured endothelial cells (Lincoln & Burnstock, 1990) in response to increased flow. Endothelial cells from different vascular beds have been shown to release ATP during shear stress induced by fast flow (Milner *et al.*, 1990a, 1990b). These studies suggest that physicochemical stimuli may cause release of vasoactive agents from endothelial cells which then act on adjacent cells to give rise to the phenomenon of flow-induced dilation.

1.3.5 Shear stress and the endothelium

Haemodynamic shear stress plays an important role in both the physiology and pathobiology of the vascular endothelium: the long axis and nuclei of endothelial cells become aligned in the direction of blood flow (Flaherty *et al.*, 1972; Langille & Adamson, 1981), vascular permeability is affected (Woolf, 1983; Svendsen & Tindall, 1988) and there is evidence that turbulent shear stress induces vascular endothelial turnover (Davies *et al.*, 1986). It has been suggested that the diameter of the lumen of the arteries is controlled continuously by the blood flow rate in an endothelium-dependent manner and that this control is ensured by the ability of the endothelium to perceive shear stress. This has been demonstrated in the canine femoral artery where flow caused vasodilatation and induced the release of EDRF (Hull *et al.*, 1986; Rubanyi *et al.*, 1986; Rubanyi, 1988). This dilatation was blocked by inhibitors of guanylate cyclase and of lipoxygenase-cyclooxygenase (Kaiser *et al.*, 1986) and attenuated by removal of the endothelium (Smiésko *et al.*, 1985). Flow-dependent, endothelium-mediated vasodilatation which is unaffected by cyclooxygenase inhibition, has been demonstrated in canine epicardial coronary arteries (Holtz *et al.*, 1984). Flow-induced vasodilatation has been shown to occur at the microvascular level in rat mesenteric arterioles (Smiésko *et al.*, 1987) in isolated resistance vessels from the rabbit brain and ear, from the cat lingual circulations (Bevan & Joyce, 1988) and in the rabbit ear arteries *in situ* (Griffith *et al.*, 1987).

1.4 CONTROL OF BLOOD PRESSURE TONE BY PURINES

Drury and Szent-györgyi (1929) showed that adenosine 5'-monophosphate (AMP), isolated from extracts of heart muscle, brain, kidney and spleen, and adenosine, prepared from yeast, caused bradycardia, lowering of arterial blood pressure, vasodilatation of the coronary blood vessels and inhibition of intestinal movements. Subsequently, it was shown that systemic administration of these compounds had profound effects on the blood pressure of a number of different species and that adenosine and probably ATP are released into the circulation after ischaemia or hypoxia (Gillespie, 1933; Green & Stoner, 1950; Berne, 1963; Haddy & Scott, 1968; Berne *et al.*, 1974). Purines also affect a large number of isolated blood vessels from numerous vascular beds including the cerebral, coronary, gastroduodenal, renal, hindlimb and forelimb vascular beds (Burnstock, 1980; Burnstock & Brown, 1981; Su, 1981, 1985).

1.4.1 Receptors for adenosine and adenine nucleotides

The first indication that adenine nucleosides and nucleotides may have different pharmacological actions were presented by Gillespie (1933), who described ATP as being more potent than its non-phosphorylated derivatives in causing relaxation of the guinea-pig ileum, and adenosine as being more potent than its phosphorylated derivatives in causing coronary vasodilatation, or inducing hypotension in cats and rabbits. In

1978, Burnstock suggested a division into P_1- and P_2-purinoceptors based on several criteria. At the P_1-purinoceptor an agonist potency order of adenosine > AMP > ADP > ATP was proposed, methylxanthine derivatives, such as theophylline and caffeine, were proposed to be selective antagonists and occupation of the P_1-purinoceptor was suggested to lead to changes in intracellular cAMP. At the P_2-purinoceptor, an agonist potency order of ATP > ADP > AMP > adenosine has been proposed, methylxanthines were not antagonists at the P_2-purinoceptor and there was no change in intracellular cAMP levels after occupation of the P_2-purinoceptor. This terminology has gained general acceptance and now subclassifications of P_1-and P_2-purinoceptors are recognised.

1.4.1 (i) P_1-Purinoceptors

Extracellular adenosine-receptors, i.e. P_1-purinoceptors, were subdivided into A_1- and A_2-receptors on the basis of inactivation and activation, respectively, of adenylate cyclase (Van Calker *et al.*, 1979). The A_1-purinoceptor has a greater affinity for adenosine than the A_2-purinoceptor, and at the A_1-purinoceptor N^6-substituted analogues, such as N^6-cyclopentyladenosine (CPA) and N^6-phenylisopropyladenosine (PIA), are more potent than 5'-substituted analogues, such as 5'-N-ethyl-carboxamido-adenosine (NECA). For A_2-purinoceptors the converse rank order of potency occurs (Bruns, 1990a, 1990b; Daly, 1990). In the majority of blood vessels, adenosine causes a vasodilatation by a direct action on P_1-purinoceptors,

specifically A_2-purinoceptors, located on the smooth muscle membrane (Collis & Brown, 1983; Burnstock & Buckley, 1985; Snyder, 1985; White & Angus, 1987). However endothelium-dependent relaxations to adenosine have been demonstrated in pig, rat and guinea-pig aorta (Gordon & Martin, 1983; Yen *et al.*, 1988; Headrich & Berne, 1990), sheep and canine coronary arteries (Rubanyi & Vanhoutte, 1985; Kwan *et al.*, 1989) and in the basilar, lingual, pulmonary and central ear artery of the rabbit (Frank & Bevan, 1983; Kennedy & Burnstock, 1985a). Adenosine has been shown to be released from coronary endothelial cells (Deussen *et al.*, 1986) and at low doses it has been shown to induce relaxation via receptors on endothelial cells (Corr & Burnstock, 1990; Balcells *et al.*, 1992). Des Rosiers and Nees (1987) have provided functional evidence for the presence of A_2-purinoceptors on cultured coronary endothelial cells. However, in the guinea-pig coronary artery P_1-purinoceptors appear to be limited to the smooth muscle (Keff *et al.*, 1992).

Adenosine has been shown to induce vasoconstriction, however this has been attributed to the secondary release of 5-HT in the rat femoral vascular bed (Sakai & Akima, 1977, 1978) and tail artery (Brown & Collis, 1981) and to angiotensin II in the dog kidney (Osswald *et al.*, 1979). The major functional role of adenosine in the nervous system appears to be that of a modulator. It is a powerful inhibitor of several transmitters, an effect mediated predominantly by adenosine receptors of the A_1 subtype, and it may also influence the activity of transmitters at the effector cell level (see Snyder, 1985; Fredholm *et al.*, 1987).

An A_3 subclass of P_1-purinoceptors has also been described (Ribeiro & Sebastião, 1986; Sebastião & Ribeiro, 1988; 1989). This receptor is not linked to an adenylate cyclase transduction mechanism and affects mobilisation of calcium instead. Typically these receptors mediate prejunctional inhibition of release of transmitter and negative tropisms in the heart by inhibiting transmembrane calcium ion fluxes (Ribeiro & Sebastião, 1986).

1.4.1 (ii) P_2-Purinoceptors

ATP can both relax and constrict isolated blood vessels (Kennedy & Burnstock, 1985b; Burnstock & Warland, 1987; Houston *et al.*, 1987; Reilly *et al.*, 1987) and studies with novel P_2-purinoceptor agonists and antagonists suggested that the P_2-purinoceptor does not form a homogeneous group. Consequently, Burnstock and Kennedy (1985) proposed a subdivision of the P_2-purinoceptor into P_{2X} and P_{2Y} subtypes. At the P_{2X}-purinoceptor, analogues of ATP in which the phosphate chain had been altered, such as α,β-methylene ATP (α,β-meATP) and β,γ-methyleneATP (β,γ-meATP), were found to be more potent than analogues of ATP in which the purine nucleus had been modified, such as 2-meSATP. On this basis the contractile responses to ATP in the rat coronary vasculature are mediated via action at P_{2X}-purinoceptors (Hopwood & Burnstock, 1987). ArylazidoaminopropionylATP ($ANAPP_3$) has been shown to block responses to ATP or its analogues mediated via P_{2X}-purinoceptors (Hogaboom *et al.*, 1980; Fedan *et al.*, 1982; Westfall

et al., 1983), but has not been shown to antagonise ATP in P_{2Y}-systems (Frew & Lundy, 1982a, 1982b; Westfall *et al.*, 1982). However, $ANAPP_3$ may interact with P_1-purinoceptors (Frew & Lundy, 1986). α,β-MeATP selectively desensitizes P_{2X}-purinoceptors (Kasakov & Burnstock, 1983).

At the P_{2Y}-purinoceptors the rank order of ATP-analogue potency is reversed: 2-meSATP is more potent than α,β-meATP or β,γ-meATP (Kennedy & Burnstock, 1985b). The relaxant responses to ATP in many blood vessels are typically mediated via P_{2Y}-purinoceptors (Kennedy & Burnstock, 1985b; Mathieson & Burnstock, 1985; Burnstock & Warland, 1987; Fleetwood & Gordon, 1987; Hopwood & Burnstock, 1987; Reilly *et al.*, 1987). In some blood vessels the P_{2Y}-purinoceptor is located on the endothelium rather than on the vascular smooth muscle and activation of the receptor stimulates release of a relaxant factor (Kennedy *et al.*, 1985; Martin *et al.*, 1985; Houston *et al.*, 1987; Hopwood *et al.*, 1989; Taylor *et al.*, 1989). ATP has been shown to induce release of prostaglandins from perfused vascular beds and isolated blood vessels (Needleman *et al.*, 1974; Schwartzman *et al.*, 1981; Boeynaems & Galand, 1983), prostacyclin in particular, from cultured endothelial cells of pig aorta, bovine adrenal medulla and human umbilical vein (Pearson *et al.*, 1983; Forsberg *et al.*, 1987; Needham *et al.*, 1987; Carter *et al.*, 1988). ATP has also been shown to cause the release of prostaglandin I_2 (PGI_2) from the heart (Needleman *et al.*, 1974) and Brown *et al.* (1991) have shown that ATP induced coronary vasodilatation is partially due to prostanoid production. Activation of P_{2Y}-purinoceptors can also

stimulate the production of NO, and the amounts of NO formed in the guinea-pig heart stimulated by ATP are effective for vasodilatation (Kelm & Schrader, 1990). In the guinea-pig coronary vasculature the vasodilatory properties of ATP require the presence of an intact endothelium (Hopwood *et al.*, 1989; Lee *et al.*, 1990). However, in the rabbit mesenteric artery (Mathieson & Burnstock, 1985) and portal vein (Kennedy & Burnstock, 1985) relaxations evoked by ATP are unaffected by the removal of the endothelium. Even in the coronary artery of the rabbit, ATP produces vasodilatation by a direct action of P_{2Y}-purinoceptors on the smooth muscle (Corr & Burnstock, 1991). Reactive blue 2, the anthraquinone sulphonic acid derivative, has been used as an antagonist of responses mediated via P_2-purinoceptors (Kerr & Krantis, 1979; Manzini *et al.*, 1985; 1986). Unfortunately, it is only useful within a narrow range of concentrations above which it has non-specific effects. Reactive blue 2 can selectively inhibit P_{2Y}-mediated responses in some blood vessels (Manzini *et al.*, 1985; Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Houston *et al.*, 1987; Reilly *et al.*, 1987; Hopwood *et al.*, 1989; Taylor *et al.*, 1989). The trypanocidal drug, suramin, is also a P_2-purinoceptor antagonist, however, it does not show selectivity for either P_{2X}- or P_{2Y}-purinoceptors (Hoyle *et al.*, 1990).

1.4.2 Progress since the P_{2X}/P_{2Y} division

In 1986, Gordon also came to the conclusion that the P_2-

purinoceptor does not form a homogeneous group. Using and extending the P_{2Y}- and P_{2X}-purinoceptor nomenclature, he proposed further P_2-purinoceptor subtypes. The P_{2T}-purinoceptor exists on platelets and was proposed to mediate platelet aggregation. It was said to be activated specifically by ADP rather than ATP. Again as defined by Gordon (1986), the P_{2Z}-purinoceptor is activated by ATP^{4-} and action at this receptor mediates secretion from mast cells (Cockroft & Gomperts 1979a, 1979b, 1980; Bennett *et al.*, 1981) and causes inhibition of activity of natural killer lymphocytes and monocyte-derived macrophages (Cameron, 1984; Schmidt *et al.*, 1984).

P_{2S}- and P_3-purinoceptors have also been proposed. In the guinea-pig ileum, 2-meSATP and α,β-meATP are roughly equipotent at causing contraction of the longitudinal muscle, and are more potent than ATP itself (Wiklund & Gustafsson, 1988a, 1988b). The contractions were not affected by either reactive blue 2 or α,β-meATP desensitization and therefore it was proposed that this receptor represents a non-P_{2X}, non-P_{2Y} subclass, named P_{2S} (Wiklund & Gustafsson, 1988b). In the rat tail artery, adenosine and ATP, and their analogues 2-chloroadenosine (2-CA) and β,γ-meATP, all inhibit release of NA from sympathetic nerves (Shinozuka *et al.*, 1988). The effect of all these compounds are antagonised by the P_1-purinoceptor antagonist 8-(*p*-sulphophenyl)theophylline (8-PSPT). This methylxanthine-sensitive adenine nucleotide receptor was termed P_3 (Shinozuka *et al.*, 1988).

It has recently become apparent that many responses to

5'-nucleotides do not fall into the above classification, including those that are insensitive to both 2-meSATP and β,γ -meATP (Fine *et al.*, 1989; Allsup & Boarder, 1990). In some cases these non-P_{2X}, non-P_{2Y} responses can also be elicited by uridine 5'-triphosphate (UTP) (Demolle *et al.*, 1988; Fine *et al.*, 1989; Davidson *et al.*, 1990; Pfeilschifter, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). While it has been argued that some responses are mediated by separate pyrimidinoceptors (for UTP) and purinoceptors (Seifert & Schultz, 1989) there is now compelling evidence for a nucleotide receptor which responds to both UTP and the purines (O'Connor *et al.*, 1991). The characteristics for receptor activation which appear to distinguish the nucleotide receptor from others are high sensitivity to both UTP and ATP and low sensitivity to other key agonist probes such as α,β-meATP and 2-meSATP: i.e. potency order UTP = ATP > adenosine-5'-O-(3-thiodiphosphate (ADPγS) > 2-meSATP, α,β-meATP (O'Connor *et al.*, 1991). The best examples are inositol phosphate production in sheep pituitary cells (Davidson *et al.*, 1990) and PGI_2 production from bovine aortic smooth muscle cells (Demolle *et al.*, 1988). This receptor has been shown to be coupled to phospholipase C (Brown *et al.*, 1991; Murrin & Boarder, 1992). However, P_{2Y}-purinoceptors may also be coupled to phospholipase C as best illustrated by the P_{2Y}-purinoceptor in turkey erythrocytes (Dainty *et al.*, 1990). Existence of a common second messenger system has probably contributed to the incorrect attribution of some nucleotide receptor containing systems as P_{2Y}-like (Boeynaems & Pearson, 1990; Kennedy, 1990). The relaxant effect of ATP on

isolated rabbit aorta is not exclusively due to the activation of P_{2Y}-purinoceptors at the endothelial level, but also to the activation of a different receptor that can be identified as a nucleotide receptor. Evidence suggests that this receptor is present on both the endothelium and the smooth muscle (Chinellato *et al.*, 1992). Nucleotide receptors have also been found, along with P_{2Y}-purinoceptors, on bovine aortic endothelial cells (Wilkinson *et al.*, 1993). Stimulation of PGI_2 production from piglet aortic endothelial cells may be another example of a mixed receptor population (Needham *et al.*, 1987).

Chapter 2

GENERAL METHODOLOGY

2.1 PHARMACOLOGY

2.1.1 Animals Used.

With the majority of experiments guinea-pigs (250-400 g) of either sex were used. These were injected with heparin (2,500 units i.p.) 15 minutes before being killed by cervical dislocation. For one set of experiments male Sprague-Dawley rats (200-500 g) were used. These were injected with heparin (2,500 units i.p.) 15 minutes before being killed by a blow to the head and exsanguination.

2.1.2 Methods

Once the animal had been killed, the heart was quickly removed and placed in cold krebs (4° C) to arrest the beating. Extraneous fat and large vessels were removed, the heart was cannulated via the aorta and the coronary circulation perfused by the method of Langendorff with a modified Krebs-Henseleit solution containing (mM): NaCl 115.3, KCl 4.6, $MgSO_4.7H_2O$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5 and glucose 11.1. Albumin (0.5 $g.l^{-1}$) was also added to the solution to increase the oncotic pressure and reduce oedema. The Krebs solution was maintained at 37° C and aerated with 95% O_2 and 5% CO_2. A water filled silicon rubber balloon, connected to a pressure transducer (Viggo-Spectramed

Bilthoven, model P23XL), was placed in the left ventricle for the measurement of left ventricular pressure. The left ventricular systolic pressure did not exceed 10 mm Hg. Perfusion pressure was monitored using a pressure transducer connected via a side arm to the cannula. A pair of platinum electrodes were placed in the right ventricle and the heart was paced at 4 Hz with electrical pulses of 5 ms duration at supramaximal voltage (usually around 20 V). The flow was gradually increased to obtain a starting perfusion pressure of 50-60 mm Hg, using a masterflex constant flow roller pump (Cole-Palmer Instruments Co., Chicago). The heart was left to equilibrate for at least 20 minutes before commencing the experiment.

2.1.3 Administration of Drugs

When dose-response relationships were being ascertained, the agonists under examination were given as a bolus of 50 µl, injected over 3 seconds into the superfusing solution close to the heart. At least 5 minutes was left between the administration of each dose of agonist. The duration of each individual experiment was no longer than 3 hours. Due to this time restriction the effect of all the agonists in a particular series of experiments could not be tested on the same heart. For this reason the agonists were chosen randomly and not more than four agonists were used on a particular heart. The order of exposure of the agonists to the heart was also random to minimise effects due to time-

dependent changes and preparation variability. When the effect of antagonists was examined control dose-response relationships for the agonists were first obtained and then the antagonist was added to the perfusing solution and allowed to equilibrate for 20 minutes. The dose-responses were then repeated in the presence of the antagonist.

2.2 BIOCHEMICAL ASSAYS

2.2.1 ATP assay

The ATP released was assayed using the luciferin-luciferase technique described by Stanley and Williams (1969). Firefly luciferase catalyses the oxidation of D(-) luciferin in the presence of ATP, magnesium and O_2 to generate oxyluciferin and light. Luciferin luciferase (50 mg) was added to 15 ml of distilled water and left in the dark for 2 hours to equilibrate. In these experiments the assay was capable of measuring ATP levels as low as 25 fmol. A standard calibration curve was prepared from measurement of the chemiluminescent intensities of various concentrations of ATP, ranging from 10^{-7} to 10^{-11} M. ATP quantification was performed on a Packard luminometer by addition of 200 µl of luciferase-luciferin (3.33 $mg.ml^{-1}$) to 100 µl samples of standard solution or perfusate. The krebs solution which was assayed for background ATP content prior to each experiment acted

as a blank. All perfusate samples were collected in ice-cold vials and directly placed on ice before being assayed for ATP.

2.2.2 Lactate dehydrogenase (LDH) activity measurements

From each sample of perfusate 0.5 ml was taken and incubated at 37° C in 1 ml of phosphate-buffered saline containing pyruvate (0.75 mM) and NADH (1.28 mM). After 30 minutes, 1 ml of colour reagent was added and the samples were incubated for 20 minutes at 20° C; 5 ml of 0.4 M NaOH was then added to each sample. Absorbance was read at 442 nm with a spectrophotometer.

2.3 ANALYSIS OF RESULTS

Results are expressed as mean ± standard error of the mean (S.E.M.). Log dose-response curves were constructed at various concentrations of agonist. Results were analysed using Student's t-tests. A probability of $P < 0.05$ was considered to be significant. Analysis of variance and Tukey's tests were used to assess relative order of potency of the adenosine analogues (these tests were used on both the pD_2 (-log EC_{50}) values and the responses to individual concentrations of the agonists).

2.4 SOURCES OF DRUGS, ENZYMES, PEPTIDES AND CHEMICALS

Adenosine, hemisulphate: Sigma

Adenosine 5'-triphosphate, sodium salt: Sigma

L-Arginine: Sigma

Bradykinin: Sigma

2-[*p*-(2-carboxyethyl)phenylethylamino]-
5'-N-ethylcarboxamidoadenosine: Research Biochemicals Inc.

2-Chloroadenosine: Sigma

N^6-Cyclopentyladenosine: Research Biochemicals Inc.

Cytidine 5'-triphosphate: Sigma

Dimethylsulfoxide: Sigma

1,3-Dipropy-8-cyclopentylxanthine: Cookson Chemicals

5'-N-ethyl-carboxamidoadenosine: Sigma

Guanosine 5'-triphosphate: Sigma

Heparin: Sigma

5-Hydroxytryptamine, creatine sulphate: Sigma

Indomethacin: Sigma

Inosine 5'-triphosphate: Sigma

Lactate dehydrogenase colour reagent: Sigma

Luciferase-luciferin: Sigma

α,β-Methylene ATP: Sigma

β,γ-Methylene ATP: Sigma

2-Methylthio ATP, sodium salt: Research Biochemicals Inc.

N^G-nitro-L-arginine: Sigma

N^G-nitro-L-arginine methyl ester: Sigma

R-N^6-phenylisopropyl-adenosine: Sigma

S-N^6-phenylisopropyl-adenosine: Sigma

Sodium nitroprusside: Sigma

Substance P: Sigma

8-(*p*-sulphophenyl)theophylline: Research Biochemicals Inc.

Suramin: Bayer

Thymidine 5'-triphosphate: Sigma

Uridine 5'-triphosphate: Sigma

S-PIA, R-PIA, 2-CA, D-NECA, CPA, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) and 1,3-dipropy-8-cyclopentylxanthine (DPCPX) were made up as stock solutions (10^{-2} M) in dimethylsulfoxide (DMSO). Indomethacin was made up as a stock solution (10^{-2} M) in sodium carbonate (10^{-2} M) solution. All other drugs were made up in distilled water. Dilutions of all stock solutions were carried out using distilled water. A 50 µl bolus injection of distilled water caused no change in perfusion pressure other than a small injection artefact.

Drugs given as bolus injections of a fixed volume (50 µl) are expressed as the 'dose' (the number of moles (mol) within the bolus). When drugs were added to the perfusate reservoir the final concentration (M) of the solution is stated.

SECTION A

Chapter 3

Effects of NO synthase inhibitors, L-NA and L-NAME, on responses to vasodilators of the guinea-pig coronary vasculature.

3.1 SUMMARY

1) The effects of L-NA and L-NAME on vasodilatation induced by ATP, substance P, 5-HT, bradykinin and sodium nitroprusside (SNP) were examined in the guinea-pig coronary bed, using a Langendorff technique. The effects of these inhibitors of NO synthesis were assessed on their ability to inhibit both the amplitude and the area of the vasodilator response.

2) The vasodilator responses evoked by low doses of 5-HT ($5x10^{-10}$ - $5x10^{-8}$ mol) were almost abolished by L-NAME and L-NA (both at 10^{-5}, $3x10^{-5}$ and 10^{-4} M), although L-NA ($3x10^{-3}$ M) was significantly less potent than L-NAME ($3x10^{-5}$ M) as an inhibitor of vasodilator responses to 5-HT ($5x10^{-8}$ mol).

3) The vasodilator responses evoked by substance P ($5x10^{-12}$ - $5x10^{-9}$ mol) were reduced in the presence of L-NAME and L-NA (both at 10^{-5} and $3x10^{-5}$ M). The response to substance P was almost abolished by L-NAME and L-NA (both at 10^{-4} M).

4) The amplitude of the vasodilator responses to ATP ($5x10^{-11}$ and $5x10^{-9}$ - $5x10^{-7}$ mol) was little affected by either L-NAME or L-NA (both at 10^{-5}, $3x10^{-5}$ and 10^{-4} M). However, the area of the response to ATP ($5x10^{-10}$ - $5x10^{-7}$ mol) was inhibited by L-NAME (10^{-5}, $3x10^{-5}$ and 10^{-4} M) and to a lesser extent by L-NA (10^{-5} and 10^{-4} M).

5) The amplitude and area of the vasodilator responses to

bradykinin ($5x10^{-12}$ - $5x10^{-11}$ mol) were reduced, but not abolished, by L-NA and L-NAME.

6) Neither the amplitude nor area of the responses to SNP ($5x10^{-10}$ - $5x10^{-7}$ mol) were inhibited by either L-NAME or L-NA (both at 10^{-5} and $3x10^{-5}$ M).

7) It is concluded that in the guinea-pig coronary vasculature, the vasodilatation evoked by substance P and low doses of 5-HT is mediated almost exclusively via NO, whereas the vasodilatations evoked by ATP and bradykinin appear to involve other mechanisms in addition to the release of NO. L-NAME was a more effective agent than L-NA in inhibiting the vasodilator actions of 5-HT and ATP in this preparation.

3.2 INTRODUCTION

Endothelial cells play a key role in the control of vascular tone by virtue of their ability to synthesise and release EDRF's. Various agents, including substance P, ATP, 5-HT, ACh and bradykinin, elicit vasodilatation in the coronary bed via an action at receptors located on endothelial cells, leading to the release of these factors (Cohen *et al.*, 1983; Saeed *et al.*, 1986; Hopwood & Burnstock, 1987; Hopwood *et al.*, 1989; Hoover, 1990; Lamontagne *et al.*, 1991). These factors include prostacyclin (Moncada & Vane, 1979) and EDRF (Furchgott & Zawadzki, 1980a). Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) which

can be released from endothelial cells by a variety of stimuli including bradykinin, AA, thrombin, the ionophore A23187, ATP and histamine (Pearson *et al.*, 1983; Weksler *et al.*, 1978b). In some blood vessels the release of EDRF also mediates the vascular relaxation evoked by certain vasodilators including ACh, ATP, substance P and bradykinin (Furchgott & Zawadzki, 1980a; Cherry *et al.*, 1982; Furchgott, 1983, 1984).

It has been proposed that EDRF is the free radical of NO (Ignarro *et al.*, 1986; Furchgott *et al.*, 1987) and indeed, EDRF has chemical and pharmacological properties identical to those of NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987b). NO is released tonically from isolated, perfused guinea-pig hearts, and ACh, bradykinin, 5-HT and ATP stimulate further release (Kelm & Schrader, 1988; Kelm & Schrader, 1990).

In endothelial cells, NO is produced from the conversion of the semi-essential amino acid L-arginine into L-citrulline by NO synthase (Palmer *et al.*, 1988b; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). Analogues of L-arginine are, therefore, potentially specific inhibitors of EDRF-mediated effects on vascular tone. In fact, it has been shown that L-NMMA inhibits dilator responses due to ACh, A23187, substance P and L-arginine in the rabbit aorta (Rees *et al.*, 1989). L-NA is also an inhibitor of the generation of NO and has been shown to be more potent than L-NMMA (Moore *et al.*, 1990). L-NA has been shown to inhibit vasodilator responses to ACh and 5-HT in the isolated perfused rabbit heart (Lamontagne *et al.*, 1991), and to attenuate the duration of the vasodilator response to bradykinin in the

isolated rat heart (Baydoun & Woodward, 1991). Similarly, L-NAME is an inhibitor of enzymatic synthesis of NO from L-arginine (Rees *et al.*, 1990a).

The purpose of this study was to compare the effects of the NO synthase inhibitors L-NA and L-NAME and to ascertain the role of NO in the vasodilator responses to various agonists in the guinea-pig coronary bed.

3.3 METHODS (See Chapter 2.1.2)

Guinea-pigs (250-400 g) of either sex were used. The heart was removed and cannulated via the aorta for perfusion according to the method of Langendorff (see Chapter 2.1.2). The mean flow rate was 22.6 ± 0.75 ml min^{-1} (n = 76).

In order to look at the effect of the antagonists L-NA and L-NAME, control dose-response relationships to agonists were first determined. An inhibitor was then added to the perfusing solution and the preparation allowed to equilibrate for 20 min. Dose-response relationships were re-evaluated in the presence of the inhibitor. Agonists were given as 50 µl boluses, injected over 3 seconds into the superfusing solution close to the heart. At least 5 min were left between administration of each agonist. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis program on an Apple II computer.

At the end of each experiment the heart was removed from the cannula, blotted and weighed. The mean weight was 2.3 ± 0.1 g (n = 76).

3.3.1 Materials See Chapter 2.4

3.3.2 Statistics See Chapter 2.3

3.4 RESULTS

Effect of L-NAME on vasodilator responses of the guinea-pig coronary bed.

The effect of L-NAME on the maximum amplitude of the vasodilator response (Figure 3.1a-e), on perfusion pressure trace response (Figure 3.2a-c) and on the area of the vasodilator response (Figure 3.4a,c,e) to various agents is demonstrated. L-NAME (10^{-5}, $3x10^{-5}$ and 10^{-4} M) was a potent inhibitor of vasodilator responses due to 5-HT virtually abolishing the effects of lower doses of this agent (Figures 3.1a and 3.2c). The maximum amplitude and area of the vasodilator responses due to substance P were significantly inhibited by L-NAME (10^{-5}, $3x10^{-5}$ and 10^{-4} M; Figures 3.1b and 3.2b) and at 10^{-4} M it almost abolished the responses to substance P.

L-NAME (10^{-5} M) did not affect the amplitude of the

dilatation evoked by ATP across its dose-range, and at $3x10^{-5}$ and 10^{-4} M it inhibited only a low dose of ATP ($5x10^{-10}$ mol; Figure 3.1c). In contrast, the area of the vasodilator response to ATP was significantly inhibited by L-NAME (10^{-5}, $3x10^{-5}$ and 10^{-4} M; Figure 3.4a), reflecting an attenuation of the duration of the response (Figure 3.2a). The area of the vasodilator response to the highest dose of ATP was not inhibited by L-NAME (10^{-4} M).

The responses evoked by bradykinin were significantly inhibited by L-NAME ($3x10^{-5}$ and 10^{-4} M; Figures 3.1d and 3.4c). Neither the amplitude (Figure 3.1e) nor the area (Figure 3.4e) of the vasodilator responses to SNP were affected by L-NAME.

Effect of L-NA on vasodilator responses of the guinea-pig coronary bed.

The effect of L-NA on the maximum amplitude of the vasodilator response (Figure 3.3a-b) and on the area of the vasodilator response (Figure 3.4b,d,f) to various agents is shown. The amplitude of the responses evoked by 5-HT ($5x10^{-10}$ - $5x10^{-9}$ mol) was significantly reduced by L-NA (10^{-5}, $3x10^{-5}$ and 10^{-4} M; Figure 3.3a). The area of the responses to 5-HT ($5x10^{-10}$ - $5x10^{-9}$ mol) was also reduced by L-NA (10^{-5}, $3x10^{-5}$ and 10^{-4} M, data not shown). L-NA ($3x10^{-5}$ M) was significantly less effective than L-NAME ($3x10^{-5}$ M) at reducing the maximum amplitude of the vasodilatation due to 5-HT ($5x10^{-8}$ mol); 84.1 ± 4.2 and 48.7 ± 13.34% inhibition of the response to 5-HT by L-NAME and L-NA (both at $3x10^{-5}$ M), respectively.

The inhibition of the responses to substance P by L-NA (10^{-5}, $3x10^{-5}$ and 10^{-4} M; data not shown) was similar to that of L-NAME (10^{-5}, $3x10^{-5}$ and 10^{-4} M).

L-NA did not inhibit the amplitude of the responses to ATP (data not shown). L-NA (10^{-5} and 10^{-4} M) only significantly attenuated the area of the response evoked by intermediate doses of ATP ($5x10^{-10}$ - $5x10^{-8}$ mol; Figure 3.4b). The mid concentration of L-NA ($3x10^{-5}$ M) did not have any inhibitory effect on the responses to ATP.

The amplitude of the responses evoked by bradykinin was significantly inhibited by L-NA ($3x10^{-5}$ and 10^{-4} M; data not shown), although the lower concentration of L-NA (10^{-5} M) had no effect on the amplitude of the response to this agent. L-NA (10^{-5}, $3x10^{-5}$ and 10^{-4} M) significantly inhibited the area of the response to bradykinin (Figure 3.4d). Responses (amplitude and area) due to SNP (Figure 3.3b and 3.4f) were not affected by L-NA.

3.5 DISCUSSION

The results of this study show that in the guinea-pig coronary vasculature, dilator responses evoked by substance P and low concentrations of 5-HT were dependent largely upon the synthesis of NO. In contrast, vasodilator responses elicited by bradykinin and ATP appeared to be only partially dependent upon the synthesis of NO, while SNP elicited vasodilatation by a mechanism that was independent of the generation of NO.

Few studies have compared the effects of both L-NAME and

L-NA as inhibitors of NO synthase. L-NAME has been shown to be equipotent with L-NA as a prejunctional inhibitor of NANC transmission in the rat anococcygeus (Hobbs & Gibson, 1990). L-NAME and L-NA at the same concentration, have been shown to produce similar inhibition of vasodilatation evoked by ACh in the rabbit aorta (Moore *et al.*, 1990). However, the present study demonstrates that, although L-NAME and L-NA exhibit similar inhibitory properties to vasodilator responses to bradykinin and substance P in the guinea-pig coronary bed, there are differences in the extent of inhibition of vasodilator responses due to 5-HT and ATP.

Responses (both the amplitude and area) due to low concentrations of 5-HT were virtually abolished by L-NAME. Consistent with this, it has been shown in the canine coronary artery that 5-HT does not act via endothelial production of prostacyclin, or following breakdown to active products by endothelial monoamine oxidase (Cohen *et al.*, 1983). Together these results suggest that the response to low doses of 5-HT was dependent almost exclusively on the production of NO. 5-HT is localised in endothelial cells in the rat heart, from where it may be released during hypoxic conditions (Burnstock *et al.*, 1988). Following release from endothelial cells, 5-HT can then act on receptors on the endothelium to elicit vasodilatation via production of NO. The higher dose of 5-HT was not abolished by L-NAME even at its highest concentration, suggesting another mode of action of 5-HT.

L-NAME did not alter the peak vasodilatation induced by

ATP, suggesting that at least this part of the response is not due to the generation of NO. However, the duration of the response was reduced by L-NAME, and therefore, part of the action of ATP occurs via the release of NO. This is supported by work on the release of NO in the guinea-pig coronary bed (Kelm & Schrader, 1990), which has shown that ATP does induce release of NO. The lack of effect of L-NA (at $5x10^{-4}$ M) on vasodilator responses of the guinea-pig coronary bed to ATP has also been found by Brown *et al.* (1991), using a constant pressure system. Thus although part of the vasodilator response to ATP is via NO, other mechanisms are also involved. Although it is not possible to extrapolate directly from studies on different species and vascular beds, the information obtained may provide clues to the other possible mechanisms of action of ATP. For example, prostacyclin production could be involved, as ATP has been shown to stimulate prostacyclin production from various perfused beds and from endothelial cells in culture (Boeynaems & Galand, 1983; Hellewell & Pearson, 1984; Needleman *et al.*, 1974). Alternatively or additionally, ATP could be acting directly on the smooth muscle. It has been shown, for example, in the coronary artery of the rabbit, that ATP produces vasodilatation by a direct action on P_{2Y}-receptors on the smooth muscle (Corr & Burnstock, 1991). ATP could also be metabolised to adenosine by highly active ectonucleotidases (Fleetwood *et al.*, 1989) and as such cause relaxation via action at P_1-purinoceptors (Burnstock & Kennedy, 1986).

Bradykinin is a potent vasoactive agent which relaxes

several vascular smooth muscle preparations via an endothelium-dependent mechanism (Cherry *et al.*, 1982); it also causes release of NO from isolated guinea-pig hearts (Kelm & Schrader, 1988). L-NAME and L-NA significantly inhibited the vasodilatatory response to bradykinin, suggesting that at least part of its action was via the release of NO from endothelial cells. The maximum inhibition of the peak response to bradykinin by L-NAME ($3x10^{-5}$ M) was reached without abolishing the response, suggesting that bradykinin exerts its effect by a combination of mechanisms. In addition to acting via NO, it could also act via activation of EDHF (Boulanger *et al.*,1989) or via release of prostacyclin, although it has been shown in the porcine coronary artery that vasodilator responses to bradykinin are maintained in the presence of indomethacin (Richard *et al.*, 1990). In the isolated perfused rat heart, bradykinin mediates vasodilator effects via activation of the kinin B_2-receptor (Baydoun & Woodward, 1991), which introduces another possible vasodilator contribution in the guinea-pig coronary bed.

L-NAME and L-NA (both at 10^{-4}M) almost abolish the response to substance P, suggesting that substance P induces relaxation almost exclusively via NO release. It has been shown in various isolated coronary arteries that substance P is endothelium-dependent (Berkenboom *et al.*, 1987; Gulati *et al.*, 1987). If it is assumed that NO is released from endothelial cells then it appears that substance P also acts by an endothelial dependent mechanism in the guinea-pig coronary vasculature.

The action of SNP was not affected by either L-NAME or

L-NA. Formation of NO from SNP is probably the mode for direct or indirect activation of soluble guanylate cyclase, resulting in relaxation of vascular smooth muscle (Feelisch & Noack, 1987). Thus, SNP should not be, and was not affected by these NO synthase inhibitors as it bypasses NO formation in endothelial cells.

This study has revealed that in the guinea-pig coronary bed L-NAME and L-NA exhibited similar inhibitory effects on vasodilator responses, although L-NAME tended to be more potent at inhibiting relaxant responses evoked by 5-HT and ATP. The use of L-NAME and L-NA has established a better understanding of the role that NO plays in the relaxant response to various vasodilators of the guinea-pig coronary vasculature.

FIGURE 3.1a The amplitude of the vasodilator responses evoked by 5-HT, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

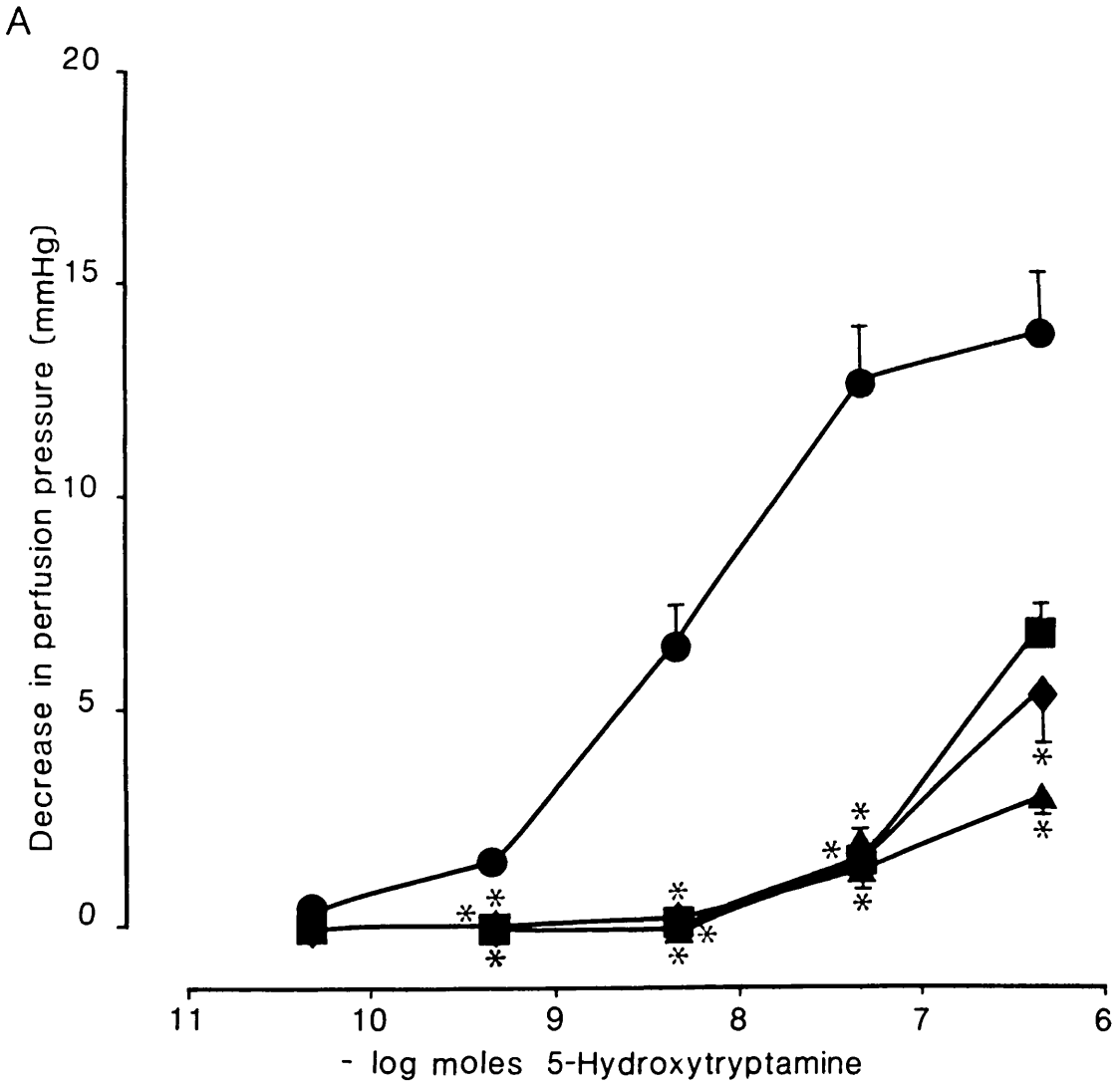

A
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
- log moles 5-Hydroxytryptamine

FIGURE 3.1b The amplitude of the vasodilator responses evoked by substance P, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

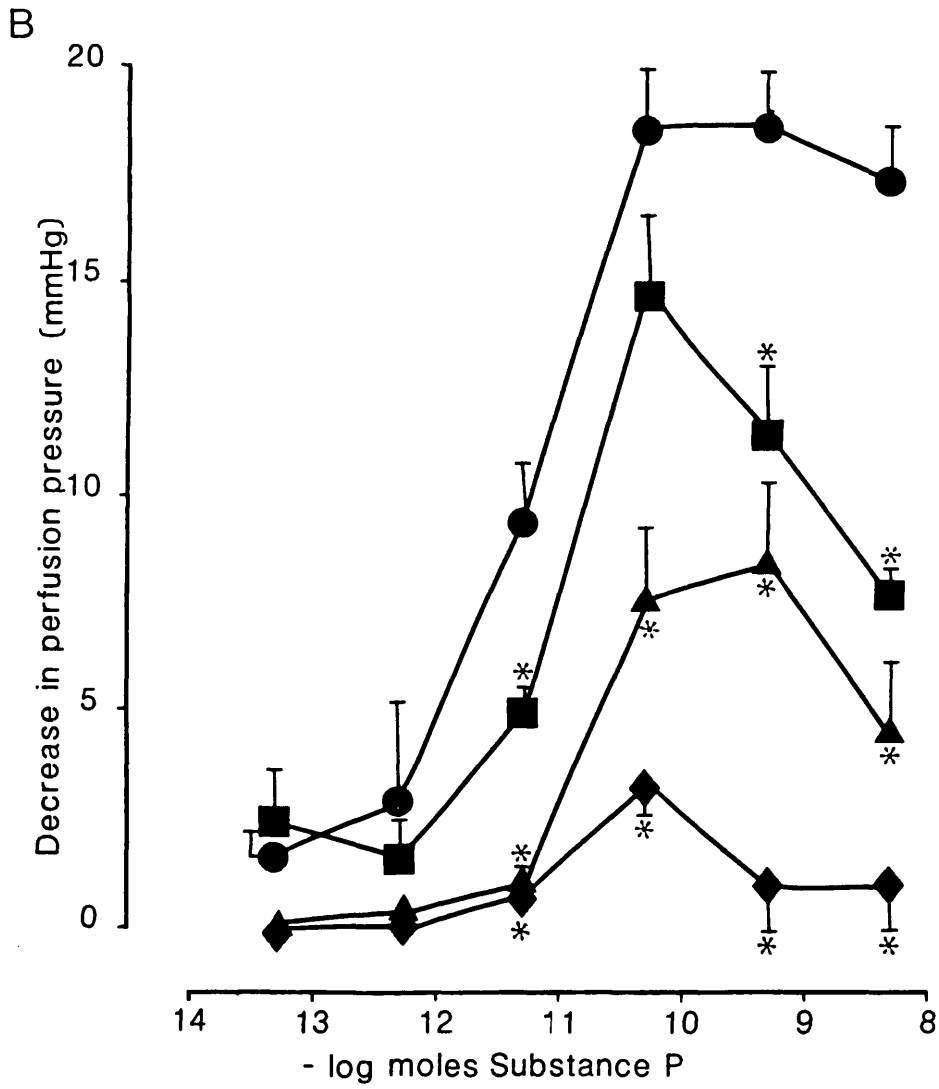

B
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
14
13
12
11
10
9
8
- log moles Substance P

FIGURE 3.1c The amplitude of the vasodilator responses evoked by ATP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3×10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

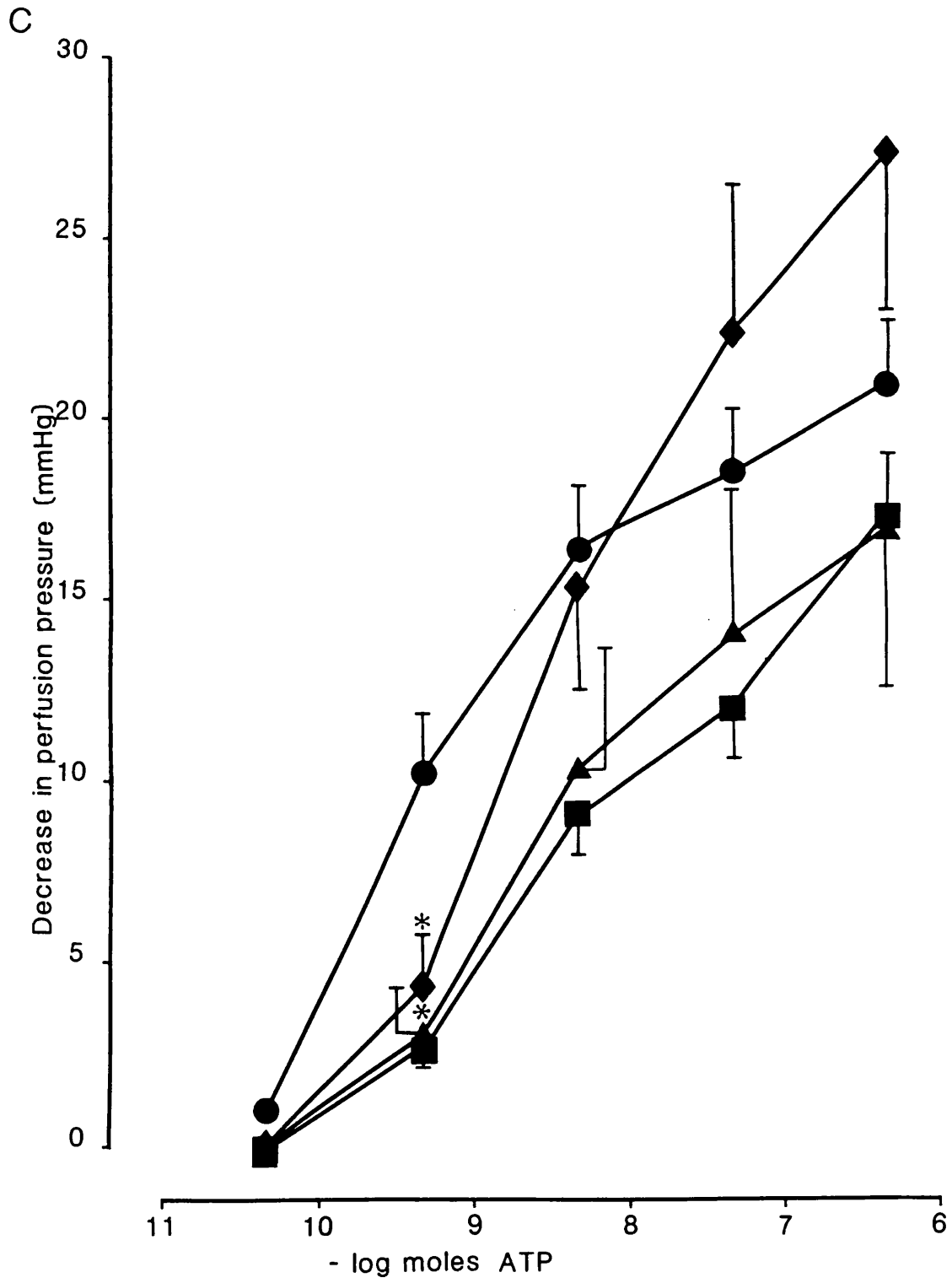

C
30
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
*
*
11
10
9
8
7
6
- log moles ATP

FIGURE 3.1d The amplitude of the vasodilator responses evoked by bradykinin, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e mean indicated by vertical bars. The significant differences are * $P<0.05$.

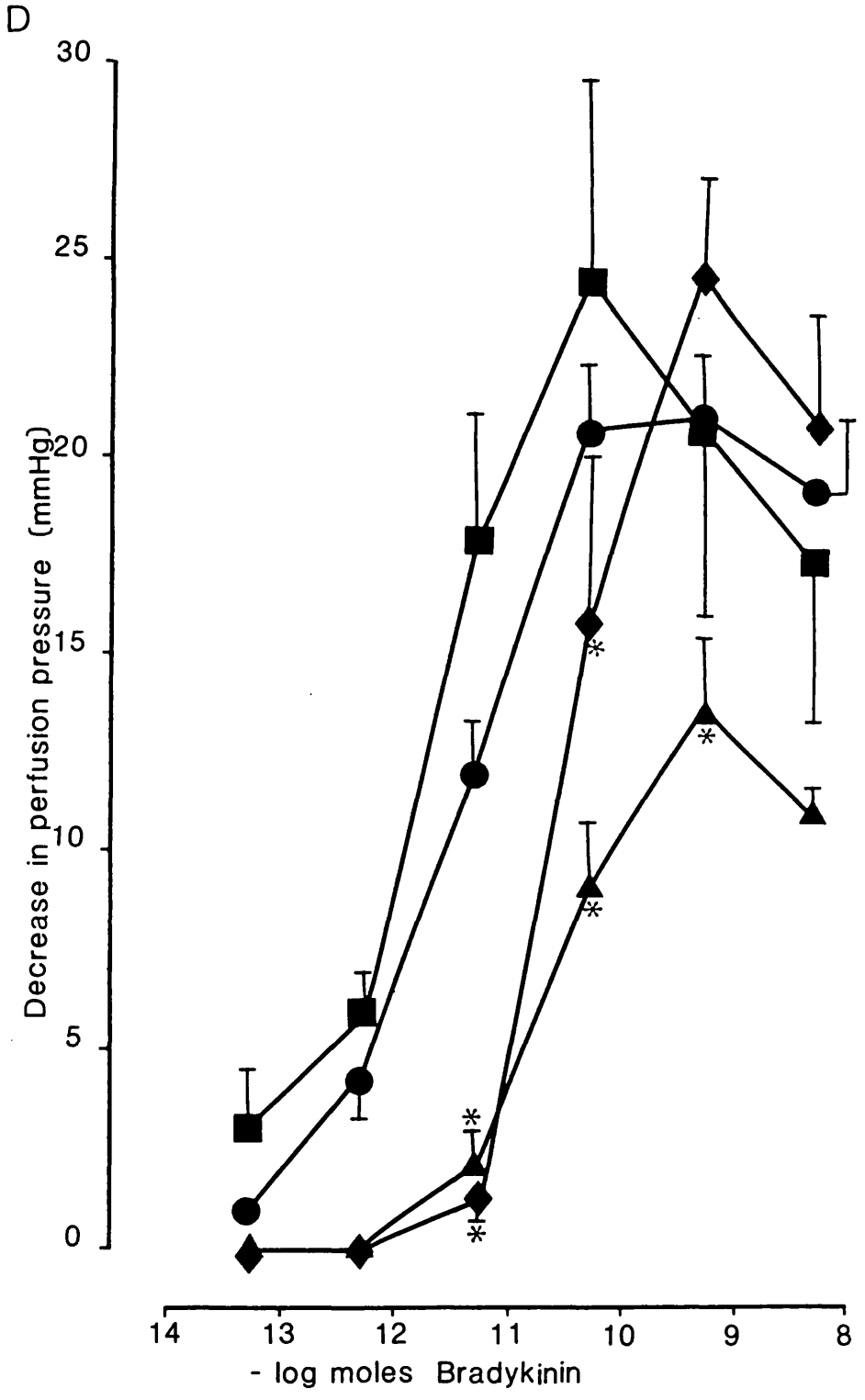

D
30
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
14
13
12
11
10
9
8
- log moles Bradykinin
*
*
*
*
*

FIGURE 3.1e The amplitude of the vasodilator responses to SNP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

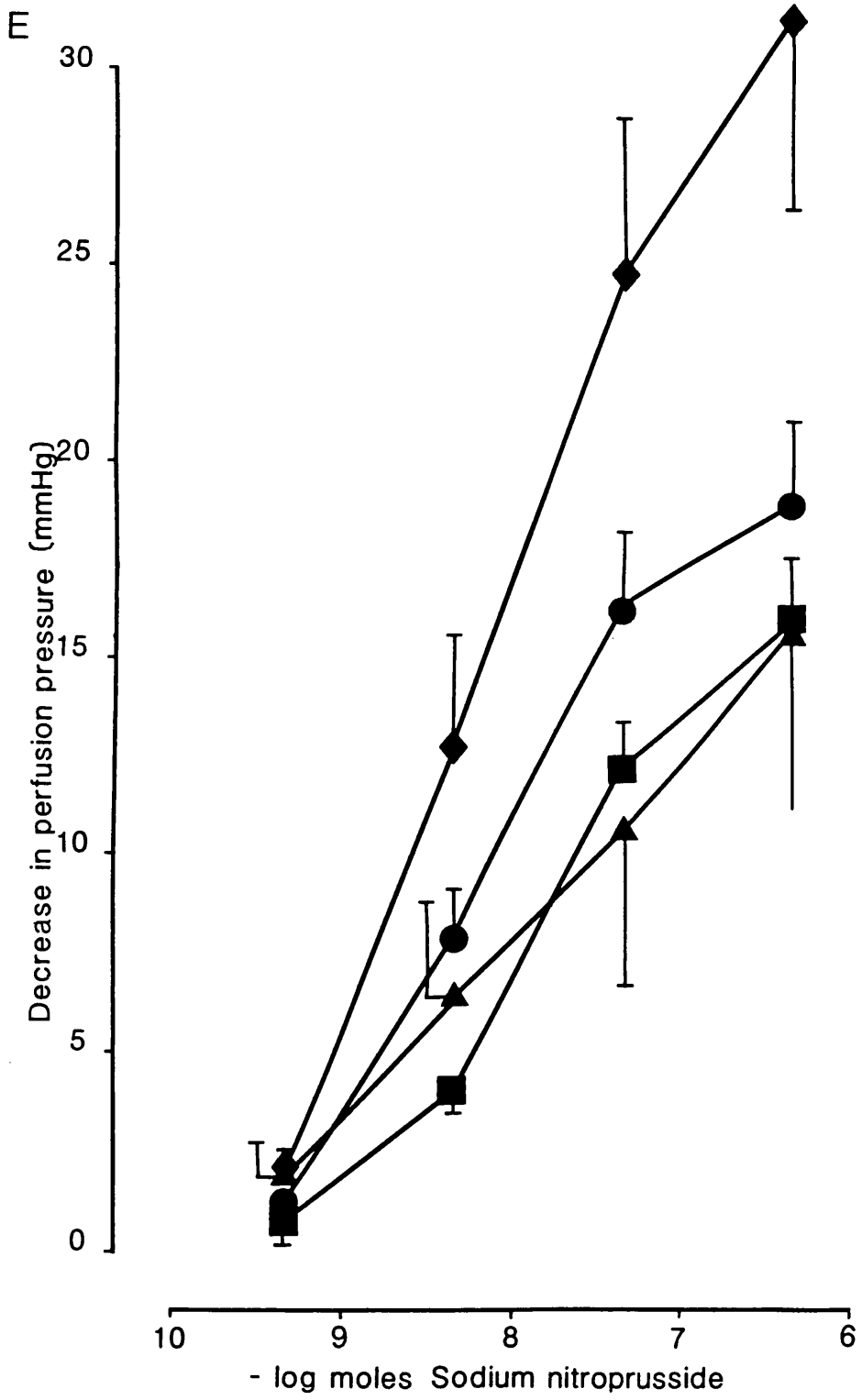

E
30
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
10
9
8
7
6
- log moles Sodium nitroprusside

FIGURE 3.2 Typical perfusion pressure traces, obtained from isolated perfused guinea-pig hearts, showing the effects of (a) ATP, (b) substance P and (c) 5-HT in the absence and presence (after ▼) of L-NAME. The dose stated is the number of moles of vasodilator agonist that is injected into the perfusion system close to the heart.

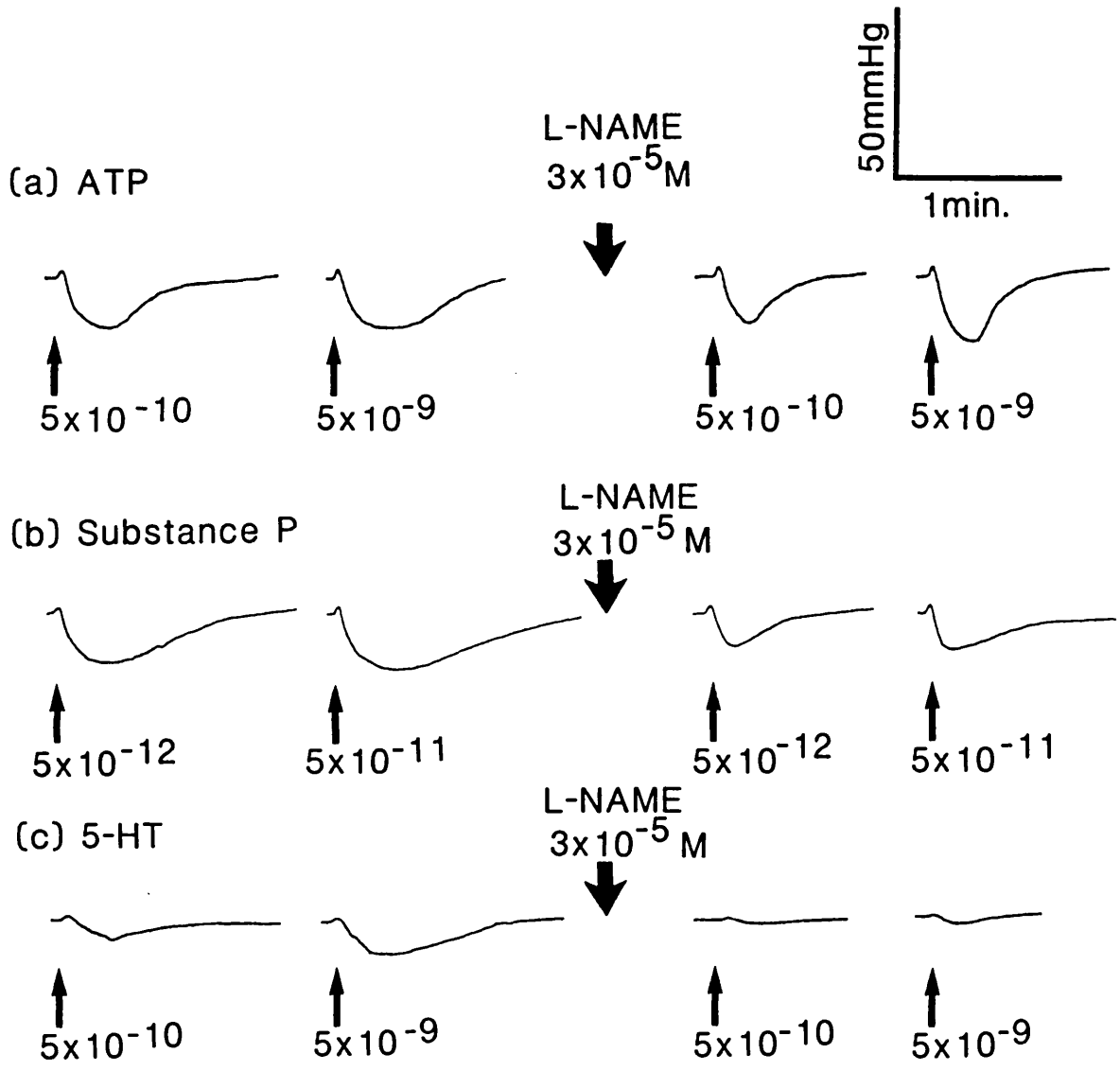

50mmHg
1min.
(a) ATP
L-NAME
3x10-5M
5x10-10
5x10-9
5x10-10
5x10-9
(b) Substance P
L-NAME
3x10-5 M
5x10-12
5x10-11
5x10-12
5x10-11
(c) 5-HT
L-NAME
3x10-5 M
5x10-10
5x10-9
5x10-10
5x10-9

FIGURE 3.3a The amplitude of the vasodilator responses evoked by 5-HT, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NA 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

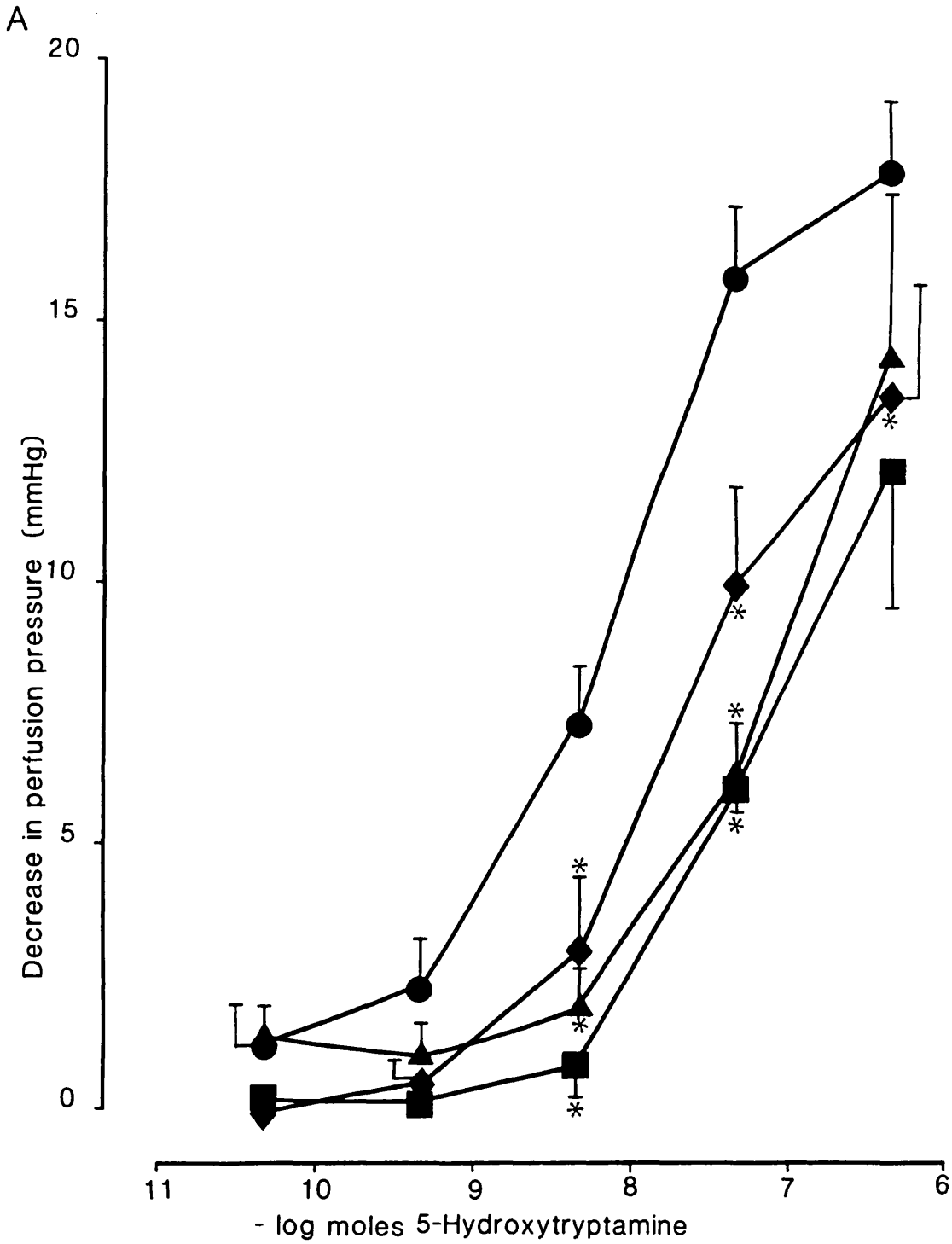

A
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
- log moles 5-Hydroxytryptamine

FIGURE 3.3b The amplitude of the vasodilator responses evoked by SNP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NA 10^{-5} M (■), 3×10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

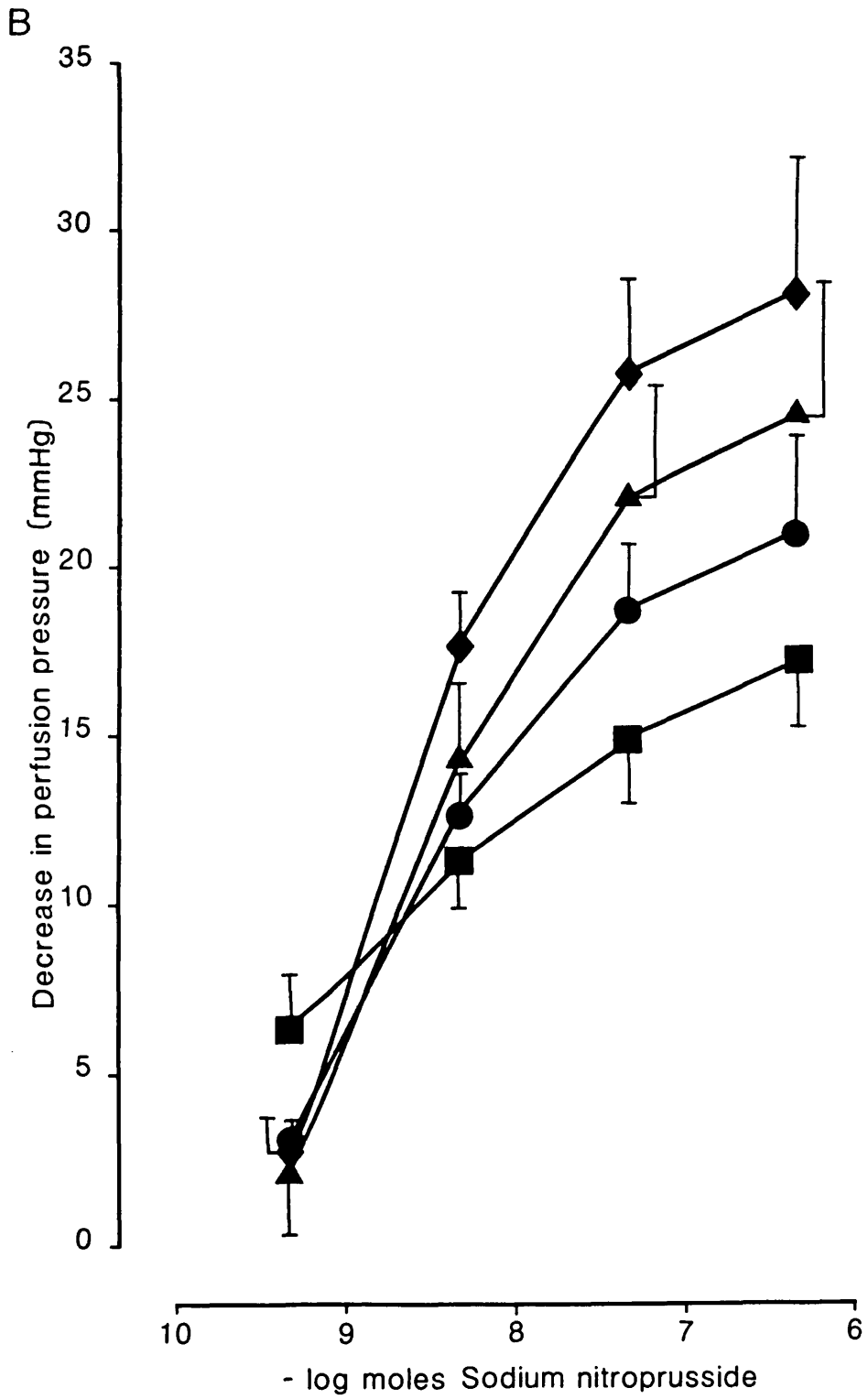

B
35
30
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
10
9
8
7
6
- log moles Sodium nitroprusside

FIGURE 3.4a Area of the vasodilator response to ATP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

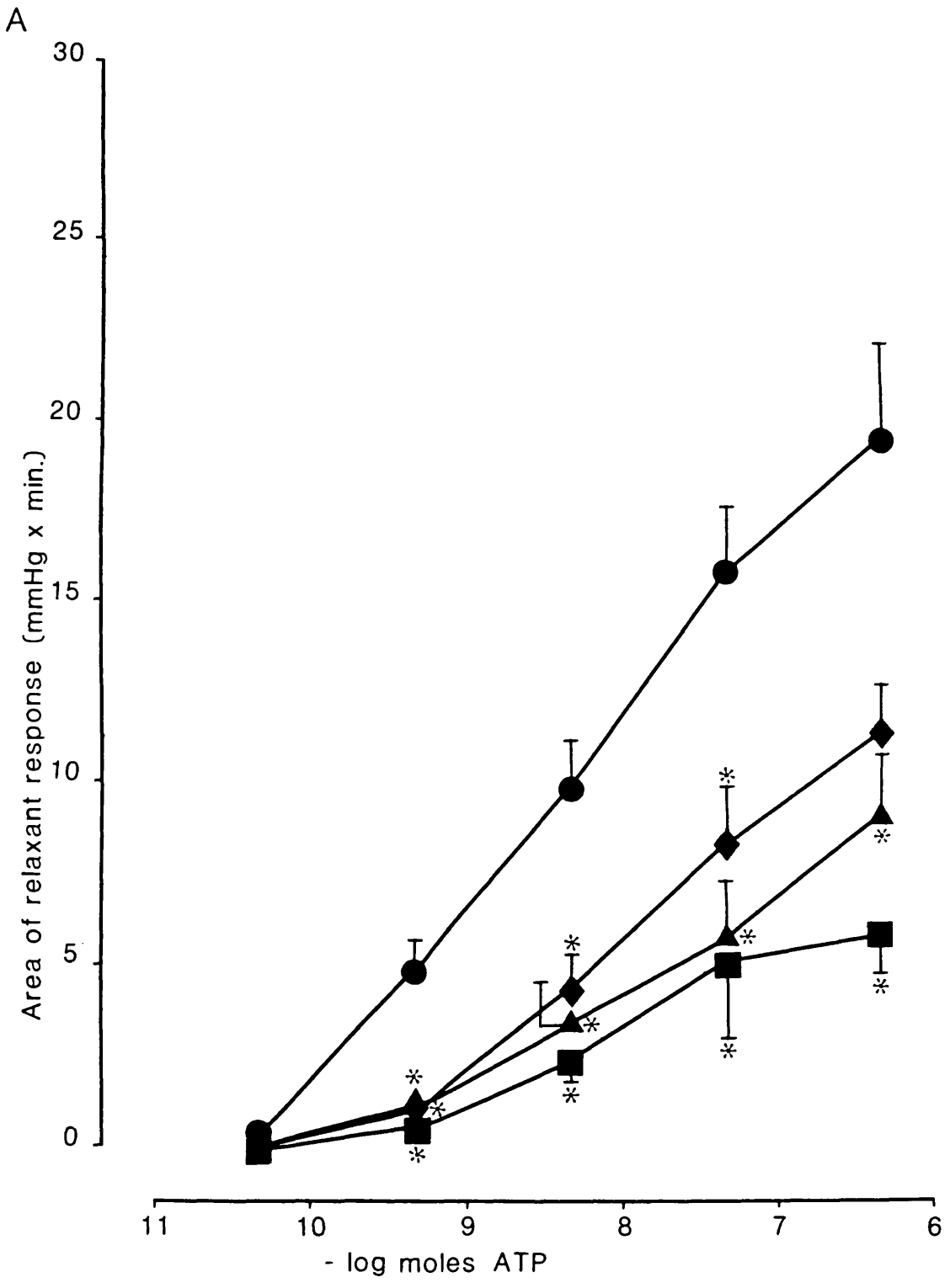

A
30
25
20
15
10
5
0
Area of relaxant response (mmHg x min.)
11
10
9
8
7
6
- log moles ATP

FIGURE 3.4b Area of the vasodilator response to ATP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NA 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

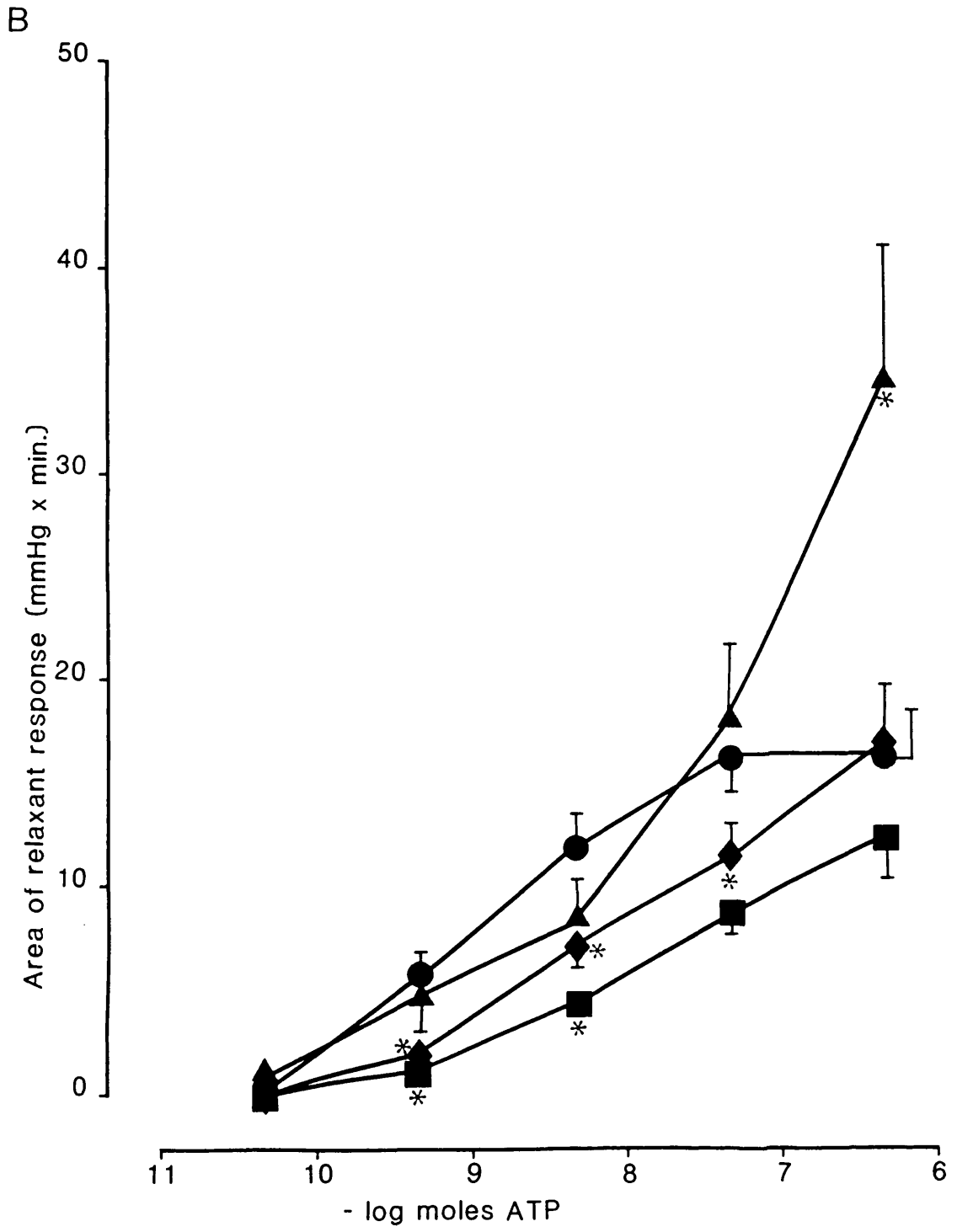

B
50
40
30
20
10
0
Area of relaxant response (mmHg x min.)
11
10
9
8
7
6
- log moles ATP

FIGURE 3.4c Area of the vasodilator response to bradykinin, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e mean indicated by vertical bars. The significant differences are * $P<0.05$.

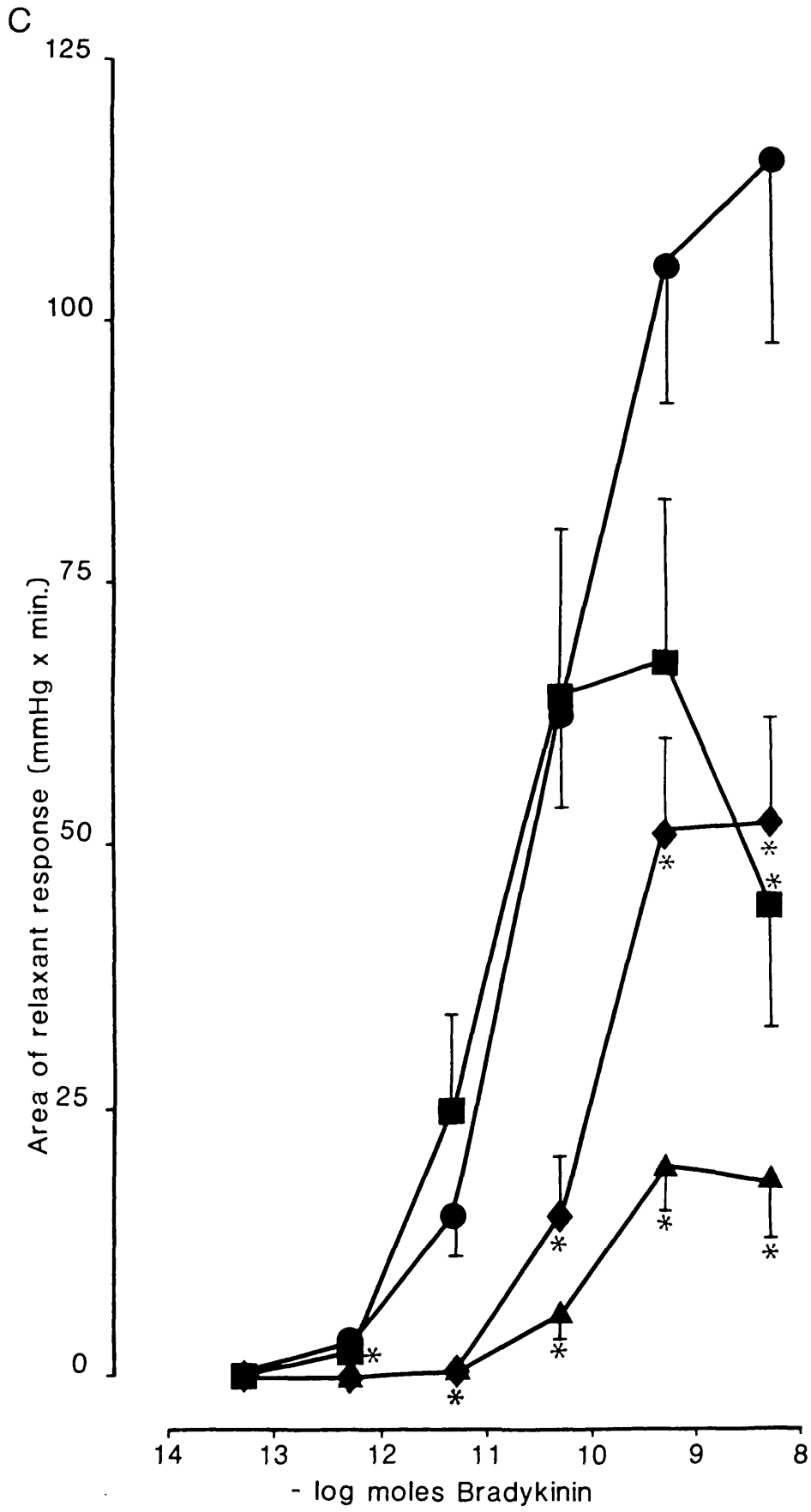

C
125
100
75
50
25
0
Area of relaxant response (mmHg x min.)
14
13
12
11
10
9
8
- log moles Bradykinin

FIGURE 3.4d Area of the vasodilator response to bradykinin, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NA 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

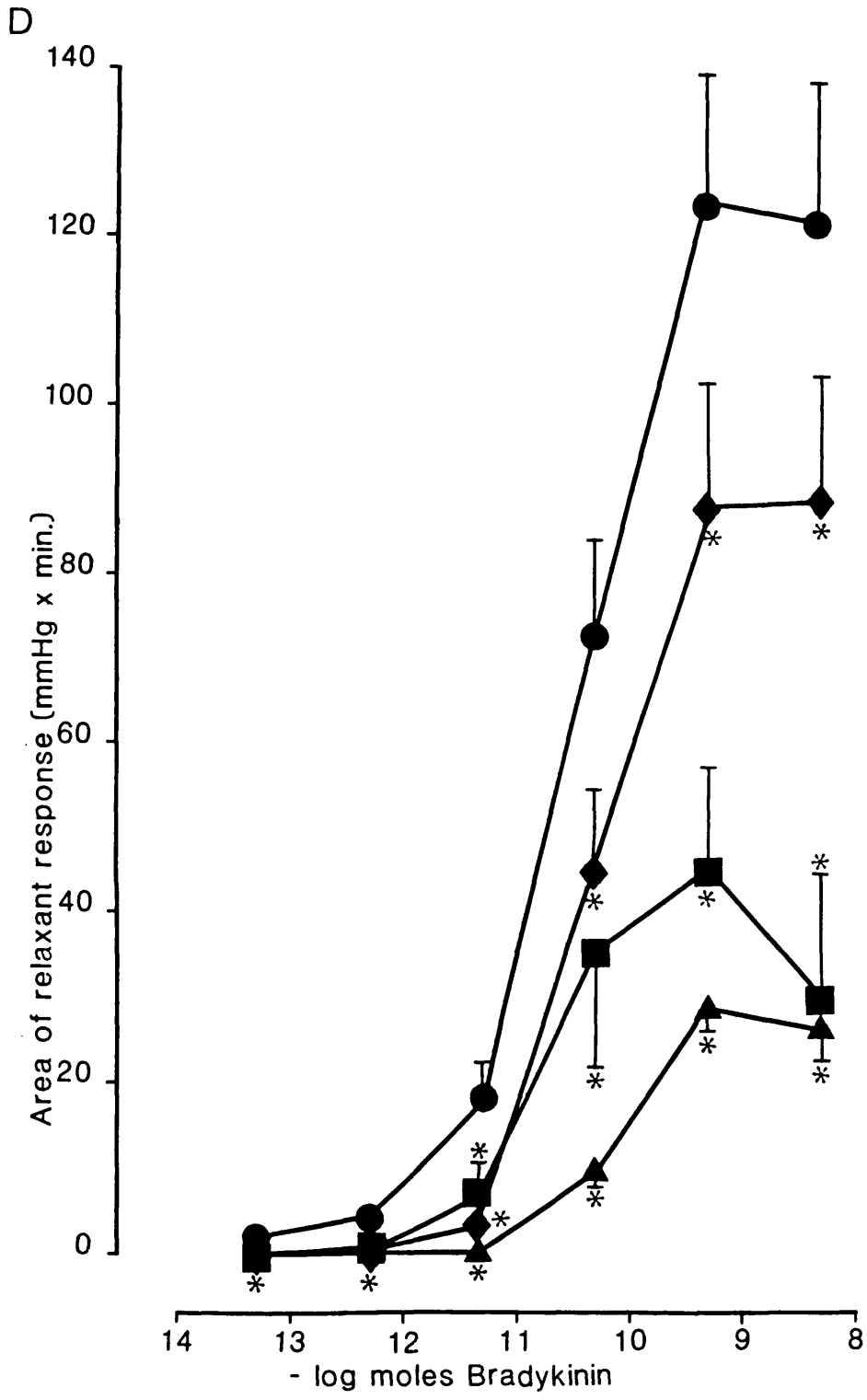

D
140
120
100
80
60
40
20
0
Area of relaxant response (mmHg x min.)
14
13
12
11
10
9
8
- log moles Bradykinin

FIGURE 3.4e Area of the vasodilator response to SNP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

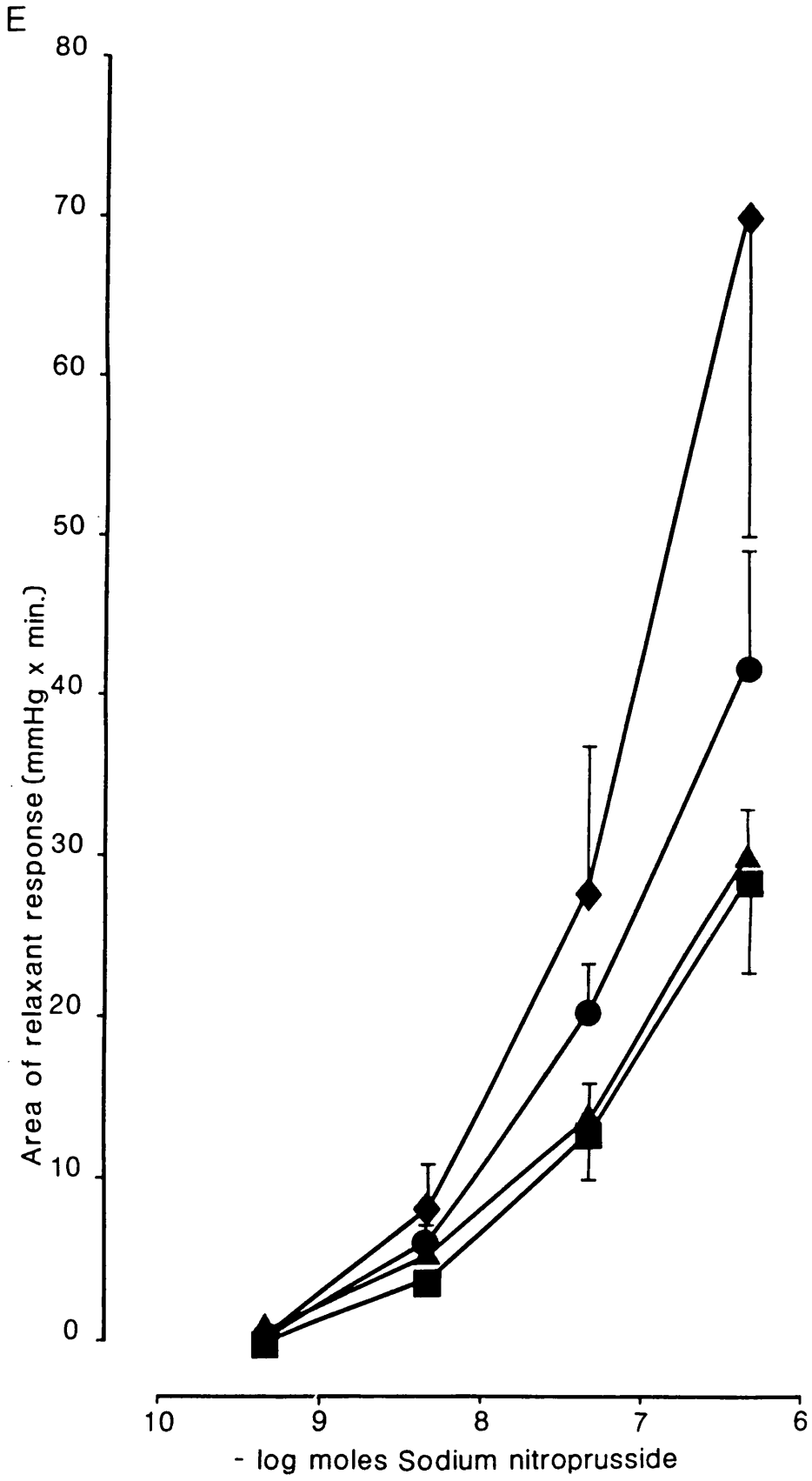
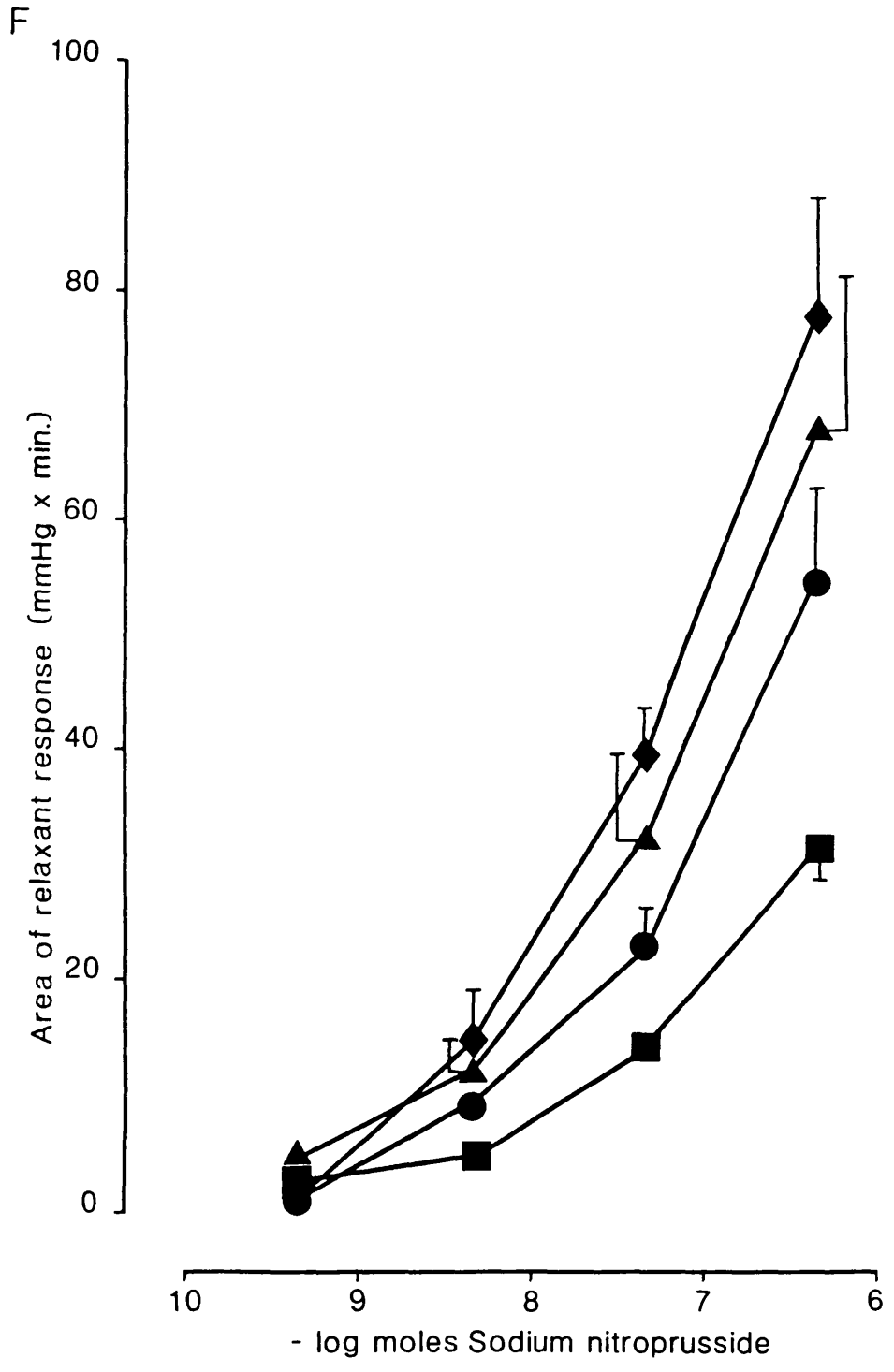

E
80
70
60
50
40
30
20
10
0
Area of relaxant response (mmHg x min.)
10
9
8
7
6
- log moles Sodium nitroprusside

FIGURE 3.4f Area of the vasodilator response to SNP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NA 10^{-5} M (■), 3 x 10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.


F
100
80
60
40
20
0
Area of relaxant response (mmHg x min.)
10
9
8
7
6
- log moles Sodium nitroprusside

SECTION B

Chapter 4

A_2-Purinoceptor-mediated relaxation in the guinea-pig coronary vasculature: a role for NO.

4.1 SUMMARY

1) The Langendorff heart preparation was used to investigate the mechanism of action of the endothelium-dependent vasodilatation evoked by adenosine and its analogues in the guinea-pig coronary vasculature.

2) The relative order of potency of adenosine and its analogues in causing a reduction in perfusion pressure was NECA = CGS 21680 > R-PIA = adenosine = 2-CA > S-PIA = CPA; thus suggesting the presence of A_2-purinoceptors in this preparation.

3) 8-PSPT ($3x10^{-5}$ M) significantly reduced both the maximum amplitude and area of the vasodilatation produced in response to adenosine ($5x10^{-10}$ - $5x10^{-8}$ mol) without having any effect on the response to the P_2-purinoceptor agonist 2-meSATP. The relaxation induced by adenosine ($5x10^{-12}$ - $5x10^{-8}$ mol) was unaffected by the selective A_1-purinoceptor antagonist DPCPX (10^{-8} M). This antagonist profile suggests that only A_2-purinoceptors are present in the guinea-pig coronary vasculature.

4) The areas of the vasodilator response to adenosine ($5x10^{-10}$ - $5x10^{-7}$ mol), NECA ($5x10^{-12}$ - $5x10^{-7}$ mol) and CGS 21680 ($5x10^{-12}$ - $5x10^{-10}$ mol) were significantly reduced by L-NAME ($3x10^{-5}$ M). The amplitude of the responses to low concentrations of adenosine ($5x10^{-10}$ - $5x10^{-9}$ mol), NECA ($5x10^{-11}$ mol) and CGS 21680 ($5x10^{-11}$ - $5x10^{-9}$ mol) were significantly reduced by L-NAME ($3x10^{-5}$ M).

5) L-Arginine ($1.5x10^{-3}$ M) significantly reversed the inhibition by L-NAME ($3x10^{-5}$ M) of the relaxant response to adenosine ($5x10^{-8}$ mol), NECA ($5x10^{-9}$ mol) and CGS 21680 ($5x10^{-11}$ mol).

6) Indomethacin (10^{-6} M) did not inhibit the response to adenosine, except at low doses ($5x10^{-11}$ - $5x10^{-10}$ mol).

7) It is concluded that in the guinea-pig coronary vasculature, while a major part of the vasodilator action of adenosine is probably directly via A_2-receptors on the smooth muscle, activation of a subpopulation of A_2-purinoceptors on endothelial cells by adenosine and its analogues induces relaxation via production of NO; prostanoids appear to play a minimal role in the relaxation induced by adenosine as in most other preparations.

4.2 INTRODUCTION

Adenosine, a potent vasodilator (Drury & Szent-Györgyi, 1929), has been proposed as a regulator of coronary flow (Berne 1963, 1980). The pharmacological effects of adenosine are mediated by specific receptors specified as P_1-purinoceptors (Burnstock, 1978). P_1-Purinoceptors have been subdivided into A_1 and A_2 receptors on the basis of different structure-activity relationships for agonists and antagonists. At A_1 receptors N^6-substituted adenosine analogues such as CPA and PIA are more potent than NECA, whereas on A_2 receptors the potency order is reversed (Bruns, 1990a,b; Daly, 1990).

On the basis of the relative order of potency of NECA, R- and S-PIA it has been determined that the vasodilator action of adenosine in the guinea-pig coronary vasculature is mediated via the A_2 subclass of the P_1-purinoceptor (Leung *et al.*, 1985). In most blood vessels the vasodilatatory effect of adenosine has been shown to be largely due to action at A_2-purinoceptors on the vascular smooth muscle and not the endothelium (Collis & Brown, 1983; White & Angus, 1987). Adenosine has been shown to be released from coronary endothelial cells (Deussen *et al.*, 1986) and at low doses it has been shown to induce relaxation via receptors on endothelial cells (Balcells *et al.*, 1992; Corr & Burnstock, 1990). Des Rosiers and Nees (1987) have provided functional evidence for the presence of A_2-purinoceptors on cultured coronary endothelial cells. The action at such receptors on the endothelium could stimulate the synthesis and release of EDRF's, such as prostacyclin (Moncada & Vane, 1979) or NO (Furchgott & Zawadzki, 1980a; Ignarro *et al.*, 1986; Moncada *et al.*, 1991).

The purpose of this study was to investigate the possible mechanisms of action of the endothelial response to adenosine and some of its analogues by using inhibitors of the enzymatic synthesis of NO and prostaglandins. L-NAME is a competitive inhibitor of the synthesis of NO from L-arginine (Rees *et al.*, 1990a) and is effective in inhibiting vasodilator responses to various agents in the guinea-pig coronary vasculature (see Chapter 3), while indomethacin is a prostaglandin-I synthase inhibitor (Vane, 1971) which will therefore prevent the formation of

prostanoids. In addition studies of the relative order of potency of adenosine analogues in the guinea-pig coronary vasculature have been expanded upon to include more specific adenosine analogues such as CGS 21680. CGS 21680 is an adenosine agonist which is 140-fold more selective for A_2-purinoceptors than A_1-purinoceptors (Hutchison *et al.*, 1989).

4.3 METHODS (See Chapter 2.1.2)

Guinea-pigs (250-400 g) of either sex were used. The heart was removed and cannulated via the aorta for perfusion according to the method of Langendorff (see Chapter 2.1.2). The average starting left ventricular systolic pressure was 27.3 ± 1.3 mm Hg (n=71). The flow rate was determined by collecting the effluent over a period of time and the average rate was 14.1 ± 0.28 ml min^{-1} (n=71). When the perfusion pressure had reached a steady state, adenosine and its analogues were given as 50 µl boluses, injected over 3 seconds into the superfusing solution close to the heart. The duration of each individual experiment was no longer than 3 hours. Due to this time restriction the effects of all the agonists could not be tested on the same heart. For this reason the agonists were chosen randomly and not more than three agonists were used on a particular heart. The order of exposure of the agonists to the heart was also random to minimise differences due to time-dependent changes in the preparation. At least 5 min was left between the administration of each dose of agonist. When the effect of antagonists was examined control

dose-response relationships for adenosine and its analogues were first obtained and L-NAME, indomethacin, 8-PSPT or DPCPX added to the perfusing solution and allowed to equilibrate for 20 min. The dose-responses were then repeated in the presence of the antagonist. After inhibition by L-NAME, L-arginine was added to the perfusing solution after the dose-response relationship had been established to determine whether the inhibition could be reversed. The preparations were allowed to equilibrate for a further 20 min before a submaximal dose (in terms of area of response) was repeated. DPCPX was not used above 10^{-8} M as the purpose of using this antagonist was to establish if there are any A_1-purinoceptors present in the guinea-pig coronary vasculature and it has been shown on guinea-pig tissue that above this concentration DPCPX also inhibits effects mediated via A_2-purinoceptors (Collis *et al.*, 1989). When the effects of adenosine were examined before and after antagonist, the highest dose used to obtain the dose-response curve (Fig. 4.1) was not used as the resting perfusion pressure tended to find a new lower level after this high dose was applied. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis program on an Apple II computer. At the end of the experiment the heart was removed from the cannula, blotted and weighed. The mean wet weights were 1.54 ± 0.02 g (n=71).

4.3.1 Materials See Chapter 2.4

4.3.2 Statistics See Chapter 2.3

4.4 RESULTS

Dose-response relationships to adenosine and analogues.

Bolus injections of NECA, CGS 21680, CPA, 2-CA, R-PIA, S-PIA and adenosine produced dose-dependent vasodilatation in the guinea-pig coronary vasculature. Dose-response curves for the 7 agonists are illustrated in Figure 4.1. The maximum responses produced by NECA and CGS 21680 were similar and the pD_2 values for both were not significantly different from one another (6.25 ± 0.16 (n=21) and 6.16 ± 0.11 (n=11) respectively). The pD_2 values for adenosine and 2-CA were 5.38 ± 0.03 (n=23) and 5.44 ± 0.06 (n=11) respectively. The pD_2 values for R-PIA, S-PIA and CPA were 5.54 ±0.09 (n=23), 4.35 ± 0.08 (n=13) and 4.68 ±0.1 (n=10) respectively, although there are limitations in the accuracy of these values as the maximum responses were not always achieved in all the preparations due to the limited solubility of these agents. Their potency expressed relative to the pD_2 value of adenosine was in the ratio 7 : 6 : 1.4 : 1.2 : 1 : 0.2 : 0.1 respectively. A similar rank order of potency was obtained when individual doses of agonists were compared by analysis of variance and Tukey's tests. There was a small but unsignificant fall in the left ventricular systolic pressure on bolus administration of agonists at the high doses.

Effect of 8-PSPT and DPCPX.

8-PSPT ($3x10^{-5}$ M) significantly reduced the maximum amplitude and area of the vasodilatation produced in response to adenosine ($5x10^{-10}$ - $5x10^{-8}$ mol, Figure 4.2a; data for area not shown). As a control, neither the maximum amplitude nor the area of the response to 2-meSATP ($5x10^{-13}$ - $5x10^{-9}$ mol, Figure 4.2b; data for area not shown) were affected by 8-PSPT ($3x10^{-5}$ M). DPCPX (10^{-8} M) did not have any effect on the response to adenosine ($5x10^{-12}$ - $5x10^{-8}$ mol; data not shown). 8-PSPT ($3x10^{-5}$ M) did not significantly affect the resting perfusion pressure or left ventricular pressure of the preparations.

Effect of L-NAME and L-Arginine.

L-NAME ($3x10^{-5}$ M) significantly reduced the amplitude of the response to adenosine ($5x10^{-10}$ - $5x10^{-9}$ mol, Figure 4.3a). However, L-NAME ($3x10^{-5}$ M) had a greater effect and significantly attenuated the area of the response to adenosine ($5x10^{-10}$ - $5x10^{-7}$ mol, Figure 4.3b). The inhibition of the response to adenosine ($5x10^{-8}$ mol, Table 4.1), by L-NAME ($3x10^{-5}$ M), was significantly reversed in the presence of L-arginine ($1.5x10^{-3}$ M). L-NAME ($3x10^{-5}$ M) only significantly reduced the amplitude of the response to a low concentration of NECA ($5x10^{-11}$ mol, data not shown) and two concentrations of CGS 21680 ($5x10^{-12}$ and $5x10^{-9}$ mol, data not shown). In the case of both NECA ($5x10^{-12}$ - $5x10^{-7}$ mol, Figure 4.4a) and CGS 21680 ($5x10^{-12}$ - $5x10^{-9}$ mol, Figure 4.4b) the area of the responses were significantly attenuated by

L-NAME ($3x10^{-5}$ M). The attenuation of the area of the response to NECA ($5x10^{-9}$ mol, Table 4.1) and CGS 21680 ($5x10^{-11}$ mol, Table 4.1) by L-NAME ($3x10^{-5}$ M) was significantly reversed in the presence of L-arginine ($1.5x10^{-3}$ M). There was only partial reversal but the concentration of L-arginine could not be increased because a higher dose reduced the perfusion pressure of the preparation and therefore affected the degree of relaxation that could be obtained. The concentration of NECA and CGS 21680 used to show reversal of inhibition of L-NAME by L-arginine was chosen as a dose that gave a similar submaximal response in terms of area. The resting perfusion and left ventricular pressure of the preparations were unaffected by the addition of L-NAME ($3x10^{-5}$ M) into the perfusate.

Effect of Indomethacin.

Indomethacin (10^{-6} M) significantly reduced the amplitude and area of the response to only two low concentrations of adenosine ($5x10^{-11}$ and $5x10^{-10}$ mol, Figure 4.5; data for area not shown). As a control, both the maximum amplitude and area of the vasodilatation produced in response to bradykinin ($5x10^{-12}$ - $5x10^{-9}$ mol, data not shown) were significantly reduced by indomethacin (10^{-6} M). Indomethacin (10^{-6} M) did not significantly affect either the resting perfusion pressure or the left ventricular pressure of the preparations.

4.5 DISCUSSION

The relative order of potency of adenosine and its analogues in the guinea-pig coronary vasculature is characteristic of P_1-purinoceptors of the A_2 sub-type (Collis, 1985; Burnstock & Kennedy, 1986; Williams, 1987). 8-PSPT inhibited the response to adenosine whereas DPCPX had no effect. This also suggests the presence of A_2-purinoceptors. The response to adenosine is at least in part mediated via NO, whereas prostaglandins play a minimal role.

Previous work involving the use of NECA, R- and S-PIA has shown that A_2-purinoceptors are present in the guinea-pig coronary vasculature (Leung *et al.*, 1985). Additional information obtained using the selective A_2-purinoceptor agonist CGS 21680 (Hutchinson *et al.*, 1989) and A_1-purinoceptor agonist CPA (Moos *et al.*, 1985) have provided a clearer picture of the receptor profile in the guinea-pig coronary vasculature. CPA is significantly less potent than adenosine and 2-CA whereas R-PIA is not, and CGS 21680 is equipotent with NECA. The agonist profile of CGS 21680 and CPA strongly implies the involvement of A_2-purinoceptors. The non-selective P_1-purinoceptor antagonist 8-PSPT (Collis *et al.*, 1987) inhibited the response to adenosine at a concentration which had no effect on the response to 2-meSATP confirming the fact that adenosine was acting on P_1-purinoceptors. DPCPX has been shown at low concentrations to be a highly selective A_1-purinoceptor antagonist (Haleen *et al.*, 1987; Lohse *et al.*, 1987) in guinea-pig and rat tissues (Collis *et al.*, 1987; 1989; Collis, 1990). In the guinea-pig coronary vasculature DPCPX had no effect on the response to adenosine suggesting that the relaxation was not

mediated by A_1-purinoceptors. The effects of the antagonists also suggest the presence of A_2-purinoceptors. Thus, it can be concluded that even with the use of more selective agonists there does not appear to be a role for A_1-purinoceptors in the relaxation of the guinea-pig coronary vasculature by adenosine analogues.

L-NAME inhibited the area, although not the maximum amplitude (except at low concentrations of adenosine, NECA and CGS 21680), of the responses to adenosine, NECA and CGS 21680. This suggests that at least part of the response induced by these vasodilators is mediated via NO. It has been shown in isolated perfused guinea-pig hearts that the endothelium is a highly active metabolic barrier for adenosine (Nees *et al.*, 1985), such that at low concentrations adenosine would be avidly metabolised and therefore unable to reach the smooth muscle. Therefore the response to low doses of adenosine would be due to action on the endothelium. There is evidence for the presence of adenosine A_2-purinoceptors in cultured coronary endothelial cells (Des Rosiers & Nees, 1987), although in the guinea-pig coronary artery P_1-purinoceptors appear to be limited to the smooth muscle (Keef *et al.*, 1992). In contrast, in the rabbit coronary artery an endothelial role to part of the response to adenosine has been reported (Corr & Burnstock, 1990). This information suggests that at least some A_2-purinoceptors are located on endothelial cells on resistance vessels in the guinea-pig coronary bed which, when activated, induce relaxation via NO. The fact that L-arginine at least partially reversed the inhibition of the response to

adenosine, NECA and CGS 21680 by L-NAME substantiates these claims in that it shows that L-NAME was selectively inhibiting the enzyme NO synthase. NO synthase converts L-arginine into L-citrulline with the additional production of NO (Palmer *et al.*, 1988b; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). It has been demonstrated in the perfused rabbit heart that inhibition by the NO synthase inhibitor L-NMMA of the effect of ACh was due to shortening of the response rather than to an effect on peak fall in perfusion pressure (Smith *et al.*, 1992). It was suggested that NO was involved in the sustained relaxation induced by ACh but not in the initial event. Similarly, in the anaesthetized guinea-pig L-NMMA decreased the duration of the hypotensive response to ACh without affecting the magnitude of the response (Aisaka *et al.*, 1989). The same might be suggested about the vasodilator action of these A_2-receptor agonists in that there appears to be two overlapping phases, a brief early one which appears to be insensitive to L-NAME and is responsible for the initial peak depressor response, followed by a second phase of longer duration that is abbreviated by L-NAME.

Prostacyclin is a powerful vasodilator and platelet anti-aggregatory compound (Gryglewski *et al.*, 1976). In the perfused guinea-pig heart the endothelium-dependent dilatation to ACh was significantly attenuated by indomethacin (Lee *et al.*, 1990), suggesting that prostanoid production can be induced. In the isolated rabbit heart the same adenosine receptors that elicit relaxation also induce release of prostacyclin (Karwatowska-Prokopoczuk *et al.*, 1988). Therefore it could be conceivable that

adenosine induces relaxation via release of prostanoids. However indomethacin, at a concentration that significantly inhibited the response to bradykinin, only attenuated the area and maximum amplitude of the responses to a few individual doses of adenosine. Therefore it is apparent that prostanoids do not play a major role in the relaxation induced by adenosine.

In conclusion it can be stated that there is a population of A_2-purinoceptors present in the guinea-pig coronary vasculature which when activated by adenosine, NECA and CGS 21680 induce relaxation that is partly mediated via NO. Prostanoids play only a minor role if any in the relaxation induced by administration of adenosine.

TABLE 4.1 The area of the relaxation obtained in response to adenosine, NECA and CGS 21680 in the isolated perfused guinea-pig heart. The effect of L-NAME (3×10^{-5} M; in the perfusate) on the response to these agonists and the effect of L-arginine (L-ARG (1.5×10^{-3} M); in the perfusate along with L-NAME) on the inhibition by L-NAME is demonstrated. The areas are expressed as the mean ± s.e. mean ($n \geq 6$). Significant differences from control are * $P<0.05$. Significant differences from responses obtained in presence of L-NAME are + $P<0.05$.

Agonist	Dose (moles)	Area of relaxant response (mmHg x min.)		
		Control	After addition of L-NAME	L-NAME & L-ARG
Adenosine	$5x10^{-8}$	26.17±6.3	10.41±2.0*	18.20±3.8+
NECA	$5x10^{-9}$	109.63±15.2	43.04±9.9*	56.91±12.8+
CGS21680	$5x10^{-11}$	117.27±26.7	10.98±4.3*	39.02±5,8+

FIGURE 4.1 The amplitude of the vasodilatation evoked by (a) NECA (●), (b) CGS 21680 (■), (c) R-PIA (○), (d) adenosine (▲), (e) 2-CA (◆), (f) S-PIA (□) and (g) CPA (△) in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 10$) with s.e. mean indicated by vertical bars.

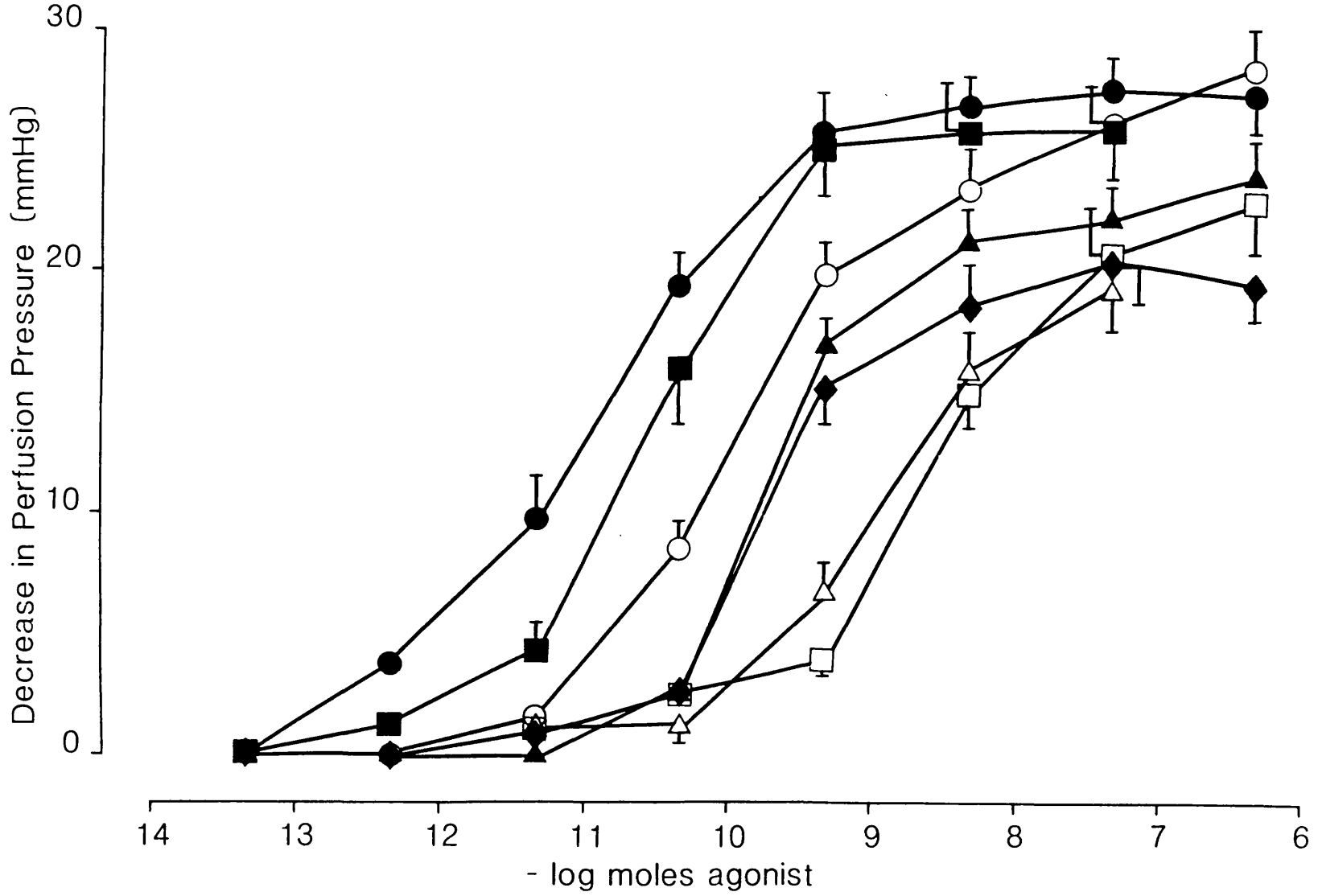

30
20
10
0
Decrease in Perfusion Pressure (mmHg)
14
13
12
11
10
9
8
7
6
- log moles agonist

FIGURE 4.2a The amplitude of the vasodilator responses evoked by adenosine, in the absence (●) and presence (○) of 8-PSPT (3×10^{-5} M), in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

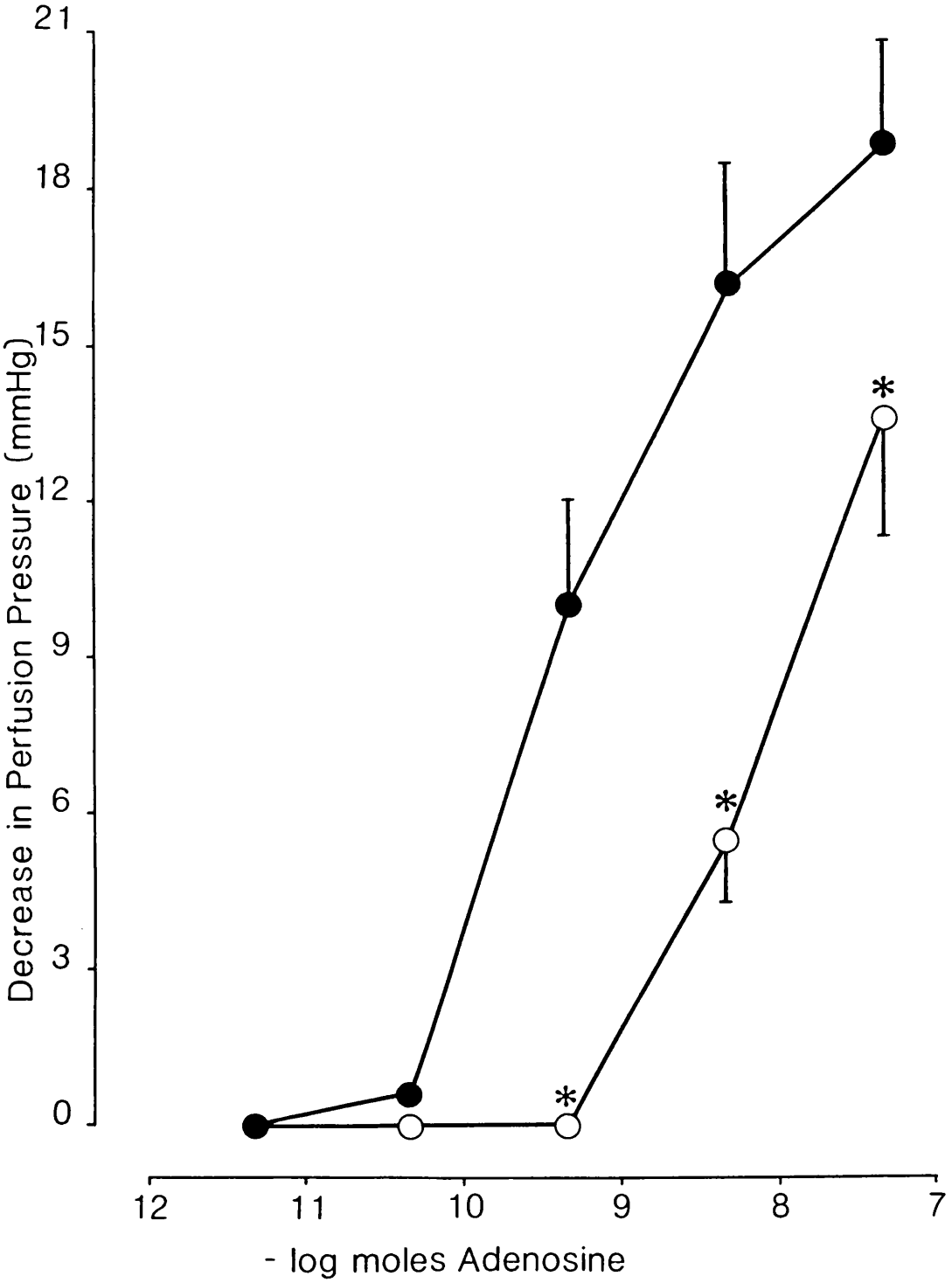

21
18
15
12
9
6
3
0
Decrease in Perfusion Pressure (mmHg)
*
*
*
12
11
10
9
8
7
- log moles Adenosine

FIGURE 4.2b The amplitude of the vasodilator responses evoked by 2-meSATP, in the absence (■) and presence (□) of 8-PSPT (3×10^{-5} M), in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

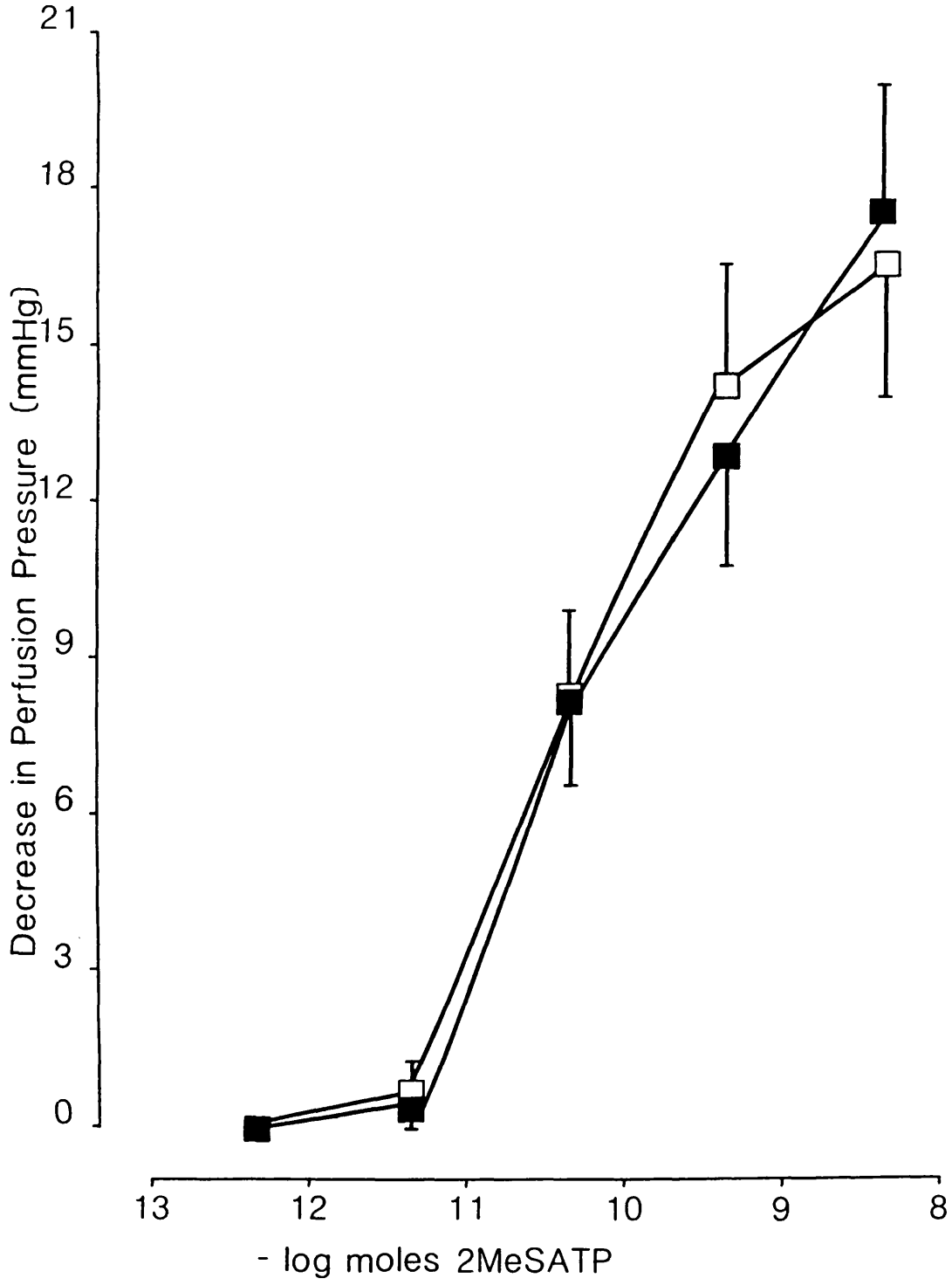

21
18
15
12
9
6
3
0
Decrease in Perfusion Pressure (mmHg)
13
12
11
10
9
8
- log moles 2MeSATP

FIGURE 4.3a The amplitude of the vasodilatation obtained in response to adenosine, in the absence (●) and presence (○) of L-NAME (3×10^{-5} M), in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

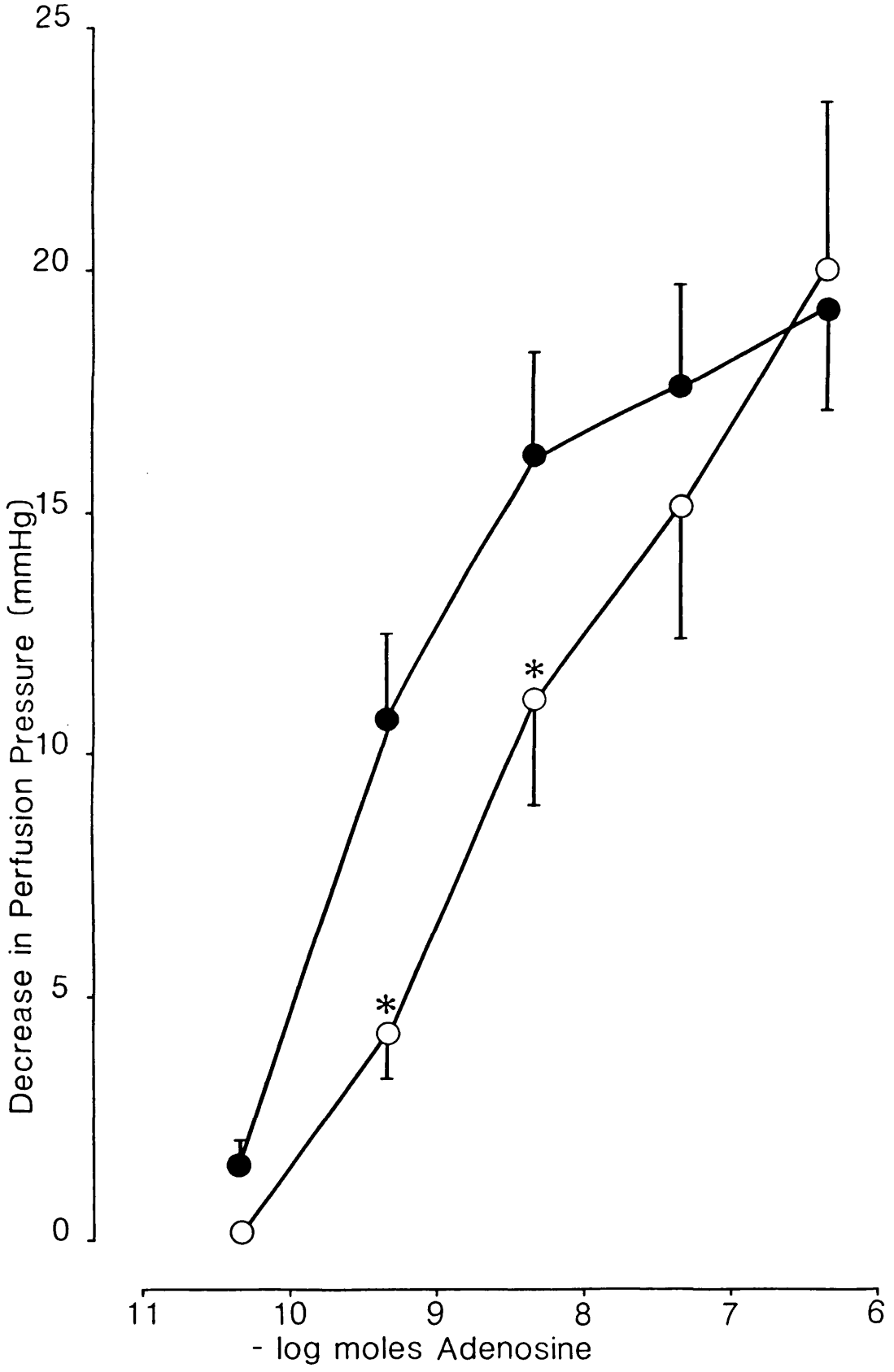

25
20
15
10
5
0
Decrease in Perfusion Pressure (mmHg)
*
*
11
10
9
8
7
6
- log moles Adenosine

FIGURE 4.3b The area of the vasodilatation obtained in response to adenosine, in the absence (■) and presence (□) of L-NAME (3×10^{-5} M), in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

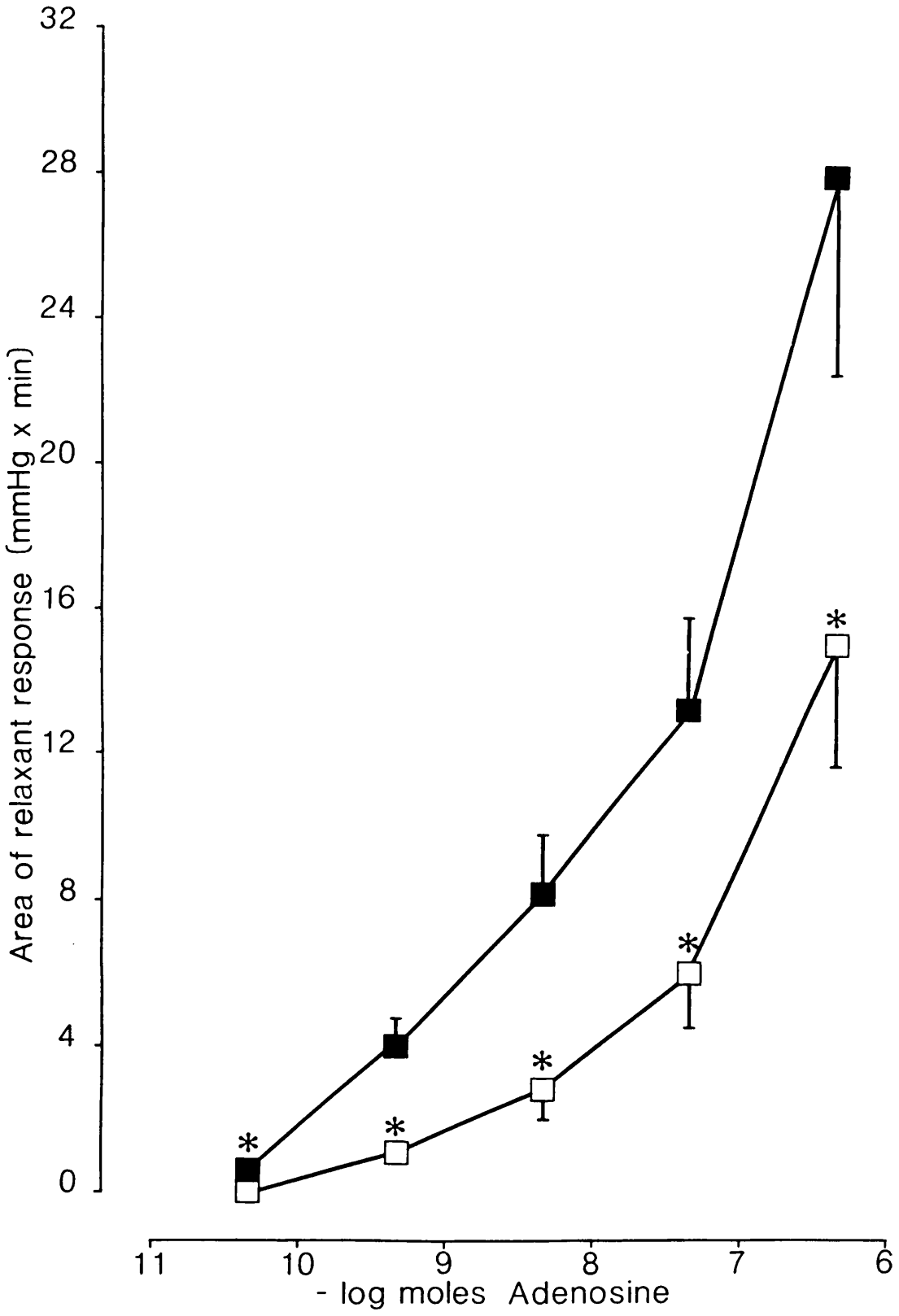

32
28
24
20
16
12
8
4
0
Area of relaxant response (mmHg x min)
11
10
9
8
7
6
- log moles Adenosine
*
*
*
*
*

FIGURE 4.4a The area of the relaxant response to NECA, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

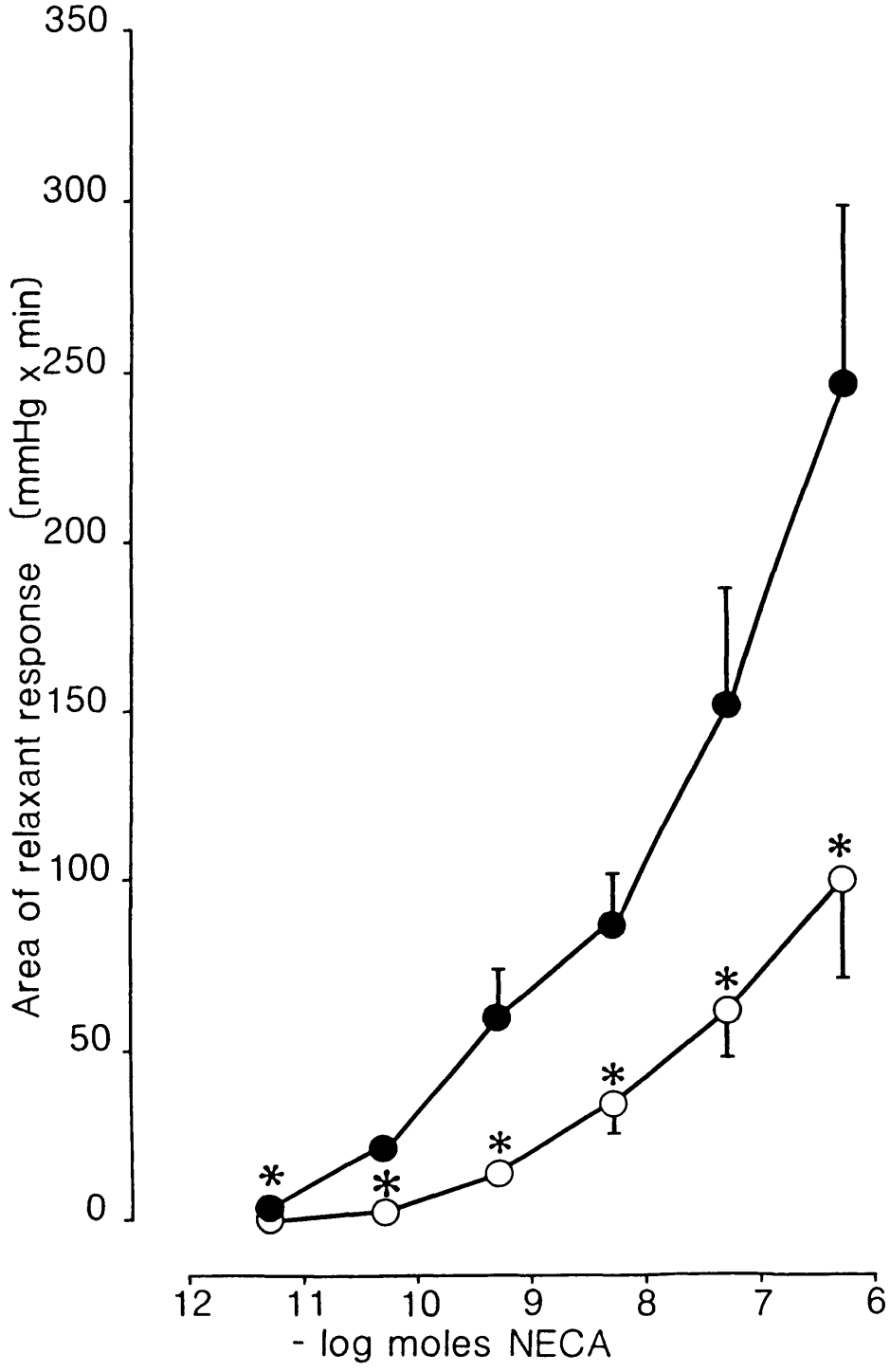

350
300
250
200
150
100
50
0
Area of relaxant response (mmHg x min)
12
11
10
9
8
7
6
- log moles NECA
*
*
*
*
*
*

FIGURE 4.4b The area of the relaxant response to CGS 21680, in the isolated perfused guinea-pig heart, in the absence (■) and presence (□) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

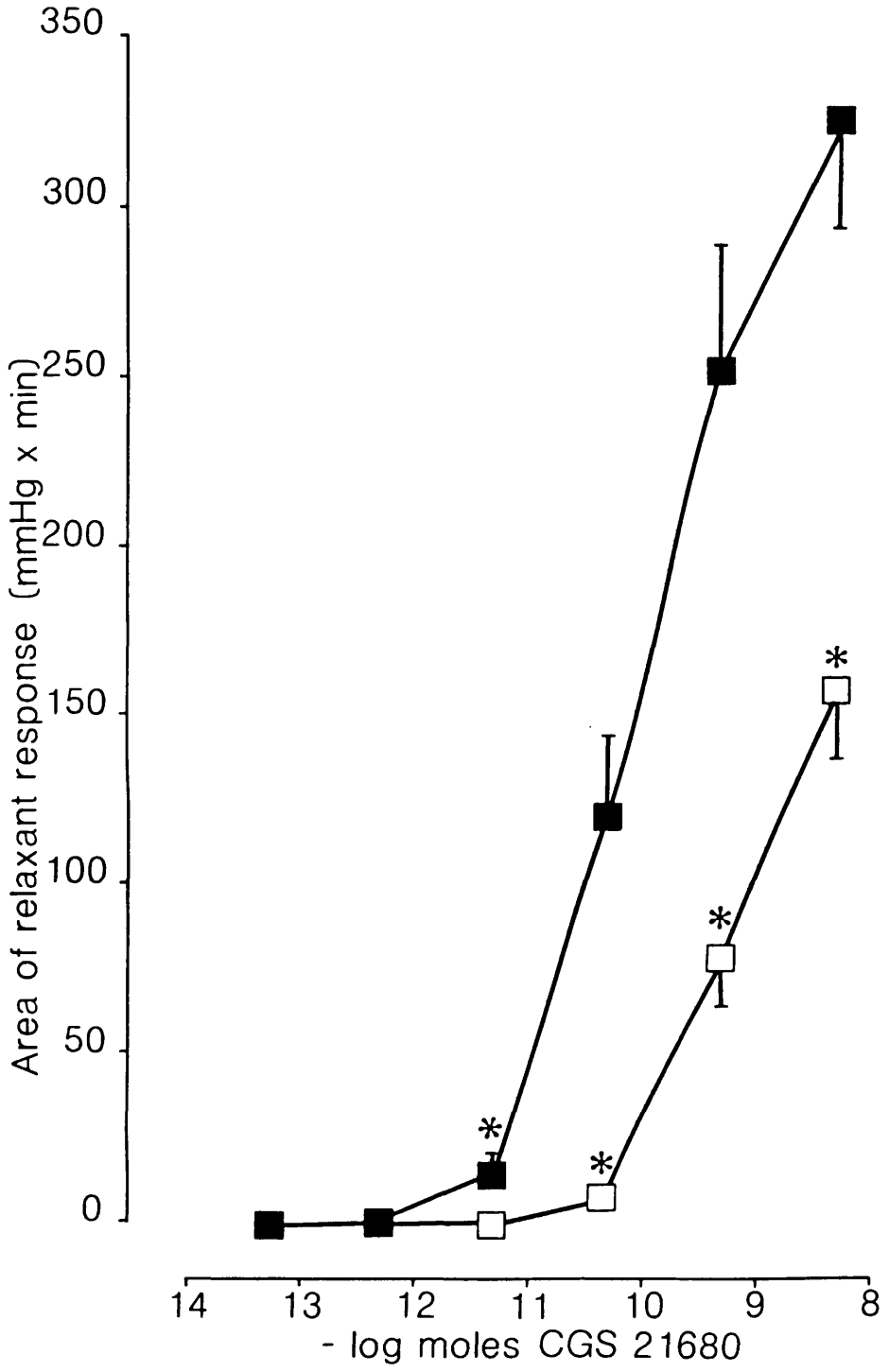
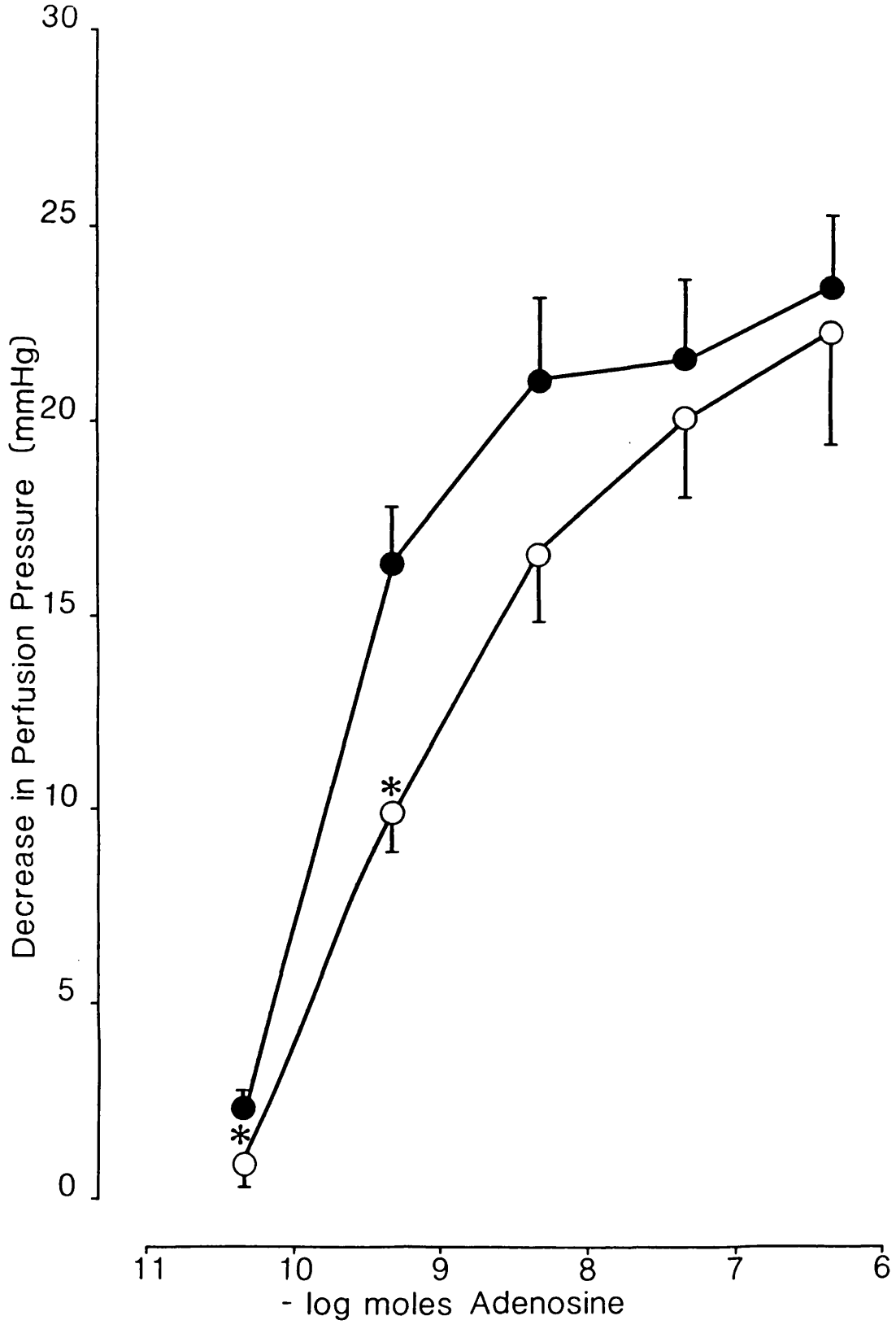

350
300
250
200
150
100
50
0
Area of relaxant response (mmHg x min)
*
*
*
*
14
13
12
11
10
9
8
- log moles CGS 21680

FIGURE 4.5 The amplitude of the vasodilatation obtained in response to adenosine in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.


30
25
20
15
10
5
0
Decrease in Perfusion Pressure (mmHg)
*
*
11
10
9
8
7
6
- log moles Adenosine

Chapter 5

Differential effects of ATP and 2-meSATP induced relaxation in the guinea-pig coronary vasculature.

5.1 SUMMARY

1) The Langendorff heart preparation was used to investigate the mechanism of action of the vasodilatation evoked by ATP and its analogues in the guinea-pig coronary vasculature.

2) The relative order of potency of ATP and its analogues in causing a reduction in perfusion pressure was 2-meSATP > ATP > β,γ -meATP ≥ α,β-meATP; thus establishing the presence of P_{2Y}-purinoceptors in this preparation.

3) L-NAME ($3x10^{-5}$ M) significantly attenuated both the area and amplitude of the vasodilatation produced in response to 2-meSATP ($5x10^{-12}$ - $5x10^{-9}$ mol). However, for ATP ($5x10^{-7}$ - $5x10^{-10}$ mol), L-NAME ($3x10^{-5}$ M) significantly attenuated the area of the response, but did not reduce the maximum amplitude, except at one low dose ($5x10^{-10}$ mol).

4) L-Arginine ($1.5x10^{-3}$ M) significantly reversed the inhibition of the area of the response to 2-meSATP ($5x10^{-10}$ mol) and ATP ($5x10^{-8}$ mol) by L-NAME ($3x10^{-5}$ M).

5) The amplitude of the area of the response to ATP ($5x10^{-10}$ - $5x10^{-7}$ mol) was significantly attenuated in the presence of indomethacin (10^{-6} M). In contrast, indomethacin (10^{-6} M) did not reduce the response to 2-meSATP and adenosine, except at low doses ($5x10^{-11}$ and $5x10^{-11}$ - $5x10^{-10}$ mol respectively).

6) 8-PSPT ($3x10^{-5}$ M) did not affect either the amplitude or area of the response to ATP, except at low doses ($5x10^{-10}$ mol). 8-PSPT ($3x10^{-5}$ M) significantly reduced both the maximum amplitude and area of the vasodilatation produced in response to adenosine ($5x10^{-10}$ - $5x10^{-8}$ mol) without having any effect on the response to 2-meSATP.

7) It is concluded that in the guinea-pig coronary vasculature 2-meSATP acts at P_{2Y}-purinoceptors on the endothelial cells to induce relaxation via production or release of NO. ATP is less potent than 2-meSATP at this receptor but has an additional vasodilator action via prostanoids.

5.2 INTRODUCTION

The vascular actions of purine nucleotides and nucleosides have been recognised for many years (Drury & Szent-Gyorgyi, 1929; Green & Stoner, 1950; Haddy & Scott, 1968; Burnstock, 1980; Su, 1981). Two types of receptors mediating responses to purines have been categorised, P_1- and P_2-purinoceptors, with selectivity for adenosine and ATP, respectively. Burnstock and Kennedy (1985) proposed a subdivision of P_2 receptors into P_{2X} and P_{2Y} subtypes, based largely on the rank order of potency of structural analogues of ATP, which are involved, respectively, with contraction and relaxation of smooth muscle. At the P_{2X}-purinoceptor, the order of potency of the agonists was found to be α,β-meATP, β,γ-meATP > ATP = 2-meSATP.

The second P_2-purinoceptor subtype (P_{2Y}) showed a potency order of 2-meSATP >> ATP > α,β-meATP, β,γ-meATP. Experimental evidence suggests that the distribution of P_2-purinoceptors on blood vessels varies between species and different vascular beds. Thus, in rabbit mesenteric artery and portal vein both P_{2X}- and P_{2Y}-purinoceptor subtypes are present on the smooth muscle (Kennedy & Burnstock, 1985b; Mathieson & Burnstock, 1985). By contrast, in rat aorta, mesenteric artery and dog coronary artery, P_{2X}- and P_{2Y}- purinoceptors are located on the smooth muscle and endothelium, respectively (White *et al.*, 1985; Houston *et al.*, 1987; Ralevic & Burnstock, 1988).

Hopwood & Burnstock (1987) have demonstrated that ATP and 2-meSATP mediate coronary vasodilatation via P_{2Y}-purinoceptors in the isolated rat heart. In the guinea-pig coronary vasculature the vasodilatory properties of ATP require the presence of an intact endothelium (Hopwood *et al.*, 1989; Lee *et al.*, 1990). Stimulation of endothelial cells by ATP leads to release of EDRF (Kelm & Schrader, 1988; Boeynaems & Pearson, 1990), identified as NO (Palmer *et al.*, 1988b) which in turn relaxes the subjacent smooth muscle. In the guinea-pig coronary vasculature at least part of the response to ATP is via NO (see Chapter 3), and the amounts of NO formed in the guinea-pig heart stimulated with ATP are effective for vasodilation (Kelm & Schrader, 1990). ATP also causes the release of prostaglandin I_2 (PGI_2) from the heart (Needleman *et al.*, 1974), presumably in large part from the endothelium (Needham *et al.*, 1987). It may also be possible that vasodilatation produced by ATP is due to its degradation to

adenosine (Moody *et al.*, 1984), although this theory has been disclaimed in the guinea-pig coronary vasculature (Brown *et al.*, 1991).

Recently, it has been proposed that ATP-induced relaxation could, in part, be caused by another separate 'nucleotide' receptor which is different from the classical P_{2Y}-purinoceptor (O'Connor *et al.*, 1991) and found to be located on both the endothelium and smooth muscle of the rabbit aorta (Chinellato *et al.*, 1992).

The purpose of this study was to investigate the possible mechanisms of action of the vasodilator response to ATP and 2-meSATP by using inhibitors of the enzymatic synthesis of NO and prostaglandins, and thus determine whether ATP acts on the same receptors as the classical P_{2Y}- purinoceptor agonist 2-meSATP to initiate relaxation. The inhibitors used were L-NAME, a competitive inhibitor of the synthesis of NO from L-arginine (Rees *et al.*, 1990), and indomethacin, a prostaglandin-synthase inhibitor (Vane, 1971) which prevents the formation of prostanoids.

5.3 **METHODS** See Chapter 2.1.2

Guinea-pigs (250-350 g) of either sex were used. The heart was removed and cannulated via the aorta for perfusion according to the method of Langendorff (see Chapter 2.1.2). The average starting left ventricular systolic pressure was 28.1 ± 1.2 mm Hg (n=69). The flow rate was determined by collecting the

effluent over a set period of time, and the average rate was 14.3 ± 0.26 ml min^{-1} (n=69). A 20 min equilibration period was allowed before the commencement of each experiment. When the perfusion pressure had reached a steady state, ATP and its analogues were given as 50µl boluses, injected over 3 seconds into the superfusing solution close to the heart. At least 5 min were left between each dose of agonist administered. When the relative order of potency was determined for ATP and its analogues the order of exposure of the agonists to the heart was random in order to minimise differences due to time-dependent changes in the preparation. For a given response both the maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis programme on an Apple II computer.

Control dose-response relationships to agonists were first determined. An inhibitor or antagonist (L-NAME, indomethacin or 8-PSPT) was then added to the perfusing solution and the preparation allowed to equilibrate for 20 min Dose-response relationships were then re-evaluated in the presence of the inhibitor. After inhibition by L-NAME, L-arginine was added to the perfusing solution to determine whether the inhibition could be reversed. The preparations were allowed to equilibrate for a further 20 min. before a submaximal dose (in terms of area of the response) was repeated. At the end of each experiment the heart was removed from the cannula, blotted and weighed. The mean wet weights were 1.52 ± 0.03 g (n=69).

5.3.1 Materials See Chapter 2.4

5.3.2 Statistics See Chapter 2.3

5.4 RESULTS

Dose-response to ATP and analogues.

Bolus injections of ATP and 2-meSATP produced a dose-dependent vasodilatation in the guinea-pig coronary vasculature. Dose-response curves for ATP, 2-meSATP, β,γ-meATP and α,β-meATP are illustrated in figure 5.1. The pD_2 value for 2-meSATP is significantly greater than the pD_2 value for ATP; 6.65 ± 0.11 and 5.39 ± 0.08 respectively. The maximum vasodilatation produced by ATP and 2-meSATP was not significantly different. α,β-meATP and β,γ-meATP only induced relaxation in the guinea-pig coronary vasculature when used at relatively high doses. There was a small but insignificant fall in the left ventricular systolic pressure on bolus administration of agonists at the high doses.

Effect of L-NAME on the response to ATP and 2-meSATP.

The effect of L-NAME ($3x10^{-5}$ M) on the area and amplitude of the response to 2-meSATP and ATP in the guinea-pig coronary vasculature is illustrated in figure 5.2a,b. L-NAME ($3x10^{-5}$ M) significantly attenuated both the area and amplitude of the vasodilatation produced in response to 2-meSATP ($5x10^{-12}$ - $5x10^{-9}$

mol; Figure 5.2a,b). However, in the case of ATP, L-NAME ($3x10^{-5}$ M) significantly attenuated the area of the response to ATP ($5x10^{-7}$ - $5x10^{-10}$ mol; Figure 5.2d) but only significantly reduced the amplitude of one low dose of ATP ($5x10^{-10}$ mol, Figure 5.2c).

L-Arginine ($1.5x10^{-3}$ M) significantly reversed the inhibition of the area of the response to 2-meSATP ($5x10^{-10}$ mol; Table 5.1) and ATP ($5x10^{-8}$ mol; Table 5.1) by L-NAME ($3x10^{-5}$ M). In the case of 2-meSATP ($5x10^{-10}$ mol; data not shown) the reduction in the amplitude of the response to 2-meSATP in the presence of L-NAME ($3x10^{-5}$ M) was also significantly reversed in the presence of L-arginine ($1.5x10^{-3}$ M). L-NAME ($3x10^{-5}$ M) and L-arginine ($1.5x10^{-3}$ M) did not significantly affect the resting perfusion pressure or left ventricular pressure of the preparations.

Effect of Indomethacin on the response to ATP, 2-meSATP and adenosine.

The effect of indomethacin (10^{-6} M) on the area and amplitude of the vasodilatation produced in response to 2-meSATP and ATP is illustrated in figure 5.3a,b. Indomethacin (10^{-6} M) only significantly reduced the amplitude of the response to one dose of 2-meSATP ($5x10^{-11}$ mol, Figure 5.3a). The area of the relaxant response to 2-meSATP (data not shown) was not reduced in the presence of indomethacin (10^{-6} M). The amplitude of the response to ATP ($5x10^{-10}$ - $5x10^{-7}$ mol, Figure 5.3b) was significantly attenuated in the presence of indomethacin (10^{-6} M), whereas the area of the response to ATP was only significantly

reduced at one intermediate dose ($5x10^{-9}$ mol; data not shown). Indomethacin (10^{-6} M) did not reduce the vasodilator response to adenosine, except at low doses ($5x10^{-11}$ - $5x10^{-10}$ mol; data not shown). The resting perfusion pressure and left ventricular pressure of the preparations were unaffected by the addition of indomethacin (10^{-6} M).

Effect of 8-PSPT on the response to ATP, 2-meSATP and Adenosine.

The effect of 8-PSPT ($3x10^{-5}$ M) on the amplitude of the vasodilatation produced in response to ATP, 2-meSATP and adenosine is illustrated in figure 5.4a,b,c. 8-PSPT ($3x10^{-5}$ M) did not affect either the amplitude or area of the response to 2-meSATP ($5x10^{-9}$ - $5x10^{-13}$ mol; Figure 5.4a, data for area not shown). In the case of ATP only the amplitude and area of the response to a low dose of ATP ($5x10^{-10}$ mol; Figure 5.4b, data for area not shown) was significantly attenuated by 8-PSPT ($3x10^{-5}$ M). The same concentration of 8-PSPT significantly attenuated the amplitude and area of the response to adenosine ($5x10^{-8}$ - $5x10^{-10}$ mol; Figure 5.4c, data for area not shown). 8-PSPT ($3x10^{-5}$ M) did not significantly affect either the resting perfusion pressure or the left ventricular pressure of the preparations.

5.5 DISCUSSION

The relative order of potency of ATP and its analogues, for inducing relaxation in the guinea-pig coronary vasculature is characteristic of P_2-purinoceptors of the P_{2Y} subtype (Burnstock &

Kennedy, 1985). 8-PSPT failed to inhibit the responses to 2-meSATP and the majority of the responses to ATP at a concentration that significantly attenuated the relaxant response to adenosine. A major part of the response to 2-meSATP has been found to be mediated via NO, whereas prostanoids do not appear to play a role in the relaxant response. In the case of the relaxant response to ATP both NO and prostanoids appear to play a role.

ATP is a powerful systemic vasodilator, and studies on a variety of isolated preparations have shown that relaxation requires the presence of an intact endothelium (Furchgott & Zawadzki, 1980b; DeMey & Vanhoutte, 1981, 1982), although, in the coronary artery of the rabbit, ATP produces vasodilatation by a direct action on P_{2Y}-purinoceptors on the smooth muscle (Corr & Burnstock, 1991). In the guinea-pig coronary vasculature responses (both amplitude and area) due to 2-meSATP were significantly reduced by L-NAME. This implies that there is a population of P_{2Y}-purinoceptors on the endothelium which when activated induce relaxation via NO. L-NAME did not alter the peak vasodilator response to ATP, suggesting that at least this part of the response was not due to the generation of NO. However, the duration of the response was reduced by L-NAME, and therefore, part of the action of ATP occurs via the release of NO. This is supported by work on the release of NO in the guinea-pig coronary bed, which has shown that ATP does induce release of NO (Kelm & Schrader, 1990). Therefore, both ATP and 2-meSATP act on a population of P_{2Y}-purinoceptors on the endothelium to elicit relaxation via NO. The fact that L-arginine reversed the

inhibition of the response to ATP and 2-meSATP substantiates these claims in that it shows that L-NAME was selectively inhibiting the enzyme NO synthase. NO synthase converts L-arginine into L-citrulline with the additional production of NO (Palmer *et al.*, 1988b; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). However, particularly with ATP, relaxation of the coronary vasculature is not exclusively via activation of this population of P_{2Y}-purinoceptors.

ATP also causes the release of PGI_2 from the heart (Needleman *et al.*, 1974), presumably in large part from the endothelium (Needham *et al.*, 1987). In the guinea-pig coronary vasculature the prostaglandin synthesis inhibitor indomethacin inhibited the relaxant response to ATP but did not affect the relaxant response to 2-meSATP. The activation of P_{2Y}-purinoceptors by 2-meSATP does not induce release of prostanoids. This is consistent with the prostaglandin-mediated rebound contraction of the taenia coli which can be induced by ATP but not the analogues in which the phosphate chain was altered (Brown & Burnstock, 1981). Brown *et al.* (1991) have also shown that ATP induced coronary vasodilatation is partially due to prostanoid production. P_{2Y}-purinoceptors on cultured porcine endothelial cells stimulate prostaglandin production (Pearson *et al.*, 1983), but in this case 2-meSATP was more potent than ATP at inducing dose-dependent prostacyclin secretion. In the guinea-pig coronary vasculature, in contrast to the response to 2-meSATP, the ATP response is mediated at least in part via a prostanoid.

There is always the possibility that the vasodilatation

produced in response to exposure to ATP is due to its degradation to adenosine (Moody *et al.*, 1984). However, the adenosine receptor antagonist 8-PSPT did not affect the response to ATP (except at one low dose) at a concentration that significantly inhibited the response to adenosine. The relaxant response to ATP in the guinea-pig coronary vasculature has also been shown to be unaffected by the adenosine receptor antagonist xanthine amine congener (XAC) (Brown *et al.*, 1991). To substantiate this theory adenosine was also unaffected by indomethacin at a concentration that significantly inhibited the response to ATP.

It has been demonstrated in the rabbit aorta that the relaxant effect of ATP is mediated not only by P_{2Y}-purinoceptors, as the endothelial-dependent relaxant effect due to ATP is greater in comparison to the effect of 2-meSATP, the most potent agonist at P_{2Y}-purinoceptors (Chinellato *et al.*, 1992). This may be explained by the present study, which has shown a reduction in the ATP-mediated, but not 2-meSATP-mediated, vasodilatation by indomethacin. In addition, it has been shown that exposure of pig aortic endothelial cells to ATP causes the release of prostacyclin, whereas, 2-meSATP was much less effective (Needham *et al.*, 1987). It is difficult to postulate from this study how ATP was inducing synthesis or release of prostanoids, although it appears that it was not via activation of P_{2Y}-purinoceptors.

In conclusion, this study has shown the presence of P_{2Y}-receptors in the guinea-pig coronary vasculature which when activated elicit relaxation via NO. In addition to ATP acting at this P_{2Y}-purinoceptor to induce relaxation via NO, ATP also

induces relaxation via a prostanoid.

TABLE 5.1 The area of the relaxation obtained in response to 2-meSATP and ATP in the isolated perfused guinea-pig heart. The effect of L-NAME (3×10^{-5} M; in the perfusate) on the response to these agonists and the effect of L-arginine (1.5×10^{-3} M; in the perfusate along with L-NAME) on the inhibition by L-NAME is demonstrated. The areas are expressed as the mean ± s.e. mean ($n \geq 6$). Significant differences from control are * $P<0.05$. Significant differences from responses obtained in the presence of L-NAME are ** $P<0.05$.

Agonist	Dose (moles)	Area of relaxant response (mmHg x min.)		
		Control	After addition of: L-NAME	L-NAME & L-ARG
2-meSATP	5×10^{-10}	10.65 ± 1.58	2.12 ± 0.47*	8.58 ± 1.58**
ATP	5×10^{-8}	24.57 ± 3.78	10.52 ± 2.07*	20.52 ± 4.24**

FIGURE 5.1 The amplitude of the vasodilatation evoked by 2-meSATP (●), ATP (■), β,γ-meATP (▲) and α,β-meATP (◆) in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 7$) with s.e. mean indicated by vertical bars.

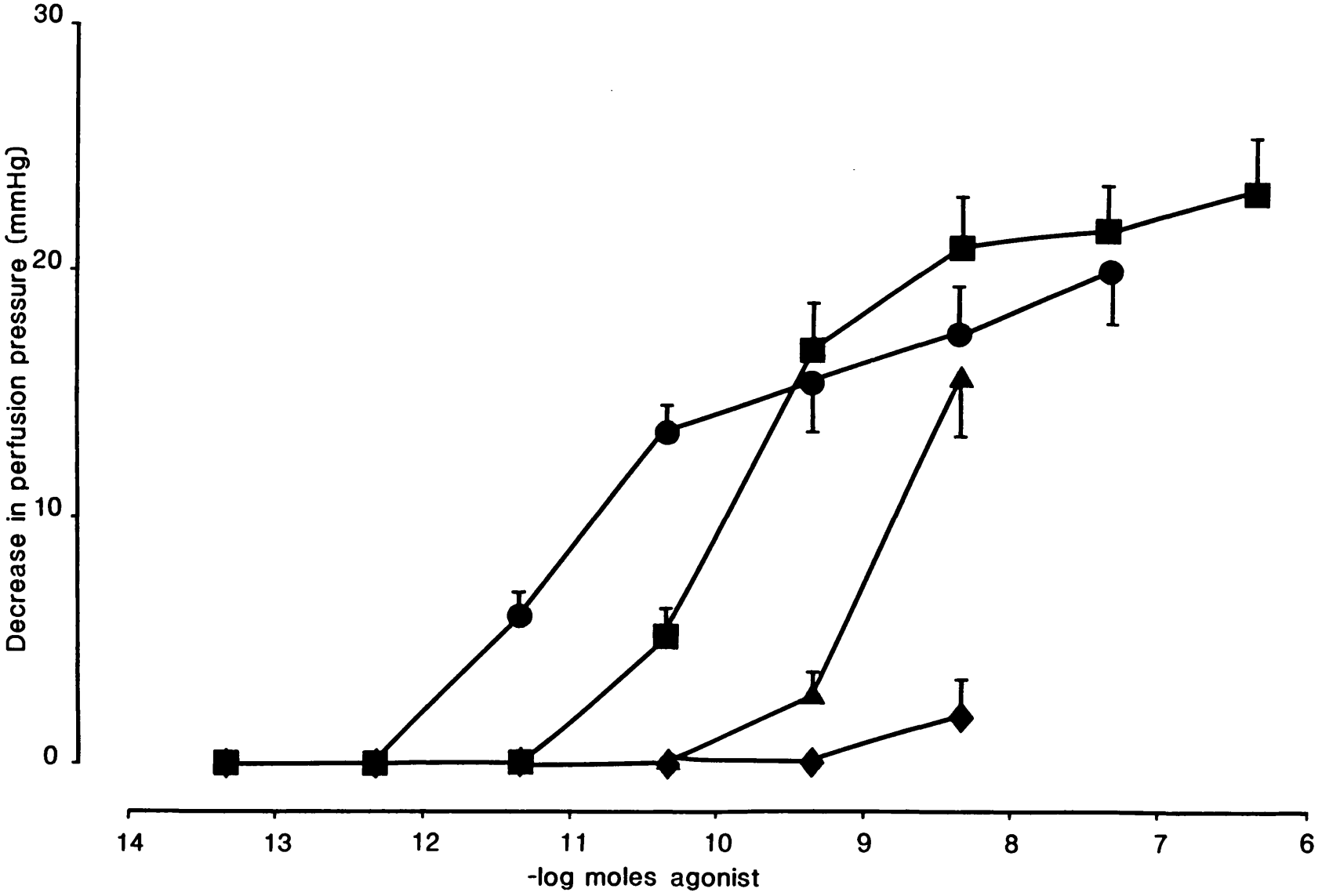

30
20
10
0
Decrease in perfusion pressure (mmHg)
14
13
12
11
10
9
8
7
6
-log moles agonist

FIGURE 5.2a The amplitude of the vasodilatation obtained in response to 2-meSATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

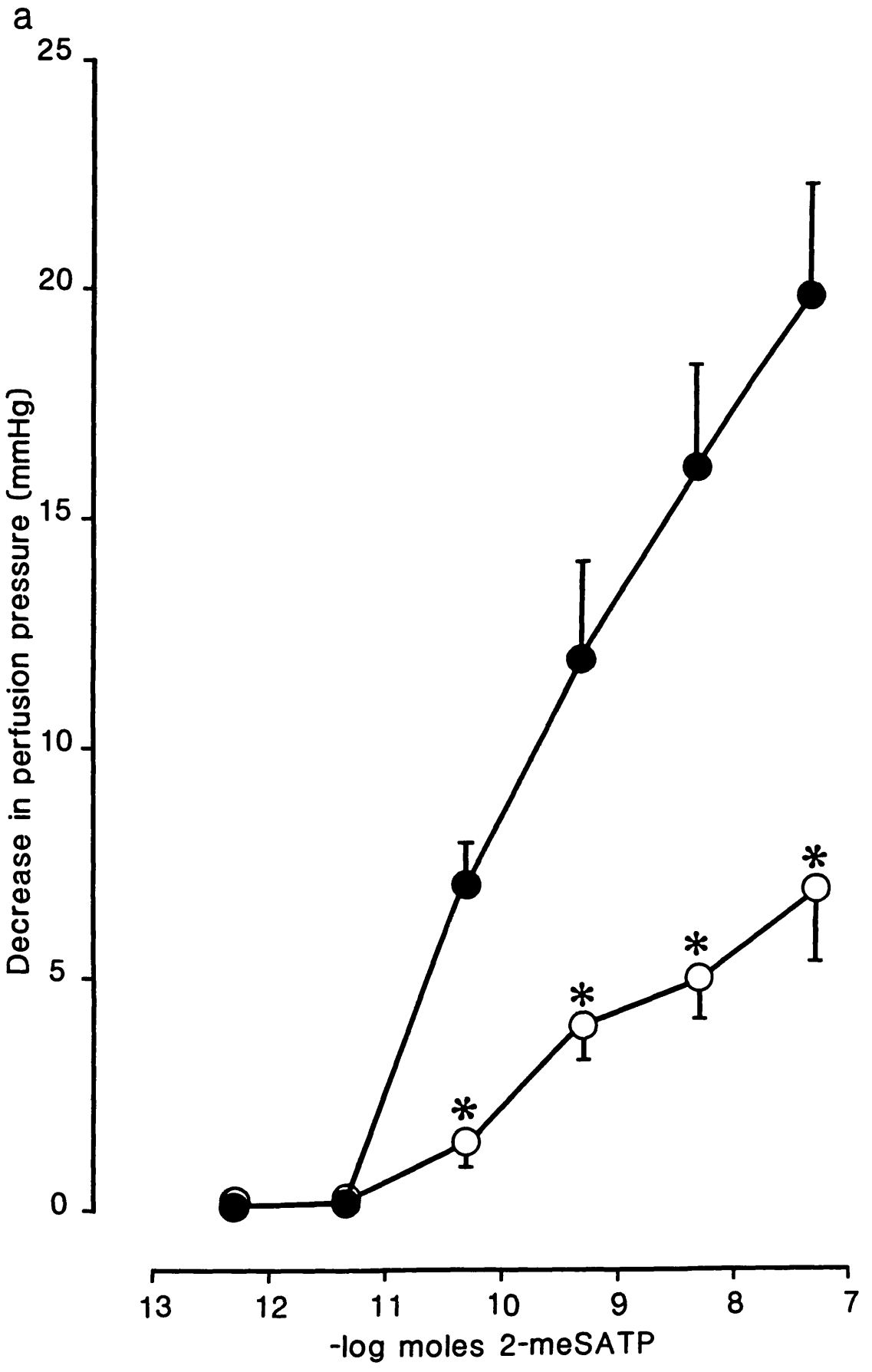

a
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
13
12
11
10
9
8
7
-log moles 2-meSATP
*
*
*
*

FIGURE 5.2b The area of the vasodilatation obtained in response to 2-meSATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e mean indicated by vertical bars. The significant differences are * $P<0.05$.

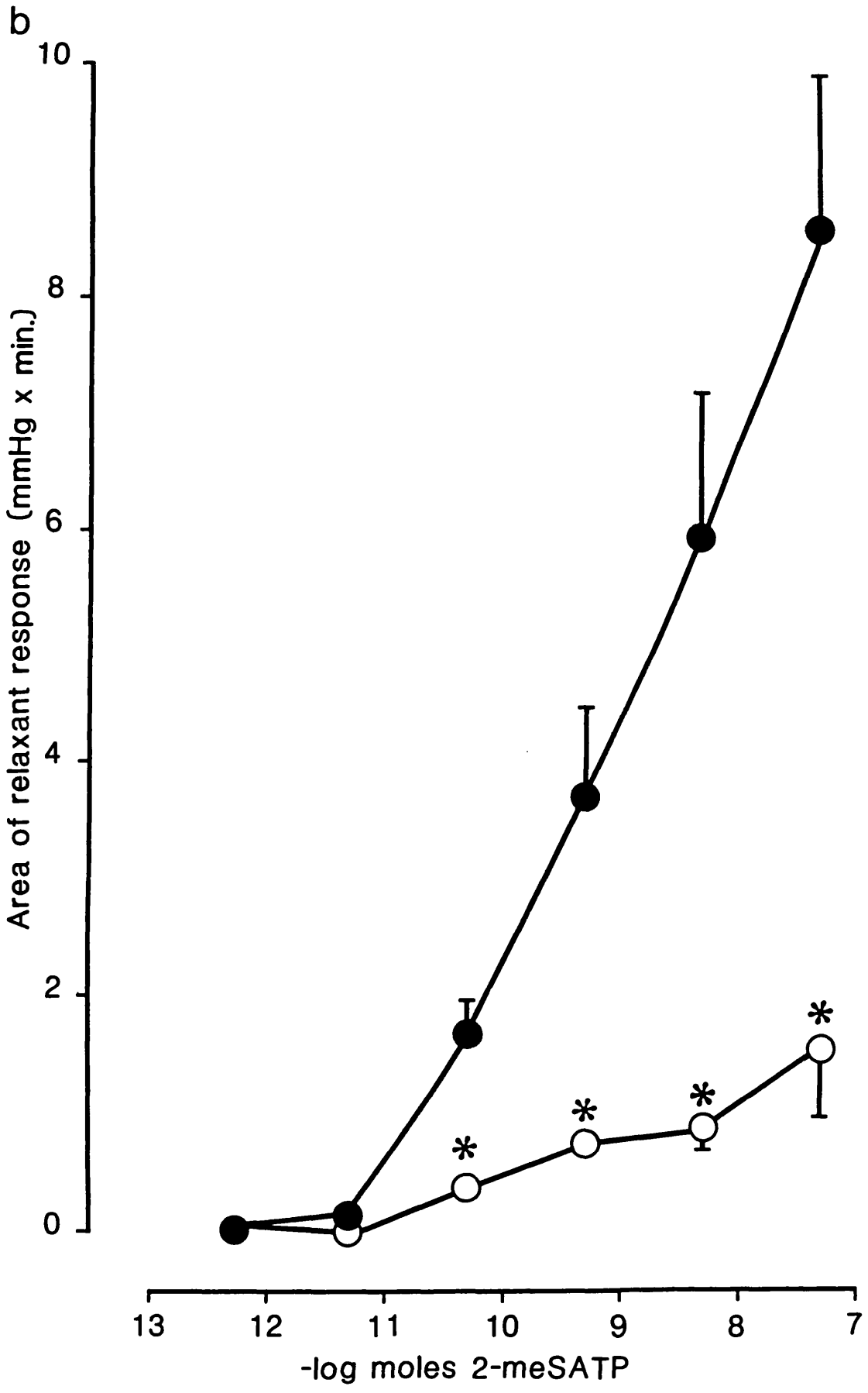

b
10
8
6
4
2
0
Area of relaxant response (mmHg x min.)
*
*
*
*
13
12
11
10
9
8
7
-log moles 2-meSATP

FIGURE 5.2c The amplitude of the vasodilatation obtained in response to ATP, in the isolated perfused guinea-pig heart, in the absence (■) and presence (□) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

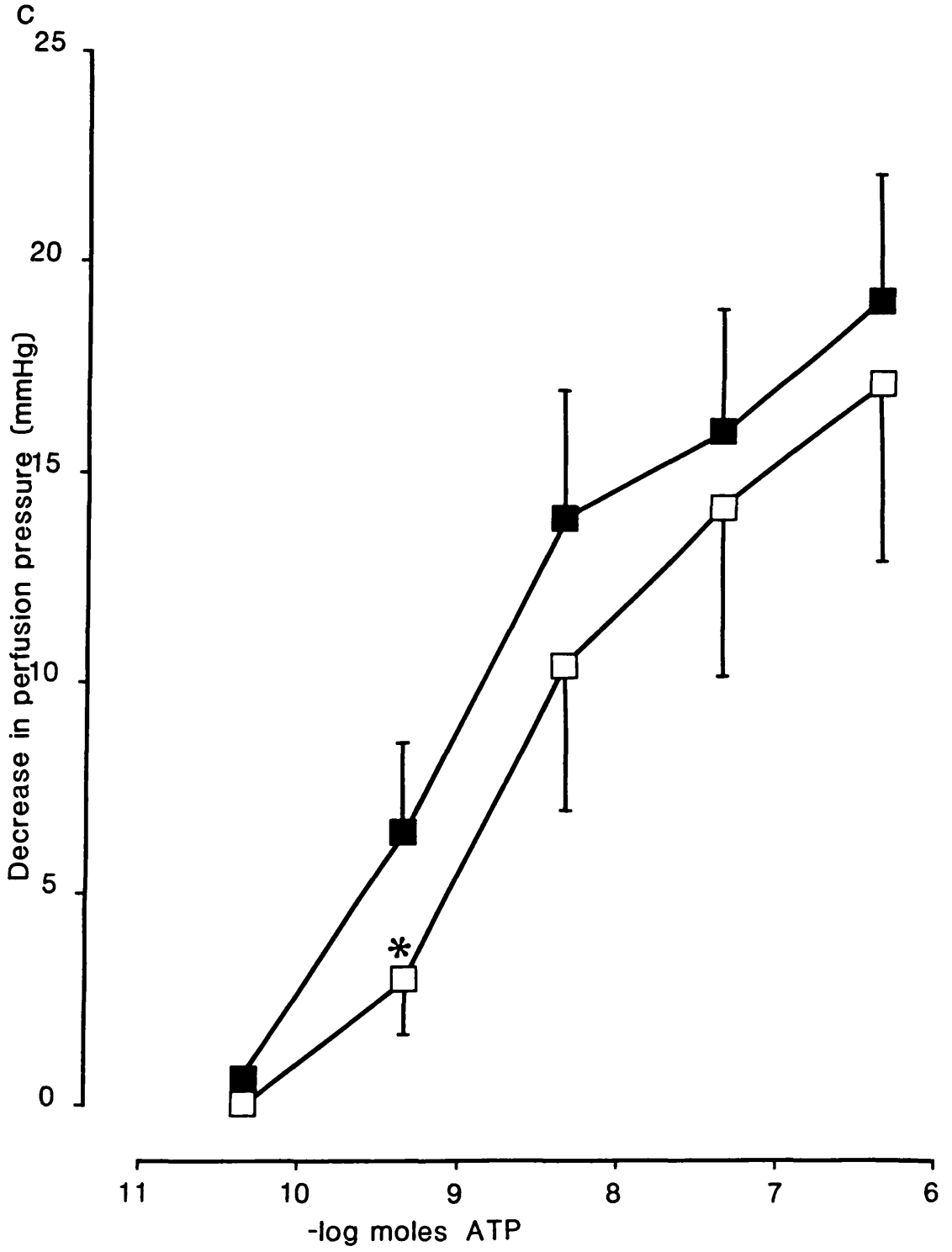

C
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
*
11
10
9
8
7
6
-log moles ATP

FIGURE 5.2d The area of the vasodilatation obtained in response to ATP, in the isolated perfused guinea-pig heart, in the absence (■) and presence (□) of L-NAME (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

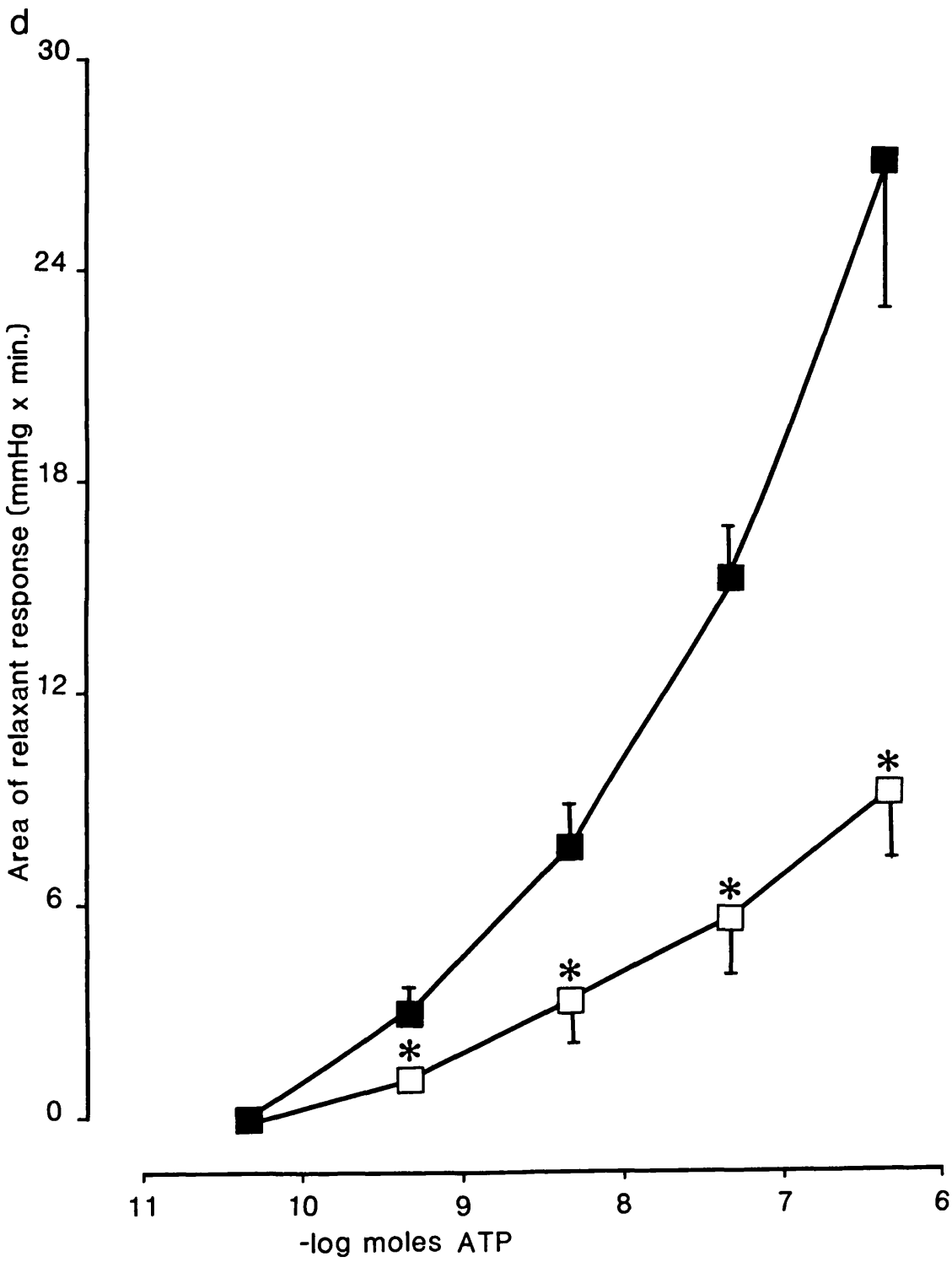

d
30
24
18
12
6
0
Area of relaxant response (mmHg x min.)
11
10
9
8
7
6
-log moles ATP
*
*
*
*

FIGURE 5.3a The amplitude of the vasodilator responses evoked by 2-meSATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

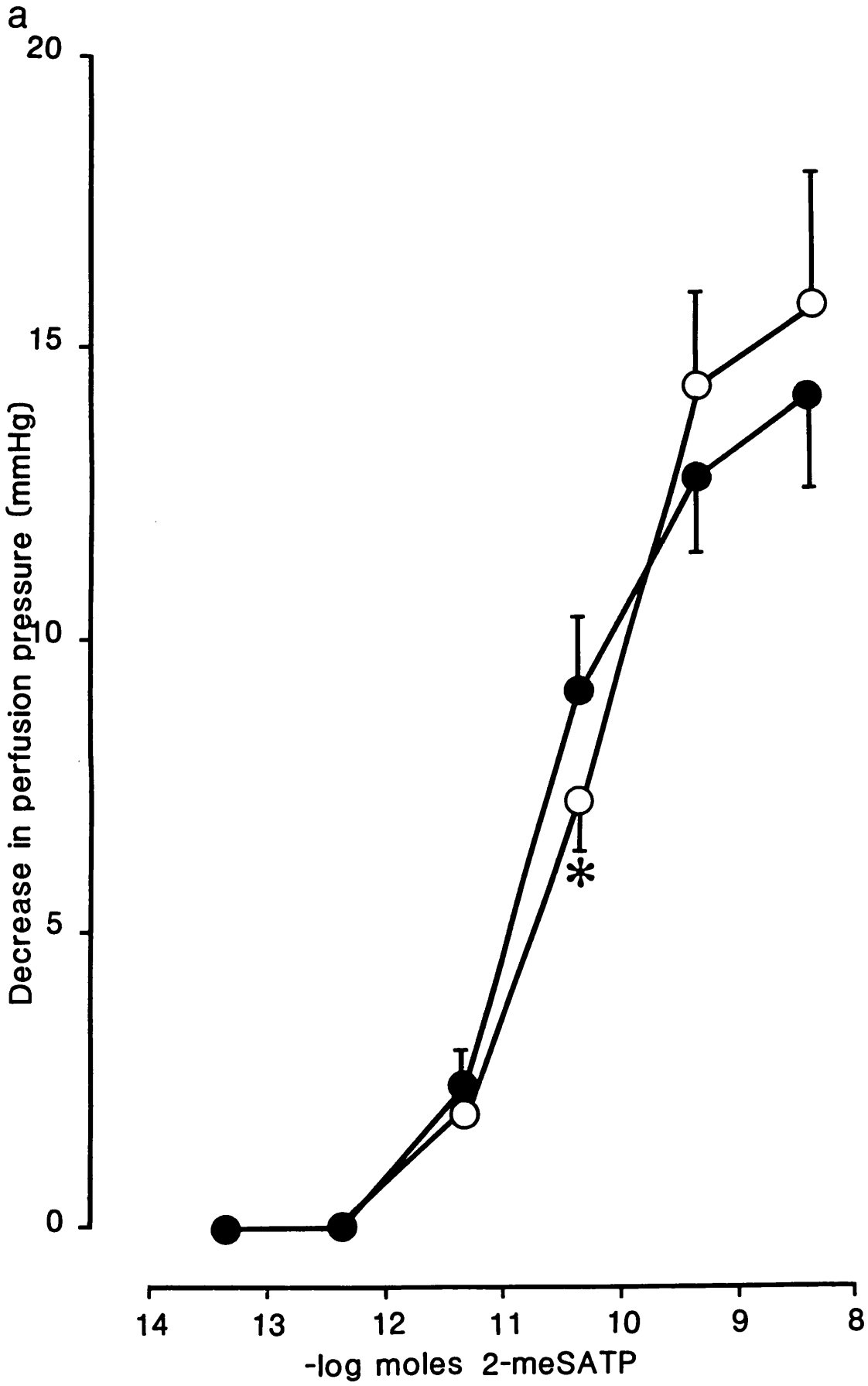

a
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
*
14
13
12
11
10
9
8
-log moles 2-meSATP

FIGURE 5.3b The amplitude of the vasodilator responses evoked by ATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

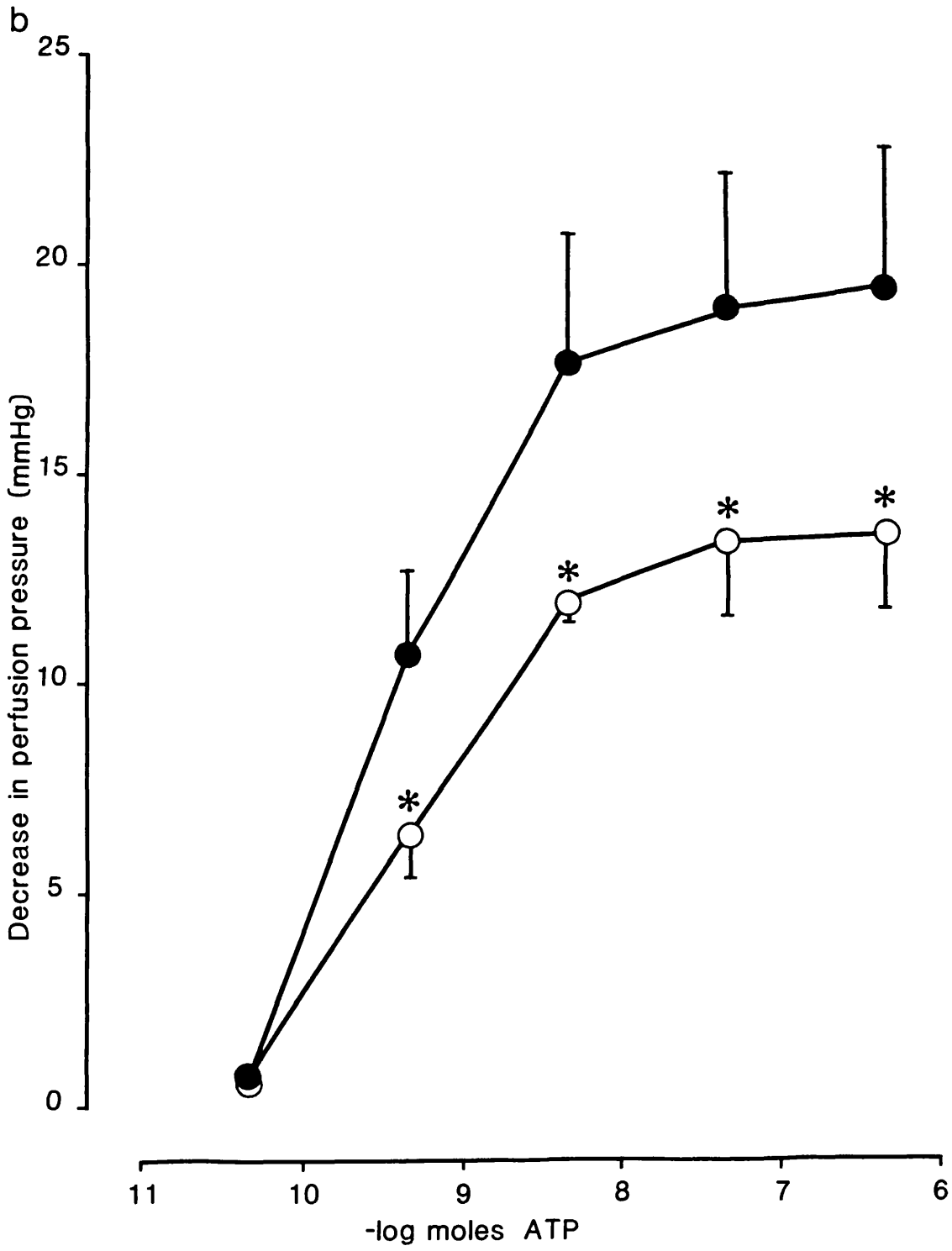

b
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
*
*
*
*
11
10
9
8
7
6
-log moles ATP

FIGURE 5.4a The amplitude of the vasodilator responses evoked by 2-meSATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of 8-PSPT (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

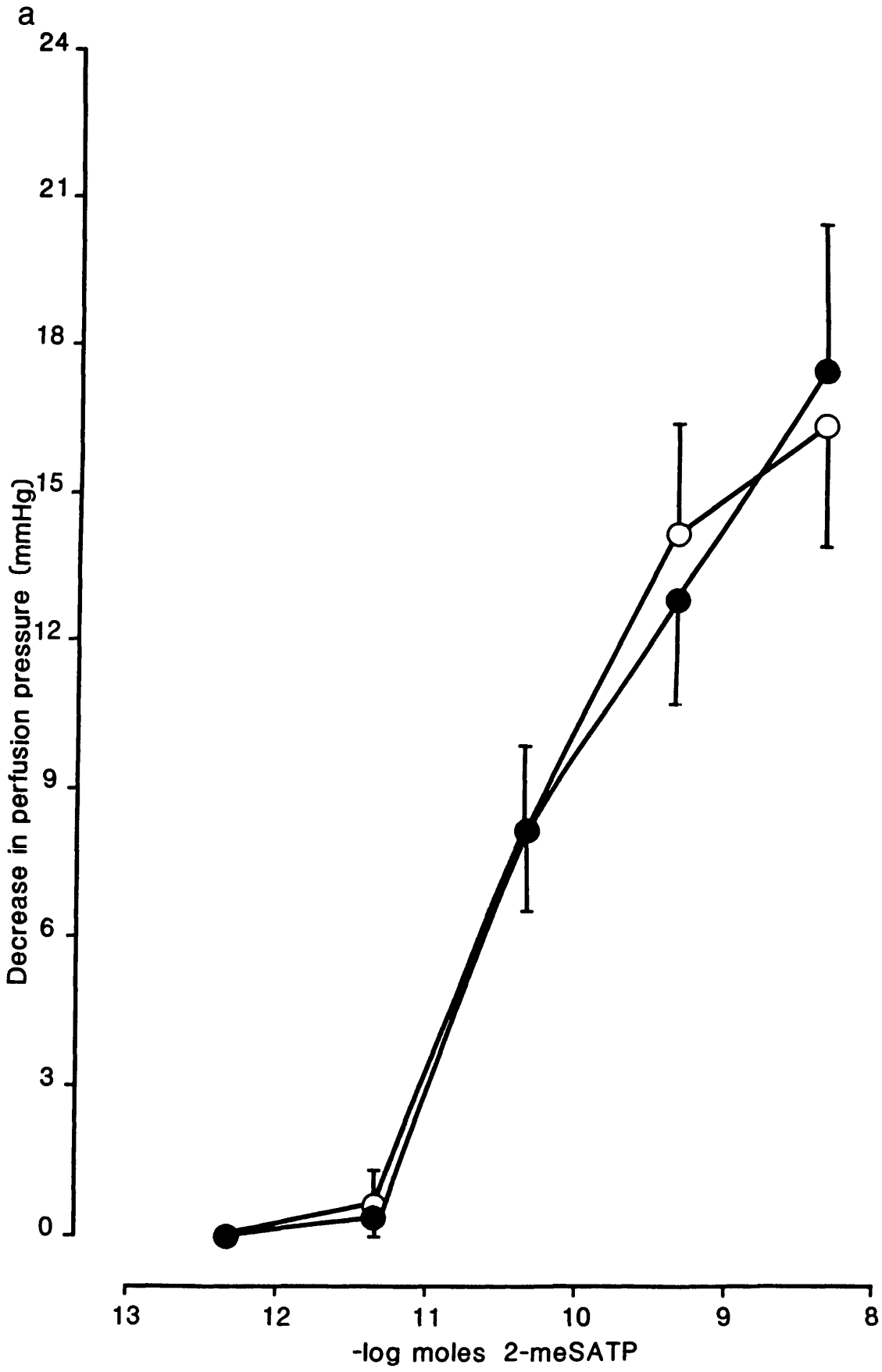

a
24
21
18
15
12
9
6
3
0
Decrease in perfusion pressure (mmHg)
13
12
11
10
9
8
-log moles 2-meSATP

FIGURE 5.4b The amplitude of the vasodilator responses evoked by ATP, in the isolated perfused guinea-pig heart, in the absence (■) and presence (□) of 8-PSPT (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

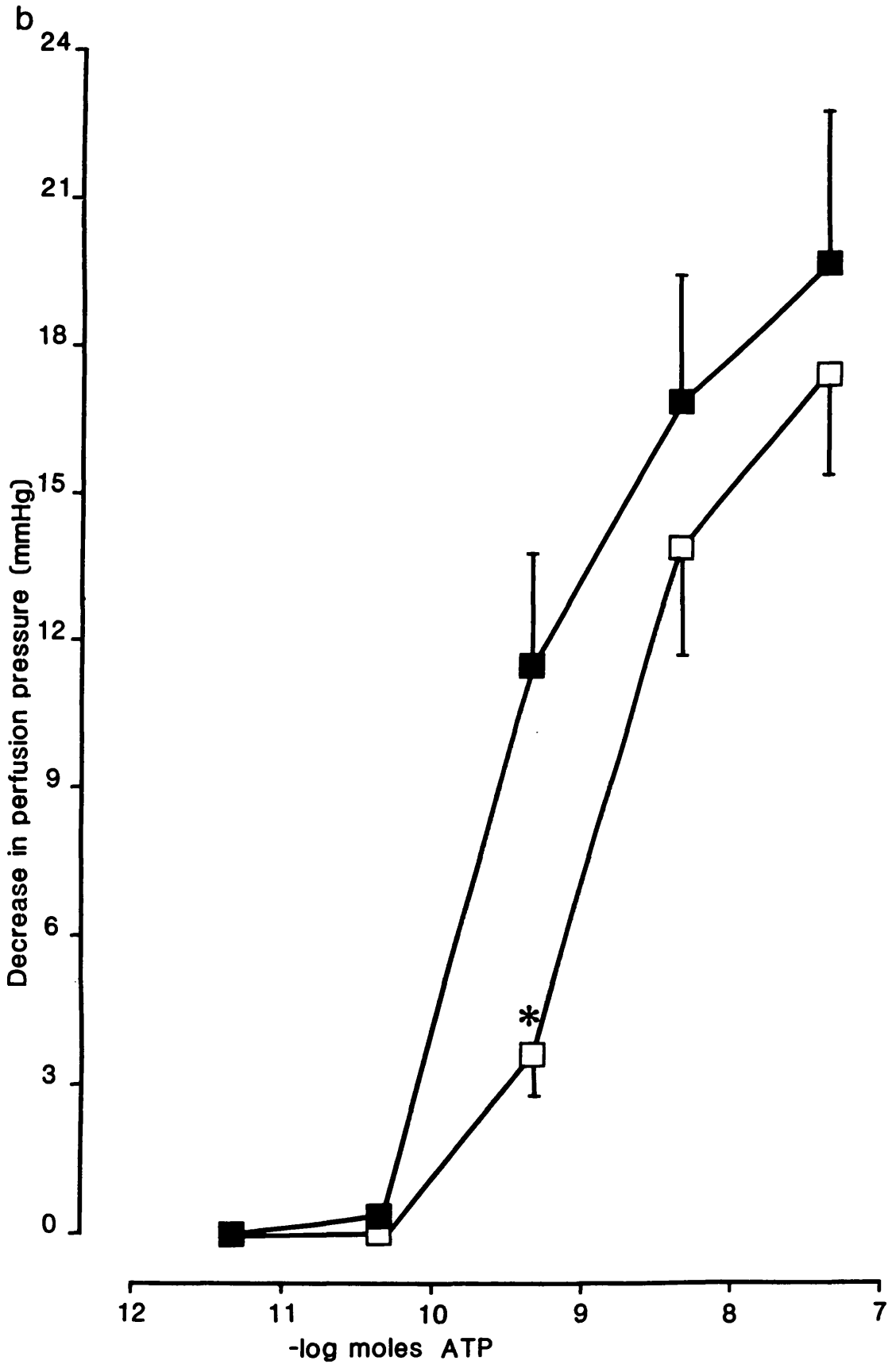
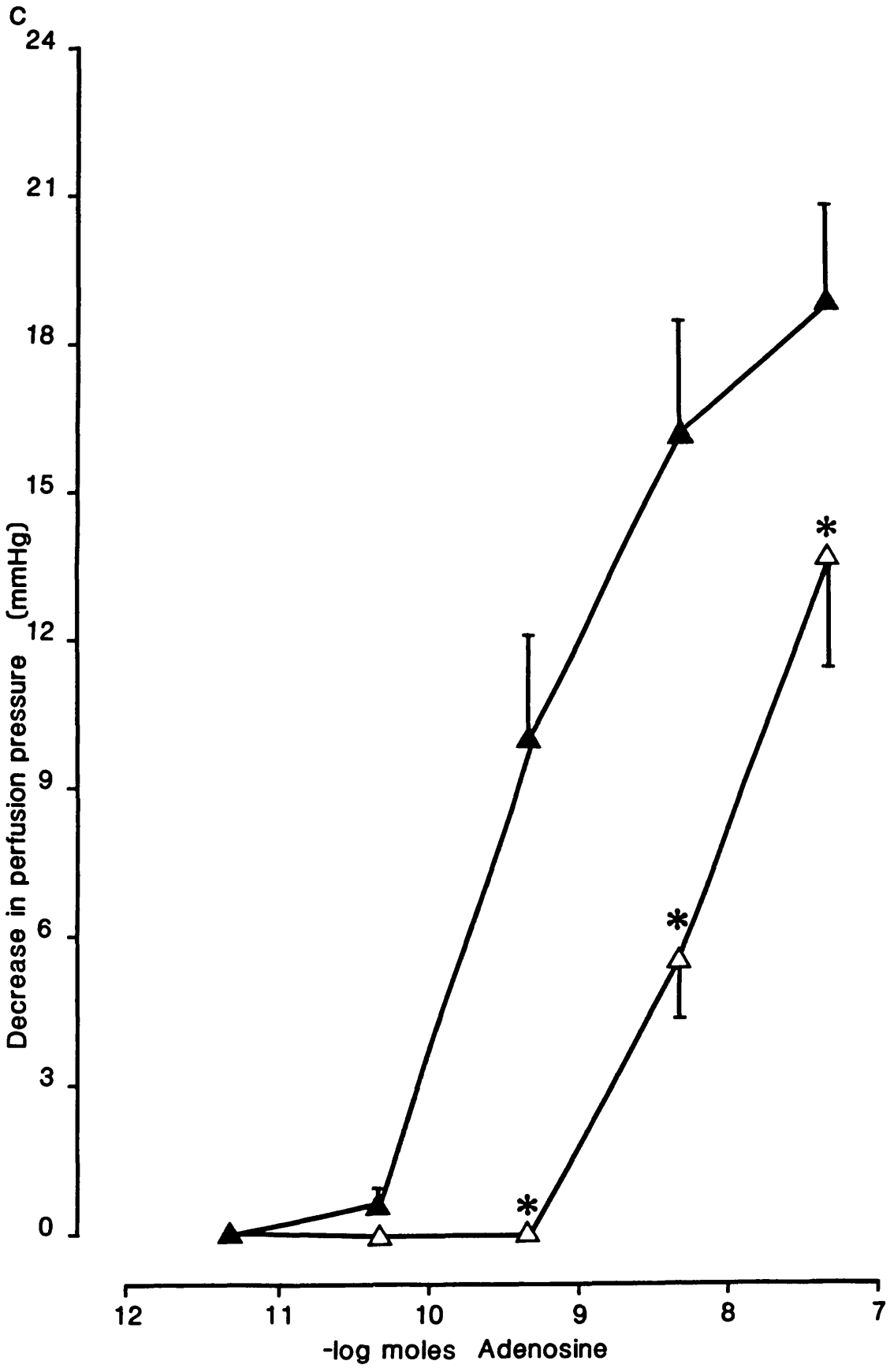

b
24
21
18
15
12
9
6
3
0
Decrease in perfusion pressure (mmHg)
*
12
11
10
9
8
7
-log moles ATP

FIGURE 5.4c The amplitude of the vasodilator responses evoked by adenosine, in the isolated perfused guinea-pig heart, in the absence (▲) and presence (△) of 8-PSPT (3×10^{-5} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.


C
24
21
18
15
12
9
6
3
0
Decrease in perfusion pressure (mmHg)
12
11
10
9
8
7
-log moles Adenosine
*
*
*

Chapter 6

The effect of suramin on vasodilator responses to ATP and 2-meSATP in the Sprague-Dawley rat coronary vasculature.

6.1 SUMMARY

1) The effects of suramin on the vasodilator response to ATP, 2-meSATP and adenosine were examined in the Sprague-Dawley rat coronary vasculature using the Langendorff heart preparation.

2) Relaxations induced by 2-meSATP (5×10^{-12} - 5×10^{-9} mol) were significantly inhibited by suramin. Only responses to low doses of adenosine (5×10^{-11} mol) and ATP (5×10^{-11} - 5×10^{-10} mol) were inhibited by suramin (3×10^{-5} M).

3) 8-PSPT (3×10^{-5} M) did not affect the relaxant response to ATP and 2-meSATP at a concentration that significantly inhibited the response to adenosine (5×10^{-10} - 5×10^{-8} mol).

4) It is concluded that 2-meSATP acts via P_{2Y}-purinoceptors while ATP appears to be acting largely through a different mechanism. It is not acting via a P_1-purinoceptor because ATP was not inhibited by the P_1-purinoceptor antagonist 8-PSPT.

6.2 INTRODUCTION

The potent cardiovascular actions of extracellular adenine nucleotides and adenosine have been known for many years. It has been demonstrated that the rat coronary vasculature exhibits purinoceptors of at least two types: a vasodilator P_2-

purinoceptor (P_{2Y}) and a P_2-purinoceptor mediating vasoconstriction (P_{2X}; Hopwood & Burnstock, 1987). Recently, O'Connor *et al.* (1991) proposed that ATP-induced relaxation could also be caused by another separate 'nucleotide' receptor, different from the classical P_{2Y}-purinoceptor. In the isolated rabbit aorta relaxation by ATP has been shown to be induced both via the P_{2Y}-purinoceptor and via a 'nucleotide' receptor (Chinellato *et al.*, 1992).

It was reported that suramin, introduced in the treatment of trypanosomas, is a reversible P_2-purinoceptor antagonist in the mouse vas deferens (Dunn & Blakely, 1988), and it was subsequently shown to block P_{2X}- and P_{2Y}-purinoceptors in the guinea-pig urinary bladder and taenia coli respectively (Hoyle *et al.*, 1990).

In this study, the effects of suramin on the relaxation induced by ATP and its analogue 2-meSATP in the perfused rat coronary vasculature are reported.

6.3 METHODS (See Chapter 2.1.2)

Male Sprague-Dawley rats (200-500 g) were used. The heart was removed and cannulated via the aorta for constant flow perfusion (the mean flow was 12.3 ± 0.29 ml min^{-1}; n=32) according to the method of Langendorff. A 200 µl bolus of 10^{-4} M ergotamine was given over 5 s. This raised the perfusion pressure by at least 10 mm Hg (mean increase 34.44 ± 2.40 mm Hg). If no arterial constriction occurred the heart was rejected. When the perfusion

pressure had reached a steady level, doses of nucleosides and nucleotides were given in 50 µl boluses, delivered over 3 s. The order of doses of 2-meSATP, adenosine and ATP was random.

When the effect of antagonists was examined, control dose-response relationships to the purines were obtained and then suramin or 8-PSPT was added to the perfusing solution and allowed to equilibrate in the organ for 30 min. The dose-response relationships were then re-established in the presence of the antagonist. Suramin ($3x10^{-5}$ M) lowered the perfusion pressure of the preparation by 38.63 ± 2.64 mmHg (n=32). To increase the perfusion pressure a 200 µl bolus of 10^{-4} M ergotamine was given over 5 s. This raised the pressure such that the perfusion pressure was not significantly different from the perfusion pressure of the preparation before suramin was added to the perfusate. At the end of the experiment the heart was removed from the cannula, blotted and weighed. The mean weight of the heart was 1.27 ± 0.01 g (n=32).

6.3.1 Materials See Chapter 2.4

6.3.2 Statistics See Chapter 2.3

6.4 RESULTS

The effect of suramin (3×10^{-5} M; Figure 6.1a-c) and 8-PSPT (3×10^{-5} M; Figure 6.1d-f) on the vasodilator response to 2-meSATP, ATP and adenosine in the rat coronary vasculature is demonstrated. Suramin (3×10^{-5} M) significantly attenuated the vasodilator response to 2-meSATP (5×10^{-12} - 5×10^{-9} mol; Figure 6.1a). Only the vasodilator response to low doses of ATP (5×10^{-11} - 5×10^{-10} mol; Figure 6.1b) and adenosine (5×10^{-11} mol; Figure 6.1c) was inhibited by suramin (3×10^{-5} M). Suramin (3×10^{-5} M) did not significantly affect the left ventricular pressure of the preparations. The perfusion pressure was significantly reduced by suramin (3×10^{-5} M) from 79.65 ± 0.53 to 41.02 ± 2.34 mm Hg.

The vasodilator response to adenosine (5×10^{-10} - 5×10^{-8} mol; Figure 6.1f) was almost abolished by 8-PSPT (3×10^{-5} M). The inhibition of the responses to adenosine by 8-PSPT (3×10^{-5} M) was overcome at high doses of adenosine (5×10^{-7} moles; Figure 6.1f). The vasodilator response to 2-meSATP (5×10^{-13} - 5×10^{-9} mol; Figure 6.1d) and ATP (5×10^{-12} - 5×10^{-8} mol; Figure 6.1e) was unaffected by 8-PSPT (3×10^{-5} M). 8-PSPT (3×10^{-5} M) did not significantly effect either the resting perfusion pressure or left ventricular pressure of the preparations.

6.5 DISCUSSION

The results of this study show that in the Sprague-Dawley rat coronary vasculature, suramin inhibited the vasodilator response to 2-meSATP but not those to ATP (except at low doses). The vasodilator responses to ATP and 2-meSATP were not inhibited by 8-PSPT at a concentration that significantly inhibited the response to adenosine.

Effects of ATP are mediated by P_2-purinoceptors, subdivided into P_{2X}- and P_{2Y}-subtypes on the basis of agonist potency order and functional response (Burnstock & Kennedy, 1985). The order α,β-meATP = β,γ-meATP > ATP = 2-meSATP is characteristic of P_{2X}-purinoceptors, while 2-meSATP > ATP > α,β-meATP = β,γ-meATP is taken to indicate the presence of P_{2Y}-purinoceptors. Recently, O'Connor *et al.* (1991) proposed that ATP-induced relaxation could also be caused by another separate 'nucleotide' receptor, different from the classical P_{2Y}-purinoceptor. It has been suggested that the agonist potency order for this receptor is UTP = ATP > ADP > α,β-meATP, 2-meSATP (O'Connor *et al.*, 1991). These 'nucleotide' receptors were found to be located on the endothelium and vascular smooth muscle of isolated rabbit aorta (Chinellato *et al.*, 1992). Suramin has been shown to have very little effect on these 'nucleotide' receptors located on bovine aortic endothelial cells (Wilkinson *et al.*, 1993). One possible explanation why the vasodilator response to ATP (except at low doses) was not inhibited by suramin is that ATP was acting at these newly defined 'nucleotide' receptors which are unaffected by suramin. The fact that suramin inhibited the

relaxation evoked by 2-meSATP in this study suggests that it is an effective inhibitor of P_{2Y}-purinoceptors in the rat coronary vasculature. ATP could also be acting at P_{2Y}-purinoceptors, but because ATP has an additional mechanism of action the concentration of suramin used may not be sufficient to show inhibition. The concentration of suramin used could not be increased as higher concentration lowered the perfusion pressure such that it could not be raised again.

Alternatively, ATP could be inducing relaxation via a prostanoid. Preliminary studies on the guinea-pig coronary vasculature have suggested that ATP-induced relaxation is not only due to action at P_{2Y}-purinoceptors but also via prostanoid synthesis or release. Fleetwood and Gordon (1987) have demonstrated that high concentrations of ATP can induce prostacyclin release from the Sprague-Dawley rat coronary vasculature. Therefore it is conceivable that in addition to its action at P_{2Y}-purinoceptors ATP might also induce relaxation via prostanoids. Indeed, ATP activation of prostanoid synthesis may explain many of the differences in the responses observed between ATP and the selective P_2-purinoceptor agonists. For example, the prostaglandin-mediated rebound contraction of the taenia coli can be induced by ATP but not the analogues in which the phosphate chain was altered (Brown & Burnstock, 1981).

It is also possible that ATP is metabolised to adenosine and as such causes relaxation via action at P_1-purinoceptors (Burnstock & Kennedy, 1986). However, the adenosine receptor antagonist 8-PSPT did not affect the response to ATP and 2-meSATP

at a concentration that significantly inhibited the response to adenosine.

In conclusion, we have shown that suramin is effective at inhibiting the vasodilator response to 2-meSATP and thus presumably responses mediated via P_{2Y}-purinoceptors in the Sprague-Dawley rat coronary vasculature. However, the vasodilator response to ATP was not inhibited by suramin suggesting that ATP was acting via a different mechanism.

FIGURE 6.1a Vasodilator responses evoked by 2-meSATP, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of suramin (3×10^{-5} M). The points show the mean ($n \geq 5$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

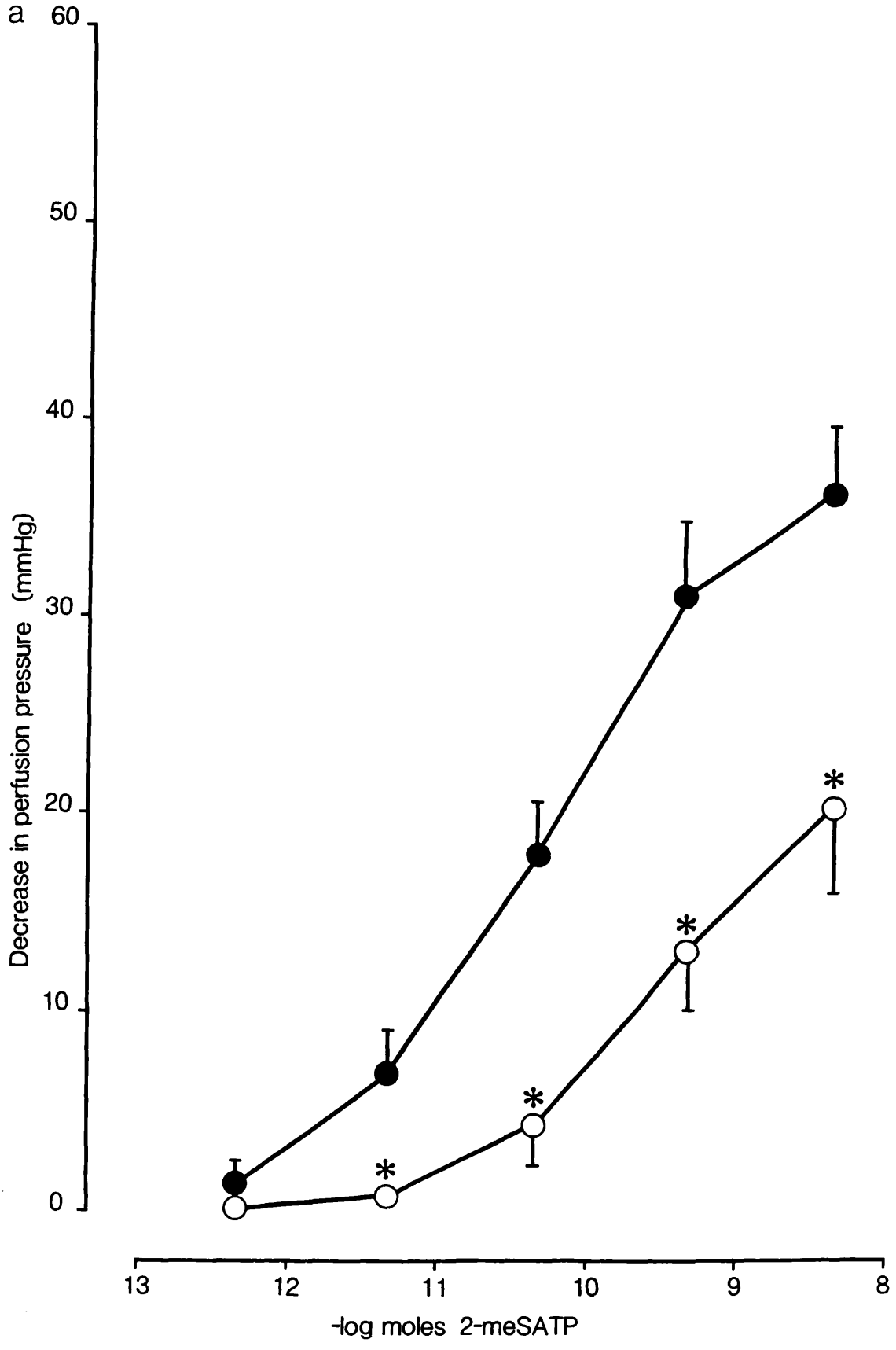

a
60
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
*
*
*
*
13
12
11
10
9
8
-log moles 2-meSATP

FIGURE 6.1b Vasodilator responses evoked by ATP, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of suramin (3×10^{-5} M). Points show the mean ($n \geq 5$) with s.e. mean indicated by the vertical bars. The significant differences are * $P<0.05$.

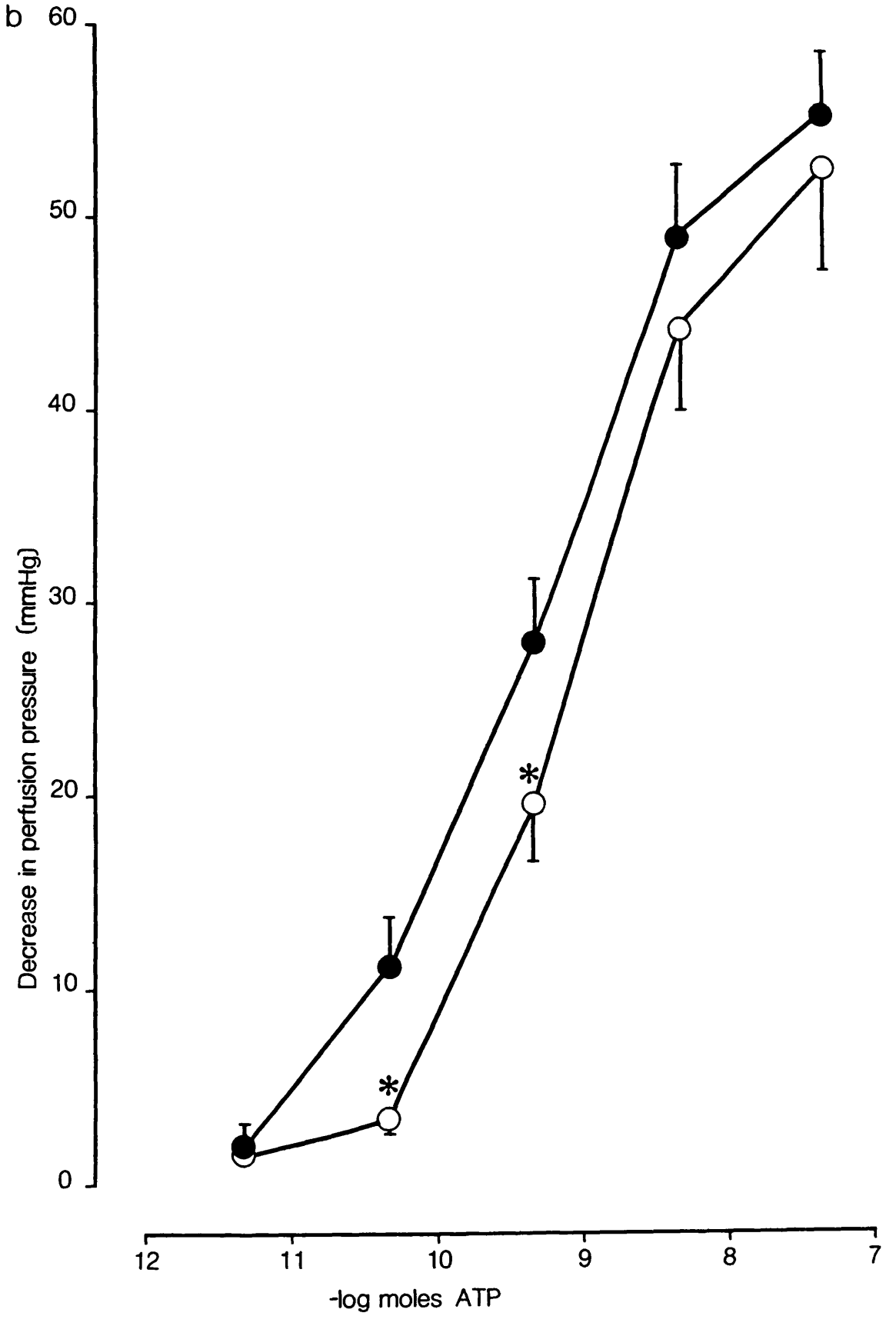

b
60
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
12
11
10
9
8
7
-log moles ATP
*
*

FIGURE 6.1c Vasodilator responses evoked by adenosine, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of suramin (3×10^{-5} M). The points show the mean ($n \geq 5$) with s.e. mean indicated by the vertical bars. The significant differences are * $P<0.05$.

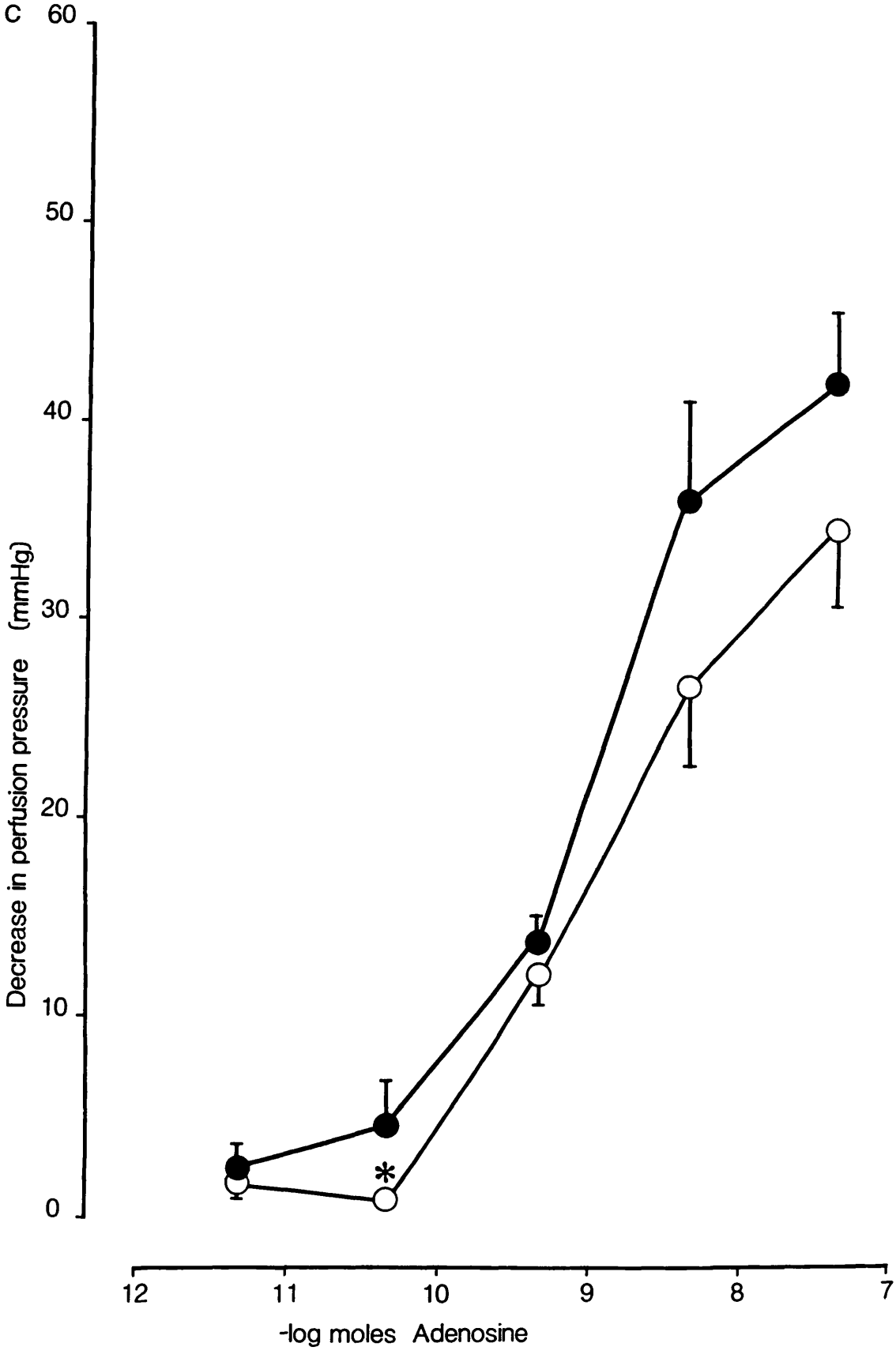

C
60
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
*
12
11
10
9
8
7
-log moles Adenosine

FIGURE 6.1d Vasodilator responses evoked by 2-meSATP, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of 8-PSPT (3×10^{-5} M). The points represent the mean ($n \geq 5$) with s.e. mean indicated by the vertical bars. The significant differences are * $P<0.05$.

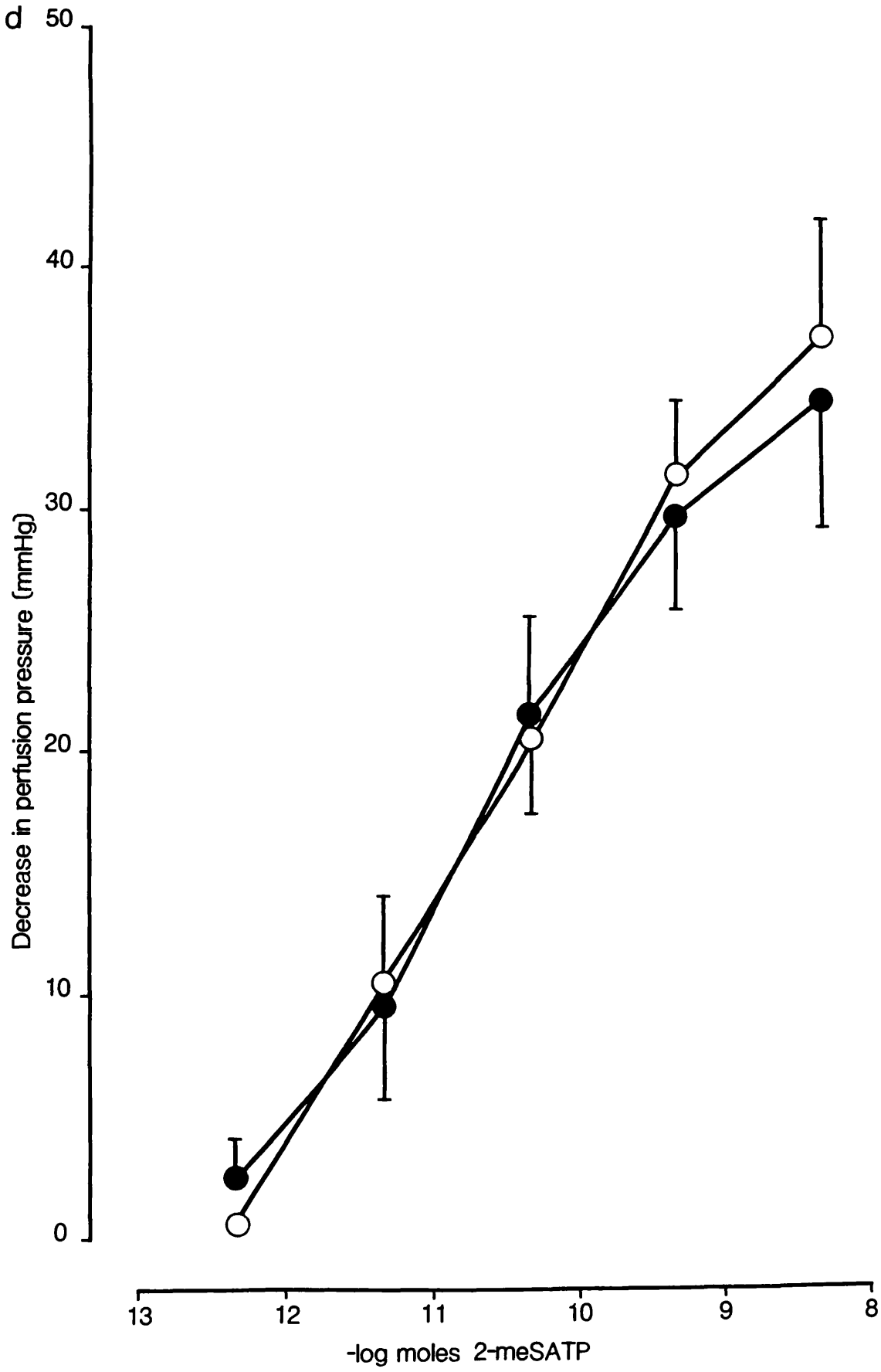

d
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
13
12
11
10
9
8
-log moles 2-meSATP

FIGURE 6.1e Vasodilator responses evoked by ATP, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of 8-PSPT (3×10^{-5} M). The points represent the mean ($n \geq 5$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

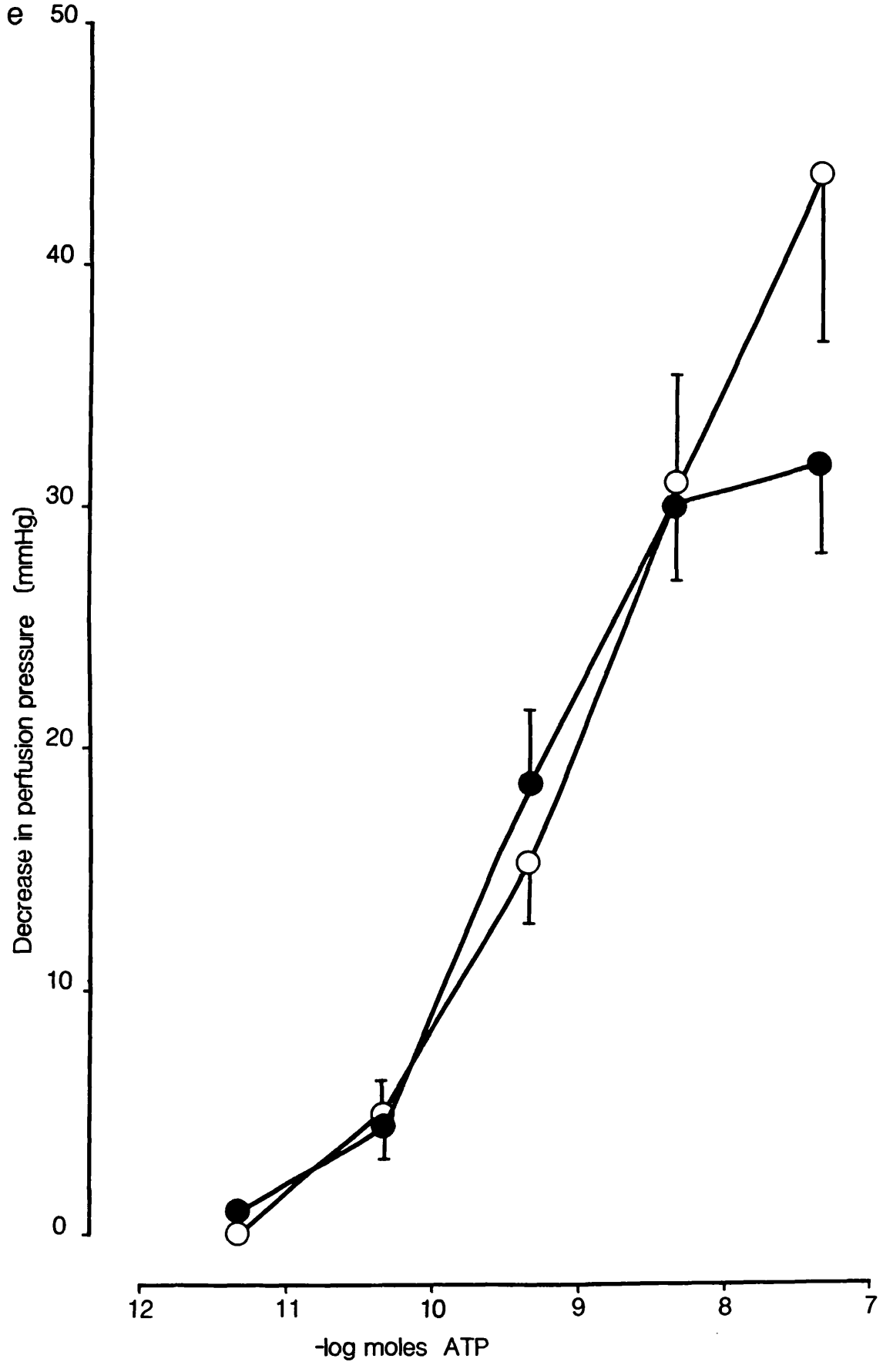
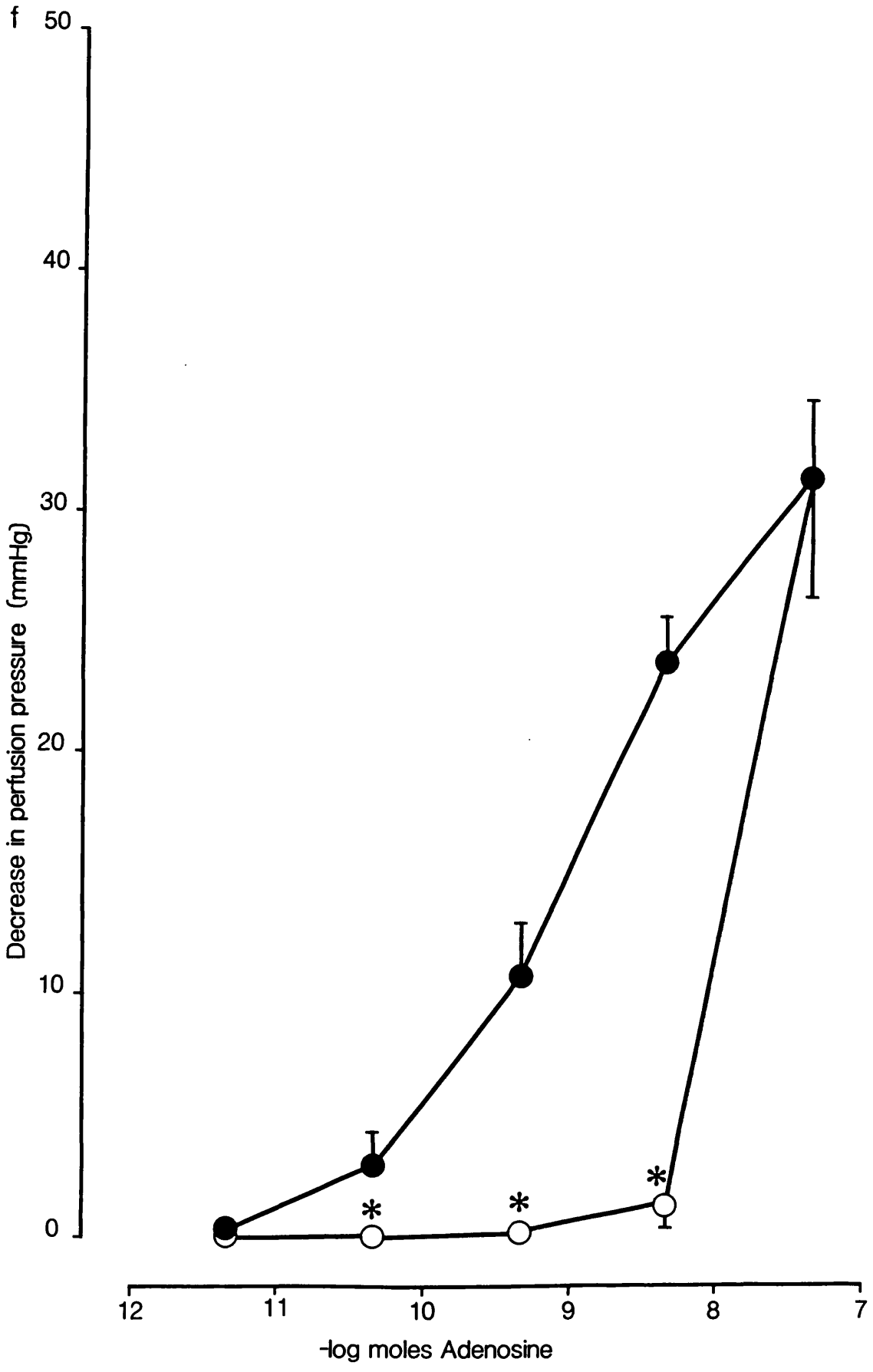

e
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
12
11
10
9
8
7
-log moles ATP

FIGURE 6.1f Vasodilator responses evoked by adenosine, in the isolated perfused Sprague-Dawley rat heart, in the absence (●) and presence (○) of 8-PSPT (3×10^{-5} M). The points represent the mean ($n \geq 5$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.


f
50
40
30
20
10
0
Decrease in perfusion pressure (mmHg)
*
*
*
12
11
10
9
8
7
-log moles Adenosine

Chapter 7

Effects of pyrimidines on the guinea-pig coronary vasculature.

7.1 SUMMARY

1) The effects of the pyrimidines, UTP, thymidine 5'-triphosphate (TTP) and cytidine 5'-triphosphate (CTP), were examined in the guinea-pig coronary bed, by use of a Langendorff technique. Comparisons were made with the actions of the purines ATP, inosine 5'-triphosphate (ITP) and guanosine 5'-triphosphate (GTP). The effect of the NO synthase inhibitor L-NAME and the prostaglandin synthase inhibitor indomethacin on the vasodilator response to these purines and pyrimidines was examined. The effects of these inhibitors were assessed on their ability to inhibit both the amplitude and the area of the vasodilator response.

2) The relative order of potency of the purines and pyrimidines studied was ATP > UTP > ITP >> GTP, TTP, CTP.

3) The maximum amplitude and area of the vasodilator response to the pyrimidines, UTP ($5x10^{-10}$ - $5x10^{-7}$ mol), TTP ($5x10^{-8}$ - $5x10^{-7}$ mol) and CTP ($5x10^{-7}$ mol), and purines, ITP ($5x10^{-9}$ - $5x10^{-7}$ mol) and GTP ($5x10^{-8}$ - $5x10^{-7}$ mol), were significantly reduced by L-NAME ($3x10^{-5}$ and 10^{-4} M).

4) The inhibition of the response to ATP ($5x10^{-8}$ mol), UTP ($5x10^{-8}$ mol), ITP ($5x10^{-8}$ mol), TTP ($5x10^{-7}$ mol), CTP ($5x10^{-7}$ mol) and GTP ($5x10^{-7}$ mol) by L-NAME ($3x10^{-5}$ M) was significantly reversed by L-arginine ($1.5x10^{-3}$ M).

5) L-NAME ($3x10^{-5}$ and 10^{-4} M) only inhibited the amplitude of the

vasodilator response to a low dose of ATP ($5x10^{-10}$ mol), although the area of the vasodilator response to ATP ($5x10^{-11}$ - $5x10^{-7}$ mol) was significantly reduced by L-NAME ($3x10^{-5}$ and 10^{-4} M).

6) The maximum amplitude of the vasodilator response to ATP ($5x10^{-10}$ - $5x10^{-7}$ mol) was significantly reduced by indomethacin (10^{-6} M), although the area of the vasodilator response to ATP was only significantly reduced at one intermediate dose ($5x10^{-9}$ mol). Indomethacin (10^{-6}M) did not affect the maximum amplitude or area of the vasodilator responses to UTP ($5x10^{-11}$ - $5x10^{-7}$ mol), ITP ($5x10^{-10}$ - $5x10^{-7}$ mol), CTP ($5x10^{-7}$ mol), TTP ($5x10^{-8}$ - $5x10^{-7}$ mol) and GTP ($5x10^{-8}$ - $5x10^{-7}$ mol).

7) It is concluded that in the guinea-pig coronary vasculature, the vasodilatation evoked by the pyrimidines, UTP, TTP and CTP, was mediated in large part via NO, as were the vasodilatations evoked by the purines ITP and GTP. The vasodilatations evoked by ATP, however, appear to involve prostanoids in addition to the release of NO.

7.2 INTRODUCTION

ATP produces powerful systemic effects; it influences many biological processes being released from nerve endings, platelets and endothelial cells in physiological and pathophysiological processes (Burnstock & Kennedy, 1986). In the

cardiovascular system its ability to cause vasoconstriction or vasodilatation is mediated through activation of subtypes of P_2-purinoceptor (Burnstock & Kennedy, 1985; Hoyle, 1992). In the rat coronary vasculature ATP causes vasoconstriction via P_{2X}-purinoceptors and vasodilatation via P_{2Y}-purinoceptors (Hopwood & Burnstock, 1987). The naturally occurring nucleotides, CTP, TTP and UTP which are pyrimidines, and GTP, which is a purine, have also been shown to have effects on the vasculature. Of these, probably the most studied has been UTP which may be released from blood platelets (Goetz *et al.*, 1971; Urquilla, 1978). In many tissues, including the piglet aorta (Martin *et al.*, 1985), 2-meSATP is a potent agonist producing a similar maximum relaxant response to ATP. The relative order of agonist potencies of these two compounds is consistent with that conventionally described for P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985): 2-meSATP >> ATP = ADP >> α,β-meATP, UTP. However there is also a variety of tissues in which ATP causes phospholipase C activation but to which the above agonist potency order does not apply; because of the common second messenger system in these tissues they have been loosely linked with the P_{2Y}-subtype (Kennedy, 1990; Boeynaems & Pearson; 1990). In these tissues 2-meSATP has little or no activity, for example, although ATP induces prostaglandin I_2 (PGI_2) production in bovine aortic smooth muscle cells, 2-meSATP does not (Demolle *et al.*, 1988). This indicates that there is a subpopulation of phospholipase C-linked P_2-purinoceptors that are insensitive to 2-meSATP. As such, these sites cannot correctly be classified as P_{2Y}-purinoceptors. This pattern is strengthened by the

observation that UTP has similar agonist potency to ATP in many of the tissues that are unresponsive to 2-meSATP. Davidson and colleagues (1990) introduced the term 'nucleotide' receptor for the ATP/UTP-sensitive site on sheep pituitary cells. This convention was adopted and it was proposed that a nucleotide receptor may be characterized by the following agonist potency order: UTP = ATP > ADP > α,β-meATP, 2-meSATP (O'Connor *et al.*, 1991). Tissues like rat aorta (Dainty *et al.*, 1990) may contain a heterogeneous population of receptor types (possibly both P_{2Y} and 'nucleotide' receptors), activation of which results in the same functional response, in this case endothelium-dependent relaxation. To support this two separate co-existing receptor populations have been demonstrated (P_{2Y}-purinoceptors and nucleotide receptors) located on bovine aortic endothelial cells (Motte *et al.*, 1993; Wilkinson *et al.*, 1993).

Endothelial cells play a key role in the control of vascular tone by virtue of their ability to synthesize and release EDRF's. ATP has been shown to elicit vasodilatation in the coronary bed via an action at receptors located on endothelial cells, leading to release of these factors (Hopwood & Burnstock, 1987; Hopwood *et al.*, 1989). These factors include prostacyclin (Moncada & Vane, 1979) and EDRF (Furchgott & Zawadzki, 1980a). Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) which can be released from endothelial cells by a variety of stimuli including UTP and ATP (Pearson *et al.*, 1983; Demolle *et al.*, 1988). In the guinea-pig coronary vasculature the release of EDRF, believed to be NO (Ignarro *et al.*, 1986; Furchgott *et al.*,

1987) mediates relaxation evoked by ATP (see Chapter 3) and in the rat mesenteric arterial bed UTP also induces relaxation via NO (Ralevic & Burnstock, 1991).

This study investigates the relaxant effects of pyrimidine nucleoside triphosphates, UTP, CTP and TTP and to compare them with the relaxant effects of the purine nucleoside triphosphates, ATP, inosine 5'-triphosphate (ITP) and GTP, on the guinea-pig coronary vasculature by using inhibitors of the enzymatic synthesis of NO and prostaglandins. L-NAME is a competitive inhibitor of the synthesis of NO from L-arginine (Rees *et al.*, 1990a) and is effective in inhibiting vasodilator responses to various agents including ATP in the guinea-pig coronary vasculature (see Chapter 3), while indomethacin is a prostaglandin synthase inhibitor (Vane, 1971) which will therefore prevent the formation of prostanoids.

7.3 METHODS (See Chapter 2.1.2)

Guinea-pigs (250-400 g) of either sex were used. The heart was removed and the coronary circulation perfused by the method of Langendorff (see Chapter 2.1.2). The flow rate was determined by collecting the effluent, over a period of time, and the average rate was 13.26 ± 0.35 ml min^{-1} (n=57). The average starting left ventricular systolic pressure was 41.92 ± 1.21 mm Hg (n=57).

When the perfusion had reached a steady state, the purines and pyrimidines were given as a bolus of 50 µl, injected

over 3 s into the superfusing solution close to the heart. The duration of each individual experiment was no longer than 3 h. Due to this time restriction the effects of all the agonists could not be tested on the same heart. For this reason the agonists were chosen randomly and not more than four agonists were used on a particular heart. The order of exposure of the agonists to the heart was also random to minimise effects due to time-dependent changes and preparation variability. At least 5 min was left between the administration of each dose of agonist. When the effect of antagonists was examined control dose-response relationships for the purines and pyrimidines were first obtained and L-NAME or indomethacin added to the perfusing solution and allowed to equilibrate for 20 min. The dose-responses were then repeated in the presence of the antagonist. After inhibition with L-NAME, L-arginine was also added to the perfusing solution to determine whether the inhibition could be reversed. The preparations were allowed to equilibrate for a further 20 min before a submaximal dose of agonist was repeated. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis program on an Apple II computer. The rank order of potency of the purines and pyrimidines was determined empirically as maximum responses to these agents were not obtained and therefore pD_2 values could not be calculated. At the end of the experiment the heart was removed from the cannula, blotted and weighed. The mean wet weights were 1.46 ± 0.04 g (n = 57).

To test for the presence of ATP as a contaminant of GTP,

TTP, CTP and ITP solutions (10^{-2}M) of these agents were assayed for ATP using the luciferin-luciferase technique described by Stanley and Williams (1969) (see Chapter 2.2.1). Negligible amounts of ATP were found in any of these solutions.

7.3.1 Materials See Chapter 2.4

7.3.2 Statistics See Chapter 2.3

7.4 RESULTS

Dose-response to pyrimidines and purines.

Bolus injections of ATP, UTP, ITP, GTP, CTP, and TTP produced dose-dependent vasodilatation in the guinea-pig coronary vasculature. Dose-response curves for the six agonists are illustrated in Figure 7.1. Due to the fact that maximum responses to these agents were not obtained an arbitrary decrease in perfusion pressure of 10 mm Hg was used to determine the relative order of potency. The potency order of these agonists was ATP > UTP >> ITP >> TTP > GTP > CTP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. There was a small but insignificant fall in the left ventricular systolic pressure on bolus administration of agonists at the high doses.

Effect of L-NAME and L-arginine.

The effect of L-NAME on the maximum amplitude (Figure

7.2a-c), area (Figure 7.3a-c) and perfusion pressure trace (Figure 7.5) of the vasodilator response to ATP, UTP and ITP is demonstrated. The maximum amplitude and area of the vasodilator responses due to UTP (5×10^{-10} - 5×10^{-7} mol) and ITP (5×10^{-9} - 5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; Figure 7.2a,b, 7.3a,b and 7.5a). L-NAME (3×10^{-5} and 10^{-4} M) only inhibited the amplitude of the vasodilator response to a low dose of ATP (5×10^{-10} mol; Figure 7.2c). In contrast, the area of the vasodilator response to ATP (5×10^{-11} - 5×10^{-7} mol; Figure 7.3c and 7.5b) was significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M), reflecting an attenuation of the duration of the response. The maximum amplitude and area of the vasodilator responses to GTP (5×10^{-8} - 5×10^{-7} mol), CTP (5×10^{-7} mol) and TTP (5×10^{-8} - 5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; data not shown). The inhibition of the response to ATP (5×10^{-8} mol), UTP (5×10^{-8} mol), ITP (5×10^{-8} mol), TTP (5×10^{-7} mol), CTP (5×10^{-7} mol) and GTP (5×10^{-7} mol) by L-NAME (3×10^{-5} M) was significantly reversed by L-arginine (1.5×10^{-3} M; Table 7.1). L-NAME (3×10^{-5} and 10^{-4} M) and L-arginine (1.5×10^{-3} M) did not significantly affect the resting perfusion pressure or left ventricular pressure of the preparations.

Effect of indomethacin.

Indomethacin (10^{-6} M) did not affect the maximum amplitude or area of the vasodilator responses to UTP (5×10^{-11} - 5×10^{-7} mol; Figure 7.4a and 7.5a), ITP (5×10^{-10} - 5×10^{-7}

mol; Figure 7.4b), CTP (5×10^{-7} mol; data not shown), TTP (5×10^{-8} - 5×10^{-7} mol; data not shown) and GTP (5×10^{-8} - 5×10^{-7} mol; data not shown). In contrast, the maximum amplitude of the vasodilator response to ATP (5×10^{-10} - 5×10^{-7} mol; Figure 7.4c and 7.5b) was significantly reduced by indomethacin (10^{-6} M). The area of the response to ATP was only significantly reduced at one intermediate dose (5×10^{-9} mol; data not shown). The resting perfusion pressure and left ventricular pressure of the preparations were unaffected by the addition of indomethacin (10^{-6} M).

7.5 DISCUSSION

The results of this study revealed that the rank order of potency of the pyrimidines and purines in the guinea-pig coronary vasculature was ATP > UTP > ITP >> TTP> GTP>, CTP. The pyrimidines UTP, CTP and TTP induce relaxation in a similar way to the purine compounds ITP and GTP in that the vasodilator responses to these pyrimidines and purines were dependent largely upon the synthesis of NO. In contrast, vasodilator responses evoked by ATP were only partially dependent upon the synthesis of NO. Prostanoids also play a role in the relaxation induced by ATP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. Contamination of these compounds could explain the responses obtained to these agents. Although minimal amounts of ATP were detected in solutions (10^{-2} M) of these compounds there is always the possibility that UTP is the contaminant.

It has been shown in the rat mesenteric arterial bed, that relaxations induced by ATP, TTP, UTP and GTP are dependent upon an intact endothelium (Ralevic & Burnstock, 1991). In the pig aorta (Martin *et al.*, 1985) and human pial vessels (Hardebo *et al.*, 1987) relaxation to UTP is also endothelium-dependent. ATP can stimulate prostanoid production from perfused vascular beds and from endothelial cells in culture (Pearson & Gordon, 1985) and in the guinea-pig coronary vasculature it has been shown to induce release of NO (Kelm & Schrader, 1990). UTP has also been shown to stimulate prostacyclin production in endothelial cells (Forsberg *et al.*, 1987) and in the perfused rat liver (Haussinger *et al.*, 1988). In the rat mesenteric arterial bed vasodilatation to UTP is in large part due to release of NO (Ralevic & Burnstock, 1991). We used this information to investigate and possibly to distinguish between the vascular mechanisms of pyrimidines and purines by using L-NAME, an inhibitor of the conversion of L-arginine to NO (Rees *et al.*, 1990a), and indomethacin, a prostaglandin- synthase inhibitor (Vane, 1971). A more direct approach to distinguish between P_{2Y}-purinoceptor-mediated and 'nucleotide' receptor-mediated relaxations could not be adopted because of the absence of specific antagonists to the 'nucleotide' receptor and because the P_{2Y}-purinoceptor antagonist, reactive blue 2 (Burnstock & Warland, 1987), caused rapid deterioration of the tissue.

The maximum amplitude and area of the vasodilator responses to the pyrimidines, UTP, CTP and TTP, and the purines, ITP and GTP, were significantly reduced by L-NAME. These results

suggest that the relaxant response to UTP, CTP, TTP, ITP and GTP take place largely through the formation and release of NO. In the rat mesenteric arterial bed, the vasodilator response to UTP is also largely dependent on the release of NO (Ralevic & Burnstock, 1991). The fact that L-arginine reversed the inhibition of the response to the pyrimidines and purines by L-NAME substantiates these claims in that it shows that L-NAME was selectively inhibiting the enzyme NO synthase. NO synthase converts L-arginine into L-citrulline with the additional production of NO (Palmer *et al.*, 1988b; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). It therefore appears that the pyrimidines and purines studied induce relaxation in a similar manner with the exception of ATP. In the guinea-pig coronary vasculature it has been shown that ATP induces release of NO (Kelm & Schrader, 1990). However, as previously demonstrated (see Chapter 3 & 5), while L-NAME reduced the duration of the vasodilatation induced by ATP, it did not alter the peak response, suggesting that at least this part of the response is not due to the generation and release of NO.

Indomethacin, the prostaglandin synthase inhibitor significantly reduced the maximum amplitude of the vasodilator response to ATP. This suggests that prostanoids are also involved in part of the response to ATP. ATP has been shown to stimulate prostacyclin production from various beds and endothelial cells in culture (Needleman *et al.*, 1974; Boeynaems & Galand, 1983; Hellewell & Pearson, 1984). In the guinea-pig coronary vasculature adenosine induced relaxation was not mediated via

prostanoids (see Chapter 4). Therefore the involvement of prostanoids in the relaxant response to ATP was not due to its breakdown to adenosine by highly active ectonucleotidases (Fleetwood *et al.*, 1989). The vasodilator responses to the pyrimidines UTP, TTP and CTP or the purines ITP and GTP were unaffected by the presence of indomethacin. Therefore prostanoids do not play a role in the vasodilatation produced in response to exposure to these pyrimidines and purines. In contrast UTP has been shown to induce prostacyclin production from bovine pulmonary artery endothelial cells (Lustig *et al.*, 1992).

In conclusion, we have demonstrated that the pyrimidines, UTP, TTP and CTP, and purines, ITP and GTP, induce relaxation in the guinea-pig coronary bed via formation and release of NO. ATP induces relaxation in the guinea-pig coronary vasculature via a combination of mechanisms involving both NO and prostanoids. Whether 'nucleotide' receptors are also present in the guinea-pig coronary vasculature is unclear. If they are present then action at these receptors induces relaxation via NO and not prostanoids. Selective antagonists will need to be established before a clear receptor profile in the guinea-pig coronary vasculature can be determined.

TABLE 7.1 The area of the relaxation obtained in response to ATP, UTP, ITP, CTP, GTP and TTP in the isolated perfused guinea-pig heart. The effect of L-NAME (3×10^{-5} M; in the perfusate) on the response to these agonists and the effect of L-arginine (L-ARG) (1.5×10^{-3}M; in the perfusate along with L-NAME) on the inhibition by L-NAME is demonstrated. The areas are expressed as the mean ± s.e. mean ($n \geq 6$). Significant differences from control are * $P<0.05$. Significant differences from responses obtained in the presence of L-NAME are ** $P<0.05$.

Agonist	Dose (moles)	Area of relaxant response (mmHg x min)		
		Control	After addition of: L-NAME	L-NAME & L-ARG
ATP	$5x10^{-8}$	22.32 ± 3.40	10.53 ± 2.45 *	20.85 ± 5.00 **
UTP	$5x10^{-8}$	8.30 ± 1.13	3.14 ± 0.75 *	5.63 + 1.18 **
ITP	$5x10^{-8}$	6.24 ± 1.27	1.00 ± 0.18 *	4.10 ± 1.17 **
CTP	$5x10^{-7}$	2.87 ± 0.78	0.03 ± 0.03 *	1.74 ± 0.66 **
GTP	$5x10^{-7}$	3.61 ± 0.40	0.83 ± 0.16 *	3.58 ± 0.66 **
TTP	$5x10^{-7}$	6.90 ± 0.98	1.49 ± 0.34 *	3.37 ± 0.36 **

FIGURE 7.1 The amplitude of the vasodilatation evoked by ATP (●), UTP (○), ITP (■), TTP (□), GTP (▲) and CTP (△) in the isolated perfused guinea-pig heart. The graph shows the mean ($n \geq 8$) with s.e. mean indicated by vertical bars.

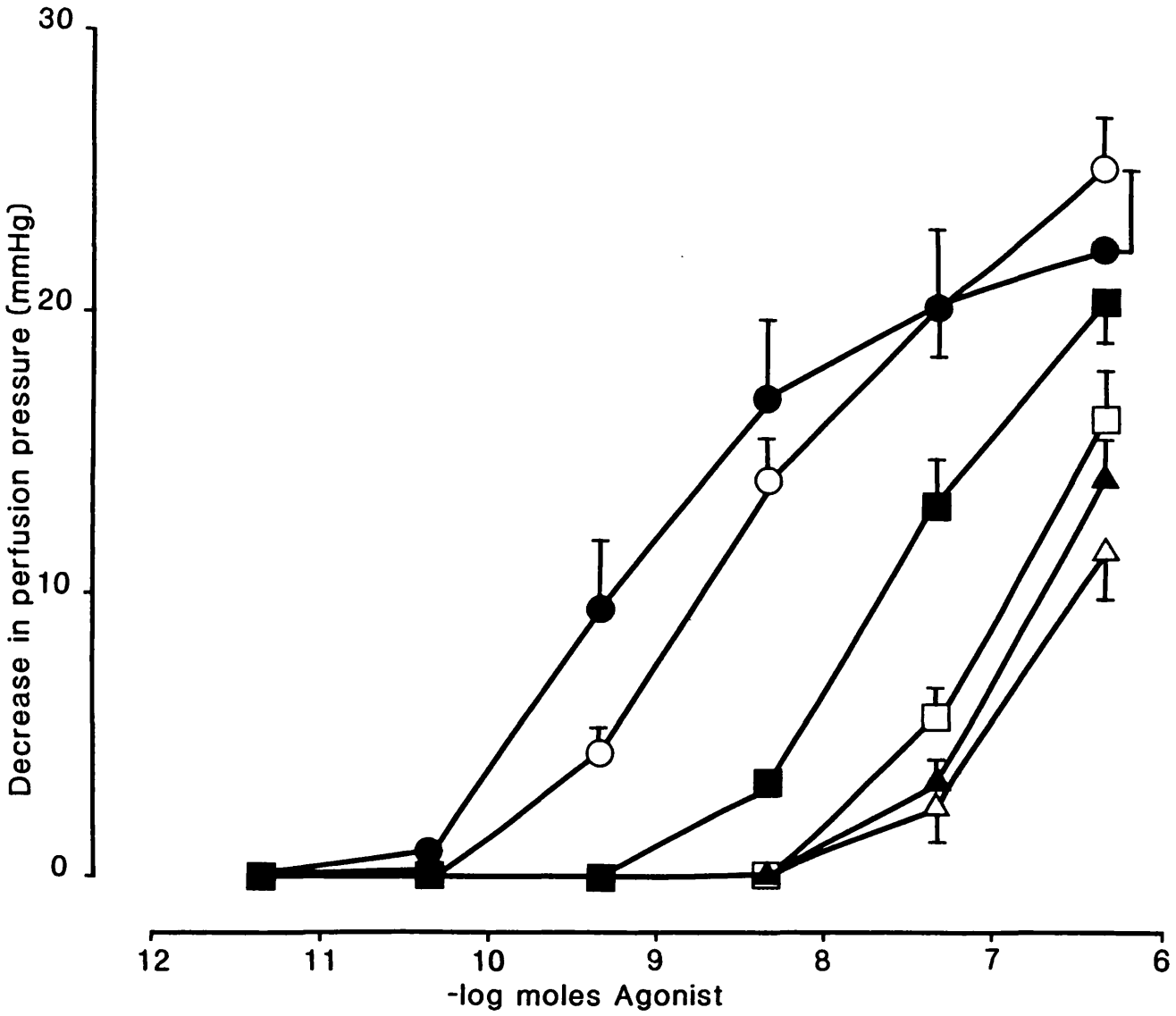

30
20
10
0
Decrease in perfusion pressure (mmHg)
12
11
10
9
8
7
6
-log moles Agonist

FIGURE 7.2a The amplitude of the vasodilatation obtained in response to UTP, in the isolated perfused guinea-pig heart, in the absence (● ; mean of all controls) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

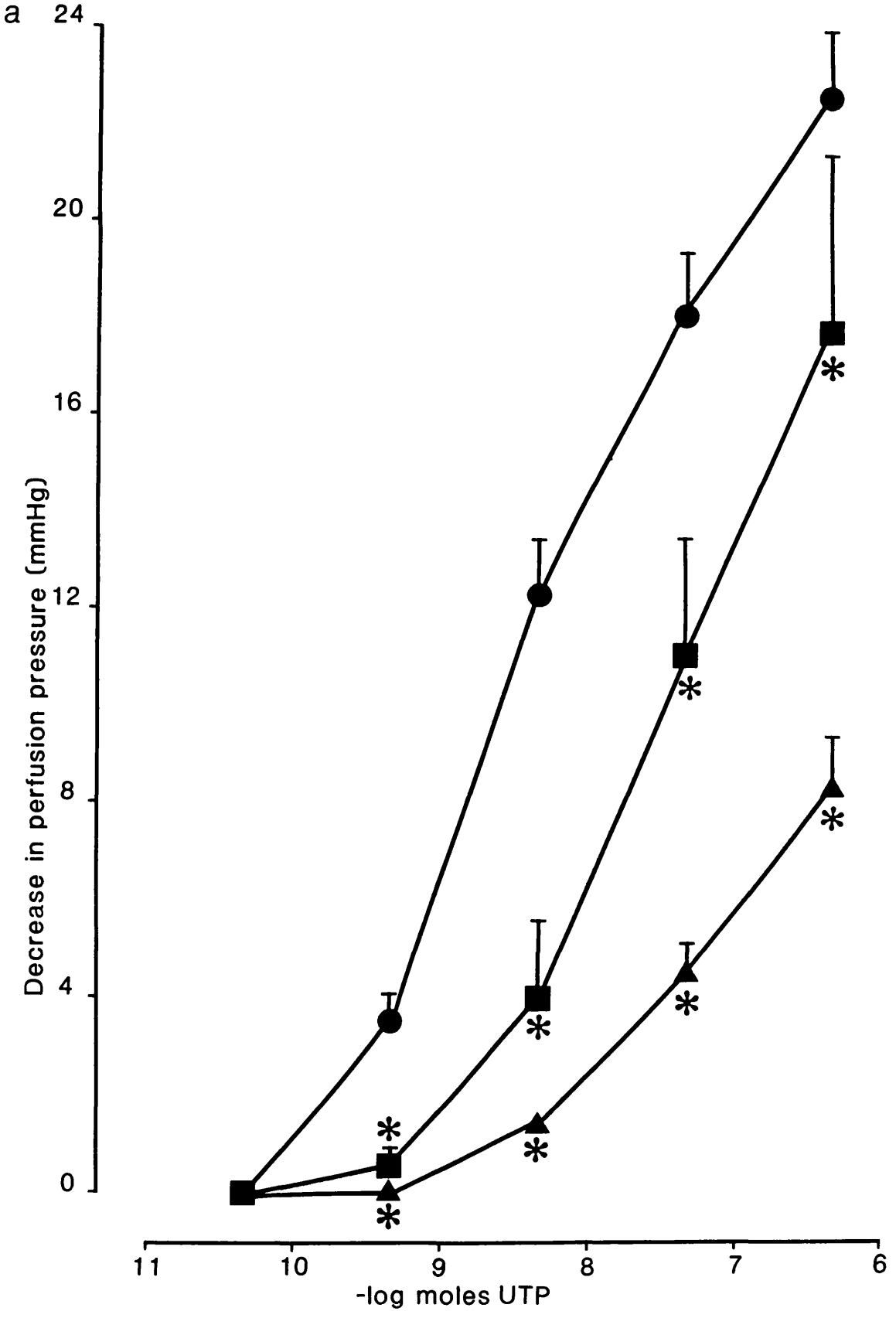

a
24
20
16
12
8
4
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles UTP

FIGURE 7.2b The amplitude of the vasodilatation obtained in response to ITP, in the isolated perfused guinea-pig heart, in the absence (●) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

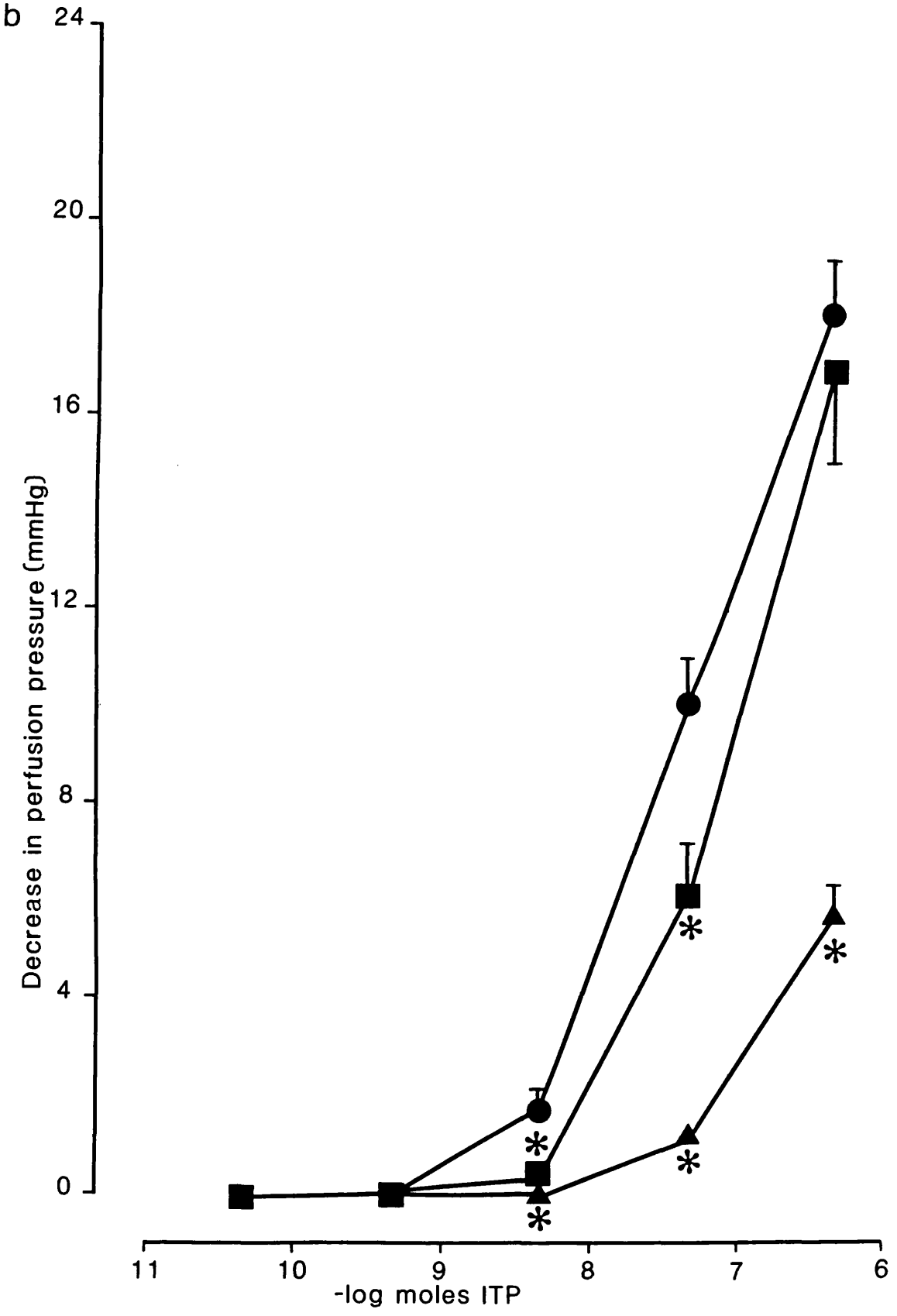

b
24
20
16
12
8
4
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles ITP
*
*
*
*
*

FIGURE 7.2c The amplitude of the vasodilatation obtained in response to ATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

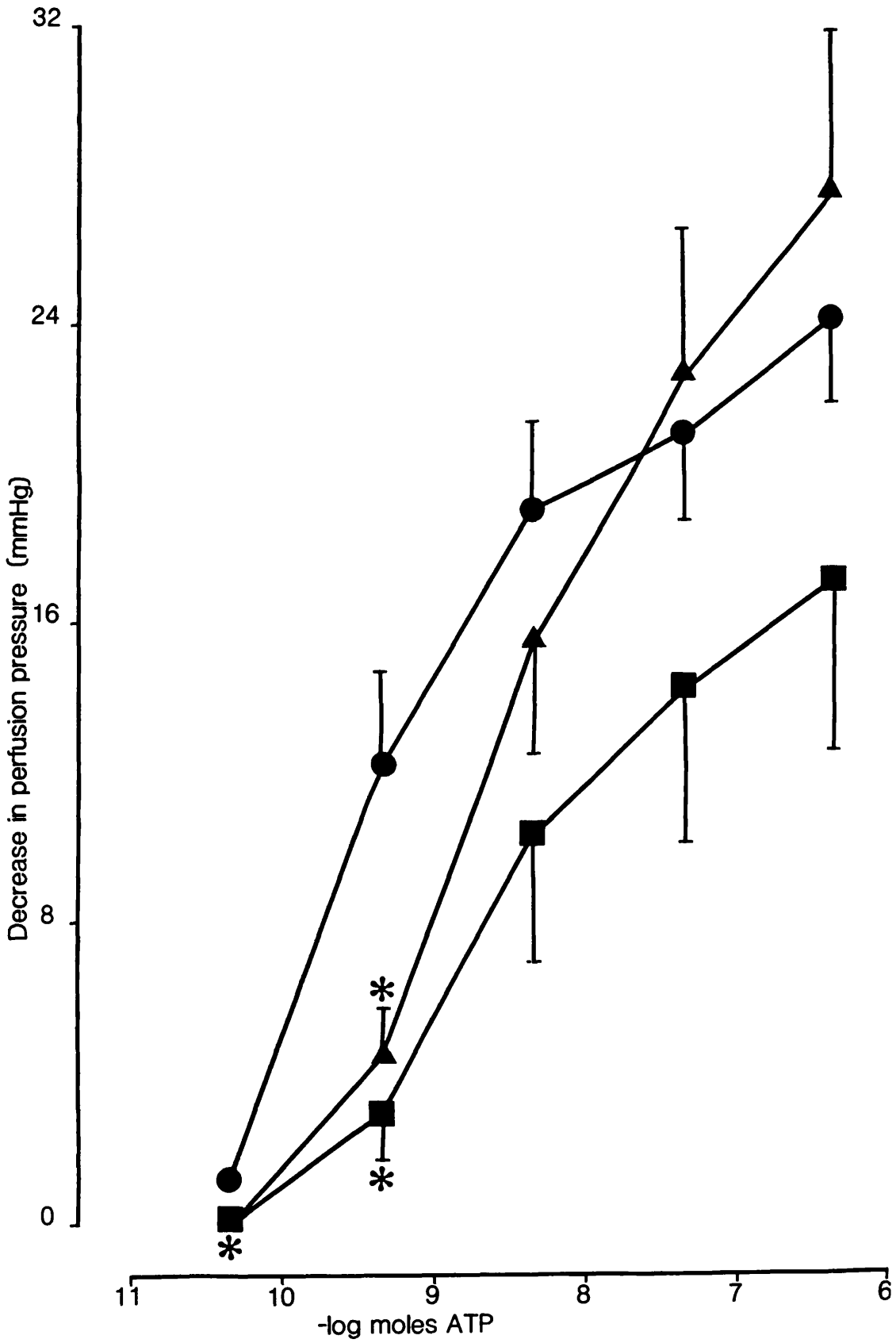

C
32
24
16
8
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles ATP
*
*
*

FIGURE 7.3a The area of the vasodilatation obtained in response to UTP, in the isolated perfused guinea-pig heart, in the absence (●) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

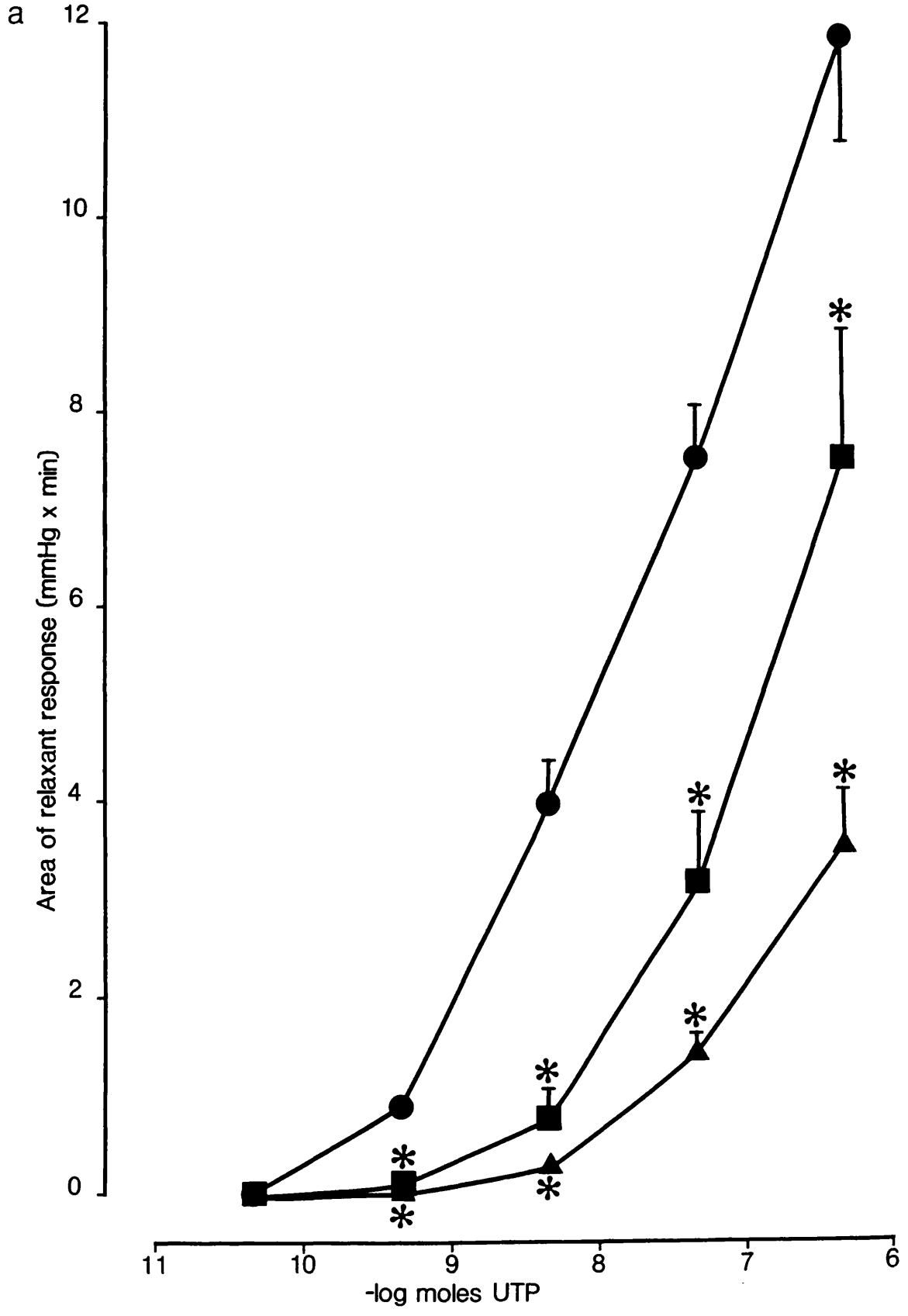

a
12
10
8
6
4
2
0
Area of relaxant response (mmHg x min)
11
10
9
8
7
6
-log moles UTP

FIGURE 7.3b The area of the vasodilatation obtained in response to ITP, in the isolated perfused guinea-pig heart, in the absence (●) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

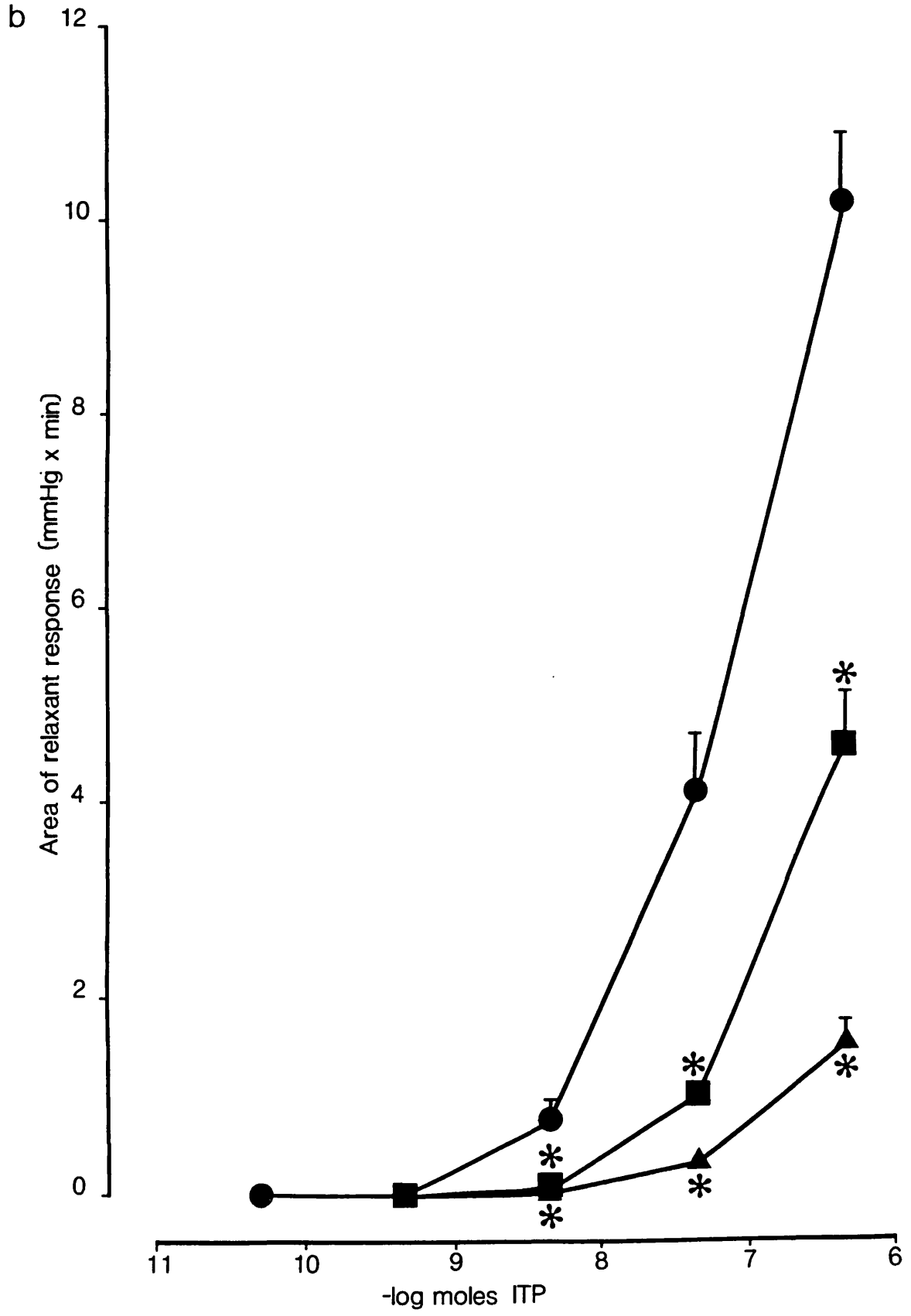

b
12
10
8
6
4
2
0
Area of relaxant response (mmHg x min)
11
10
9
8
7
6
-log moles ITP
*
*
*
*
*
*

FIGURE 7.3c The area of the vasodilatation obtained in response to ATP, in the isolated perfused guinea-pig heart, in the absence (●) and presence of L-NAME (3×10^{-5} M (■) and 10^{-4} M (▲)). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

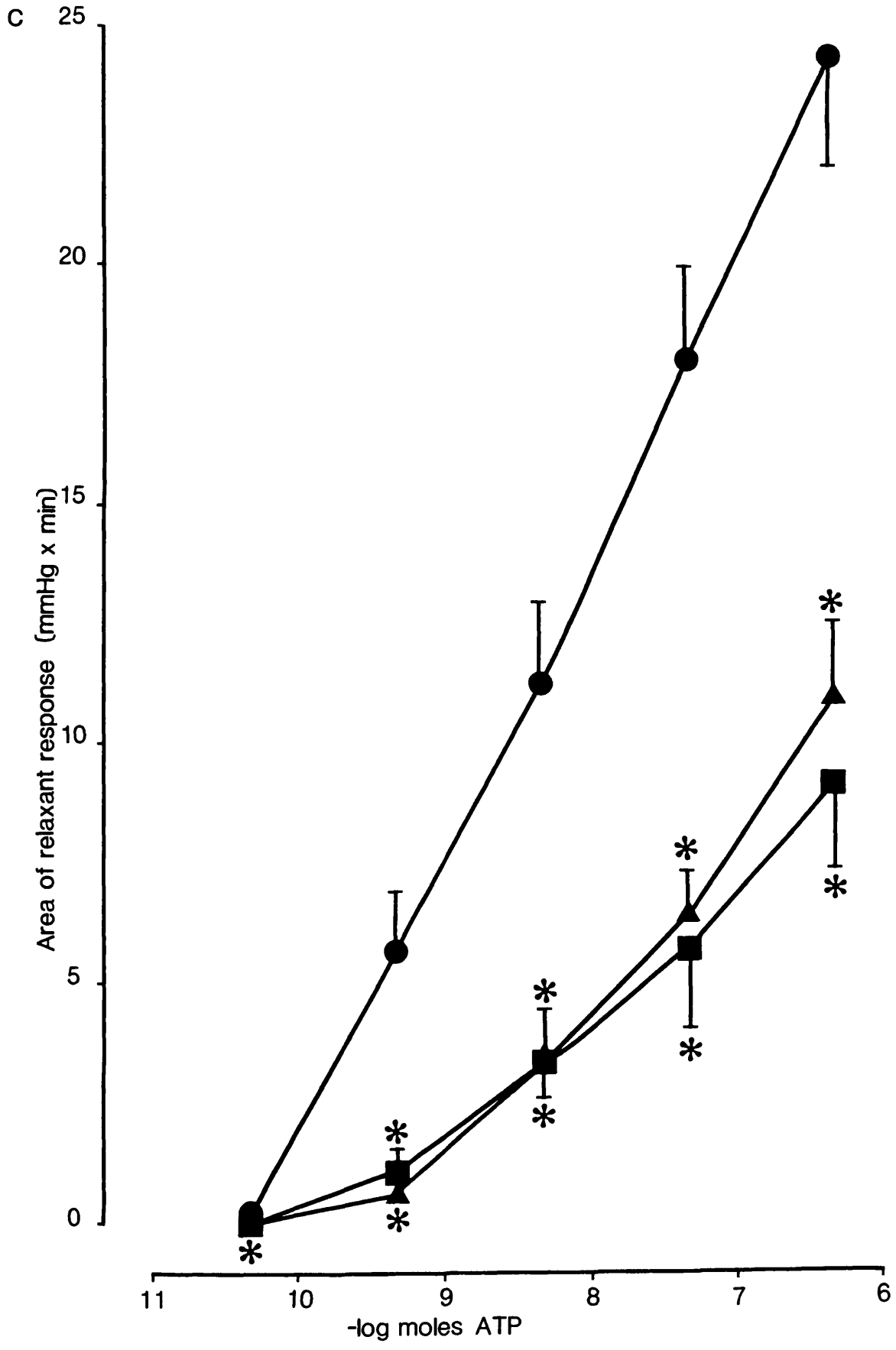

C
25
20
15
10
5
0
Area of relaxant response (mmHg x min)
11
10
9
8
7
6
-log moles ATP

FIGURE 7.4a The amplitude of the vasodilator responses evoked by UTP, in the isolated perfused guinea-pig heart, in the absence (●) and presence (○) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P<0.05$.

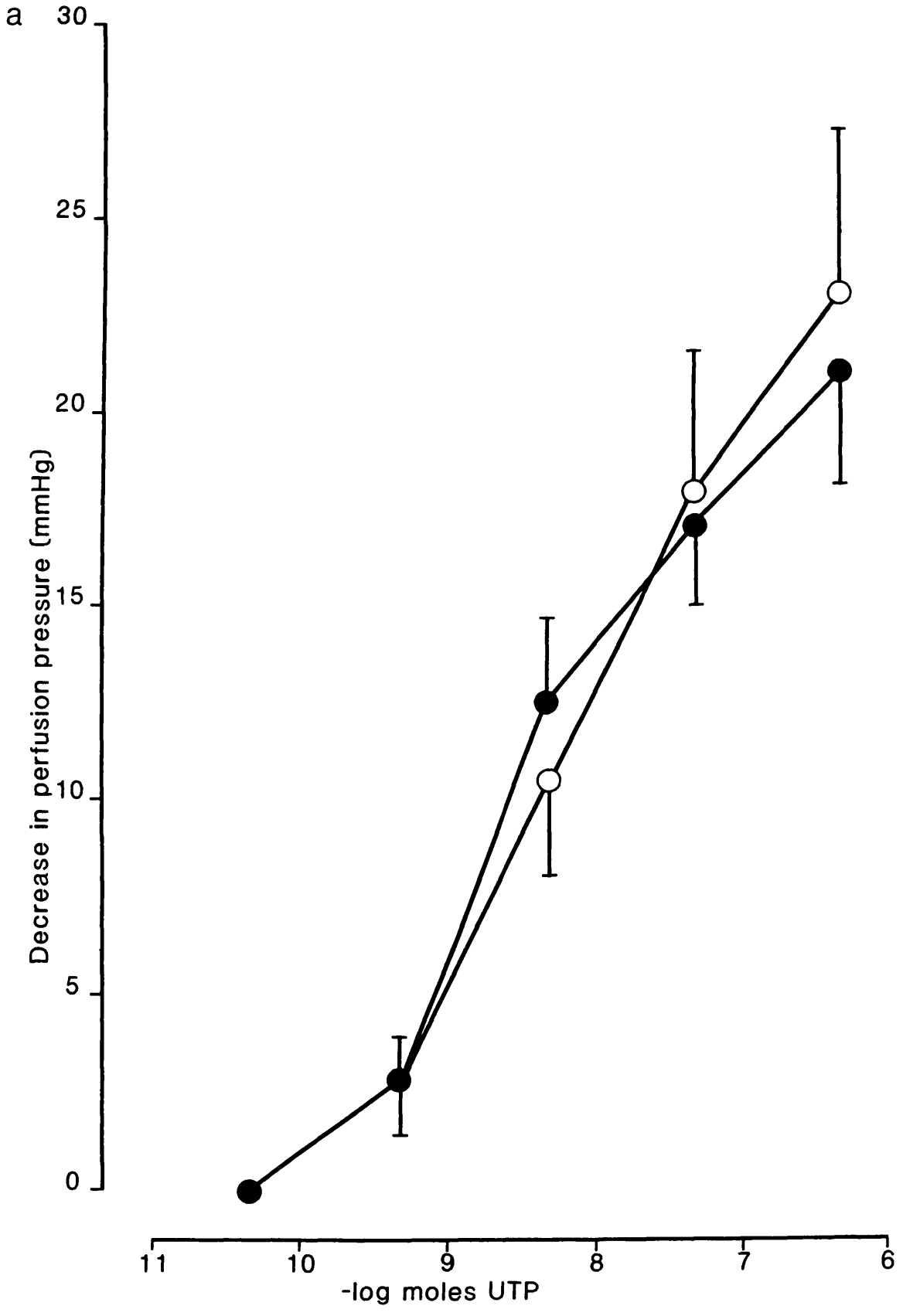

a
30
25
20
15
10
5
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles UTP

FIGURE 7.4b The amplitude of the vasodilator responses evoked by ITP, in the isolated perfused guinea-pig heart, in the absence (■) and presence (□) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

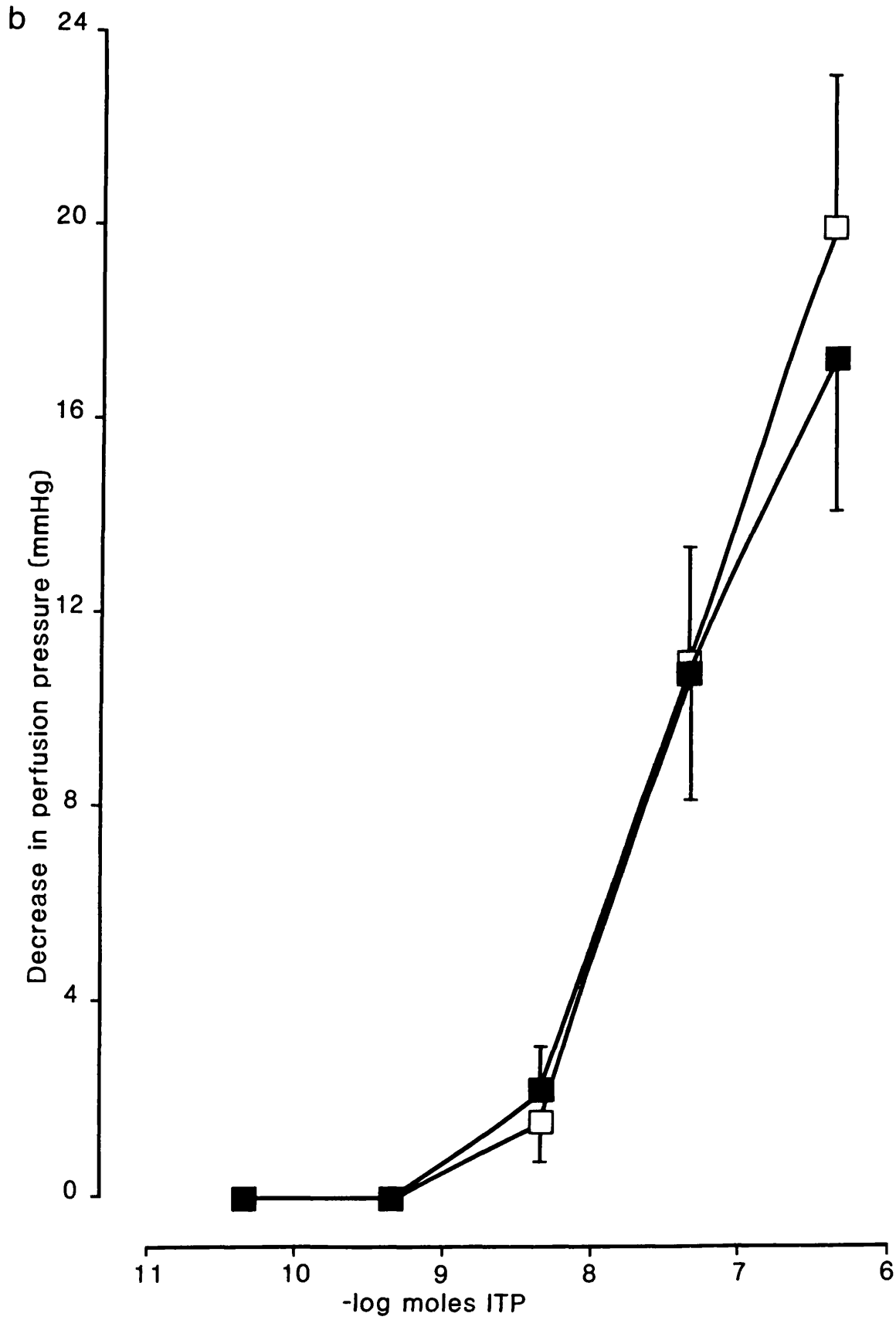

b
24
20
16
12
8
4
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles ITP

FIGURE 7.4c The amplitude of the vasodilator responses evoked by ATP, in the isolated perfused guinea-pig heart, in the absence (▲) and presence (△) of indomethacin (10^{-6} M). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences from control are * $P<0.05$.

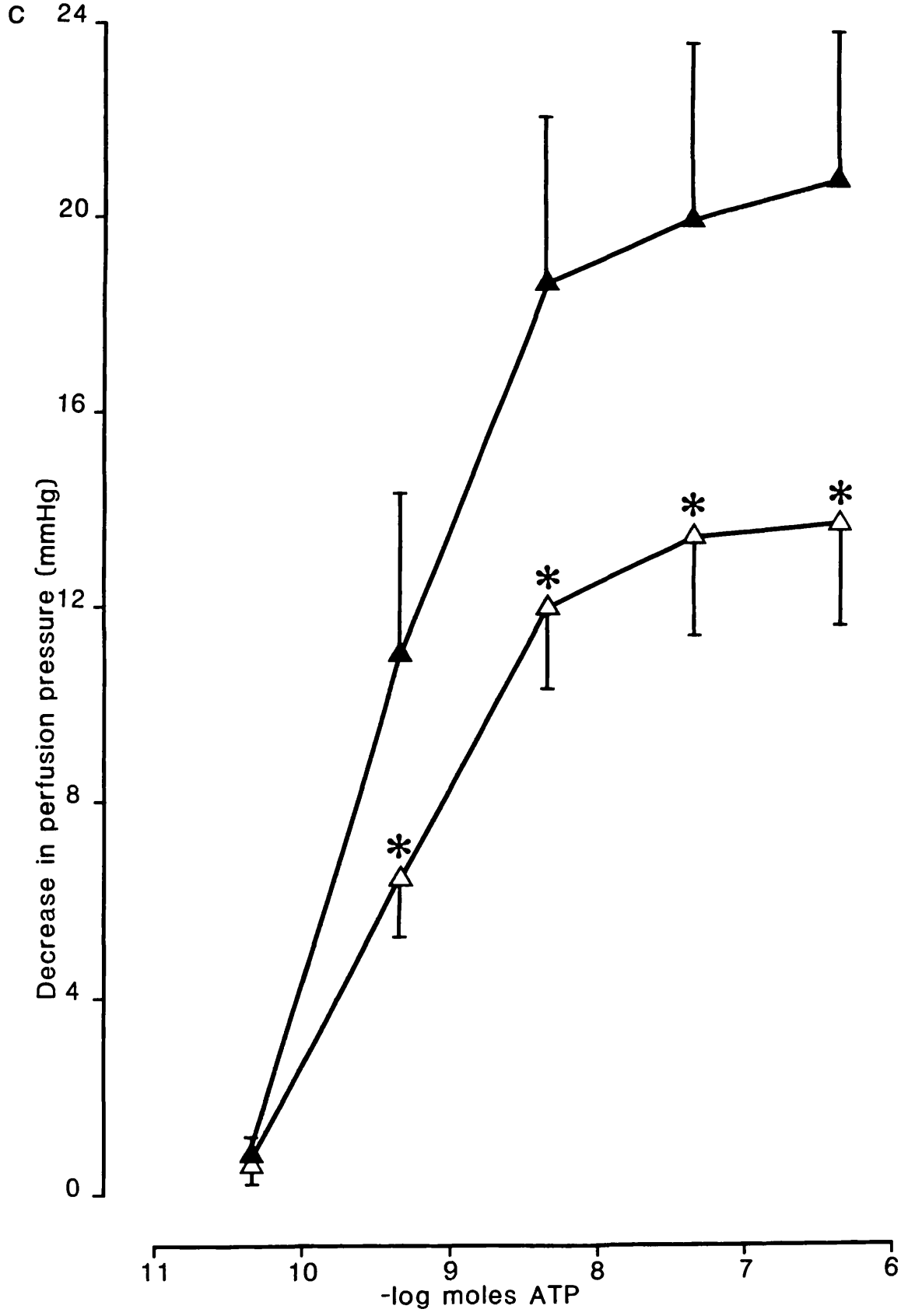
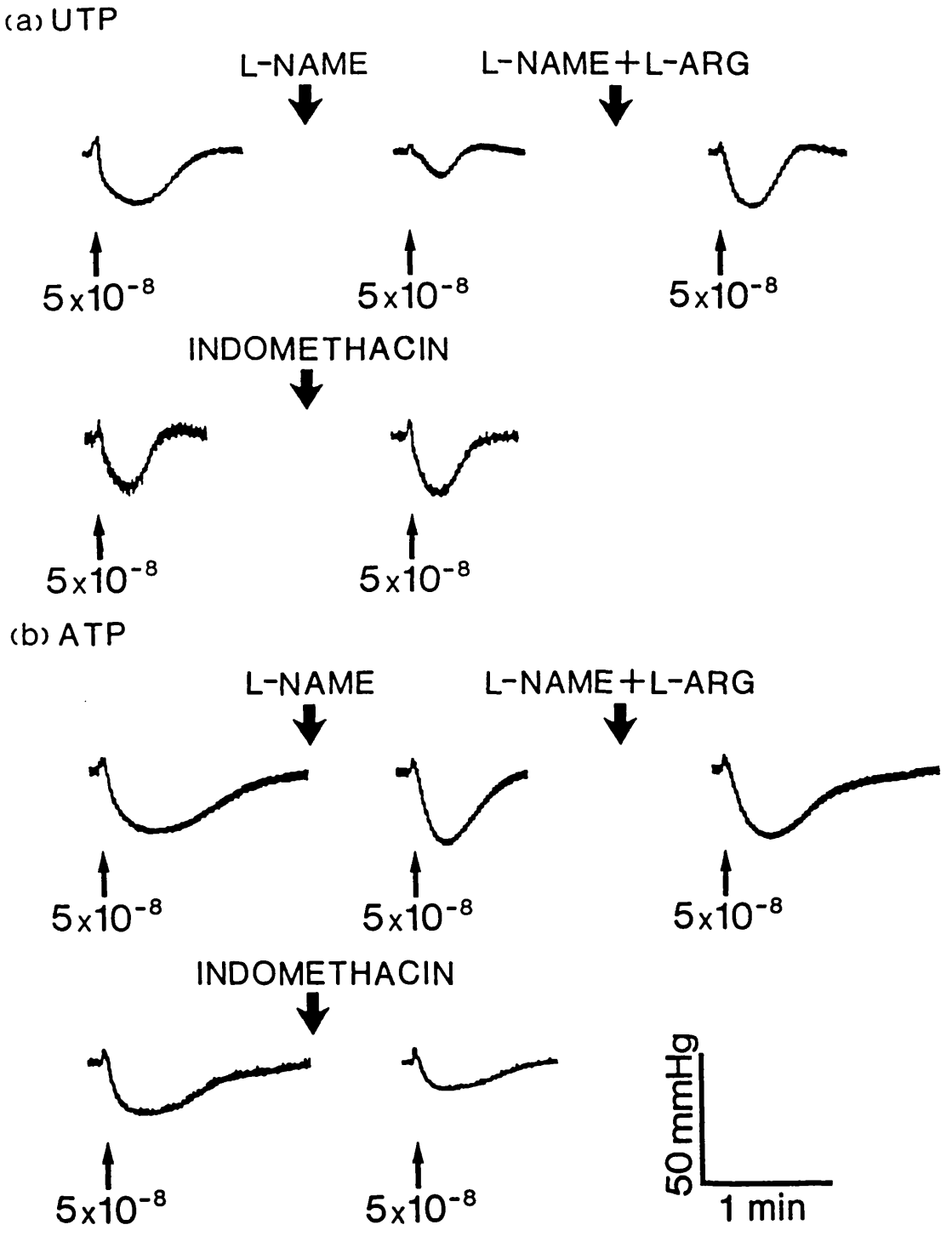

C
24
20
16
12
8
4
0
Decrease in perfusion pressure (mmHg)
11
10
9
8
7
6
-log moles ATP
*
*
*
*

FIGURE 7.5 Typical perfusion pressure traces, obtained from guinea-pig isolated perfused hearts, showing the effects of (a) UTP and (b) ATP. The vasodilator response to these agents in the absence or presence (after ▼) of L-NAME (3×10^{-5} M) and indomethacin (10^{-6} M) is demonstrated. Reversal of the inhibition of the vasodilator response of UTP and ATP, in the presence of L-NAME, by L-arginine (L-ARG, 1.5×10^{-3}M) is also demonstrated. The dose stated is the number of moles of vasodilator agonist that is injected into the perfusion pressure system close to the heart.


(a) UTP
L-NAME
L-NAME+L-ARG
5x10-8
5x10-8
5x10-8
INDOMETHACIN
5x10-8
5x10-8
(b) ATP
L-NAME
L-NAME+L-ARG
5x10-8
5x10-8
5x10-8
INDOMETHACIN
5x10-8
5x10-8
50 mmHg
1 min

SECTION C

Chapter 8

ATP release from the isolated perfused guinea-pig heart in response to increased flow.

8.1 SUMMARY

1) The Langendorff heart preparation was used to investigate the release of ATP from the guinea-pig heart in response to increased coronary flow. The luciferin-luciferase firefly technique was used to determine levels of ATP in the perfusate.

2) During periods of increased flow ATP release was rapidly and significantly increased from 4.28 ± 0.97 pmoles min^{-1} (n=9) to 40.50 ± 13.6 pmoles min^{-1} (n=9; for the first increase in flow) and 16.40 ± 3.3 pmoles min^{-1} (n=9; for the second increase in flow).

3) The pressure/flow ratio was reduced in increased flow conditions suggesting that coronary vasodilatation occurred.

4) It is concluded that ATP release from the guinea-pig heart is increased under raised flow conditions. It is suggested that this ATP release induces coronary vasodilatation.

8.2 INTRODUCTION

Blood flow through the coronary arteries is precisely regulated and any imbalance between the oxygen supply and the oxygen demand of the heart can lead to angina pectoris or to heart failure. Flow-induced vasodilatation is a well known phenomenon,

which is endothelium-dependent and coincides with release of EDRF and prostacyclin (Rubanyi *et al.*, 1986). A study by Paddle and Burnstock (1974), later confirmed by Clemens and Forrester (1981), showed that ATP is released from the guinea-pig coronary vasculature during hypoxic stimulation and provided early, although indirect, evidence for a role for ATP as a physiological modulator of coronary flow. More recently, Hopwood *et al.* (1989) showed that hydroquinone, an antagonist of EDRF-mediated effects and reactive blue 2, a selective P_{2Y}-purinoceptor antagonist, attenuated both the vasodilatation evoked by 2-meSATP at the P_{2Y}-purinoceptor and the hypoxic relaxation, implicating both ATP and EDRF in the cardiac dilatation to hypoxia. In the guinea-pig coronary vasculature vasodilatation evoked by ATP is partly mediated via NO (see Chapter 3, 5 & 7). During shear stress induced by fast flow, endothelial cells from different vascular beds have been shown to release ATP both in culture and *in situ* (Milner *et al.*, 1990a; Ralevic *et al.*, 1992).

In the present study, the release of ATP from isolated perfused guinea-pig hearts submitted to increased flow was investigated, using the sensitive luminometric luciferin-luciferase firefly technique.

8.3 METHODS (See Chapter 2.1.2)

Guinea-pigs (250-300 g) of either sex were used. The heart was removed and cannulated via the aorta for perfusion (initial mean flow was 13.9 ± 0.4 ml min^{-1}; n = 9) according to

the method of Langendorff (see Chapter 2.1.2). The heart was allowed to equilibrate for 30 min prior to increased flow conditions. Perfusate samples for assaying were collected 1 min prior to increased flow, 1 min during increased flow and 1 min after increased flow. This collection procedure was immediately repeated. During increased flow the flow was raised to a mean flow rate of 24.9 ± 0.6 ml min^{-1} (n = 9).

8.3.1 ATP assay. See Chapter 2.2.1

8.3.2 LDH activity measurements. See Chapter 2.2.2

8.3.3 Materials. See Chapter 2.4

8.3.4 Statistics. See Chapter 2.3

8.4 RESULTS

The effect of increased flow on the release of ATP from the perfused guinea-pig heart is demonstrated (Figure 8.1). During low flow (mean flow 13.9 ± 0.4 ml min^{-1}; n = 9) the mean basal release of ATP was 4.28 ± 0.97 pmoles min^{-1} (n = 9). During periods of increased flow (mean high flow 24.9 ± 0.6 ml min^{-1}; n = 9) ATP release was rapidly and significantly increased to 40.5 ± 13.6 pmoles min^{-1} (n = 9; for the first increase in flow) and 16.4 ± 3.3 pmoles min^{-1} (n = 9; for the second increase in flow).

One of the fundamental equations of circulation

physiology is: pressure (V) = flow (I) x resistance (R). Therefore resistance is directly proportional to the pressure/flow ratio. The pressure/flow ratio before any increase in flow was 14.40 ± 0.73 mmHg min ml^{-1} (n = 9). When the flow was increased for the first time the pressure/flow ratio was significantly reduced to 10.42 ± 0.30 mmHg min ml^{-1} (n = 9). When the flow was reduced the pressure/flow ratio returned to 14.04 ± 0.88 mmHg min ml^{-1} (n = 9). On the second increase in flow the pressure/flow ratio was again significantly reduced to 10.16 ± 0.54 mmHg min ml^{-1} (n = 9). Therefore the resistance of the guinea-pig coronary vasculature was reduced on increased flow suggesting that coronary vasodilatation occurred.

LDH activity was measured in the perfusate. It was not detectable in the perfusate at either the high or low flow rate.

8.5 DISCUSSION

In the present study we provide evidence that under raised flow conditions ATP was released from the isolated perfused guinea-pig heart. Although the perfusion pressure increased, the pressure/flow ratio was reduced in increased flow conditions suggesting that coronary vasodilatation occurred.

It is not possible to attribute the source of ATP to any single tissue in a perfusion experiment such as the one performed in this study. Apart from the vascular endothelium ATP could be released from nerves, the myocardial cells, the vascular smooth muscle or some other unknown source. Isolated aortic endothelial

cells have been shown to release ATP under high flow conditions (Milner *et al.*, 1990a). Therefore, although it is not possible to extrapolate directly from studies on different species and tissue, it is likely that under raised flow conditions ATP could be released from the endothelial cells of the guinea-pig coronary vasculature. Although isolated smooth muscle cells from the rabbit aorta released ATP under normal flow conditions there was no increase in release of ATP from these cells under high flow conditions (Bodin *et al.*, 1991). Vascular smooth muscle cells in the guinea-pig coronary vasculature may also play a minimal role in the ATP released under high flow conditions from this vascular bed. Adult heart cells isolated from the rat ventricle released ATP upon exposure of the cells to an hypoxic environment (Forrester and Williams, 1977). It is therefore possible that some contributory ATP is released from these cells under shear stress conditions such as high flow.

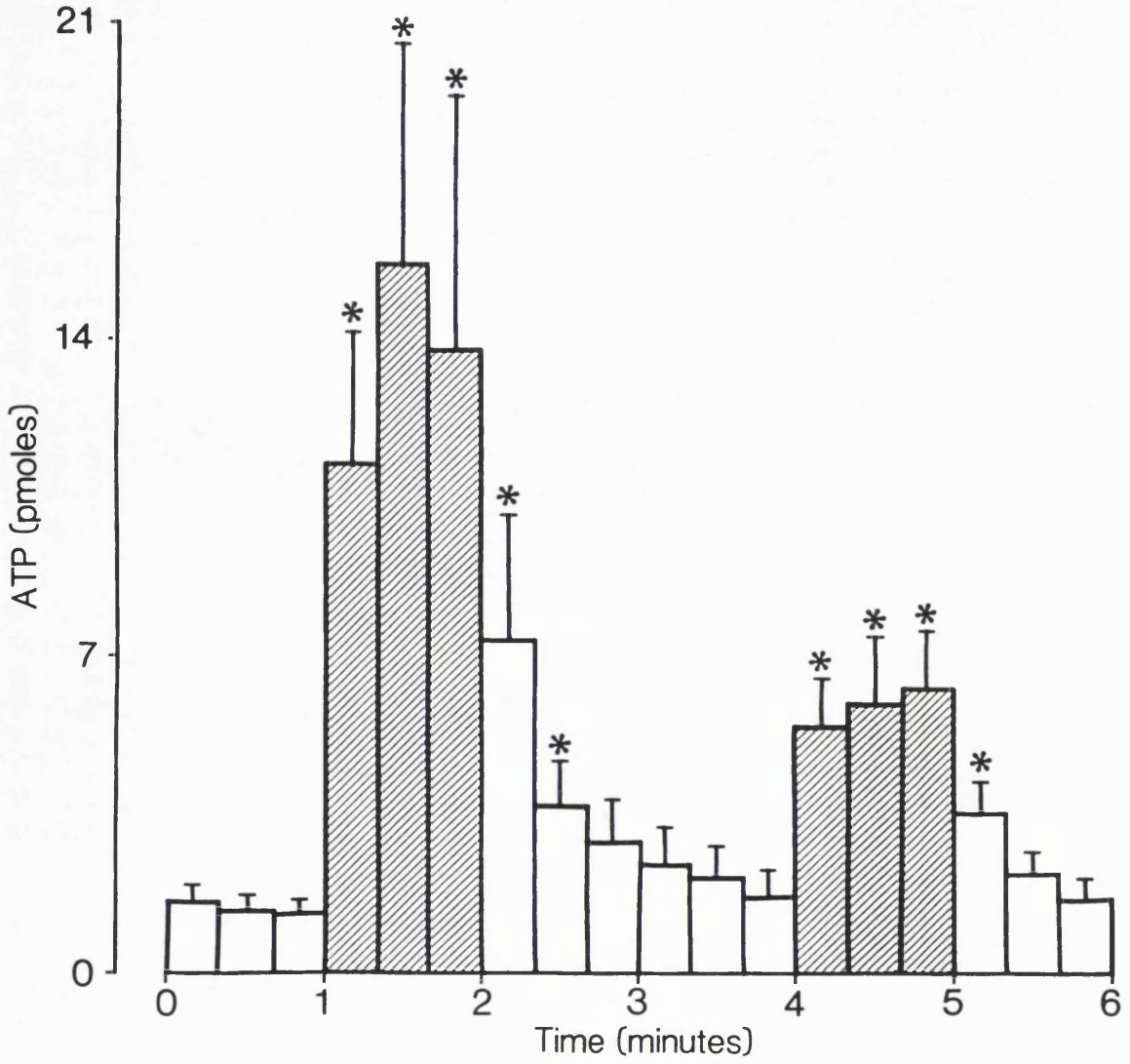
We have previously shown ATP to induce relaxation in the guinea-pig coronary vasculature (see Chapter 3, 5 & 7); in addition we have shown that this relaxation is partially mediated via NO (see Chapter 3, 5 & 7). This vasodilator action is via action at P_{2Y}-purinoceptors rather than P_1-purinoceptors since vasodilator responses to ATP in the guinea-pig coronary vasculature were reduced by over 40% by reactive blue 2 (Hopwood *et al.*, 1989). It is suggested that shear stress induced by high flow causes release of ATP which then acts at P_{2Y}-purinoceptors on the endothelium to initiate relaxation via release of NO.

It is conceivable that the ATP release during high flow

was due to tissue trauma. However, the intracellular marker enzyme LDH was not detected in the perfusate during low or high flow perfusion. This suggests that the high flow conditions did not cause tissue trauma in the guinea-pig coronary vasculature.

In conclusion, we have demonstrated that under raised flow conditions ATP release from the isolated perfused guinea-pig heart was rapidly and significantly increased. The resistance of the vasculature was also significantly reduced under high flow conditions suggesting that coronary vasodilatation occurred.

FIGURE 8.1 The release of ATP from the isolated perfused guinea-pig heart. The effect of increased flow (shaded columns) from 13.87 ± 0.42 ml min^{-1} (n=9) to 24.85 ± 0.61 ml min^{-1} (n=9) on the release of ATP is demonstrated. The amount of ATP released is expressed as the mean (n=9) with s.e. mean indicated by vertical bars. Significant differences from control (mean of first three control values) are * $P<0.05$.


21
14
7
0
ATP (pmoles)
0
1
2
3
4
5
6
Time (minutes)

Chapter 9

GENERAL DISCUSSION

For many years vascular tone was thought to be predominantly influenced by effects exerted by the autonomic nervous system through perivascular nerves. However, since Furchgott and Zawadzki (1980a) showed that the endothelium played an important role in the regulation of peripheral resistance and local blood flow, it has become increasingly apparent that there is a dual control of vascular resistance in that vascular tone is determined by both perivascular nerves and endothelial cells. In this discussion the role played by the endothelium in the control of coronary vascular tone is considered. The general implications of endothelial control of vascular tone are important because under certain pathophysiological conditions the endothelium is prone to structural and/or functional changes. For example, it is now well established that endothelium-mediated vasodilatation via EDRF is diminished in atherosclerosis in man and in several experimental models of the disease (see Verbeuren & Herman, 1988). Impaired vasodilatation may be the outcome of any one of several mechanisms, including functional abnormalities of the endothelium (which can occur in the absence of any obvious damage to the endothelium itself) (Ross, 1988) or vascular smooth muscle. An understanding of how vasoactive agents elicit relaxation via endothelial cells is therefore important. Endothelial cells have also been shown to store and release vasoactive substances such as ATP, substance P, 5-HT and ACh (Paddle & Burnstock, 1974; Burnstock *et al.*, 1988; Ralevic *et al.*, 1989; Milner *et al.*, 1990a, 1990b). Therefore the role of endothelial cells as both effectors of, as well as a source of vasoactive agents, will be

discussed. Before the importance of vascular endothelial cells in control of vascular tone was established (Furchgott & Zawadzki, 1980a) it was widely believed that coronary vasodilatation induced by hypoxaemia was due to adenosine released directly from myocardial cells, as proposed by Berne (1963). The current status of Berne's hypothesis is reviewed in this discussion and an endothelial role in hypoxic vasodilatation is proposed. Since the P_{2X}/P_{2Y}-purinoceptor subclassification proposed by Burnstock & Kennedy (1985), several further subtypes have been proposed. Therefore the findings in this thesis concerning the actions of purines and pyrimidines on coronary vessels (Chapter 5 & 6) are discussed in the context of current purinoceptor subclassification.

9.1 ENDOTHELIAL CELLS.

By virtue of their position at the intimal surface of blood vessels endothelial cells are subject to a different host of stimuli than are perivascular nerves. Endothelial cells act as sensors to a wide range of vasoactive substances via specific endothelial receptors, and also to humoral changes (see Furchgott, 1983; Vanhoutte *et al.*, 1986; Lüscher, 1988). Endothelial cells have also been shown to store and release vasoactive substances such as substance P, ATP, 5-HT and ACh (Paddle & Burnstock, 1974; Burnstock *et al.*, 1988; Ralevic *et al.*, 1989; Milner *et al.*, 1989; 1990a, 1990b).

The importance of the endothelium is highlighted by the fact that the same substance which causes endothelium-dependent vasodilatation causes vasoconstriction via receptors on the underlying smooth muscle when the endothelium is damaged or removed. For example, NA released from sympathetic nerves is primarily responsible for tone in peripheral blood vessels by constrictor effects mediated by postjunctional α_1- and α_2-adrenoceptors (Langer, 1974). However, NA acting at the endothelial α_2 receptor causes vasodilatation mediated by the release of EDRF (Cocks & Angus, 1983; Matsuda *et al.*, 1985; Miller & Vanhoutte, 1985a). Similarly, ATP acting abluminally following release as a co-transmitter from sympathetic nerves (see Burnstock, 1988a) exerts contractile effects following activation of P_{2X}-purinoceptors, while its action at the endothelial P_{2Y}-purinoceptor is to cause vasodilatation. In the isolated perfused rat heart ATP mediates coronary vasoconstriction via P_{2X}-purinoceptors and coronary vasodilatation via P_{2Y}-purinoceptors (Hopwood & Burnstock, 1987). Coronary vasodilatation via P_{2Y}-purinoceptors has been confirmed and extended upon in the present study (see Chapters 5 & 6 & later in discussion).

Many studies looking at endothelial mediated responses have examined vasodilator responses of isolated vessels or vascular beds in the absence and presence of an intact endothelium. Removal of the endothelium from vascular beds is technically more difficult than from isolated vessels, but this has been achieved in a variety of different ways. In the perfused rat hind limb, removal of the endothelium from large arteries was

achieved by a combination of high flow together with controlled application of air (Ralevic *et al.*, 1989). Alternatively, the detergent 3-[(3-cholamidopropyl)-dimethylammino]-1-propanesulfonate (CHAPS) has been used to remove the endothelium of the guinea-pig coronary vasculature (McLeod & Piper, 1992). Unfortunately, selective removal of endothelial cells from the resistant vessels of the guinea-pig or rat coronary vasculature while retaining a functionally viable preparation was not possible in the present study using either of these approaches. The endothelial cells of the capillaries showed some degree of damage even when some of the resistant vessels were still intact. Oedema and diminishment of all the vasodilator responses via endothelium and smooth muscle was also observed. Therefore, in order to examine endothelium-dependent vasodilatation inhibitors of the synthesis of various EDRF's were used. The advantage of this approach is that it is possible to distinguish between the different EDRF's and therefore define more accurately the mechanisms of vasodilatation induced by various vasoactive agents. The disadvantage with this protocol is that the inhibitors may not be totally selective for endothelium-dependent mechanisms. For example, endocardial cells are ontogenetically similar to the endothelium and the L-arginine:NO pathway may play a role in myocardial contractility (Lewis *et al.*, 1990; Smith *et al.*, 1991). Therefore inhibitors of endothelial NO synthesis, such as L-NAME, may also affect the endocardial cell L-arginine:NO pathway and therefore influence myocardial contractility.

This study has shown that in the guinea-pig coronary

vasculature the NO synthase inhibitor L-NAME attenuates vasodilator responses to substance P, UTP, CTP, TTP, ITP, GTP and 5-HT. Therefore, it may be assumed that the vasodilatation initiated by these agents is mediated via NO (Chapters 3 & 7). However, although NO does play a role in the vasodilator responses to ATP, bradykinin and adenosine other mechanisms are also involved. (Chapters 3, 4 & 5). The prostaglandin synthesis inhibitor indomethacin also inhibited vasodilator responses to ATP and bradykinin (but not to adenosine) indicating a role for prostanoids in vasodilator responses to these agents (Chapters 5 & 7). A direct action of adenosine at A_2-purinoceptors on the smooth muscle is likely to account for the L-NAME-resistant component of the vasodilator response to adenosine (Chapter 4).

Under physiological conditions it is likely that endothelial cells are primarily responsible for maintaining homeostasis by altering vascular tone in response to fluctuations in pH, oxygen tension and flow. There is much evidence to suggest that dilatation of blood vessels evoked by increased flow is mediated by EDRF (see Introduction). During hypoxia the perfused coronary circulation releases ATP (Paddle & Burnstock, 1974; Hopwood & Burnstock, 1987), substance P and ACh (Burnstock *et al.*, 1988) in addition to adenosine (Berne, 1963; Sollevi, 1986) and in the present study during increased flow the perfused guinea-pig heart released ATP (Chapter 8). As mentioned earlier, the nature of this preparation has made it difficult to remove the endothelium (and still maintain a viable preparation) as a means of establishing the source of these substances. However, electron

microscopy coupled with immunocytochemistry has identified the presence of substance P and ChAT (the ACh synthesizing enzyme) in endothelial cells (Burnstock *et al.*, 1988; Loesch & Burnstock, 1988; Milner *et al.*, 1989). Further, ATP has been shown to be contained within and may be released from cultured coronary endothelial cells (Nees & Gerlach, 1983), thus a source of ATP release during increased flow or hypoxia may be from endothelial cells. The released ATP could then act on specific endothelial receptors on the coronary vasculature which trigger the release of NO or a prostanoid.

Under normal conditions vasoactive substances such as ATP, 5-HT and substance P may act on their respective endothelial receptors to mediate vasodilatation, via NO or a prostanoid, following release from endothelial cells. A defective release and/or effect of vasoactive substances from endothelial cells may be responsible for a variety of clinical disorders including one highly relevant to the coronary circulation, namely atherosclerosis (Harrison, 1988; Verbeuren & Herman, 1988). A characterisation of this disease is the formation of plaques in the subendothelial space and both morphological and pharmacological studies predict the participation of a dysfunctional endothelium (Woolf, 1983; Rosenfeld *et al.*, 1987; Lüscher, 1988). Several studies have demonstrated that atherosclerotic blood vessels are very susceptible to the development of vasospasm: an increase in sensitivity of vascular smooth muscle to various agents including 5-HT, histamine, NA, phenylalanine and angiotensin II has been demonstrated *in vivo* and

in vitro in hypercholesterolemic and atherosclerotic animals (Heistad *et al.*, 1984; Tozzi *et al.*, 1985; Hof & Hof, 1988). Conversely, endothelium-dependent relaxations to ACh, substance P, bradykinin, ATP and ADP have been shown to be attenuated in atherosclerotic vessels (Chappell *et al.*, 1985; Jayakody, 1985; Freimann *et al.*, 1986; Ibengwe & Suzuki, 1986; Verbeuren *et al.*, 1986a, 1986b; Forstermann *et al.*, 1988; Hof & Hof, 1988). It is likely that there are multiple abnormalities of endothelium-dependent relaxation since bioassay studies have shown both a normal (Verbeuren *et al.*, 1986a) and a decreased (Harrison *et al.*, 1988) luminal release of EDRF in atherosclerotic vessels. In dogs with experimental stenosis of the femoral and carotid artery and intact endothelial cells, prostacyclin production increases with increased luminal narrowing in stenosed segments (Qvarfordt *et al.*, 1985). While this may represent a normal physiological response of endothelial cells to prevent platelet deposition and encourage vasodilatation in the stenosed segments, it is conceivable that with damaged endothelial cells covering an atherosclerotic plaque this mechanism may not be fully operative

9.2 CORONARY VASODILATATION IN HYPOXIA: REVISION OF BERNE'S HYPOTHESIS.

Oxygen deficiency in the blood tissue is called hypoxia. Hypoxia is usually classified into four categories: *hypoxic* hypoxia, in which the oxygen tension in the arterial blood is low;

anaemic hypoxia, in which the oxygen carrying capacity of the blood is low; *stagnant* or *ischaemic* hypoxia, due to circulatory inadequacy; and *histotoxic* hypoxia, in which the tissues cannot utilize the oxygen supplied to them even though the amount of this is normal.

Hypoxic hypoxia is due to a low tension of oxygen in the arterial blood resulting in a low oxygen content. Anaemic hypoxia is due to a reduction in the haemoglobin available for oxygen carriage, but the blood oxygen tension is normal and occurs in anaemia, methaemaglobinaemia and carbon monoxide poisoning. In stagnant hypoxia, the oxygen tension in the blood leaving the lungs is normal, as is the haemoglobin content of the blood, but the circulation is so sluggish that insufficient oxygen may be available for the tissues' needs. It may occur in heart failure, haemorrhage and shock. The cells are poisoned in histotoxic hypoxia and are unable to utilize the oxygen supplied to them. For example, cyanide inhibits the enzyme cytochrome oxidase and interferes with tissue oxidation.

An oxygen deficiency in the blood is termed hypoxaemia. Berne (1963) proposed that a reduction in myocardial oxygen tension by hypoxaemia, decreased coronary blood flow, or increased oxygen utilization by the myocardial cells leads to the breakdown of heart muscle adenine nucleotides to adenosine. The adenosine diffuses out of the cell and reaches the coronary arterioles via the interstitial fluid and produces arteriolar dilation. It was proposed that the resultant increase in coronary blood flow (Berne, 1963) elevates myocardial oxygen tension, thereby reducing

the rate of degradation of adenine nucleotides, and decreasing the interstitial fluid concentration of adenosine by washout and enzymatic destruction. Berne (1963) suggested that this feedback mechanism serves to adjust coronary blood flow to meet the new metabolic requirements and a new steady state is reached. Restoration of normal myocardial oxygen tension prevents the degradation to adenosine, and permits the intrinsic vascular tone to reduce coronary blood flow to control levels. This hypothesis, although old, is still widely accepted. However, the discovery of the importance of vascular endothelial cells in the control of vascular resistance demands that this hypothesis should be reviewed.

In the types of hypoxia in which the myocardial cells are hypoxic before the blood, such as in stagnant or ischaemic hypoxia then Berne's hypothesis may still be important. On the other hand however, in hypoxic hypoxia or anaemic hypoxia, where the blood supply is hypoxic before the myocardial cells, the cells that are in direct contact with the blood, i.e. endothelial cells, would be more appropriately positioned to detect and respond to the conditions of the blood. Several lines of evidence support this view. Busse *et al.* (1983) have shown that endothelial cells are important in mediating hypoxic vasodilatation. In the guinea-pig coronary vasculature, hypoxic vasodilatation has been reduced by hydroquinone (Hopwood *et al.*, 1989), a compound which, by virtue of its free-radical quenching properties, destroys EDRF (Moncada *et al.*, 1986). There is evidence for the presence of adenosine A_2-purinoceptors in cultured coronary endothelial cells (Des

Rosiers & Nees, 1987) and the present study (Chapter 4) has shown that part of the vasodilator response to adenosine is mediated via NO. It has also been reported that cultured coronary endothelial cells release adenosine during exposure to hypoxia and hypercarbia (Nees *et al.*, 1980). This adenosine could then act on endothelial cell A_2-purinoceptors to induce relaxation via NO.

However, methylxanthines, the antagonists of P_1-purinoceptors are virtually ineffective at reducing the vasodilatation seen during hypoxia or reactive hyperaemia at concentrations where they fully block the vasodilatations due to adenosine (Spark & Gorman, 1987). Further, Ishibashi *et al.* (1985) showed that the time course of adenosine release from hypoxic heart was too late for the elicited vasodilatation to be due to adenosine. This raises the possibility that substances in addition to adenosine contribute to hypoxic vasodilatation.

Our understanding of the role played by endothelial cells in vascular homeostasis has been greatly improved by the availability of endothelial cell cultures. This technique has allowed investigations in different aspects of nucleotide metabolism exerted by the endothelium, such as extra- and intracellular catabolism, synthesis, uptake and release of purines in the intravascular space (Pearson & Gordon, 1985). Purines are stored presumably as ATP (see Pearson & Gordon, 1985) and can be released from coronary endothelial cells under various conditions (Nees & Gerlach, 1983). ATP has been shown to be released from the Langendorff heart in response to hypoxia (Paddle & Burnstock, 1974; Clemens & Forrester, 1981). It has been proposed

(Burnstock, 1989; Hopwood *et al.*, 1989) that during reactive hyperaemia following ischaemia, ATP is released into the blood from endothelial cells, and then acts on P_{2Y}-purinoceptors (Martin *et al.*, 1985; Burnstock & Warland, 1987) leading to the release of EDRF and a subsequent vasodilatation. The present study has demonstrated that in the guinea-pig coronary vasculature ATP induced relaxation is through activation of P_{2Y}-purinoceptors on the endothelium which initiates relaxation via NO (Chapter 5). Additionally, ATP induces relaxation via prostanoids (Chapter 5 & 7).

The ATP released from endothelial cells is rapidly broken down to adenosine (see Pearson & Gordon, 1985) by an ectoenzyme system located on the endothelial cell surface, which represents a protective mechanism limiting the extent of vasodilatation caused by circulating ATP. Adenosine thus: **a**) acts on A_2-purinoceptors on the endothelium to initiate relaxation via NO (Chapter 4); **b**) diffuses through the intimal layer to occupy P_1-purinoceptors on the subjacent smooth muscle cells; **c**) may reach the adventitia to act on P_1-purinoceptors on perivascular sympathetic nerve terminals to reduce the release of NA and thereby minimize vasoconstrictor actions and **d**) is taken up by the endothelium via an endothelial adenosine carrier system, where it will be converted to ATP. As such, the ectoenzyme and the endothelial adenosine uptake systems, in combination, form a biochemical cycle for the control of vascular tone.

Other vasodilators have also been shown to be present inside endothelial cells of several blood vessels. For example,

5-HT is localized in endothelial cells of rat coronary arteries and it has been shown to be released from the perfused rat heart during hypoxia (Burnstock *et al.*, 1988). The present study has demonstrated that low doses of 5-HT induce relaxation almost exclusively via NO (Chapter 3). Ultrastructural localisation of substance P in vascular endothelial cells in rat femoral, mesenteric and coronary arteries has also been demonstrated (Loesch & Burnstock, 1988; Milner *et al.*, 1989). Substance P has also been shown to be released from rat coronary arteries during hypoxia (Milner *et al.*, 1989). This released substance P could then initiate relaxation via NO as is demonstrated in the guinea-pig coronary vasculature in the present study (Chapter 3).

Hence it is proposed that, since endothelial-derived substances can be released from endothelial cells and act on specific endothelial receptors to elicit vasodilatation during hypoxia, these substances and the endothelium feature crucially in any consideration of hypoxic vasodilator mechanisms.

9.3 PURINOCEPTOR SUBTYPES IN THE CORONARY CIRCULATION

9.3.1 P_2-Purinoceptors

Shortly after endothelium-dependent vasodilatation was identified, it was recognized that in many blood vessels the powerful dilator actions of ATP and ADP are mediated in this way (De Mey & Vanhoutte, 1981). Stimulation of endothelial cells by ATP leads to release of the labile, potent dilator EDRF (Kelm &

Schrader, 1988; Boeynaems & Pearson, 1990), now identified as NO (Palmer *et al.*, 1987), which relaxes subjacent smooth muscle. These observations provided the first indication that P_2-purinoceptors are present on endothelial cells and it has been shown directly that ATP releases NO from cultured aortic endothelial cells (Kelm *et al.*, 1988). P_{2Y}-purinoceptors are usually characterized by an agonist potency order where 2-meSATP is more potent than ATP (Burnstock & Kennedy, 1985). Using this potency order it was determined that P_{2Y}-purinoceptors are present on the endothelium of canine coronary blood vessels (Houston *et al.*, 1987). In the present study the relative order of potency of ATP and its analogues for inducing relaxation in the guinea-pig coronary vasculature is characteristic of P_{2Y}-purinoceptors. A major part of the vasodilator response to 2-meSATP was found to be mediated via NO (Chapter 5). This implies that there is a population of P_{2Y}-purinoceptors on the endothelium which when activated induce relaxation via NO. Although L-NAME did not alter the peak vasodilator response to ATP the duration of the response was reduced (Chapter 3 & 5). This suggests that at least part of the vasodilator response to ATP is via release of NO. Therefore, both ATP and 2-meSATP act on a population of P_{2Y}-purinoceptors on the endothelium to elicit relaxation via NO.

ATP and ADP also induce the release of prostacyclin (PGI_2) from endothelial cells (Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984). In the guinea-pig coronary vasculature the prostaglandin synthesis inhibitor indomethacin inhibited the relaxant response to ATP but did not

affect the relaxant response to 2-meSATP (Chapter 5). Therefore activation of P_{2Y}-purinoceptors by 2-meSATP does not induce release of prostanoids. ATP induced release of prostanoids is therefore not via activation of the P_{2Y}-purinoceptors that 2-meSATP acts on. The exclusive role of P_{2Y}-purinoceptors in mediating responses to ATP is therefore challenged. Significant differences between the production of PGI_2 and NO in response to ATP have been reported. In particular, PGI_2 release is rapid in onset, transient in the continued presence of ATP and followed by a period of refractoriness to a new challenge (Toothill *et al.*, 1988). NO release is also rapid, but can be sustained for many minutes and cumulative dose-response curves can be constructed (Martin *et al.*, 1985; Kelm *et al.*, 1988). These differences presumably reflect control by different elements of the cellular signalling pathway.

It has become increasingly apparent that many responses to 5'-nucleotides do not fit exactly into the P_{2X}/P_{2Y} classification, including those that are insensitive to both 2-meSATP and β,γ-meATP (Fine *et al.*, 1989; Allsup & Boarder, 1990). In some cases these non-P_{2X} and non-P_{2Y} responses can also be elicited by UTP (Demolle *et al.*, 1988; Fine *et al.*, 1989; Davidson *et al.*, 1990; Pfeilschifter, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). For example, 2-meSATP induced a smaller release of prostacyclin from pig aortic endothelial cells than did ATP, while the pyrimidine nucleotide UTP appeared to be active and as potent as ATP (Needham *et al.*, 1987). While it has been argued that some responses are mediated by separate pyrimidinoceptors

(for UTP) and purinoceptors (for ATP and ADP) (Seifert & Schultz, 1989) there is now evidence for a 'nucleotide or P_{2U}-receptor which responds to both UTP and the purines (O'Connor *et al.*, 1991). In the guinea-pig coronary vasculature the vasodilator response to UTP was attenuated by the NO synthase inhibitor L-NAME suggesting that UTP induced relaxation was mediated in large part via NO (Chapter 7). The vasodilator response to UTP was not inhibited by indomethacin and therefore it can be surmised that prostanoids do not play a role in the vasodilator response to UTP. Whether 'nucleotide'/P_{2U}-receptors are present in the guinea-pig coronary vasculature is unclear. If they are present then action at these receptors induces relaxation via NO and not prostanoids. However, one thing is apparent, ATP induced relaxation via prostanoids is not due to its action at these newly defined P_{2U}-receptors in the guinea-pig coronary vasculature.

The question arises that if ATP is not inducing prostanoid release via P_{2Y}- or P_{2U}- receptors then what is the mechanism of action? One school of thought may be that the vasodilatation produced in response to exposure to ATP maybe in part due to its degradation to adenosine (Moody *et al.*, 1984). However there are several experimental factors that argue against this possibility. First, in the present study, the adenosine receptor antagonist 8-PSPT did not affect the response to ATP at a concentration that significantly inhibited the response to adenosine (Chapter 5). In addition, the relaxant response to ATP in the guinea-pig coronary vasculature has also been shown to be unaffected by the adenosine receptor antagonist XAC (Brown *et al.*,

1991). Secondly, this study has also demonstrated that the vasodilator response to adenosine is unaffected by the prostaglandin synthesis inhibitor indomethacin (Chapter 4). It is difficult to postulate from this study how ATP was inducing synthesis or release of prostanoids. The mechanism may not even be receptor mediated. The lack of any highly effective P_{2Y}-purinoceptor antagonists complicates the situation. In the rat coronary vasculature (chapter 6) the P_2-purinoceptor antagonist suramin attenuated the vasodilator response to 2-meSATP but not ATP. This implies that, at least in the rat coronary vasculature, ATP does not induce relaxation via the same receptor as 2-meSATP (Chapter 6).

9.3.2 P_1-Purinoceptors

In most blood vessels the vasodilator effect of adenosine has been shown to be largely due to action at A_2-purinoceptors on the vascular smooth muscle and not the endothelium (Collis & Brown, 1983; White & Angus, 1987). However at low doses adenosine has been shown to induce relaxation via receptors on endothelial cells in coronary vessels (Corr & Burnstock, 1990; Balcells *et al.*, 1992). In addition, Des Rosiers & Nees (1987) have provided functional evidence for the presence of A_2-purinoceptors on cultured coronary endothelial cells, although in the guinea-pig coronary artery, P_1-purinoceptors appear to be limited to the smooth muscle (Keef *et al.*, 1992). The present study (Chapter 4)

suggests that there is a population of A_2-purinoceptors on the endothelium of the guinea-pig coronary vasculature which, when activated, induce relaxation via NO. It has been demonstrated in the rabbit perfused heart that inhibition by the NO synthase inhibitor L-NMMA of the effect of ACh was due to a shortening of the response rather than to an effect on peak fall in perfusion pressure (Smith *et al.*, 1992). It was suggested that NO was involved in the sustained relaxation induced by ACh but not in the initial event. The same might be suggested about the vasodilator action of A_2-receptor agonists in that there appears to be two overlapping phases, a brief early phase which appears to be insensitive to L-NAME and is responsible for the initial peak depressor response, followed by a second phase of longer duration that is attenuated by L-NAME.

9.4 CONCLUDING COMMENTS

The work presented in this thesis contributes to the understanding of the mechanisms in which various vasoactive agents induce vasodilatation in the coronary vasculature. The importance of NO in mediating coronary vasodilatation is emphasised. An intriguing feature of the control of vascular tone is that some vasoactive substances, such as ATP and bradykinin, initiate relaxation via more than one mechanism: both NO and prostanoids appear to play a role in the vasodilatation observed in response to ATP and bradykinin.

The ability of the endothelium to generate a powerful vasodilator(s) that controls vessel wall diameter and consequently plays a role as a determinant of blood flow and blood pressure should be considered in relationship to phenomena such as hyperaemia, autoregulation, flow-dependent dilation, blood pressure regulation and mechanisms for oedema formation in the cardiovascular system. Clearly, a dysfunction of the endothelium, as might occur in atherosclerosis, would upset the normal sequence of events.

A greater understanding of the mechanisms involved in control of blood vessel tone will facilitate development and treatment of conditions resulting from abnormalities associated with these mechanisms.

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