AN INVESTIGATION INTO THE SIGNAL TRANSDUCTION PATHWAYS MEDIATING CALCITONIN GENE-RELATED PEPTIDE-INDUCED VASORELAXATION

A Thesis submitted by
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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a vasodilator, having at least two mechanisms of eliciting vasorelaxation, these being dependent on the presence of an intact endothelium in some tissues, but independent in others. The work presented in this thesis is an attempt to clarify the signal transduction pathways mediating CGRP vasorelaxation.

Results presented show that CGRP acts in an endothelium-dependent manner in rat thoracic aortic rings via a $CGRP_{(8-37)}$-insensitive subtype of receptor. The pharmacological profile of inhibition e.g. sensitivity to haemoglobin, methylene blue and the nitric oxide synthase inhibitors but not cyclo-oxygenase inhibitors, sodium/potassium ATPase inhibitors or ATP-sensitive potassium channel blockers, indicates that CGRP and acetylcholine release nitric oxide as their endothelium-derived relaxant factor (EDRF). While both vasodilators increase cyclic GMP levels, CGRP, in contrast to acetylcholine, also causes elevations in cyclic AMP. The elevation in cyclic GMP is probably a result of the release of nitric oxide, but the role of the cyclic AMP required further clarification.

Evidence is given indicating that CGRP may belong to a class of drugs, including β-adrenoreceptor agonists and forskolin, which are capable of stimulating nitric oxide synthase by elevating cyclic AMP levels.

The endothelium-independent relaxations induced by CGRP in the pig coronary artery are mediated by a $CGRP_{(8-37)}$-sensitive subtype of receptor and are associated with selective rises in cyclic AMP.

In summary the endothelium-dependent and -independent vasorelaxant actions of CGRP in the tissues studied appear to be mediated by two different

receptor subtypes. While both receptors appear to be linked to activation of adenylate cyclase, the endothelium-dependent mechanism involves activation of nitric oxide synthase, release of nitric oxide, stimulation of guanylate cyclase and subsequent relaxation. The endothelium-independent mechanism only involves activation of adenylate cyclase and not guanylate cyclase.

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For my parents

PUBLICATIONS

Some of the data presented in this thesis have been published in the following papers:

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(2) GRAY, D.W. & MARSHALL, I. (1990b) L-N^G-monomethylarginine and N^G-nitro-L-arginine inhibit the responses to human α-calcitonin gene-related peptide in rat aortic rings. *Blood Vessels*, **27**, 38

(3) FOULKES, R., SHAW, N., MARSHALL, I., GRAY, D.W. & HUGHES B. (1990c) Differential regional sensitivity to calcitonin gene-related peptide in pig left anterior descending coronary arteries. *J. Mol. Cell Cardiol.*, **22**, Supplement 3, PT6

(4) GRAY, D.W. & MARSHALL, I. (1991a) Isoprenaline relaxation of rat thoracic aorta is endothelium-dependent, releases nitric oxide and raises cyclic GMP and cyclic AMP. *Br. J. Pharmacol.*, **102**, 125P

(5) GRAY, D.W. & MARSHALL, I. (1991b) Human α-calcitonin gene-related peptide relaxes rat thoracic aorta by releasing nitric oxide and raising cyclic AMP and cyclic GMP. *Br. J. Pharmacol.*, **102**, 126P

(6) GRAY, D.W., MARSHALL, I., BOSE, C., FOULKES, R. & HUGHES B. (1991c) Subtypes of the calcitonin gene-related peptide receptor in vascular tissues. *Br. J. Pharmacol.*, **102**, 189P

(7) GRAY, D.W. & MARSHALL, I. (1991d) Human α-calcitonin gene-related peptide relaxes pig coronary artery in an endothelium-independent manner and elevates cyclic AMP. *Br. J. Pharmacol.*, **102**, 190P

(8) GRAY, D.W. & MARSHALL, I. (1991e) Effect of the 8-37 fragment of calcitonin gene-related peptide on relaxation and second messenger accumulations to calcitonin gene-related peptide in pig left anterior descending coronary artery. *Fund. & Clin. Pharmacol.*, **5**, 452P

(9) GRAY, D.W. & MARSHALL, I. (1991f) A pharmacological profile of the EDRF released by calcitonin gene-related peptide in rat aorta. *Regul. Peps.*, **34**, 51P

10) GRAY, D.W. & MARSHALL, I. Nitric oxide synthesis inhibitors attenuate calcitonin gene-related peptide endothelium-dependent vasorelaxation in rat aorta. *Eur. J. Pharmacol.* (manuscript submitted)

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12) GRAY, D.W. & MARSHALL, I. Isoprenaline stimulates guanylate cyclase and adenylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide. *Br. J. Pharmacol.* (manuscript submitted)

13) GRAY, D.W., MARSHALL, I., BOSE, C., FOULKES, R. & HUGHES B. Subtypes of the CGRP receptor in vascular tissue. *Br. J. Pharmacol.* (manuscript submitted)

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ABBREVIATIONS

ACH	Acetylcholine
BK	Bradykinin
CGRP	Calcitonin gene-related peptide
cyclic AMP	Adenosine 3',5'-cyclic monophosphate
cyclic GMP	Guanosine 3,'5'-cyclic monophosphate
D-ARG	D-isomer of arginine
DMSO	Dimethyl sulphoxide
EDRF	Endothelium-derived relaxant factor
EtOH	Ethanol
FOR	Forskolin
HCL	Hydrochloric acid
IP_3	Inositol 1,4,5-trisphosphate
ISO	Isoprenaline
L-ARG	L-isomer of arginine
L-ARG/NO SYNTHASE	Nitric oxide synthase
L-NMMA	L-N^G-monomethyl arginine
L-NOARG	L-N^G-nitroarginine
NO	Nitric oxide
O_2^-	Superoxide anion
SAL	Salbutamol
s.e. mean	Standard error of the mean
SNP	Sodium nitroprusside
U	International units

CHAPTER 1

INTRODUCTION

The calcitonin gene-related peptides (CGRPs) are a series of evolutionally highly conserved peptides produced as alternative products of the gene coding for the calcium homoeostasis hormone, calcitonin. Their discovery occurred in unusual circumstances. Historically, bioactive substances have been shown to exist because of the observation of inexplicable (by current knowledge) phenomena, mostly from the application of homogenised tissue extracts to smooth muscle or other preparations e.g. bradykinin and prostaglandins. The substance responsible is then purified and, in the case of a peptide, sequenced.

The existence of CGRP, however, was predicted in 1981 by Rosenfeld *et al.* after the observation that a rat medullary thyroid carcinoma cell line produced multiple messenger ribonucleic acids (mRNAs) and that the alterations in the levels of these were associated with the switching of the cells from high to low calcitonin production. It was postulated that two mRNAs were being transcribed from the calcitonin gene and that the differential control of the genetic material results in either calcitonin or another product i.e. CGRP being favoured for expression.

Purification and sequencing of the mRNAs as well as the *in vitro* translation and the presence of the mRNAs on polyribosomes *in vivo* allowed the primary amino acid structure and existence of CGRP to be predicted

before the isolation or pharmacology had been confirmed (Amara *et al.*, 1982).

Further study in rats showed that the peptide was indeed being produced and that its structure was as predicted (Rosenfeld *et al.*, 1983). Shortly after this a second rat CGRP sequence, closely related to the first, was isolated and sequenced (Rosenfeld *et al.*, 1984) and so they were termed α- and β-CGRP respectively. The presence of a human CGRP was demonstrated in human medullary thyroid carcinoma and this peptide proved to be closely related to the rat versions (Morris *et al.*, 1984) though there is less homology between any of the CGRPs and calcitonin. A second human CGRP has also been described (Steenbergh *et al.*, 1985) and is expressed (Conlon *et al.*, 1989).

Structure

The primary structures of the various species and forms of CGRP differ only marginally (**Table 1.1**). However, the differences in amino acid sequence between all of the CGRPs and calcitonin is much greater. Structural alterations to the amino acid sequence of CGRP have indicated that it has two basic structural requirements for agonist activity, these being an amphiphilic α-helix starting at residue 8 and the disulphide bond between residues cys 2 and cys 7 (Tippins *et al.*, 1986; Lynch & Kaiser, 1988) (though some subtypes of CGRP receptor may not require the latter element (Dennis *et al.*, 1989)). In this respect the CGRPs are similar to calcitonin in that it has two analogous basic structural requirements, these being an amphiphilic α-helix between residues 8 and 22, and the disulphide bridge between cys 1 and cys 7 (Moe & Kaiser, 1985; Green *et al.*, 1987).

	S----S
Human α-CGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
Human β-CGRP	A N M S K
Rat α-CGRP	S N V D E
Rat β-CGRP	S N V D K
	S-----S
Human calcitonin	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP
Salmon calcitonin	S V GKLS ELH LQ Y R NT S T

Table 1.1 Table showing the primary amino acid structures of human α-CGRP, human calcitonin and some related peptides. Amino acid residues are represented by the single letter code and S---S indicates a disulphide link between two cysteine residues.

Localisation

Attempts have been made to clarify the distribution of the peptide within the body. CGRP proves to have an extremely wide distribution in both the central and peripheral nervous systems as well as in some non-neuronal tissues. **Tables 1.2** and **1.3**, although by no means comprehensive, illustrate this point.

Co-localisation

While the above studies were being carried out many research groups considered the possibility that CGRP was co-localised in neurons and other tissues with other neurotransmitters or neuromodulators. Double immunoreactivity studies, utilising two antibodies recognising CGRP and the putative co-localised substance respectively, were performed and images obtained compared for overlap of immunoreactivity. From these studies it appears that CGRP is co-localised with a range of known and putative neurotransmitters or neuromodulators. These are presented in **Table 1.4.**

PHARMACOLOGY OF CGRP

a) Cardiovascular Effects

It became apparent quite soon after the discovery of CGRP that application of exogenous CGRP caused a number of actions on the cardiovascular system. These proved to be complex and varied considerably depending on the site of administration. These effects can be conveniently divided into the effects of central and peripheral administration.

TISSUE	SPECIES	REFERENCES
AMYGDALOID NUCLEUS	RAT	INAGAKI *et al.*, 1988
HYPOTHALAMUS	RAT TELEOST FISH	INAGAKI *et al.*, 1988 BATTEN & COMBRE, 1989
NUCLEUS TRACTUS SOLITARIUS	RAT	KUBOTA *et al.*, 1988 KAWAI *et al.*, 1985
GRACILE NUCLEUS	RAT	KUBOTA *et al.*, 1988
CUNEATE NUCLEUS	RAT	KUBOTA *et al.*, 1988
GENICULATE NUCLEUS	RAT	KUBOTA *et al.*, 1988
TRIGEMINAL GANGLION	GUINEA PIG CAT RABBIT RAT	UDDMAN *et al.*, 1986 EDVINSSON *et al.*, 1987 McCULLOCH *et al.*, 1986 EDVINSSON *et al.*, 1987 EDVINSSON *et al.*, 1987 EDVINSSON *et al.*, 1987
PERIVASCULAR NERVES IN ALL MAJOR BLOOD VESSELS.	CAT RAT GUINEA PIG RABBIT	McCULLOCH *et al.*, 1986 EDVINSSON *et al.*, 1987 SAITO *et al.*, 1989 EDVINSSON *et al.*, 1987 EDVINSSON *et al.*, 1987 EDVINSSON *et al.*, 1987
SPINAL CORD	PIG MAN	KIMURA *et al.*, 1987 GIBSON *et al.*, 1984

Table 1.2 Table showing the extensive distribution of CGRP-like immunoreactivity in the brain.

TISSUE	SPECIES	REFERENCES
DORSAL ROOT GANGLION	RAT GUINEA PIG	GIBSON *et al.*, 1984 UDDMAN *et al.*, 1986
FIBRES IN DORSAL HORN	MAN	TSCHOPP *et al.*, 1985
CEREBROSPINAL FLUID	MAN	WIMALAWANSA & MACINTYRE, 1987
PERIVASCULAR NERVES IN ALL VASCULAR BEDS	GUINEA PIG RABBIT RAT	LUNDBERG *et al.*, 1985 BAUERFIEND *et al.*, 1989 KAWASAKI *et al.*, 1988 MIYAUCHI *et al.*, 1988
FIBRES IN THE HEART	RAT GUINEA PIG	LUNDBERG *et al.*, 1985 MIYAUCHI *et al.*, 1988 GIBBINS *et al.*, 1985 ISHIKAWA *et al.*, 1987
FIBRES IN THE LUNG	HAMSTER	KEITH & EKMAN, 1988
MYENTERIC NEURONS	CAT RAT	PARKMAN *et al.*, 1989 CLAGUE *et al.*, 1985
MOTOR NEURONS	FROG RAT MOUSE	MATTEOLI *et al.*, 1988 FRIED *et al.*, 1989 TAKAMI *et al.*, 1985
THYROID	RAT	ZAIDI *et al.*, 1986
MERKEL CELLS OF ORAL MUCOSA	CAT	GAUWEILER *et al.*, 1988

Table 1.3 Table showing the extensive distribution of CGRP-like immunoreactivity in the periphery.

	TISSUE	REFERENCES
SUBSTANCE P	SMALL DIAMETER SENSORY FIBRES	GIBSON *et al.*, 1984 GAZELIUS *et al.*, 1987
	PERIVASCULAR NEURONS	LUNDBERG *et al.*, 1985 UDDMAN *et al.*, 1986 EDVINSSON *et al.*, 1987
	DORSAL ROOT GANGLION	GIBBINS *et al.*, 1985
5-HYDROXYTRYPTAMINE	LUNG	KEITH & EKMAN, 1988
ACETYLCHOLINE	MOTOR NEURONS	TAKAMI *et al.*, 1985 NEW & MUDGE, 1986

Table 1.4 Table showing substances known to be co-localised with CGRP.

i) Effects of central administration.

In the central nervous system, CGRP has a number of different effects on the vasculature. These effects can be ascribed to CGRP acting on systems responsible for the maintenance of blood pressure. The earliest studies used intracerebroventricular administration of rat α-CGRP in the rat to assess the central effects of CGRP. This relatively non-specific method of applying the peptide caused a transient increase in blood pressure accompanied by a longer lasting tachycardia (Fischer *et al.*, 1983). The increased levels of plasma noradrenaline observed in this study were thought to be sufficient to account for all these observations.

More recent studies, however, (Gardiner *et al.*, 1988a) have questioned whether CGRP causes a non-specific, centrally mediated increase in sympathetic outflow. While at high concentrations of CGRP, this effect is present, at lower concentrations, the pharmacological effects are seen without the accompanying general increase in circulating catecholamines. It has been suggested that this is due to a more subtle effect of CGRP, perhaps mediating selective increases in sympathetic output to individual vascular beds (Gardiner *et al.*, 1988a).

When specific areas of the brain were targeted for administration of CGRP, more complex effects were observed. In the amygdala of the rat, where the cardiovascular and autonomic changes associated with the 'fight or flight' response are thought to be mediated, the effects of rat α-CGRP application were analogous to those seen on intracerebroventricular administration i.e. increase in mean arterial pressure, heart rate and levels of circulating noradrenaline (Brown & Gray, 1988). However, when applied in the nucleus tractus solitarius of the rat, responsible for the relay of visceral sensory information, a different pattern of response was observed with lower

doses causing a decrease in mean arterial pressure accompanied by a marked bradycardia and higher doses causing a pressor response without affecting heart rate (Vallejo *et al.*, 1988). With both these areas of the central nervous system containing neurons immunoreactive to CGRP, it is possible that CGRP is involved in the central control of cardiovascular homeostasis.

ii) Effects of peripheral administration of CGRP

The response to administration of a variety of forms of CGRP in the periphery is simpler with all groups showing a pronounced hypotensive effect with an associated tachycardia in both rats and dogs (Lappe *et al.*, 1987; Gardiner *et al.*, 1988b; 1989; Xu *et al.*, 1989; Verburg *et al.*, 1989). The use of the pithed rat preparation, with the loss of central reflexes, indicated that the tachycardia was only partially a reflex response since the response was reduced, but still present (Haass & Skofitsch, 1985). Addition of the standard cholinergic and adrenergic antagonists in anaesthetised rats confirmed this observation as the tachycardia, while again being reduced, was still present. The Langendorff rat perfused heart preparation also clearly shows a tachycardia (Marshall *et al.*, 1986b).

Recently, the use of new techniques has allowed the *in vivo* alterations in blood flow to be measured in different vascular beds. These studies appear to indicate a degree of selectivity in the vasodilation induced by CGRP. In the rat and dog the renal artery appears to be particularly sensitive to both human α-CGRP and human β-CGRP, while the mesentery normally shows a vasoconstriction to low doses of CGRP, only higher doses giving the relaxant effect (Gardiner et al., 1988b; 1989; Villareal et al., 1988). The limited studies carried out so far in man seem to indicate that human α-CGRP has some selectivity for the cerebral circulation (MacDonald *et al.*, 1989).

While there is an apparent selectivity *in vivo* for CGRP, the results of all the *in vitro* studies demonstrates clearly that CGRP is a vasodilator of all vascular beds, including both the renal artery and the perfused mesentery (Marshall *et al.*, 1986a; 1988). These results indicate that the vasoconstriction seen *in vivo* in the mesentery is a result of compensatory reflexes.

Initially, there was some dispute over the role of the endothelium in the relaxant response induced by CGRP in isolated vessels. In the rat aorta (Brain *et al.*, 1985; Uddman *et al.*, 1986; Grace *et al.*, 1987) and mesenteric artery (Al-Kazwini *et al.*, 1987; McGrath *et al.*, 1988) the relaxant response to rat α-CGRP and human α-CGRP was wholly endothelium-dependent whereas in the rat perfused mesentery (Han *et al.*, 1990) and bovine coronary artery (Greenberg *et al.*, 1987), it appears to be independent of the presence of the endothelium. It has since become apparent that CGRP has two mechanisms of inducing vasorelaxation, one endothelium-dependent, and the other endothelium-independent. Further, they could both exist within the same vascular bed.

Although the smaller vessels are more likely to exhibit an endothelium-independent relaxant effect to CGRP and the larger vessels an endothelium-dependent relaxant effect (**Table 1.5**), this is not a strict trend. There are a number of exceptions and considerable species variation. The only vascular bed which exhibits a consistency is the cerebral vasculature which is reported to be endothelium-independent in all species studied so far (Edvinsson *et al.*, 1985; 1987; Marshall, 1989).

The cardiovascular effects of CGRP described above coupled with the distribution of CGRP immunoreactive neurons and circulating CGRP suggests that, as well as a central role, CGRP may also have an important role in the control of blood pressure in the periphery.

ENDOTHELIUM-DEPENDENT		
SPECIES	VESSEL	REFERENCES
RAT	AORTA	BRAIN *et al.*, 1985
RAT	MESENTERIC ARTERY	AL-KAZWINI *et al.*, 1988
RAT	CORONARY ARTERY	PRIETO *et al.*, 1991

ENDOTHELIUM-INDEPENDENT		
SPECIES	VESSELS	REFERENCES
MAN	PIAL ARTERIES	MARSHALL, 1989
MAN	EPICARDIAL ARTERIES	FRANCO-CERECEDA, 1991
MAN	SKELETAL MUSCLE ARTERIES	PERNOW, 1989
COW	CORONARY ARTERY	GREENBERG *et al.*, 1987
PIG	CORONARY ARTERY	FRANCO-CERECEDA, 1987
PIG	SPLENIC ARTERY	PERNOW, 1989
CAT	CEREBRAL ARTERIES	EDVINSSON *et al.*, 1985
RAT	PERFUSED MESENTERY	HAN *et al.*, 1990
RAT	CAUDAL ARTERY	FISCUS *et al.*, 1991
RAT	CORONARY ARTERY	PRIETO *et al.*, 1991

Table 1.5 Table showing the distribution of the endothelium-dependent and -independent mechanisms of vascular relaxation.

b) Gastrointestinal actions

CGRP has a number of complex actions on the gastrointestinal tract. As with the cardiovascular effects, these can be divided into central and peripheral effects.

i) Effects of central administration.

Intracerebroventricular administration of human α-CGRP and rat α-CGRP in the rat causes decreased gastric acid secretion, decreased bicarbonate secretion (Lenz *et al.*, 1989) and restoration of the cyclical pattern of electrical activity in the rat jejunum to the 'fasted' state (Fargeas *et al.*, 1985). These affects are associated with a decreased food intake (Krahn *et al.*, 1984; Tannenbaum & Goltzman, 1985). Although not conclusively disproved, a centrally mediated sedative effect of CGRP causing these events seems unlikely as water intake remains at the normal levels. No localisation of the various systems mediating these effects of CGRP have been reported. However, these actions of CGRP are closely mirrored by the effects of calcitonin administered in the same fashion. A novel receptor has been described in the central nervous system having equal affinity for CGRP and calcitonin whereas peripherally, these peptides act on distinct receptors (see Chapter 7). In view of the low levels of calcitonin mRNA in the brain (Jacobs *et al.*, 1982) but much higher levels of CGRP mRNA, it seems likely that any endogenous ligand mediating the central effects on gastrointestinal function is CGRP.

ii) Effects of peripheral administration

Peripheral administration of human α-CGRP in rabbits, like central administration in rats, causes inhibition of gastric acid secretion (Bauerfiend *et al.*, 1989) and ion transport (McCulloch & Cooke, 1989), though at least a component of these effects is mediated indirectly through release of

somatostatin (Cox *et al.*, 1989). *In vitro*, CGRP causes relaxation of many gastrointestinal smooth muscle types including gastric smooth muscle cells (Maton *et al.*, 1988) and sphincters (Parkman *et al.*, 1989) from a variety of species.

The gastrointestinal system, like the cardiovascular system, is densely innervated with neurons immunoreactive for CGRP. *In situ* hybridisation experiments have indicated that the predominant form in enteric neurons is β-CGRP (Mulderry *et al.*, 1988).

c) Role in sensory perception.

In the dorsal root ganglion of the rat certain small diameter C-fibres thought to mediate nociception are immunoreactive for CGRP and express CGRP mRNA, predominantly of the α-CGRP type (Mulderry *et al.*, 1988). Exogenously applied rat α-CGRP enhances the calcium current (Ono *et al.*, 1989) while antisera against CGRP has an antinociceptive effect in this region (Kuraishi *et al.*, 1988). It therefore seems possible that CGRP is involved physiologically as a sensory neuropeptide.

d) Neurotransmission/modulation

A number of criteria have been derived empirically which must be satisfied before a substance can be defined as a neurotransmitter. On the basis of these, CGRP may have a role as a transmitter substance. It is synthesised in neurons and can be shown to be stored within a distinct population of vesicles (Gazelius *et al.*, 1987). Neuronal stimulation and various noxious stimuli including ischaemia can release the peptide from the neurons (Belia & Burnstock, 1988; Miyauchi *et al.*, 1988; Franco-Cereceda *et al.*, 1989). Specific receptors have been identified for CGRP (see Chapter 7). Exogenous application of CGRP mimics the effects of neuronal

stimulation in a number of situations (Kawasaki et al., 1988). CGRP is metabolised into C terminal fragments (Mulderry *et al.*, 1987) and can also be rendered physiologically inactive in some situations by removal from the active site into the blood (Zaidi *et al.*, 1985). Finally, agents which inhibit CGRP actions also inhibit the effects of neuronal stimulation (Kuraishi *et al.*, 1988; Maggi *et al.*, 1991).

In view of CGRP satisfying a substantial number of the classical criteria for identification of a neurotransmitter it may play a physiological/pathophysiological role in mediating some of the pharmacological effects described above.

CGRP also appears to be a neuromodulator, altering the effects of other transmitters, particularly those with which it is co-localised (**Table 1.4**). In the isolated rat diaphragm, it has been reported that CGRP is capable of increasing the twitch response to phrenic nerve stimulation (caused by acetylcholine release) but does not have a direct action itself on the quiescent muscle (Ohhashi & Jacobwitz, 1988). After blockade of the nicotinic receptors with d-tubocurarine, CGRP enhanced the twitch response to direct muscle stimulation. This suggests that CGRP acts post-junctionally to directly affect the transduction of the impulse to muscular contraction.

Another post-junctional neuromodulatory site of action for CGRP is postulated in the mouse vas deferens (Al-Kazwini *et al.*, 1986). In this tissue it has been shown that both human α-CGRP and rat α-CGRP, in contrast to in the phrenic nerve/diaphragm described above, inhibit the contractile response to field stimulation. This effect is blocked by neither propranolol nor idazoxan and is not due to any effect on noradrenergic uptake.

e) Trophic effects

There have been a number of reports describing the trophic effects of CGRP. These include regulation of the α-subunit of the nicotinic receptor in cultured chick myocytes (Fontaine *et al.*, 1984), an effect thought to be mediated through increased expression of the α-subunit mRNA. Recently, it has been shown that CGRP is the endogenous substance which causes conversion of a number of interneurones in the rat olfactory bulb from a GABA-ergic to a dopaminergic phenotype (Denis-Donini, 1989).

Summary

CGRP has a wide distribution in neuronal tissues throughout both the central and peripheral nervous systems. CGRP-immunoreactive fibres exist in regions where application of exogenous CGRP causes a range of effects on the cardiovascular, gastrointestinal and sensory systems. The classical criteria for identification of a neurotransmitter are largely satisfied by CGRP. This may mean that CGRP has a number of important physiological roles.

Aims

The aims of this project were to try to elucidate the signal transduction mechanisms responsible for both the endothelium-dependent and -independent vasorelaxant actions of exogenously applied CGRP. Initially this involved the setting up two vascular bioassay systems exhibiting endothelium-dependent and -independent relaxation to CGRP. The pharmacology of exogenously applied CGRP was investigated in these tissues before the intracellular biochemical events associated with the relaxant response were studied.

CHAPTER 2

MATERIALS AND METHODS

In order that the mechanisms by which CGRP induces its vasorelaxant responses could be studied, two model systems displaying the endothelium-dependent and independent modes of CGRP relaxation respectively were required. A number of tissues were investigated including the rat isolated perfused mesentery, rabbit isolated aortic rings, rat aortic rings and pig coronary artery rings. The two most appropriate in terms of mode of CGRP relaxation and suitability for cyclic nucleotide study were used throughout the rest of the project. These proved to be the rat thoracic aortic ring preparation (endothelium-dependent) and the pig coronary artery preparation (endothelium-independent).

ISOMETRIC RECORDING

Endothelium-dependent relaxation - Rat thoracic aorta

Male Sprague-Dawley rats (300-450g) were stunned and killed by cervical dislocation. The thoracic aortae were removed, cleared of fat and connective tissue, and cut into rings of approximately 3mm in length. The endothelium was removed in some experiments by gently abrading the intimal surface with fine wires. The failure of acetylcholine (10^{-6}M) to elicit a relaxant response in the presence of tone induced by noradrenaline (10^{-7}M) was taken as an indication of endothelium removal. This was confirmed in some experiments histologically (see below).

The rings were mounted on tungsten wires (0.125mm diameter) under 0.5g (5mN) resting tension in 10 ml organ baths in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11 at 37°C and oxygenated with 95% O_2/5% CO_2. Tension was measured with Grass FT.O3 isometric transducers and recorded on a Grass (series 7) polygraph. The tissues were allowed to equilibrate for 75 minutes before being contracted with noradrenaline (10^{-7}M), a concentration which preliminary experiments indicated gave rise to approximately 80% of the maximum possible tone elicited by noradrenaline. The stability of the spasmogen response was assessed before successive cumulative concentration/effect curves to various vasodilators (Appendix 1) were constructed with at least 20 minutes between each curve (**Figure 2.1**).

Superfusion studies

In order to show release of a chemical factor from the endothelium a superfusion system was set up. This involved the preparation of a rat thoracic aortic ring denuded of endothelium as described above confirmed by lack of relaxation to acetylcholine (10^{-6}M).

Female New Zealand White rabbits (2.5-3.0 kg) were killed by overdose with pentobarbitone. The thoracic aorta was removed into Krebs solution and cannulated at both ends. The aorta was then perfused with oxygenated Krebs solution at 37°C at a flow rate of 4 ml/minute using a Watson-Marlow peristaltic pump (502-S). After a 30 minute equilibration period the perfusate from the rabbit aorta was directed to superfuse the rat aortic ring. A further 15 minute period of equilibration followed. The rat aortic ring was constricted by constant superfusion with noradrenaline 10^{-7}M through the rabbit aorta. Drugs were then added as bolus injections either over the rat aortic ring directly or through the rabbit aorta, the resulting

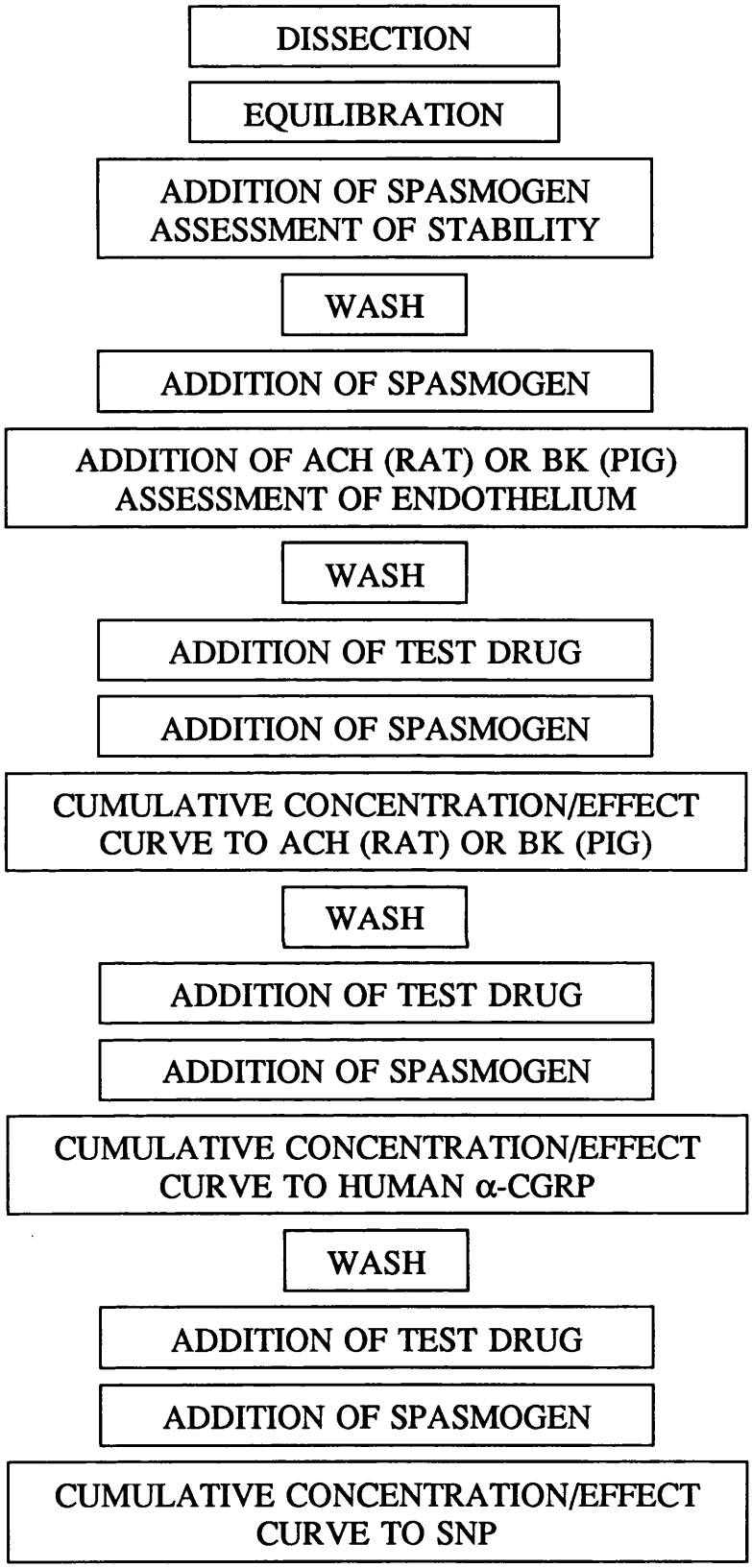


Figure 2.1 Schematic indicating the standard protocol used for pharmacological studies in both rat aortic rings and pig coronary artery rings. Controls followed a similar time-cycle but no test drug was added. (ACH is acetylcholine, BK is bradykinin and SNP is sodium nitroprusside).

effects on the rat aortic ring being measured via an isometric transducer on a polygraph.

Endothelium-independent relaxation - Pig coronary artery

Pig hearts from mixed strain and sex pigs (6-12 months) were obtained fresh from the abattoir. The left descending coronary artery was removed from just below the branch of the left circumflex artery to close to the base of the heart (**Figure 2.2**) and transported back to the laboratory in chilled Krebs solution. Two different ring segments were taken these being termed the left anterior descending coronary artery and the anterior interventricular artery.

Left anterior descending coronary artery

The larger preparation, termed the left anterior descending coronary artery (internal diameter 4mm), was taken between the top of the artery and the first branch, the conus artery (**Figure 2.2**). The artery was cut into rings of approximately 3mm in length. Initially, the endothelium was removed in some experiments by gently abrading the intimal surface with fine wires. The failure of bradykinin (10^{-6}M) to elicit a relaxant response in the presence of tone induced by U46619 (10^{-8}M) was taken as an indication of endothelium removal. This was confirmed in some experiments histologically (see below). After confirming an endothelium-independent relaxant mechanism for CGRP in this tissue the endothelium was routinely removed.

The rings were mounted on steel wires (0.40 mm diameter) under 1.0g (10 mN) resting tension in 10 ml organ baths in Krebs solution. Tension was measured with Grass FT.O3 isometric transducers and recorded on a Grass (series 7) polygraph. The tissues were allowed to equilibrate for 60 minutes before being contracted with U46619 (10^{-8}M), a concentration which

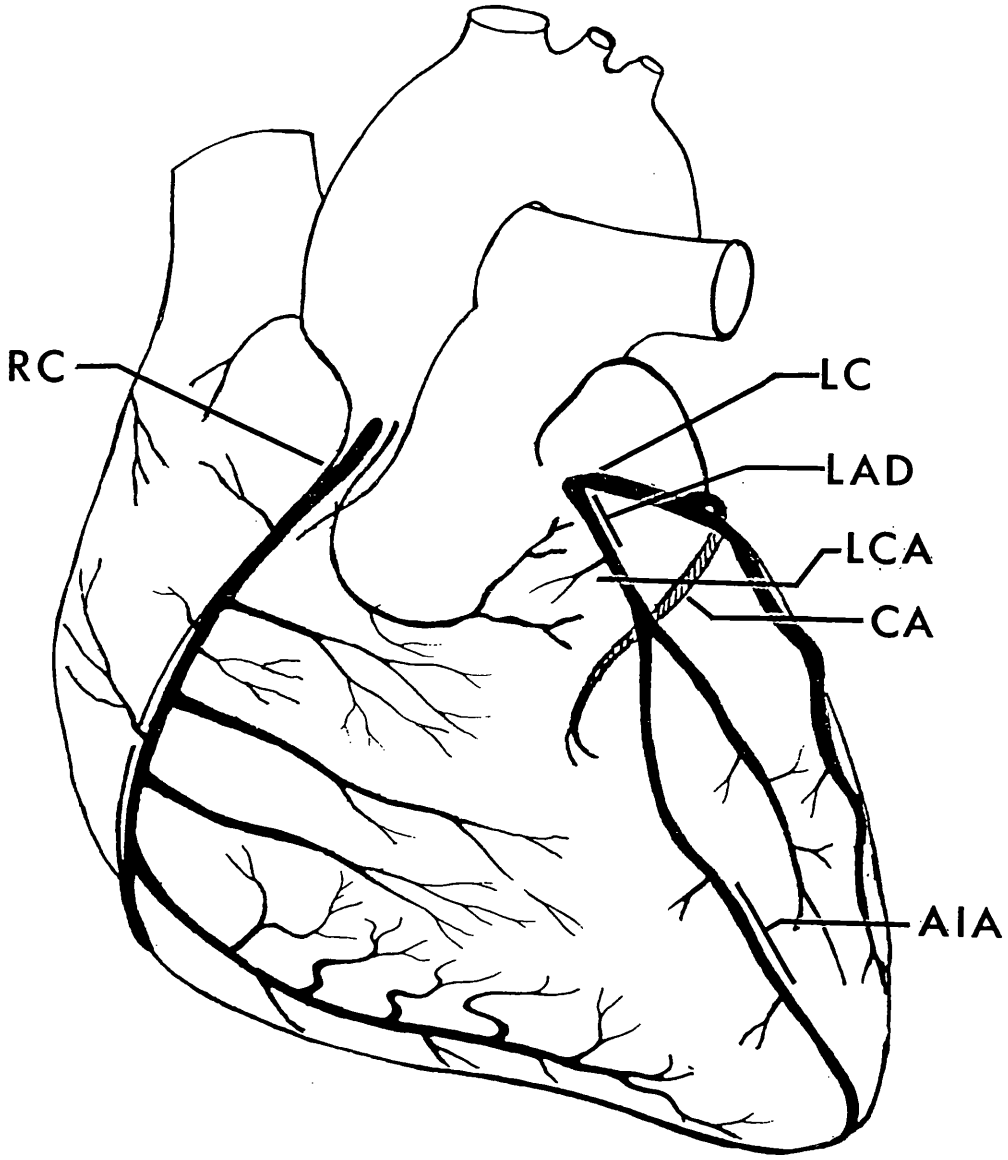


Figure 2.2 Figure showing the main arteries of the heart. RC is the right coronary artery and LC is the left coronary artery. LCA is the left conus artery and CA the circumflex artery. LAD and AIA show the regions from which the left anterior descending coronary artery and anterior interventricular artery preparations were derived.

preliminary experiments indicated gave rise to approximately 80% of the maximum possible tone elicited by U46619. The stability of the spasmogen response was assessed before successiv cumulative concentration/effect curves to various vasodilators (Appendix 1) were constructed with at least 20 minutes between each curve (**Figure 2.1**).

Anterior interventricular artery

The smaller preparation, termed the anterior interventricular artery (internal diameter 1mm), was taken from as far down the arterial trunk as possible (**Figure 2.2**). The artery was cut into rings of approximately 3mm in length. Initially, the endothelium was removed in some experiments by gently abrading the intimal surface with fine wires. The failure of bradykinin (10^{-6}M) to elicit a relaxant response in the presence of tone induced by acetylcholine ($2x10^{-7}$M) was taken as an indication of endothelium removal. This was confirmed in some experiments histologically (see below). After confirming an endothelium-independent relaxant mechanism for CGRP in this tissue the endothelium was routinely removed.

The rings were mounted on steel wires (0.40 mm diameter) under 1.0g (10 mN) resting tension in 10 ml organ baths in Krebs solution. Tension was measured with Grass FT.O3 isometric transducers and recorded on a Grass (series 7) polygraph. The tissues were allowed to equilibrate for 60 minutes before being contracted with acetylcholine ($2x10^{-7}$M), a concentration which preliminary experiments indicated gave rise to approximately 80% of the maximum possible tone elicited by acetylcholine. The stability of the spasmogen response was assessed before successive cumulative concentration/effect curves to various vasodilators (Appendix 1) were constructed with at least 20 minutes between each curve (**Figure 2.1**).

CYCLIC NUCLEOTIDE DETERMINATION

Extraction

Rings of each preparation were removed at specific time-points after experimentation into liquid nitrogen (**Figure 2.3**). The extraction of the cyclic nucleotides was carried out in a similar fashion to that of Grace et al. (1987). Briefly, rings were individually ground in ice-cold 95% ethanol at pH 3.0 in a mortar and pestle. The ground tissues were left overnight for extraction of the cyclic nucleotides. The remaining protein was then pelleted by centrifugation at 9000 x g for 1 minute. The ethanol containing the cyclic nucleotides was decanted off with the tissue being retained for protein determination (see below). The ethanol was then evaporated to dryness at 50°C under nitrogen. The sample was reconstituted in sodium acetate (50mM pH 5.0) and the sample split into two aliquots for simultaneous determination of levels of cyclic AMP and cyclic GMP by scintillation proximity assay using Amersham kits (**Figure 2.4** and Appendix 2).

Scintillation proximity assay

In an aqueous environment, the weak β particles (Auger electrons) emitted by ^{125}iodine require to be extremely close to the scintillant in order for them to produce light. This physical property has been used in scintillation proximity assay. In this technique, microspheres are impregnated with scintillant and are coated with protein A, a protein which binds all antibodies of the G type. Immunoglobin G is then raised against the required substance, in this case either cyclic AMP or cyclic GMP.

A fixed quantity of radioactive ligand is added with an unknown quantity of the same ligand from the sample. A competition is set up for the available binding sites on the immunoglobin. Fixed amounts of antibody and

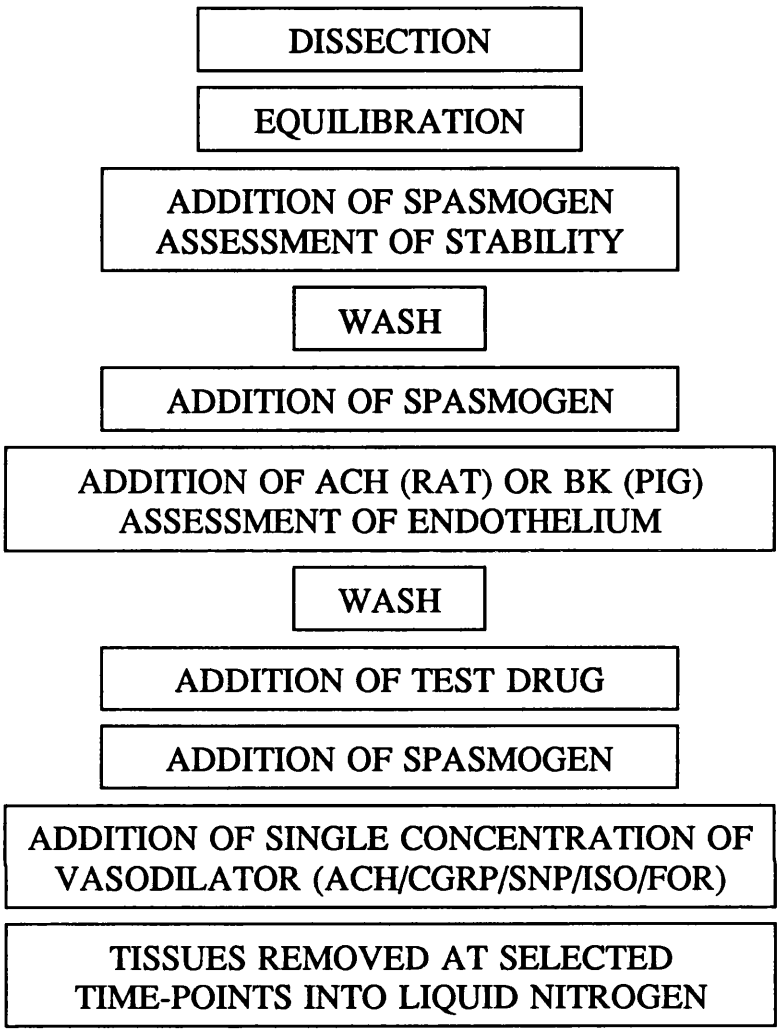


Figure 2.3 Schematic indicating the standard protocol used for preparation of both rat aortic rings and pig coronary artery rings for cyclic nucleotide studies. Controls followed a similar time-cycle but no test drug was added. (ACH is acetylcholine, SNP sodium nitroprusside, ISO is isoprenaline and FOR is forskolin).

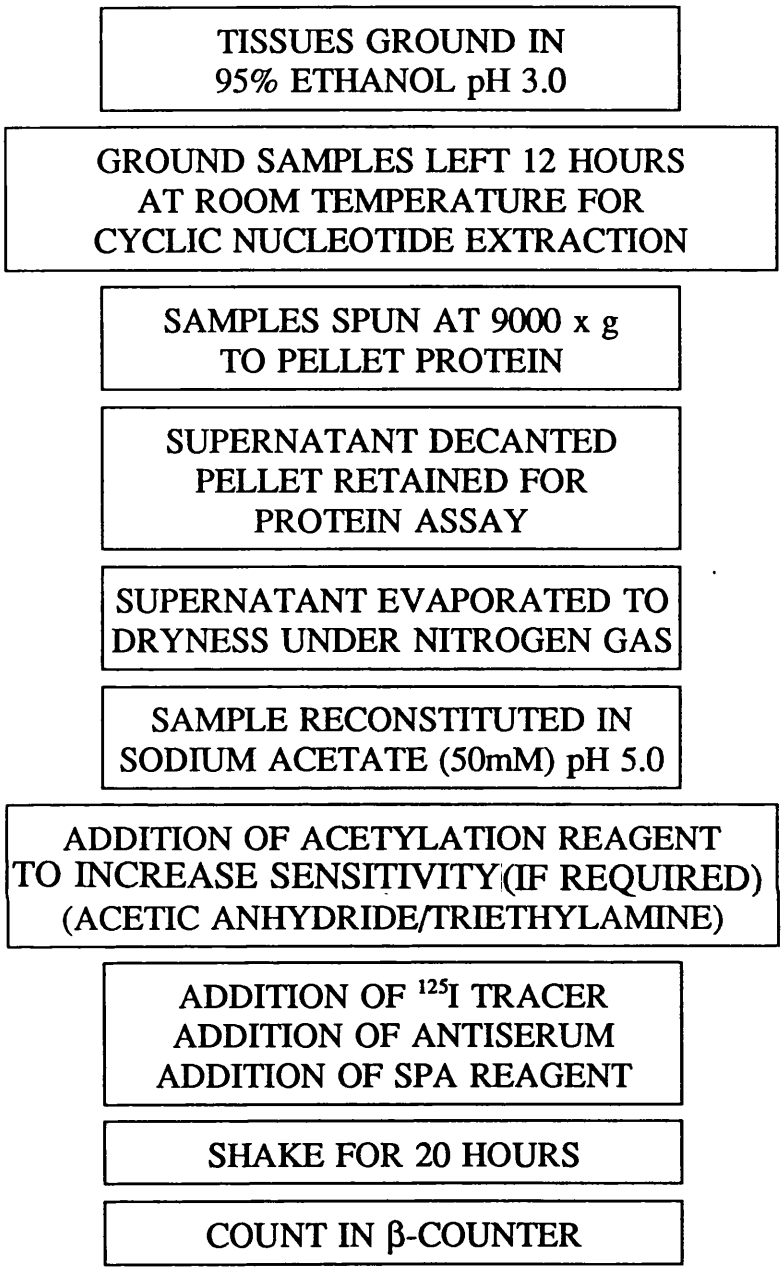


Figure 2.4 Schematic indicating the protocol for acetylation scintillation proximity assay of both cyclic AMP and cyclic GMP using Amersham kits. The non-acetylation protocol did not include addition of the acetylation reagent. (SPA is the scintillation proximity assay reagent).

radiolabelled ligand means that the amount of radioligand bound will be inversely proportional to the concentration of unlabelled ligand from the sample.

This can then be quantified in a β-counter since only the radiolabelled ligand which is bound to the immunoglobin and thus to the microspheres via protein A is close enough to the scintillant to cause fluorescence.

Levels of cyclic AMP and cyclic GMP were measured in rat aortic rings utilising the acetylation protocol. In the pig coronary artery levels were measured using the non-acetylation protocol for cyclic AMP and the acetylation protocol for cyclic GMP (Appendix 2).

PROTEIN DETERMINATION

The protein content of various tissues was determined using the method of Lowry et al., (1951). This process involves the reaction of proteins in an alkali medium with copper and then, subsequently, the reduction of the phosphomolybdic-phosphotungstic reagent (Folin reagent) by the copper treated protein. The resulting blue colour was quantified in a colorimeter (Gallenkamp 4S-800) at 670nm and compared with a known control, in this case bovine serum albumin (Appendix 3).

HISTOLOGY

The technique used to show the presence or absence of endothelium on the rings was the *en face* silver staining method of Poole et al., (1958) as modified by Griffiths et al., (1984b). After the experiment, tissues were bathed in 1% silver nitrate for a period of 3 minutes. This was followed by bathing in a solution containing 3% cobalt bromide and 3% ammonium bromide for a similar period. The rings were then opened and viewed under a microscope.

As seen in **Figures 2.5** and **2.6**, the deposited silver outlines the endothelial cells which run in the direction of the blood flow. The elastic lamina exposed by removal of the endothelium stained darker than the endothelium and the cells ran at 90° to those of the endothelium.

CALCULATION OF RESULTS AND STATISTICS

Figures, points and bars given on graphs represent the mean with the error bars being the standard error of the mean. In the isometric tension recording experiments the level of relaxation (expressed as a percentage of the tone induced by the spasmogen) is plotted against $\log_{10}$[concentration of vasodilator]. The EC_{50} values (concentration required to give a half-maximal response) for each vasodilator were calculated by fitting a sigmoid curve to results from individual tissues using a commercially available curve-fitting program (GraphPad version 3.1). These values were then manipulated to give a mean EC_{50}. Analysis of Variance was used to assess the significance of differences between various concentration/effect

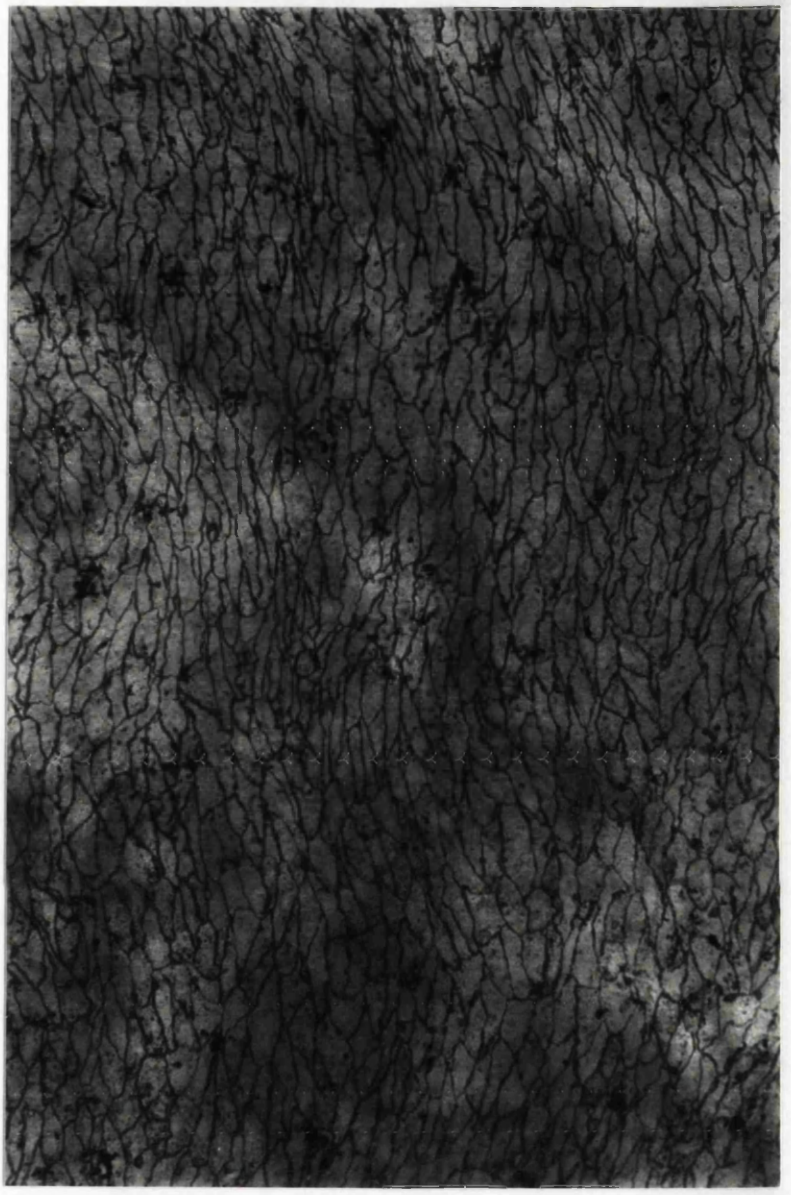


Figure 2.5 Photograph showing *en face* silver staining of a rat aortic ring with intact endothelium.

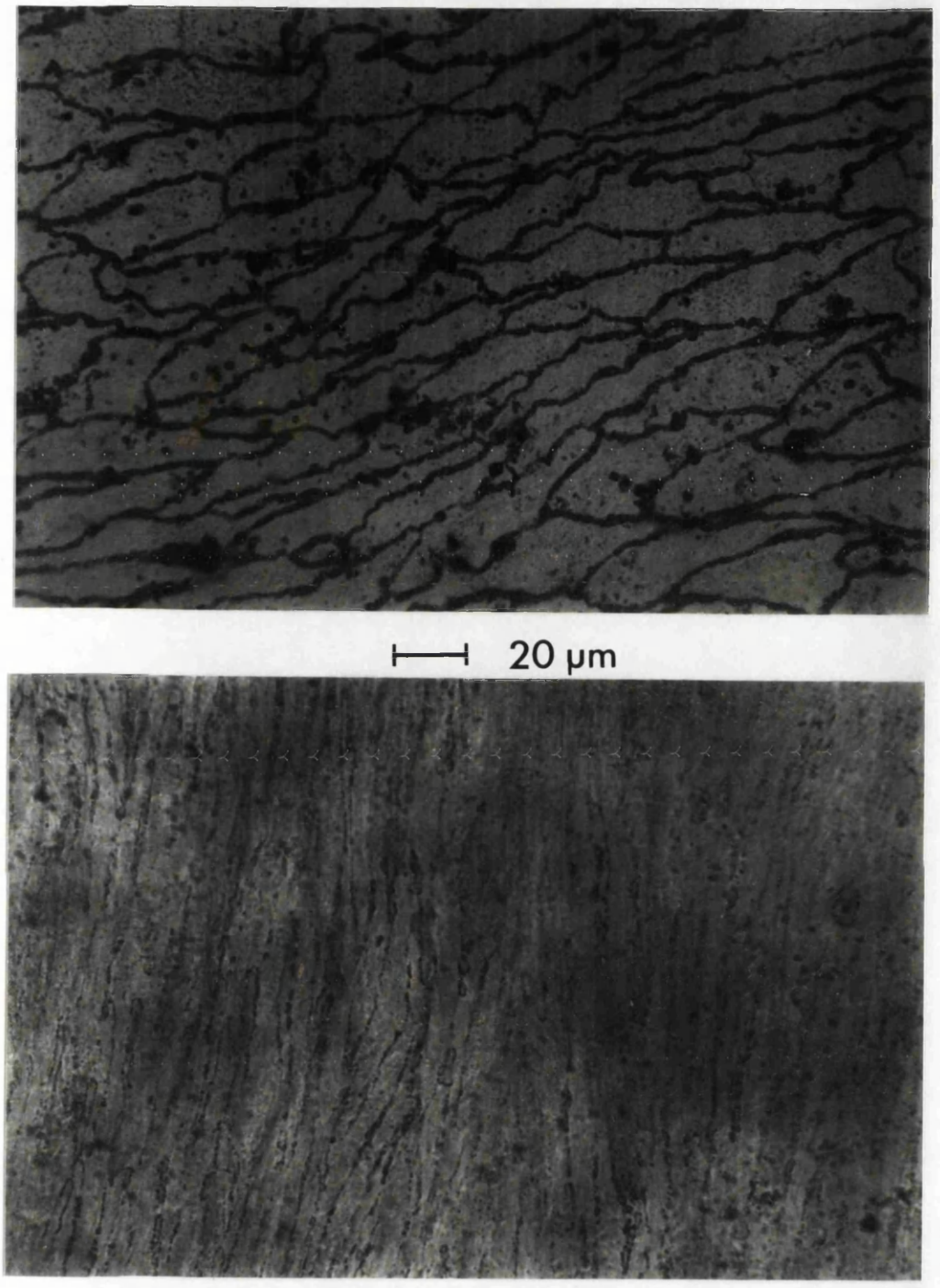


Figure 2.6 Photographs showing *en face* silver staining of rat aortic rings with intact endothelium (top) and denuded of endothelium (bottom). The endothelial cells are outlined to a much greater extent than the smooth muscle cells. Photographs are aligned as the two cell types would be in the vessel with the endothelial cells lining up along the axis of blood flow and the smooth muscle cells at 90° to the endothelial cells.

curves, $P<0.05$ being taken as statistically significant.

In the studies using antagonists the mean EC_{50} for the vasodilator in the absence of the antagonist was compared with results in individual tissues treated with antagonist. This gives a series of dose ratios, defined as in **Equation 2.1.**

$$Dose\ Ratio = \frac{[EC_{50}\ with\ antagonist\ present]}{[EC_{50}]}$$

Equation 2.1

Where single concentrations of the antagonist were used a pA_2 value was calculated assuming a Schild slope of 1 according to the formula derived by Van Rossum (1977) given in **Equation 2.2.**

$$pA_2 = -\log[B] + \log\left(\frac{[A]_2}{[A]_1} - 1\right)$$

where [B] is the concentration of the antagonist

$[A]_2$ is the EC_{50} of the agonist in the presence of [B]

$[A]_1$ is the EC_{50} of the agonist in the absence of antagonist

Equation 2.2

Where multiple concentrations of the antagonist were used a Schild plot of log [Dose Ratio - 1] against log [B] was plotted and linear regression carried out using GraphPad to derive the pA_2 value and the Schild slope.

In the cyclic nucleotide studies the levels of cyclic AMP, cyclic GMP

and associated relaxations were compared to time-matched controls using Student's t-test to determine the significance of differences, $P<0.05$ being taken as statistically significant.

CHAPTER 3

INTRODUCTION

Endothelium-derived relaxant factor (EDRF)

In 1980, Furchgott and Zawadzki demonstrated that the relaxation of rabbit aorta to acetylcholine and other muscarinic agonists required the presence of an intact endothelium. Removal of the endothelium by abrasion or with collagenase abolished the relaxant response to acetylcholine and this was replaced at higher concentrations of acetylcholine by a contractile response. The nature of the preparation required that the endothelium had to be releasing some chemical substance, termed an endothelium-derived relaxant factor (EDRF), which was then diffusing into the smooth muscle and initiating the relaxant response. A number of other agonists have since been found to be capable of causing endothelium-dependent relaxations, amongst them CGRP (Brain *et al.*, 1985; Grace *et al.*, 1987; McGrath *et al.*, 1988; Al-Kazwini *et al.*, 1987).

EDRF as nitric oxide

It has been shown that EDRF is nitric oxide (Palmer *et al.*, 1987a; 1988a) and that it is synthesised from the terminal guanidino nitrogen of the amino acid, L-arginine. The enzyme carrying out this reaction, termed nitric oxide synthase, can be inhibited by a number of N-guanidino substituted analogues of L-arginine including N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989) and N^G-nitro-L-arginine (L-NOARG) (Moore *et al.*, 1990; Ishii *et al.*, 1990). This inhibition can be reversed in a stereospecific manner

by the L-, but not the D- isomers of arginine and, to a lesser extent, citrulline. Nitric oxide is inactivated by interactions with superoxides (Wei *et al.*, 1985; Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986b; Pieper *et al.*, 1989).

Nitric oxide stimulates the soluble form of guanylate cyclase (EC 4.6.1.2) leading to increases in the intracellular level of cyclic GMP (Gruetter *et al.*, 1981; Griffith *et al.*, 1985). It has been suggested that this represents the mechanism by which nitric oxide exerts its vasorelaxant effect and there are a number of observations supporting this theory. Firstly, the membrane soluble analogues of cyclic GMP, such as 8-bromo-cyclic GMP (Lincoln, 1983), and the selective cyclic GMP phosphodiesterase inhibitor, M & B 22 948 (Zaprinast) (Griffith *et al.*, 1985), cause vascular relaxation. Also, a good, positive correlation between levels of cyclic GMP and level of relaxation exists for a number of endothelium-dependent vasodilators (Rapoport & Murad, 1983; Ignarro *et al.*, 1984; Griffith *et al.*, 1985). The pattern of protein phosphorylation is reported to be similar for acetylcholine endothelium-dependent and sodium nitroprusside endothelium-independent relaxation (Rapoport *et al.*, 1983), the nitrovasodilators also being thought to relax vascular smooth muscle by elevating levels of cyclic GMP. Finally, a number of studies comparing the effects of inhibitors of endothelium-dependent relaxation with the effects on cyclic GMP have been reported. In each case the level at which the inhibitor affected the relaxant response also gave a reduction in the associated accumulations of cyclic GMP, entirely consistent with cyclic GMP mediating the relaxant response (Martin *et al.*, 1985). Further, in all cases where an appropriate time course was utilised, the relaxant response to the vasodilator was preceded by the elevation of cyclic GMP levels, a prerequisite if the elevations in this nucleotide are causally related to the relaxant response (Gruetter *et al.*, 1981; Ignarro *et al.*, 1984).

Exactly how the elevations in cyclic GMP mediate the relaxant

response remains open to question. It appears that the first step is the activation of a cyclic GMP-dependent protein kinase. The presence of this enzyme has been confirmed in vascular tissues (Ives *et al.*, 1980), but the relevant substrate protein leading to the relaxant response has yet to be conclusively shown. In rabbit aortic muscle for example four different proteins appear to be phosphorylated (Casnellie *et al.*, 1980). The function of these proteins is unknown.

The final step in the enzyme cascade leading to vascular relaxation is also controversial. Evidence exists suggesting the existence of two possible mechanisms, both involving control of the myosin light chain kinase. This enzyme catalyses the phosphorylation of myosin, important for contraction since myosin is only capable of interacting with actin when phosphorylated. The first mechanism involves the reduction of cytosolic calcium levels thus inhibiting the calcium-dependent myosin light chain kinase. The second mechanism involves phosphorylation and therefore inactivation of myosin light chain kinase. Whether both or only one of these mechanisms predominate remains open to question.

The synthesis, transduction and inactivation of acetylcholine-induced nitric oxide is summarised on **Figure 3.1.**

Pharmacology of the EDRF released by acetylcholine

Before the identification of the EDRF being released by acetylcholine as nitric oxide, various studies had shown that a number of drugs were capable of altering endothelium-dependent relaxations. Most of these substances were shown to alter the action of exogenous nitric oxide in a similar fashion. On the basis of these observations a number of criteria now exist to aid in the identification of any endothelium-dependent response as

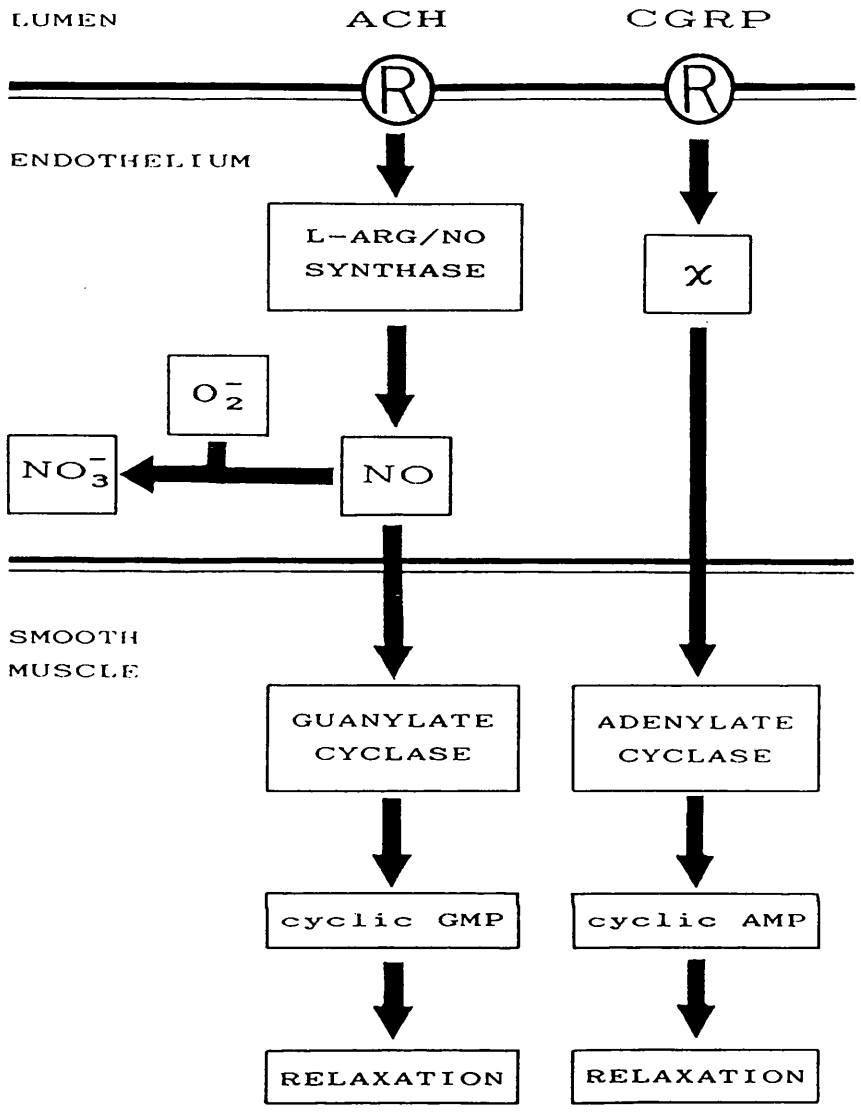


Figure 3.1 Diagram summarising the transduction pathways mediating acetylcholine (left) and CGRP (right) endothelium-dependent relaxations. Acetylcholine acts on a muscarinic receptor (R) and activates nitric oxide synthase (L-ARG/NO SYNTHASE). Nitric oxide (NO) is either inactivated by interaction with superoxides (O_2^-) or induces vascular smooth muscle relaxation by activating soluble guanylate cyclase. CGRP, thought to activate its own receptor (R), releases some unknown substance (x) from the endothelium. This substance induces vascular relaxation by activating adenylate cyclase.

being mediated by nitric oxide.

As well as the nitric oxide synthase inhibitors mentioned above, and the stereospecific reversal of their inhibitory effects by L-, but not D-arginine, haemoglobin (known to bind nitric oxide (Gibson & Roughton, 1957)) and methylene blue (a soluble guanylate cyclase inhibitor (Murad *et al.*, 1978; Gruetter *et al.*, 1979; 1981) and oxidising agent) inhibit endothelium-dependent relaxations. In certain systems it has been found that superoxide dismutase (an oxygen radical scavenger (McCord & Fridovich, 1969)) augments endothelium-dependent relaxations (Rubanyi & Vanhoutte, 1986a), though this is not universal (Bhardwaj *et al.*, 1988). The EDRF released by acetylcholine is known to elevate cyclic GMP levels and have a biological half-life of between 5 and 50 seconds (Griffith *et al.*, 1984a; Gryglewski *et al.*, 1986a; Angus and Cocks, 1987; Myers *et al.*, 1990).

Although most of the pharmacological profile of the EDRF released by acetylcholine is shared by exogenous nitric oxide, a number of disparities have been noted. These include differences in the behaviour of this EDRF and nitric oxide on various ion exchange resins and the relatively vascular selective relaxant effects of EDRF compared to the relatively non-specific smooth muscle relaxant effects of nitric oxide (Shikano *et al.*, 1988). It has also been suggested that the levels of nitrite detected by chemiluminescence (thought to be derived from EDRF) are not sufficient to account for the relaxant effect if the EDRF is nitric oxide. On the basis of this last point it has been suggested that the EDRF released by acetylcholine may not be nitric oxide itself, but a more potent nitroso containing substance, such as S-nitrosocysteine (Myers *et al.*, 1990). Although less data is currently available on the activity of the S-nitrosocysteine, its short half-life (Myers *et al.*, 1990) and ability to elevate cyclic GMP levels (Ignarro *et al.*, 1981) have been demonstrated. For the sake of clarity, the EDRF released by

acetylcholine will be referred to as nitric oxide, since it is possible that, even if the initial compound released from the endothelium is an S-nitrosothiol, the active product mediating the relaxation of the smooth muscle is nitric oxide.

Other forms of EDRF

It has been suggested that there may be more than a single form of EDRF (Rubanyi & Vanhoutte, 1987). This is based on a number of observations that certain endothelium-dependent relaxant responses can be inhibited by compounds which do not affect the synthesis, transmission or action of nitric oxide. Some of these responses can be ascribed to the release of prostanoids, particularly prostacyclin, from the endothelium. However, others require the release of novel substances. In the dog coronary artery preparation, Rubanyi and Vanhoutte (1985b) have shown that the endothelium-dependent relaxations to arachidonic acid can be inhibited by ouabain, a sodium/potassium ATPase inhibitor (Schwartz *et al.*, 1975), a mechanism which plays no part in mediating nitric oxide relaxations.

CGRP endothelium-dependent vasorelaxation

While a large body of work has been carried out to characterise the EDRF released by acetylcholine, very little has been attempted in the characterisation of the EDRF released by CGRP. However, the available evidence suggests that CGRP may be releasing an EDRF which is not nitric oxide. In contrast to nitric oxide, which has selective rises in guanosine 3', 5'-cyclic monophosphate (cyclic GMP) associated with its vasorelaxant response (Ignarro *et al.*, 1984; Griffith *et al.*, 1985), CGRP has been shown to have selective rises in levels of adenosine 3', 5'-cyclic monophosphate (cyclic AMP) associated with its endothelium-dependent vasodilation (Kubota *et al.*, 1985; Grace *et al.*, 1987; Hirata et al, 1988), suggesting that the EDRF released by CGRP cannot be nitric oxide, but must be some novel substance.

The endothelium-dependent mechanisms of inducing vasorelaxation by acetylcholine and CGRP are compared in **Figure 3.1.**

CGRP is capable of releasing prostacyclin from endothelial cells (Crossman *et al.*, 1987). As this prostaglandin is known to be a vasodilator in some vascular beds (Bunting *et al.*, 1976) and acts by elevating cyclic AMP levels (Gorman *et al.*, 1977; Tateson *et al.*, 1977), it was considered that prostacyclin represented the prime candidate for the EDRF released by CGRP. Since nitric oxide and prostacyclin have a different pharmacological profile, it was important to establish that for the EDRF being released by CGRP. This could then be compared with prostacyclin- and nitric oxide-induced vasorelaxation (using acetylcholine to release nitric oxide) in the same tissue to establish whether either are viable candidates for the EDRF released by CGRP.

This chapter outlines the characterisation of the EDRF released by CGRP. This involved the comparison of the effects of various agents on acetylcholine- and CGRP-induced endothelium-dependent relaxation. The principal aim of this chapter was to construct a pharmacological profile of the EDRF released by human α-CGRP. Further, by comparing this with the profile obtained for acetylcholine in the same tissue, the possibility of investigating the existence of a second EDRF released by CGRP was examined.

MATERIALS AND METHODS

To study the endothelium-dependent relaxations induced by acetylcholine and human α-CGRP, two techniques were used, superfusion and bathed preparations. In the superfusion studies rat thoracic aortic rings were used as the bioassay tissue, being prepared as described in Chapter 2,

removing the endothelium from all rings. The protocol used was as described in Chapter 2. Tissues were superfused, directly over the rings, with Krebs solution containing noradrenaline (10^{-7}M) to elicit a contractile response and superoxide dismutase (100U/ml) to prolong the half-life of nitric oxide. Once it had been established by lack of relaxation to a bolus injection of acetylcholine (10^{-8} mols) that the tissues were denuded of endothelium, a section of rabbit aorta (10 cm long) was cannulated at both ends and the bioassay rings superfused with the effluent from the rabbit aorta.

Bolus concentrations of either acetylcholine or human α-CGRP or sodium nitroprusside were administered before and after the donor aorta, the responses being assessed on the bioassay ring.

In the bathed preparations, rat thoracic aortic rings were prepared as described in Chapter 2, removing the endothelium from a number of rings. Experiments were carried out using the protocol detailed in Chapter 2. Cumulative dose/response curves to acetylcholine, human α-CGRP and sodium nitroprusside were carried out after a stable contraction had developed to noradrenaline (10^{-7}M). The effects of a number of test drugs on the contractions elicited by noradrenaline and the relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside were studied. After each cumulative curve, the Krebs solution and the relevant test drug were replaced.

Test drugs

Ibuprofen and glibenclamide were prepared in 100% DMSO to give 10^{-1}M and 10^{-2}M stock solutions, respectively. Subsequent dilutions were carried out using Krebs solution. Preliminary experiments indicated that, at the diluted concentrations of the drugs used, the concentration of DMSO did

not affect either the contractile or relaxant properties of the tissues to any of the vasoconstrictors or vasodilators studied.

Haemoglobin as supplied commercially contains a mixture of oxyhaemoglobin and the oxidised derivative, methaemoglobin. Since pure oxyhaemoglobin was required this was prepared according to the method of Martin *et al.*, (1985). Briefly, this involves adding a 10-fold molar excess of sodium dithionite to a 10^{-3}M solution of bovine haemoglobin. This was then dialysed for 2 hours at 4°C and then stored in aliquots at -20°C.

N^G-nitro-L-arginine (L-NOARG) was prepared in 1M hydrochloric acid at 50mg/ml before being diluted to 10^{-3}M and neutralised to pH 7.0.

Tissues were preincubated in ouabain ($5x10^{-6}$M) for 1 hour or with ibuprofen (10^{-5}M) or glibenclamide (10^{-5}M) for a period of 30 minutes before starting the experiment.

All other drugs; ouabain, superoxide dismutase, haemoglobin, methylene blue, and the analogues and enantiomers of arginine were dissolved in Krebs solution and incubated for 15 minutes before each cumulative curve to any vasodilator was started.

All control values were obtained using the same time-course as the pretreated values, but without adding the test drug under study.

RESULTS

Superfusion studies

Although it proved possible to measure the release of an EDRF induced by bolus doses of acetylcholine from the donor aorta, human α-CGRP in bolus doses up to (10^{-7} mols) proved incapable of causing measurable relaxations of the bioassay rings. It was found in bathed rings of rabbit aorta that human α-CGRP was capable of inducing endothelium-dependent relaxations (data not shown). It is likely, therefore, that the lack of relaxant responses to human α-CGRP does not reflect a lack of CGRP receptors in the rabbit aorta. Further, as the relaxations to human α-CGRP in the bathed rabbit aortic ring preparation were entirely dependent on an intact endothelium it seems likely that some form of EDRF is being released. The lack of transfer of the compound released by human α-CGRP to the bioassay ring in the superfusion studies could reflect different kinetics of release of the same substance by human α-CGRP and acetylcholine, or the release of two different EDRFs by these drugs. Since this technique proved unsuitable for the study of the EDRF released by human α-CGRP, all further experiments were carried out using the bathed rat thoracic aortic ring preparation.

Bathed tissues - role of endothelium

In the rat thoracic aorta with intact endothelium noradrenaline 10^{-7}M induced a sustained contractile response of 1.1 ± 0.1g. Removal of the endothelium significantly increased this response to 1.6 ± 0.1g (**Figure 3.2**).

Figure 3.3 shows that acetylcholine ($3x10^{-9}$-10^{-7}M) and human α-CGRP ($3x10^{-9}$-$3x10^{-7}$M) elicited concentration-dependent relaxations only in preparations where an intact endothelium was present (confirmed histologically). The EC_{50} (the concentration of the drug giving 50% of the

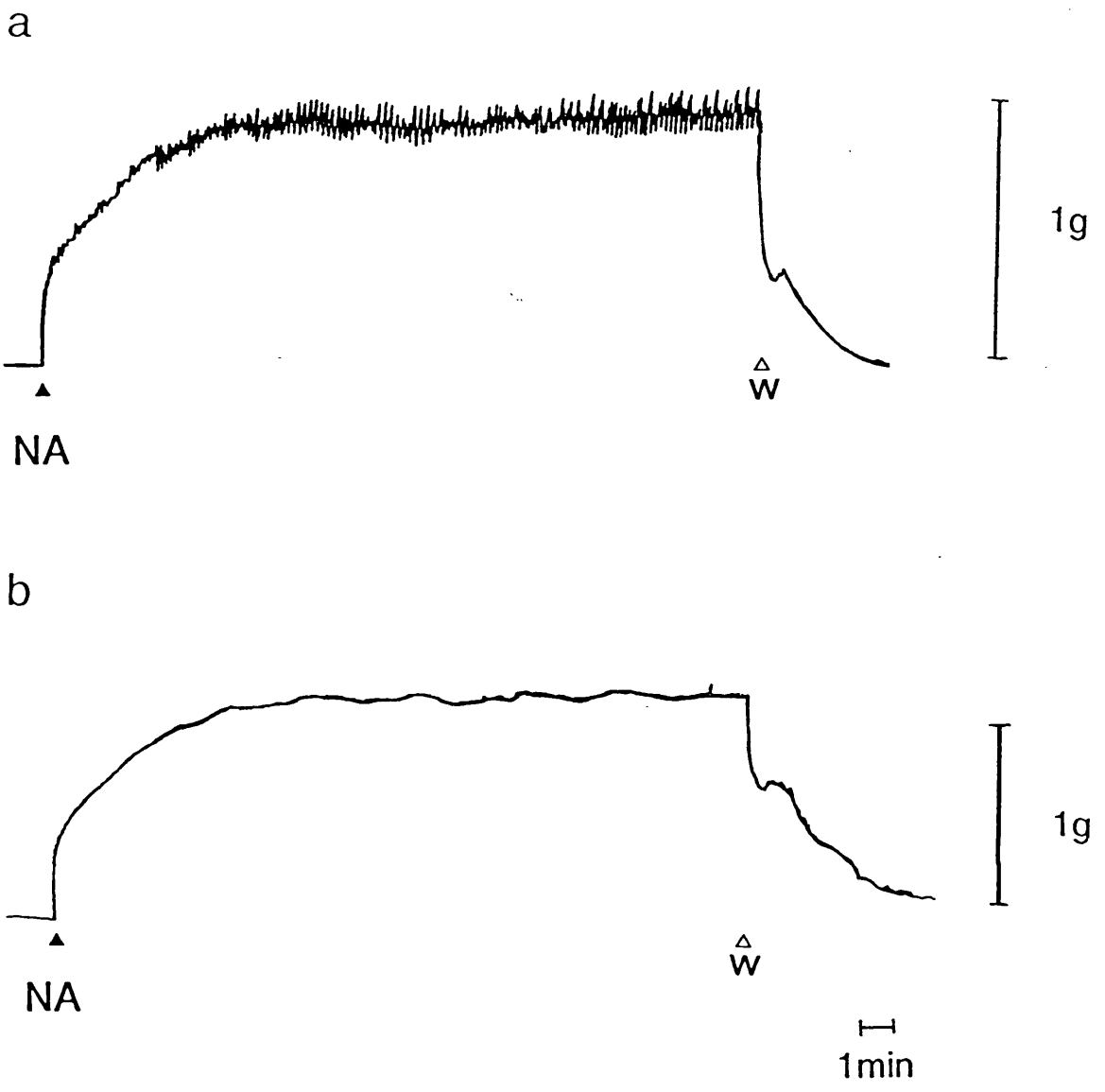


Figure 3.2 Traces showing contractile activity of 10^{-7}M noradrenaline (NA) in rings of rat thoracic aorta with a) intact endothelium and b) denuded of endothelium. Note the absence of spontaneous activity in the ring denuded of endothelium when compared to the intact ring.

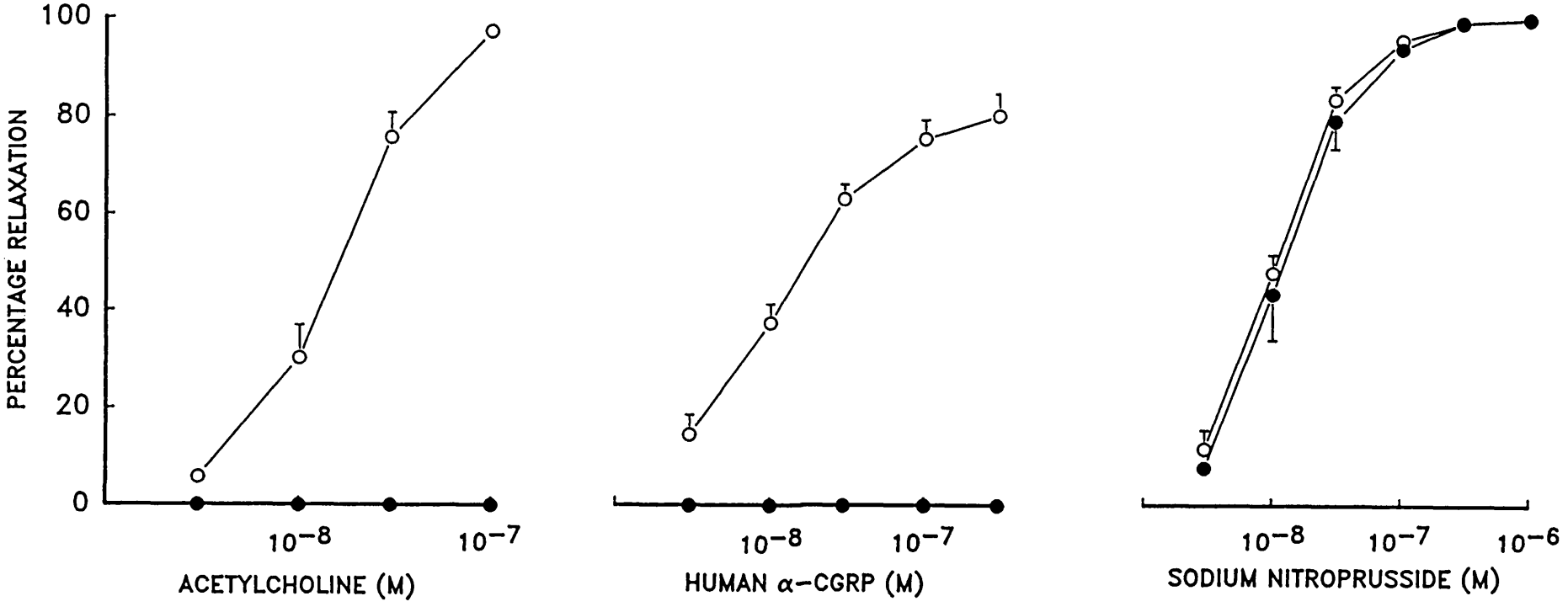


Figure 3.3 Effect of removal of endothelium on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O are responses in intact rings, ● the responses in endothelium denuded rings for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

maximal relaxation for that drug) for acetylcholine, $1.6 \pm 0.4 \times 10^{-8}$M, was similar to that of human α-CGRP ($1.1 \pm 0.2 \times 10^{-8}$M) but their maximum relaxations were quite different, being 100 and 80% respectively of the contraction induced by noradrenaline (10^{-7}M). Neither of these dilators displayed signs of tachyphylaxis on repeat administration (data not shown).

Sodium nitroprusside (3×10^{-9}-10^{-6}M) relaxed rat aortic rings independently of the presence of endothelium, the EC_{50} being $7.2 \pm 0.4 \times 10^{-8}$M with the maximum relaxation being 100% of the noradrenaline-induced contraction.

The traces illustrated in **Figure 3.4** show marked temporal differences in the onset and equilibration of the relaxant response. Acetylcholine and sodium nitroprusside displayed rapid onset and equilibration (approx. 5s and 45s respectively for both drugs) with human α-CGRP having a much slower onset and equilibration (approx. 15s and 200s respectively). This time-course for relaxation to CGRP is similar to that noted by Brain *et al.*, (1985). In most endothelium-intact rings spontaneous activity was observed which was absent in all endothelium-denuded rings (**Figures 3.2** and **3.4**). This phenomenon was also described by Brain *et al.*, (1985).

Effect of test drugs on noradrenaline-induced vasoconstriction

The effect of the various drugs used to obtain a pharmacological profile of the EDRF released by CGRP on noradrenaline-induced contractions was assessed using two consecutive contractions to noradrenaline. The test drugs were added between these contractions. The level of contraction to noradrenaline in the presence of the drugs was expressed as a percentage of the first contraction to noradrenaline in the same tissue. This percentage

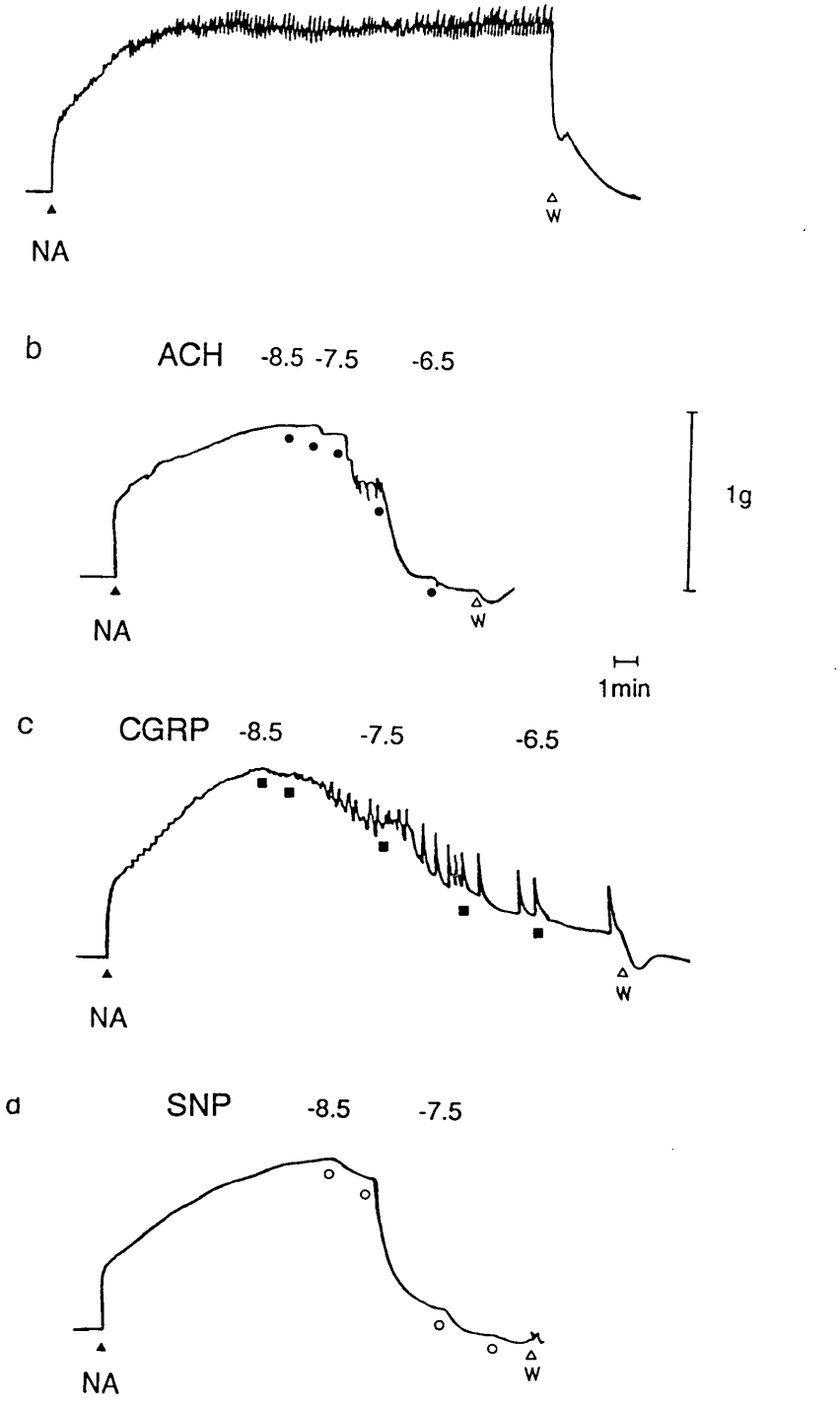


Figure 3.4 Traces showing a) contractile activity of 10^{-7}M noradrenaline (NA) and typical relaxant effects of cumulative addition of b) acetylcholine (ACH), c) human α-CGRP (CGRP) and d) sodium nitroprusside (SNP) on rings of rat thoracic aorta with intact endothelium. All rings were constricted with noradrenaline (10^{-7}M) at the point labelled NA. The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

result was then compared to a control tissue where two consecutive contractions to noradrenaline in the absence of any other drug were carried out.

Ibuprofen (10^{-5}M), ouabain ($5x10^{-6}$M), glibenclamide (10^{-5}M) and superoxide dismutase (100U/ml) had no significant effect on the contractile response to noradrenaline. Haemoglobin (10^{-6}M), reported to bind nitric oxide, and methylene blue (10^{-5}M), a soluble guanylate cyclase inhibitor and producer of superoxides, increased the tone induced by noradrenaline (10^{-7}M) by 30 and 36% respectively (**Figure 3.5**). The increase in tone was maintained over a period of 15 minutes in both cases. These data are consistent with there being a basal release of nitric oxide in this tissue as described by Martin *et al.*, (1986a).

Pharmacological comparison of the endothelium-dependent relaxant responses elicited by acetylcholine and human α-CGRP

Ibuprofen

Ibuprofen (10^{-5}M), a cyclo-oxygenase inhibitor (Greaves & MacDonald-Gibson, 1973), did not significantly affect the relaxant responses to any of the vasodilators studied (**Figure 3.6**). This result suggests that none of the prostaglandins, and in particular, prostacyclin, are involved in mediating the relaxant effects of CGRP. Prostacyclin itself, as in the pig aorta (Gordon & Martin, 1983) and the rabbit aorta (Gryglewski *et al.*, 1986a), was found to be incapable of eliciting a relaxant effect in this tissue whether the endothelium was present or absent (data not shown).

The main candidate for the EDRF released by CGRP, prostacyclin, has

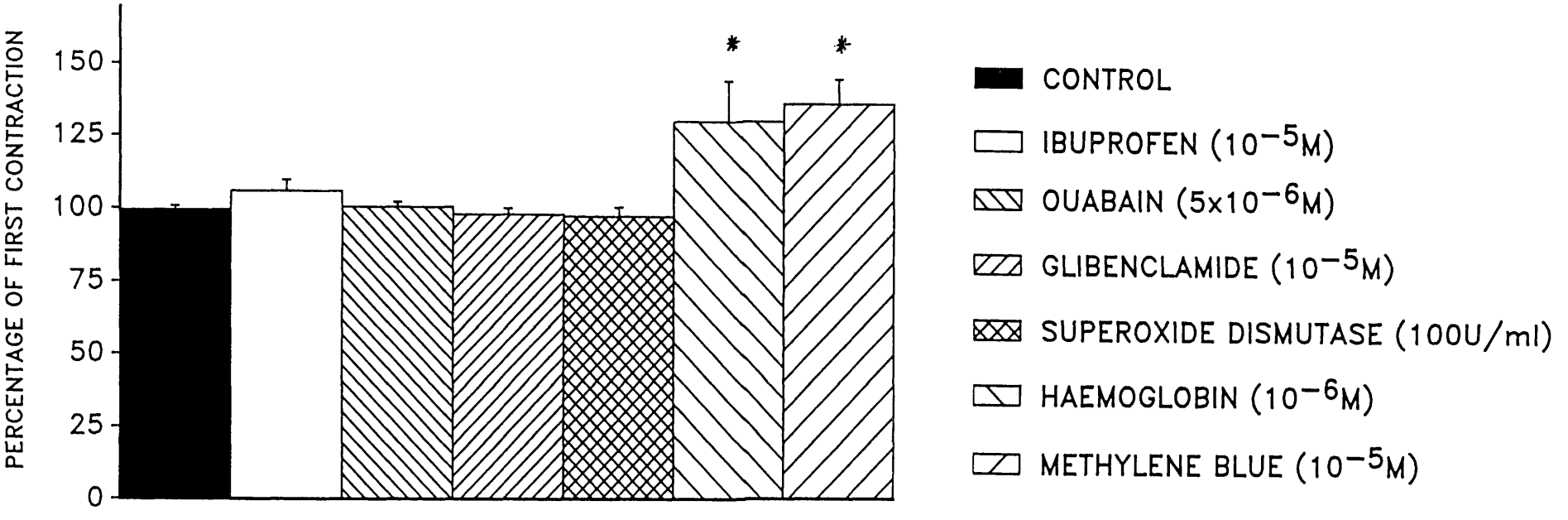


Figure 3.5 Effect of ibuprofen (10^{-5}M), ouabain ($5x10^{-6}$M), glibenclamide (10^{-5}M), superoxide dismutase (100U/ml), haemoglobin (10^{-6}M) & methylene blue (10^{-5}M) on contractions induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. The results were assessed using two consecutive contractions to noradrenaline. The various drugs were added between these contractions. The level of contraction to noradrenaline in the presence of the various drugs was expressed as a percentage of the first contraction to noradrenaline in the same tissue. Control tissues received two consecutive contractions to noradrenaline in the absence of any other drug. Blocks represent the mean ± s.e. mean of 4 separate experiments. (* indicates a significant difference from control ($P<0.05$).

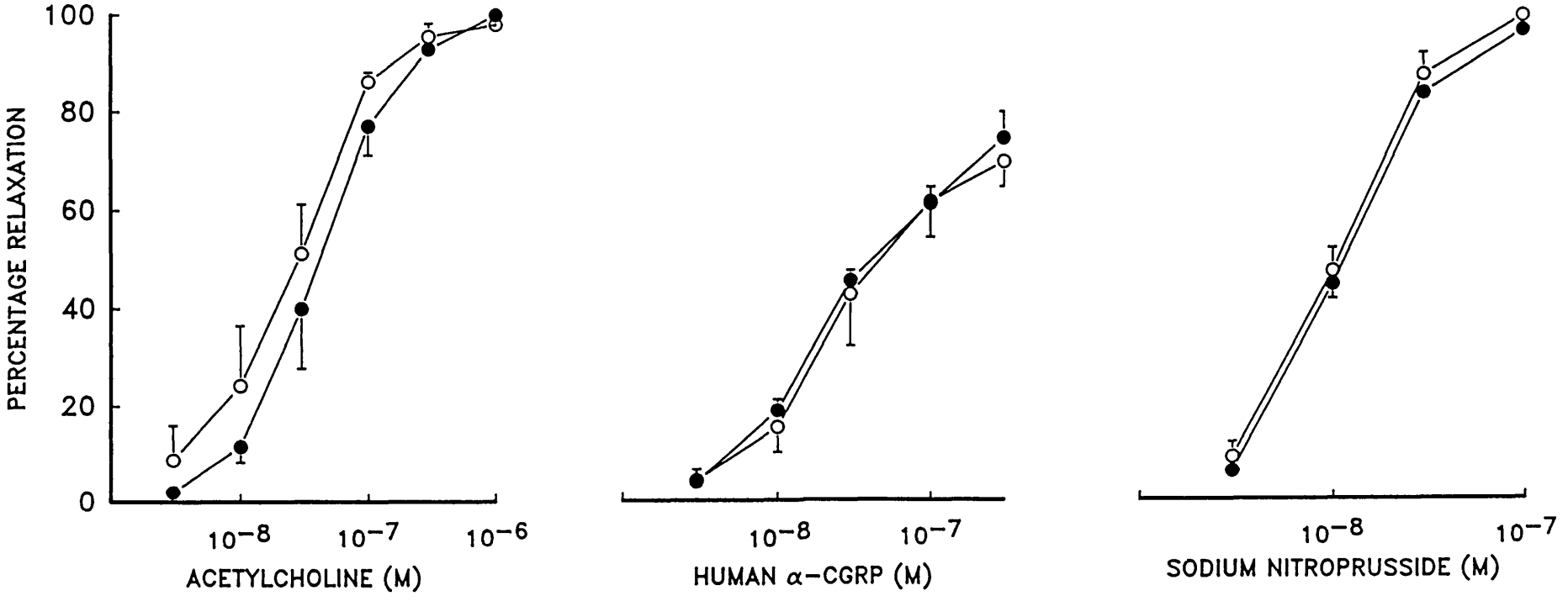


Figure 3.6 Effect of ibuprofen (10^{-5}M) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the ibuprofen pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

thus been eliminated since it is not a vasorelaxant in this tissue and the inhibitor of its synthesis had no effect on the relaxant response induced by human α-CGRP.

Ouabain

In the dog coronary artery a second EDRF has been described which acts by activating the sodium/potassium ATPase system (Rubanyi & Vanhoutte, 1985b). This study indicated that ouabain ($5x10^{-6}$M) significantly inhibited the endothelium-dependent component of the relaxations to arachadonic acid. It appears unlikely that a similar factor is being released in the rat aorta by either acetylcholine or CGRP as ouabain ($5x10^{-6}$M), the same concentration used in the dog coronary artery, did not inhibit endothelium-dependent or endothelium-independent relaxant responses. **(Figure 3.7)**.

Glibenclamide

In the rat thoracic aorta, glibenclamide had no significant effect on the relaxant response induced by any of the vasodilators studied **(Figure 3.8)**. This result conflicts with the recent findings of Nelson *et al.*, (1990) who reported that glibenclamide, an ATP-sensitive potassium channel blocker (Sturgess *et al.*, 1985) inhibited the relaxations and potassium channel opening induced by CGRP in the rat mesenteric artery. This artery is known to have an endothelium-dependent mechanism of relaxation to CGRP (Al-Kazwini *et al.*, 1987; McGrath *et al.*, 1988). However, as the electrophysiology was carried out on isolated smooth muscle cells of this artery without endothelial cells in the above report, it seems clear that the observed potassium currents seen on addition of CGRP (Nelson *et al.*, 1990) play no role in the mediation of CGRP endothelium-dependent relaxation. It must also be questioned whether the inhibition by glibenclamide of these currents is responsible for the

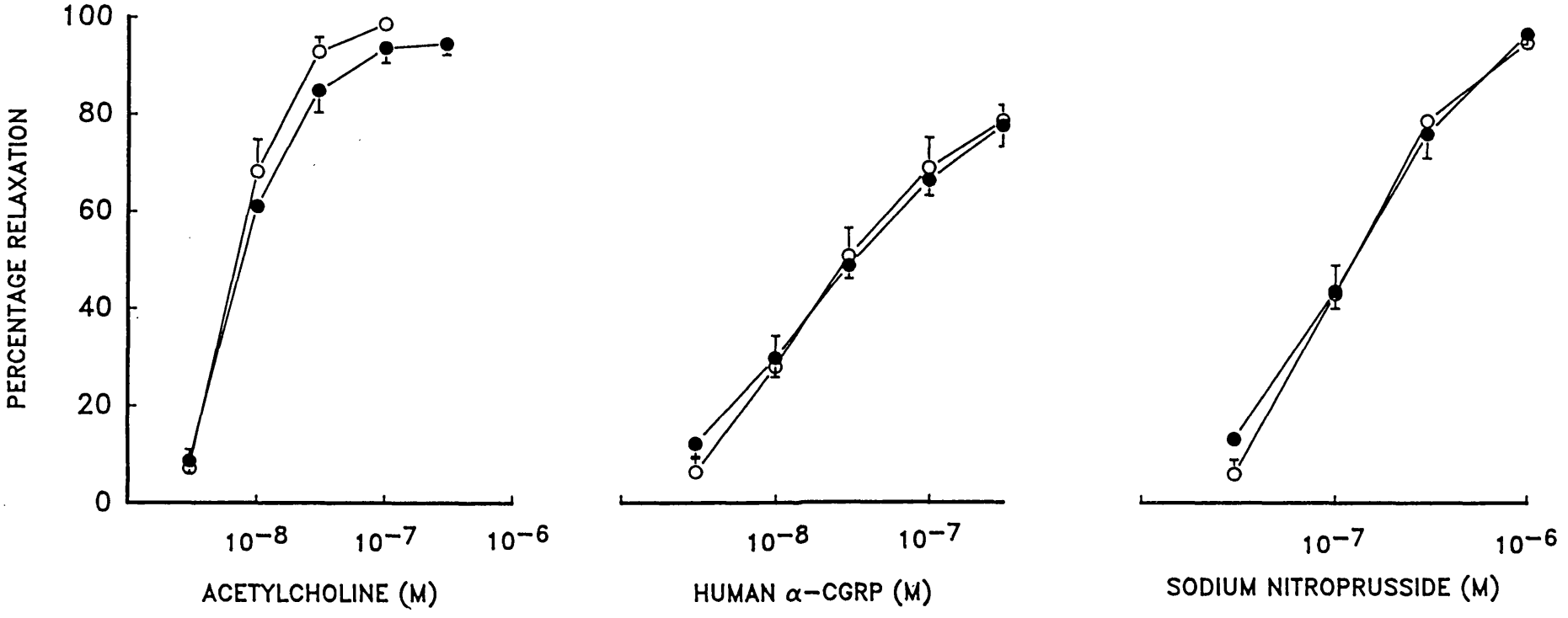


Figure 3.7 Effect of ouabain ($5x10^{-6}$M) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the ouabain pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

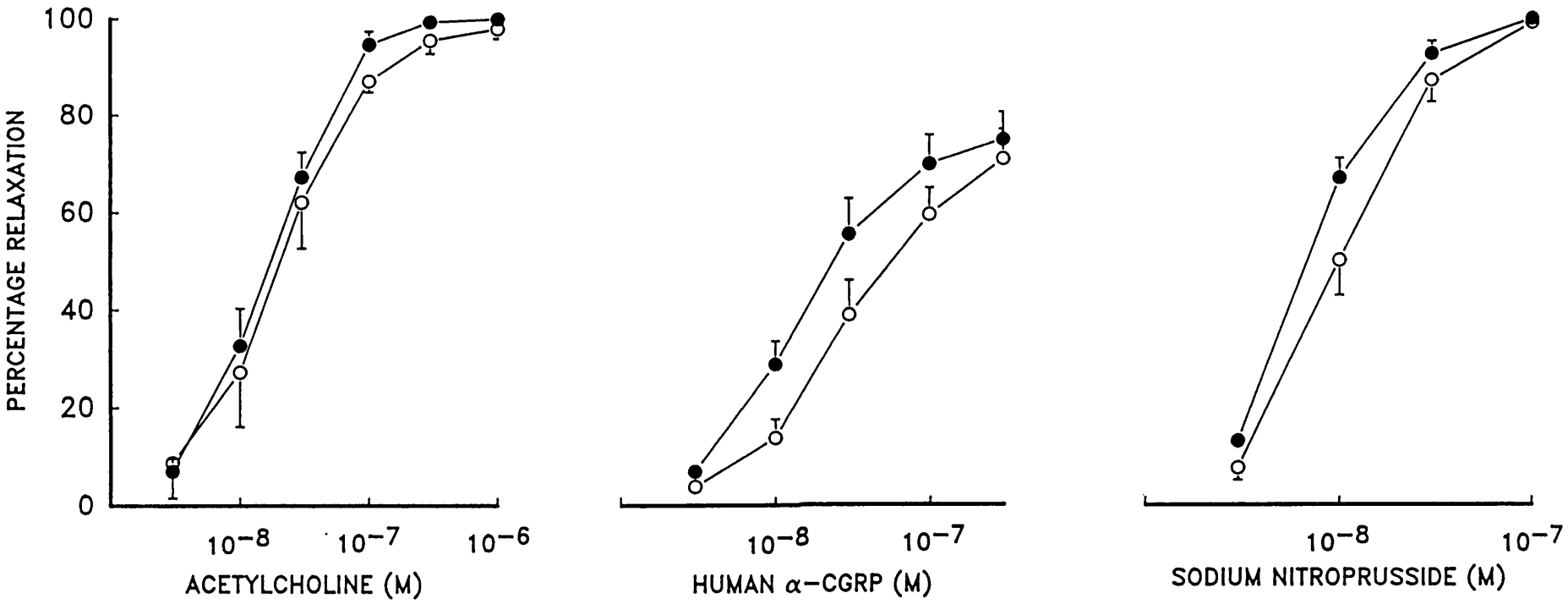


Figure 3.8 Effect of glibenclamide (10^{-5}M) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the glibenclamide pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments. Glibenclamide had no significant effects on any of the vasodilators studied the P values being greater than 0.5 for acetylcholine, between 0.1 and 0.3 for human α-CGRP and greater than 0.5 for sodium nitroprusside, respectively.

inhibitory effect of this drug on CGRP-induced vasorelaxation in the rat mesenteric artery.

Superoxide dismutase (E.C 1.1.15.1)

Superoxide dismutase (100U/ml), known to scavenge superoxides (McCord & Fridovich, 1969) and thus prolong the half-life of nitric oxide (see **Figure 3.1**), had no significant effect on the endothelium-dependent relaxant responses induced by either acetylcholine or human α-CGRP (**Figure 3.9**). Relaxant responses to sodium nitroprusside were also unaffected. While some studies have shown that in bathed tissues superoxide dismutase is capable of augmenting the relaxant responses to acetylcholine (Rubanyi & Vanhoutte 1986b) other studies found it ineffective (Bhardwaj *et al.*, 1988). It has generally been found that it is considerably more effective when used in superfused systems. This could be due to some form of directional release of nitric oxide in the direction of the smooth muscle. Problems of access for superoxide dismutase to the required site between the endothelium and the smooth muscle and the relatively high oxygen partial pressures used in the experiments described above could also account for the lack of effect of superoxide dismutase.

Haemoglobin

Haemoglobin (10^{-6}M), known to bind nitric oxide (Gibson & Roughton, 1957), caused approximately a ten fold parallel, rightward shift in the concentration/effect curve to acetylcholine ($P<0.05$) (**Figure 3.10**). This is entirely consistent with the results achieved by Martin *et al.*, (1985) where haemoglobin at 10^{-6}M reduced, and at 10^{-5}M completely abolished, the relaxant response to acetylcholine. The response to human α-CGRP was almost completely abolished by haemoglobin (10^{-6}M) ($P<0.05$). It is clear, therefore, that the relaxations induced by human α-CGRP are inhibited to a greater

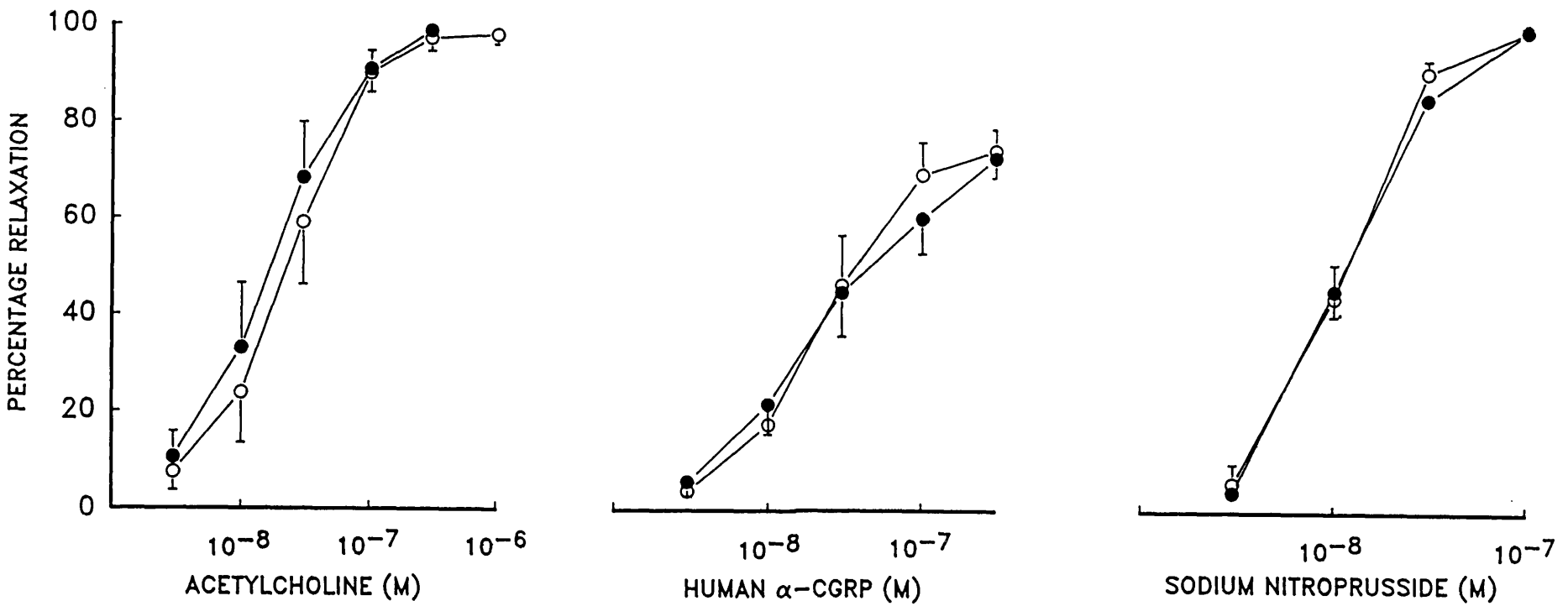


Figure 3.9 Effect of superoxide dismutase (100U/ml) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the superoxide dismutase pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

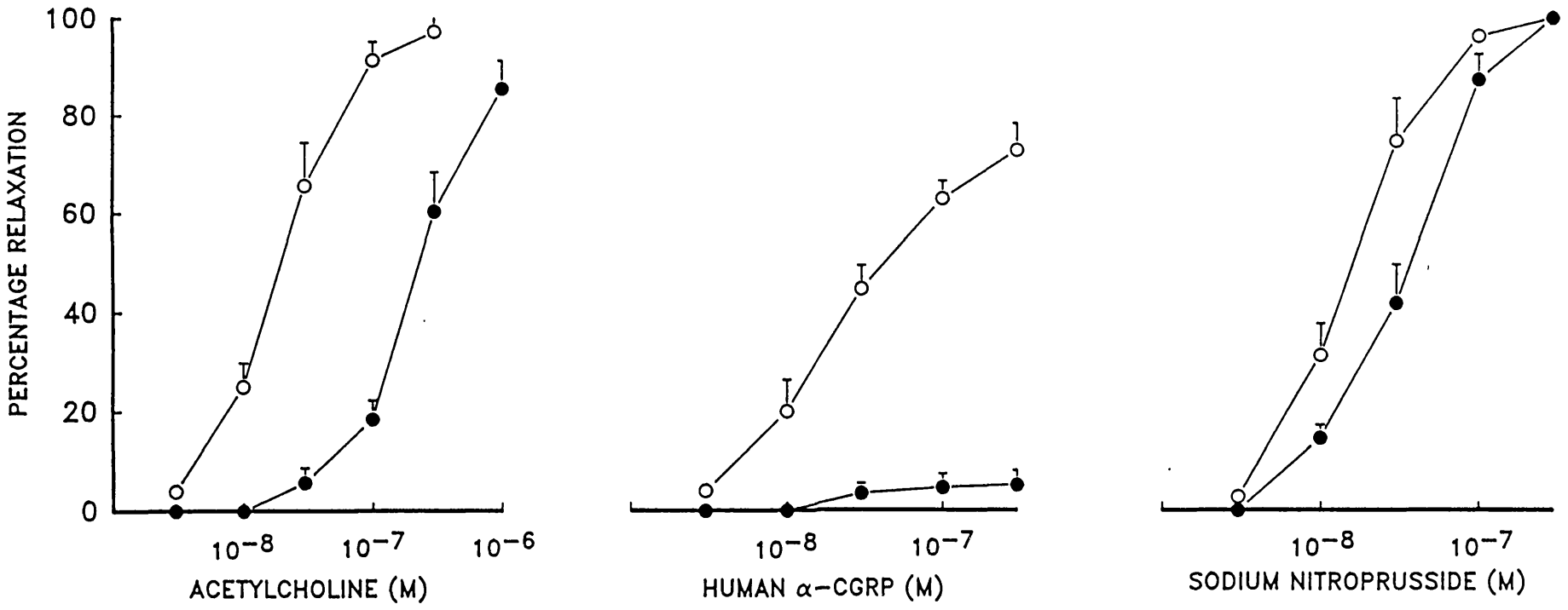


Figure 3.10 Effect of haemoglobin (10^{-6}M) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the haemoglobin pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

degree by haemoglobin than those of acetylcholine. Haemoglobin (10^{-5}M) also significantly inhibited the relaxant effects of the endothelium-independent vasodilator, sodium nitroprusside, ($P<0.05$) though to a lesser extent than any of the endothelium-dependent vasodilators studied. This result is contrary to the reported mechanisms of both haemoglobin and sodium nitroprusside. One possible explanation is that the shift in the sodium nitroprusside curve occurs as a consequence of the greater tone induced by noradrenaline (10^{-7}M) when haemoglobin (10^{-5}M) is present (**Figure 3.5**). However, this seems unlikely given that methylene blue (10^{-5}M) (**Figure 3.5**), L-NMMA (10^{-4}M) (**Figure 3.12**) and L-NOARG ($3x10^{-6}$M) (**Figure 3.13**) elevate the contraction induced by noradrenaline (10^{-7}M) to a similar extent without affecting the relaxant responses to sodium nitroprusside. Indeed, it is reported that increasing the level of contraction increases the sensitivity to sodium nitroprusside (Shirasaki & Su, 1985).

Methylene blue

Methylene blue (10^{-5}M), known to inhibit soluble guanylate cyclase (Murad *et al.*, 1978; Gruetter *et al.*, 1979; 1981), shifted the acetylcholine curve to the right in a parallel manner, approximately three fold in this case (**Figure 3.11**). This is again in line with the results obtained by Martin *et al.*, (1985). The concentration/effect curve to human α-CGRP was shifted to the right by methylene blue with the maximum response being greatly reduced. The lack of any significant inhibition of the relaxation induced by sodium nitroprusside tends to suggest that the majority of the inhibition of endothelium-dependent vasorelaxations by methylene blue is due to the production of superoxides which break down nitric oxide and not through direct inhibition of guanylate cyclase.

None of the above test drugs caused significant alterations in the speed

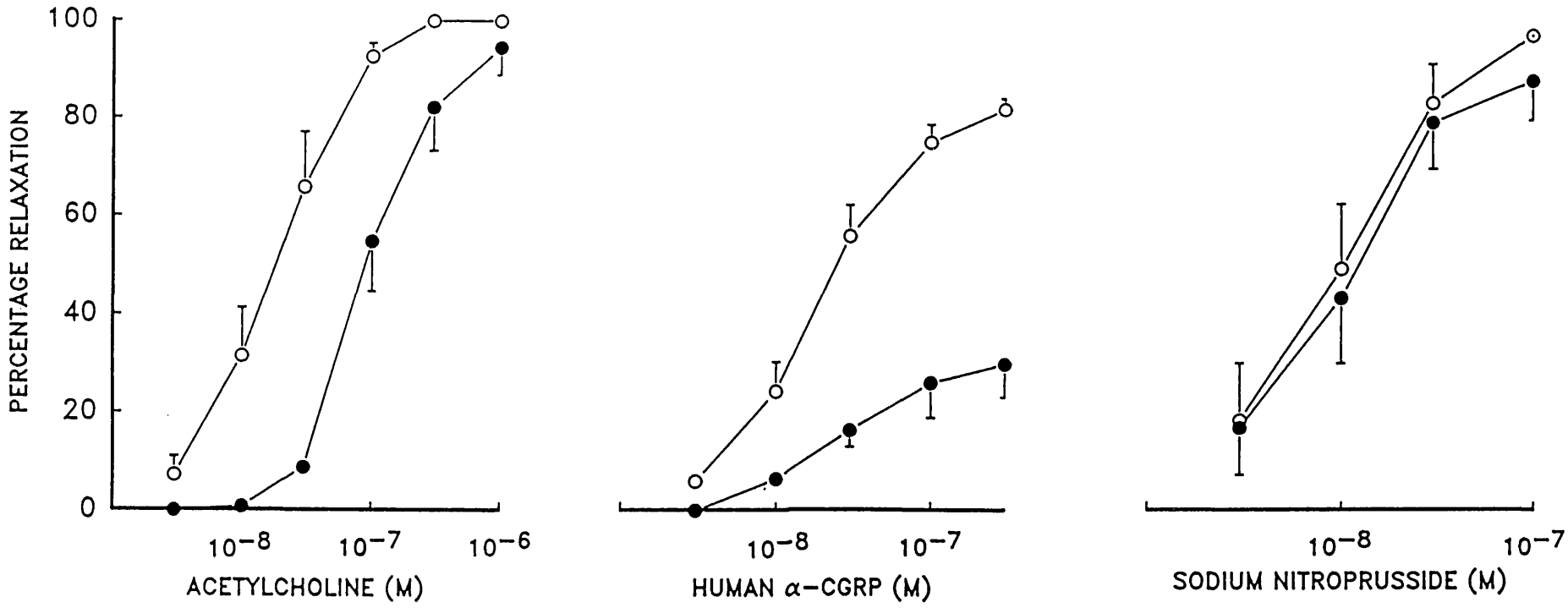


Figure 3.11 Effect of methylene blue (10^{-5}M) on relaxations induced by acetylcholine, human α-CGRP and sodium nitroprusside in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the methylene blue pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

of onset or equilibration of the relaxant response to acetylcholine, human α-CGRP or sodium nitroprusside.

Nitric oxide synthase inhibitors

N^G-monomethyl-L-arginine (L-NMMA) has been shown to inhibit the synthesis of nitric oxide. Further, this inhibition can be reversed in a stereospecific manner by the L- but not the D-enantiomer of the amino acid, arginine (Rees *et al.*, 1989). Other analogues of arginine, which inhibit nitric oxide formation, have also been synthesised of which N^G-nitro-L-arginine is reported to be one of the more potent examples (Moore *et al.*, 1990; Ishii *et al.*, 1990).

Effect of L-NMMA and L-NOARG on the noradrenaline contraction.

Increasing concentrations of L-NMMA ($3x10^{-5}$M and 10^{-4}M) caused significant elevations in the tone elicited by noradrenaline (10^{-7}M) (**Figure 3.12**), but were without contractile activity on their own. This result is consistent with a basal release of nitric oxide in this tissue being inhibited by this compound. $P<0.05$

This enhancement of the noradrenaline-induced contraction by L-NMMA was not significantly altered by concomitant addition of L- or D-arginine (both at 10^{-4}M). L- and D-arginine at this concentration were also devoid of any significant effects when added on their own in the presence or absence of noradrenaline.

L-NOARG (10^{-6}M and $3x10^{-6}$M) caused significant concentration-related increases in the tone elicited by noradrenaline (10^{-7}M) (**Figure 3.13**) at 10^{-6}M and $3x10^{-6}$M. Variations in the control responses to acetylcholine $P<0.05$

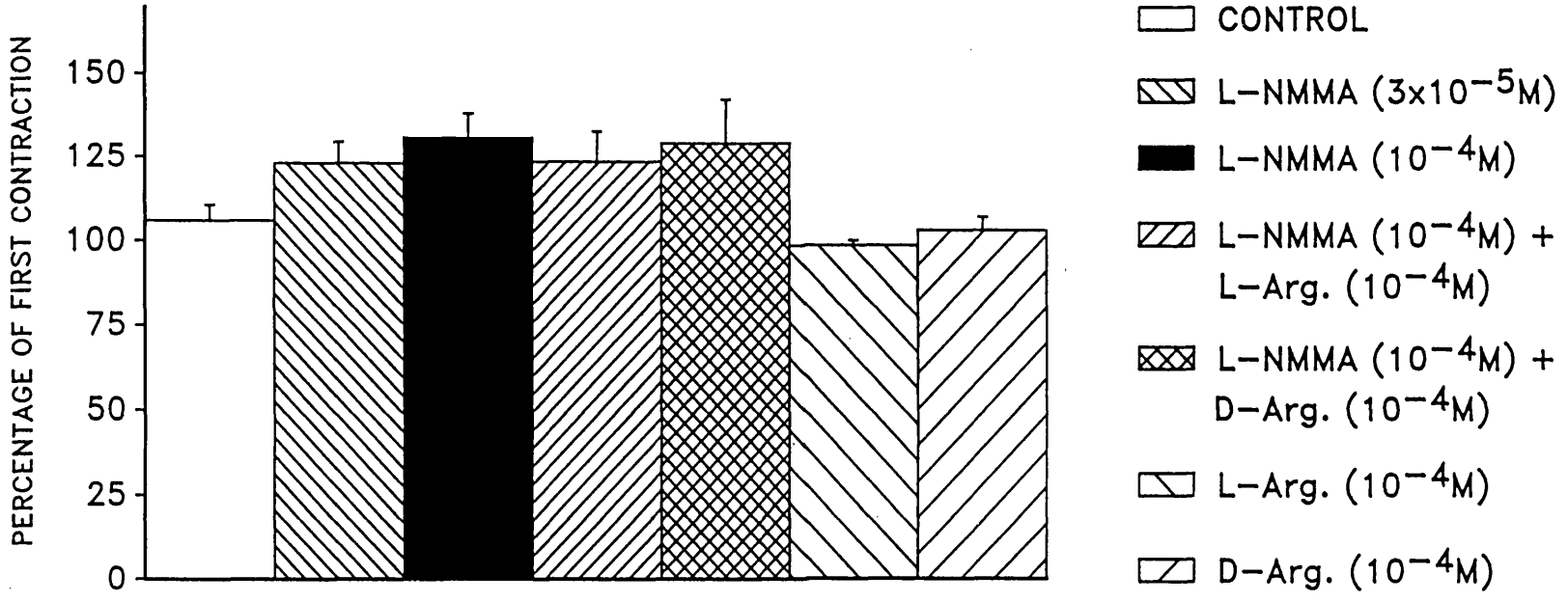


Figure 3.12 Effect of L-NMMA, L-arginine, D-arginine and combinations of these drugs on contractions induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. The results were assessed using two consecutive contractions to noradrenaline. The arginine analogues and enantiomers were added between these contractions. The level of contraction to noradrenaline in the presence of these drugs was expressed as a percentage of the first contraction to noradrenaline in the same tissue. Control tissues received two consecutive contractions to noradrenaline in the absence of any other drug. Blocks represent the mean ± s.e. mean of 4 separate experiments.

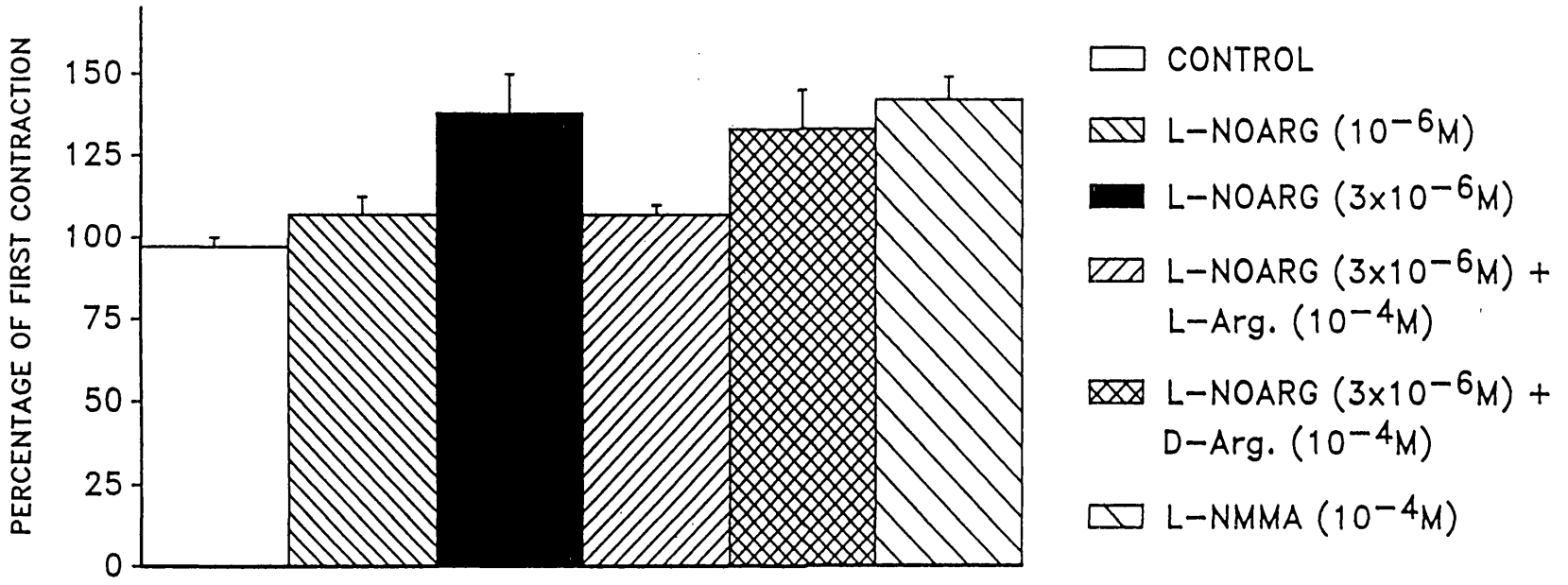


Figure 3.13 Effect of L-NOARG, L-NMMA and combinations of L-NOARG, L-arginine and D-arginine on contractions induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. The results were assessed using two consecutive contractions to noradrenaline. The arginine analogues and enantiomers were added between these contractions. The level of contraction to noradrenaline in the presence of these drugs was expressed as a percentage of the first contraction to noradrenaline in the same tissue. Control tissues received two consecutive contractions to noradrenaline in the absence of any other drug. Blocks represent the mean ± s.e. mean of 4 separate experiments.

and CGRP between the two groups of animals used to study L-NMMA and L-NOARG respectively (**Figure 3.14a and 3.14b**), led to the inclusion of L-NMMA (10^{-4}M) in both groups. As in the previous experiment it gave a significant increase in the tone induced by noradrenaline (10^{-7}M). $P<0.05$

The enhancement of the noradrenaline-induced contraction by L-NOARG ($3x10^{-6}$M) was significantly reduced ($P<0.05$) in a stereospecific manner by concomitant addition of L- but not D-arginine (both at 10^{-4}M).

Effect of L-NMMA and L-NOARG on endothelium-dependent relaxation.

L-NMMA ($3x10^{-5}$M and 10^{-4}M) and L-NOARG (10^{-6}M and $3x10^{-6}$M) caused significant concentration related inhibition of the endothelium-dependent relaxation induced by both acetylcholine and CGRP (**Figure 3.14**). Variations in sensitivity to acetylcholine and CGRP in the two groups of animals studied made a direct potency ratio impossible to calculate. However, because L-NMMA (10^{-4}M) was included in both groups of experiments, a comparison can be made suggesting that L-NOARG is between 10 and 30-fold more potent than L-NMMA.

The inhibition caused by L-NMMA (10^{-4}M) or L-NOARG ($3x10^{-6}$M) could be partially reduced by simultaneous addition of L-arginine (10^{-4}M) but was unaffected by concomitant addition of D-arginine (10^{-4}M) (**Figure 3.15**). Neither L- nor D-arginine on their own, at this concentration, had any significant effects on the relaxation induced by any of the vasorelaxants studied.

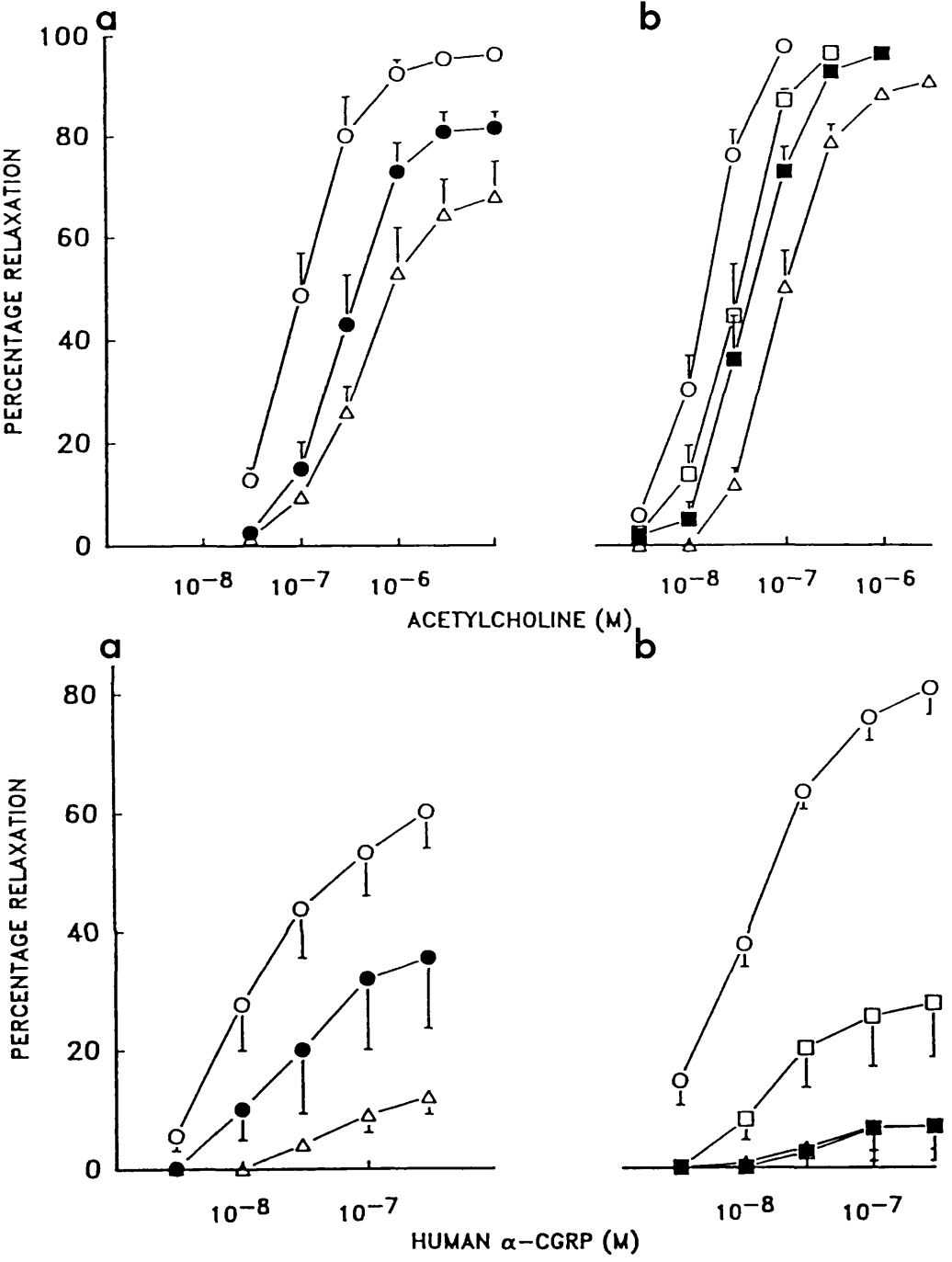


Figure 3.14 Inhibitory effect of L-NMMA and L-NOARG on relaxations induced by acetylcholine and human α-CGRP in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7}M). Relaxant effect of acetylcholine (top panel) and human α-CGRP (bottom panel) in the presence of a) L-NMMA and b) L-NOARG. Control values for acetylcholine and human α-CGRP induced relaxations (○) and in the presence of $3x10^{-5}$M L-NMMA (●), 10^{-4}M L-NMMA (△), 10^{-6}M L-NOARG (□) and $3x10^{-6}$M L-NOARG (■). Points represent the mean ± s.e. mean of between 4 and 6 experiments.

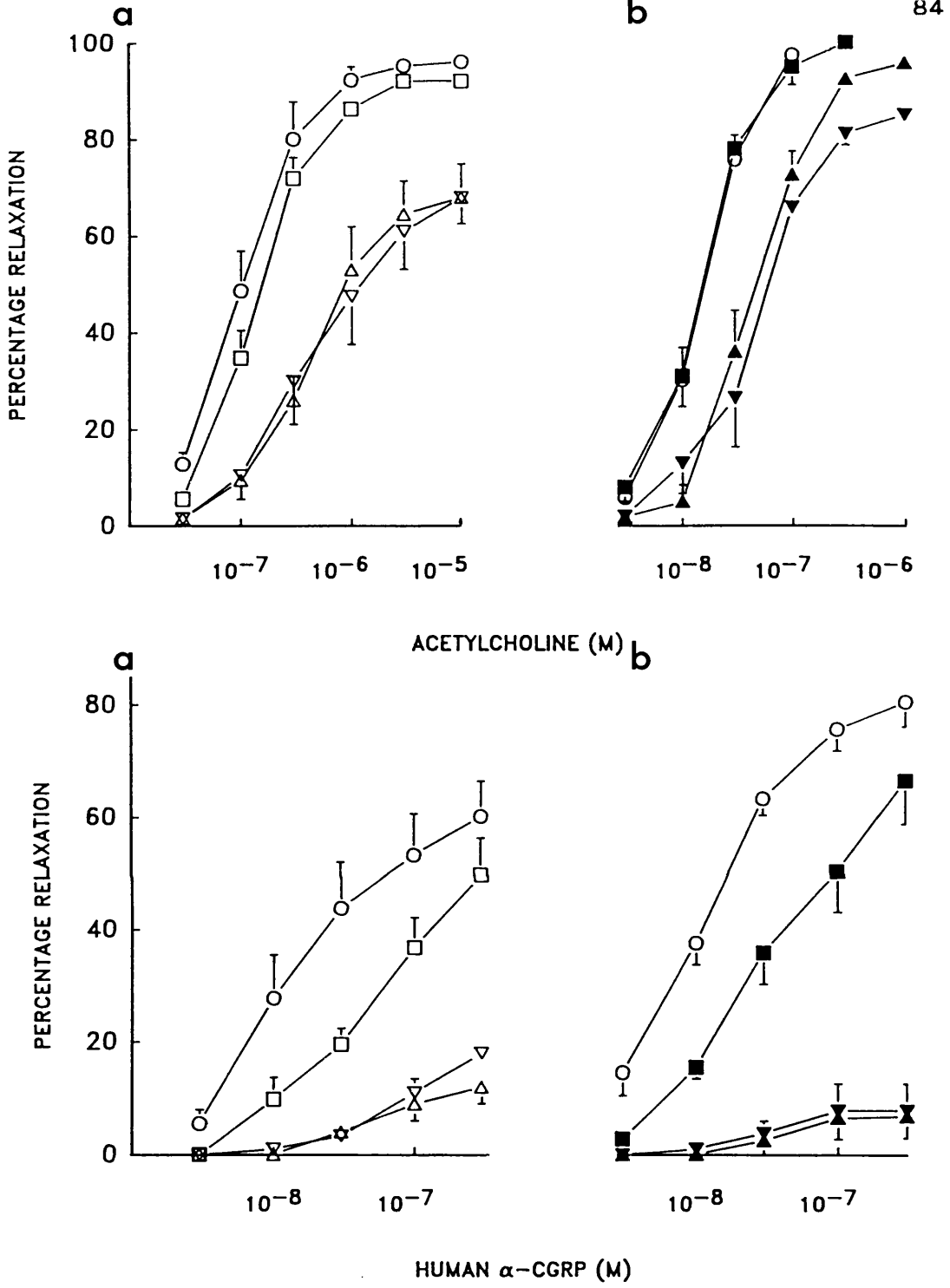


Figure 3.15 Stereospecific reversal by L- but not D-arginine of inhibition by a) L-NMMA and b) L-NOARG of acetylcholine (top panel) and human α-CGRP (bottom panel) induced relaxation in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7}M). Control values for acetylcholine and human α-CGRP alone (o) and in the presence of 10^{-4}M L-NMMA (△), 10^{-4}M L-NMMA plus 10^{-4}M L-arginine (□), 10^{-4}M L-NMMA plus 10^{-4}M D-arginine (▽), $3x10^{-6}$M L-NOARG (▲), $3x10^{-6}$M L-NOARG plus 10^{-4}M L-arginine (■) and $3x10^{-6}$M L-NOARG plus 10^{-4}M D-arginine (▼). Points represent the mean ± s.e. mean of between 4 and 6 experiments.

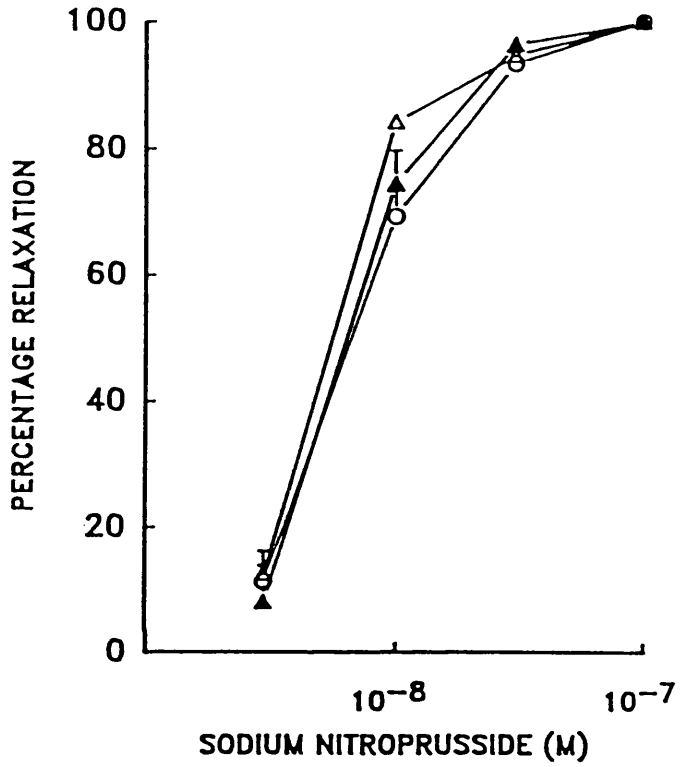


Figure 3.16 Lack of effect of L-NMMA or L-NOARG on sodium nitroprusside induced relaxations in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7}M). Control values for sodium nitroprusside alone (o) and in the presence of 10^{-4}M L-NMMA (△) and $3x10^{-6}$M L-NOARG (▲). Points represent the mean ± s.e. mean of between 4 and 6 experiments.

Effect of L-NMMA and L-NOARG on endothelium-independent relaxation

L-NMMA (10^{-4}M) and L-NOARG ($3x10^{-6}$M) had no effect on the relaxations induced by sodium nitroprusside (**Figure 3.16**) indicating that they are specific as inhibitors for endothelium-dependent vasorelaxants.

DISCUSSION

In this chapter it was confirmed that in the rat thoracic aortic ring preparation, human α-CGRP and acetylcholine are capable of inducing a relaxant response only when an intact endothelium is present. The results for human α-CGRP are markedly different from the, admittedly conflicting, results reported by Brain *et al.*, (1985), Kubota *et al.*, (1985), Uddman *et al.*, (1986) and Grace *et al.*, (1987) in the rat thoracic aorta. The concentration range ($3x10^{-9}$-$3x10^{-7}$M) at which CGRP elicits effects as shown in **Figure 3.2** is similar to the range shown by Kubota *et al.*, (1985), Uddman *et al.*, (1986) and Grace *et al.*, (1987). However, this differs by almost 1000-fold from the results recorded by Brain *et al.*, (1985). It seems unlikely that the differing forms of CGRP are responsible for this observation, since differences in the structures of the various rat and human CGRPs have been shown to have only minor effects on the potency of CGRP (Marshall *et al.*, 1986a; Holman *et al.*, 1986) in the vasculature.

While the range at which relaxation to CGRP occurs in this study is more akin to that achieved by Kubota *et al.*, (1985), Uddman *et al.*, (1986) and Grace *et al.*, (1987) the level of relaxation more closely resembles that of Brain *et al.*, (1985) being approximately 80% of the tone induced by the spasmogen as opposed to 20-25% seen in the other studies.

Sodium nitroprusside elicits a relaxant response which is independent of the presence of the endothelium. Characterisation of the EDRF released by CGRP was attempted and the profile of inhibition with a number of drugs compared to that achieved for acetylcholine within the same tissues.

On the basis of the reported differences in cyclic nucleotide accumulations associated with acetylcholine (cyclic GMP) and CGRP (cyclic AMP) (vide supra) induced relaxations, the most obvious alternative candidate for the EDRF released by CGRP is prostacyclin. However, this drug proved incapable of eliciting a relaxant effect in this tissue whether the endothelium was present or absent. This rules out prostacyclin as a candidate for the EDRF released by CGRP. The participation of other prostaglandins in mediating the relaxant response to CGRP can no longer be regarded as tenable since ibuprofen, a cyclo-oxygenase inhibitor, did not affect the relaxations induced by CGRP.

Other compounds postulated to inhibit other forms of EDRF and CGRP-induced relaxation in other tissues, namely ouabain (Rubanyi & Vanhoutte, 1985b) and glibenclamide (Nelson *et al.*, 1990) respectively, were also without effect on the relaxations induced by human α-CGRP in the rat thoracic aorta. This means that there is no pharmacological evidence, in this tissue, to suggest the existence of an alternative form of EDRF. On the contrary, there are many results supporting nitric oxide as the EDRF released by CGRP.

Haemoglobin, a compound known to bind nitric oxide, inhibits the relaxant response to acetylcholine and human α-CGRP. Further, methylene blue, a soluble guanylate cyclase inhibitor and producer of superoxides, also inhibited the vasorelaxation induced by acetylcholine and CGRP. These results are entirely consistent with CGRP releasing nitric oxide.

It was also shown that L-NMMA and L-NOARG, nitric oxide synthase inhibitors, as well as inhibiting the endothelium-dependent relaxations induced by acetylcholine are also capable of inhibiting the vasorelaxation induced by CGRP. Indeed, in common with haemoglobin and methylene blue, they appear to be more potent at inhibiting the relaxant effect of CGRP rather than that of acetylcholine. This may reflect a quantitative variation in the amount of EDRF being released by these vasodilators.

Further, it is clear that some of the structural requirements for reversal of the L-NMMA- and L-NOARG-induced inhibition are shared for both acetylcholine and CGRP-induced endothelium-dependent vasorelaxation i.e. the L-isomer of arginine causes partial reversal with the D-isomer being ineffective. This observation again tends to suggest that CGRP is releasing nitric oxide as acetylcholine does. Alternatively, if CGRP is releasing a second EDRF then it is synthesised from L-arginine, is similar to nitric oxide but stimulates adenylate cyclase.

However, a second EDRF appears very unlikely on the basis of the present results. For example, the inhibitors of the synthesis of nitric oxide, L-NOARG and L-NMMA, appeared to produce effects in the present experiments entirely consistent with this mechanism of action. In their presence the contraction to noradrenaline was increased suggesting that they were reducing the synthesis and therefore the basal release of EDRF. Furthermore, this effect of the inhibitors was reduced by L-arginine but not by the D-arginine. In addition their inhibition of the acetylcholine relaxation, reversed in a stereospecific manner, and their lack of effect against sodium nitroprusside are all consistent with their postulated mechanism of action as inhibitors of nitric oxide production (Amezcua *et al.*, 1989; Rees *et al.*, 1989; Moore *et al.*, 1990; Ishii *et al.*, 1990).

Although a number of studies exist giving a pharmacological profile of nitric oxide (Griffith *et al.*, 1984a; Gryglewski *et al.*, 1986a; Shikano *et al.*, 1988), only limited information is available for the CGRP-EDRF (Kubota *et al.*, 1985; Grace *et al.*, 1987; Hirata *et al.*, 1988). This indicates that the only difference between nitric oxide and the CGRP-EDRF exists at the level of signal transduction. Aside from the reported differences in second messenger accumulations associated with acetylcholine and CGRP, all the data currently available is consistent with the EDRF released by CGRP being nitric oxide. There are a number of possibilities exist to explain the biochemical data.

It is possible that the accumulations of the respective cyclic nucleotides reported for both acetylcholine and CGRP are causally unrelated to their relaxant. This seems improbable particularly for acetylcholine, as the accumulation of cyclic GMP precedes the relaxant response to acetylcholine (Ignarro *et al.*, 1984), there is a good correlation between levels of cyclic GMP and the level of relaxation (Holzman, 1982; Rapoport & Murad, 1983; Ignarro *et al.*, 1984) and membrane soluble analogues of cyclic GMP (Lincoln *et al.*, 1983) and selective cyclic GMP phosphodiesterase inhibitor, M & B 22 948, are vasodilators (Griffith *et al.*, 1985). With such strong evidence linking cyclic GMP to the relaxant response induced by acetylcholine this hypothesis seems unlikely.

The best possible explanation is that CGRP, like acetylcholine, releases nitric oxide as its EDRF and that, for technical reasons, this was missed in the published reports. These reports comprise two different experimental setups.

The first, Hirata *et al.*, (1988), involves the attempt to measure the levels of cyclic nucleotides in cultured endothelial cells. Although a rise in cyclic AMP was observed in these experiments, no alterations in cyclic GMP levels were observed. These results must be considered as compromised by

their use of cultured endothelial cells. It is known that a number of different agonists, while capable of inducing endothelium-dependent relaxations in isolated blood vessels, are incapable of inducing release of EDRF in cultured endothelial cells. These include acetylcholine (Gryglewski *et al.*, 1986a) and CGRP (J.A. Mitchell, private communication; Crossman *et al.*, 1991). Since these cells do not release a relaxant factor of any kind when challenged with CGRP, the fact that no rises in cyclic GMP were observed in these studies is irrelevant to the identification of the EDRF released by CGRP.

The second group, Grace *et al.*, (1987), measured levels of cyclic GMP in rat thoracic aorta. In this study, no elevations in cyclic GMP were seen on addition of CGRP, although elevations in levels of cyclic GMP were seen on addition of both acetylcholine and sodium nitroprusside. A possible explanation of this result is the suggestion that in this study the endothelium may have been partially removed. There is some evidence supporting this view. Although the relaxation induced by sodium nitroprusside was similar to the results achieved above, the endothelium-dependent relaxations induced by acetylcholine and, in particular, CGRP, were very different. Acetylcholine showed a maximum response of 100% relaxation of the tone induced by the spasmogen as achieved above, but this response was obtained at a ten-fold higher concentration. The CGRP relaxant effect was even more affected displaying a maximum relaxation of only 20-25%.

Variations in the relaxant responses to acetylcholine and human α-CGRP were observed in the two groups of animals used to study the nitric oxide synthase inhibitors, L-NMMA and L-NOARG (**Figure 3.14 and Figure 3.15**). These seem to be caused by partial damage to the endothelium in the first group of animals studied (**Figure 3.14**). The reduction in the maximum response to CGRP and the rightward shift in the acetylcholine curve without alterations in the sodium nitroprusside responses (data not shown) are all

consistent with this hypothesis. Further, it may also explain why the effect of L-NMMA on the noradrenaline-induced contraction could not be significantly reversed with L-arginine (**Figure 3.11**). This problem, while affecting the quantitative nature of the results did not appear to alter their qualitative nature. Thus, both L-NMMA and L-NOARG are more effective at inhibiting the response to CGRP than to acetylcholine and that the inhibition of the endothelium-dependent relaxant responses produced by both L-NMMA and L-NOARG could be stereospecifically reversed by the L-, but not the D- isomer of arginine.

It has also been demonstrated above that CGRP-induced endothelium-dependent relaxations are particularly sensitive to haemoglobin, methylene blue, L-NMMA and L-NOARG, much more so than those to acetylcholine. In addition, it was found during the course of the bathed experiments that sufficient of the endothelium could be removed to abolish the relaxant effects of CGRP without affecting the maximum response to acetylcholine. This, together with the lack of a measurable response to human α-CGRP in superfusion studies, suggests that human α-CGRP has a lesser ability to release EDRF than acetylcholine, perhaps as a result of lower receptor density on the endothelium.

In view of these observations, it is possible that so much of the endothelium has been removed in the study by Grace *et al.*, (1987) that the little of the CGRP response that remained was not sufficient to give a measurable alteration in cyclic GMP levels using current techniques.

In summary, it is impossible to separate the EDRF released by CGRP from nitric oxide, the EDRF released by acetylcholine, using the standard inhibitors. In light of this finding and the potential flaws in the studies reporting the only difference between the EDRF released by CGRP and nitric

oxide, that is, their respective cyclic nucleotide accumulations, it is essential that the levels of cyclic nucleotide associated with its endothelium-dependent relaxations are measured. Details of the results of these experiments are given in Chapter 4.

CHAPTER 4

INTRODUCTION

As described in the Introduction to Chapter 3, the existence of a second form of EDRF being released by CGRP was postulated on the basis of the reported differences in second messenger accumulations associated with nitric oxide and CGRP, these being cyclic AMP (Kubota *et al.*, 1985; Grace *et al.*, 1987; Hirata *et al.*, 1988) and cyclic GMP (Gruetter *et al.*, 1981; Griffith *et al.*, 1985), respectively. In Chapter 3 it was shown that the relaxations induced by both acetylcholine and human α-CGRP could be inhibited by L-NMMA and L-NOARG, which are reported to inhibit the synthesis of nitric oxide from the terminal guanidino nitrogen of L-arginine (Rees *et al.*, 1989; Moore *et al.*, 1990; Ishii *et al.*, 1990). Further this inhibition could be reversed in both cases by the concomitant addition of L- but not D-arginine. This implies a common synthetic pathway for nitric oxide and the EDRF released by CGRP. A number of other inhibitors of nitric oxide action also inhibited CGRP-induced endothelium-dependent vasorelaxation. In view of these results it was surprising that no alterations in levels of cyclic GMP were observed on addition of human α-CGRP in rat aorta by Grace *et al.*, (1987).

A number of possible explanations for these conflicting results were presented including: a) the possibility that the increased levels of cyclic nucleotides observed for both acetylcholine- and CGRP-induced vasorelaxation are causally unrelated to the relaxant response b) that CGRP released a second EDRF, similar in structure to nitric oxide, but which

stimulates adenylate cyclase as opposed to guanylate cyclase c) or that for technical reasons, the rise in cyclic GMP caused by CGRP releasing nitric oxide was missed.

In this chapter the cyclic nucleotide levels associated with acetylcholine, sodium nitroprusside and human α-CGRP-induced vasorelaxation in the rat aorta are measured in an attempt to clarify some of these possibilities. Further, by using inhibitors, the relationship between the levels of cyclic nucleotides and the relaxant response elicited by these vasodilators was investigated.

MATERIALS AND METHODS

To study the cyclic nucleotide accumulations associated with the endothelium-dependent relaxations induced by acetylcholine and human α-CGRP, and the endothelium-independent relaxations induced by sodium nitroprusside, rat thoracic aortic rings were prepared as described in Chapter 2, removing the endothelium from a number of rings. To determine the optimum time-point for cyclic nucleotide accumulations, single concentrations of these vasodilators giving approximately 80% relaxation of the tone induced by noradrenaline (10^{-7}M) were added and the tissues removed into liquid nitrogen at various time-points. The levels of cyclic nucleotides were then determined by scintillation proximity assay (Amersham) using the acetylation protocols (see Chapter 2 and Appendix 2).

Thereafter, the effects of various drugs on the cyclic nucleotide accumulations associated with human α-CGRP-induced endothelium-dependent vasorelaxation were studied using this optimum time-point.

Ibuprofen and glibenclamide were prepared in 100% DMSO to give

10^{-1}M and 10^{-2}M stock solutions, respectively. Subsequent dilutions were carried out using Krebs solution. Preliminary experiments indicated that, at the diluted concentrations of the drugs used, the concentration of DMSO did not affect either the contractile or relaxant properties of the tissues to any of the vasoconstrictors or vasodilators studied.

N^G-nitro-L-arginine (L-NOARG) was prepared in 1M hydrochloric acid at 50mg/ml before being diluted to 10^{-3}M and neutralised to pH 7.0.

Tissues were preincubated with ibuprofen (10^{-5}M) or glibenclamide (10^{-5}M) for a period of 30 minutes and L-NOARG (10^{-5}M) for 20 minutes before starting the experiment. All control values were obtained using the same time-course as the pretreated values, but without adding the drug under study.

RESULTS

Basal levels of cyclic nucleotides in rat thoracic aortic rings

Cyclic AMP and cyclic GMP control levels in rat thoracic aortic rings with endothelium constricted with 10^{-7}M noradrenaline were 760 ± 114 fmol/mg protein and 52 ± 9 fmol/mg protein, respectively. Removal of the endothelium did not significantly alter the level of cyclic AMP (941 ± 122 fmol/mg protein), but significantly reduced the level of cyclic GMP (22 ± 4 fmol/mg protein) (**Figure 4.1**).

Time course for relaxation and cyclic nucleotide accumulation.

Acetylcholine ($3x10^{-8}$M) caused a relaxation of 80 ± 7%. This relaxant response was rapid in onset (5s), was almost fully equilibrated at 60s and was

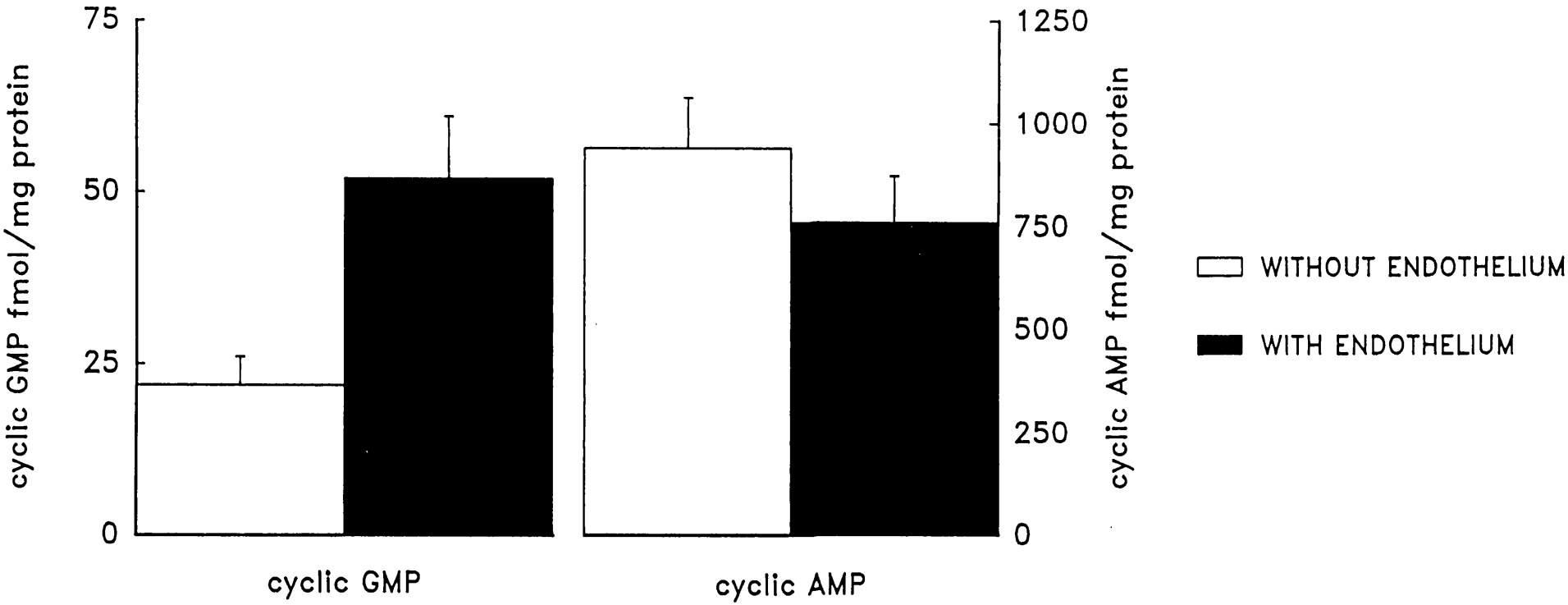


Figure 4.1 Basal levels of cyclic nucleotides in rat thoracic aortic rings with and without endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed in fmol/mg protein for cyclic GMP (left panel) and cyclic AMP (right panel). Bars represent the mean ± s.e. mean of between 4 and 10 separate experiments.

maintained for a period of at least 300s (**Figure 4.2**). While this relaxant response was developing, levels of cyclic GMP showed progressive increases, reaching a maximal 16-fold increase (853 ± 224 fmol/mg protein) at the 60s time-point (**Figure 4.3**). Levels of cyclic AMP were not significantly different from basal.

Maximum relaxation with sodium nitroprusside ($3x10^{-8}$M) was 82 ± 2% which developed over a similar time-course to acetylcholine-induced vasorelaxation, the response commencing at approximately 5s, approaching equilibration at 60s and being maintained for at least 300s (**Figure 4.2**). Sodium nitroprusside ($3x10^{-8}$M) relaxations, like acetylcholine, were associated with selective rises in cyclic GMP levels, reaching 19-fold (998 ± 209 fmol/mg protein) above basal levels at 300s. No significant increase in levels of cyclic AMP were observed at any time-point (**Figure 4.4**).

Human α-CGRP ($3x10^{-7}$M) gave a relaxation of 63 ± 3%. The response did not develop continuously but, in marked contrast to the relaxant responses induced by both acetylcholine and sodium nitroprusside, showed an initial relaxant spike (10-30s), followed by a recovery of tone (30-60s) and then a further relaxant response which achieved equilibration at 300s (**Figure 4.2**). Neither of the relaxant components or the recovery phase was affected by pretreatment with glibenclamide (10^{-5}M) (data not shown). Cyclic messenger accumulations also showed a different pattern to acetylcholine and sodium nitroprusside with progressive rises in cyclic AMP giving a maximal 3-fold rise (2443 ± 233 fmol/mg protein) at 30s and cyclic GMP giving a maximal 10-fold rise (667 ± 106 fmol/mg protein) again at 30 seconds (**Figure 4.5**).

Contrary to some of the findings of Grace *et al.*, (1987) it has been demonstrated that CGRP elevated levels of cyclic GMP, the first time an

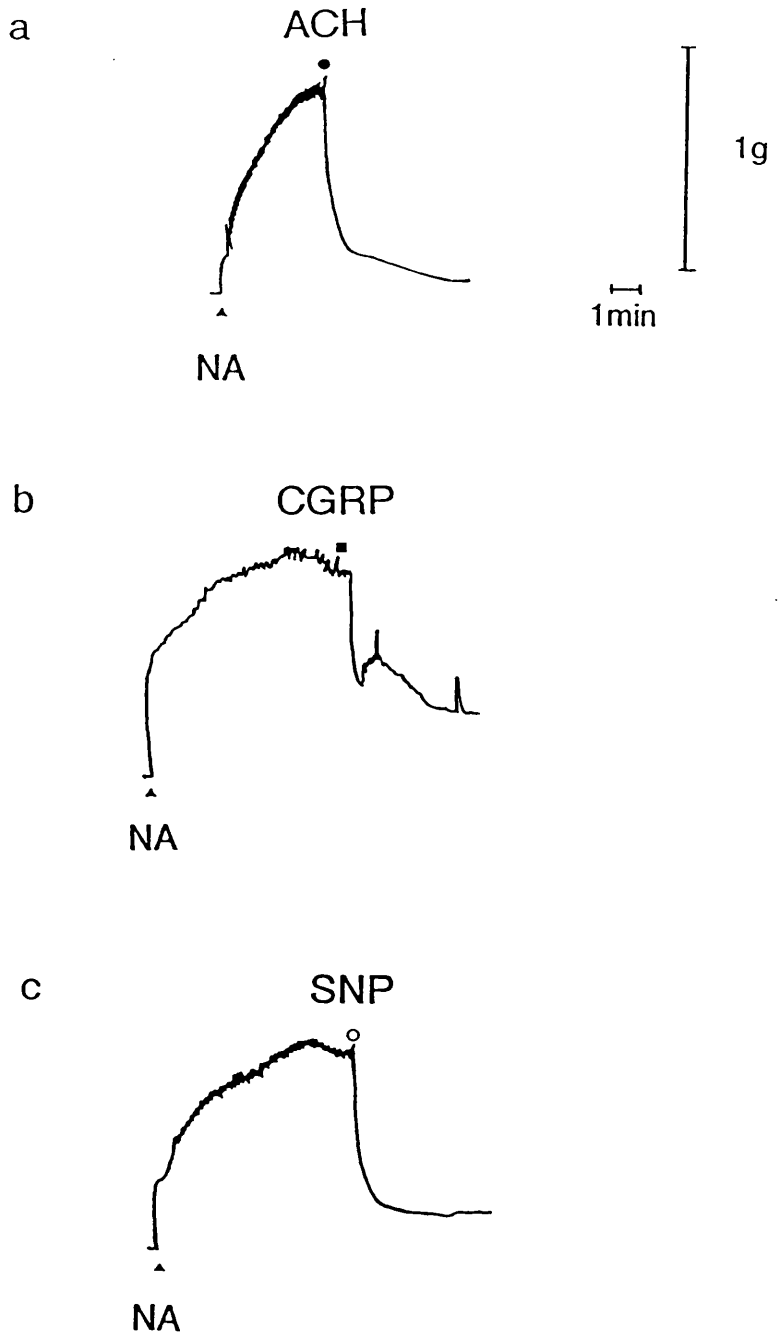


Figure 4.2 Traces showing typical relaxant effects of a) acetylcholine ($3x10^{-8}$M) (ACH) b) human α-CGRP ($3x10^{-7}$M) (CGRP) and c) sodium nitroprusside ($3x10^{-8}$M) (SNP) on rings of rat thoracic aorta preconstricted with noradrenaline (10^{-7}M) at the point labelled NA. The vasodilators were added at the points indicated.

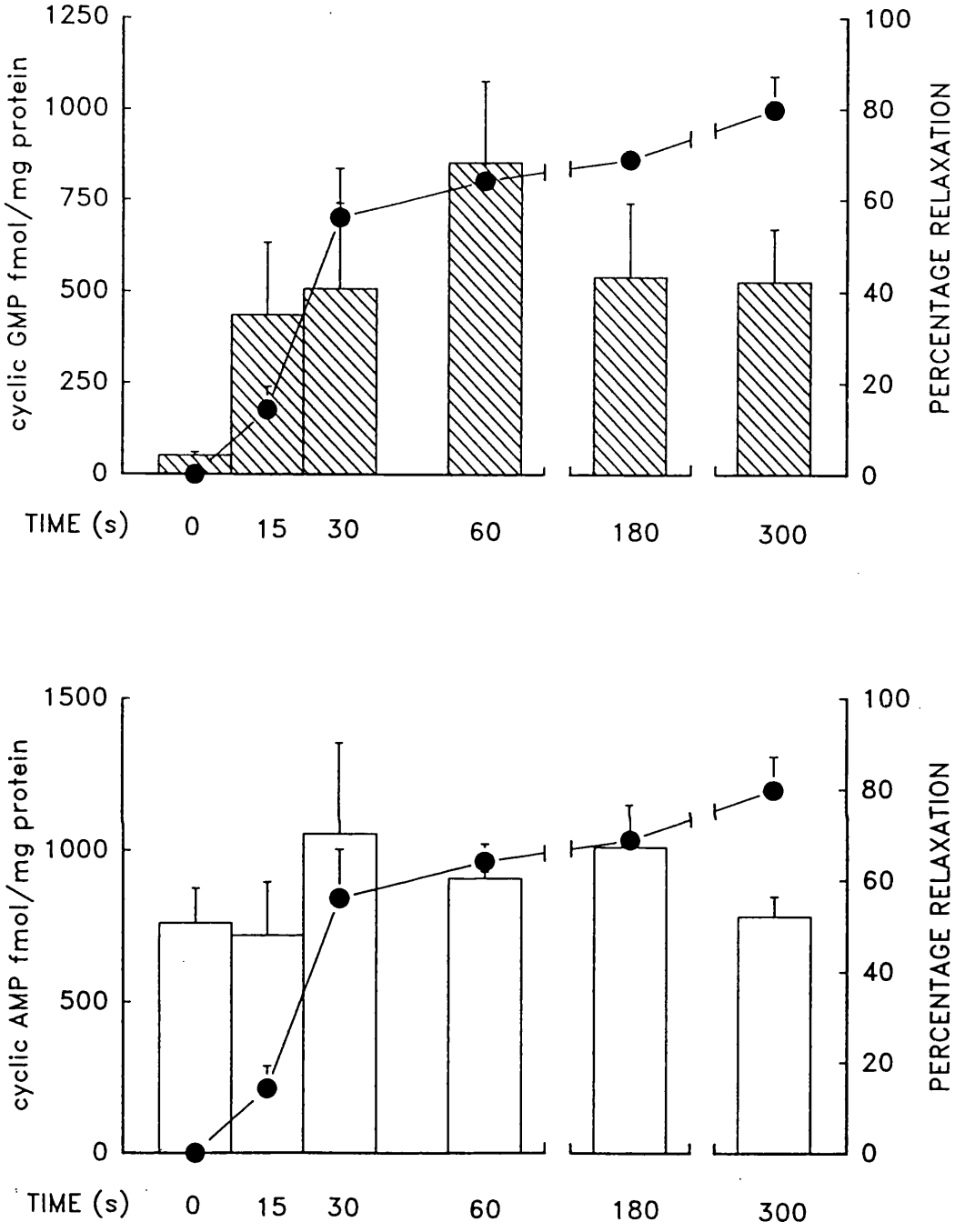


Figure 4.3 Effect of acetylcholine ($3x10^{-8}$M) on cyclic nucleotide levels and tone induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (bars) are expressed in fmol/mg protein. Relaxant responses (filled circles) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M) in the same tissues. Bars and circles represent the mean ± s.e. mean of between 3 and 10 separate experiments.

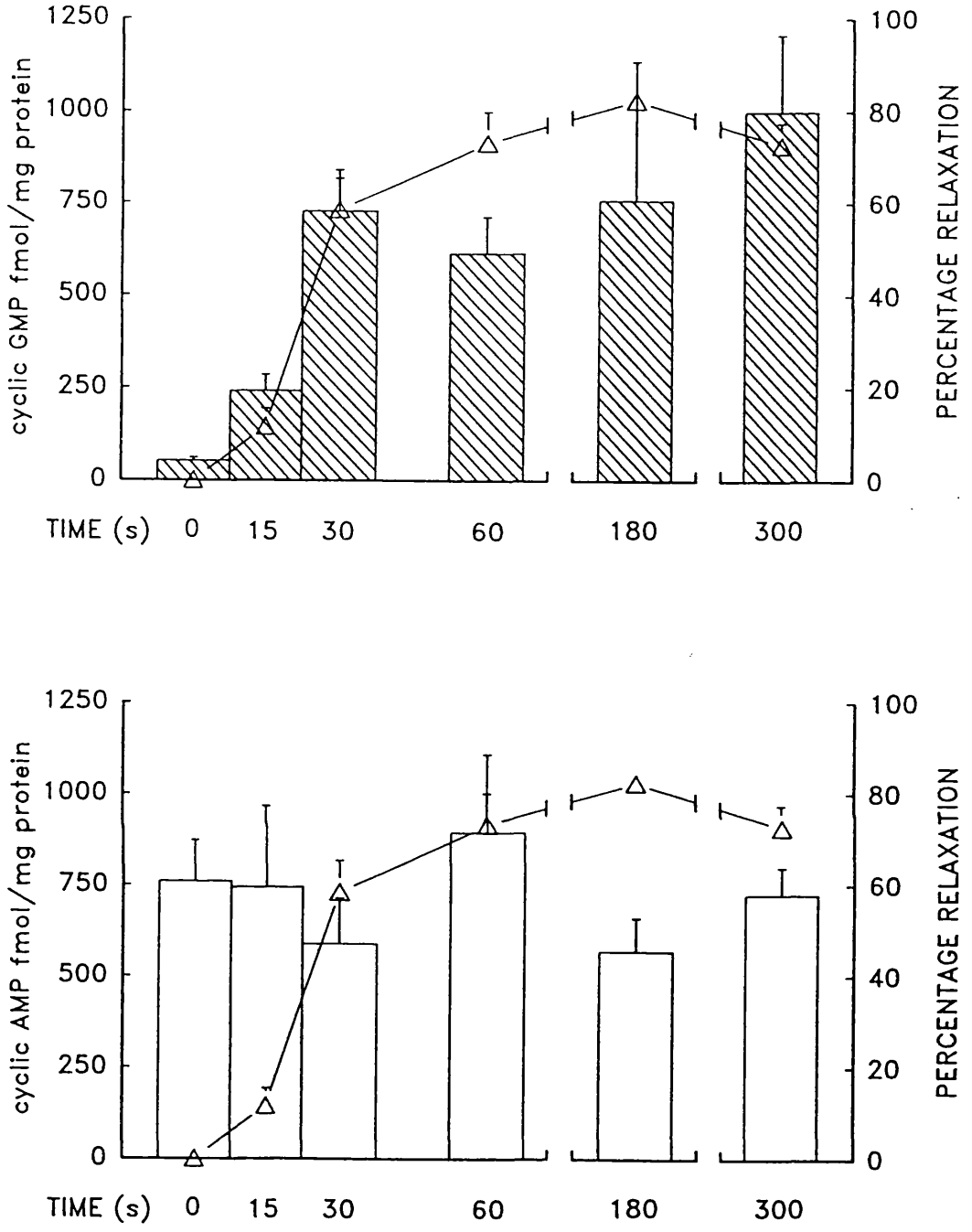


Figure 4.4 Effect of sodium nitroprusside ($3x10^{-8}$M) on cyclic nucleotide levels and tone induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (bars) are expressed in fmol/mg protein. Relaxant responses (open triangles) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M) in the same tissues. Bars and triangles represent the mean ± s.e. mean of between 3 and 10 separate experiments.

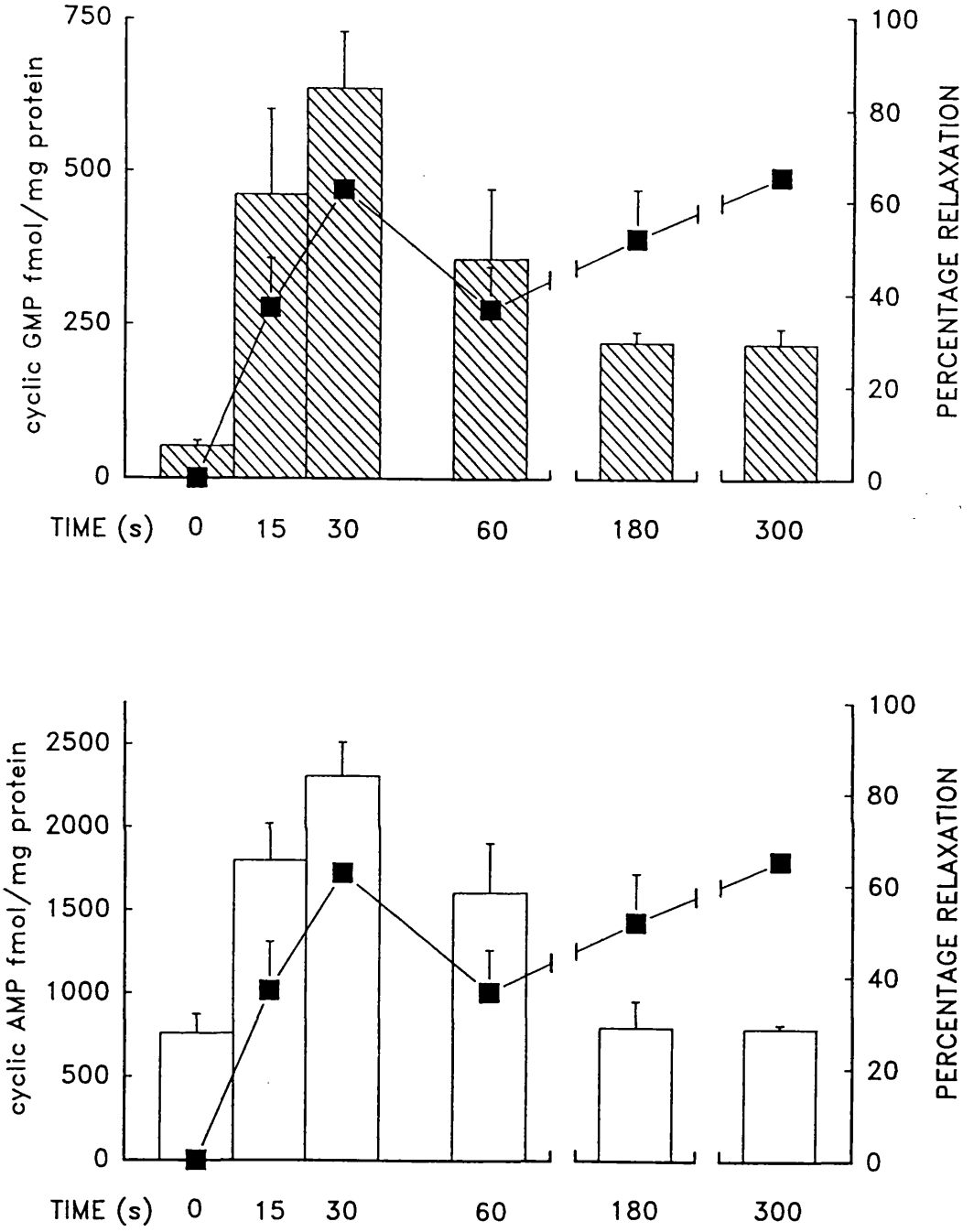


Figure 4.5 Effect of human α-CGRP ($3x10^{-7}$M) on cyclic nucleotide levels and tone induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (bars) are expressed in fmol/mg protein. Relaxant responses (filled squares) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M) in the same tissues. Bars and squares represent the mean ± s.e. mean of between 3 and 10 separate experiments.

elevation in this nucleotide has been associated with CGRP-induced vasorelaxation. This result eliminates the only reported difference between the EDRF and nitric oxide. In view of this it seems highly probable that CGRP is releasing nitric oxide as its EDRF.

Effect of endothelium removal on cyclic nucleotide accumulations associated with human α-CGRP.

Although a role has been established for nitric oxide and cyclic GMP in the CGRP signal transduction pathway the role of the elevated cyclic AMP levels remains unclear. It is possible that the rise in cyclic AMP is causally unrelated to the relaxant response elicited by human α-CGRP. If this were the case then the rise in cyclic AMP might be initiated in the smooth muscle as opposed to the rise in cyclic GMP which is initiated by EDRF released from the endothelium (**Figure 4.6**). Clearly, removal of the endothelium should abolish the relaxant response to human α-CGRP (as shown in Chapter 3) and the elevations in cyclic GMP without affecting the increases in cyclic AMP.

The effect of removing the endothelium on the cyclic nucleotide levels induced by human α-CGRP ($3x10^{-7}$M) after 30s exposure are detailed in **Figure 4.7**. There were no significant alterations in levels of either cyclic GMP (22 ± 4 fmol/mg protein: 16 ± 3 fmol/mg protein, intact: endothelium denuded) or cyclic AMP (941 ± 122 fmol/mg protein: 1050 ± 86 fmol/mg protein, intact: endothelium denuded). This result means that the pathway illustrated in **Figure 4.6** cannot exist. The elevations in cyclic AMP must be mediated by a factor released from the endothelium (**Figure 4.8**) or occur within the endothelium itself (**Figure 4.8**).

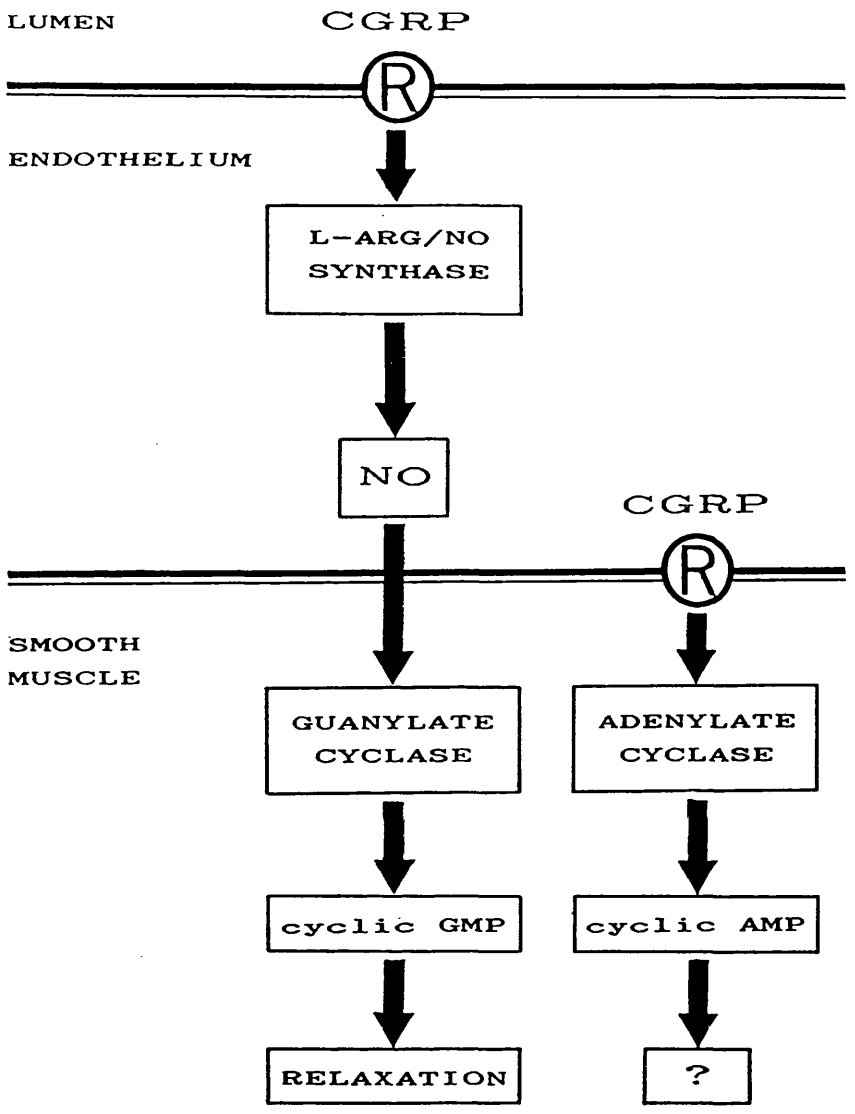


Figure 4.6 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) on both the endothelium and the smooth muscle of the vessel. The endothelial receptors activate the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation. The receptor on the smooth muscle activates adenylate cyclase. The elevations in cyclic AMP are not directly involved in relaxation.

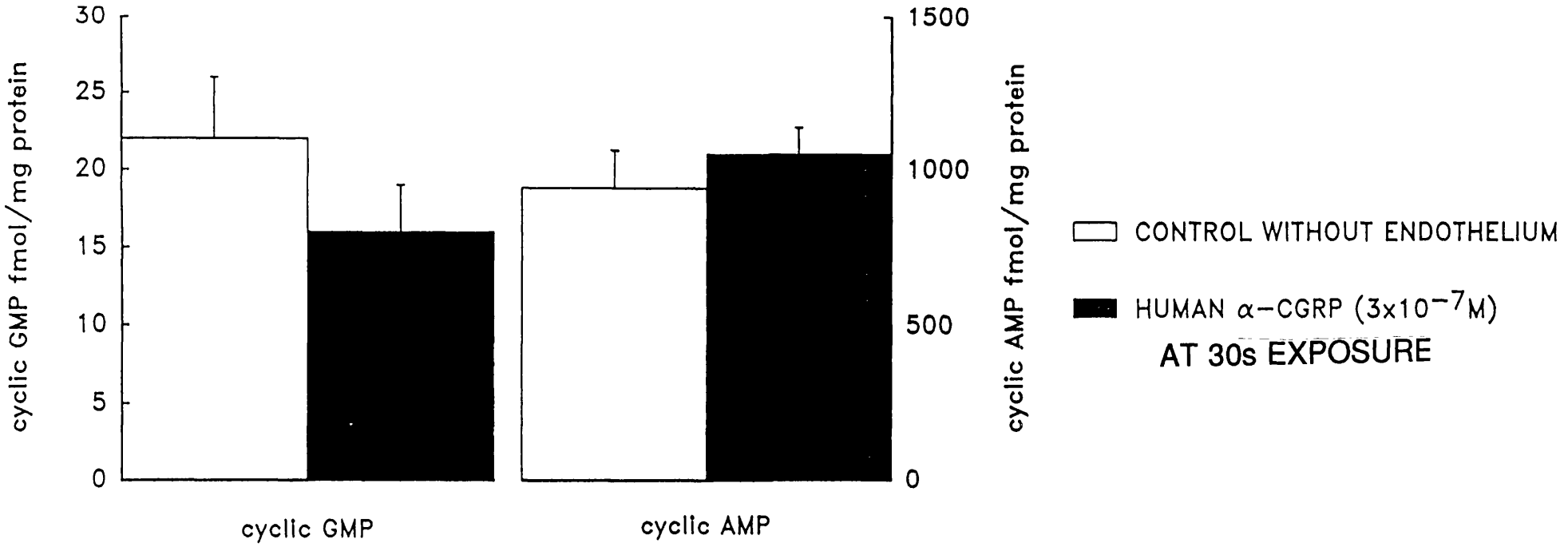


Figure 4.7 Effect of human α-CGRP ($3x10^{-7}$M) on cyclic nucleotide levels in rat thoracic aortic rings denuded of endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 5 and 10 separate experiments.

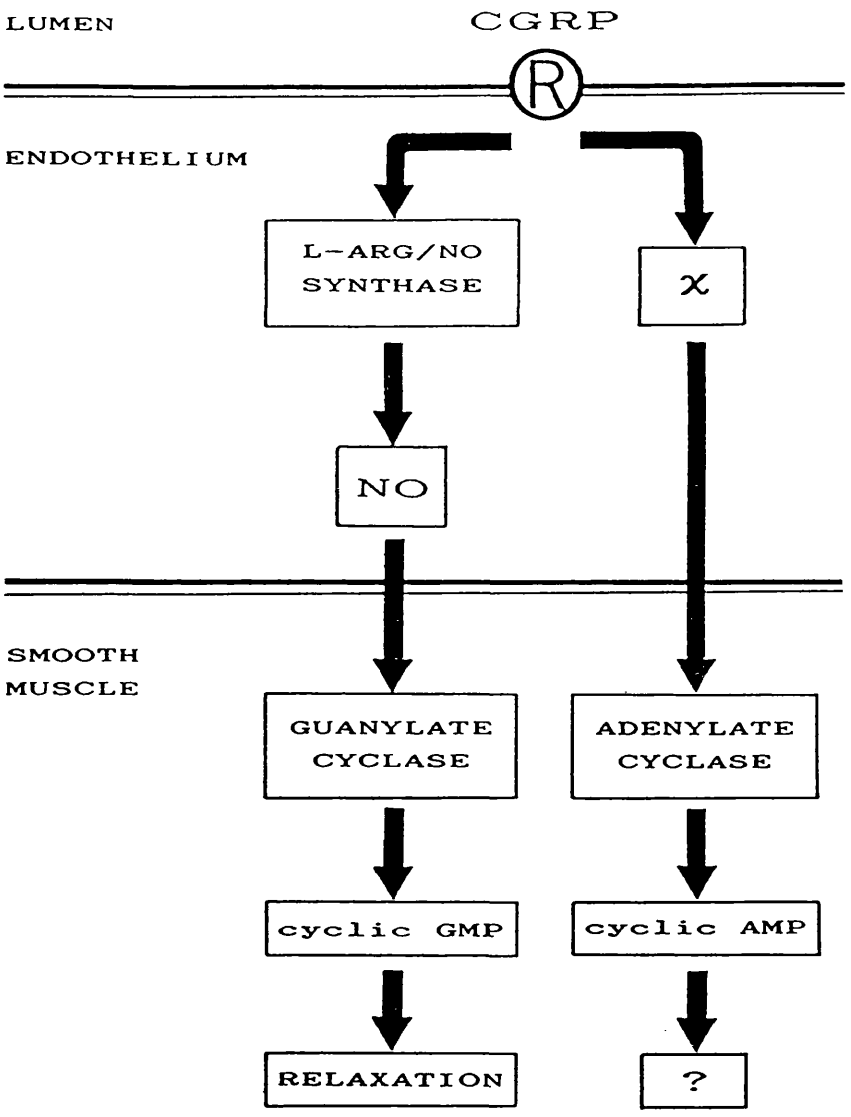


Figure 4.8 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) only on the endothelium. The endothelial receptors activate two distinct pathways: 1) the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation (left) 2) the synthesis of some other endothelium-derived substance (χ) which activates adenylate cyclase in the smooth muscle (right).

Effect of ibuprofen (10^{-5}M) on endothelium-dependent relaxations and cyclic nucleotide accumulations induced by human α-CGRP.

A number of candidates exist which would fulfil the role of a second endothelium-derived factor which activates adenylate cyclase in the smooth muscle. Despite the fact that prostacyclin does not cause relaxation in this tissue (see Chapter 3), it is capable of activating adenylate cyclase (Gorman *et al.*, 1977; Tateson *et al.*, 1977) and CGRP is capable of releasing prostacyclin from cultured endothelial cells (Crossman *et al.*, 1987). In order to establish whether any of the prostaglandins have a role in mediating any of the biochemical events seen on addition of human α-CGRP, the cyclo-oxygenase inhibitor, ibuprofen was used.

The effects of preincubating tissues in ibuprofen (10^{-5}M) on the cyclic nucleotide accumulations induced by human α-CGRP ($3x10^{-7}$M) at 30s exposure are given in **Figure 4.9**. Ibuprofen did not alter the level of relaxation (63 ± 3%: 60 ± 4%, control: pretreated) or the levels of either cyclic AMP (2443 ± 233 fmol/mg protein: 2803 ± 530 fmol/mg protein, control: pretreated) or cyclic GMP (2443 ± 233 fmol/mg protein: 2803 ± 530 fmol/mg protein, control: pretreated) evoked by human α-CGRP. This result indicates that neither prostacyclin nor any of the other prostaglandins are involved in mediating either the relaxant or the biochemical effect induced by human α-CGRP in this tissue. While the pathway in **Figure 4.8** can be considered unlikely in view of this result, the possibility of an activation of adenylate cyclase in the endothelium unrelated to the relaxant response cannot be ruled out (**Figure 4.10**).

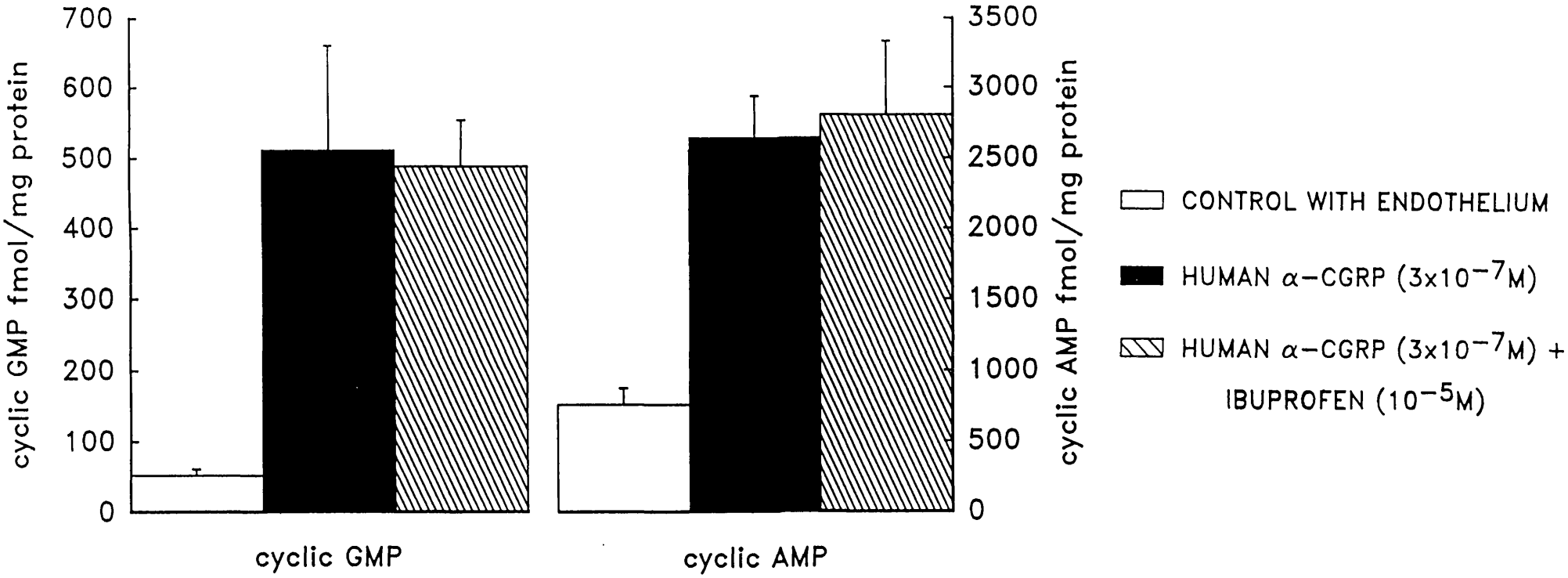


Figure 4.9 Effect of ibuprofen (10^{-5}M) on cyclic nucleotide levels induced by human α-CGRP in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 5 and 10 separate experiments.

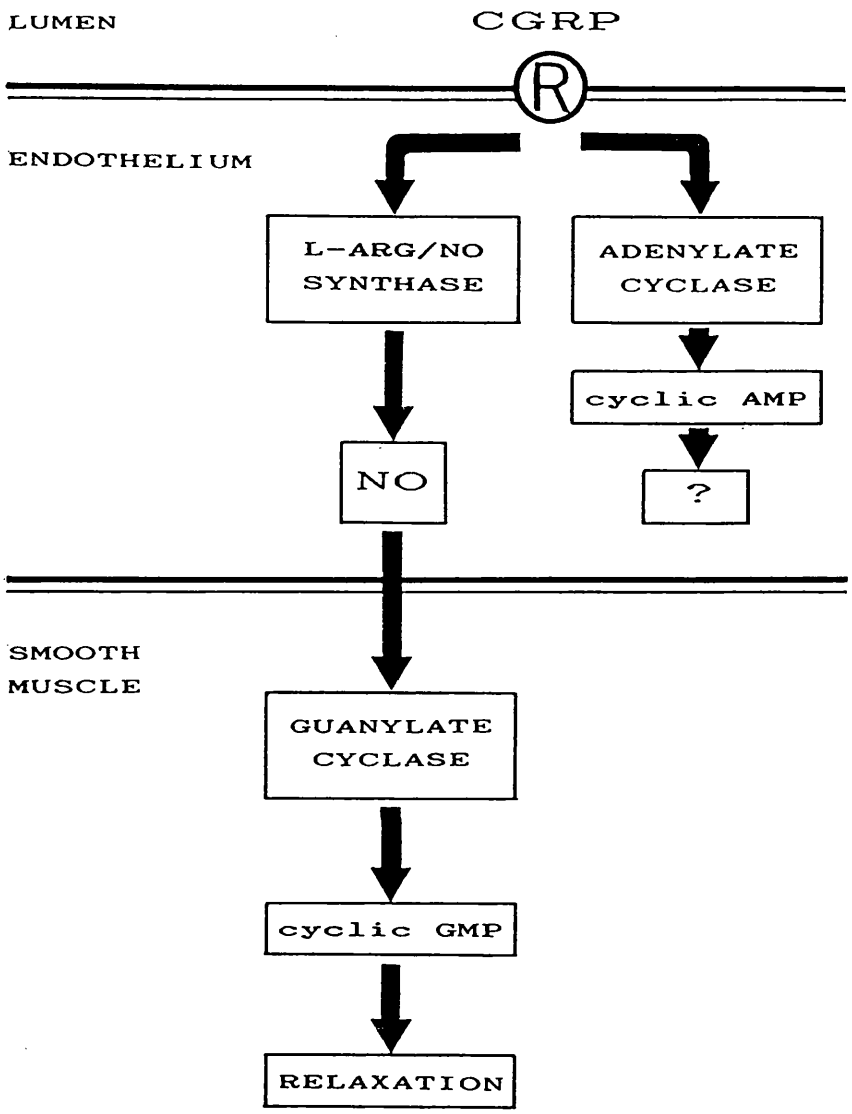


Figure 4.10 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) only on the endothelium. The endothelial receptors activate two distinct pathways: 1) the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation (left) 2) the activation of adenylate cyclase within the endothelium (right).

Effect of L-NOARG (10^{-5}M) on endothelium-dependent relaxations and cyclic nucleotide accumulations induced by human α-CGRP.

All the postulated pathways described above involve the separate activation of guanylate and adenylate cyclase in two distinct and non-interacting mechanisms. However, cyclic GMP has been shown to be capable of elevating cyclic AMP levels by inhibition of some forms of the selective cyclic AMP-phosphodiesterase, PDE IV (Yamamoto *et al.*, 1984). This could result in a sequential arrangement of a single pathway where cyclic GMP, as well as mediating the relaxant response, causes the elevations in cyclic AMP (**Figures 4.11**). However, this must be regarded as unlikely since, at the concentrations studied, acetylcholine and sodium nitroprusside increased cyclic GMP to levels above those achieved for human α-CGRP but did not alter the levels of cyclic AMP.

Alternatively, in **Figure 4.12** the elevation in cyclic AMP leads, either directly or indirectly, to the activation of nitric oxide synthase and thus to a rise in cyclic GMP.

To differentiate between these postulated transduction pathways the nitric oxide synthase inhibitor, L-NOARG was used. If the rise in cyclic GMP is responsible for the subsequent elevations in cyclic AMP (**Figure 4.11**), then inhibiting the synthesis of nitric oxide should inhibit the elevations in both cyclic nucleotides seen on addition of CGRP. If, however, the rise in cyclic AMP mediates the activation of nitric oxide synthesis (**Figure 4.12**), then only the elevations in cyclic GMP levels should be affected.

The remaining possibility is that the levels of cyclic GMP and cyclic AMP seen on addition of human α-CGRP may remain unaltered. Since it

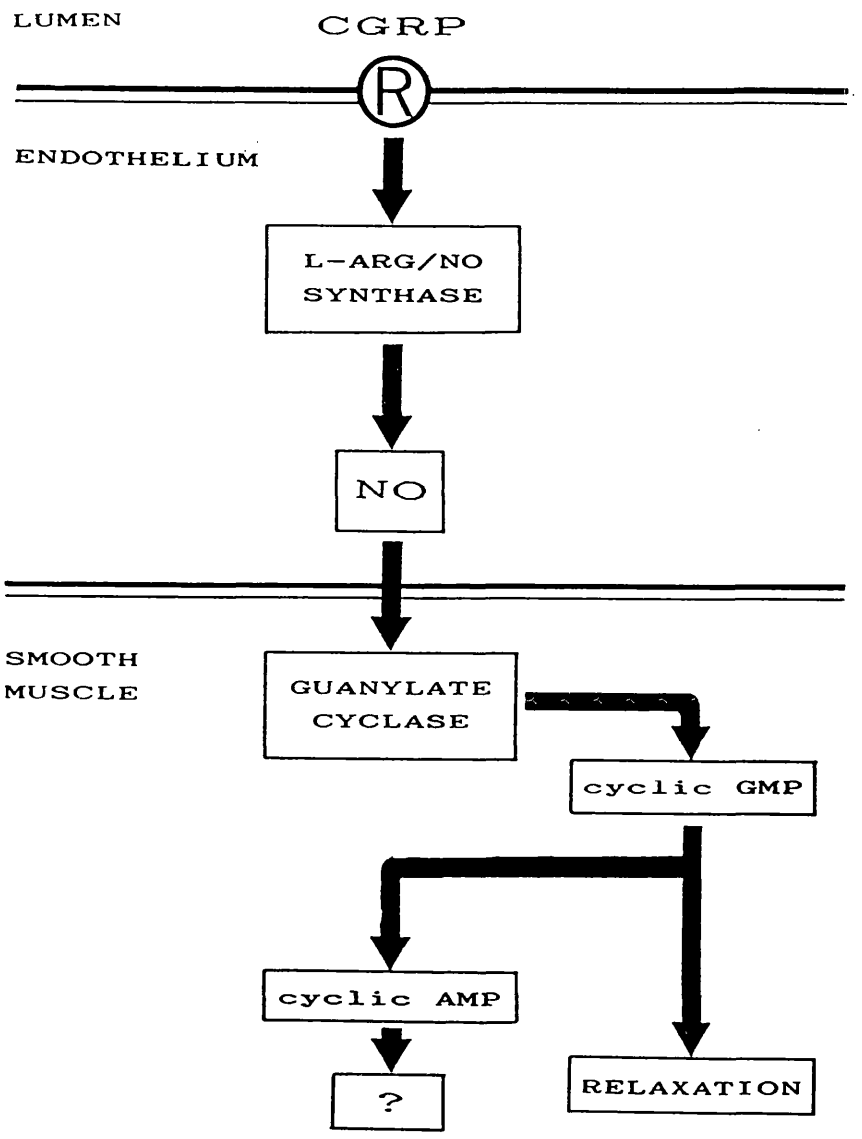


Figure 4.11 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) only on the endothelium. The endothelial receptors activate the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation. However, the elevations in cyclic GMP also cause increases in cyclic AMP.

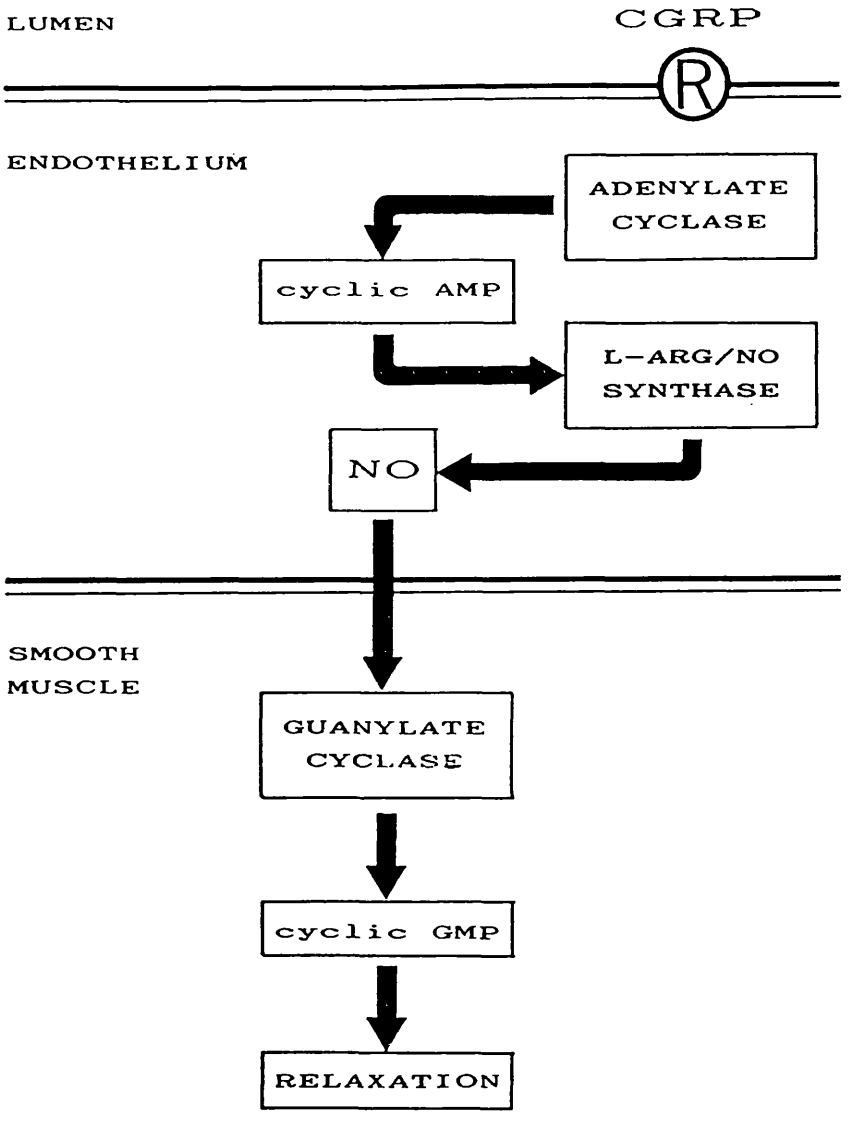


Figure 4.12 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) only on the endothelium. The endothelial receptors activate adenylate cyclase, elevating cyclic AMP levels in the endothelium. This activates the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation in the smooth muscle.

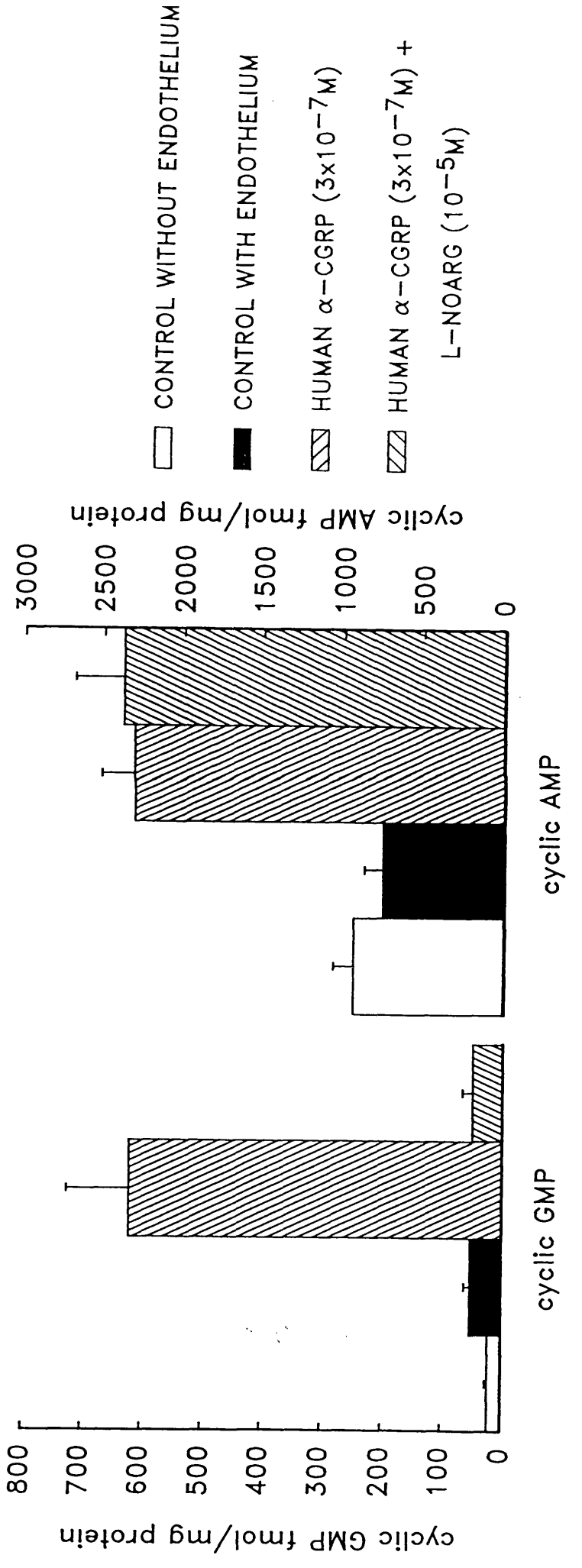


Figure 4.13 Effect of L-NOARG (10^{-5}M) on cyclic nucleotide levels induced by human α-CGRP ($3x10^{-8}$M) in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 5 and 10 separate experiments.

was shown in Chapter 3 that the L-NOARG inhibited the relaxant effect of CGRP in this tissue then this would indicate that neither cyclic nucleotide is directly involved in the transduction mechanism.

At 30 seconds, $3x10^{-7}$M human α-CGRP caused a relaxant response of 63 ± 3%. Preincubation with L-NOARG (10^{-5}M) reduced this response to 7 ± 3%. Levels of cyclic GMP were also reduced from 667 ± 106 fmol/mg protein where human α-CGRP alone was present to 49 ± 16 fmol/mg protein where L-NOARG was also present (**Figure 4.13**)

Levels of cyclic AMP were not significantly affected by pretreatment with L-NOARG (10^{-5}M), the levels being 2443 ± 233 fmol/mg protein and 2376 ± 306 fmol/mg protein for control and pretreated tissues respectively.

It is clear from the data presented above that the idea of an increase in cyclic GMP levels leading to activation of adenylate cyclase (**Figure 4.11**) is no longer tenable. L-NOARG inhibited the relaxant response to CGRP and also selectively decreased the levels of cyclic GMP, leaving the levels of cyclic AMP unaltered.

DISCUSSION

Acetylcholine and CGRP have been shown to require the presence of an intact endothelium in order to relax rat thoracic aortae (Brain *et al.*, 1985; Grace *et al.*, 1987). The endothelium-derived relaxing factor for acetylcholine has been identified as nitric oxide (Palmer *et al.*, 1987a; 1988a). In view of the reported differences in second messenger accumulation for acetylcholine and CGRP, these being cyclic GMP (Griffith *et al.*, 1985) and cyclic AMP (Kubota *et al.*, 1985; Grace *et al.*, 1987; Hirata *et al.*, 1988) respectively, it

appeared possible that another factor and not nitric oxide was being released by CGRP. The results described in Chapter 3 from the pharmacological studies indicated that the EDRF released by CGRP could not be distinguished from that released by acetylcholine. Therefore it is surprising that the published data indicates that CGRP does not give a rise in cyclic GMP.

In this chapter the cyclic nucleotide accumulations associated with acetylcholine, sodium nitroprusside and human α-CGRP endothelium-dependent relaxations were studied. As reported in the literature, acetylcholine and sodium nitroprusside selectively increased levels of cyclic GMP, levels of cyclic AMP being unaltered (Ignarro *et al.*, 1984; Griffith *et al.*, 1985). Limitations of the equipment meant that it was impossible to work to a short enough time-course to show that the rises in cyclic GMP preceded the relaxant response. However, at the first time-point (15s) relaxation was under way and cyclic GMP levels were raised for both acetylcholine and sodium nitroprusside, so there is no evidence to suggest that the relaxations are unrelated and therefore precede the increases in cyclic GMP.

In contrast to the smooth, monophasic relaxations seen on addition of single, high concentrations of acetylcholine or sodium nitroprusside, single, high concentrations of human α-CGRP induced relaxations comprising an initial rapid relaxation, then a slower recovery of tone followed by a further relaxant phase. This complex relaxation to a single high concentration of human α-CGRP is in contrast to the effects of cumulative addition (**Figure 3.2**), where relaxations, although slower in onset and equilibration than either acetylcholine or sodium nitroprusside gave a similar monophasic relaxation. It was shown in **Figure 3.7** that the ATP-sensitive potassium channel blocker, glibenclamide, did not affect the cumulative concentration/effect curve to human α-CGRP, despite reports that it inhibited CGRP-induced endothelium-dependent relaxations in the rat mesenteric artery (Nelson *et al.*, 1990). In

view of the complex relaxant effect elicited by single, high concentrations of human α-CGRP in the rat aorta, it was considered possible that one or more components of this response might prove sensitive to glibenclamide. However, glibenclamide was without effect on either of the relaxant components or the recovery phase. This rules out the involvement of the ATP-sensitive potassium channel in mediating any part of the relaxant response to human α-CGRP in this tissue.

Contrary to the findings of Grace *et al.*, (1987) it was found that human α-CGRP elevated levels of cyclic GMP. This result eliminates the only reported difference between the EDRF released by CGRP in the rat thoracic aorta and nitric oxide.

The EDRF released by human α-CGRP in the rat thoracic aorta therefore has the following properties: 1) It appears to be synthesised from L-arginine by nitric oxide synthase, since L-NMMA and L-NOARG inhibited the relaxant response to CGRP and this inhibitory effect could be partially reversed by simultaneous administration of L-, but not D-arginine. 2) Its action is inhibited by agents known to inhibit nitric oxide action but is unaffected by agents thought to inhibit novel EDRFs. 3) Its relaxant effect is associated with rises in cyclic GMP, like nitric oxide. In view of these properties, it seems highly probable that CGRP is releasing nitric oxide as its EDRF.

One difference remains between the endothelium-dependent relaxations induced by human α-CGRP and acetylcholine. While acetylcholine selectively increases levels of cyclic GMP, CGRP elevates levels of both cyclic GMP and cyclic AMP. If, as seems likely from the data presented above, CGRP is releasing nitric oxide as its EDRF, then the stimulation of guanylate cyclase resulting in increased cyclic GMP levels is readily

explained. However, the role of elevations in cyclic AMP levels is unclear.

There are reports that in cultured endothelial cells and cultured vascular smooth muscle cells CGRP elicits selective increases in cyclic AMP (Kubota *et al.*, 1985; Hirata *et al.*, 1988). In contrast to this, it was shown above that in rings of rat aorta, elevations in both cyclic AMP and cyclic GMP occurred but only when the endothelium was present. This conflicting result is unlikely to be due to the species differences, but possibly is accounted for by the observed detrimental effects of culturing endothelial cells on responses to a number of agonists including acetylcholine (Gryglewski *et al.*, 1986b) and human α-CGRP (J.A. Mitchell, private communication; Crossman *et al.*, 1991). Further, it has recently been shown that the smooth muscle of the rat thoracic aorta does not display high affinity binding sites for CGRP, but that these develop when the smooth muscle cells are cultured (Connat *et al.*, 1991). In view of these observations it seems likely that the rises in cyclic AMP seen in cultured smooth muscle cells on challenge with CGRP represent an artifact of the culturing process.

The observation that no elevations in cyclic AMP occurred when the endothelium was absent, although ruling out the existence of receptors for CGRP mediating the rise in cyclic AMP on the vascular smooth muscle, does not necessarily require the cyclic AMP increases to occur solely within the endothelium. It is possible that a second endothelium-derived factor is being released from the endothelium by CGRP which activates adenylate cyclase within the smooth muscle. This second factor need only mediate the rise in cyclic AMP since the simultaneous release of nitric oxide by CGRP would be sufficient to account for any relaxant response. However, the lack of effect of ibuprofen (**Figure 4.8**) on the relaxations or cyclic nucleotide accumulations induced by human α-CGRP rules out the involvement of the prostaglandins and prostacyclin in particular as mediating the effects induced

by CGRP.

Although there are a number of mechanisms by which cyclic GMP elevations could subsequently lead to rises in cyclic AMP, for example, activation of adenylate cyclase or inhibition of phosphodiesterase by cyclic GMP, it is highly improbable that the rise in cyclic GMP mediates the subsequent rise in cyclic AMP for a number of reasons. Acetylcholine and sodium nitroprusside at the concentrations used elevated cyclic GMP levels above those achieved by human α-CGRP but did not cause any alterations in the levels of cyclic AMP. Further, the nitric oxide synthase inhibitor, L-NOARG inhibits the relaxation and rise in cyclic GMP induced by CGRP without affecting the accumulation of cyclic AMP. This result, while being entirely consistent with the postulated mechanism of action of L-NOARG and with CGRP releasing nitric oxide as its EDRF, requires the elevations in cyclic AMP to precede the activation of nitric oxide synthase, a novel mechanism for the activation of this enzyme, if they are involved in the relaxant response induced by CGRP at all.

There have been a number of reports of vasodilators known to act by increasing levels of cyclic AMP having an endothelium-dependent component to their relaxant responses. These include forskolin and prostacyclin *in vitro* in the pig coronary artery, both vasodilators having an element of their relaxant response which can be inhibited by haemoglobin in this tissue (Shimokawa *et al.*, 1988), and salbutamol *in vivo* in the rat (Gardiner *et al.*, 1991a) which is partially inhibited with L-NOARG. In view of these results, it appears likely that CGRP and a number of other drugs exert their endothelium-dependent relaxant effects through the release of nitric oxide by the novel signal transduction pathway given in **Figure 4.11.** This postulated mechanism, while consistent with the known initial step in the transduction mechanisms of action of prostacyclin, forskolin, β-adrenoreceptor agonists

and, indeed, human α-CGRP, conflicts with results in cultured endothelial cells where it has been shown that neither cyclic AMP nor cyclic GMP activate the synthesis or release of nitric oxide (Kuhn *et al.*, 1991).

In view of these conflicting results, it was considered possible that a number of classical adenylate cyclase activating, endothelium-independent vasodilators might display an endothelium-dependent relaxant component to their relaxations in the rat aorta.

In Chapter 5, this hypothesis was studied utilising a number of β-adrenoreceptor agonists and the diterpene, forskolin in the rat thoracic aorta. Their effect was clarified using both pharmacological and biochemical techniques.

CHAPTER 5

INTRODUCTION

Since the discovery in 1980 by Furchgott and Zawadzki that the endothelium was capable of releasing a relaxant factor it has become customary to categorise vasodilators as either endothelium-dependent, for example, acetylcholine, substance P and bradykinin or endothelium-independent, for example, the nitrovasodilators.

It is apparent from the Introduction to this thesis that CGRP belongs to a third category, those vasorelaxants having both an endothelium-dependent and -independent mechanism of inducing vasorelaxation. From the results presented in Chapters 3 and 4, it is clear that CGRP endothelium-dependent relaxations have an unusual signal transduction pathway involving nitric oxide but rises in both cyclic AMP and cyclic GMP.

Prostacyclin and forskolin (Shimokawa *et al.*, 1988) and β-adrenergic agonists (Rubanyi & Vanhoutte, 1985a) in the pig coronary artery and salbutamol *in vivo* (Gardiner *et al.*, 1991a) in the rat have been shown to owe at least part of their vasodilatory activity to an endothelium-dependent mechanism. The first step mediating the vasorelaxant effects of these drugs is known to be activation of adenylate cyclase. It is possible that a general mechanism for activation of the enzyme nitric oxide synthase might exist, the first stage being the activation of adenylate cyclase.

If this were true then other agonists known to act by elevating cyclic AMP levels might also have an endothelium-dependent component to their relaxant effects. The β-adrenoreceptor agonists, isoprenaline and salbutamol, were chosen since the receptors have been cloned and are known to be linked to adenylate cyclase activation (Nahorski *et al.*, 1975) via the guanine-nucleotide binding protein, G_s (Gilman, 1986), and the receptors are known to be present on endothelial cells (Stephenson & Summers, 1987; Molenaar *et al.*, 1988). Forskolin was chosen as it activates adenylate cyclase directly (Miller & Baer, 1983; Karnushina *et al.*, 1983).

MATERIALS AND METHODS

To study the relaxations induced by isoprenaline, salbutamol and forskolin, endothelium-denuded and -intact rings of rat thoracic aorta were prepared as described in Chapter 2. Cumulative dose/response curves to isoprenaline, salbutamol and forskolin were carried out in the presence of noradrenaline (10^{-7}M). The effects of a number of drugs on the contractions elicited by noradrenaline and the relaxations induced by isoprenaline and forskolin were studied.

Alternatively, to study the cyclic nucleotide accumulations associated with the relaxations induced by isoprenaline and forskolin, rat thoracic aortic rings were mounted for isometric recording of tension in organ baths. To determine the optimum time-point for cyclic nucleotide accumulations, a single concentration of isoprenaline giving approximately 80% relaxation of the tone induced by noradrenaline (10^{-7}M) was added and the tissues removed into liquid nitrogen at various time-points. Cyclic nucleotide levels were determined by scintillation proximity assay (Amersham) using the acetylation protocol. Thereafter, the effects of endothelium removal and L-NOARG on

the cyclic nucleotide accumulations associated with isoprenaline-vasorelaxation were studied using this optimum time-point. The effect of L-NOARG on forskolin-induced vasorelaxation was also studied utilising a similar protocol to that described for isoprenaline but removing the rings after 60s exposure to forskolin.

Test drugs

N^G-nitro-L-arginine (L-NOARG) was prepared in 1M hydrochloric acid at 50mg/ml before being diluted to 10^{-3}M and neutralised to pH 7.0. Propranolol and ICI 118551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were prepared in Krebs solution as 10^{-2}M stock solutions. Tissues were preincubated with these drugs for a period of 30 minutes before starting the experiment. All control values were obtained using the same time-course as the pretreated values, but without adding the drug under study.

RESULTS

In the tissues used for this series of experiments, noradrenaline (10^{-7}M) evoked an increase in tone of the rat aortic rings with intact endothelium to 1.2 ± 0.1g. In endothelium-denuded rings the contractile response was significantly increased (2.0 ± 0.2g). Intact rings were relaxed by acetylcholine (10^{-6}M) by greater than 80%, while endothelium denuded rings displayed no response.

Endothelium-dependent relaxation.

Isoprenaline ($3x10^{-8}$-10^{-5}M), salbutamol ($3x10^{-7}$-10^{-4}M) and forskolin ($3x10^{-9}$-$3x10^{-7}$M) caused concentration-dependent relaxations in rat thoracic

aorta which were wholly dependent on the presence of an intact endothelium (**Figure 5.1**). The EC_{50} (concentration required to give a half maximal response) for isoprenaline, 1.5 ± 0.1x10^{-7}M, was intermediate, that of salbutamol being lower at 4.6 ± 0.2x10^{-6}M, with that of forskolin being higher at 2.6 ± 0.3x10^{-8}M. All three vasodilators induced a maximum response which was approximately 100% of the tone induced by noradrenaline (10^{-7}M). Preliminary experiments indicated that relaxation to isoprenaline in rat aorta from Wistar rats over the same range (3x10^{-8}-10^{-5}M) was also endothelium-dependent and reached a similar maximum level of relaxation, but relaxation in rabbit aorta (10^{-7}-10^{-5}M) occurred where the endothelium had been removed.

The traces illustrated in **Figure 5.2** depict the relaxant responses elicited by cumulative concentrations of isoprenaline, salbutamol and forskolin. All three vasodilators have a similar time-course for the onset and equilibration of the relaxant response, these being approximately 15s and 180s respectively. Considerable spontaneous activity was seen in endothelium-intact rings but this was absent in all endothelium-denuded rings.

Effect of propranolol and ICI 118551 on isoprenaline-induced endothelium-dependent relaxation

Neither propranolol (3x10^{-7}M) nor ICI 118551 (10^{-7}M) had any significant effect on the noradrenaline (10^{-7}M)-induced contraction. The concentration/effect curve to isoprenaline was shifted by propranolol (3x10^{-7}M) to the right in a parallel fashion (**Figure 5.3**). Preliminary experiments with higher concentrations of propranolol gave larger shifts (3x10^{-6}M propranolol giving approximately a 100-fold rightward parallel shift with no decrease in the maximum response), indicating that propranolol is a competitive antagonist at this site. The degree of shift, approximately ten-fold

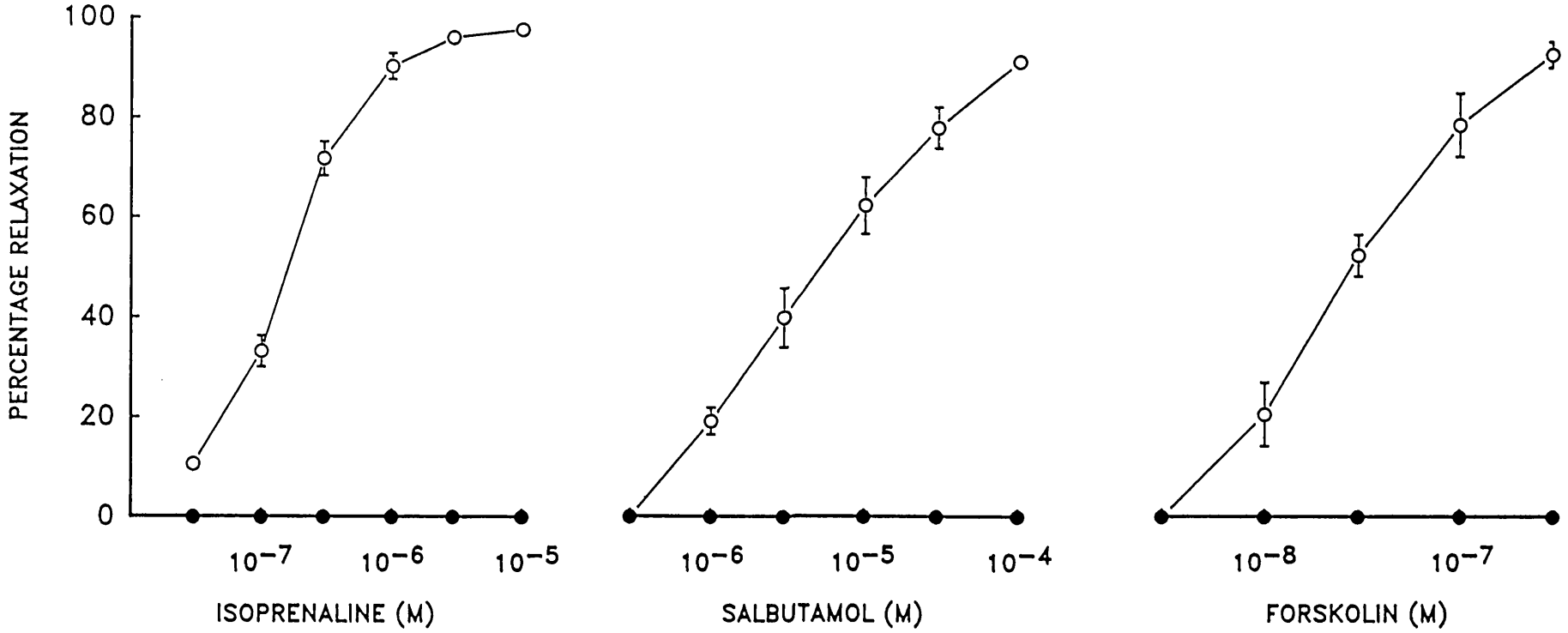


Figure 5.1 Effect of removal of endothelium on relaxations induced by isoprenaline, salbutamol and forskolin in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O are responses in intact rings, ● the responses in endothelium denuded rings for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

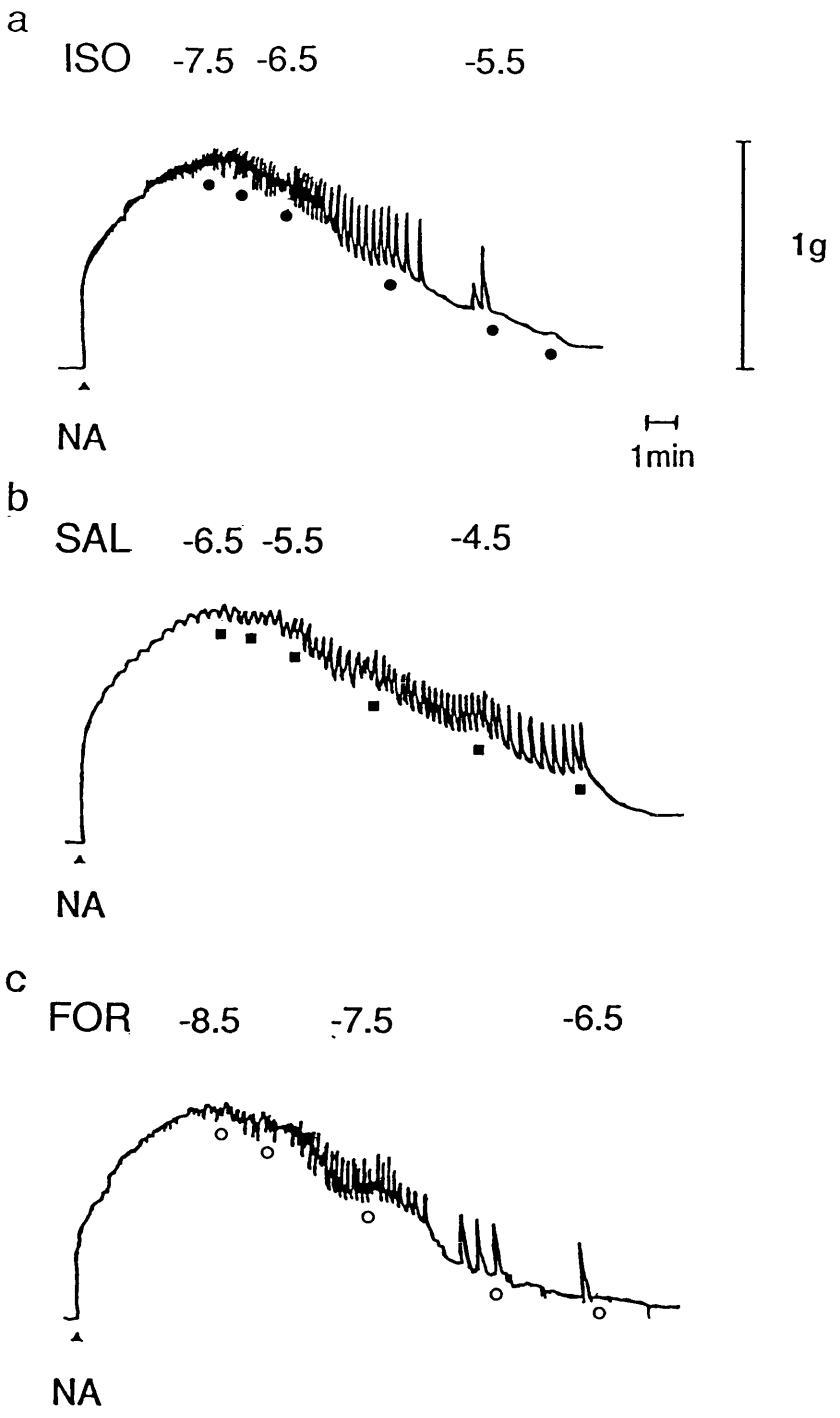


Figure 5.2 Traces showing typical relaxant effects of cumulative addition of a) isoprenaline (ISO), b) salbutamol (SAL) and c) forskolin (FOR) on rings of rat thoracic aorta with intact endothelium. All rings were constricted with noradrenaline (10^{-7}M) at the point labelled NA. The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

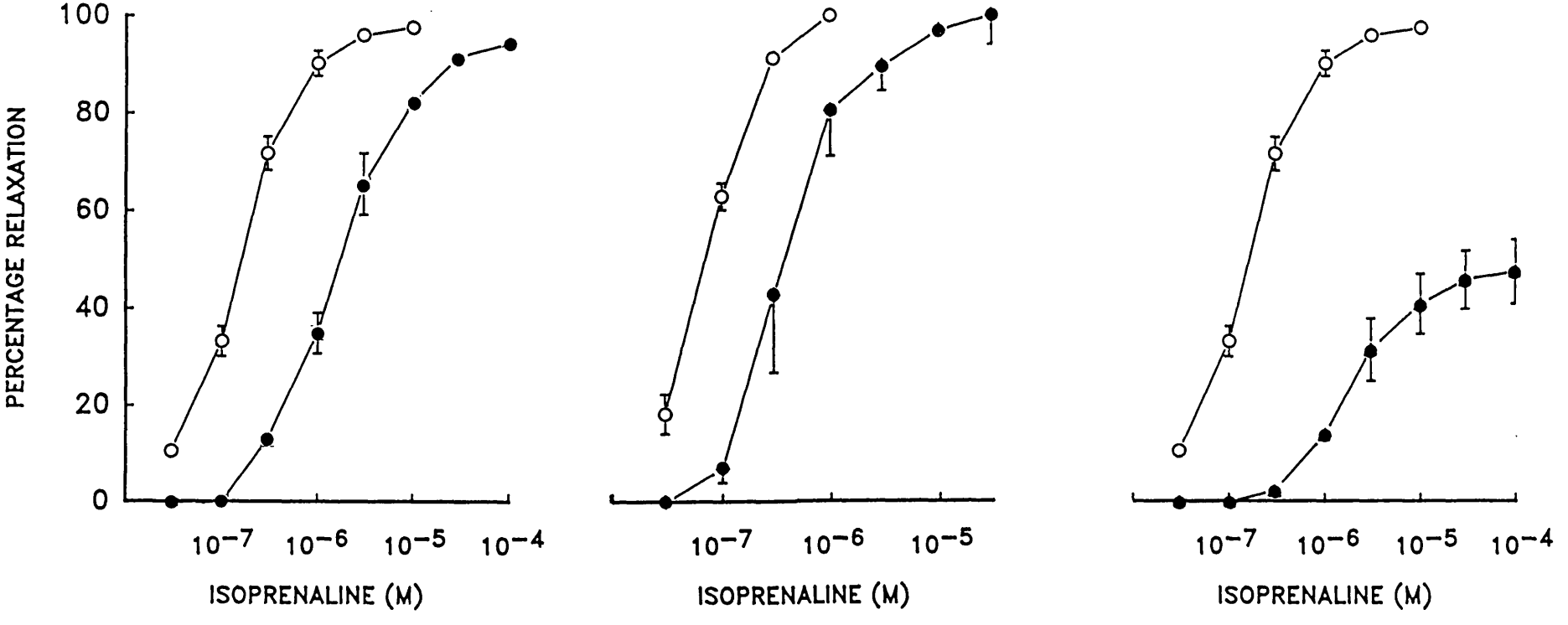


Figure 5.3 Effect of propranolol ($3x10^{-6}$M) (left), ICI 118551 (10^{-7}M) (middle) and L-NOARG (10^{-5}M) (right) on relaxations induced by isoprenaline in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the pretreated values for each vasodilator. Points represent the mean ± s.e. mean of 4 separate experiments.

with $3x10^{-7}$M propranolol (calculated pA_2 7.5), is consistent with the known actions of both isoprenaline and propranolol, indicating that this endothelium-dependent relaxant effect of isoprenaline is being mediated via a β-adrenoreceptor.

ICI 118551 (10^{-7}M) is reported to be a selective antagonist for the β_2-subtype of adrenoreceptor (O'Donnell & Wanstall, 1980). In the rat thoracic aorta, ICI 118551 caused parallel rightward shift of the isoprenaline concentration/effect curve with no alteration in maximum response (**Figure 5.3**) (calculated pA_2 7.6).

Neither of the antagonists of the isoprenaline response caused significant alterations in the speed of onset or equilibration of the relaxant response.

Effect of L-NOARG on isoprenaline-induced endothelium-dependent relaxation

The nitric oxide synthase inhibitor, L-NOARG (10^{-5}M) significantly augmented the tone induced by noradrenaline (10^{-7}M) to 1.9 ± 0.2g in endothelium-intact rings of rat aorta. L-NOARG (10^{-5}M) shifted the relaxant response to isoprenaline to the right while decreasing the maximum response from 98% to 48% of the tone induced by noradrenaline (10^{-7}M) (**Figure 5.3**). L-NOARG (10^{-5}M) did not significantly alter the speed of onset or equilibration of the isoprenaline response.

Basal levels of cyclic nucleotides in rat thoracic aortic rings

As stated in Chapter 4, cyclic AMP and cyclic GMP control levels in rat thoracic aortic rings with endothelium constricted with noradrenaline (10^{-7}M) were 760 ± 114 fmol/mg protein and 52 ± 9 fmol/mg protein,

respectively. Removal of the endothelium did not significantly alter the levels of cyclic AMP (941 ± 122 fmol/mg protein), but significantly reduced the levels of cyclic GMP (30 ± 4 fmol/mg protein) (**Figure 4.1**).

Time course for relaxation and cyclic nucleotide accumulation

Isoprenaline (10^{-6}M) caused a relaxant response of 85 ± 5% of the tone induced by noradrenaline (10^{-7}M). This response developed in a single relaxant phase which started between 0 and 15s and reached a plateau at approximately 180s after the initial exposure to isoprenaline. As this relaxant response developed, increases in levels of both cyclic GMP and cyclic AMP were observed. These reached a maximum at 30s with levels of cyclic GMP being 12-fold (621 ± 105 fmol/mg protein) and levels of cyclic AMP being 4-fold (2751 ± 151 fmol/mg protein) above basal levels (**Figure 5.4**).

Effect of endothelium removal on cyclic nucleotide accumulations induced by isoprenaline

In rings denuded of endothelium, isoprenaline (10^{-6}M) evoked no relaxant response. Further, it caused no alterations in levels of either cyclic AMP (control; 941 ± 122 fmol/mg protein; isoprenaline treated; 924 ± 78 fmol/mg protein) or cyclic GMP (control; 22 ± 4 fmol/mg protein; isoprenaline treated; 19 ± 6 fmol/mg protein) (**Figure 5.5**).

Effect of L-NOARG on relaxation and second messenger accumulation induced by isoprenaline and forskolin

At 30 seconds, isoprenaline (10^{-6}M) caused a relaxant response of 69 ± 4%. Preincubation with L-NOARG (10^{-5}M) significantly reduced this response to 13 ± 6%. Levels of cyclic GMP were also reduced from 621 ± 105 fmol/mg protein where isoprenaline alone was present to 38 ± 15

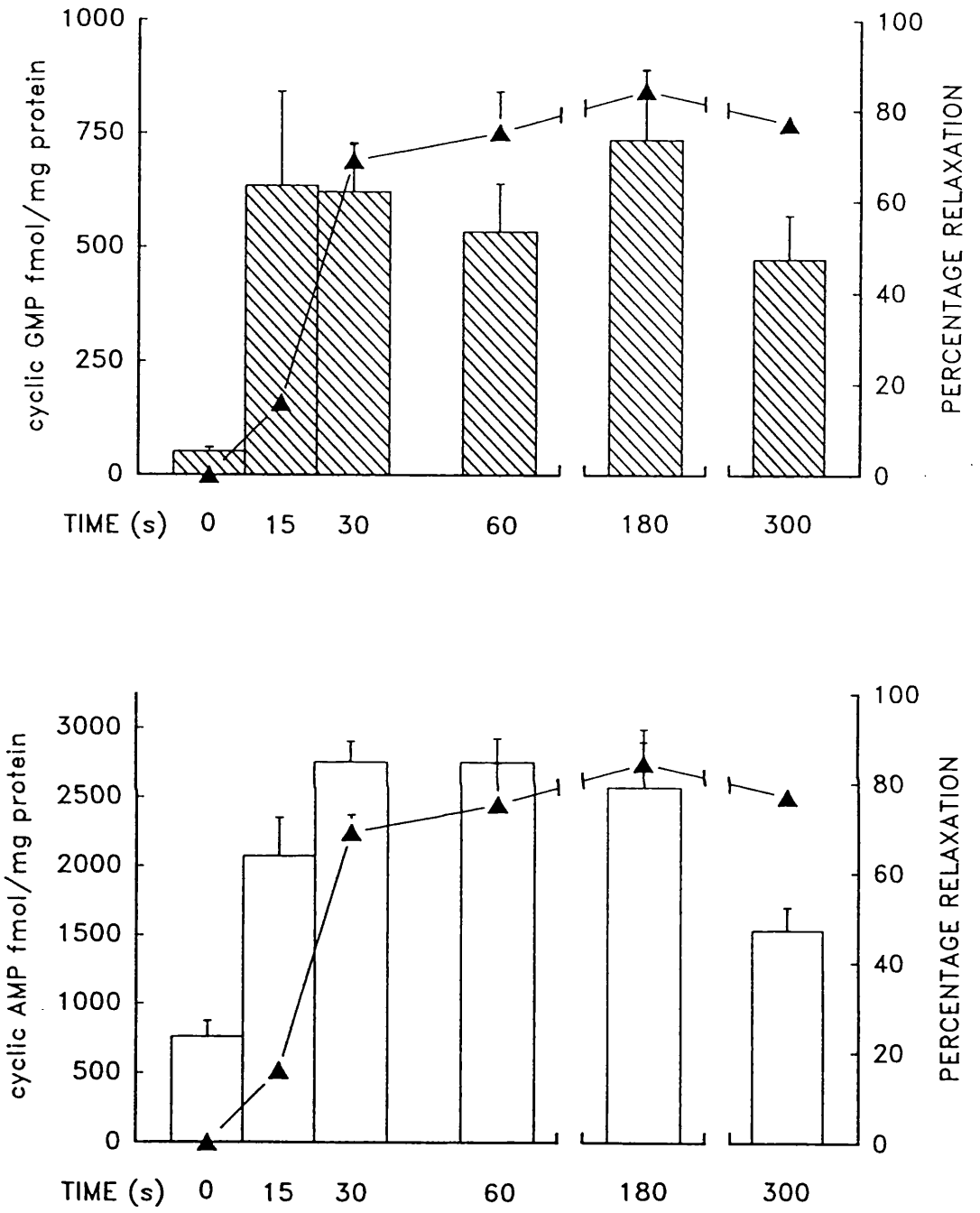


Figure 5.4 Effect of isoprenaline (10^{-6}M) on cyclic nucleotide levels and tone induced by noradrenaline (10^{-7}M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (bars) are expressed in fmol/mg protein. Relaxant responses (filled trangles) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M) in the same tissues. Bars and triangles represent the mean ± s.e. mean of between 3 and 10 separate experiments.

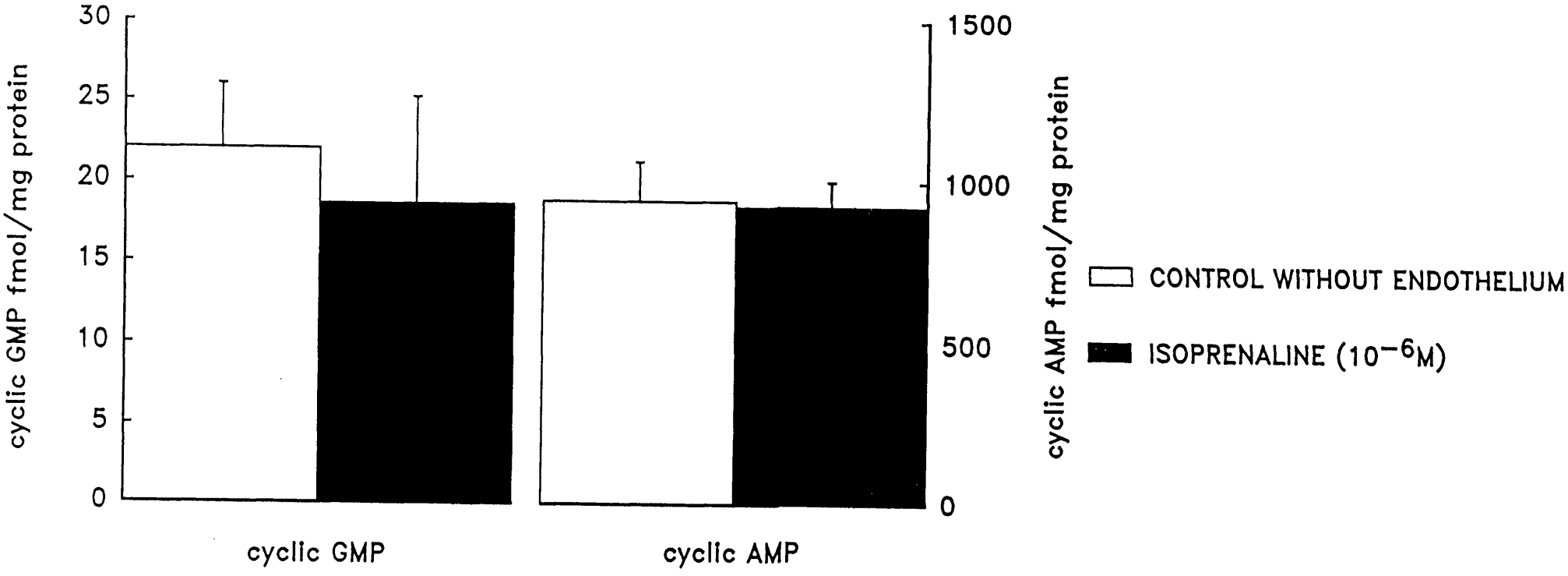


Figure 5.5 Effect of isoprenaline (10^{-6}M) at 30s exposure on cyclic nucleotide levels in rat thoracic aortic rings denuded of endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 3 and 10 separate experiments.

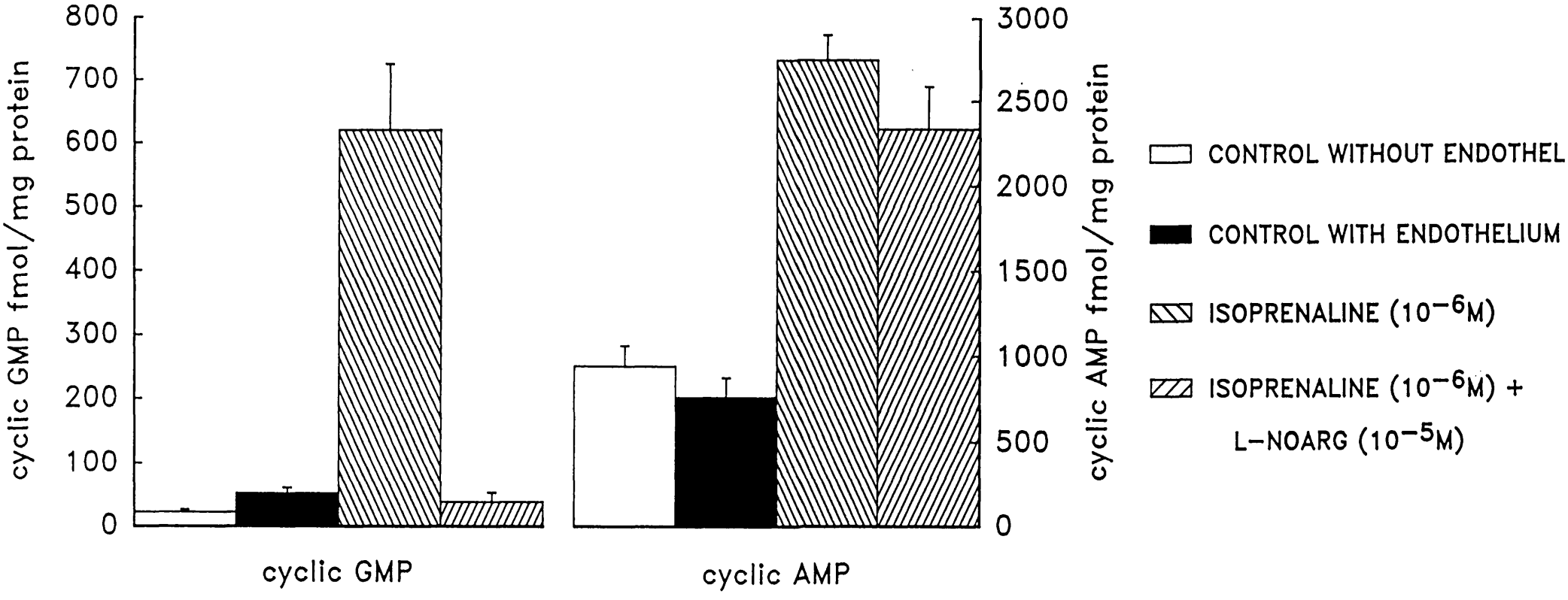


Figure 5.6 Effect of L-NOARG (10^{-5}M) on cyclic nucleotide levels induced by isoprenaline (10^{-6}M) at 30s exposure in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 5 and 10 separate experiments.

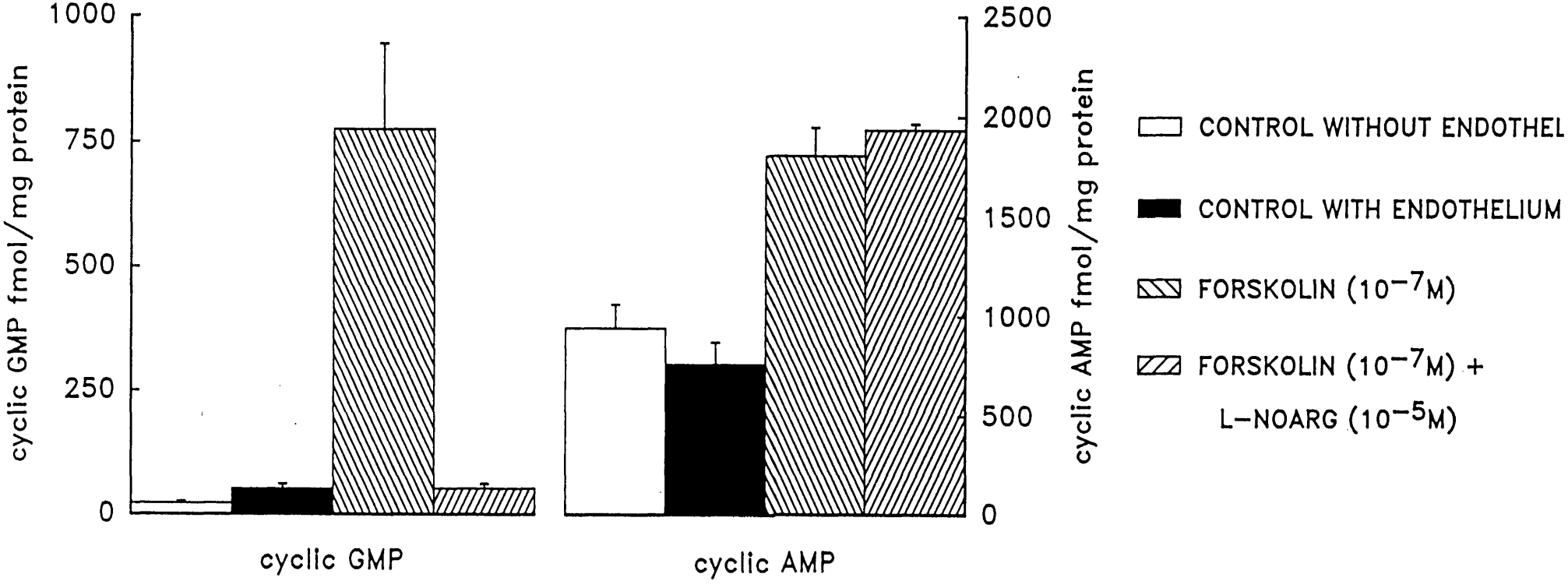


Figure 5.7 Effect of L-NOARG (10^{-5}M) on cyclic nucleotide levels induced by forskolin (10^{-7}M) at 60s exposure in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of between 5 and 10 separate experiments.

fmol/mg protein where L-NOARG was also present. Levels of cyclic AMP were not significantly affected by pretreatment with L-NOARG (10^{-5}M), the levels being 2751 ± 151 fmol/mg protein and 2336 ± 253 fmol/mg protein for control and pretreated tissues respectively (**Figure 5.6**).

At 60 seconds, forskolin (10^{-7}M) caused a relaxant response of 41 ± 5%. Preincubation with L-NOARG (10^{-5}M) completely abolished this response. Levels of cyclic GMP were also reduced from 775 ± 170 fmol/mg protein where forskolin alone was present to 52 ± 9 fmol/mg protein where L-NOARG was also present. Levels of cyclic AMP were not significantly affected by pretreatment with L-NOARG (10^{-5}M), the levels being 1808 ± 140 fmol/mg protein and 1935 ± 31 fmol/mg protein for control and pretreated tissues respectively (**Figure 5.7**).

DISCUSSION

Isoprenaline, and other β-adrenoreceptor agonists have been regarded as archetypal endothelium-independent vasodilators mediating their relaxant effects by increasing cyclic AMP (Furchgott & Martin, 1985; Furchgott & Vanhoutte, 1989). Recent reports, however, have suggested that at least part of the response to a number of classical endothelium-independent vasodilator drugs known to activate adenylate cyclase is endothelium-dependent. These drugs include prostacyclin, forskolin (Shimokawa *et al.*, 1988) and β-adrenergic agonists (Rubanyi & Vanhoutte, 1985a) *in vitro* in the pig coronary artery (where it has been shown that the response to these vasodilators can be partially inhibited with haemoglobin and endothelium removal) and salbutamol *in vivo* in the rat (Gardiner *et al.*, 1991a) (the hindquarters hyperaemia induced by this β-adrenoreceptor agonist being partially inhibited by the nitric oxide synthesis inhibitor, N^G-nitro-L-arginine

methyl ester (L-NAME)).

In this chapter it has been shown that the β-adrenoreceptor agonists, isoprenaline and salbutamol, and the adenylate cyclase activating drug, forskolin, cause relaxations in the rat thoracic aorta which are totally dependent on the presence of an intact endothelium.

The receptors mediating the relaxant response to isoprenaline appear to be β-adrenoreceptors since salbutamol also has an endothelium-dependent mechanism of inducing relaxation but is between 10 and 30-fold less potent than isoprenaline (consistent with a β_2-adrenoreceptor-mediated response) and propranolol competitively inhibits the relaxant response to isoprenaline with a potency characteristic of a typical β_2-adrenoreceptor (calculated pA_2 7.5). While the antagonism of the isoprenaline-induced relaxant response by ICI 118551 implicates the involvement of the β_2-subtype of adrenoreceptor, the degree of rightward shift, 10-fold with $3x10^{-7}M$ (calculated pA_2 7.6) appears to be too small for this subtype to be wholly responsible for all of the relaxant response to isoprenaline. It therefore seems likely that a mixed population of β-adrenoreceptor subtypes mediates the endothelium-dependent relaxant response to isoprenaline in this tissue.

Since the β-adrenoreceptor is known to be linked to adenylate cyclase (Nahorski *et al.*, 1975) via the guanine nucleotide binding protein, G_s (Gilman, 1986), it appears likely that the first stage in the signal transduction pathway mediating this relaxant response to isoprenaline is an increase in cyclic AMP within the endothelium. This hypothesis is supported by the absence of relaxation and lack of increases in cyclic nucleotides in tissues denuded of endothelium when challenged with isoprenaline.

The role of the rise in cyclic AMP appears to be activation of nitric

oxide synthase, either directly or indirectly. This is apparent as forskolin, which activates adenylate cyclase directly, causes endothelium-dependent relaxations in this tissue. Further, both isoprenaline and forskolin-induced relaxations and the associated rises in cyclic GMP , but not the increases in cyclic AMP can be inhibited by the nitric oxide synthase inhibitor, L-NOARG. If the rise in cyclic AMP is related to the relaxant response, as seems probable given the known mechanisms of action of forskolin and the β-adrenoreceptor agonists, then it must precede the activation of nitric oxide synthase.

The signal transduction pathway responsible for the relaxant effects of the β-adrenoreceptor agonists and forskolin in the rat aorta appears to share a number of characteristics with that postulated to mediate human α-CGRP relaxations in the same tissue. Firstly, all these vasodilators require the presence of the endothelium to exert their relaxant effects. Secondly, their endothelium-dependent relaxations are associated with rises in cyclic AMP and cyclic GMP. Thirdly, removal of the endothelium, as well as abolishing their relaxant effect also abolishes the rises in both cyclic nucleotides. Fourthly, the relaxant effects of these vasodilators are inhibited by L-NOARG which also selectively inhibits the accumulations in cyclic GMP without altering the increases in cyclic AMP. Finally, when compared with the traces for acetylcholine, human α-CGRP and sodium nitroprusside (**Figure 3.2**) it is clear that the relaxations induced by isoprenaline, salbutamol and forskolin (**Figure 5.2**) resemble those induced by human α-CGRP, but are slower in both onset and equilibration than either acetylcholine- or sodium nitroprusside-induced vasorelaxation in the rat aorta.

In view of these similarities, a common signal transduction mechanism may mediate the endothelium-dependent relaxant responses to human α-CGRP, the β-adrenoreceptor agonists and forskolin in the rat thoracic aorta.

Although the results presented above appear to indicate that isoprenaline-induced relaxation is endothelium-dependent and mediated by the release of nitric oxide in the rat aorta, a number of studies have reported that relaxations to this vasodilator occur independently of the endothelium in rings of rat aorta (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990; Weir *et al.*, 1991). Although different strains of rat have been used in these studies, preliminary experiments indicated that this could not account for the differences observed since both Sprague-Dawley and Wistar strain rats gave endothelium-dependent relaxations to isoprenaline. Clearly there must be some fundamental variation in the protocols used above and in the reported studies using rat aorta.

The evidence presented below suggests that in the reported studies the endothelium has not been sufficiently removed to abolish the relaxant effects of isoprenaline. In addition to the data presented in this chapter the evidence supporting this hypothesis consists of the following.

1) A number of studies have shown that 'complete' removal of the endothelium (shown by lack of a relaxant response to muscarinic agonists) alters the relaxant response to isoprenaline in the rat aorta, shifting the concentration/effect curve to the right and decreasing the maximum response (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990). Although slightly different levels of tone were induced in the above studies, there is general agreement that in rings of rat aorta with intact endothelium, isoprenaline induces a maximum relaxation of approaching 100% of the spasmogen-induced tone. However, in supposedly completely endothelium-denuded rings the maximum relaxation varies widely from approximately 30% (Grace *et al.*, 1988) to 80% of the spasmogen-induced tone (Weir *et al.*, 1991), with virtually every intermediate value being represented (Martin *et al.*, 1986b; Kamata *et al.*, 1989; Dainty *et al.*, 1990). This variability of the

isoprenaline response in the rat aorta has also been found within individual studies with maximum responses varying between 30-80% of spasmogen-induced tone in one report (Maurice *et al.*, 1991). This observation is difficult to reconcile with an endothelium-independent mechanism of relaxation but is consistent with an endothelium-dependent relaxant effect where varying proportions of the endothelium have been removed.

2) It has been reported that isoprenaline induced relaxations in the rat aorta can be inhibited by methylene blue ($3x10^{-5}M$) and haemoglobin ($10^{-5}M$) (Grace *et al.*, 1988), compounds known to inhibit the action of nitric oxide. This is in marked contrast to the rabbit aorta where these agents at similar concentrations are without effect on isoprenaline-induced vasorelaxation (Martin *et al.*, 1985)

3) The metabolic inhibitors, rotenone and 2-deoxyglucose have been shown to be capable of inhibiting endothelium-dependent relaxations in the rabbit aorta (Griffiths *et al.*, 1986; 1987). In a recent study in rat aorta, these metabolic inhibitors at the same concentrations were found to be capable of inhibiting the relaxations induced by both acetylcholine and isoprenaline (Weir *et al.*, 1991).

4) Brain *et al.*, (1985) commented that spontaneous activity sometimes appeared in constricted rings of rat aorta with intact endothelium. This observation was confirmed in the present experiments, spontaneous activity appearing in almost all rings with intact endothelium but none which were endothelium-denuded. This seems to infer that the spontaneous activity is a function of the endothelium yet in the study by Rapoport (1991), rings of rat aorta, supposedly denuded of the endothelium, display spontaneous activity and variable relaxations to isoprenaline. This is consistent with an endothelium-dependent mechanism of relaxation for isoprenaline and with

some portion of the endothelium still being intact in this study.

The tool used to confirm absence of endothelium in these studies was lack of relaxation to a muscarinic agonist, normally acetylcholine. The use of a muscarinic agonist to confirm loss of endothelium relies on the assumption that removal of the entire endothelium is required to abolish the endothelium-dependent relaxant response. This has been demonstrated in the rabbit aorta (Furchgott & Zawadzki, 1980) (where isoprenaline induces endothelium-independent vasorelaxation). However, it does not appear to be the case in the rat aorta where Grace *et al.*, (1987) using 'endothelium-denuded' rings of rat aorta still managed to get a doubling of cyclic GMP levels on challenge with acetylcholine without a relaxant response.

Despite the assumption being invalid, the use of muscarinic agonists to confirm endothelium loss in the rat aorta could still be useful if all endothelium-dependent vasodilators had an equal sensitivity to endothelium loss, since removal of the same proportion of the endothelium would abolish the responses to all endothelium-dependent vasodilators. However, this is clearly not the case, removal of a small proportion of the endothelium abolishing the relaxant response to human α-CGRP while only moving the concentration/effect curve to acetylcholine slightly to the right without diminishing the maximum response. It may be that isoprenaline requires only a very small percentage of the endothelium in the rat aorta to be intact to cause relaxation, but that acetylcholine requires a greater proportion in this tissue.

Muscarinic agonists, such as acetylcholine, can be used to determine the presence of a viable endothelium providing rigorous parameters for degree of relaxation achieved by specific concentrations are observed. However, their use appears to be wholly unsuitable for confirming the removal of the

entire endothelium in this tissue, which is best assessed histologically.

The pathway mediating human α-CGRP, isoprenaline, salbutamol and forskolin endothelium-dependent relaxations in the rat aorta, represented diagrammatically in **Figure 5.8**, appears to involve activation of adenylate cyclase either directly, as is the case for forskolin, or via receptors as is the case for the human α-CGRP and the β-adrenoreceptor agonists. The elevations in cyclic AMP, either directly or indirectly, activates nitric oxide synthase resulting in release of nitric oxide. In the smooth muscle of the blood vessel nitric oxide directly activates soluble guanylate cyclase giving rises in cyclic GMP and consequently the relaxant effect.

Whether other vasodilators can activate this signal transduction pathway remains open to question. However, there is some circumstantial evidence suggesting that both prostacyclin and vasoactive intestinal polypeptide (VIP) may exert some of their relaxant effects via this mechanism. Both are known to act on receptors which are linked to adenylate cyclase (Tateson *et al.*, 1977; Gorman *et al.*, 1977; Huang & Rorstad, 1983; Itoh *et al.*, 1985b) and have at least a component of their relaxant effect which is endothelium-dependent (Davies & Williams, 1983; Thom *et al.*, 1986; Shimokawa *et al.*, 1988).

The distribution of this particular signal transduction mechanism is also obscure. Although the evidence suggests that a number of agents act to cause endothelium-dependent relaxation via this mechanism in rat aorta very little information exists for other vessels. It is possible that prostacyclin (Shimokawa *et al.*, 1988) and β-adrenoreceptor agonists (Rubanyi & Vanhoutte, 1985a) in the pig coronary artery may activate this mechanism.

A trend has developed defining the relaxant responses to agonists in

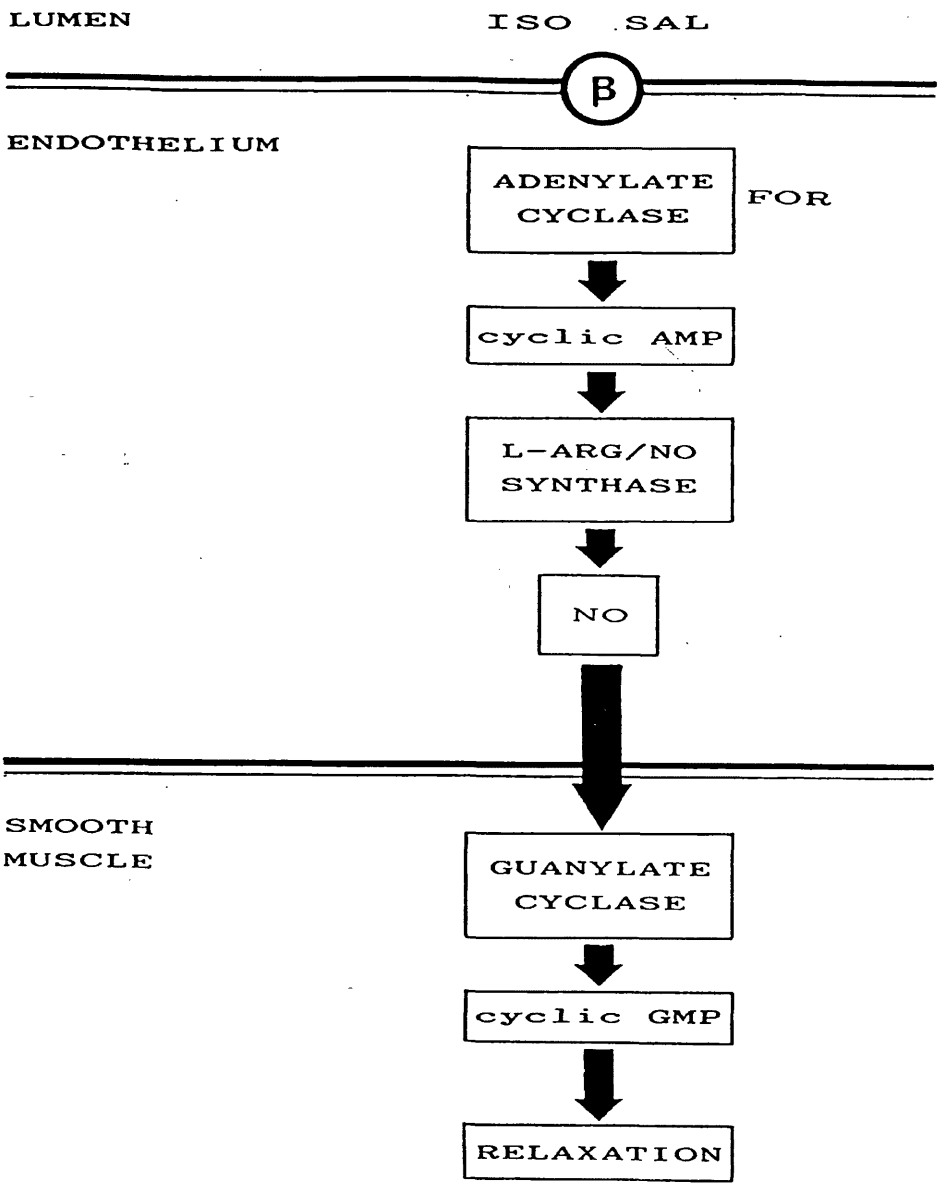


Figure 5.8 Postulated transduction pathway mediating isoprenaline (ISO), salbutamol (SAL) and forskolin (FOR) endothelium-dependent relaxations in rat aorta. Isoprenaline (ISO) and salbutamol (SAL) acting on β-adrenoreceptors on the endothelium cause activation of adenylate cyclase. Forskolin (FOR) activates endothelial adenylate cyclase directly. The elevation in cyclic AMP levels in the endothelium activates nitric oxide synthase (L-ARG/NO SYNTHASE) causing the synthesis of nitric oxide (NO). This causes activation of soluble guanylate cyclase stimulation in the smooth muscle leading to cyclic GMP accumulation and subsequently relaxation.

vascular bed as either wholly endothelium-dependent or wholly endothelium-independent. The tool used to support this categorisation is removal of the endothelium. While it is justifiable to define a response as wholly endothelium-dependent if removal of the endothelium completely abolishes the relaxant response to the agonist, it is less satisfactory to define an agonist as wholly endothelium-independent on the basis of presence of a relaxant response after removal of the endothelium. This protocol does not eliminate the possibility of a mixed response comprising endothelium-dependent and -independent components. In view of this it is possible that the endothelium-dependent components of vasorelaxation to various drugs may be masked by their endothelium-independent vasorelaxant effect.

CHAPTER 6

INTRODUCTION

Endothelium-independent relaxations

Many of the vasorelaxant effects of CGRP are not thought to be mediated via the endothelium as is the case in the rat aorta described in Chapters 3 and 4, but are due to an action of CGRP directly on the smooth muscle of the blood vessels. This appears to be true in most, but not all, of the small resistance vessels responsible for the control of blood pressure as CGRP-induced falls in blood pressure *in vivo* in the rat are not altered by pretreatment with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Gardiner *et al.*, 1991b). *In vitro*, a number of larger vessels including bovine and porcine coronary arteries (Greenberg *et al.*, 1987; Franco-Cereceda *et al.*, 1987; Beny *et al.*, 1989), the perfused rat mesenteric bed (Kawasaki *et al.*, 1988), human skeletal muscle arteries, porcine splenic arteries (Pernow, 1989) and cerebral arteries of human, feline, rabbit, guinea pig and rat (Edvinsson *et al.*, 1985; 1987) exhibit endothelium-independent relaxations to CGRP.

Since a number of these vessels have been shown to be densely innervated with CGRP-immunoreactive nerves (Edvinsson *et al.*, 1987; Kawasaki *et al.*, 1988), it has been postulated that CGRP might have some physiological role in the maintenance of blood pressure.

The signal transduction pathway mediating this endothelium-

independent vasorelaxant response to CGRP has yet to be fully elucidated. It has been demonstrated that this effect of CGRP is not associated with changes in membrane potential so is likely to be purely biochemical (Beny *et al.*, 1989). Increases in cyclic AMP levels have been shown to be associated with CGRP-induced endothelium-independent relaxations in the cerebral vasculature of the cat (Edvinsson *et al.*, 1985) although no reports have studied the possible involvement of cyclic GMP in CGRP-induced endothelium-independent vasorelaxation.

The principal aims of the experiments set out in this chapter were to confirm the existence of an endothelium-independent relaxant response to human α-CGRP and, using pharmacological and biochemical techniques, to elucidate the transduction pathways mediating this response.

MATERIALS AND METHODS

To study the relaxations induced by human α-CGRP two preparations of the pig left anterior descending coronary artery were prepared as described in Chapter 2. Briefly, the left anterior descending coronary arterial ring preparation was taken from between the junction with the circumflex and the first branch, the conus artery. It is the larger preparation having an internal diameter of approximately 4mm. The anterior interventricular arterial rings were taken from further down the same artery and had an internal diameter of approximately 1mm. Both preparations were initially set up retaining the endothelium but once the presence of an endothelium-independent relaxant response had been confirmed the endothelium was routinely removed by mild abrasion. Cumulative concentration/effect curves to bradykinin, human α-CGRP, sodium nitroprusside, and isoprenaline were carried out in the presence of U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$;

10^{-8}M) for rings of left anterior descending coronary artery and acetylcholine ($2x10^{-7}$M) for the anterior interventricular artery using the typical protocol given in Chapter 2. The effects of a number of drugs on the contractions elicited by U46619 and the relaxations induced by bradykinin, human α-CGRP and sodium nitroprusside were studied in the left anterior descending coronary artery.

Test drugs

Glibenclamide was prepared in 100% DMSO to give a 10^{-2}M stock solutions. Subsequent dilutions were carried out using Krebs solution. Preliminary experiments indicated that, at the diluted concentrations of the drug used, the concentration of DMSO did not affect either the contractile or relaxant properties of the tissues to any of the vasoconstrictors or vasodilators studied.

N^G-nitro-L-arginine (L-NOARG) was prepared in 1M hydrochloric acid at 50mg/ml before being diluted to 10^{-3}M and neutralised to pH 7.0. Tissues were preincubated for 30 minutes in L-NOARG before the start of the experiment.

All control values were obtained using the same time-course as the pretreated values, but without adding the test drug under study.

Alternatively, to study the cyclic nucleotide accumulations associated with the relaxations induced by human α-CGRP, isoprenaline and sodium nitroprusside, porcine left anterior descending coronary arterial rings were prepared as above, removing the endothelium from all rings. To determine the optimum time-point for cyclic nucleotide accumulations, single concentrations of the vasodilators giving approximately 80% relaxation of the

tone induced by U46619 (10^{-8}M) were added and the tissues removed into liquid nitrogen at various time-points. Cyclic nucleotide levels were determined by scintillation proximity assay (Amersham) using the acetylation protocol for cyclic GMP levels and the non-acetylation protocol for cyclic AMP (see Chapter 2 and Appendix 2).

RESULTS

LEFT ANTERIOR DESCENDING CORONARY ARTERY

In the left anterior descending coronary artery preparation with intact endothelium, U46619 (10^{-8}M) induced a contractile response of 2.7 ± 0.2g. Removal of the endothelium (confirmed by lack of relaxation to bradykinin (10^{-6}M) and histologically) significantly increased this to 3.7 ± 0.3g. Bradykinin (10^{-9}-$3x10^{-6}$M) induced a relaxant response (EC_{50} 4.5 ± $1.0x10^{-8}$M) only in tissues with intact endothelium (**Figure 6.1**). In contrast to its effects in the rat aorta, human α-CGRP (10^{-9}-$3x10^{-7}$M) induced relaxations in this tissue which were unaltered by removal of the endothelium (EC_{50}s intact; 1.6 ± $0.5x10^{-8}$M: denuded;1.8 ± $0.4x10^{-8}$M) (**Figure 6.1**). Sodium nitroprusside (10^{-9}-10^{-5}M) evoked a relaxant response which was also unaltered by removal of the endothelium (EC_{50}s intact; 8.7 ± $3.0x10^{-6}$M: denuded;1.2 ± $0.5x10^{-7}$M) (**Figure 6.1**).

The traces illustrated in **Figure 6.2** show the marked temporal differences in the onset and equilibration of the relaxant responses to bradykinin, human α-CGRP and sodium nitroprusside in rings of left anterior descending coronary artery with intact endothelium. Bradykinin and sodium nitroprusside displayed a rapid onset and equilibration (approx. 5s and 60s respectively) of their relaxant responses. In contrast, human α-CGRP-induced relaxations in this tissue were much slower both in onset and equilibration

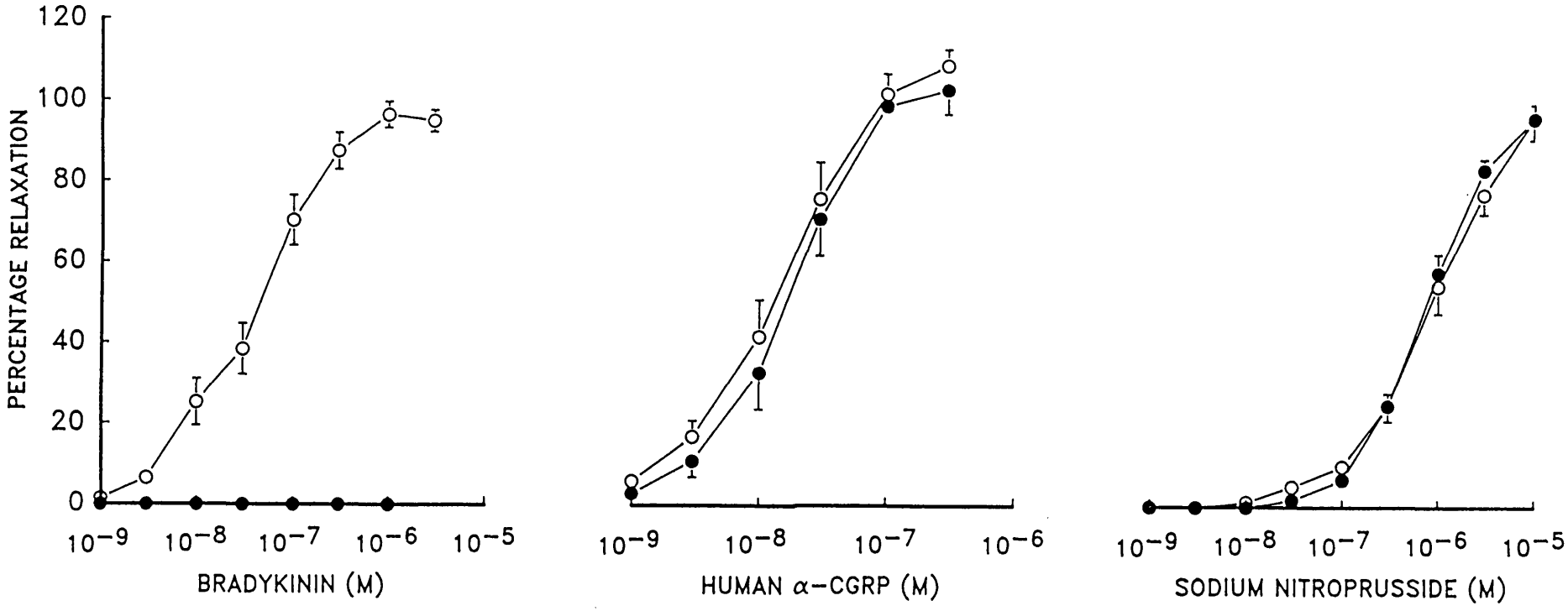


Figure 6.1 Effect of removal of endothelium on relaxations induced by bradykinin, human α-CGRP and sodium nitroprusside in pig left anterior descending coronary artery rings preconstricted with U46619 (10^{-8}M). Results are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M). O are responses in intact rings, ● the responses in endothelium denuded rings for each vasodilator. Points represent the mean ± s.e. mean of between 7 and 11 separate experiments.

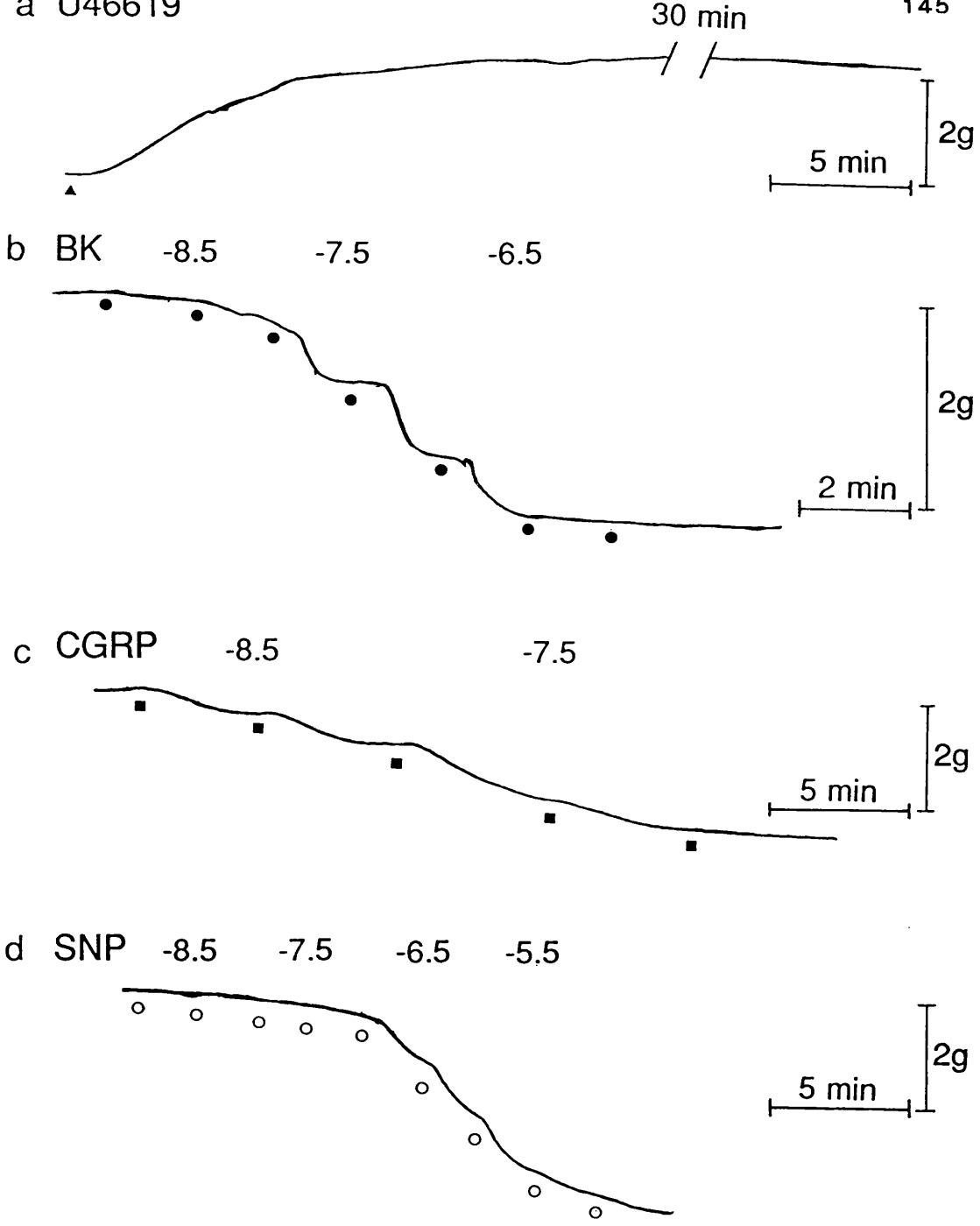


Figure 6.2 Traces showing a) the spasmogen effect of U46619 (10^{-8}M) and the typical relaxant effects of cumulative addition of b) bradykinin (BK), c) human α-CGRP (CGRP) and d) sodium nitroprusside (SNP) on rings of pig left anterior descending coronary artery. All traces, with the exception of that of bradykinin, were carried out in rings denuded of endothelium. All rings were constricted with U46619 (10^{-8}M). The vasodilators were added at the points indicated in concentrations giving half-log molar increments. All the vasodilators relaxed the vessels to 100% of the tone induced by U46619 (10^{-8}M).

(approx 20 and 300s respectively). The time-course for relaxation was similar to this in endothelium-denuded rings of left anterior descending coronary artery and anterior interventricular artery.

Effect of agents on relaxations induced by bradykinin, human α-CGRP and sodium nitroprusside.

L-NOARG

In rings of left anterior descending coronary artery with intact endothelium, the nitric oxide synthase inhibitor, L-NOARG (10^{-5}M), augmented the tone induced by U46619 (10^{-8}M) to 5.2 ± 0.3g. This increase in spasmogen-induced tone by L-NOARG was not observed in rings denuded of endothelium. The relaxations to bradykinin in endothelium intact rings and the relaxations to human α-CGRP in both endothelium intact and denuded rings were unaltered by preincubation with L-NOARG (10^{-5}M) (**Figure 6.3**).

Glibenclamide

The ATP-sensitive potassium channel blocker, glibenclamide (10^{-5}M) (Sturgess et al., 1985), when added to endothelium-denuded rings of left anterior descending coronary artery preconstricted with U46619 (10^{-8}M) caused a very slow vasorelaxation, probably due to antagonism of TxA_2 receptors as reported by Cocks *et al.*, 1990. However, addition of glibenclamide (10^{-5}M) after a submaximal relaxation to human α-CGRP ($3x10^{-8}$M) had reached equilibration did not give the rapid reversal of the human α-CGRP-induced relaxant response as was reported in the rat mesenteric artery (Nelson *et al.*, 1990).

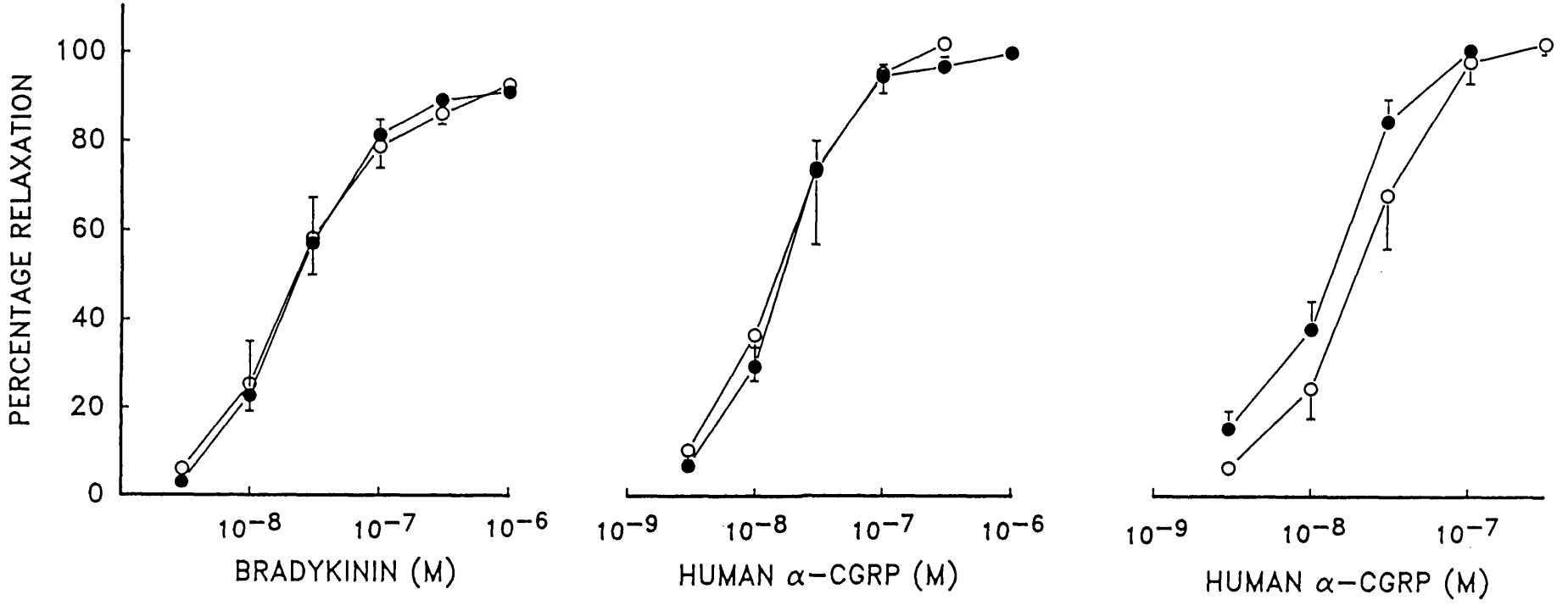


Figure 6.3 Effect of L-NOARG (10^{-5}M) on relaxations induced by bradykinin (left) and human α-CGRP (middle) in endothelium-intact rings and human α-CGRP (right) in endothelium-denuded rings of pig left anterior descending coronary artery rings preconstricted with U46619 (10^{-8}M). Results are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M). O represents the control and ● the L-NOARG pretreated values for each vasodilator. Points represent the mean ± s.e. mean of between 5 and 7 separate experiments.

Effect of repeat administration of isoprenaline and human α-CGRP

Isoprenaline ($3x10^{-9}$-10^{-6}M) induced a relaxant response (EC_{50} 2.1 ± $0.2x10^{-8}$M) in endothelium-denuded rings of left anterior descending coronary artery. The traces illustrated in **Figure 6.4** show that isoprenaline has a similar time course of relaxation to human α-CGRP, both vasodilators showing an initial onset of the relaxant response at approximately 20s after the initial exposure and equilibration at approximately 300s. Consecutive concentration/effect curves to isoprenaline showed no sign of tachyphylaxis (**Figure 6.5**). In contrast, four consecutive concentration effect curves to human α-CGRP did show a tachyphylaxis with progressive rightward shifts in the concentration/effect curves (up to 30-fold at the fourth curve) and a reduction in the maximum response (**Figure 6.5**).

Basal levels of cyclic nucleotides

Cyclic AMP and cyclic GMP control levels in pig left anterior descending coronary arterial rings with intact endothelium constricted with U46619 (10^{-8}M) were 1943 ± 415 fmol/mg protein and 366 ± 27 fmol/mg protein, respectively. Removal of the endothelium significantly reduced levels of cyclic AMP (580 ± 73 fmol/mg protein) and cyclic GMP (96 ± 12 fmol/mg protein) (**Figure 6.6**).

Time-course for relaxation and second messenger accumulation

In endothelium-denuded rings of left anterior descending coronary artery, isoprenaline (10^{-6}M) induced a relaxant response of 63 ± 5% of the tone evoked by U46619 (10^{-8}M). This relaxant response was relatively slow in onset (between 15 and 30s after the initial exposure) and development (reaching a plateau at approximately 300s). Levels of cyclic AMP were

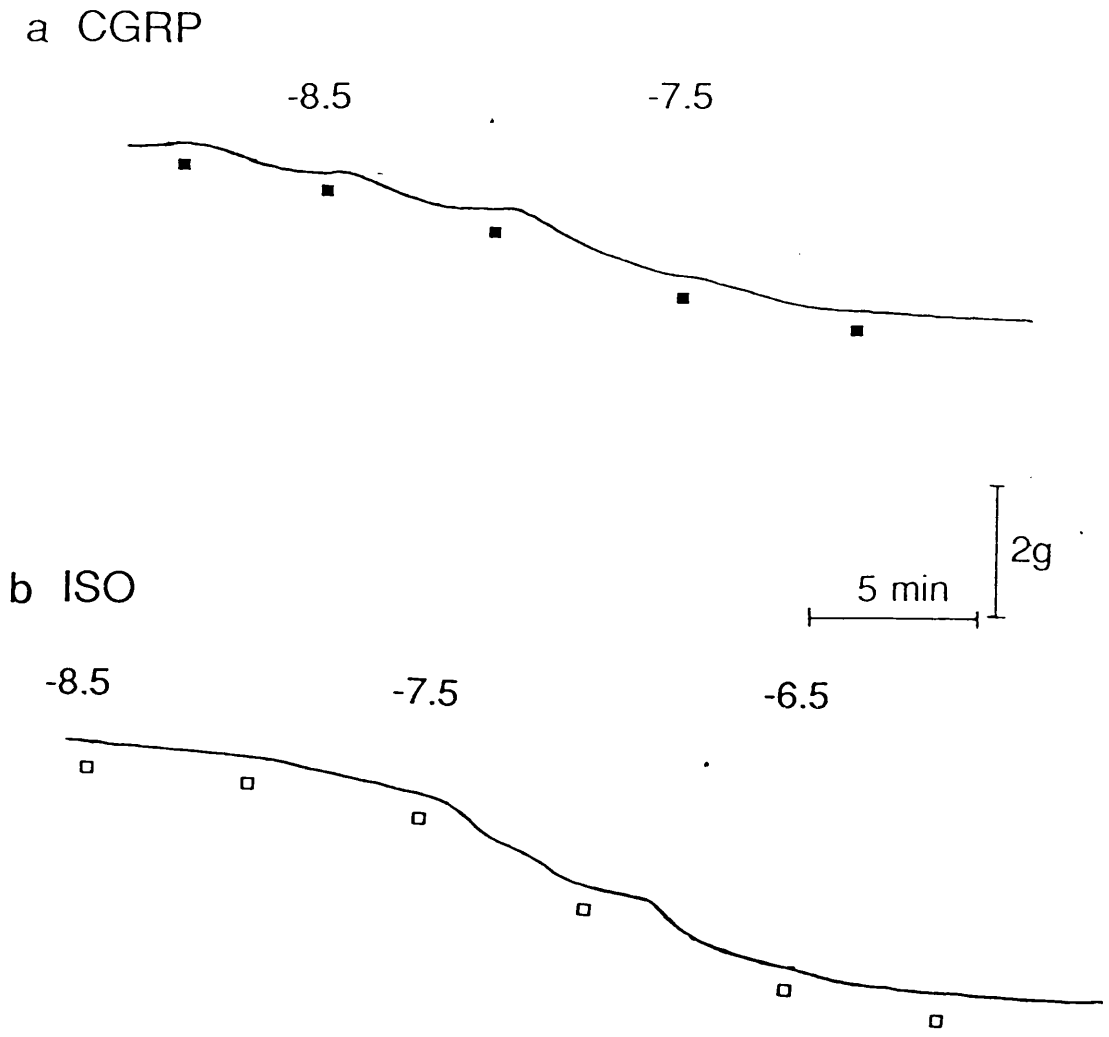


Figure 6.4 Traces showing typical relaxant effects of cumulative addition of a) human α-CGRP (CGRP) and b) isoprenaline on rings of pig left anterior descending coronary artery denuded of endothelium. All rings were constricted with U46619 (10^{-8}M). The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

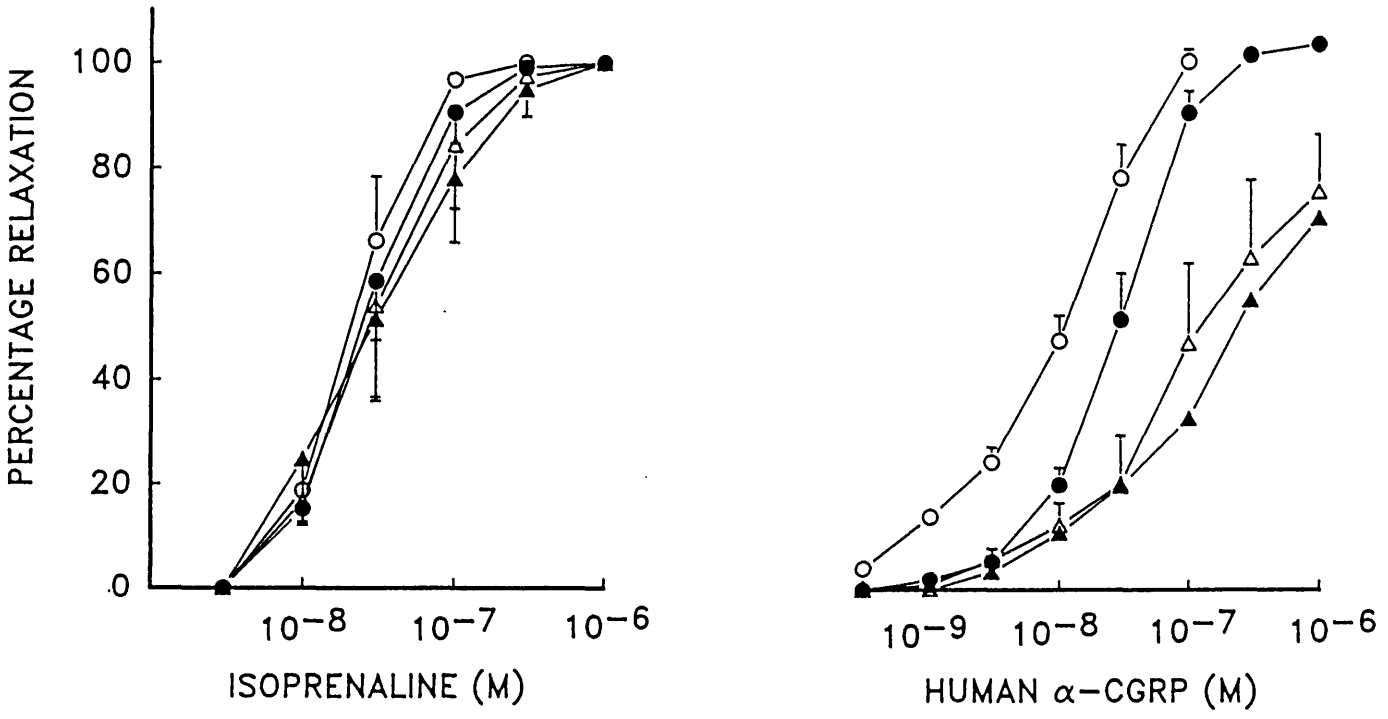


Figure 6.5 Effect of repeat concentration/effect curves to isoprenaline (left) and human α-CGRP (right) in endothelium-denuded rings of pig left anterior descending coronary artery rings preconstricted with U46619 (10^{-8}M). Results are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M). O represents the first curve, ● the second, △ the third and ▲ the fourth curve to the vasodilators. Points represent the mean ± s.e. mean of between 4 and 6 separate experiments.

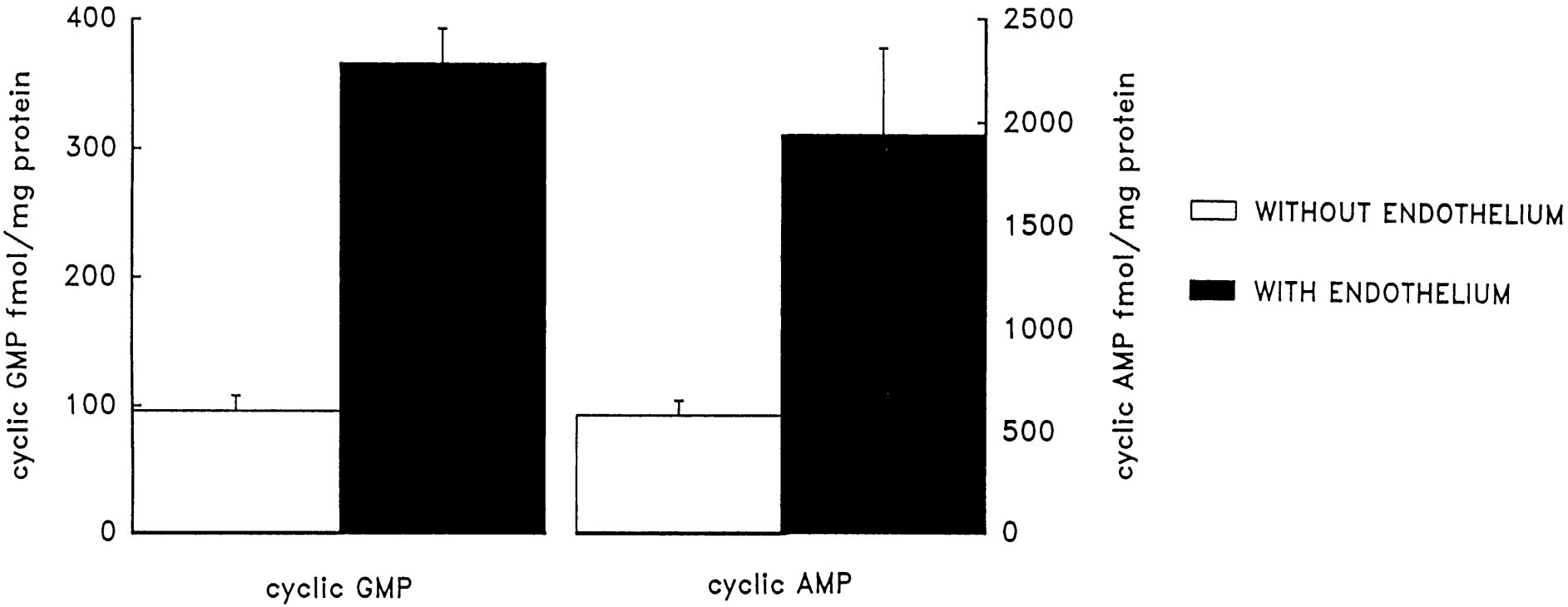


Figure 6.6 Basal levels of cyclic nucleotides in pig left anterior descending coronary artery rings with and without endothelium preconstricted with U46619 (10^{-8}M). Results are expressed in fmol/mg protein for cyclic GMP (left panel) and cyclic AMP (right panel). Bars represent the mean ± s.e. mean of between 4 and 6 separate experiments.

raised at the first time-point (15s), indicating that the rise in cyclic AMP preceded the onset of relaxation and reached a maximal 5-fold (2869 ± 239 fmol/mg protein) above basal levels at 60s (**Figure 6.7**). Levels of cyclic GMP were not significantly different from the basal levels (**Figure 6.7**).

In the same preparation sodium nitroprusside ($3x10^{-6}M$) induced a relaxant response of 91 ± 1% of the tone evoked by U46619 ($10^{-8}M$). The relaxations to this sodium nitroprusside developed more rapidly than those elicited by isoprenaline, onset occurring between 0 and 15s and the relaxation reaching a plateau at approximately 180s. In contrast to the isoprenaline-induced relaxations, sodium nitroprusside did not significantly alter levels of cyclic AMP from basal levels but increased cyclic GMP levels to a maximal 6-fold (612 ± 252 fmol/mg protein) above basal levels at 180s (**Figure 6.8**).

In the absence of the endothelium, human α-CGRP ($3x10^{-8}M$) induced a relaxation of 77 ± 5% of the tone evoked by U46619 ($10^{-8}M$). This response had a similar time-course to that induced by isoprenaline, developing initially between 15 and 30s and reaching a plateau at approximately 300s. Like isoprenaline, human α-CGRP increased levels of cyclic AMP before the onset of the relaxant response. Cyclic AMP levels reached a maximal 6-fold (3185 ± 867 fmol/mg protein) above basal levels at 180s while levels of cyclic GMP were not significantly different from basal levels (**Figure 6.9**).

ANTERIOR INTERVENTRICULAR ARTERY

In anterior interventricular arterial rings with endothelium, acetylcholine ($2x10^{-7}M$) induced a contractile response of 2.8 ± 0.3g. Removal of the endothelium increased this response to 3.4 ± 0.5g. Human α-CGRP (10^{-9}-$10^{-7}M$) induced vasorelaxation in rings denuded of endothelium. The EC_{50}, 4.8 ± $0.7x10^{-9}M$, was significantly different from that of human

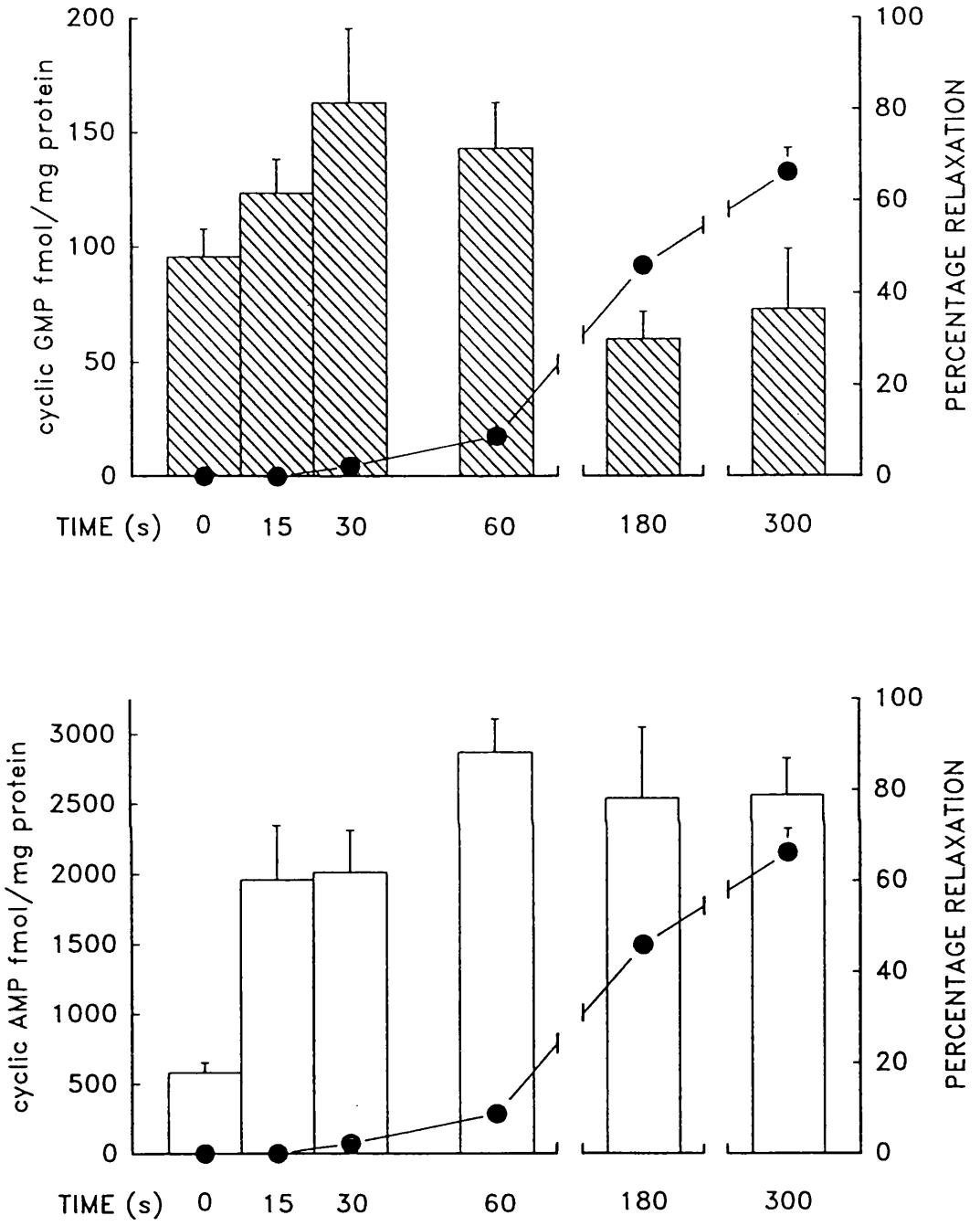


Figure 6.7 Effect of isoprenaline (10^{-6}M) on cyclic nucleotide levels and tone induced by U46619 (10^{-8}M) in endothelium-denuded rings of pig left anterior descending coronary artery. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (Bars) are expressed in fmol/mg protein. Relaxant responses (Points) are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M) in the same tissues. Bars and points represent the mean ± s.e. mean of between 3 and 6 separate experiments.

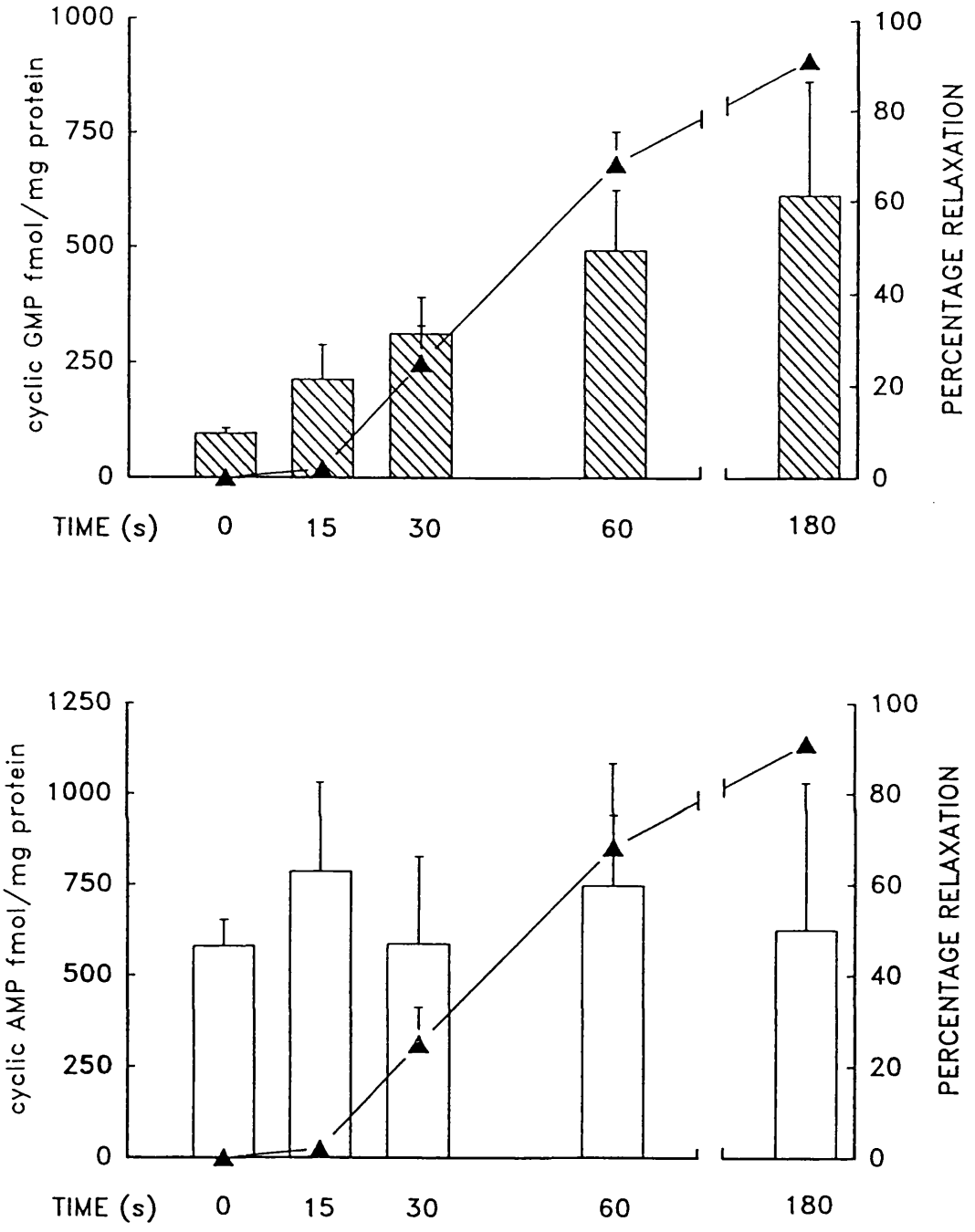


Figure 6.8 Effect of sodium nitroprusside ($3x10^{-6}$M) on cyclic nucleotide levels and tone induced by U46619 (10^{-8}M) in endothelium-denuded rings of pig left anterior descending coronary artery. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (Bars) are expressed in fmol/mg protein. Relaxant responses (Points) are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M) in the same tissues. Bars and points represent the mean ± s.e. mean of between 3 and 6 separate experiments.

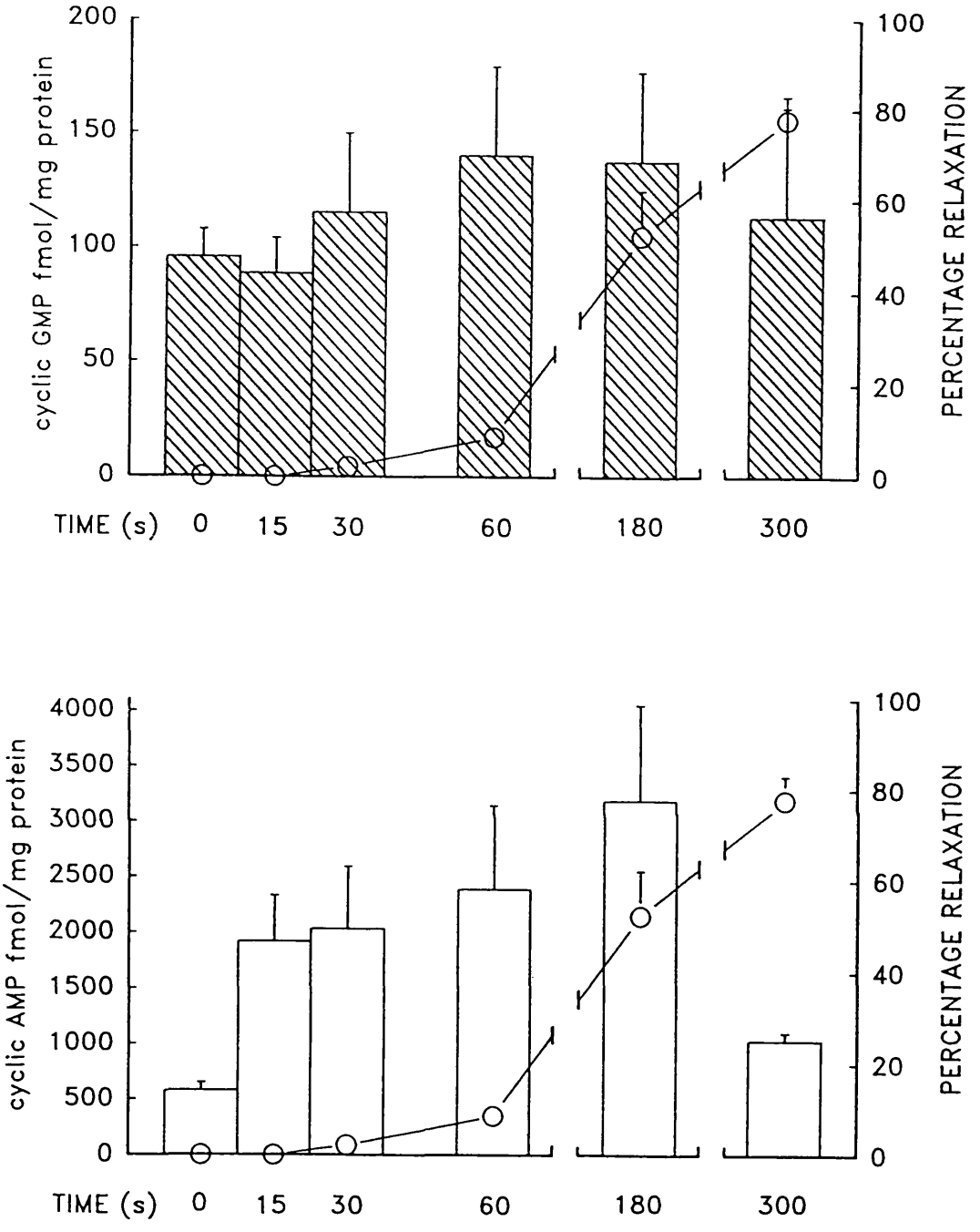


Figure 6.9 Effect of human α-CGRP ($3x10^{-8}$M) on cyclic nucleotide levels and tone induced by U46619 (10^{-8}M) in endothelium-denuded rings of pig left anterior descending coronary artery. Results are expressed in the form of a time course for accumulation of cyclic GMP (top panel) and cyclic AMP (bottom panel) levels and relaxation. Levels of cyclic nucleotides (Bars) are expressed in fmol/mg protein. Relaxant responses (Points) are expressed as a percentage relaxation of the tone induced by U46619 (10^{-8}M) in the same tissues. Bars and points represent the mean ± s.e. mean of between 3 and 10 separate experiments.

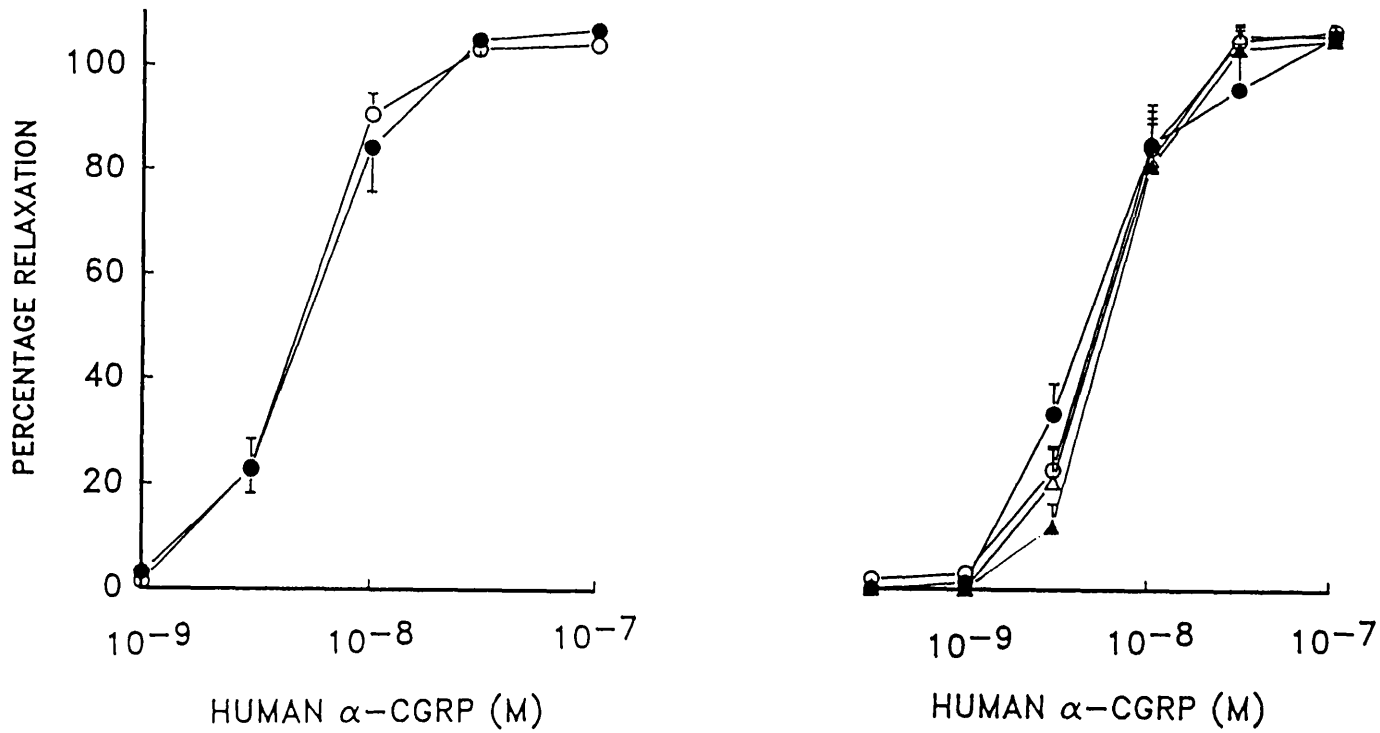


Figure 6.10 (Left) Effect of removal of endothelium on relaxations induced by human α-CGRP in pig anterior interventricular artery rings preconstricted with acetylcholine ($2x10^{-7}$M). O are responses in intact rings, ● the responses in endothelium denuded rings for each vasodilator. (Right) Effect of repeat concentration/effect curves to human α-CGRP in endothelium-denuded rings of pig anterior interventricular artery rings preconstricted with acetylcholine ($2x10^{-7}$M). O represents the first curve, ● the second, △ the third and ▲ the fourth curve to the vasodilator. Points represent the mean ± s.e. mean of between 4 and 6 separate experiments. Results are expressed as a percentage relaxation of the tone induced by acetylcholine ($2x10^{-7}$M).

α-CGRP in the larger left anterior descending coronary artery preparation indicating that CGRP was more potent in the smaller preparation. In contrast to the rings of left anterior descending coronary artery, consecutive curves to human α-CGRP displayed no sign of tachyphylaxis in rings of anterior interventricular artery (**Figure 6.10**).

DISCUSSION

It has been shown both *in vivo* and *in vitro* that CGRP-induced vasorelaxation in a number of vessels occurs independently of the endothelium. In this chapter it was shown that in two preparations of the pig coronary artery, the left anterior descending coronary artery (internal diameter 4mm) and the anterior interventricular artery (internal diameter 1mm), human α-CGRP elicits relaxations independently of the endothelium. A comparison of the essential features of CGRP-induced endothelium-dependent and -independent vasorelaxations are given in **Table 6.1**. The EC_{50} for human α-CGRP endothelium-dependent relaxation is very similar to the values found for human α-CGRP-induced relaxations in endothelium-intact rings of both preparations of the pig coronary artery. If there were a significant proportion of the relaxant response mediated via the endothelium in this tissue, then removal of the endothelium would be expected to increase the EC_{50}. This does not occur suggesting that the relaxations elicited by human α-CGRP in both preparations of the pig coronary artery are mediated substantially by an endothelium-independent mechanism. This is in agreement with other reports for this tissue (Franco-Cereceda *et al.*, 1987; Beny *et al.*, 1989).

In contrast to the results achieved in the rat aorta, the nitric oxide synthase inhibitor, L-NOARG, did not affect the relaxations induced by human α-CGRP in the left anterior descending coronary artery. This

TISSUE	RAT THORACIC AORTA	PIG LEFT ANTERIOR DESCENDING CORONARY ARTERY	PIG ANTERIOR INTERVENTRICULAR ARTERY
INVOLVEMENT OF ENDOTHELIUM	DEPENDENT	INDEPENDENT	INDEPENDENT
EC_{50}	$1.1 \pm 0.2 \times 10^{-8}$M	$1.6 \pm 0.5 \times 10^{-8}$M	$4.8 \pm 0.7 \times 10^{-9}$M
MAXIMUM RESPONSE AS A PERCENTAGE OF SPASMOGEN TONE	80%	100%	100%
TACHYPHYLAXIS	NO	YES	NO
CYCLIC AMP	ELEVATED	ELEVATED	NOT STUDIED
CYCLIC GMP	ELEVATED	UNALTERED	NOT STUDIED

Table 6.1 Table comparing some of the characteristics of CGRP induced endothelium-dependent and -independent vasorelaxation.

observation is consistent with both the mechanism of action of L-NOARG and human α-CGRP acting independently of the endothelium in this tissue. However, L-NOARG failed to inhibit the endothelium-dependent relaxations elicited by bradykinin, a vasodilator known to activate the synthesis of nitric oxide from L-arginine in cultured pig aortic endothelial cells (Palmer *et al.*, 1987; 1988a). L-NOARG augmented the tone induced by U46619 in rings of left anterior descending coronary artery with intact endothelium but not those which were endothelium denuded. This is consistent with there being a basal release of nitric oxide in this tissue and with the L-NOARG used in these experiments being active.

Recently, it was reported that another inhibitor of nitric oxide synthase, L-NMMA, while capable of inhibiting the endothelium-dependent relaxations and cyclic GMP accumulations induced by 5-hydroxytryptamine, was only able to inhibit the elevations in cyclic GMP, but not the endothelium-dependent relaxations to bradykinin in pig coronary arteries (Cowan & Cohen, 1990). The results from this study and the observations described above suggest that bradykinin may be releasing two forms of endothelium-derived relaxant factor in the pig coronary artery. The first, nitric oxide, is synthesised from L-arginine by nitric oxide synthase (a process inhibited by L-NOARG and L-NMMA) and causes vasorelaxation by stimulating the soluble guanylate cyclase in the smooth muscle. The second postulated factor is also derived from the endothelium but is not affected by L-NMMA or L-NOARG and does not appear to mediate its vasorelaxant effect by stimulating guanylate cyclase.

The ATP-sensitive potassium channel blocker, glibenclamide, was without effect on the relaxations induced by human α-CGRP in the pig coronary artery. This result is in contrast to the reported inhibitory effect of glibenclamide on CGRP-induced relaxations in the rat mesenteric artery

(Nelson *et al.*, 1990). Although the relaxations described in this paper are probably endothelium-dependent (see Chapter 3 Discussion), the lack of effect of glibenclamide against human α-CGRP-elicited relaxations in the pig coronary artery indicates that CGRP endothelium-independent relaxations in this tissue are not associated with activation of ATP-sensitive potassium channels. Indeed, it has been shown that CGRP endothelium-independent vasorelaxation in the pig coronary artery is not associated with changes in membrane potential (Beny *et al.*, 1989), indicating that the signal transduction pathway mediating this response is probably entirely biochemical.

Human α-CGRP-induced endothelium-independent relaxations were associated with selective rises in cyclic AMP, levels of cyclic GMP remaining unaltered. The rise in cyclic AMP preceded the relaxant response, thus it is possible that activation of adenylate cyclase could represent part of the transduction pathway as was suggested by Edvinsson *et al.*, (1985). This result contrasts with the endothelium-dependent vasorelaxant response elicited by human α-CGRP in the rat aorta where increases in levels of both cyclic AMP and cyclic GMP were observed.

Isoprenaline-induced endothelium-independent relaxations also seem to be associated with selective increases in cyclic AMP in contrast to the results from the rat aorta where elevations in both cyclic AMP and cyclic GMP were seen. Like human α-CGRP, the rise in cyclic AMP occurred before the relaxant response had started. These results and the fact that the β-adrenoreceptor is known to be linked to activation of adenylate cyclase, make it likely that activation of this enzyme represents the first step in the signal transduction pathway for isoprenaline endothelium-independent vasorelaxation. Given the similarities in time course for relaxation and second messenger accumulations it seems possible that the same transduction pathway mediates CGRP endothelium-independent vasorelaxation.

Unlike human α-CGRP and isoprenaline, sodium nitroprusside evoked endothelium-independent relaxations in both the pig coronary artery and the rat aorta. These relaxations were associated with selective increases in cyclic GMP, levels of cyclic AMP remaining unaltered as reported in other tissues (Gruetter *et al.*, 1981).

Two preparations of the pig coronary artery were chosen for study. Both the left anterior descending coronary artery and the anterior interventricular artery preparations exhibit endothelium-independent vasorelaxation when challenged with human α-CGRP. Further, these relaxations share a similar time-course and maximum response in both preparations though the EC_{50} is significantly higher in the smaller vessels. Despite the difference in internal diameter between the two preparations being small, the responses to repeat administration of human α-CGRP were markedly different, the left anterior descending coronary artery rings, but not the anterior interventricular artery, displaying tachyphylaxis. Differences also exist in the response to vasoconstrictors in rings of pig coronary artery taken from points only 2-3 cm apart within the same vessel and having physiologically large internal diameters (Miwa & Toda, 1984).

The tachyphylaxis to human α-CGRP in the left anterior descending coronary artery preparation appears to be mediated at, or in close proximity to, the receptor. It is unlikely that down-regulation of adenylate cyclase or changes in any of the processes distal to this enzyme in the transduction pathway play any role in the tachyphylaxis as isoprenaline, which appears to share the same transduction pathway as human α-CGRP in this preparation, does not display tachyphylaxis.

It is possible that the difference in tachyphylaxis to human α-CGRP between the two preparations of the pig coronary artery could be a

manifestation of different receptor subtypes or, alternatively, the expression of a receptor down-regulation pathway in the left anterior descending coronary artery, but not in the anterior interventricular artery.

In order to study these possibilities and classify the receptors mediating human α-CGRP endothelium-dependent and -independent vasorelaxation, the known properties of a selective antagonist were utilised. The results from these experiments are described in Chapter 7.

CHAPTER 7

INTRODUCTION

Receptors

High affinity binding sites for CGRP have been identified using autoradiography with radiolabelled CGRP. These sites have been shown to be widely distributed in many species in the brain, particularly in the Purkinje fibres of the cerebellum (Dotti-Sigrist *et al.*, 1988), and pituitary glands (Tschopp *et al.*, 1985). They have also been found in the spinal cord (Hiroshima *et al.*, 1988), the pancreas (Seifert *et al.*, 1985), vascular smooth muscle and endothelial cells (Hirata *et al.*, 1988; Connat *et al.*, 1991), gastric smooth muscle cells (Maton *et al.*, 1988), lung (Umeda *et al.*, 1989), liver (Yamaguchi *et al.*, 1988) and in human placental tissue (Foord & Craig, 1987a). The latter group also carried out a partial characterisation of the 'receptor' finding that the complex has a molecular mass of 240 kDa with subunits of between 62 and 68 kDa and that it appeared to bind to CGRP in a 1:1 ratio (Foord & Craig, 1987b).

These high affinity binding sites are thought to be receptors specific for CGRP. A number of observations support this hypothesis. Firstly, none of the standard non-CGRP antagonists affect the responses to CGRP in a variety of isolated preparations (Marshall *et al.*, 1986b; Franco-Cereceda *et al.*, 1987; Greenberg *et al.*, 1987; Kawasaki *et al.*, 1988). Secondly, CGRP, despite sharing some structural homology with calcitonin (see Chapter 1) is approximately 1000-fold less potent than calcitonin when acting on calcitonin

of calcium
receptors to inhibit resorption by cultured rat osteoclasts (Zaidi *et al.*, 1988). Further, when challenged with CGRP, the rat clonal osteogenic sarcoma cell line UMR 106-01, which does not express a functional calcitonin receptor, still displays elevations in cyclic AMP (Michelangeli *et al.*, 1986). These results are consistent with CGRP acting on different receptors from calcitonin.

A possible exception to this situation exists in the brain where it has been demonstrated that a novel calcitonin\CGRP receptor is expressed (Dotti-Sigrist *et al.*, 1988). In contrast to both calcitonin and CGRP receptors in the periphery, this receptor has a similar order of affinity for both calcitonin and CGRP. In this special case it has been postulated that both calcitonin and CGRP may act as the endogenous ligand (Tannenbaum & Goltzman, 1985).

The first step in the signal transduction pathway mediating the majority of the pharmacological effects of CGRP is therefore likely to be activation of unique CGRP receptors.

Recently there have been reports of multiple receptor subtypes for CGRP (Dennis *et al.*, 1989; Donoso *et al.*, 1990). These have been based on the properties of fragments and modified forms of human α-CGRP. The linear analogue of human α-CGRP, [cys(ACM)^{2,7}human α-CGRP] (**Table 7.1**), appears to be a weak selective agonist. While being approximately 100-fold less potent than CGRP at inhibiting the twitch response in the field stimulated isolated rat vas deferens [cys(ACM)^{2,7}human α-CGRP] does not mimic the CGRP-induced positive ino- and chronotropic response in guinea pig isolated atria (Dennis *et al.*, 1989).

The 12-37 fragment of human α-CGRP ($CGRP_{(12-37)}$) (**Table 7.1**) has been shown to antagonise CGRP actions in the guinea pig atria but not in the rat vas deferens (Donoso *et al.*, 1990). On the basis of these results CGRP

	S----S
Human α-CGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
Human α-CGRP$_{(8-37)}$	VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
Human α-CGRP$_{(12-37)}$	AGLLSRSGGVVKNNFVPTNVGSKAF
[Cys(ACM)^{2,7}) human α-CGRP]	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF

Table 7.1 Table showing the primary amino acid structures of human α-CGRP and some analogues and truncated fragments. Amino acid residues are represented by the single letter code and S---S indicates a disulphide link between two cysteine residues. [Cys(ACM)^{2,7}) human α-CGRP] is [acetamidomethyl-Cys^{2,7}] human α-CGRP is a linear analogue of human α-CGRP having no disulphide link between cysteine residues 2 and 7.

receptors have been divided into two subtypes, CGRP1 being inhibited by $CGRP_{(12-37)}$ and mediating CGRP actions in the guinea pig atria and the non-CGRP1 (sometimes referred to as the CGRP2) having [cys(ACM)^{2,7}]human α-CGRP] as a selective agonist, not being antagonised by $CGRP_{(12-37)}$ and mediating CGRP responses in the rat vas deferens.

The 8-37 fragment ($CGRP_{(8-37)}$) (**Table 7.1**) has been shown to exhibit antagonism of CGRP receptors (Chiba *et al.*, 1989), inhibiting the binding and associated rises in cyclic AMP in rat liver plasma membranes. $CGRP_{(8-37)}$ has also been shown to inhibit a number of pharmacological actions of CGRP including relaxation of preconstricted opposum internal anal sphincter (Chakder & Rattan, 1991), the fall in mean arterial pressure *in vivo* in the rat (Donoso *et al.*, 1990), the inhibition of the twitch response in the field stimulated isolated rat vas deferens and the positive inotropic effect in the isolate guinea pig left atria (Maggi *et al.*, 1991).

In this chapter $CGRP_{(8-37)}$ was used to try to further clarify the signal transduction pathway mediating CGRP endothelium-dependent and independent vascular relaxant responses.

MATERIALS AND METHODS

Rat thoracic aortic rings were prepared as described in Chapter 2, taking care to avoid damage to the endothelium. Cumulative dose/response curves to human α-CGRP were carried out after a stable contraction had developed to noradrenaline (10^{-7}M). The effects of $CGRP_{(8-37)}$ on the contractions elicited by noradrenaline and the relaxations induced by human α-CGRP were studied.

The two preparations of the pig left anterior descending coronary artery were prepared as described in Chapter 2. Briefly, the left anterior descending coronary arterial ring preparation was taken from between the junction with the circumflex and the first branch, the conus artery. It is the larger vessel being approximately 4mm in internal diameter. The anterior interventricular arterial rings were taken from further down the same artery and had an internal diameter of approximately 1mm. Both preparations had the endothelium routinely removed by mild abrasion. Cumulative concentration/effect curves to human α-CGRP were carried out in the presence of U46619 (10^{-8}M) for rings of left anterior descending coronary artery and acetylcholine ($2x10^{-7}$M) for the anterior interventricular artery. The effects of $CGRP_{(8-37)}$ on the contractions elicited by the spasmogen and the relaxations induced by human α-CGRP in both preparations of the pig coronary artery were studied.

Treated tissues were preincubated for 15 minutes in various concentrations of $CGRP_{(8-37)}$, control tissues receiving no pretreatment but following a similar time cycle. All rings were discarded after one cumulative concentration/effect curve to human α-CGRP due to the tachyphylaxis of the CGRP response in the pig left anterior descending coronary artery preparation described in Chapter 6.

To study the effect of $CGRP_{(8-37)}$ on the cyclic nucleotide accumulations associated with the endothelium-dependent and independent relaxations induced by human α-CGRP, rings of rat thoracic aortic and pig left anterior descending coronary artery were prepared as described above. Single concentrations of human α-CGRP giving approximately 80% relaxation of the tone induced by the relevant spasmogen were added and the tissues removed into liquid nitrogen at the optimum time-point for cyclic nucleotide accumulation (as determined in Chapters 4 and 6). The levels of cyclic

nucleotides were then determined by scintillation proximity assay (Amersham) using the acetylation protocols (see Chapter 2 and Appendix 2) for both cyclic AMP and cyclic GMP in the rat and the non-acetylation protocol for cyclic AMP in the pig left anterior descending coronary artery.

$CGRP_{(8-37)}$ was prepared in distilled water to form a 10^{-2}M stock solution. This was divided into aliquots and stored at -20°C. After thawing, the aliquot was diluted to the required concentration with Krebs solution. No aliquot was refrozen, the remnants being discarded.

RESULTS

Effect of $CGRP_{(8-37)}$ on human α-CGRP-induced endothelium-dependent relaxations

Rat thoracic aorta

As was shown in Chapters 3 and 4, human α-CGRP induces relaxations in the rat thoracic aorta which are wholly dependent on the presence of an intact endothelium (**Figure 3.2**). Further, these relaxations are associated with rises in levels of both cyclic AMP and cyclic GMP (**Figure 4.5**).

$CGRP_{(8-37)}$ at concentrations up to 10^{-5}M neither affected the contractile response induced by noradrenaline (10^{-7}M) nor had any relaxant effect of its own in the rat aorta. The human α-CGRP-induced relaxations were unaffected by pretreatment with $CGRP_{(8-37)}$ at concentrations up to 10^{-5}M (**Figure 7.1**) as were the endothelium-dependent and -independent relaxations induced in this tissue by acetylcholine and sodium nitroprusside, respectively.

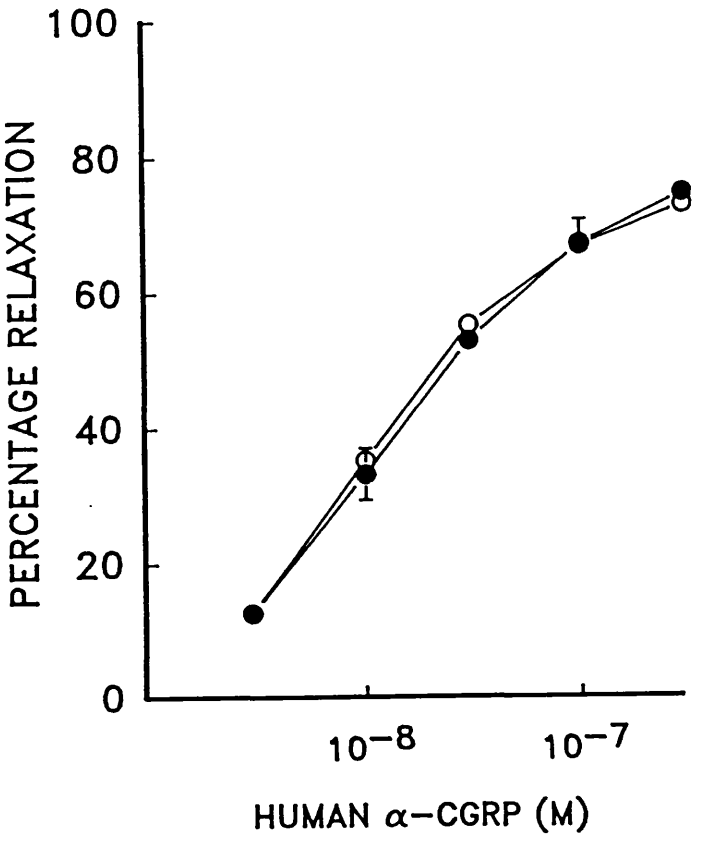


Figure 7.1 Effect of $CGRP_{(8-37)}$ (10^{-5}M) on relaxations induced by human α-CGRP in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Results are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7}M). O represents the control and ● the $CGRP_{(8-37)}$ pretreated values. Points represent the mean ± s.e. mean of 4 separate experiments.

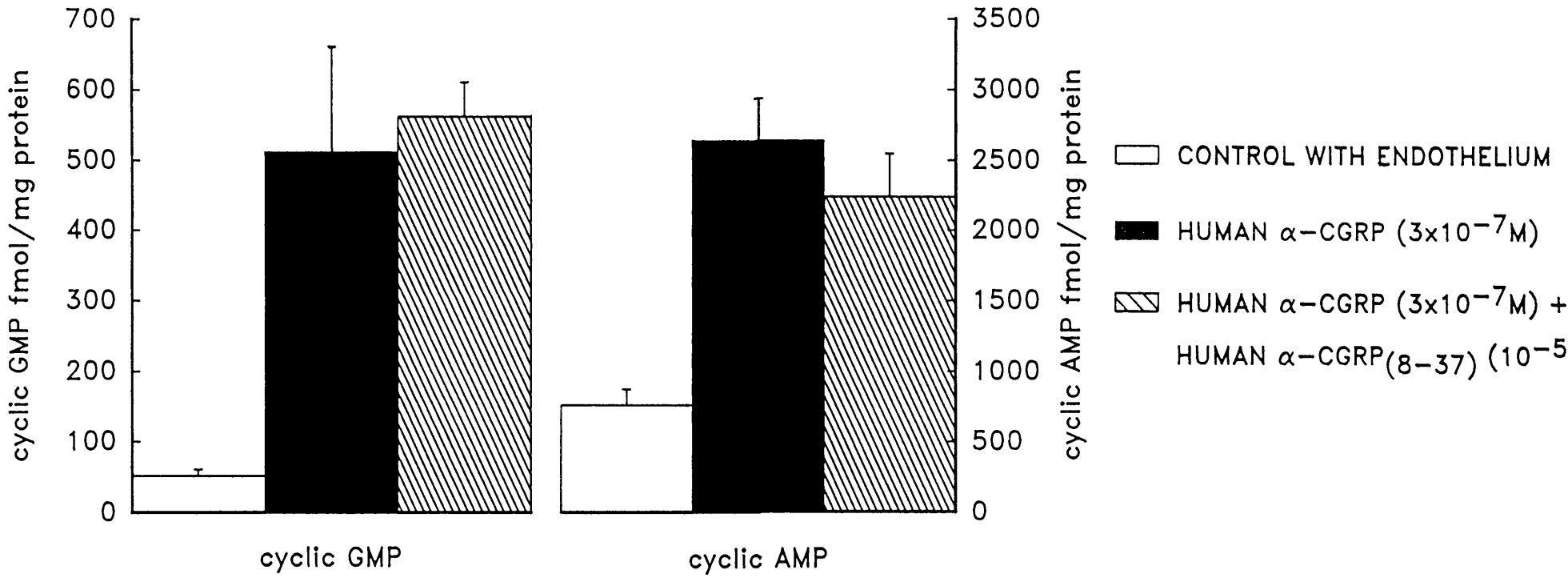


Figure 7.2 Effect of $CGRP_{(8-37)}$ (10^{-5}M) on cyclic nucleotide levels induced by human α-CGRP ($3x10^{-9}$M) in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7}M). Levels of cyclic GMP (left panel) and cyclic AMP (right panel) are expressed in fmol/mg protein. Bars represent the mean ± s.e. mean of 6 separate experiments.

Problems with the surface tension precluded the addition of any higher concentrations of $CGRP_{(8-37)}$. $CGRP_{(8-37)}$ (10^{-5}M) did not significantly affect the rise in cyclic AMP or cyclic GMP seen after 30s exposure to human α-CGRP ($3x10^{-7}$M) **(Figure 7.2)**.

Effect of $CGRP_{(8-37)}$ on human α-CGRP-induced endothelium-independent relaxations

Pig left anterior descending coronary artery

Human α-CGRP evokes relaxations in this tissue in both endothelium intact and denuded preparations with a similar level of affinity suggesting that the response in this tissue is wholly endothelium-independent **(Figure 6.1)**. In all tissues used in this experiment the endothelium was removed. $CGRP_{(8-37)}$ at concentrations up to 10^{-5}M was devoid of any relaxant effect or any effect on the tone induced by U46619 (10^{-8}M). The concentration/effect curve to human α-CGRP was shifted to the right in a parallel manner with no reduction in the maximum response by 10^{-6}M, $3x10^{-6}$M and 10^{-5}M $CGRP_{(8-37)}$ **(Figure 7.3)**. A Schild plot of this data gives a pA_2 value of 6.3 and slope not significantly different from 1 (0.96 ± 0.05; correlation coefficient 0.99) **(Figure 7.4)**

Effects of $CGRP_{(8-37)}$ on human α-CGRP-induced cyclic AMP accumulations

Human α-CGRP endothelium-independent relaxations are associated with selective increases in levels of cyclic AMP, cyclic GMP levels not being significantly altered from basal levels **(Figure 6.8)**. At 180s human α-CGRP ($3x10^{-8}$M) elicited a relaxant effect of 46 ± 4% of the tone induced by U46619 (10^{-8}M) this relaxant effect being associated with approximately a 4-fold rise in levels of cyclic AMP. $CGRP_{(8-37)}$ (10^{-7}M), a concentration below

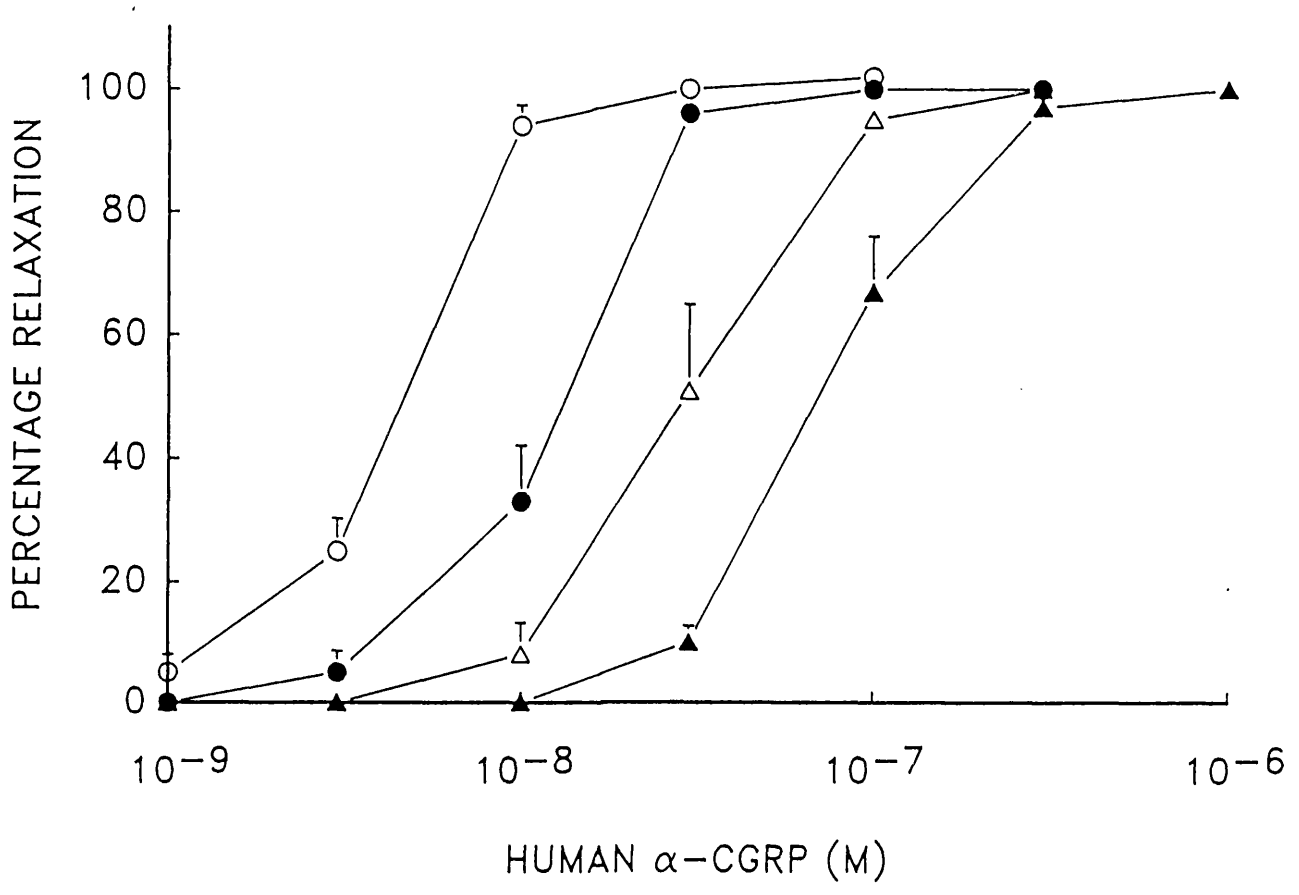


Figure 7.3 Effect of $CGRP_{(8-37)}$ on relaxations induced by human α-CGRP in pig left anterior descending coronary artery rings denuded of endothelium preconstricted with U46619 (10^{-8}M). O represents the control curve, ● in the presence of $CGRP_{(8-37)}$ (10^{-6}M), △ in the presence of $CGRP_{(8-37)}$ ($3x10^{-6}$M) and ▲ in the presence of $CGRP_{(8-37)}$ (10^{-5}M). Results are expressed as a percentage reduction in the tone induced by U46619 (10^{-8}M). Points represent the mean ± s.e. mean of between 4 and 6 separate experiments.

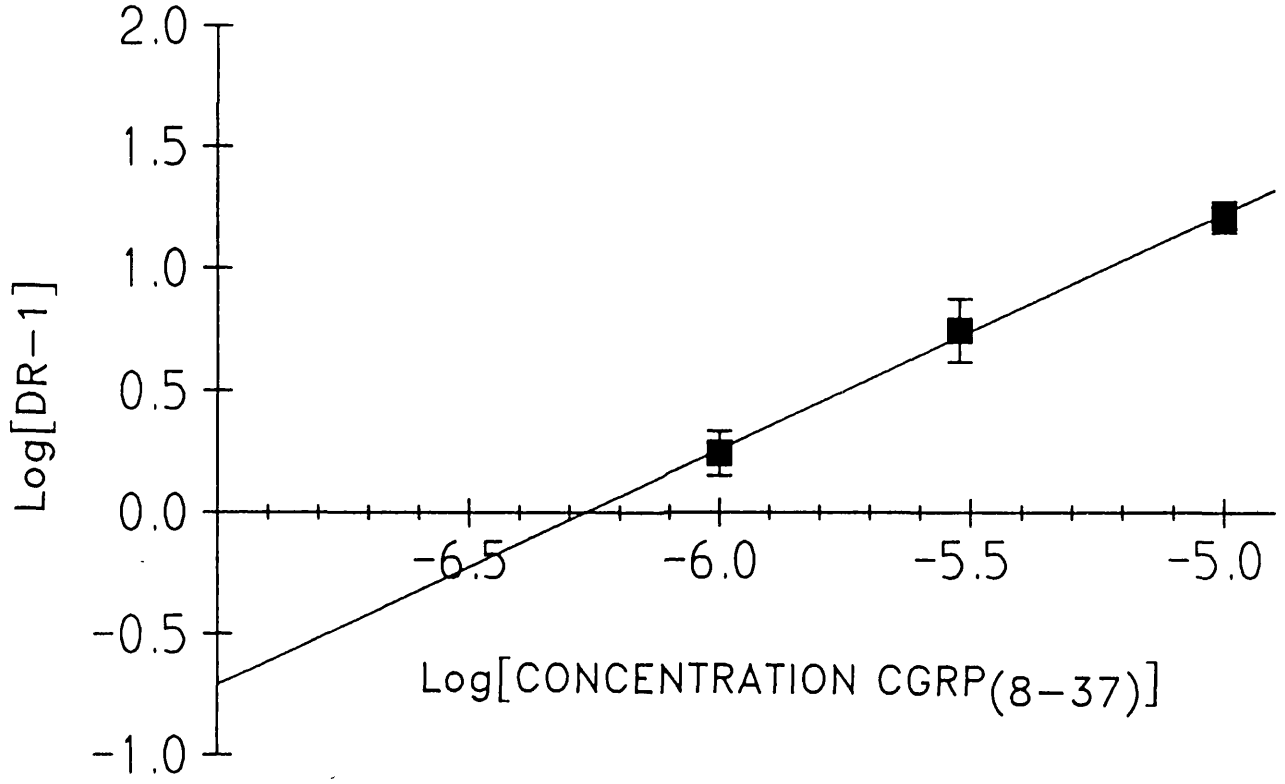


Figure 7.4 Schild plot of $CGRP_{(8-37)}$ antagonism of human α-CGRP-induced vasorelaxation in pig left anterior descending coronary artery denuded of endothelium. Points represent the mean ± s.e. mean of between 4 and 6 separate experiments.

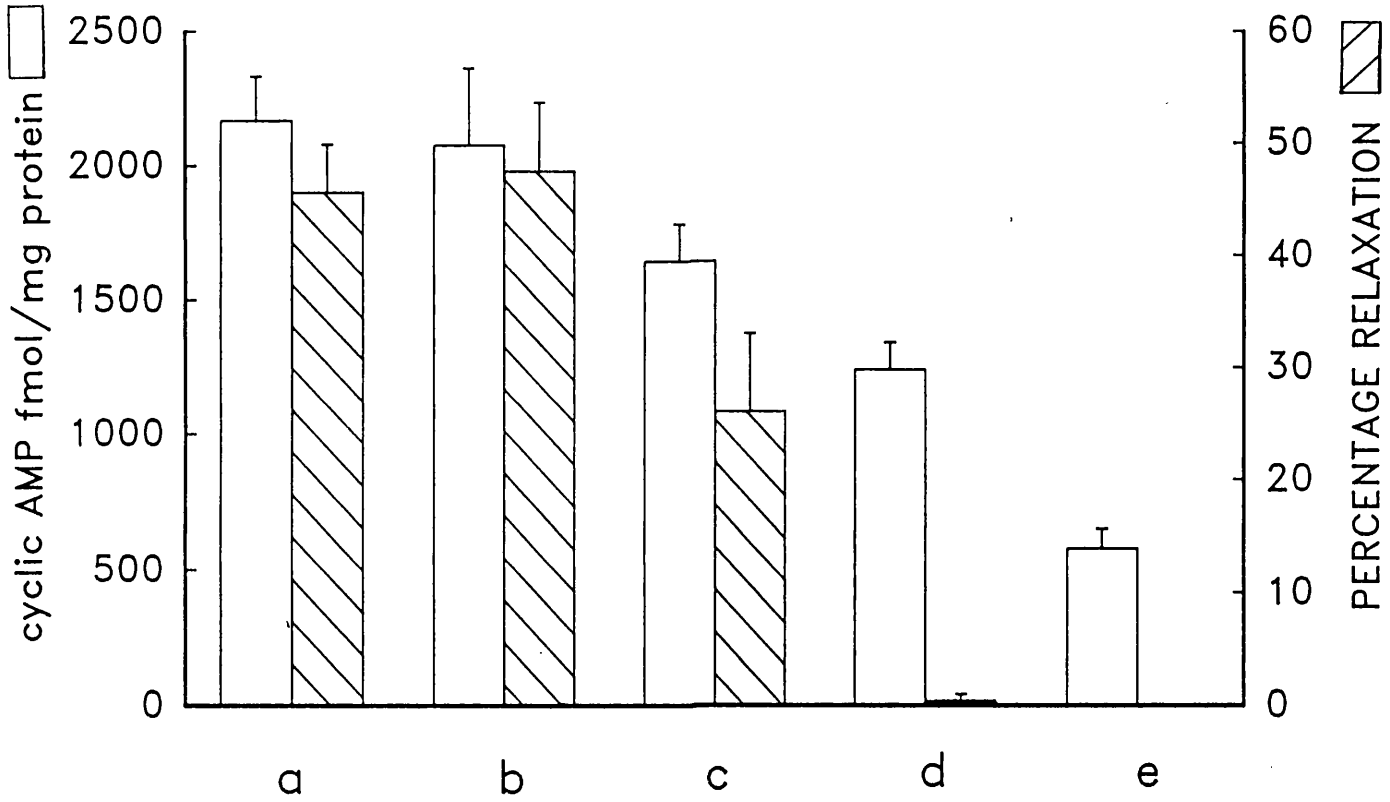


Figure 7.5 Effect of $CGRP_{(8-37)}$ on cyclic AMP accumulations and relaxations induced by human α-CGRP ($3x10^{-8}$M) at 180s exposure in pig left anterior descending coronary artery rings denuded of endothelium preconstricted with U46619 (10^{-8}M). Open bars represent levels of cyclic AMP expressed in fmol/mg protein while the hatched bars represent the level of relaxation expressed as a percentage of the tone induced by U46619 (10^{-8}M). a) human α-CGRP ($3x10^{-8}$M) alone. b) human α-CGRP ($3x10^{-8}$M) plus $CGRP_{(8-37)}$ (10^{-7}M). c) human α-CGRP ($3x10^{-8}$M) plus $CGRP_{(8-37)}$ (10^{-6}M). d) human α-CGRP ($3x10^{-8}$M) plus $CGRP_{(8-37)}$ (10^{-5}M). e) basal levels. Bars represent the mean ± s.e. mean of between 4 and 6 separate experiments.

the pA_2 value, did not significantly alter the relaxation or cyclic AMP accumulations evoked by human α-CGRP ($3x10^{-8}M$) at 180s exposure. At higher concentrations of $CGRP_{(8-37)}$, $10^{-6}M$ and $10^{-5}M$ both the relaxation and associated cyclic AMP increases evoked by human α-CGRP were reduced (**Figure 7.5**).$P<0.05$

Pig anterior interventricular artery

In this, the smaller of the two preparations of the pig coronary artery, human α-CGRP also induces a relaxant effect which is endothelium-independent (**Figure 6.9**). As with the left anterior descending coronary artery described above, $CGRP_{(8-37)}$ was without relaxant or any other effect on the spasmogen-induced tone (acetylcholine; $2x10^{-7}M$). The concentration/effect curve to human α-CGRP was significantly shifted to the right with no reduction in the maximum response by $10^{-6}M$, $3x10^{-6}M$ and $10^{-5}M$ $CGRP_{(8-37)}$. However at $10^{-7}M$, $CGRP_{(8-37)}$ had no significant effect on human α-CGRP vasorelaxation (**Figure 7.6**). A Schild plot of this data gives a pA_2 of 6.7 and slope equalling 1.0 ± 0.1 (correlation coefficient 0.99) (**Figure 7.7**).

DISCUSSION

The majority of the pharmacological actions of CGRP are thought to be mediated by receptors with high affinity for CGRP. The use of analogues and truncated fragments of human α-CGRP has indicated that subtypes of the CGRP receptor may exist (Dennis *et al.*, 1989; Donoso *et al.*, 1990). In this chapter the effects of $CGRP_{(8-37)}$ on human α-CGRP-induced endothelium-dependent and -independent vasorelaxations were studied.

In the rat aorta, human α-CGRP-induced relaxations are endothelium-

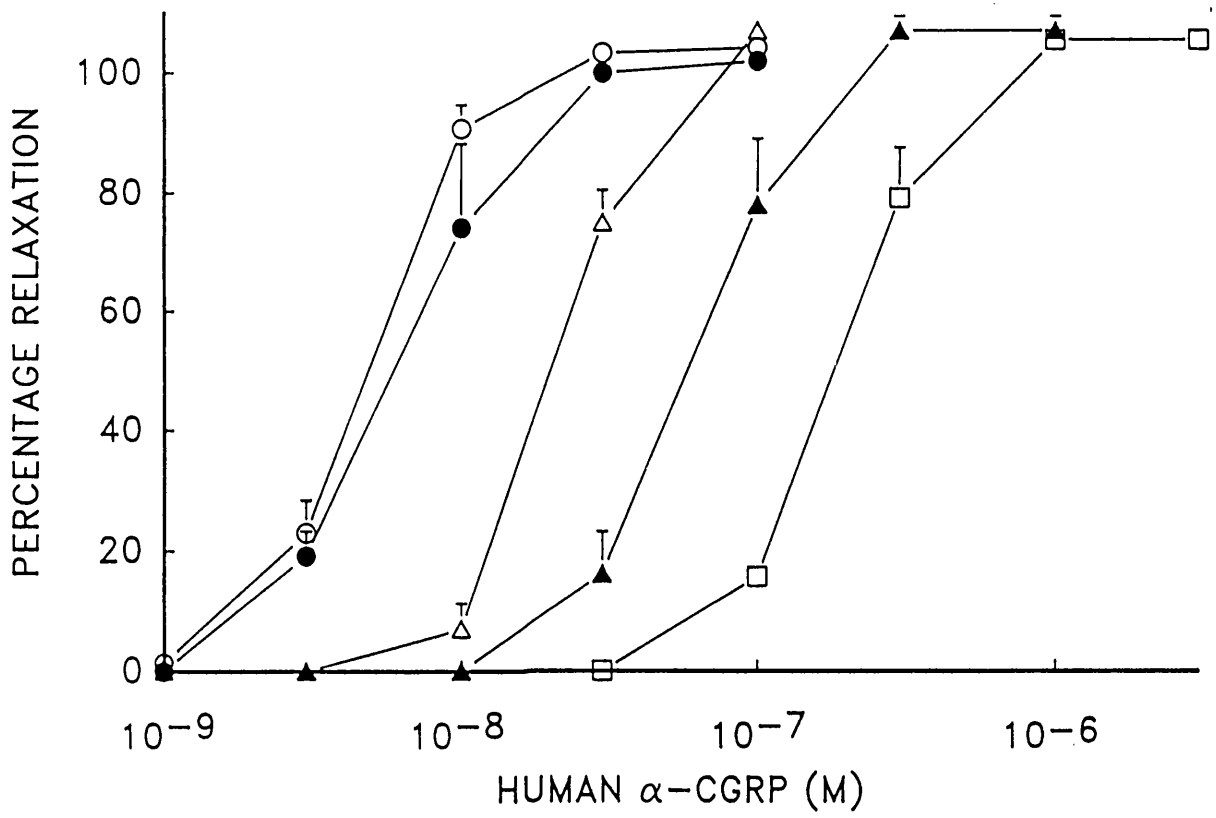


Figure 7.6 Effect of $CGRP_{(8-37)}$ on relaxations induced by human α-CGRP in pig anterior interventricular artery rings denuded of endothelium preconstricted with acetylcholine ($2x10^{-7}M$). O represents the control curve, ● in the presence of $CGRP_{(8-37)}$ ($10^{-7}M$), △ in the presence of $CGRP_{(8-37)}$ ($10^{-6}M$), ▲ in the presence of $CGRP_{(8-37)}$ ($3x10^{-6}M$) and □ in the presence of $CGRP_{(8-37)}$ ($10^{-5}M$). Results are expressed as a percentage reduction in the tone induced by acetylcholine ($10^{-7}M$). Points represent the mean ± s.e. mean of between 4 and 6 separate experiments.

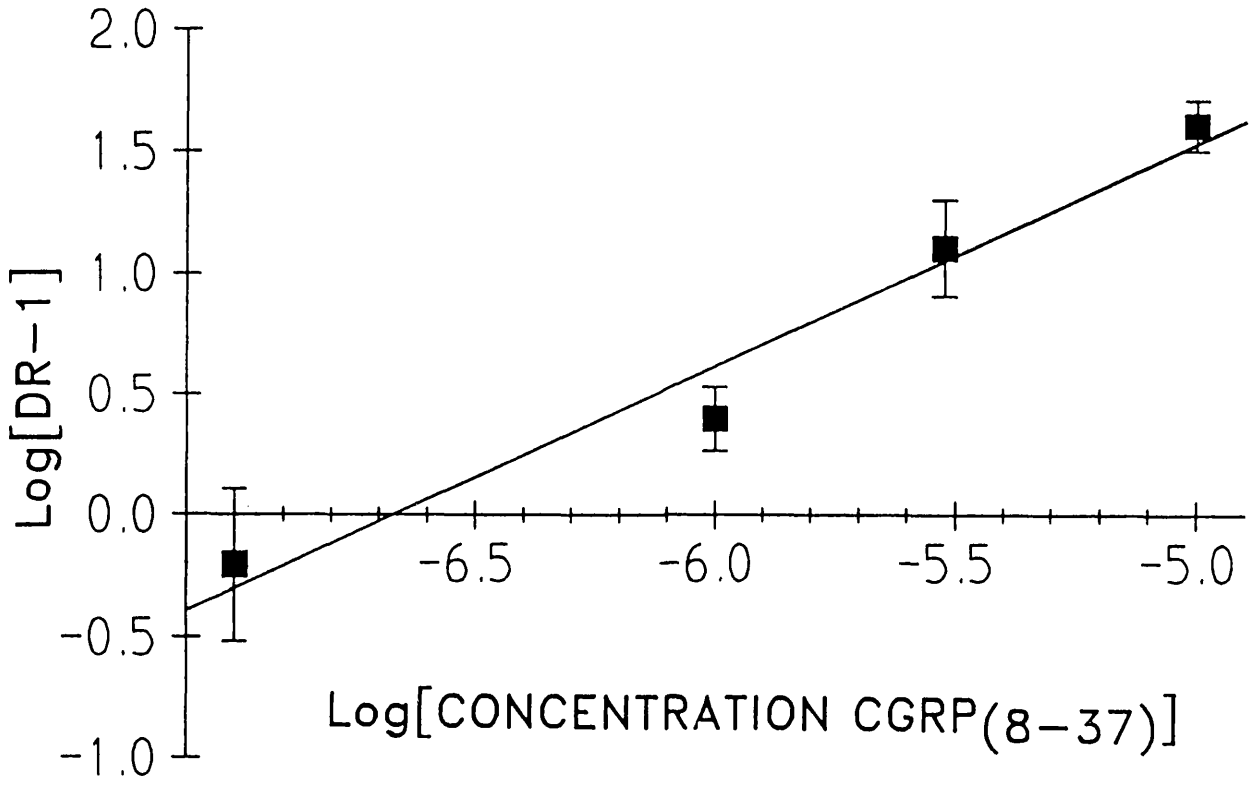


Figure 7.7 Schild plot of $CGRP_{(8-37)}$ antagonism of human α-CGRP-induced vasorelaxation in pig left anterior descending coronary artery denuded of endothelium. Points represent the mean ± s.e. mean of between 4 and 6 separate experiments.

dependent and associated with rises in cyclic AMP and cyclic GMP (Chapter 5). In this tissue $CGRP_{(8-37)}$ at concentrations up to 10^{-5}M did not affect the relaxations or cyclic nucleotide accumulations evoked by human α-CGRP. Therefore $CGRP_{(8-37)}$ is either not an antagonist at this site or has a pA_2 of less than 5.

In the pig coronary artery human α-CGRP induces relaxations which are endothelium-independent. $CGRP_{(8-37)}$ proved to be a competitive antagonist of human α-CGRP in both preparations of the pig coronary artery giving similar pA_2 values (6.3 and 6.7 in the left anterior descending coronary artery and anterior interventricular artery respectively). These values are close to those obtained by other groups on the positive inotropic effect induced by CGRP in the guinea pig isolated left atria (6.9), on CGRP-induced inhibition of the twitch response in the rat isolated field-stimulated vas deferens (6.55) (Maggi *et al.*, 1991) and on CGRP-induced vasorelaxation in the rat caudal artery (Fiscus *et al.*, 1991). Despite the difference in tachyphylaxis to CGRP observed in the two preparations described in Chapter 6 the pA_2 values for $CGRP_{(8-37)}$ are very similar.

Over a similar range at which it antagonises human α-CGRP-induced relaxations in the left anterior descending coronary artery, $CGRP_{(8-37)}$ also antagonises the accumulations of cyclic AMP evoked by human α-CGRP. On the basis of this observation, together with the observation that the rise in cyclic AMP precedes the relaxant response to human α-CGRP and the report that CGRP relaxations in the pig coronary artery are not associated with changes in membrane potential (Beny *et al.*, 1989), it seems likely that the signal transduction pathway responsible for CGRP endothelium-independent relaxations is biochemical and involves activation of adenylate cyclase.

On the basis of the selective antagonistic properties of $CGRP_{(8-37)}$ it is

possible to define two receptor subtypes for CGRP, the first mediating CGRP-endothelium-independent vasorelaxation in the pig coronary artery and the second mediating CGRP endothelium-dependent vasorelaxation in the rat aorta. The identity of these receptors within the categorisation of Dennis *et al.*, (1989) remains unclear. The CGRP1 receptor has been defined as mediating those effects of CGRP which are antagonised by $CGRP_{(12-37)}$. These include the positive ino- and chronotropic effects of CGRP in the guinea pig isolated atria, where $CGRP_{(8-37)}$ is also an antagonist (Maggi *et al.*, 1991). The non-CGRP1 subtype (or CGRP2 receptor) is defined as mediating the actions of CGRP not antagonised by $CGRP_{(12-37)}$. This subtype is reported to cause the CGRP-induced inhibition of the twitch response in the field stimulated isolated rat vas deferens (Dennis *et al.*, 1989). However, $CGRP_{(8-37)}$ has also been shown to antagonise this action of CGRP with a similar pA_2 as on the putative CGRP1 receptor (Maggi *et al.*, 1991).

The selective antagonistic action of $CGRP_{(8-37)}$ in the vascular tissues described in the present study conflicts with the apparent lack of selectivity against the CGRP response in the rat vas deferens and the guinea pig atria. These results taken at face value would result in the classification of 3 subtypes of CGRP receptor these being a) insensitive to $CGRP_{(8-37)}$ b) antagonised by $CGRP_{(8-37)}$ but not $CGRP_{(12-37)}$ and c) antagonised by both $CGRP_{(8-37)}$ and $CGRP_{(12-37)}$. However, this seems unlikely in view of the relatively low concentrations of $CGRP_{(12-37)}$ used in the study by Dennis *et al.*, (1989) in the rat vas deferens. This group used a maximum concentration of 10^{-6}M $CGRP_{(12-37)}$. Since $CGRP_{(8-37)}$ has been shown to have a pA_2 of 6.55 in this tissue (Maggi *et al.*, 1991) and has been shown to be slightly more potent as an antagonist of CGRP than $CGRP_{(12-37)}$ (Donoso *et al.*, 1990), it is possible that too little $CGRP_{(12-37)}$ was added to see a significant shift in the CGRP concentration/effect curve. If this were true then the division of the CGRP receptors into subtypes would be premature on the basis of the published data.

However, based on the results presented in this chapter, CGRP may indeed have two subtypes of receptor, one being antagonised by $CGRP_{(8-37)}$ with a pA_2 of between 6.3 and 6.7 and the other being insensitive to $CGRP_{(8-37)}$ at concentrations up to 10^{-5}M.

The finding that the receptors mediating the endothelium-dependent and -independent vasorelaxant effects of human α-CGRP appear to be mediated by two different subtypes of CGRP receptor contradicts the hypothesis that in the rat the CGRP receptors in the cardiovascular system are of a single receptor subtype (Donoso *et al.*, 1990). This hypothesis stems from the observation that *in vivo* the fall in mean arterial blood pressure evoked by CGRP can be inhibited by $CGRP_{(8-37)}$. While this result certainly defines the subtype responsible for this action of CGRP as being of the $CGRP_{(8-37)}$-sensitive subtype, it does not preclude the existence of $CGRP_{(8-37)}$-insensitive receptors in the cardiovascular system of the rat. From the results presented in this chapter it seems possible that the $CGRP_{(8-37)}$-sensitive subtype mediates CGRP endothelium-independent vasorelaxations while the $CGRP_{(8-37)}$-insensitive subtype mediates CGRP endothelium-dependent vasorelaxation. It has been shown in the rat that the small resistance vessels almost invariably exhibit endothelium-independent relaxations to CGRP (Gardiner *et al.*, 1991b), the vasorelaxant effects of CGRP being unaltered by L-NAME, a nitric oxide synthesis inhibitor. Since these vessels are responsible for the control of blood pressure it is not surprising that the reduction in mean arterial blood pressure seen on administration of CGRP in the rat appears to be solely due to $CGRP_{(8-37)}$-sensitive receptors.

It is clear from the distribution of the two mechanisms of CGRP vasorelaxation and the relative characteristics of these mechanisms that, if CGRP is involved physiologically in the control of blood pressure, it probably

involves activation of $CGRP_{(8-37)}$-sensitive receptors and endothelium-independent vasorelaxation, as opposed to the $CGRP_{(8-37)}$-insensitive receptor-mediated endothelium-dependent vasorelaxation.

CHAPTER 8

SUMMARY AND CONCLUSIONS

In this thesis an attempt has been made to elucidate the signal transduction pathways mediating CGRP-induced endothelium-dependent and -independent vasorelaxation. During the course of this study a number of novel findings were described.

Endothelium-dependent relaxations

The endothelium-dependent mechanism of vasorelaxation induced by CGRP appears to be mediated by a $CGRP_{(8-37)}$-insensitive subtype of CGRP receptor and involves a novel signal transduction pathway. It was found that this action of CGRP was associated with rises in both cyclic AMP and cyclic GMP, the latter never previously being shown to be associated with any of the pharmacological actions of the peptide. Using a number of pharmacological agents it was concluded that CGRP releases nitric oxide as its EDRF. The nitric oxide stimulation of soluble guanylate cyclase accounts for the rise in cyclic GMP. However, the role of the elevations in cyclic AMP appears to be unusual as it appears to mediate the stimulation of nitric oxide synthase **(Figure 8.1)**.

The evidence for this is as follows. Firstly, removal of the endothelium abolishes the relaxant response and rises in both cyclic nucleotides induced by CGRP. This is consistent with the elevations in cyclic AMP either occurring within the endothelium or being caused by some

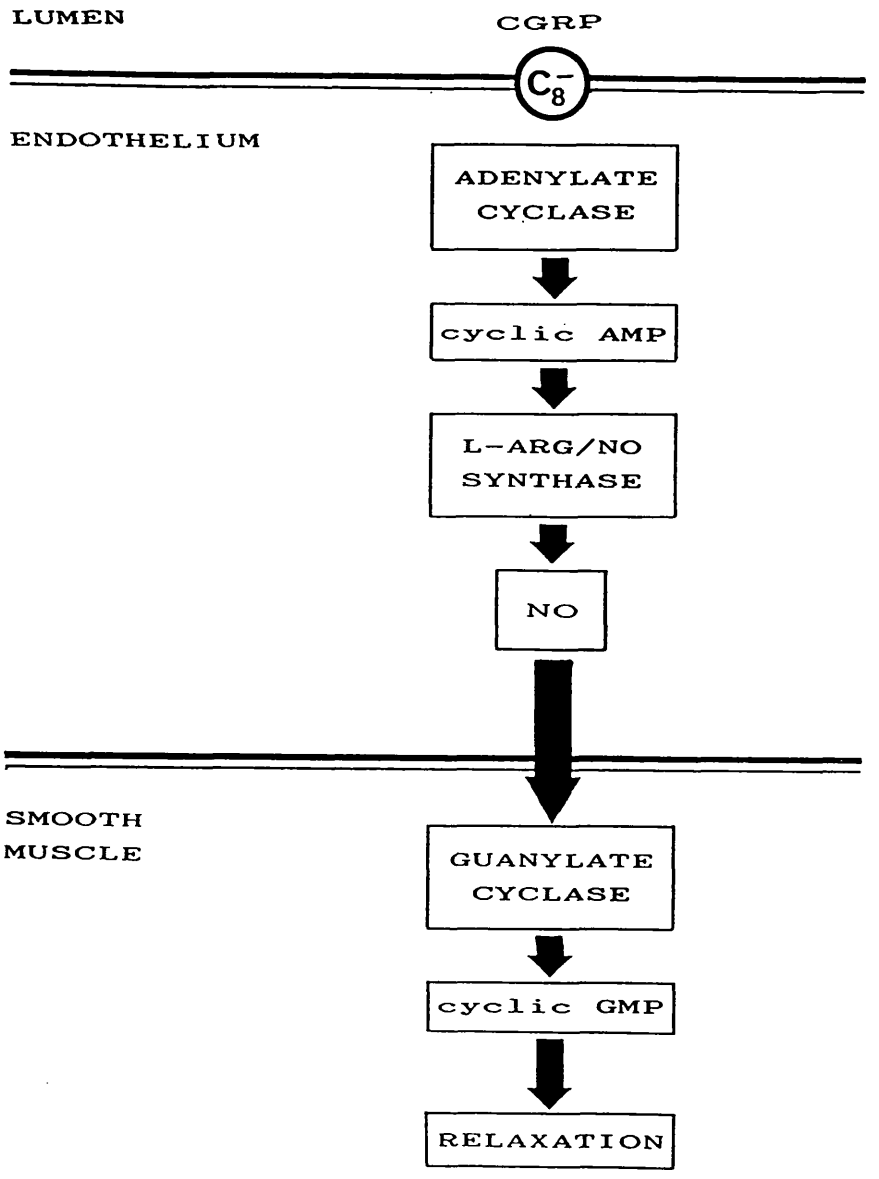


Figure 8.1 Postulated transduction pathway mediating human α-CGRP endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors insensitive to the antagonist $CGRP_{(8-37)}$ (C_8-) only on the endothelium. The endothelial receptors activate adenylate cyclase. The elevation in cyclic AMP levels in the endothelium activates nitric oxide synthase (L-ARG/NO SYNTHASE) causing the synthesis of nitric oxide (NO). This causes activation of soluble guanylate cyclase stimulation in the smooth muscle leading to cyclic GMP accumulation and subsequently relaxation.This activates the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation in the smooth muscle.

substance released from the endothelium. Secondly, the nitric oxide synthase inhibitor, L-NOARG, inhibited the relaxations and elevations in cyclic GMP induced by CGRP without significantly altering the elevations in cyclic AMP induced by the peptide. This observation requires the elevation in cyclic AMP to precede the activation of nitric oxide synthase or be unrelated to the relaxant response. Finally, a number of other agents known to stimulate adenylate cyclase, including the β-adrenoreceptor agonist, isoprenaline, and the diterpene, forskolin, caused endothelium-dependent relaxations in the rat aorta. Further, these relaxations shared a similar pharmacological profile to those induced by CGRP, being inhibited by L-NOARG, associated with elevations in both cyclic AMP and cyclic GMP (the latter but not the former being inhibited by L-NOARG) and the relaxations and associated cyclic nucleotide accumulations being abolished by removal of the endothelium.

This represents strong circumstantial evidence that some agents which can elevate cyclic AMP levels can activate nitric oxide synthase via this cyclic nucleotide (**Figure 8.2**).

The question of how cyclic AMP might activate nitric oxide synthase remains unanswered. There are a number of possible mechanisms including the activation of a cyclic AMP-dependent protein kinase causing phosphorylation and thus activation of nitric oxide synthase. It is interesting to note that the recent cloning of brain nitric oxide synthase (although different from the vascular form) has revealed a cyclic AMP-dependent protein kinase consensus site (Bredt *et al.*, 1991). Alternatively, the activation of the enzyme might be mediated indirectly through elevations in intracellular calcium levels. This could be caused by cyclic AMP increasing IP_3 levels (as it does in other preparations (Laufer & Changeux, 1989)) or by phosphorylation and activation of the IP_3 receptor/channel complex on the endoplasmic reticulum.

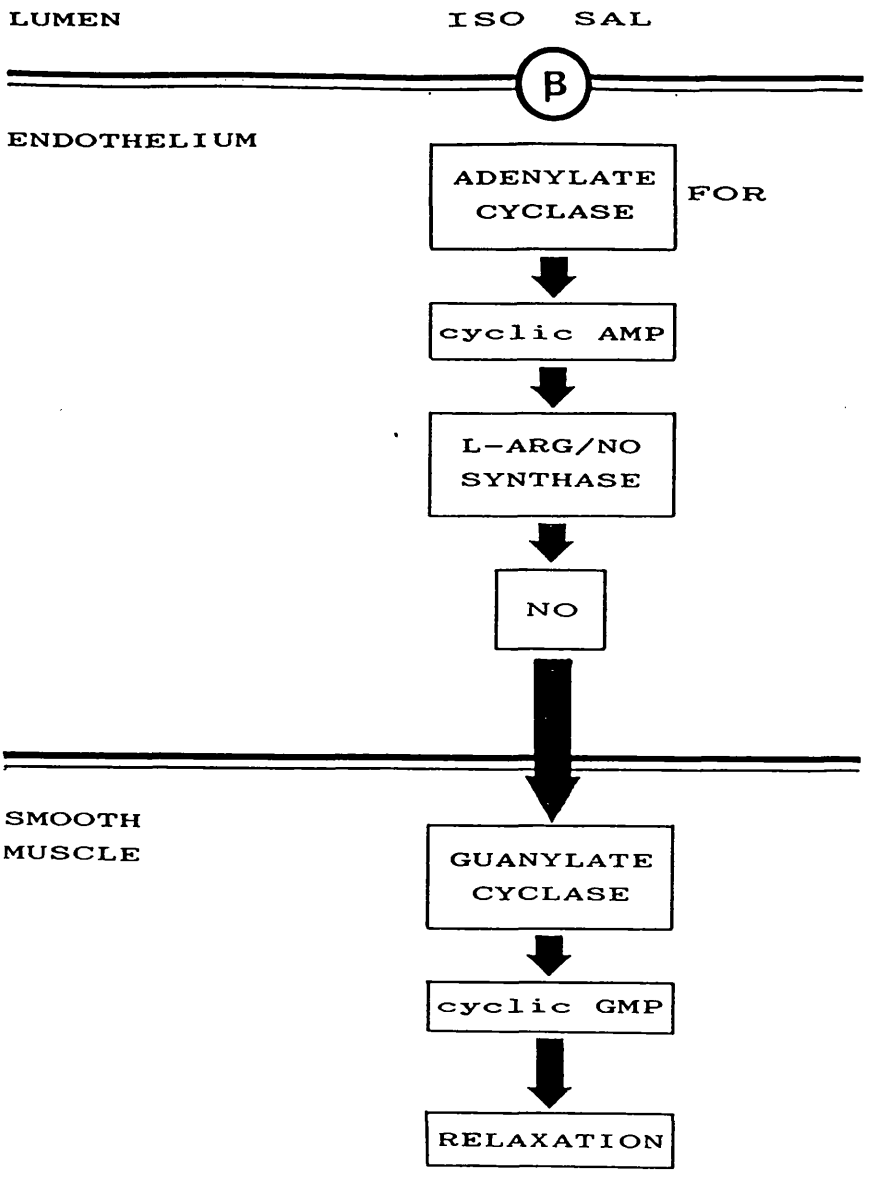


Figure 8.2 Postulated transduction pathway mediating isoprenaline (ISO), salbutamol (SAL) and forskolin (FOR) endothelium-dependent relaxations in rat aorta. Isoprenaline (ISO) and salbutamol (SAL) acting on β-adrenoreceptors on the endothelium cause activation of adenylate cyclase. Forskolin (FOR) activates endothelial adenylate cyclase directly. The elevation in cyclic AMP levels in the endothelium activates nitric oxide synthase (L-ARG/NO SYNTHASE) causing the synthesis of nitric oxide (NO). This causes activation of soluble guanylate cyclase stimulation in the smooth muscle leading to cyclic GMP accumulation and subsequently relaxation.

While the activation of nitric oxide synthase by cyclic AMP represents a possible signal transduction pathway mediating CGRP endothelium-dependent relaxations in the rat thoracic aorta different mechanisms may exist in other vascular beds. The possible involvement of ATP-sensitive potassium channels can not be ruled out in other tissues, notably the mesenteric artery (Nelson *et al.*, 1990).

Both CGRP and β-adrenergic agonists also have an endothelium-independent mechanisms of inducing vasorelaxation (although not in the rat thoracic aorta). However, the relative potency and maximum response of the endothelium-independent as opposed to the endothelium-dependent vasorelaxant effect of these vasodilators make it likely that, if there is any physiologically-relevant vasorelaxation, then it is mediated independently of the endothelium. While this is the case for these vasodilators, the physiological relevance of the activation of nitric oxide synthase by cyclic AMP remains unanswered. It is possible that other agents which activate adenylate cyclase may preferentially act on the endothelium as opposed to the smooth muscle of the blood vessel.

Endothelium removal

The observation that isoprenaline causes endothelium-dependent vasorelaxation in the rat aorta (but not the rabbit aorta) was entirely unexpected. This species difference apparently contradicts a number of papers in which isoprenaline is reported to be an endothelium-independent vasodilator in the rat aorta. However, most of these studies found that removal of the endothelium in this tissue caused a marked (though variable even within single reports) reduction in the isoprenaline response. This apparent impasse appears to be due to tools used to define whether a tissue has been denuded of endothelium.

In most cases acetylcholine is used, lack of relaxation to a high concentration being taken as an indicator of *complete* endothelium removal. While the use of acetylcholine for this purpose has been verified in the rabbit aorta using histological means (Furchgott & Zawadzki, 1980), the same study has not been carried out in the rat aorta. In contrast to the rabbit aorta, there are indications that only a portion of the endothelium must be removed in the rat aorta to abolish the relaxant response to acetylcholine. This differing sensitivity to endothelium removal in different species probably accounts for the conflicting results of isoprenaline.

In view of this observation it would be beneficial to carry out parallel studies in a number of vessels considering the physiological involvement of the endothelium when different proportions have been removed (determined histologically).

Cultured cells versus intact tissues

During the course of this study it has become apparent that reports investigating the endothelium-dependent vasorelaxant actions of CGRP and the transduction pathways mediating this effect within isolated cells achieved conflicting results to those using intact tissues. These include the following observations:-

a) CGRP does not release an EDRF from cultured endothelial cells derived from pig or bovine aorta (J.A. Mitchell, private communication; Crossman *et al.*, 1991), but apparently does in some intact vessels (Brain *et al.*, 1985; Al-Kazwini *et al.*, 1987 and Chapter 3).

b) CGRP receptors are present in cultured smooth muscle cells of rat aorta,

but are not present on the smooth muscle in the intact tissue (Connat *et al.*, 1991).

c) CGRP causes increases in cyclic AMP in cultured endothelial cells (Hirata *et al.*, 1988; Crossman *et al.*, 1991), but rises in both cyclic AMP and cyclic GMP in the intact tissue (see Chapter 4).

d) CGRP causes increases in cyclic AMP in cultured vascular smooth muscle cells derived from rat and bovine aorta (Kubota *et al.*, 1985; Hirata *et al.*, 1988; Crossman *et al.*, 1991), but does not in endothelium-denuded rat aorta (see Chapter 4).

e) Cyclic nucleotides do not modulate the release of nitric oxide in cultured bovine aortic endothelial cells (Kuhn *et al.*, 1991), but in intact tissues cyclic AMP appears to promote the synthesis of nitric oxide (Chapters 4 & 5) and cyclic GMP may inhibit the synthesis of nitric oxide (Evans *et al.*, 1988).

In view of the extensive differences between cultured systems and the intact tissue it would appear that cultured cells do not represent an appropriate tool for the study of the endothelium-dependent vascular relaxant effects of CGRP.

Endothelium-independent relaxations

The endothelium-independent vasorelaxations induced in the pig coronary artery by CGRP appear to have a less complex transduction pathway involving a $CGRP_{(8\text{-}37)}$-sensitive receptor (pA_2 between 6.3 and 6.7) and activation of adenylate cyclase. This leads to accumulations of cyclic AMP and presumably to activation of a cyclic AMP-dependent protein kinase and phosphorylation and inactivation of the myosin light chain kinase. This is

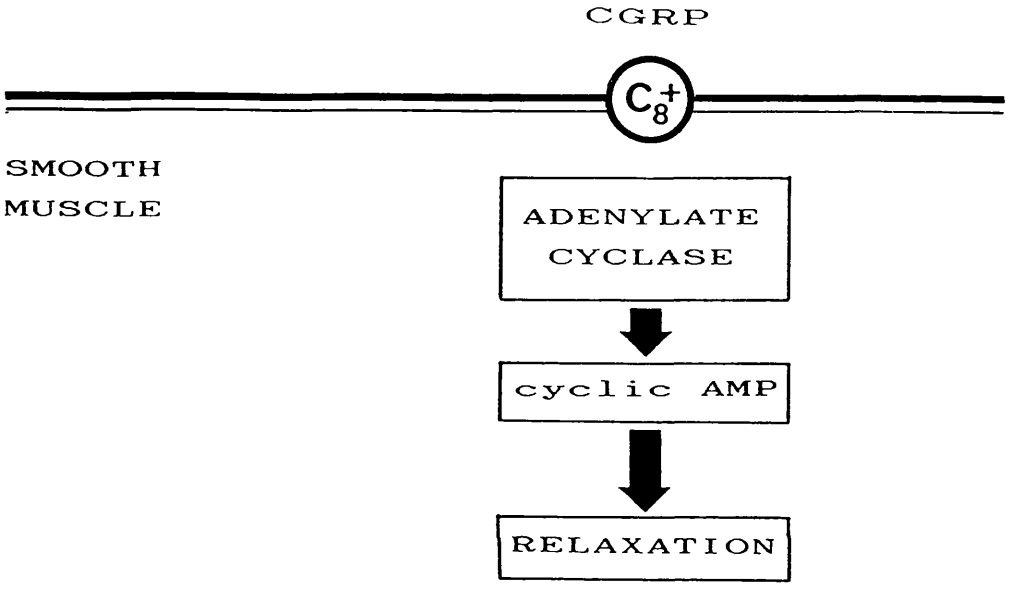


Figure 8.3 Postulated transduction pathway mediating human α-CGRP endothelium-independent relaxations in pig left anterior descending coronary artery. Human α-CGRP has receptors which are blocked by the antagonist $CGRP_{(8\text{-}37)}$ (C_8^+). The receptors activate adenylate cyclase, elevating cyclic AMP levels. This, by some mechanism, possibly the activation of a cyclic AMP-dependent protein kinase and phosphorylation of the myosin light chain kinase, causes the relaxant response.

represented in **Figure 8.3.**

While this pathway probably represents the only signal transduction pathway mediating CGRP endothelium-independent vasorelaxation in the pig coronary artery, it is possible that ATP-sensitive potassium channels mediate some of this action of CGRP in other vessels. This remains to be investigated.

While the studies presented in this thesis have attempted to separate the endothelium-dependent from the endothelium-independent vasorelaxant effects of CGRP, these mechanisms coexist *in vivo* in some vascular beds. Interactions between these two mechanism of vasorelaxations may be important physiologically and merits further investigation.

APPENDIX 1 DRUGS

The drugs used in this study were obtained from the following sources and dissolved in the solvent shown.

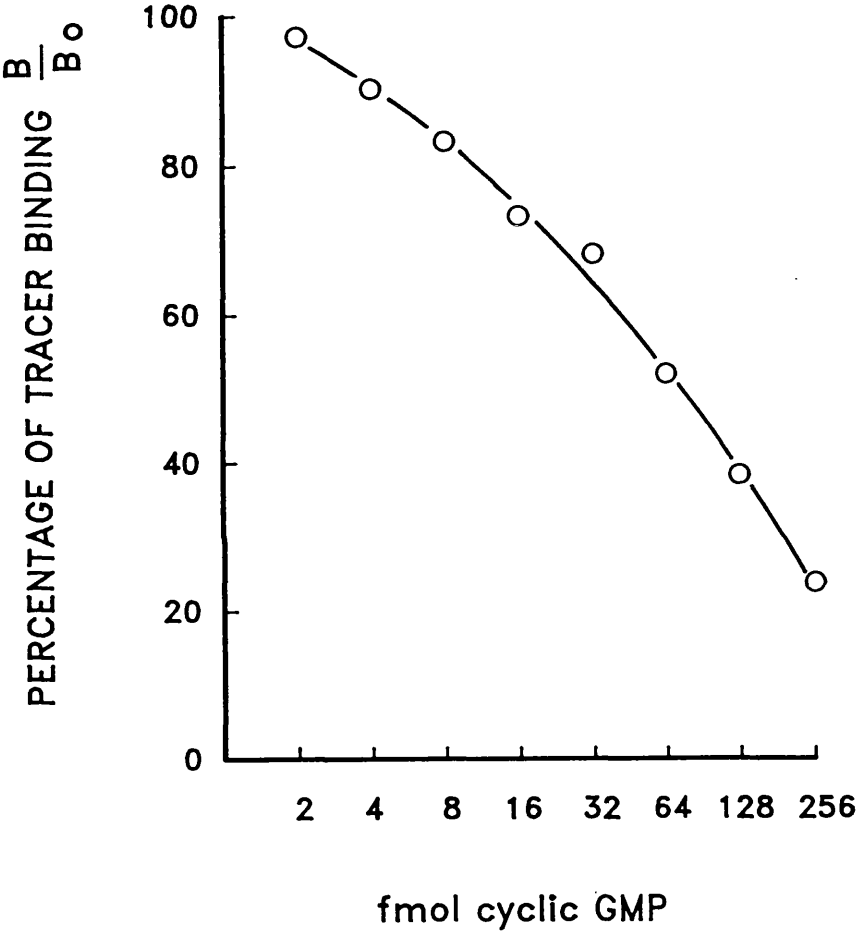
COMPOUND	SOLVENT	SOURCE
Acetylcholine chloride	DDW	Sigma
D-arginine (freebase)	DDW	Sigma
L-arginine (freebase)	DDW	Sigma
Bradykinin acetate	DDW	Sigma
U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$)	EtOH (100%)	Sigma
Human α-CGRP	DDW	Bachem
Human α-$CGRP_{(8-37)}$	DDW	Celltech
Forskolin	EtOH (70%)	Sigma
Glibenclamide	DMSO (100%)	Hoechst
Ibuprofen	DMSO (100%)	Sigma
ICI 118551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol)	DDW	ICI
Isoprenaline hemisulphate	DDW	Sigma
Methylene blue	DDW	Sigma
L-N^G-monomethylarginine (L-NMMA)	DDW	Wellcome
L-N^G-nitroarginine (L-NOARG)	1M HCl	Sigma
Noradenaline bitartrate	DDW	Sigma
Ouabain octahydrate	DDW	Sigma
Prostaglandin I_2 (Prostacyclin)	EtOH (100%)	Sigma
Propranolol	DDW	ICI
Salbutamol	DDW	Sigma
Sodium nitroprusside	DDW	Sigma
Superoxide dismutase	DDW	Sigma

DDW is distilled deionised water, EtOH is ethanol, HCl is hydrochloric acid and DMSO is dimethyl sulphoxide

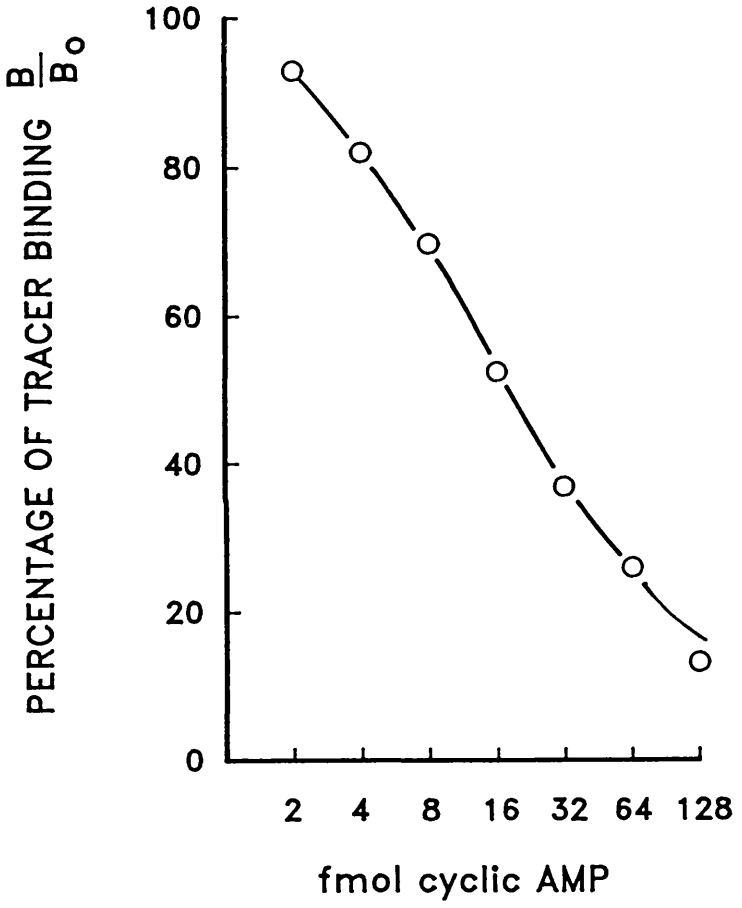
APPENDIX 2 SCINTILLATION PROXIMITY ASSAY

Below are presented data and calculations for the standard curve derived from an experiment involving the cyclic GMP scintillation proximity assay kit (Amersham) using the acetylation protocol. **Graph 1** shows the standard curve for the cyclic GMP acetylation protocol while **Graph 2** shows the standard curve for the cyclic AMP acetylation protocol. **Graph 3** shows the standard curve for the cyclic AMP non-acetylation protocol.

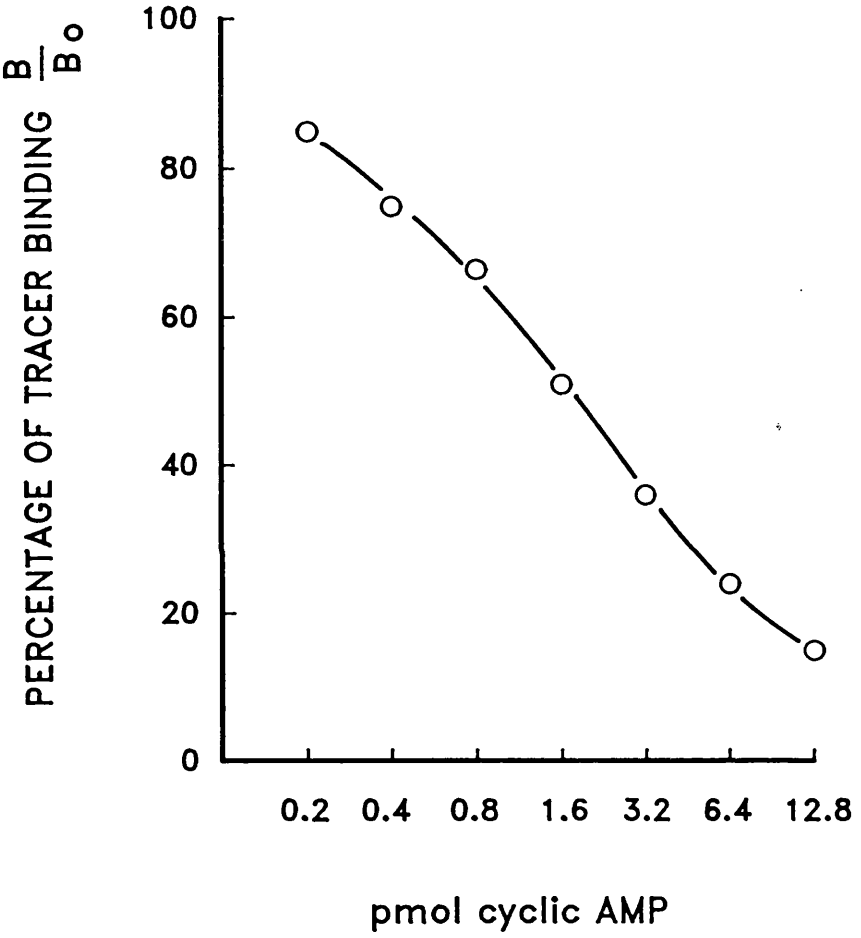
	MEAN COUNTS PER MINUTE (CPM)	CPM - NSB (B^*)	B^*/B_o x 100
NON SPECIFIC BINDING (NSB)	171.0	-	-
B_o	2229.5	2058.5	-
2 fmol cyclic GMP	2173.3	2002.3	97.3
4 fmol cyclic GMP	2032.0	1861.0	90.4
8 fmol cyclic GMP	1888.5	1715.5	83.4
16 fmol cyclic GMP	1681.5	1510.5	73.4
32 fmol cyclic GMP	1577.8	1406.8	68.3
64 fmol cyclic GMP	1243.5	1072.5	52.1
128 fmol cyclic GMP	961.3	790.3	38.4
256 fmol cyclic GMP	663.8	492.8	23.9



Graph 1 Graph showing the standard curve for the cyclic GMP acetylation protocol achieved using scintillation proximity assay.

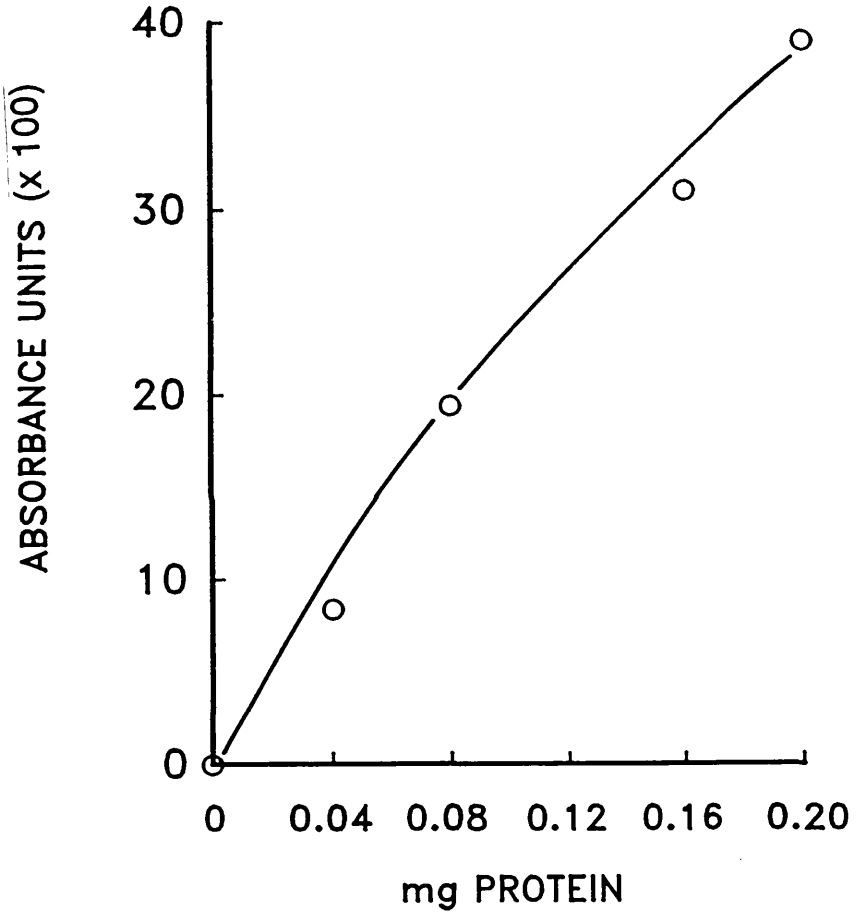


Graph 2 Graph showing the standard curve for the cyclic AMP acetylation protocol achieved using scintillation proximity assay.



Graph 3 Graph showing the standard curve for the cyclic AMP non-acetylation protocol achieved using scintillation proximity assay.

APPENDIX 3 PROTEIN DETERMINATION



Graph 4 Graph showing the standard curve for protein determination using the protocol of Lowry *et al.*, 1951 with bovine serum albumin as control.

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