FUNCTIONAL EVIDENCE FOR ATYPICAL β-ADRENOCEPTORS IN RAT ISOLATED ARTERIES

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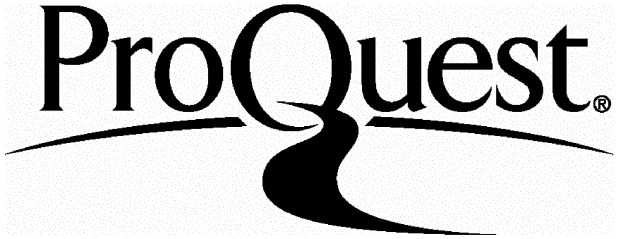

Abstract

Since the original classification by Lands *et al.* (1967a), it has become evident that not all β-adrenoceptor mediated responses can be classified as either β_1 or β_2, with the existence of an additional β-adrenoceptor subtype, referred to as the atypical or β_3-adrenoceptor. This β-adrenoceptor subtype has been identified in adipose and gastrointestinal tissue, as well as skeletal muscle and airway smooth muscle. The work presented in this thesis demonstrates the presence of atypical β-adrenoceptors in rat vasculature.

The present *in vitro* results show that relaxations to isoprenaline in the rat thoracic aorta, mesenteric and pulmonary artery, are antagonized by propranolol in a non-competitive manner. In the thoracic aorta and mesenteric artery, relaxant responses to isoprenaline comprised a propranolol-sensitive (β_2-adrenoceptor mediated) and -insensitive component. In the pulmonary artery, propranolol produced surmountable antagonism of isoprenaline-induced relaxations, but the antagonism did not satisfy the criteria for competitive antagonism. The β_3-adrenoceptor agonists, ZD 7114 and BRL 37344, relaxed all three tissues and in addition, CGP 12177 and alprenolol were agonists, adding support to the hypothesis that atypical β-adrenoceptors are present in the vasculature.

Although this β-adrenoceptor in rat vasculature has certain features in common with the β_3-adrenoceptor in adipose and gut tissues, they do not appear to be identical. For example, the potency of BRL 37344 appears to be particularly low in rat vasculature compared with its relaxation of several gastrointestinal preparations, where it is one of the most potent β_3-adrenoceptor agonists. Also, the β_1-/β_2-adrenoceptor antagonist, alprenolol which has been shown to be an antagonist at the β_3-adrenoceptor in various preparations did not antagonize responses to isoprenaline (carried out in the presence of 10^{-6}M propranolol) or ZD 7114. Furthermore, β_3-adrenoceptor mRNA, which is consistently found in adipose and gut tissues, was not detected in rat vasculature, independently of adipose tissue.

In summary, a third β-adrenoceptor population, referred to as the atypical β-adrenoceptor, exists in rat vasculature and this receptor has certain features which differ from the β_3-adrenoceptor.

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For my parents

Publications

1. Sooch, S. & Marshall, I. (1994). Isoprenaline relaxes rat isolated mesenteric artery rings via endothelium-dependent and -independent mechanisms. *Br. J. Pharmacol.*, **112**, 209P.

2. Sooch, S. & Marshall, I. (1995). An atypical β-adrenoceptor mediates relaxation of the rat isolated mesenteric artery. *Br. J. Pharmacol.*, **114**, 22P.

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Abbreviations

ACH	acetylcholine
APAT	aryloxypropanolaminotetralin
ATP	adenosine 5'-triphosphate
β_3-AR	β_3-adrenoceptor
bp	base pair
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
cyclic AMP	adenosine 3':5'-cyclic monophosphate
cyclic GMP	guanosine 3':5'-cyclic monophosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EFS	electrical field stimulation
hprt	hypoxanthine phosphoribosyltransferase
Iso	isoprenaline
K_D	dissociation binding constant
L-NAME	N^G-nitro-L-arginine methylester
L-NOARG	N^G-nitro-L-arginine
L-NMMA	N^G-monomethyl-L-arginine
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
NANC	non-adrenergic non-cholinergic
NO	nitric oxide
PEAT	phenylethanolaminotetraline
PCR	polymerase chain reaction
PE	phenylephrine
RNA	ribonucleic acid
RT	reverse transcription
s.e.mean	standard error of the mean
SPA	scintillation proximity assay
u	unit
UCP	uncoupling protein
UV	ultraviolet
WAT	white adipose tissue

Chapter 1

Introduction

1.1 The adrenoceptors

The endogenous catecholamines, adrenaline and noradrenaline mediate their physiological and pharmacological effects by interacting with adrenoceptors. Adrenoceptors are found in nearly all peripheral tissues and on many neuronal populations within the central nervous system. These receptors belong to the superfamily of receptors that contain seven transmembrane helices, are coupled to guanine nucleotide regulatory proteins (G-proteins) and are involved in the transmission of signals across cell membranes. Adrenoceptors mediate a variety of functions and have been of major interest for many years as targets for drug action. Control of blood pressure, myocardial contractile force and rate, airway reactivity and a variety of metabolic functions are in part regulated by catecholamines.

1.2 History

In 1948, Ahlquist proposed the existence of two adrenoceptor subtypes, based on different rank orders of potency of a series of structurally related natural and synthetic agonists, when responses to these agonists were measured in different tissues. Ahlquist designated these two subtypes of adrenoceptors as α and β. This was followed by a further subdivision of β-adrenoceptors into β_1- and β_2-adrenoceptors by Lands and co-workers (1967a). Lands *et al.* (1967a) found that the relative potencies of 15 catecholamine agonists correlated well for stimulation of heart rate, stimulation of white adipocyte lipolysis and relaxation of rabbit jejunum. Good correlations of relative potency for these same compounds were also obtained for responses in guinea pig airways, rat uterus and diaphragm, and for vasodepression in the dog, but cross correlations of agonist potency between the two sets were poor. The receptor mediating responses in the heart was designated β_1, and the receptor responsible for relaxation of vascular, uterine and airway smooth muscle was labelled β_2.

Subsequent studies with selective antagonists confirmed the existence of two β-adrenoceptor subtypes (Daly, 1981; Ariens, 1981). This subdivision was further supported by later receptor binding studies in which functionally selective agonists or antagonists displayed two affinities for the displacement of non-selective radiolabelled ligands from their membrane binding site (Minneman *et al.,* 1981; Raper, 1987).

1.3 Further heterogeneity of β-adrenoceptors

During the past few years, studies have suggested that not all β-adrenoceptor mediated responses can be classified as either β_1 or β_2, with the existence of an additional β-adrenoceptor subtype, referred to as the atypical or β_3-adrenoceptor (see review, Bylund *et al.,* 1994). A common feature of atypical β-adrenoceptors is their resistance to blockade by propranolol and other β-adrenoceptor antagonists. Furthermore, several novel agonists producing selective stimulation of the β_3-adrenoceptor have been identified, including BRL 37344 (Arch *et al.,* 1984), ZD 7114 (Holloway *et al.,* 1991), SR 58611A (Croci *et al.,* 1988) and CL 316243 (Bloom *et al.,* 1992).

1.4 β_3-adrenoceptors in adipose tissue

1.4.1 Functional studies

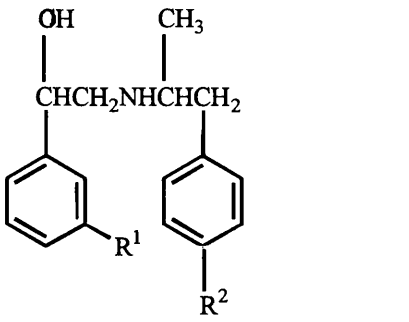
The classification by Lands *et al.* (1967a) of the rat white adipocyte β-adrenoceptor as a β_1 subtype was questioned by Harms *et al.* (1974) and De Vente *et al.* (1980). They proposed that the receptor mediating lipolysis was a hybrid or atypical β-adrenoceptor which had characteristics of both the β_1- and β_2-subtypes and had low affinity for β-adrenoceptor antagonists. Also, fat cell β-adrenoceptors showed lower stereoselectivity ratios for antagonist enantiomers compared with 'typical' β_1-(cardiac) and β_2-adrenoceptors (skeletal muscle; Harms *et al.,* 1977). This proposal was supported by Tan & Curtis-Prior (1983) who suggested that the receptor should be termed 'beta-hybrid' or 'β_3-adrenoceptor'. In addition, the introduction of a series

of novel β-adrenoceptor agonists provided important support for the concept of an atypical β-adrenoceptor mediating lipolysis.

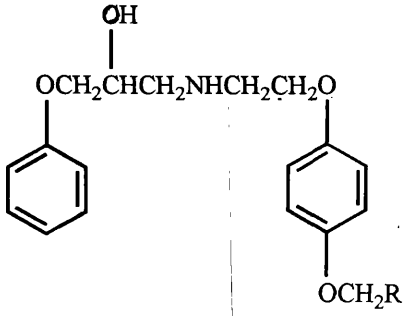
In 1984, Wilson *et al.* reported on the activities of three novel arylethanolamine β-adrenoceptor agonists, BRL 28410, BRL 35113 and BRL 35135 (see **Figure 1.1** for chemical structures), which were found to be more potent at stimulating lipolysis than conventionally used agonists and had a rank order of potency which did not correlate with either a β_1- or β_2-adrenoceptor classification. Wilson *et al.* (1984) also pointed out that the hybrid β_1-/β_2-adrenoceptor proposed by Harms *et al.* (1974) and De Vente *et al.* (1980) adequately explained the results obtained by these authors but that the model did not readily explain their own results. For example, it is difficult to explain a lipolytic receptor having both β_1- and β_2-adrenoceptor characteristics, when BRL 35113 is a poor agonist at β_1- and β_2-adrenoceptors yet is a potent agonist at the lipolytic β-adrenoceptor.

Concurrent studies by Arch *et al.* (1984) showed that BRL 28410, BRL 35113 and BRL 37344 (the active metabolite of BRL 35135) stimulated rat brown as well as white adipocyte lipolysis selectively relative to stimulation of rat atrial rate (β_1-adrenoceptor mediated) and relaxation of guinea pig tracheal tension (β_2-adrenoceptor mediated). These results were supported by Stock & Sudera (1988) who measured rat adipocyte respiration rather than lipolysis and demonstrated that propranolol, atenolol (β_1-selective) and ICI 118551 (β_2-selective) have low pA_2 values against both isoprenaline and BRL 37344.

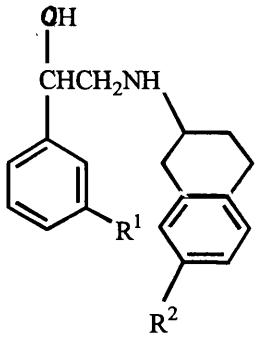
The atypical β-adrenoceptor of brown adipocytes is not confined to the rat. The results of Mohell *et al.* (1983) indicate that the hamster adipocyte receptor is atypical and Jones *et al.* (1989) obtained pA_2 values of 6.8 for antagonism of isoprenaline-stimulated rabbit brown adipocyte respiration and lipolysis by propranolol.



	R^1	R^2
BRL 26830	H	CO_2CH_3
BRL 28410	H	CO_2H
BRL 35135	Cl	$OCH_2CO_2CH_3$
BRL 37344	Cl	OCH_2CO_2H
BRL 35113	CF_3	CO_2H



	R
ZD 7114	$CONHCH_2CH_2OCH_3$



	R^1	R^2
SR 58306A	H	OH
SR 58375A	H	OH
SR 58339A	Cl	OH
SR 58611A	Cl	$OCH_2COOC_2H_5$

Figure 1.1 Chemical structures of selective β_3-adrenoceptor agonists.

Hollenga & Zaagsma (1989) investigated the effects of CGP 20712A (β_1-selective) and ICI 118551 on BRL 37344- and isoprenaline-induced lipolysis in rat white adipocytes. They reported that BRL 37344 acts solely through atypical β-adrenoceptors, whilst the main effect of isoprenaline on lipolysis is attributable to the activation of atypical β-adrenoceptors with β_1-adrenoceptors having a small, minor role. Hollenga *et al.* (1991) subsequently obtained similar results by measuring adenylate cyclase activation as the functional response, also in rat white adipocytes.

1.4.2 Receptor binding studies

Receptor binding techniques have, for the most part, failed to detect atypical β-adrenoceptors in brown adipocytes. Some groups have suggested that the brown adipocyte receptor is of the β_1 subtype (Bukowiecki *et al.,* 1978; Senault *et al.,* 1984), although one group (Baresi *et al.,* 1986) has argued for the β_2 subtype. However, two studies showed the presence of both β_1- and β_2-adrenoceptors in ratios varying from 59:41 (Rothwell *et al.,* 1985) to 80:20 (Levin & Sullivan, 1986) in rat brown adipose tissue. Arch (1989) pointed out the problems associated with such binding studies: firstly, these receptors studied by binding methods may not be the ones that mediate the functional response, and secondly, the radioligands would not have bound, at the concentrations used, to a significant proportion of atypical β-adrenoceptors.

The introduction of selective β_3-adrenoceptor agonists led to further attempts to identify atypical β-adrenoceptors in rat brown adipocytes. Binding studies using the β-adrenoceptor radioligand [^{3}H]-CGP 12177 (Levin & Sullivan, 1986; Mohell & Nedergaard, 1989), failed to detect binding of BRL compounds to atypical β-adrenoceptors, presumably because the concentration of [^{3}H]-CGP 12177 used (i.e., 1nM) was suitable for labelling β_1- and β_2-adrenoceptors, rather than β_3-adrenoceptors. However, further studies using higher concentrations of [^{3}H]-CGP 12177 revealed the presence of an atypical binding site in rat brown adipocytes (Muzzin *et al.,* 1992). They found that [^{3}H]-CGP 12177 bound to a low affinity site (pK_B 7.5), which constituted 70% of total binding. More recently, D'Allaire *et al* (1995) confirmed the presence of low affinity (-)-[^{3}H]-CGP 12177 binding sites

(corresponding to β_3-adrenoceptors) together with a smaller population of high affinity sites representing β_1-adrenoceptors in rat brown adipose tissue.

Receptor binding techniques have also been used to investigate rat white adipocyte subtypes. While functional studies on rat white adipocytes revealed a major role for the atypical β-adrenoceptor, ligand binding studies failed to detect atypical β-adrenoceptors and identified mainly β_1-adrenoceptors with a small population (15-40%) of β_2-adrenoceptors (Bojanic & Nahorski, 1983; Lacasa *et al.,* 1985). Binding experiments using CGP 20712A and ICI 118551, to displace up to four different radioligands in adipocyte membranes, confirmed the identity of mainly β_1-adrenoceptor binding sites, CGP 20712A having considerably higher affinities (K_D 0.5-3.4nM) than ICI 118551 (K_D 0.5-3.4nM; Bahouth & Malbon, 1988). However, this difference was not seen in parallel functional experiments, with CGP 20712A and ICI 118551 exhibiting IC_{50} values for inhibition of adrenaline-stimulated cyclic AMP accumulation and lipolysis at micromolar concentrations, far greater than their K_D values at β_1- and β_2-adrenoceptors (Bahouth & Malbon, 1988). Langin *et al.* (1991) using the radioligand [^{125}I]-cyanopindolol ([^{125}I]-CYP), also failed to identify atypical β-adrenoceptors in rat white adipose tissue, even after the concentration of [^{125}I]-CYP was increased from 50pM (normally used to detect β_1- and β_2-adrenoceptors) to 4nM. However, a predominant β_3-adrenoceptor population has been detected in adipocytes differentiated from mouse 3T3 F442A cells using [^{125}I]-ICYP and (-)-[^{3}H]-CGP 12177 (Feve *et al.,* 1991).

1.4.3 Molecular biology studies

An important contribution to the debate about β-adrenoceptor subtypes was made by the isolation of a human gene that encodes for a receptor protein having 51% and 46% homology with the β_1- and β_2-adrenoceptors, respectively, and referred to as the β_3-adrenoceptor gene (Emorine *et al.,* 1989). Detailed pharmacological evaluation of the β_3-adrenoceptor has mainly been achieved by investigations with stably transfected Chinese hamster ovary (CHO) cells that express a high copy number of one β-adrenoceptor subtype (Tate *et al.,* 1991). CHO cells transfected with the β_3-

adrenoceptor gene (CHO-β_3) were analyzed by adenylate cyclase activation and ligand binding with the β-adrenoceptor ligand [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) and compared with similarly prepared CHO-β_1 and CHO-β_2 cells. These studies showed that the human β_3-adrenoceptor when expressed in CHO cells is related to the atypical β-adrenoceptor characterized in rodent adipose tissue. Also, classical β-adrenoceptor antagonists displayed very low affinity for the β_3-adrenoceptor expressed in CHO cells. However, differences between the cloned human β_3-adrenoceptor and the rat atypical β-adrenoceptor suggest there may be species differences between the two β-adrenoceptors. For example, the potency and intrinsic activity of BRL 37344 in the cloned human β_3-adrenoceptor appears to correspond more closely to that of the rat adipocyte than that of the human adipocyte (Zaagsma & Nahorski, 1990).

Recently, the genes encoding for a β_3-adrenoceptor were isolated in mouse (Nahmias et al., 1991) and rat (Granneman *et al.*, 1991; Muzzin *et al.*, 1991). Experiments with the cloned rat β_3-adrenoceptor suggest that its pharmacological properties are similar to those of the atypical β-adrenoceptor in rat brown fat, but differs in several respects from those reported for the cloned human β_3-adrenoceptor (Emorine *et al.*, 1989; Emorine *et al.*, 1992; Tate *et al.*, 1991).

1.4.4 Terminology

There has been a tendency to use the term β_3-adrenoceptor for cloned receptors and in situations, e.g., lipolysis, where the response is thought to be mediated by these receptors, whereas functional responses not mediated by β_1- and/or β_2-adrenoceptors have been referred to as 'atypical'. It is now generally accepted that the β-adrenoceptor in rat adipose tissue is of an atypical (i.e., non-β_1, non-β_2) nature and is referred to as the 'β_3-adrenoceptor'. The term 'β_3-adrenoceptor' was initially used by Tan & Curtis-Prior (1983) to describe the rat adipocyte β-adrenoceptor. In addition, Emorine *et al.* (1989) has used the term β_3-adrenoceptor to describe an atypical β-adrenoceptor expressed by a clone from a human genomic library, whilst

McLaughlin & MacDonald (1990) have used it to describe β-adrenoceptors in rat colon and rabbit jejunum. However, some authors have been more cautious, preferring to use the term 'atypical' (Norman & Leathard, 1990; Blue *et al.,* 1990) or referring to a 'third type' of β-adrenoceptor (Kaumann, 1989). However, Arch (1989) has suggested that atypical β-adrenoceptors in a number of tissues are sufficiently similar, in terms of pharmacological properties, to be described as β_3-adrenoceptors. In this thesis the original authors' nomenclature of 'atypical' or 'β_3-adrenoceptor' is used.

Although atypical β-adrenoceptors in various tissues have a number of features in common, differences are also apparent. Therefore, the term atypical β-adrenoceptor may be more appropriate in such situations until these receptors have been characterized further, and it is possible that some further subdivision may prove necessary in the future.

1.5 Tissue distribution of atypical β-adrenoceptors

There is now increasing evidence that atypical or β_3-adrenoceptors are present in other sites apart from adipose tissue. They have been shown to exist in a number of gastrointestinal preparations, e.g., guinea pig ileum (Bond & Clarke, 1987; 1988), rabbit jejunum (Norman & Leathard, 1990), rat colon (Bianchetti & Manara, 1990; McLaughlin & MacDonald, 1990), as well as skeletal muscle (Challis *et al.,* 1988) and heart and blood vessels (Kaumann, 1989; Oriowo, 1994; Tavernier *et al.,* 1992). However, the main focus of this introduction will be evidence supporting the existence of atypical β-adrenoceptors in smooth muscle.

1.5.1. Gut β-adrenoceptors

(a) Guinea pig

(i) Ileum

In addition to adipose tissue, functional studies in gastrointestinal tissues such as guinea pig ileum have revealed that a component of the response to isoprenaline is resistant to blockade with propranolol. Bond & Clarke (1987) demonstrated that the

isoprenaline-induced inhibitory response in electrically-stimulated or histamine-contracted guinea pig ileum comprised a propranolol-sensitive (β_1- and/or β_2-adrenoceptor mediated) and -insensitive response. Subsequently, BRL 37344 was shown to be 2.5-fold more potent than isoprenaline in eliciting these propranolol-resistant responses, indicating that they are mediated by an atypical β-adrenoceptor (Bond & Clarke, 1988; **Table 1.1**). In addition to isoprenaline and BRL 37344, ZD 7114 (**Figure 1.1**) has also been shown to produce maximal relaxation of guinea pig ileum, these responses being poorly antagonized by propranolol (Growcott *et al.*, 1993a; **Table 1.1**).

Blue *et al.* (1990) have further characterized the atypical β-adrenoceptor in guinea pig ileum by showing that while isoprenaline responses were resistant to antagonism by propranolol, they were competitively antagonized by (-)-alprenolol and (-)-dihydroalprenolol with pA_2 values of 6.47 and 6.43, respectively. These values are lower than those, i.e., 8.2 and 8.81, respectively, for antagonism at β_1-adrenoceptors. (-)-Alprenolol also exerted agonist activity at the atypical β-adrenoceptor in guinea pig ileum (Blue *et al.*, 1990).

(ii) Gastric fundus

Guinea pig gastric fundus has been studied in less detail than guinea pig ileum, but evidence suggests that it possesses atypical β-adrenoceptors. BRL 35135 was almost as potent as isoprenaline in inhibiting $PGF_{2\alpha}$-induced contractions and 10^{-5}M propranolol produced shifts of only 33- and 5-fold in the concentration-response curves to isoprenaline and BRL 35135, respectively (Coleman *et al.*, 1987) giving pK_B values of 6.5 and 5.6. Low pA_2 values were also observed for alprenolol for antagonism of responses to isoprenaline (pA_2 6.0) and BRL 35135 (pA_2 6.8; Nials *et al.*, 1992). Also, the shift of the salbutamol concentration-response curve was similar to that of BRL 35135, suggesting that the two agonists act via the same receptor populations (Coleman *et al.*, 1987).

Table 1.1 Rank order of potency of β-adrenoceptor agonists for relaxation of gastrointestinal tissues

In parentheses pEC_{50} (-log concentration which produces 50% of the maximum response) or pEC_{30}* (-log concentration which produces 30% of the maximum response; Ad, adrenaline; Iso, isoprenaline; NA, noradrenaline.

Animal models	*Results*	*References*
Guinea pig		
Ileum	(-)-Iso (7.7) ≥ BRL 37344 (7.6) > (-)-NA (7.2) > Ad (6.6) > Fenoterol (6.4) BRL 37344 (7.3) > (-)-Iso (6.7) = ZD 7114 (6.7) > (-)-NA (6.4)	Bond & Clarke, 1988* Growcott *et al.*, 1993a*
Gastric fundus	Iso (7.3) ≥ BRL 35135 (7.2) > NA (6.7) > Salbutamol (5.7)	Coleman *et al.*, 1987
Rat		
Gastric fundus	(-)-Iso (7.7) > (-)-NA (6.7) > BRL 37344 (5.2)	McLaughlin & MacDonald, 1991
Jejunum	(-)-Iso (6.9) > BRL 37344 (6.7) > (-)-NA (5.9) > (-)-Ad (5.5)	MacDonald *et al.*, 1994
Distal colon	(-)-Iso (7.7) > BRL 37344 (7.3) > (-)-NA (6.3) Iso (7.3) ≥ BRL 37344 (7.2) > Denopamine (4.8) > Salbutamol (4.1)	McLaughlin & MacDonald, 1990 Kirkham & Kelly, 1992
Proximal colon	(-)-Iso (9.3) > SR 58611A (8.5) > (-)-NA (8.0) ≥ (-)-Ad (7.8) > Ritodrine (7.2) > Salbutamol (6.5)	Bianchetti & Manara, 1990
Oesophagus	BRL 37344 (8.3) > (-)-Iso (7.5) > Fenoterol (7)	de Boer *et al.*, 1993
Ileum	BRL 37344 (7.3) > (-)-Iso (6.3) > (-)-NA (5.6)	Growcott *et al.*, 1993b
Rabbit		
Jejunum	NA (7.4) > Ritodrine (6.2) > Salbutamol (5.3)	Norman & Leathard, 1990
Human		
Colon	Iso (7.6) > NA (7.1) ≥ Ad (6.9) Iso (6.4) ≥ Ad (6.2) > NA (5.9) > Salbutamol (>4.5) = BRL 37344 (lack of effect) Iso (6.5) > CGP 12177A (6.1) > BRL 37344 (lack of effect)	McLaughlin *et al.*, 1988 McLaughlin *et al.*, 1991 De Ponti *et al.*, 1996

(b) Rat

(i) Distal colon, gastric fundus and jejunum

β-Adrenoceptors have been characterized in rat distal colon (McLaughlin & MacDonald, 1990), gastric fundus (McLaughlin & MacDonald, 1991) and jejunum (MacDonald *et al.,* 1994; **Table 1.1**). In all three tissues, propranolol was weakly effective against isoprenaline and particularly BRL 37344. In the colon, the Schild plot slope against isoprenaline was only 0.44, therefore consistent with the presence of more than one population of β-adrenoceptors. In the presence of an α-adrenoceptor antagonist and 10^{-6}M propranolol to block α-adrenoceptors and β_1- and β_2-adrenoceptors, BRL 37344 was more potent than isoprenaline in the jejunum (MacDonald *et al.,* 1994), almost as effective in the colon (McLaughlin & MacDonald, 1990), but tenfold less effective in gastric fundus (McLaughlin & MacDonald, 1991). The relatively low potency of BRL 37344 in the rat gastric fundus compared with other tissues may be due to differences in coupling efficiency or may indicate receptor heterogeneity.

In common with other groups (Kirkham & Kelly, 1992; Blue *et al.,* 1989), MacDonald and McLaughlin (McLaughlin & MacDonald, 1990; 1991; MacDonald *et al.,* 1994) have found that cyanopindolol (CYP) is a moderately potent antagonist of the rat gut atypical β-adrenoceptor (pA_2 6.7-7.3). Significantly lower pA_2 values were obtained against BRL 37344 than against isoprenaline in both the colon and gastric fundus. In addition, the β_3-adrenoceptor agonist ZD 7114 was found to have no agonist activity in this preparation and antagonized responses to isoprenaline (pA_2 7.29; MacDonald & Lamont, 1993).

McLaughlin and MacDonald have also reported tachyphylaxis to BRL 37344 in both the rat colon (McLaughlin & MacDonald, 1990) and rat gastric fundus (McLaughlin & MacDonald, 1991). Other agonists did not induce tachyphylaxis, but exposure to BRL 37344 between concentration-response curves reduced responses to isoprenaline and noradrenaline. Tachyphylaxis has also been reported for BRL 35135 in guinea pig gastric fundus (Coleman *et al.,* 1987). The significance of the tachyphylaxis is unclear.

(ii) Proximal colon

The phenylethanolaminotetraline (PEAT) β_3-adrenoceptor agonists designed by a group of the Sanofi company have also been used to identify the atypical nature of the β-adrenoceptor in the rat colon. Compounds such as SR 58306A, SR 58339A, SR 58375A and SR 58611A (**Figure 1.1**), were at least 20-fold more potent in relaxing the rat proximal colon spontaneous contractions than relaxation of guinea pig trachea (β_2-adrenoceptor mediated) and stimulation of guinea pig right atrial rate (β_1-adrenoceptor mediated; Croci *et al.,* 1988; Bianchetti & Manara, 1990; **Table 1.1**). In these studies, alprenolol and propranolol gave pA_2 values of around 7.5 and 6.5, respectively, against the PEAT agonists. Alprenolol also had a low pA_2 value against responses to isoprenaline and ritodrine but this value was still a log unit higher than that in guinea pig ileum, leading to the suggestion that the receptors in the two tissues differ (Bianchetti & Manara, 1990). Also, CGP 20712A and ICI 118551, either alone or in combination, did not antagonize rat colon motility inhibition by the representative PEAT SR 58611A.

The selectivity of SR 58375A and two other PEAT agonists, SR 58572A and SR 58539B, relative to isoprenaline, ritodrine and salbutamol was also demonstrated *in vivo* in the rat by comparing doses that inhibited colonic motility, increased heart rate (β_1-adrenoceptor mediated) and lowered blood pressure (β_2-adrenoceptor mediated; Giudice *et al.,* 1989).

(iii) Oesophagus

Studies of oesophageal muscularis mucosae in rat have shown that it also contains an atypical β-adrenoceptor (Ford *et al.,* 1992). Low pA_2 values were obtained with a range of antagonists and the tissue relaxed to BRL 37344. The high sensitivity of rat oesophagus smooth muscle to BRL 37344 was later confirmed by de Boer *et al.* (1993; **Table 1.1**). They also demonstrated using CGP 20712A and ICI 118551, that BRL 37344-induced relaxation of rat oesophagus smooth muscle is mediated solely through β_3-adrenoceptors, whilst isoprenaline-induced relaxations are mediated predominantly via β_3-adrenoceptors, with a small β_2-adrenoceptor component. More recent studies by the same group have shown that (-)-noradrenaline,

like BRL 37344, behaves as a full agonist acting entirely through β_3-adrenoceptors (de Boer *et al.*, 1995).

(iv) Ileum

Evidence for an atypical β-adrenoceptor in rat ileum has been reported by Growcott *et al.* (1993b; **Table 1.1**). BRL 37344 was a partial agonist in this tissue being nine times more potent than isoprenaline in relaxing carbachol ($5x10^{-7}M$) contracted rat ileum, but producing a maximum response (i.e. 71±5%) which was significantly lower than that obtained with isoprenaline. Propranolol weakly antagonized responses to isoprenaline and BRL 37344 giving pK_B values of 5.7 and 5.5, respectively. Alprenolol also had a low pA_2 value against responses to isoprenaline (6.5) and a similar pK_B value was obtained when BRL 37344 was used as the agonist (6.4). Although ZD 7114 was a full agonist in the guinea pig ileum (Growcott *et al.*, 1993a), the relaxant effects of isoprenaline and BRL 37344 were antagonized by ZD 7114 yielding pA_2 and pK_B values of 6.3 and 6.7, respectively in the rat ileum. The authors' suggested that these differences could be explained by the guinea pig and rat tissues containing different levels of β_3-adrenoceptor reserves, such that in the rat ileum, the β_3-adrenoceptor reserve is sufficiently low to allow ZD 7114 to act as a β_3-adrenoceptor antagonist and for BRL 37344 to act as a partial agonist. However, more recent studies have shown that BRL 37344 is a full agonist in rat ileum (Hoey *et al.*, 1996), although they did find that ZD 7114 acted as an antagonist against BRL 37344 (pK_B 7.3).

Binding studies have identified a (-)-[^{125}I]-cyanopindolol (CYP) binding site in rat ileum which is resistant to blockade by $10^{-7}M$ propranolol and is located predominantly in the mucosa and to a lesser extent in the muscularis (Roberts *et al.*, 1995). A small population of β_2-adrenoceptors were detected, located mainly in the longitudinal and circular smooth muscle layers. The (-)-[^{125}I]-CYP binding site was resistant to downregulation by isoprenaline, indicating that this site is distinct from β_1- and β_2-adrenoceptors and has binding characteristics of the atypical β-adrenoceptor.

(v) Stomach and caecum

Both SR 58611A and BRL 37344 stimulated acid secretion in rat stomach *in vitro* (Canfield & Paraskeva, 1992). The response to SR 58611A was inhibited by propranolol (10^{-5}M) but not by a combination of practolol ($1.25x10^{-5}$M) and ICI 118551 (10^{-6}M). Also, responses to SR 58611A were not antagonized by alprenolol (10^{-5}M), previously shown to have some affinity for atypical β-adrenoceptors (Blue *et al.*, 1990). In a similar study, the same group showed that stimulation of bicarbonate secretion in rat caecum *in vitro* is mediated by atypical β-adrenoceptors (Canfield & Abdul-Ghaffar, 1992). In this case, the response to SR 58611A was reduced by alprenolol ($2x10^{-5}$M) but not by propranolol ($2x10^{-5}$M), indicating that the atypical β-adrenoceptors mediating the two secretory processes may be similar, but not identical.

(c) Rabbit

(i) Jejunum

The original classification of the rabbit jejunal β-adrenoceptor as β_1-adrenoceptor (Lands *et al.*, 1967a, b) was questioned by Furchgott (1972), who compared both the relative potencies of agonists and pA_2 values for antagonists in the jejunum and a number of other tissues. Low pA_2 values for antagonism of relaxant responses to isoprenaline by propranolol, pronethanol and practolol were obtained, compared with their pA_2 values at rabbit β_1-adrenoceptor (8.8, 7.1 and 6.9, respectively). Also, low pA_2 values have been determined for antagonism of the effects of β_2-adrenoceptor agonists, ritodrine and salbutamol, by propranolol (6.4 and 6.6, respectively; Norman & Leathard, 1990; **Table 1.1**). The authors' have suggested that the inhibition of spontaneous activity in the rabbit jejunum is unlikely to be mediated by β_2-adrenoceptors because ritodrine was approximately eight times as potent as salbutamol, whereas ritodrine is less potent than ritodrine on conventional β_2-adrenoceptors in tissues such as the rat uterus or guinea pig lung strip. Therefore, in the rabbit jejunum, ritodrine and salbutamol may act via an atypical β-adrenoceptor. The Schild plot slopes of propranolol against these agonists were close to unity, whereas that against noradrenaline was only 0.28, indicating that another

receptor subtype, perhaps β_1-adrenoceptors, may co-exist with atypical β-adrenoceptors in this tissue.

(ii) Stomach fundus and colon

Furchgott (1972) also suggested that the rabbit stomach fundus possesses an atypical β-adrenoceptor, but one that is different from the jejunal atypical β-adrenoceptor. The pA_2 value obtained for practolol against isoprenaline (<3.5) was even lower in gastric fundus compared with jejunum. Rabbit colon also appears to possess atypical β-adrenoceptors, with propranolol giving a pK_B value of 6.3 against isoprenaline (Gillespie & Khoyi, 1977).

(d) Dog

(i) Colon

Low antagonist potencies have also been reported for dog colon - pA_2 values of 6.6, 5.4 and 5.2 were obtained for antagonism of isoprenaline relaxations by propranolol, sotalol and practolol (Grivegnée *et al.,* 1984). More recent studies have demonstrated that SR 58611A inhibits canine colonic motility both *in vivo* and *in vitro* (De Ponti *et al.,* 1995). The inhibitory effect of a single dose of SR 58611A (100μg kg^{-1}, i.v.) on the gastrocolonic response was reversed by alprenolol, but was resistant to CGP 20712A and ICI 118551. Similarly, responses were competitively antagonized by alprenolol (pA_2 7.1) but resistant to CGP 20712A or ICI 118551 *in vitro.*

(e) Human

(i) Colon

Little work has been conducted on the classification of the β-adrenoceptor in the human gut. McLaughlin *et al.* (1988) using circular strips of human colon exhibiting spontaneous activity, tested several agonists (**Table 1.1**). They reported a pK_B value of 7.7 for antagonism of isoprenaline responses by propranolol. This value is higher than that obtained in gut from laboratory animals (**Table 1.1**) but lower than pK_B values for human atrial β_1- (8.6-8.7) and β_2-adrenoceptor (9.1) for propranolol (Gille *et al.,* 1985). A lower pK_B value was obtained against noradrenaline (6.2), but

it was suggested that this was due to noradrenaline overcoming the effect of phentolamine (10^{-6}M) which was present to block α-adrenoceptors (McLaughlin *et al.*, 1988).

Subsequent work showed that the isoprenaline response was antagonized by betaxolol but not ICI 118551, and that BRL 37344 had no agonist activity in the human colon (McLaughlin *et al.*, 1991; **Table 1.1**). These results seemed to indicate that the human colonic β-adrenoceptor is of the β_1-adrenoceptor subtype, yet the apparent pA_2 value for betaxolol (7.7) was below that expected at β_1-adrenoceptor (pA_2 8.5), though not as low as the value in rat stomach (7.1). The lack of effect of BRL 37344 is similar to its weak partial agonist activity in human adipose tissue. One possibility is that the human gut β-adrenoceptor is atypical but differs from β_3-adrenoceptor in failing to bind to BRL 37344 or alternatively, the human colon may have a low β_3-adrenoceptor reserve such that BRL 37344 has limited efficacy.

More recent experiments have investigated the effect of a novel β_3-adrenoceptor antagonist SR 59230A, on agonist-induced relaxation of human colonic muscle strips (β_3-adrenoceptor antagonists discussed later; De Ponti *et al.*, 1996). CGP 12177A (a β_1-/β_2-adrenoceptor antagonist and β_3-adrenoceptor partial agonist) relaxed the human colon while BRL 37344 as before, had no agonist activity. The authors suggested that BRL 37344 may be an inadequate tool for investigating the human native β_3-adrenoceptor. This observation is consistent with the many differences in agonists' affinity and efficacy that have been reported in cultured cells transfected with genes coding for either human or rodent β_3-adrenoceptors (Liggett, 1992; Granneman *et al.*, 1993; Blin *et al.*, 1994), although due to the high level of receptor expression in transfected cells, results from such studies may not be directly comparable with functional assays.

1.5.2. Airways

β_2-Adrenoceptors are believed to be the predominant β-adrenoceptor in airway smooth muscle of a number of species, but β_1-adrenoceptors also play some role. For

example, neuronally released noradrenaline relaxes bovine tracheal smooth muscle mainly through β_1-adrenoceptor (Lemoine *et al.,* 1989).

Evidence for an atypical β-adrenoceptor in airway smooth muscle has been demonstrated in a detailed study which compared the potencies of (-)- and (+)-pindolol as partial agonists in guinea pig trachea (Walter *et al.,* 1984). The (-)-enantiomer was shown to produce relaxation via both high- and low-sensitivity components, whereas the (+)-enantiomer exhibited a nearly monophasic concentration-response curve. Using subtype-selective antagonists (i.e., (-)-bisoprolol and ICI 118551), the relaxant effect of (+)-pindolol and the high-sensitivity component of (-)-pindolol were attributed to β_2-adrenoceptors. The antagonist activities of both enantiomers were also mediated through β_2-adrenoceptors. However, the low-sensitivity component of the agonist effect of (-)-pindolol was poorly antagonized by both (-)-bisoprolol and ICI 118551 and also bupranolol, suggesting this component may be mediated via an atypical β-adrenoceptor.

(a) Non-adrenergic non-cholinergic

Itabashi *et al.* (1992) showed that BRL 37344 and BRL 35135 reduced non-adrenergic non-cholinergic (NANC) contractions of the guinea pig bronchus induced by electrical field stimulation (EFS) or capsaicin, and this inhibitory effect was not significantly affected by β_1-/β_2-adrenoceptor or β_1-selective antagonists. While these agonists inhibited the contractile response to EFS, they did not inhibit the contractile response to substance P, suggesting that a β_3-adrenoceptor might mediate a prejunctional inhibitory action on NANC contractions. However, since these authors did not use selective β_2-adrenoceptor agonists or antagonists, the involvement of inhibitory β_2-adrenoceptors on sensory nerve endings cannot be excluded.

In a more recent study, the effects of SR 58611A and BRL 37344 were compared with those of salbutamol on the guinea pig bronchus (Martin *et al.,* 1993). The results demonstrated that the two β_3-adrenoceptor agonists and salbutamol produced different effects in this tissue. Salbutamol inhibited both cholinergic and

NANC components of the neurally-mediated biphasic response observed following EFS, while SR 58611A and BRL 37344 produced an inhibition of the NANC response predominantly at a prejunctional level. The effects of salbutamol were inhibited by both propranolol and ICI 118551, showing that the effects of salbutamol are due to stimulation of β_2-adrenoceptors. However, the inhibitory effects of BRL 37344 were partially inhibited by propranolol or ICI 118551, whereas those of SR 58611A were unaffected by treatment with either β-adrenoceptor antagonist. The partial inhibition of the effects of the effects of BRL 37344 by the two β-adrenoceptor antagonists implies that a β_2-adrenoceptor component is involved in the action of BRL 37344.

(b) Smooth muscle

Tamaoki *et al.* (1993) showed that in dog, BRL 37344 produced relaxation of isolated bronchi precontracted with acetylcholine. These relaxations were not antagonized by ICI 118551, suggesting that β_3-adrenoceptors may be present on canine airway smooth muscle.

In a separate study, SR 58611A was found to produce a slight fall in tension in precontracted bronchi of human, guinea pig and sheep, but this decrease in tension was not significantly different from the spontaneous and weak relaxation observed with saline addition during the experiment (Martin *et al.,* 1994). These relaxations were not affected by either propranolol or ICI 118551. In contrast, BRL 37344 produced significant concentration-dependent relaxations in each species. Its relaxant effects were inhibited by both antagonists in human and guinea pig airways, whereas in the isolated sheep bronchus BRL 37344-induced relaxations were only slightly, but significantly, reduced with either propranolol or ICI 118551. Salbutamol and isoprenaline induced potent relaxations of guinea pig, human and sheep airway smooth muscle *in vitro*, which were antagonized by both propranolol and ICI 118551. The authors concluded that β_3-adrenoceptors do not mediate relaxation in these preparations, and that a β_2-adrenoceptor agonist component may be involved in the relaxant effects of BRL 37344. The differences observed between these two studies appear to be species-related, since the experimental conditions were similar in both.

(c) Epithelial cells

Evidence for an atypical β-adrenoceptor in ferret tracheal epithelium has been reported by Webber & Stock (1992). BRL 37344 was 10,000 times more potent than salbutamol, which was slightly more potent than prenalterol (β_1-selective) as a stimulant of methacholine-induced albumin secretion. The weak antagonism by ICI 118551 of the response to BRL 37344 (pK_B 5.6) was consistent with this response being mediated by an atypical β-adrenoceptor.

1.5.3 Cardiovascular system

(a) Cardiac β-adrenoceptors

The coexistence of an atypical β-adrenoceptor population, in addition to β_1- and β_2-adrenoceptors in mammalian hearts has been suggested. The proposal was based on the *in vitro* cardiostimulant effects of non-conventional partial agonists defined as β-adrenoceptor blocking agents that exhibit agonist effects at concentrations much greater than those causing blockade of β_1- and β_2-adrenoceptors (Kaumann 1973; 1989; Kaumann & Blinks, 1980). The dissociation between stimulation of inotropic as well as chronotropic effects has been observed in particular with pindolol (a β_1-/β_2-adrenoceptor antagonist) and related compounds in isolated heart tissues of rat, guinea pig and cat (Kaumann, 1989).

Pindolol has been shown to cause tachycardia in man (Man In't Veld & Schalekamp, 1981) and it was suggested that this may partly be due to the activation of a third β-adrenoceptor population (Kaumann, 1989). However, Kaumann & Lobnig (1986) failed to detect any positive inotropic effects of (-)-pindolol in human isolated atrium. This was suggested to be due to the low efficacy of (-)-pindolol for cardiac atypical β-adrenoceptors compared to other non-conventional partial agonists (Kaumann, 1989; Arch & Kaumann, 1993). More recently, Kaumann (1996) has demonstrated that the non-conventional partial agonist (-)-CGP 12177 produces positive inotropic effects in human atrial myocardium, which are resistant to blockade by (-)-propranolol, but antagonized by (-)-bupranolol, previously shown to have

antagonist activity at the β_3-adrenoceptor (Langin *et al.*, 1991; Blin *et al.*, 1994) which is consistent with the existence of atypical β-adrenoceptors in this tissue.

(b) Vasular smooth muscle

β-Adrenoceptors in vascular smooth muscles were initially classified as β_2-adrenoceptors (Lands *et al.*, 1967b). However, later studies using more selective agonists and antagonists have shown that vasodilatation may be mediated via β_1- or β_2-adrenoceptors. For example, β_2-adrenoceptors predominate in the guinea pig pulmonary artery (O'Donnell & Wanstall, 1985) and human saphoneous vein (Ikezono *et al.*, 1987), whereas in the rabbit facial vein (McPherson & Bevan, 1987), coronary and cerebral arteries (Edvinsson & Owman, 1974; Edvinsson *et al.*, 1976) β_1-adrenoceptors appear to predominate. In most other blood vessels, isoprenaline-induced vasorelaxation is mediated largely by β_2-adrenoceptors with a small β_1-adrenoceptor component (Taira *et al.*, 1977; Cohen & Wiley, 1978; O'Donnell & Wanstall, 1985). However, there is also some evidence for the existence of atypical β-adrenoceptors in vasculature.

(i) Rat

Pindolol was found to cause relaxation of rat isolated aortic rings (Doggrell, 1990). These responses were not altered by 10^{-6}M ICI 118551, suggesting that they may be mediated by atypical β-adrenoceptors. Oriowo (1994) has also reported that atypical β-adrenoceptors may mediate vasorelaxation in the rat common carotid artery. Although propranolol shifted the isoprenaline concentration-response curve to the right, the Schild plot slope was less than unity (0.74). Furthermore, relaxations to CGP 12177 and BRL 37344 were unaffected by 10^{-7}M propranolol, providing additional support for the presence of atypical β-adrenoceptors in the carotid artery.

(ii) Dog

Evidence for atypical β-adrenoceptors being present in canine vasculature comes from a study by Clark & Bertholet (1983) who observed that pindolol was a full agonist in isolated perfused mesenteric vessels. The concentration of propranolol

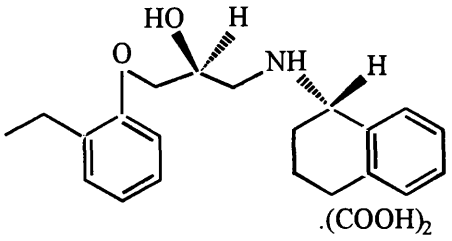
required to produce a measurable shift of the pindolol concentration-response curve (i.e., 10^{-7}M) was found to produce a much greater inhibition of isoprenaline responses. Also, β_3-adrenoceptor agonists and/or non-conventional partial agonists have been shown to have vasodilator effects in the dog *in vivo* in a number of studies (Tavernier *et al.*, 1992; Berlan *et al.*, 1994; Shen *et al.*, 1994).

1.6 β_3-Adrenoceptor antagonists

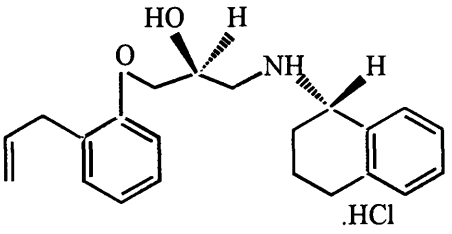
The most important problem in detecting and pharmacologically characterizing the β_3-adrenoceptor has been the absence of a selective β_3-adrenoceptor antagonist. Arch (1989) pointed out that there are a number of limitations in classifying receptors solely on the basis of rank order of potencies of agonists and that characterization of a novel receptor type requires both an agonist that acts, at least in part, via this receptor and an antagonist that has a different affinity for the novel receptor compared with known receptors.

Recently, the Sanofi group have synthesized antagonists selective for the β_3-adrenoceptor. The aryloxypropanolaminotetralins (APATs) are the first representatives of such antagonists (see **Figure 1.2** for chemical structures). *In vitro* studies have shown that the three representative APATs, SR 588944A, SR 59230A and SR 59396A concentration-dependently inhibited the inhibition of spontaneous motility in the rat colon mediated by the β_3-adrenoceptor agonist SR 58611A (pA_2 values ranging from 8.1-8.8; Manara *et al.*, 1995a; 1996). In contrast, antagonism of guinea pig tracheal relaxation by salbutamol (β_2-adrenoceptor mediated; pA_2 6.5-6.9) and the atrial chronotropic response by isoprenaline (β_1-adrenoceptor mediated; pA_2 6.7-7.3) by the three representative APATs was non-competitive in nature (i.e., Schild plot slope < 1 and/or reduction in the maximal agonist effect).

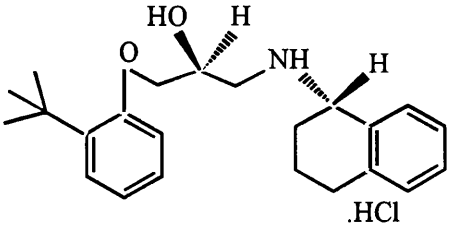
In vivo, the inhibition of colonic motility and the thermogenic response of brown adipose tissue elicited in rats by SR 58611A and BRL 37344 were reduced by SR 59230A at oral doses ($\leq$ 5mg kg^{-1}) well below those half maximally effective (ID_{50}, antagonist doses causing 50% inhibition of agonist responses) for preventing



SR 59230A



SR 58894A



SR 59396A

Figure 1.2 Chemical structures of representative aryloxypropanolaminotetralins (APATs).

β_1-(isoprenaline tachycardia $\geq$ 80mg kg^{-1}) or β_2-(salbutamol bronchodilatation, 44mg kg^{-1}) mediated responses (Manara *et al.,* 1996).

1.7 Cloned β_3-adrenoceptors

The species homologues for the human (Emorine *et al.,* 1989), mouse (Nahmias *et al.,* 1991), rat (Granneman *et al.,* 1991; Muzzin *et al.,* 1991) and also bovine (Piétri-Rouxel *et al.,* 1995) β_3-adrenoceptor have now been cloned. The human and rodent β_3-adrenoceptors were the first adrenoceptors, with the α_{1B} receptor, shown to contain introns (Granneman *et al.,* 1992; Van Spronsen *et al.,* 1993; Lelias *et al.,* 1993). The human β_3-adrenoceptor has a single intron separating the first exon from the final six residues and the polyadenylation signal which are encoded by a second exon, whereas the rodent receptors are split over three exons, the third one not coding for any amino acid residues (Granneman *et al.,* 1992; Van Spronsen *et al.,* 1993; **Figure 1.3**).

The primary protein structure deduced from the nucleotide sequence shows that β_3-adrenoceptors display features common for all adrenoceptors: a single polypeptide chain comprising amino- and carboxy-terminal regions, three intracellular and three extracellular loops, and seven conserved hydrophobic domains. The degree of amino acid sequence identity between human, mouse, rat and bovine β_3-adrenoceptors is about 80-90% (Emorine *et al.,* 1994; Piétri-Rouxel *et al.,* 1995), the homology between species being even greater in the transmembrane regions. Several residues located in these functional domains are specific to β_3-adrenoceptors and are not found in other β-adrenoceptor subtypes (Emorine *et al.,* 1991). The β_3-adrenoceptors have a short carboxy-terminal tail which lacks recognition sites for both the cyclic AMP-dependent kinase or β-adrenoceptor kinase, which are involved in desensitization of the β_2-adrenoceptor, therefore suggesting that the regulation of the β_3-adrenoceptor may differ from that of other subtypes (Strosberg, 1993).

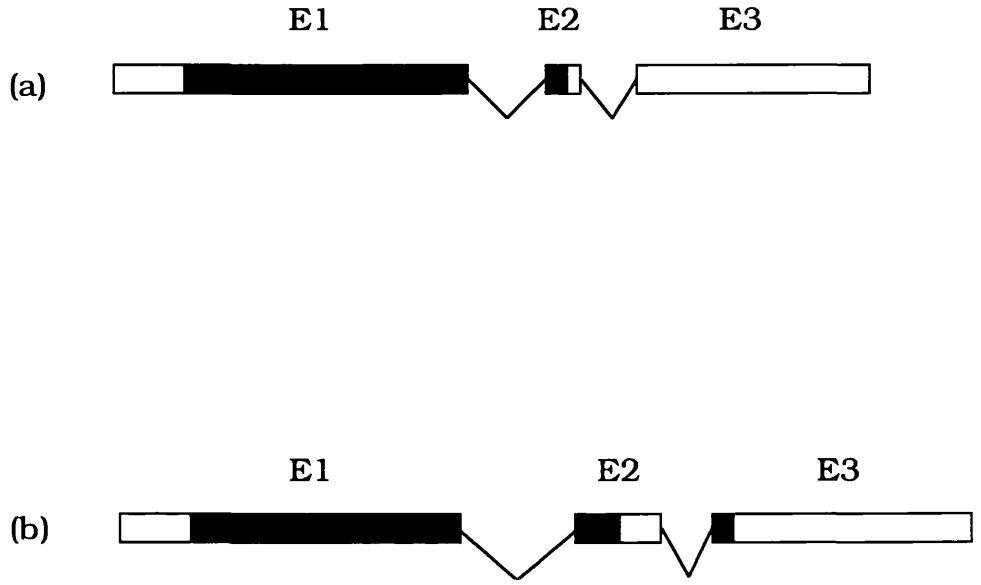


Figure 1.3 Schematic representation of (a) the rat (Granneman *et al.*, 1992) and (b) human (Lelias *et al.*, 1993) β_3-adrenoceptor genes with mRNA as blocks and the coding sequence filled (E: exon). The lines connecting the exons depict the intron sequences.

1.7.1 Molecular biology studies

Isolation of the β_3-adrenoceptor gene has enabled a means of detecting the presence of the corresponding mRNA in tissues known to possess atypical β-adrenoceptors. In all species examined, β_3-adrenoceptor mRNA is expressed in both white and brown adipose tissue (Granneman *et al.,* 1991; Nahmias *et al.,* 1991; Granneman *et al.,* 1993; Krief *et al.,* 1993). Northern analysis of RNA from mouse tissues failed to detect any expression of β_3-adrenoceptor mRNA in non-adipose tissues, including ileum and colon (Nahmias *et al.,* 1991). In the rat, mRNA for the β_3-adrenoceptor has been detected in ileum (Granneman *et al.,* 1991), colon (Bensaid *et al.,* 1993) and more recent studies have demonstrated β_3-adrenoceptor mRNA expression in the fundus (Granneman & Lahners, 1994; Cohen *et al.,* 1995), supporting functional studies for β_3-adrenoceptors in gastrointestinal tissues.

In man, expression of β_3-adrenoceptor mRNA has been demonstrated in the gallbladder, colon, ileum and heart (Krief *et al.,* 1993; Granneman *et al.,* 1993), although the presence of uncoupling protein (UCP) mRNA in both ventricles and atria suggests that the observed cardiac expression of β_3-adrenoceptor may be due to intrinsic fat deposits (Krief *et al.,* 1993). β_3-Adrenoceptor mRNA is also found in the human neuroblastoma cell line SK-N-MC (Esbenshade *et al.,* 1992). However, expression of β_3-adrenoceptor mRNA was not detected in human skeletal muscle, lung, liver, kidney, thyroid or lymphocytes by the reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Krief *et al.,* 1993).

1.8 Summary

The existence of β-adrenoceptors that differ from both the β_1- and β_2-adrenoceptors has now been demonstrated in a range of mammalian tissues by functional studies, radioligand binding and molecular biology studies. These β_3- or atypical β-adrenoceptors are characterized by a low affinity for conventional β-adrenoceptor antagonists such as propranolol, and agents such as BRL 37344 have been shown to selectively stimulate these receptors. The recent introduction of

selective antagonists for the β_3-adrenoceptor (Manara *et al.,* 1995a) will enable further characterization of this receptor.

In addition to adipose tissue, atypical β-adrenoceptors have been shown to exist in a number of gastrointestinal smooth muscle preparations, e.g. guinea pig ileum (Bond & Clarke, 1987; 1988), rat distal colon (McLaughlin & MacDonald, 1990), rat proximal colon (Croci *et al.,* 1988), rat jejunum (van der Vliet *et al.,* 1990) and rat gastric fundus (McLaughlin & MacDonald, 1991). There is also evidence for the presence of atypical β-adrenoceptors in other tissues including skeletal muscle (Challiss *et al.,* 1988), tracheal epithelium (Webber & Stock, 1992), and heart and blood vessels (Kaumann, 1989; Oriowo, 1994; Tavernier *et al.,* 1992).

The β_3-adrenoceptor isolated from human, rat and mouse genomic or cDNA libraries and expressed in mammalian cells has been shown to possess the pharmacological properties expected of an atypical β-adrenoceptor (Emorine *et al.,* 1989; Granneman *et al.,* 1991; Nahmias *et al.,* 1991; Blin *et al.,* 1994).

1.9 Aims

The present studies were designed to determine the possible existence of atypical β-adrenoceptors in vascular smooth muscles of the rat, i.e., mesenteric artery, thoracic aorta and pulmonary artery. This involved functional studies where the effects of various agonists and antagonists on these three vessels was examined. Also, β_3-adrenoceptor mRNA expression in a number of rat and human tissues was determined by means of the RT-PCR assay.

Chapter 2

Materials and methods

2.1 Functional studies

2.1.1 Methods

Male Sprague-Dawley rats (300-450g) were stunned and killed by cervical dislocation. The thoracic aortae, mesenteric or pulmonary arteries were removed, cleared of fat and connective tissue and cut into rings of approximately 3-4mm length. In some experiments the endothelium was removed by gentle abrasion of the intimal surface with fine wires. The rings were suspended on tungsten wires (0.125mm diameter) in 10ml organ baths under 0.5g resting tension in Krebs solution, maintained at 37^0C and oxygenated with $95\%O_2/5\%CO_2$. The composition of the Krebs solution was as follows (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. Tension was recorded with Grass FT.03 isometric transducers connected to a Grass polygraph.

Tissues were allowed to equilibrate for 75 min following dissection. Arterial rings were contracted with phenylephrine (giving between 60-80% of the maximum response) and the contraction assessed for stability over a period of 20-25 min. For experiments with endothelium-intact rings, the functional integrity of the endothelium was assessed by the presence of acetylcholine-induced relaxation (10^{-6}M) of phenylephrine-induced tone. Any rings not showing at least 80% relaxation of phenylephrine-induced tone to acetylcholine (10^{-6}M) were discarded as having damaged endothelium. For endothelium-denuded rings, the failure of acetylcholine to elicit a relaxant response in the presence of tone induced by phenylephrine was taken as an indication of endothelium removal. Arterial rings were contracted again with phenylephrine and half log molar cumulative concentration-response curves to various agonists were constructed. Following a 30 min interval, tissues were equilibrated with a given concentration of antagonist for 30 min, after which arterial rings were contracted again and a second cumulative concentration-response curve to

the various relaxants was obtained. The various drugs used in this study are shown in **Table 2.1**. The β_3-adrenoceptor agonists, ZD 7114 and ICI 215001, and the K_{ATP}-channel blocker, glibenclamide were prepared in 3.3% and 100% DMSO respectively, to give stock solutions of 10^{-2}M. Subsequent dilutions were carried out using distilled water. Preliminary experiments demonstrated 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.

2.2 Cyclic AMP studies

2.2.1 Methods

For the cyclic AMP studies, aortic rings were prepared as described above with either the endothelium intact or denuded and set up in 10ml organ baths in Krebs solution. The state of the endothelium in all tissues was assessed using acetylcholine (10^{-6}M). Some rings were contracted with phenylephrine ($3x10^{-7}$M) and once the spasmogen response had reached a plateau, these rings were removed into liquid nitrogen. In some experiments, rings were precontracted with phenylephrine and a single concentration of isoprenaline (10^{-6}M) or ZD 7114 (10^{-5}M) was added, after which the tissues were removed into liquid nitrogen at various time-points for cyclic nucleotide determination. Some tissues were incubated for 15 min with propranolol (10^{-6}M) before being contracted with phenylephrine ($3x10^{-7}$M) and relaxed with a single concentration of either isoprenaline or ZD 7114. These rings were removed into liquid nitrogen to determine the effect of propranolol on cyclic AMP accumulation.

2.2.2 Cyclic AMP determination

Frozen tissues were individually ground in 95% ethanol (pH 3.0) in a mortar and pestle and left overnight for extraction of cyclic AMP (**Figure 2.1**). The samples were centrifuged at 9000xg for 1 min to pellet the residual tissue fragments. The supernatant was decanted and evaporated to dryness under nitrogen. The sample was then resuspended in sodium acetate (50mM at pH 5.0) for measurement of cyclic AMP

Table 2.1 The following drugs were used in this study and dissolved in the solvents shown to give a stock solution of 10^{-2}M. Subsequent dilutions were then carried out in distilled water.

Compound	*Solvent*	*Source*
Acetylcholine chloride	DDW	Sigma
Alprenolol hydrochloride	DDW	RBI
Atenolol	DDW	RBI
BRL 37344	DDW	SmithKline Beecham
CGP 12177A hydrochloride	DDW	RBI
Glibenclamide	DMSO (100%)	Sigma
ICI 118551	DDW	ICI
ICI 215001	DMSO (3.3%)	Zeneca Pharmaceuticals
Isoprenaline hemisulfate	DDW	Sigma
N^G-nitro-L-arginine (L-NOARG)	DDW	Sigma
Noradrenaline bitartrate	DDW	Sigma
Phenylephrine hydrochloride	DDW	Sigma
Procaterol	DDW	Sigma
Propranolol hydrochloride	DDW	Sigma
Salbutamol hemisulfate	DDW	Sigma
ZD 2079	DDW	Zeneca Pharmaceuticals
ZD 7114	DMSO (3.3%)	Zeneca Pharmaceuticals

DDW is distilled deionized water and DMSO is dimethyl sulphoxide

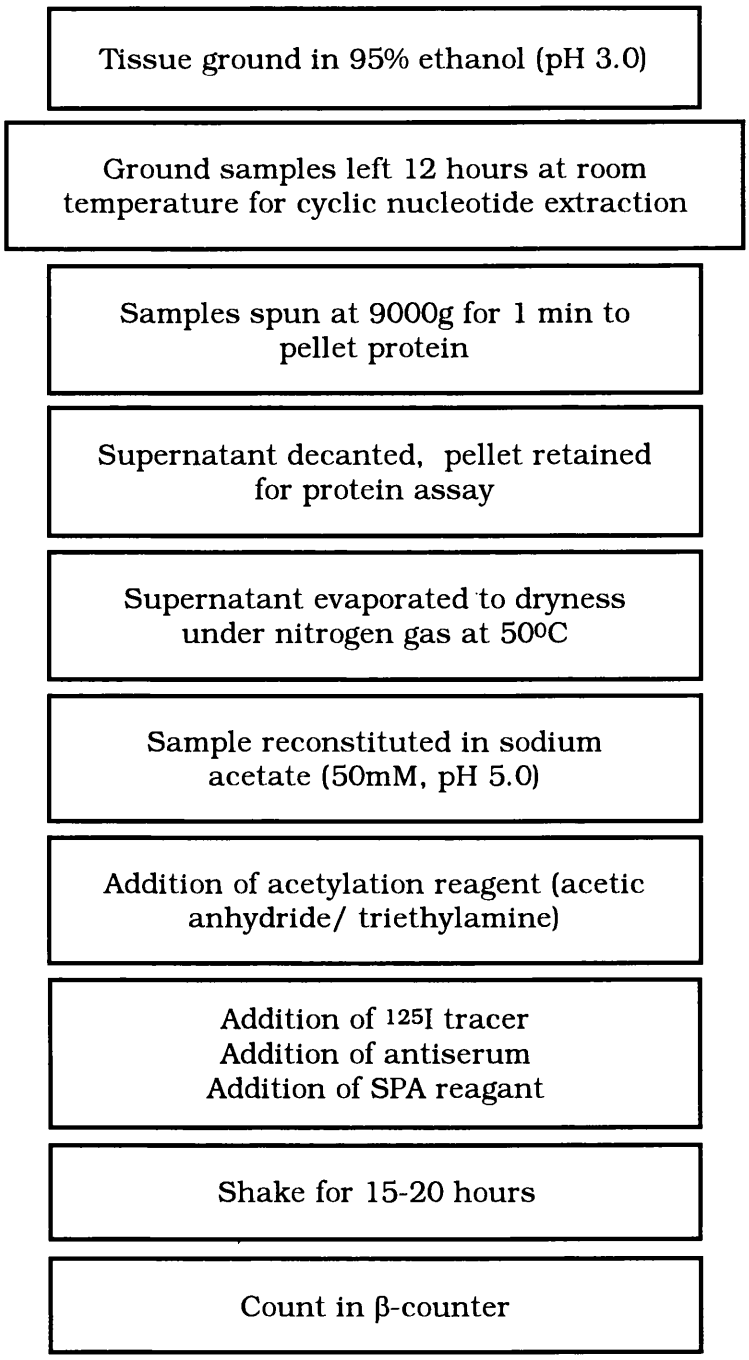


Figure 2.1 Schematic showing the protocol for acetylation scintillation proximity assay of cyclic AMP using Amersham kits. (SPA is the scintillation proximity assay reagant.)

by Scintillation Proximity Assay (Amersham) using the acetylation protocol for increased sensitivity.

2.2.3 Protein determination

The tissue residue obtained following centrifugation was dissolved in sodium hydroxide solution (0.5M) and the protein content determined using the method of Lowry *et al.* (1951). This process involves the reaction of proteins in an alkali medium with copper, followed by reduction of the phosphomolybdic-phosphotungstic (Folin reagent) by the copper treated proteins. The resulting blue colour was quantified in a colorimeter and compared with a standard, which in this case was bovine serum albumin (**Appendix 1**).

2.3 Molecular biology studies

2.3.1 Methods

(a) Animal tissues

Male Sprague-Dawley rats (300-450g) were used to obtain tissue RNA for analysis. Human tissues were obtained at medical autopsy within 24-48 hours of death, except for adipose tissue which was obtained at operations. Following removal, the tissues were transferred in Krebs solution to the laboratory where they were frozen in liquid nitrogen and stored at -70^{0}C. **Table 2.2** lists the tissues studied. Any fat surrounding non-adipose tissues was carefully dissected away.

(b) Preparation of RNA

Total RNA was extracted using TRI REAGENT (Sigma), based on the single-step RNA isolation method developed by Chomczynski & Sacchi (1987). Tissue samples were homogenized in TRI REAGENT (1ml per 50-100mg tissue) using a Polytron. To avoid any cross-contamination, the Polytron was washed thoroughly between each sample (**Figure 2.2**). Following isolation the yield of the RNA was assessed by measuring absorbance at 260 and 280nm. To check the quality of the RNA, 1μg of each sample was electrophoresed through a 1.2% agarose-formaldehyde denaturing gel and viewed over UV light (**Figure 2.3**). Only samples showing clear

Table 2.2 Rat and human tissues from which total RNA was derived for RT-PCR studies

Rat	*Human*
Adipose tissue	Adipose tissue
Kidney	Lung
Spleen	Bladder
Lung	
Liver	
Bladder	
Heart	Carotid artery
Aorta	Aorta
Mesenteric artery	Mesenteric artery
	Pulmonary artery
Oesophagus	
Colon	
Jejunum	
Ileum	
Duodenum	
Stomach	
Whole brain	

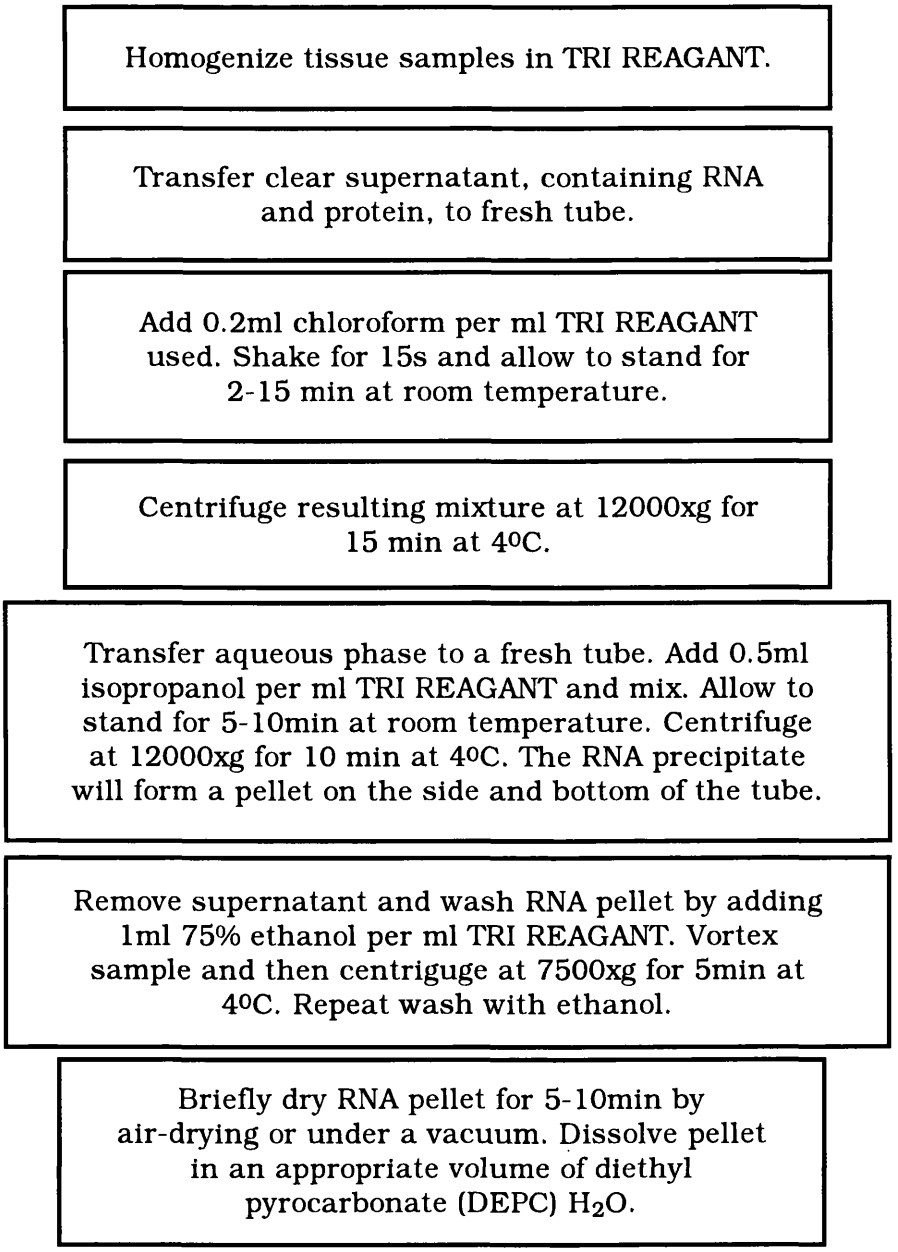


Figure 2.2 Schematic showing the protocol for RNA isolation from rat and human tissues. TRI REAGANT consists of guanidium thiocyanate buffer (containing 4M guanidium thiocyanate, 25M sodium citrate (pH 7.0), 0.5% sarcosyl and 0.72% β-mercaptoethanol), phenol and 2M sodium acetate (pH 4.0) in a ratio of 10:10:1.

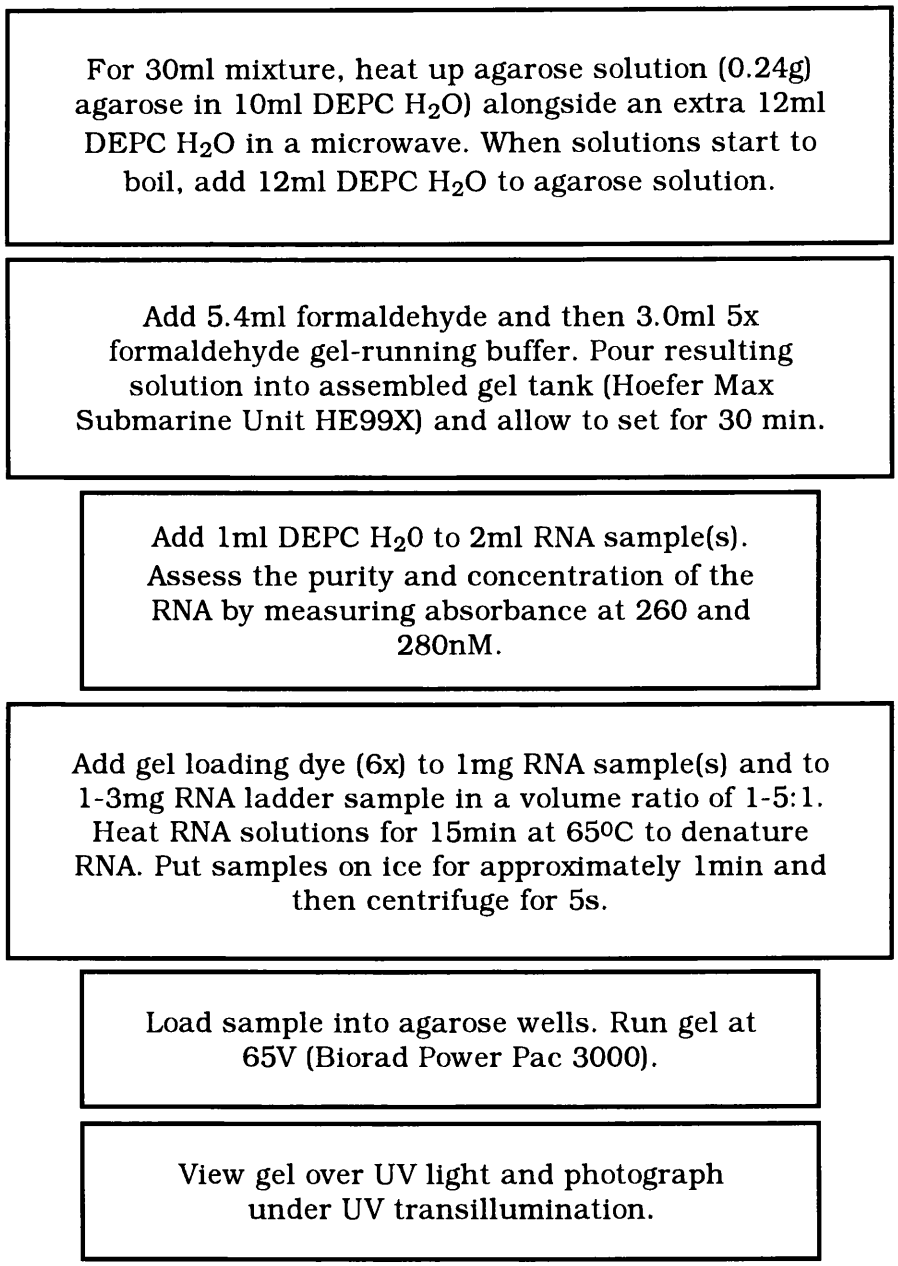


Figure 2.3 Schematic showing the protocol for RNA agarose gel preparation and gel electrophoresis. 5 x formaldehyde gel-running buffer consists of 0.1M MOPS (pH 7.0), 40mM sodium acetate and 5mM EDTA (pH 8.0).

RNA bands with minimal smearing were used subsequently for mRNA and cDNA synthesis.

(c) mRNA synthesis

For human and rat adipose tissue and rat mesenteric artery, total RNA was isolated as described above and poly A^+ RNA was purified from total RNA using an oligo-dT cellulose column (QUIAGEN kit).

(d) Reverse transcription-polymerase chain reaction (RT-PCR)

RNA (1μg) was reverse transcribed to cDNA using a 'Ready to Go T-Primed First-Strand Kit' (Pharmacia Biotech). This kit utilises the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and an oligo $(dt)_{15}$ primer to generate first strand cDNA. The RNA in a volume of 33μl was initially heated to 65^0C for 5 min and then at 37^0C for a further 5 min. The reaction mixture was simultaneously heated at 37^0C for 5 min before addition of the RNA. Following incubation at 37^0C for 5 min, the reactions were centrifuged briefly. This was followed by incubation at 37^0C for 60 min. To inactivate the reverse transcriptase, the reactions were heated at 95^0C for 5 min. The completed reverse transcriptase reactions were stored at -20^0C.

PCR amplification was carried out on cDNA equivalent to 100ng of starting RNA, using oligonucleotide primers specific for β_3-adrenoceptor, hprt and adipsin (**Table 2.3**; synthesized at Zeneca Pharmaceuticals and R&D systems). PCR mixes contained 1u of Taq polymerase, 0.25mM dNTP, 1μM reverse primer, 1μM forward primer, 2.5μl of 10x Taq polymerase buffer (containing 100mM Tris-HCl (pH 8.8), 500mM KCl, 15mM $MgCl_2$ and 1.0% Triton X-100) and cDNA in a volume of 25μl. For each specific primer pair, a single reaction mix containing all components except the cDNA was prepared for the entire PCR mix and aliquoted, therefore ensuring minimal variations between samples. As well as sample cDNAs, each PCR experiment included a negative control consisting of a reaction with no cDNA, and also a positive control, in this case rat and human adipose tissue cDNA. After initial heating of samples at 95^0C for 5 min, each cycle of amplification consisted of 30s at 95^0C (denaturing step), 30s at an annealing temperature appropriate for the primers

Table 2.3 Oligonucleotides used as PCR primers

Name	*Length*	*Strand*	*Sequence (5'→3')*	**Location*
Primers				
hprt	29	for	ATGAAGGAGATGGGAGGCCATCACATTGT	M63983: 258-286
hprt	28	rev	CAACAAACTTGTCTGGAATTTCAAATCC	M63983: 636-663
h.β_3	30	for	TCTTCGTCTACGCGCGGGTTTTCGTGGTGG	X72861: 1299-1328
h.β_3	30	rev	GTAGAGTGTCACAGCCGGGGAATCCCATGG	X72861: 2966-2995
r.β_3	30	for	ATGCTCGAGTGTTCGTCGTAGCTAAGCGCC	S73473: 712-741
r.β_3	30	rev	TCCAGAAGTCAGGCTCCTTGCTAGATCTCC	S73473: 1262-1291
r.adipsin	19	for	ATGAGCAGTGGGTGCTGAG	M92059: 167-185
r.adipsin	20	rev	AGAACGTTTTCAATCCACGG	M92059: 736-755

*Genbank accession number and nucleotide numbers within corresponding entry. In the case of the hprt primers, the sequences chosen were homologous to both rat and human.

used, and 1 min at 72^{0}C (extension step). Individual annealing temperatures were 60^{0}C for β_3-adrenoceptor, 60^{0}C for hprt and 56^{0}C for adipsin. Following amplification, products were visualized on a 1.4% agarose gel.

2.4 Statistical analysis

From each concentration-response curve, an EC_{50} value (molar concentration of the drug which produces 50% of the maximum relaxation of the spasmogen-induced tone for the drug) was calculated using Graphpad Prism (version 2.0) and these values were used to determine pEC_{50} values (-log EC_{50}). Concentration-ratios were determined from EC_{50} values in a given experiment with and without antagonist. In cases where a single concentration of the antagonist was used or where the antagonist effect was shown to be non-competitive, pK_B values were derived from the equation: **pK_B = log (concentration ratio - 1) - log (antagonist concentration)** according to the method of Furchgott (1972).

Where multiple concentrations of the antagonist were used a Schild plot of **log [concentration ratio]** against **log [antagonist]** was plotted and linear regression carried out using Graphpad Prism to derive the pA_2 value and the Schild slope. If the slope of the regression line was not significantly different from unity, then the antagonism was considered to be competitive (Arunlakshana & Schild, 1959).

Results are expressed as mean±s.e.mean of *n* number of experiments. Where no error bars are shown on **Figures** the standard error lies within the dimensions of the symbol. Statistical significance was determined using Student's *t* test where $p<0.05$ was considered to be significant.

Chapter 3

Rat mesenteric artery - functional studies

3.1 Introduction

Lands *et al.* (1967b) initially classified β-adrenoceptors in vascular smooth muscle as β_2-adrenoceptors. However, later studies revealed that both β_1- and β_2-adrenoceptors may mediate relaxation in the vasculature. For example, whereas β_2-adrenoceptors predominate in the guinea pig pulmonary artery (O'Donnell & Wanstall, 1985), the coronary arteries of many species appear to possess the β_1 subtype (Purdy & Stupecky, 1986). In addition, other vessels (rat and rabbit pulmonary artery; O'Donnell & Wanstall, 1985) possess mixed populations of β-adrenoceptor subtypes, both mediating vasodilatation.

More recent studies indicate that atypical β-adrenoceptors may also be present in the vasculature where they mediate vasorelaxation. For example, Clark & Bertholet (1983) demonstrated a vasorelaxant effect of pindolol in canine isolated perfused mesenteric vessels. A similar observation was also made in the rat aorta (Doggrell, 1990). In both studies, the vasorelaxant effect of pindolol was not inhibited by propranolol (Clark & Bertholet, 1983) or ICI 118551 (Doggrell, 1990), suggesting the presence of an atypical β-adrenoceptor, distinct from the conventional β_1- and β_2-adrenoceptors. Oriowo (1994) has also reported that atypical β-adrenoceptors may mediate vasorelaxation in the rat common carotid artery. Although propranolol shifted the isoprenaline concentration-response curve to the right without suppressing the maximum response, the slope of the Schild plot (0.74) was significantly less than 1. Also relaxations to CGP 12177 and BRL 37344 were not antagonized by 10^{-7}M propranolol, further suggesting that atypical β-adrenoceptors are present in the carotid artery.

The aim of the present chapter was to determine to possible existence of an atypical β-adrenoceptor in the rat mesenteric artery. Preliminary experiments

suggested that there was a propranolol-resistant component to isoprenaline-induced relaxation in the rat mesenteric artery. Therefore, the nature of the β-adrenoceptors mediating the isoprenaline response using selective β_1- and β_2-adrenoceptor antagonists was investigated. Furthermore, the atypical nature of the β-adrenoceptor in this tissue was examined using β_3-adrenoceptor agonists.

In order to determine the atypical nature of the β-adrenoceptors in the rat mesenteric artery, the three criteria as established by Arch & Kaumann (1993) were followed: (i) the receptor is selectively stimulated by β_3-adrenoceptor agonists; (ii) the receptor is resistant to blockade by β_1-/β_2-adrenoceptor antagonists; and (iii) certain antagonists at β_1-/β_2-adrenoceptors are agonists at the receptor.

3.2 Materials and methods

3.2.1 Tissue preparation

Rat mesenteric arterial rings were prepared as described in **Chapter 2**. Agonist and antagonist studies were carried out using the protocol detailed in **Chapter 2**.

3.2.2 Agonist activity

The relaxant effects of agonist were determined by measuring the inhibition of phenylephrine (10^{-6}M)-induced tone by addition of the agonists. This concentration of phenylephrine was found to produce between 60-75% of the maximal achievable tone. Agonists were added cumulatively until a maximal relaxant effect was observed. In some cases, repeat concentration-response curves to agonists were carried out with an interval of 60 min to check for any changes in tissue sensitivity.

EC_{50} values (concentration which produced a relaxant response that was 50% of the maximal effect) for each agonist was calculated and these values were used to determine pEC_{50} values (-log EC_{50}).

3.2.3 Antagonist activity

The equilibration time for antagonists was 30 min (unless otherwise stated). Antagonist activity was determined by comparing the agonist EC_{50} value obtained in the absence of the antagonist to that obtained in its presence and calculated as a concentration-ratio.

Where single concentrations of antagonist were used or where the antagonist effect was shown to be non-competitive, pK_B values were derived from the equation: pK_B = log (concentration-ratio - 1) - log (antagonist concentration) according to the method of Furchgott (1972).

3.2.4 Statistics

Results are expressed as mean±s.e.mean with the number of observations, *n*, in parentheses. Statistical significance was determined using Student's t test where $p < 0.05$ was considered to be significant.

3.3 Results

In the rat mesenteric artery with intact endothelium, phenylephrine (10^{-6}M) induced a sustained contractile response of 0.9±0.2g (n=5) over a period of 20 min (**Figure 3.1.a**).

3.3.1 Relaxations to isoprenaline

Isoprenaline (10^{-9}M-10^{-4}M) produced a reproducible concentration-dependent relaxation of phenylephrine-induced contractions in rat mesenteric arterial rings (pEC_{50} values 6.5±0.3 and 6.2±0.2 for first and second curves, respectively; n=4; **Figure 3.2**). The relaxant response to isoprenaline began within 15s and the effect of a given concentration reached a maximum within approximately 180s after administration. **Figure 3.1.b** shows typical relaxant responses to isoprenaline.

(a) Effect of propranolol, atenolol and ICI 118551

Propranolol (10^{-8}M, 10^{-7}M, 10^{-6}M and $3x10^{-6}$M) shifted isoprenaline concentration-response curves to the right producing concentration-ratios of 2.5±0.7

(n=5), 7.2±1.1 (n=4), 5.8±2.0 (n=4) and 5.5±3.3 (n=4), respectively with no depression in the maximum response (**Figure 3.3.a**). Over this range of concentrations, antagonism of isoprenaline by propranolol was non-competitive in nature. A pK_B value derived from the lowest concentration of propranolol used was 7.9±0.2.

Isoprenaline-induced relaxations were resistant to treatment with the selective β_1-adrenoceptor antagonist, atenolol (10^{-7}M, 10^{-6}M and $3x10^{-6}$M; **Figure 3.3.b**). The shift in the isoprenaline concentration-response curves by the selective β_2-adrenoceptor antagonist, ICI 118551, while of similar magnitude to that evoked by propranolol, i.e., concentration-ratios of 8.4±3.4 (n=4), 10.7±3.9 (n=4) and 6.5±1.7 (n=4) at 10^{-8}M, 10^{-7}M and $3x10^{-6}$M, respectively (**Figure 3.3.c**) was also non-competitive. A pK_B of 8.5±0.5 was obtained from the lowest concentration of ICI 118551 used.

(b) Effect of alprenolol

The β_1-/β_2-adrenoceptor antagonist, (±)-alprenolol (10^{-6}M and $3x10^{-6}$M) caused rightward shifts of the isoprenaline concentration-response curves of 6.5±2.9 (n=4) and 14.3±8.1 (n=4), respectively with no decrease in the maximum response (**Figure 3.4**). The antagonist effect did not appear to be competitive and a pK_B value of 6.8±0.2 was obtained using the lowest concentration of alprenolol.

Since alprenolol has been shown to have some antagonist activity at the atypical β-adrenoceptor (Blue *et al.,* 1990), the effect of alprenolol was also examined in the presence of propranolol (10^{-6}M) or atenolol (10^{-6}M) and ICI 118551 (10^{-6}M) in order to eliminate any possible contribution from β_1- and β_2-adrenoceptors. Similar rightward shifts of the isoprenaline concentration-response curve were obtained in the presence of these antagonists (i.e., pEC_{50} values of 5.2±0.2 and 5.4±0.2 in the presence of either propranolol or both atenolol and ICI 118551, respectively) as had been obtained previously (see **Figure 3.3**). Relaxant responses to isoprenaline were unaffected by (±)-alprenolol either in the presence of propranolol (10^{-6}M) alone (**Figure 3.5.a**) or both atenolol (10^{-6}M) and ICI 118551 (10^{-6}M; **Figure 3.5.b**). The

effect of (-)-alprenolol (10^{-6}M and $3x10^{-6}$M) was also investigated since it has been suggested that the racemic form of alprenolol is less able to discriminate multiple sites (Blue *et al.,* 1990). However, (-)-alprenolol in the presence of propranolol (10^{-6}M, to block β_1- and β_2-adrenoceptor subtypes) had no effect on the isoprenaline concentration-response curves at 10^{-6}M and $3x10^{-6}$M (**Figure 3.6**).

(c) Effect of CGP 12177

CGP 12177, a β_1-/β_2-adrenoceptor antagonist, produced concentration-ratios of 6.4±0.7 (n=4) and 5.0±2.1 (n=4) at 10^{-7}M and 10^{-6}M, respectively (**Figure 3.7**). The antagonist effect was non-competitive and a pK_B value of 7.7±0.1 was obtained from the lowest concentration used.

3.3.2 Relaxations to salbutamol

Salbutamol (10^{-9}M-10^{-3}M) also dose-dependently relaxed rings of the mesenteric artery precontracted with phenylephrine (10^{-6}M; pEC_{50} 5.0±0.1; n=5; **Figure 3.8**). The relaxant effect of salbutamol was slow in onset with a given concentration taking about 180s to reach a maximum. The concentration-response curve to salbutamol was biphasic in nature, the initial phase of the curve occurring at lower concentrations (10^{-9}M-10^{-6}M) of salbutamol, and the second phase at high concentrations (10^{-5}M-10^{-4}M; **Figure 3.8**).

(a) Effect of propranolol and ICI 118551

Propranolol (10^{-6}M) antagonized the responses to the lower concentrations of salbutamol causing a shift in the first phase of the concentration-response curve of about 100-fold (**Figure 3.8.a**). This shift corresponded to a pK_B value of about 8.0.

ICI 118551 (10^{-6}M) also gave a shift of the first phase of the salbutamol concentration-response curve, although the degree of antagonism was not as great as with 10^{-6}M propranolol (**Figure 3.8.b**).

3.3.3 Relaxations to procaterol

The β_2-adrenoceptor agonist, procaterol (10^{-9}M-10^{-4}M) elicited concentration-dependent relaxations of phenylephrine (10^{-6}M)-contracted rings. However, procaterol was a partial agonist in the rat mesenteric artery, producing a maximum relaxation of approximately 52% at 10^{-4}M. ICI 118551 (10^{-7}M) produced a large shift (greater than 300-fold) of the procaterol concentration-response curve. Increasing the ICI 118551 concentration to 10^{-6}M produced no further shift of the procaterol concentration-response curve (**Figure 3.9**).

3.3.4 Relaxations to selective β_3-adrenoceptor agonists

ZD 7114, ICI 215001 and ZD 2079 (10^{-9}M-10^{-4}M) elicited concentration-dependent relaxations of phenylephrine-contracted rings. The relaxant response to ICI 215001 was most rapid in onset with the effect of a given concentration reaching a maximum within 120s following administration of the drug, while relaxations to ZD 2079 began within 15s and took approximately 3-4 min to reach a maximum. In contrast, the response to ZD 7114 was slowest in onset, with the effect of a single concentration taking about 5 min after administration to reach a plateau (**Figure 3.1.c**). Repeat concentration-response curves to these agonists were reproducible (**Figure 3.10.a, b, c**).

BRL 37344 (10^{-8}M-$3x10^{-5}$M) produced a concentration-dependent relaxation of rat mesenteric arterial rings. Responses to BRL 37344 began within 40s, taking about 2-3 min to plateau (**Figure 3.10.d**).

(a) Effect of propranolol

A maximal relaxation of approximately 100% of the contraction induced by phenylephrine was obtained with 10^{-4}M ZD 7114 (pEC_{50} 5.3±0.1; n=17), while ICI 215001 and ZD 2079 had not reached a maximum at the highest concentration used (10^{-4}M; relaxations of 78.3±2.4% (n=8) and 52.1±4.2% (n=8), respectively, limited supplies of these compounds precluded the use of higher concentrations). Propranolol (10^{-6}M) was without effect on the relaxant responses to ZD 7114, ICI 215001 and ZD 2079 (**Figures 3.11.a, b, c**).

At the highest concentration used ($3x10^{-5}$M), BRL 37344 produced a relaxation of 72.9±9.0% (n=5). Propranolol (10^{-6}M) produced a small shift of the bottom portion of the BRL 37344 concentration-response curve to the right (**Figure 3.11.d**), although this shift was comparable to that obtained with consecutive concentration-response curves after a 60 min interval (**Figure 3.9.d**).

(b) Effect of ICI 118551 on relaxations to ZD 7114

ICI 118551 (10^{-6}M) had no effect on the relaxant responses to ZD 7114 (pEC_{50} values 5.3±0.1 and 5.2±0.2 in the absence and presence of ICI 118551 respectively; **Figure 3.12**).

(c) Effect of alprenolol on relaxations to ZD 7114

In the presence of propranolol (10^{-6}M; **Figure 3.13.a**) or atenolol (10^{-6}M) and ICI 118551 (10^{-6}M; **Figure 3.13.b**) to block any contribution from β_1- and/or β_2-adrenoceptors, (±)-alprenolol (10^{-6}M) had no effect on the ZD 7114 concentration-response curve. With (-)-alprenolol (10^{-6}M), a small rightward shift of approximately three-fold (in the presence of 10^{-6}M propranolol) was observed (**Figure 3.14**).

3.3.5 Relaxations to (±)-alprenolol and CGP 12177

(±)-Alprenolol (10^{-9}M-10^{-4}M) and CGP 12177 (10^{-8}M-10^{-3}M) relaxed rat mesenteric arterial rings precontracted with phenylephrine (pEC_{50} values 5.6±0.1, n=9 and 5.0±0.1, n=8, respectively). The relaxant effects of (±)-alprenolol and CGP 12177 began within 30s, with responses to a given concentration reaching a plateau in about 3-5 min.

(a) Effect of propranolol

Propranolol (10^{-7}M and 10^{-6}M) had no effect on the relaxant responses to alprenolol (**Figure 3.15.a**; note that the apparent shift observed with propranolol is a result of variations in the alprenolol control curve obtained separately with 10^{-7}M and 10^{-6}M propranolol). Pretreatment with propranolol (10^{-6}M) produced a concentration-ratio of 2.3±0.3 (n=4) against CGP 12177 (**Figure 3.15.b**). However, repeat concentration-response curves to CGP 12177 in the same tissue produced a similar

concentration-ratio (i.e., 2.4±0.4; n=4), the second curve consistently being to the right of the first (data not shown).

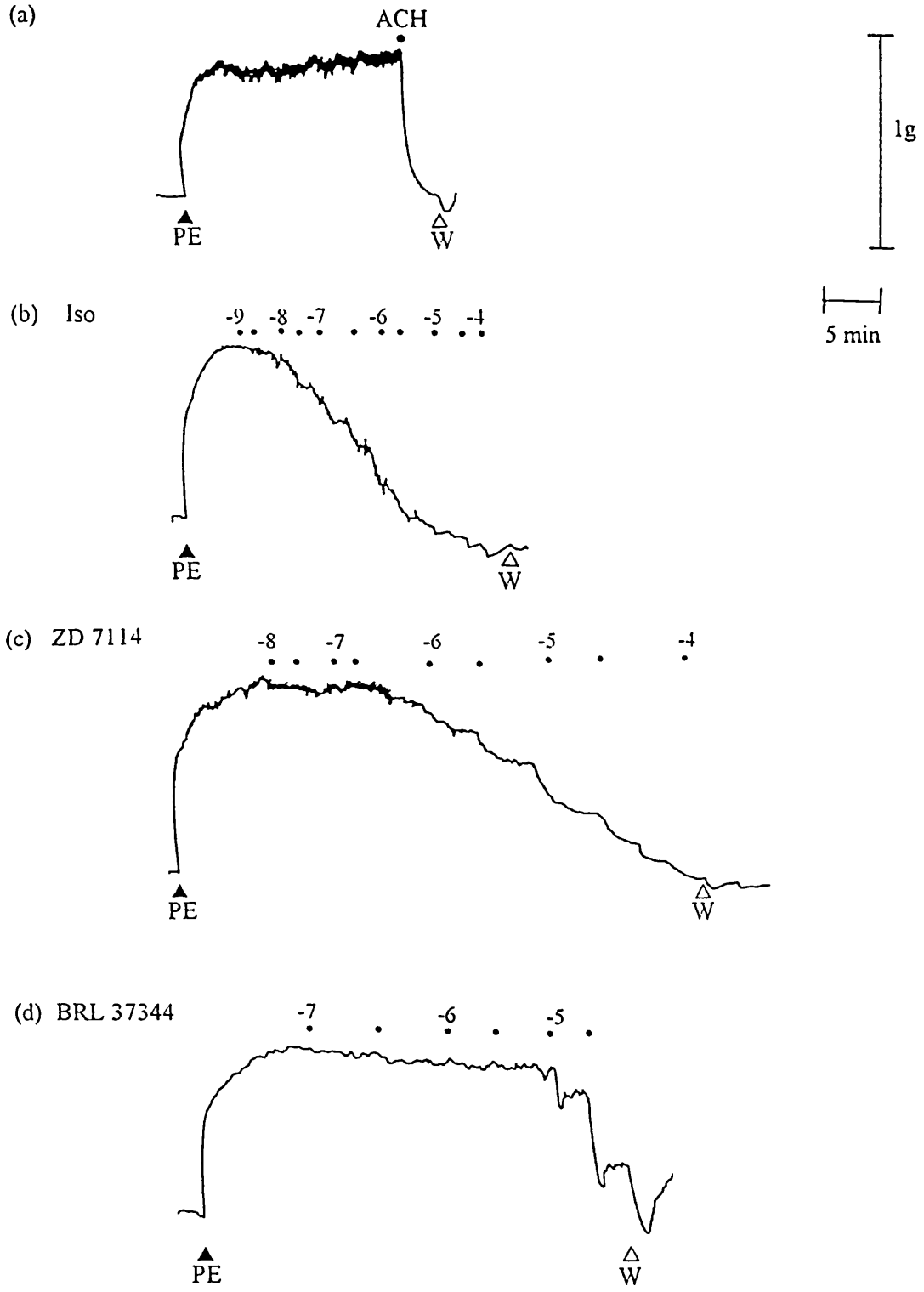


Figure 3.1 Traces showing (a) contractile activity of 10^{-6}M phenylephrine (PE) and typical relaxant effects of cumulative addition of (b) isoprenaline (Iso), (c) ZD 7114 and (d) BRL 37344 in rings of rat mesenteric artery with intact endothelium. All rings were constricted with phenylephrine (10^{-6}M) at the point labelled PE The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

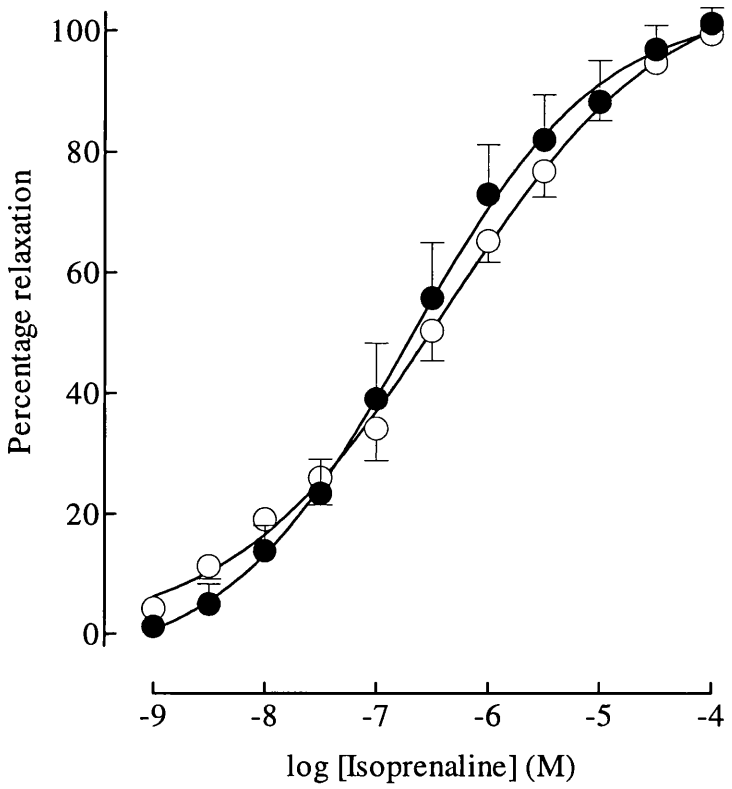


Figure 3.2 Repeat curves to isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M; ●, control; ○, repeat curve following 60 min interval). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 5 separate experiments.

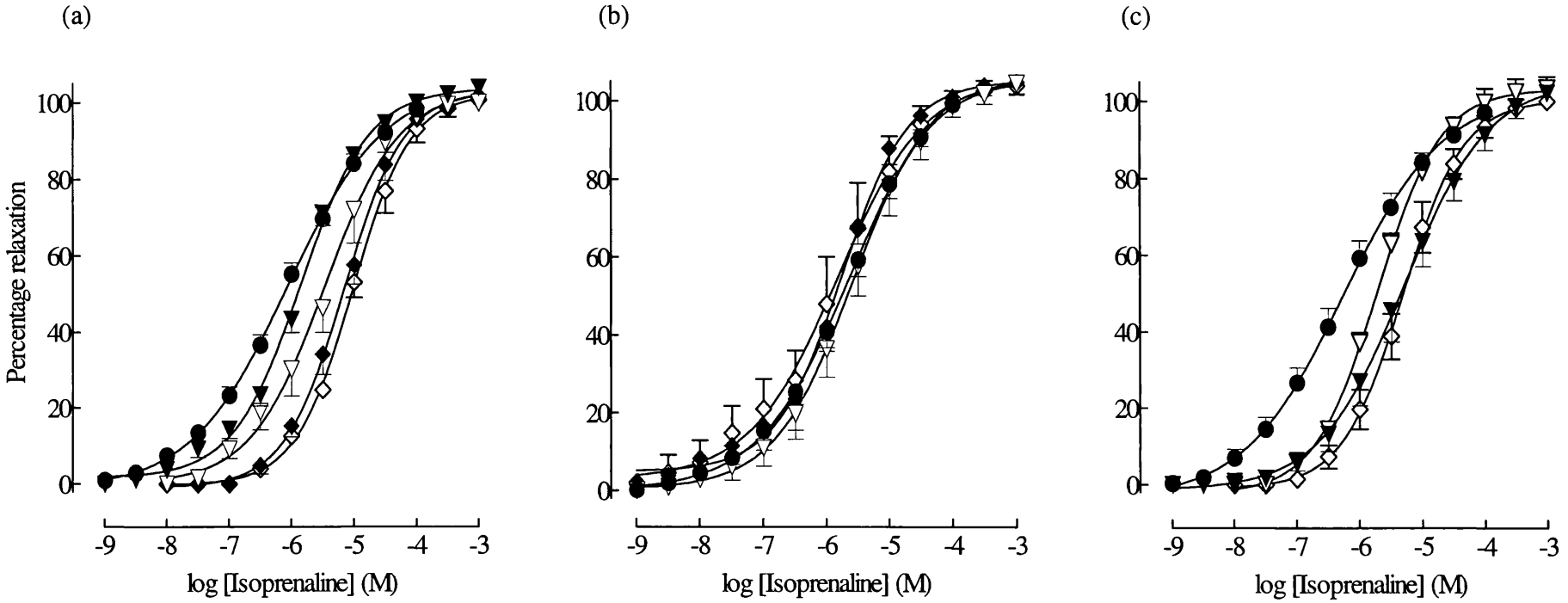


Figure 3.3 Relaxant effect of isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence and presence of (a) propranolol, (b) atenolol and (c) ICI 118551 (●, control; ▼, 10^{-8}M; ▽, 10^{-7}M; ◆, 10^{-6}M; ◇, $3x10^{-6}$M). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

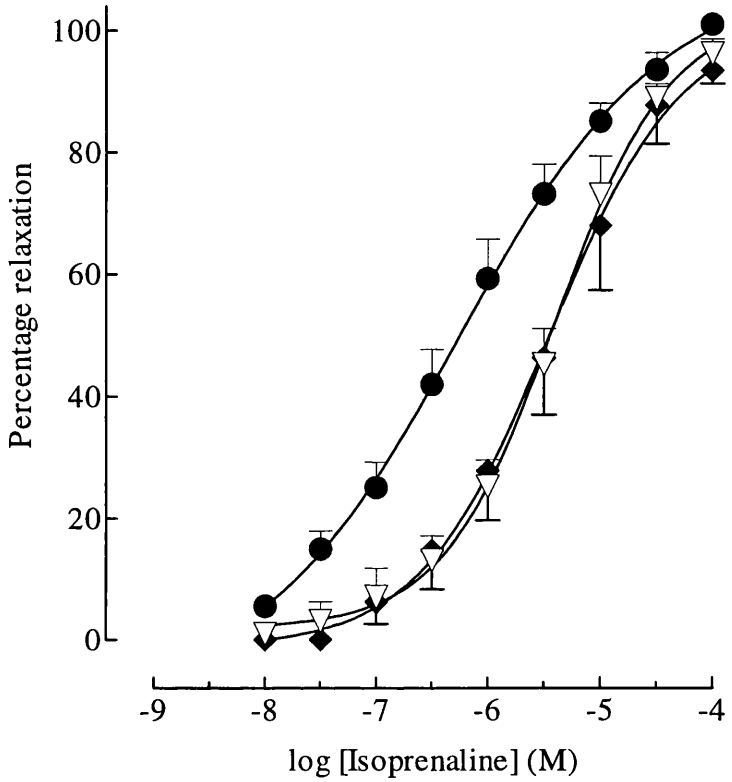


Figure 3.4 Relaxant effects of isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of (±)-alprenolol 10^{-7}M (▽) and 10^{-6}M (◆). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4 separate experiments.

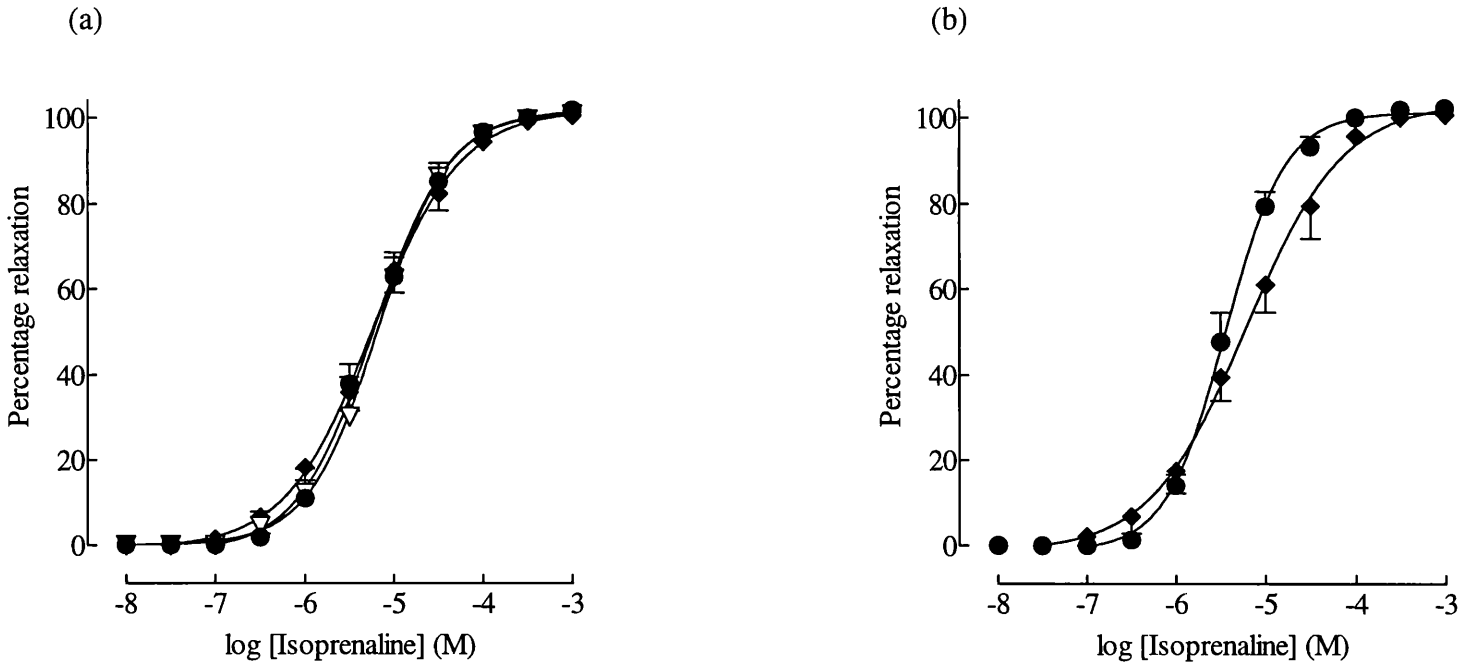


Figure 3.5 Relaxant effects of isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of (±)-alprenolol 10^{-7}M (▽) and 10^{-6}M (◆) with (a) propranolol (10^{-6}M) or (b) atenolol (10^{-6}M) and ICI 118551 (10^{-6}M) present. Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

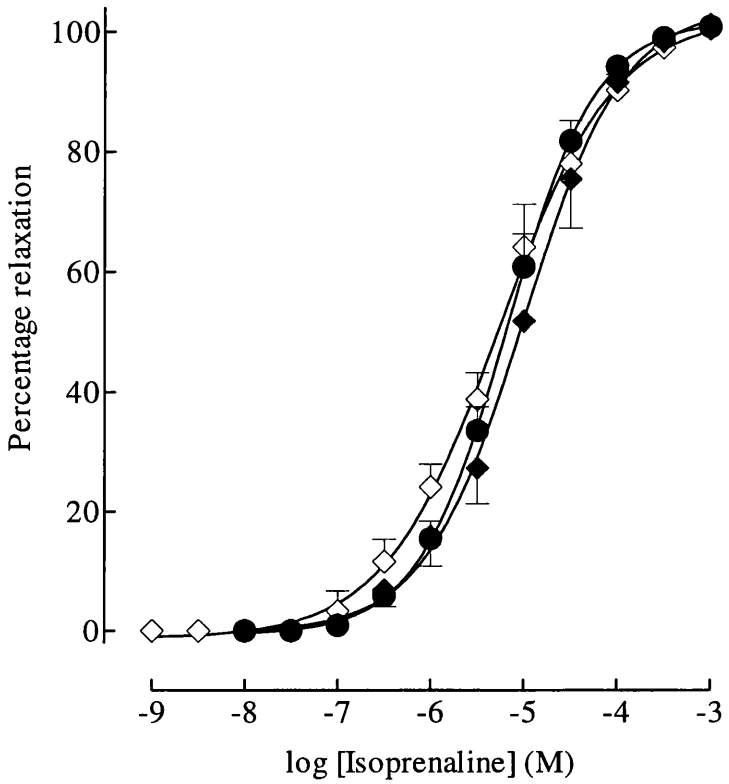


Figure 3.6 Relaxant effects of isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) with propranolol (10^{-6}M) present, in the absence (●) and presence of (-)-alprenolol 10^{-6}M (◆) and $3x10^{-6}$M (◇). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4 separate experiments.

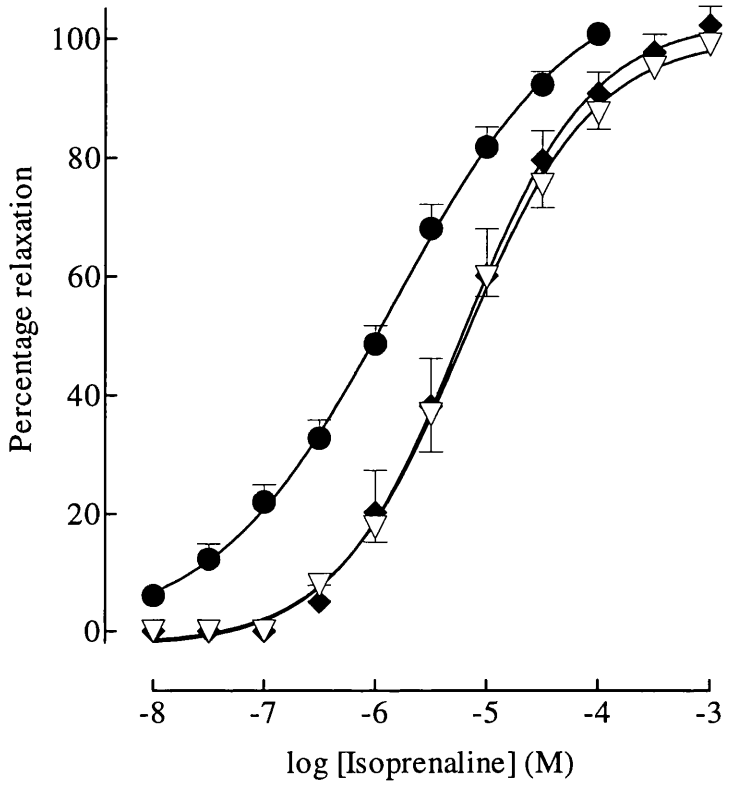


Figure 3.7 Relaxant effects of isoprenaline in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of CGP 12177 10^{-7}M (▽) and 10^{-6}M (◆). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-6 separate experiments.

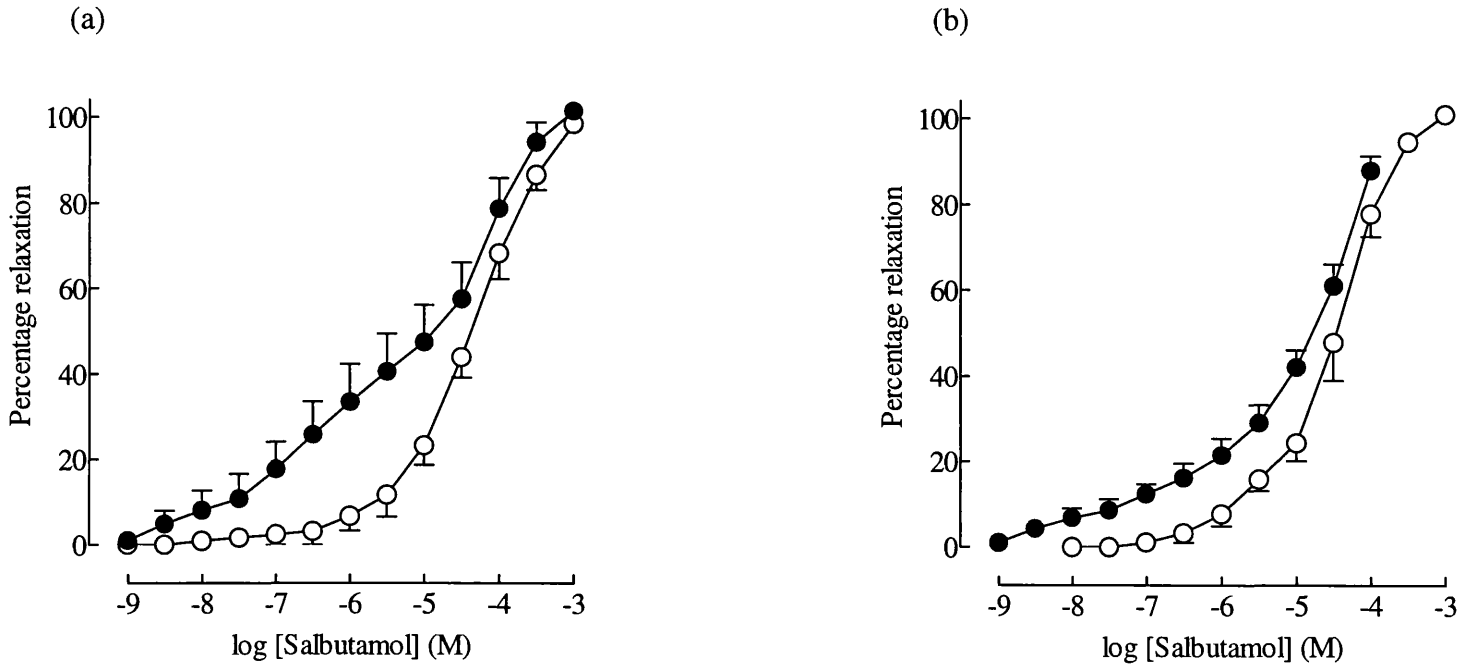


Figure 3.8 Relaxant effects of salbutamol in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of (a) propranolol 10^{-6}M and (b) ICI 118551 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

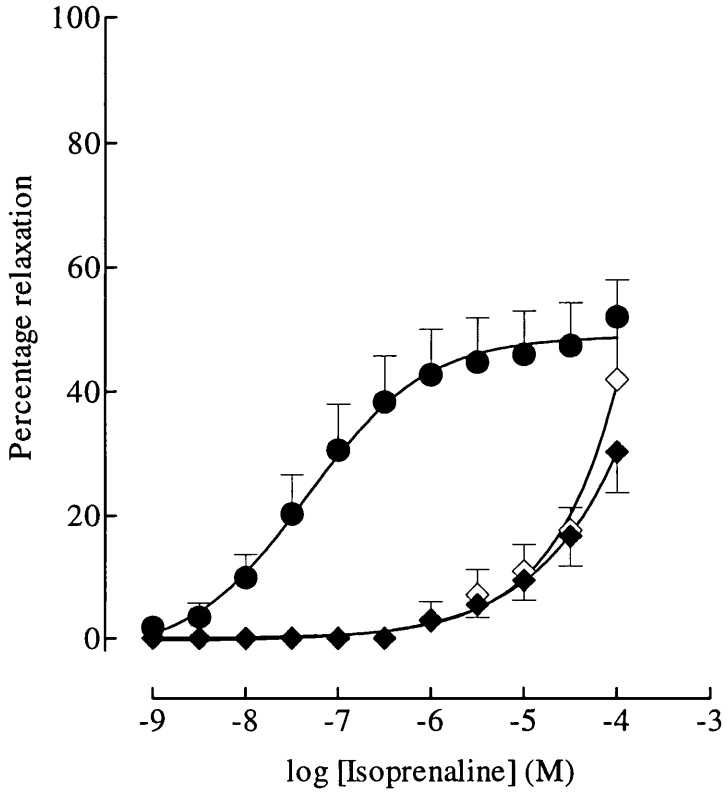


Figure 3.9 Relaxant effects of procaterol in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of ICI 118551 10^{-7}M (◇) and 10^{-6}M (◆). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4 separate experiments.

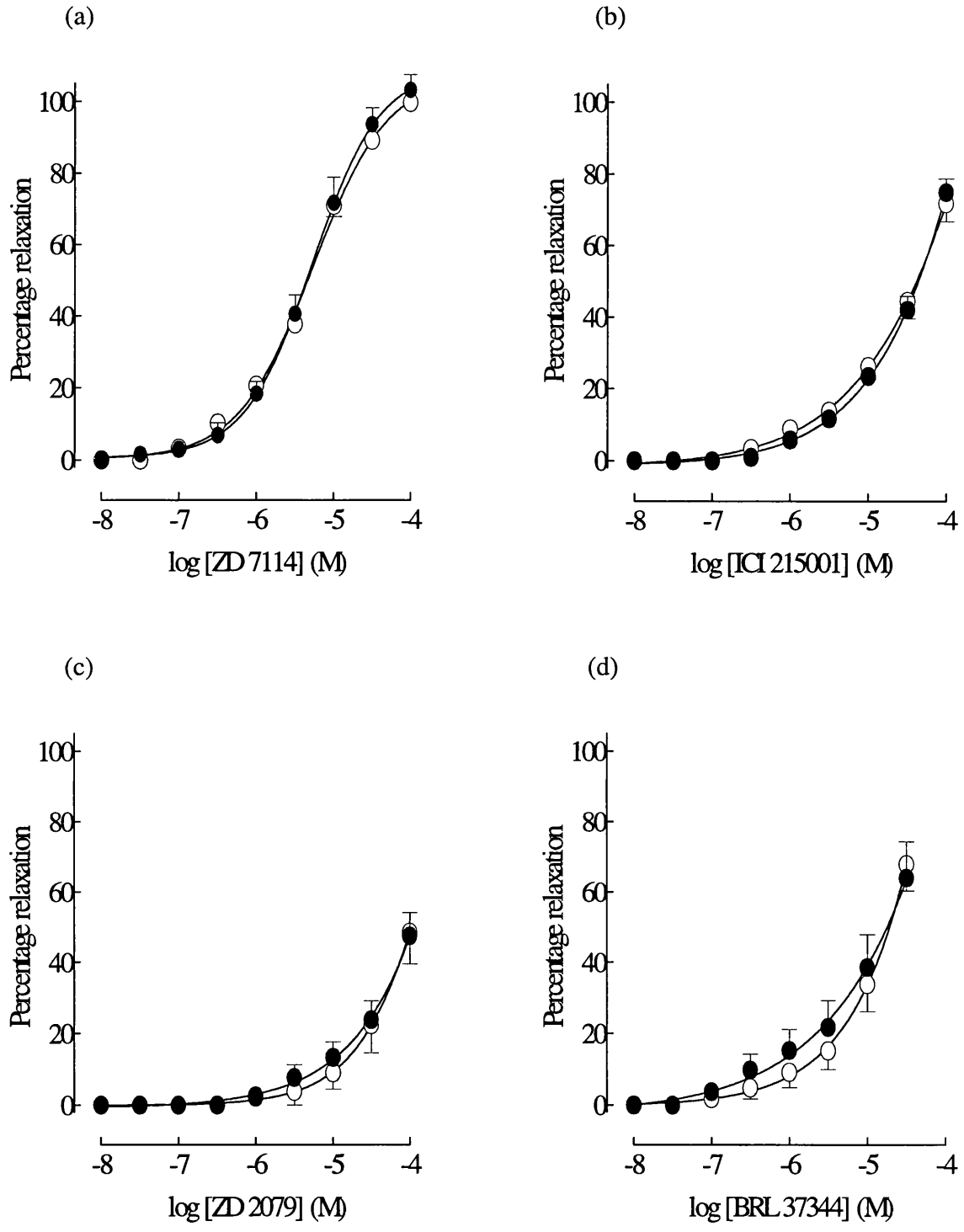


Figure 3.10 Repeat curves to (a) ZD 7114, (b) ICI 215001, (c) ZD 2079 and (d) BRL 37344 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M; ●, control; O, repeat curve following 60 min interval). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-6 separate experiments.

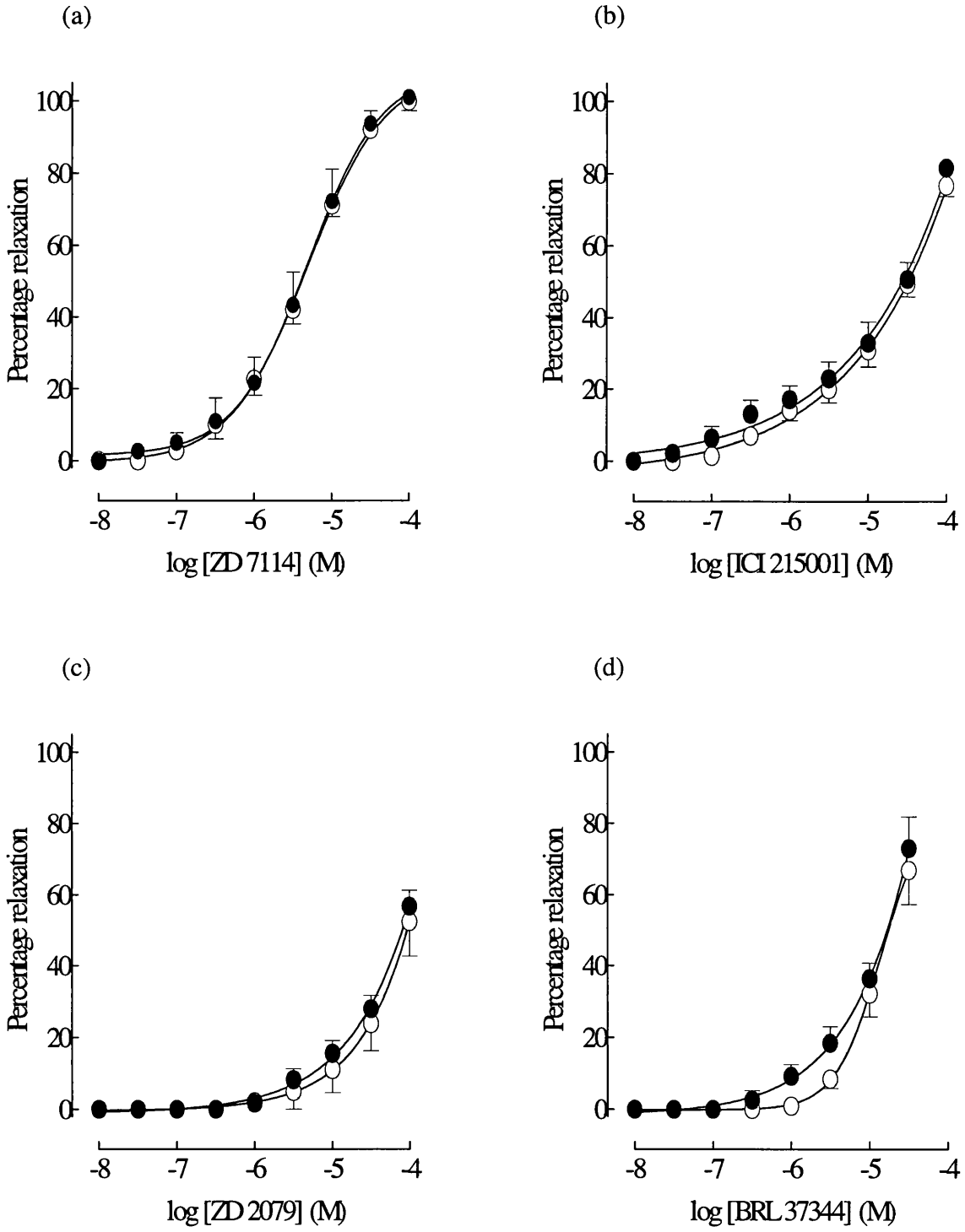


Figure 3.11 Relaxant effects of (a) ZD 7114, (b) ICI 215001, (c) ZD 2079 and (d) BRL 37344 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of propranolol 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

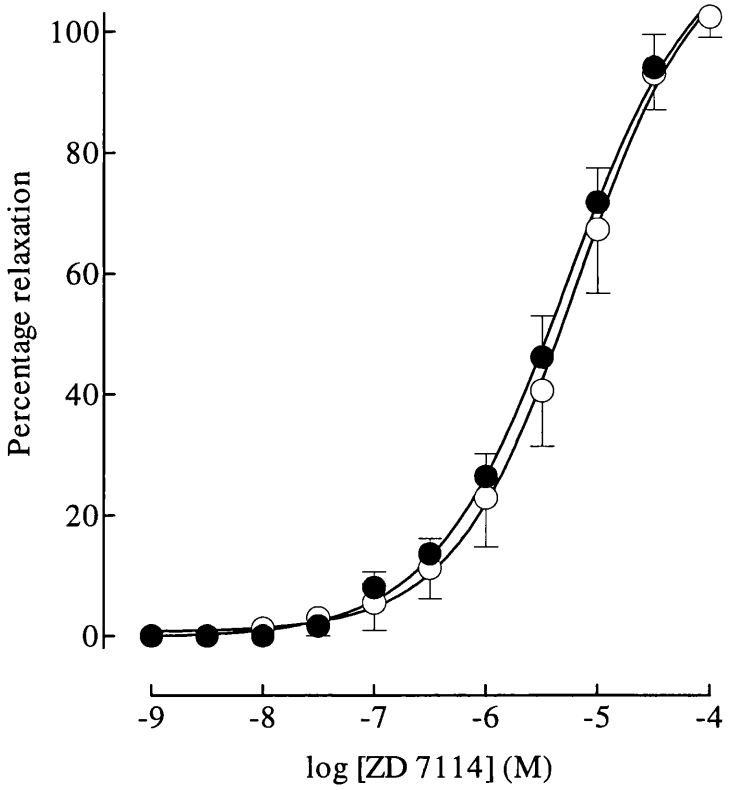


Figure 3.12 Relaxant effects of ZD 7114 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of ICI 118551 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4 separate experiments.

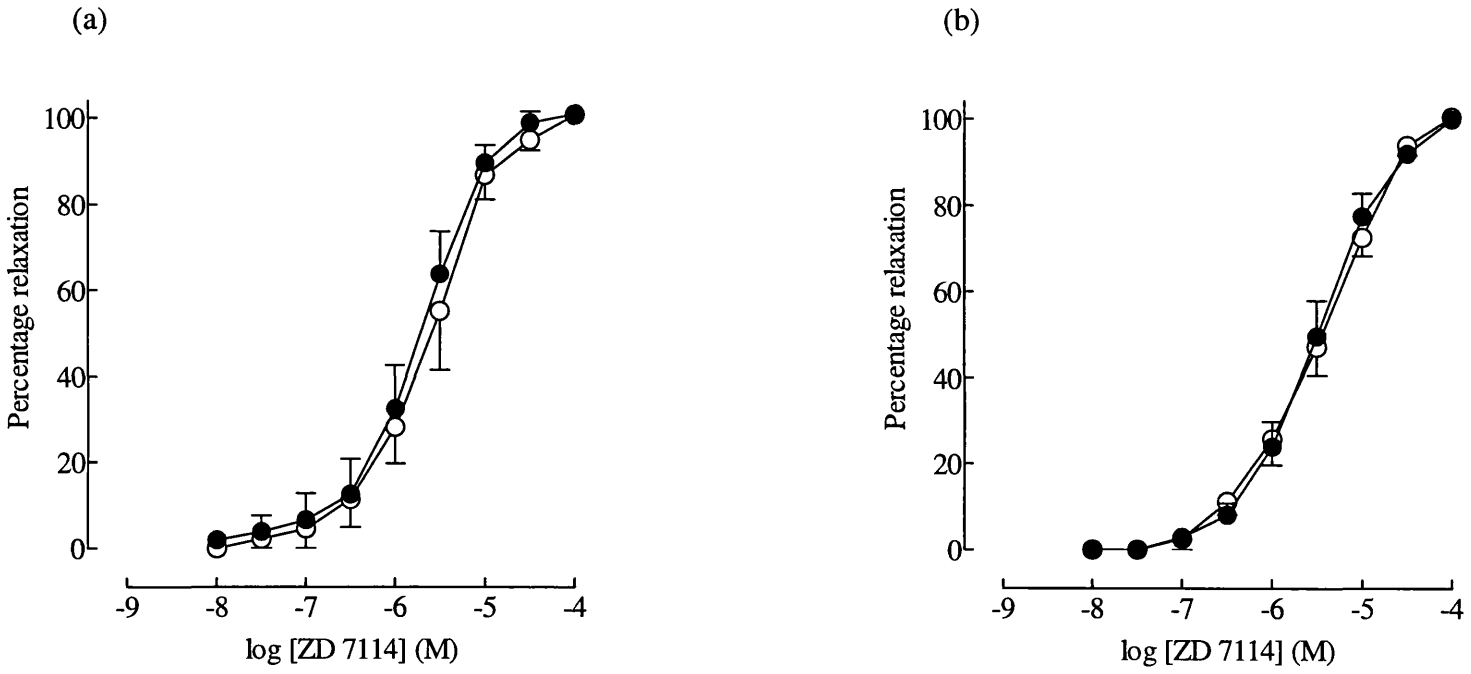


Figure 3.13 Relaxant effects of ZD 7114 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of (±)-alprenolol 10^{-6}M (○) with (a) propranolol (10^{-6}M) or (b) atenolol (10^{-6}M) and ICI 118551 (10^{-6}M) present. Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

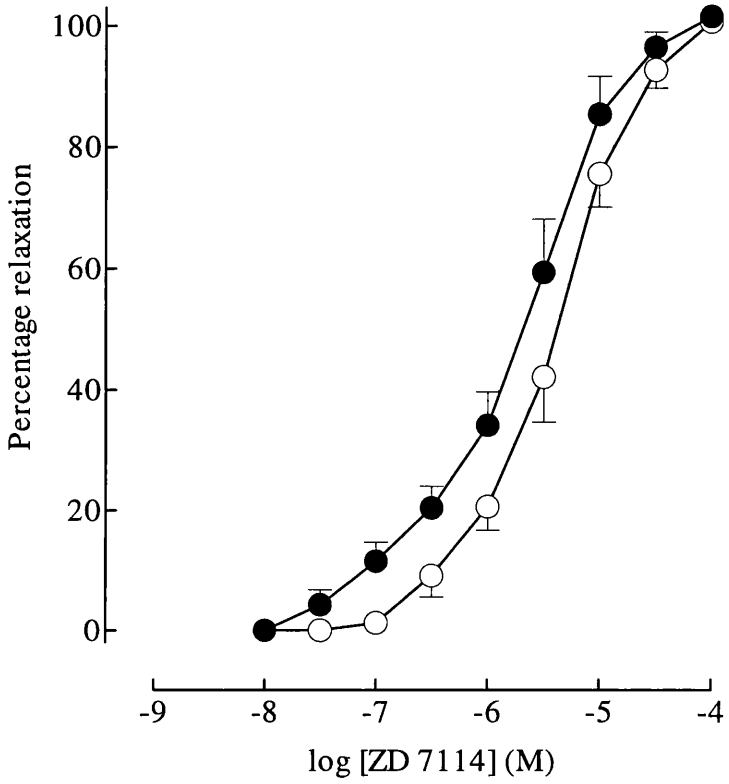


Figure 3.14 Relaxant effects of ZD 7114 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of (-)-alprenolol 10^{-6}M (O) with propranolol (10^{-6}M) present. Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 5 separate experiments.

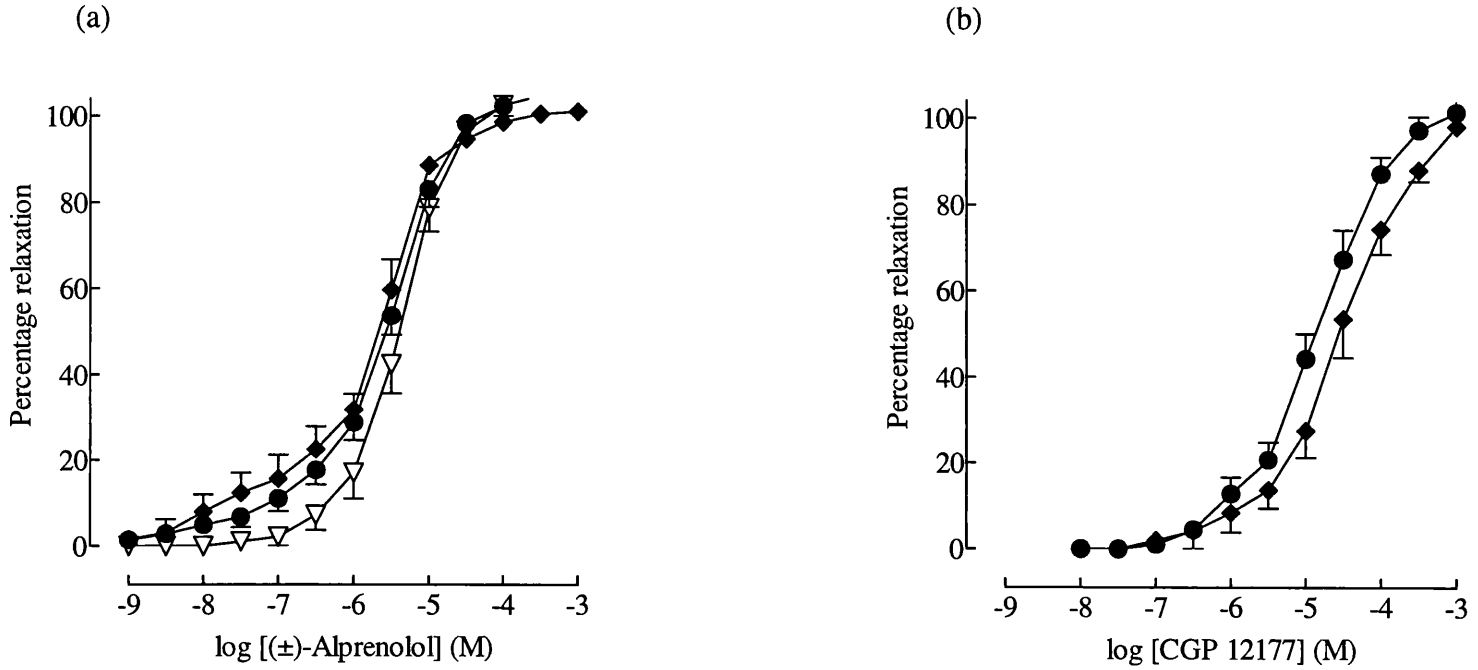


Figure 3.15 Relaxant effects of (a) (±)-alprenolol and (b) CGP 12177 in rat mesenteric arterial rings preconstricted with phenylephrine (10^{-6}M) in the absence (●) and presence of propranolol 10^{-7}M (▽) and 10^{-6}M (◆). Results are expressed as percentage relaxation of the tone induced by phenylephrine (10^{-6}M). Points represent the mean±s.e.mean of 4-5 separate experiments.

3.4 Discussion

The present chapter provides evidence for the existence of an atypical β-adrenoceptor in the rat mesenteric artery. Relaxant responses to isoprenaline were weakly antagonized by propranolol in this tissue, the antagonism being non-competitive in nature (i.e., increasing shifts of the isoprenaline concentration-response curve were not observed with increasing propranolol concentrations). The 'propranolol-sensitive' component of isoprenaline vasorelaxation may correspond to responses mediated via classical (i.e., β_1- and/or β_2-adrenoceptor) β-adrenoceptors. Similarly, other authors have also observed that while part of the response to isoprenaline is antagonized by propranolol (propranolol-sensitive), a component of the response is resistant to blockade with propranolol (propranolol-insensitive), e.g., guinea pig ileum (Bond & Clarke, 1987; 1988) and rat distal colon (MacDonald & Lamont, 1993).

The nature of the β-adrenoceptor subtype(s) involved in the propranolol-sensitive component was determined using subtype-selective antagonists. The lack of effect of atenolol indicates that β_1-adrenoceptors do not contribute to the vasorelaxant effect of isoprenaline in the rat mesenteric artery. However, ICI 118551 antagonized responses to isoprenaline, although as with propranolol the antagonism was non-competitive, suggesting that only a proportion of the isoprenaline response is mediated via β_2-adrenoceptors. These results would therefore suggest that isoprenaline may be acting via two receptor subtypes, one of which is a β_2-adrenoceptor.

The similar degree of shifts of the isoprenaline concentration-response curves over the range of antagonist concentrations used, indicates that ICI 118551 and propranolol may be blocking the same population of β_2-adrenoceptors. pK_B values for propranolol and ICI 118551 against isoprenaline (using the lowest concentration employed) were 7.9 and 8.5 respectively. Although these pK_B values are not very different from what would be expected for the effect of these antagonists at β_2-adrenoceptors, i.e., reported pK_B's for propranolol (Wilson *et al.*, 1984) and ICI

118551 (O'Donnell & Wanstall, 1980) being greater than 8.0 at β_2-adrenoceptors, they are of limited value since the antagonism did not satisfy the criteria for competitive antagonism.

pEC_{50} values for salbutamol acting at β_2-adrenoceptors vary from 7.0 in the guinea pig lung strip (Schreurs *et al.,* 1980) to 8.3 in uterine smooth muscle (Granger *et al.,* 1985). The low pEC_{50} value (approximately 5.0 obtained in the present study is therefore not consistent solely with activation of β_2-adrenoceptors. Also, concentration-response curves to salbutamol in the current study were clearly of a biphasic nature, suggesting that salbutamol was interacting with more than one site. Antagonism of the first phase of the salbutamol by propranolol (10^{-6}M) and ICI 118551 (10^{-6}M) may correspond to β_2-adrenoceptors, whereas the latter phase of the concentration-response curve (unaffected by these antagonists) may involve atypical β-adrenoceptors. The low potency of salbutamol has also been observed in the rabbit jejunum (pEC_{50} 5.3, Norman & Leathard, 1990) and rat carotid artery (pEC_{50} 5.1, Oriowo, 1994), therefore leading these authors to conclude that salbutamol may be interacting with atypical β-adrenoceptors rather than conventional β_2-adrenoceptors. Another selective β_2-adrenoceptor agonist, procaterol, has been reported to be a useful drug for detecting a functional population of β_2-adrenoceptors in tissues, whether they are the predominant or the minor receptor subtype present (Hedberg & Mattson, 1981; O'Donnell & Wanstall, 1985). Procaterol was a partial agonist in the mesenteric artery, which would be consistent with the idea that there is a low β_2-adrenoceptor reserve in this tissue.

To investigate the 'propranolol-resistant' component of isoprenaline-induced vasorelaxation in the rat mesenteric artery further, the effects of a number of β_3-adrenoceptor agonists were examined. ZD 7114 has previously been shown to stimulate atypical β-adrenoceptors in rat isolated brown adipocytes leading to increased whole body oxygen consumption (Holloway *et al.,* 1991). In addition to its metabolic effects, ZD 7114 has been shown to act as agonist at atypical β-adrenoceptors in guinea pig ileum (pEC_{50} 6.7; Growcott *et al.,* 1993a), but is an

antagonist to isoprenaline at atypical β-adrenoceptors in rat ileum (pA_2 6.3; Growcott *et al.,* 1993b) and distal colon (pA_2 7.3; MacDonald & Lamont, 1993). In the present study, ZD 7114 was a full agonist (pEC_{50} 5.3), being approximately 15-fold less potent than isoprenaline (pEC_{50} 6.5). Relaxations to another β_3-adrenoceptor agonist, ICI 215001 (acid metabolite of ZD 7114) were also observed in the mesenteric artery. In a recent study, Tesfamariam & Allen (1994) demonstrated that ICI 215001 produced relaxations of the longitudinal smooth muscle of guinea pig ileum. These authors observed that as well as possessing agonist activity at atypical β-adrenoceptors, ICI 215001 exhibited antagonist activity at β_1- (guinea pig atrium) and β_2-adrenoceptors (guinea pig trachea). ZD 2079, which has been reported to be a full agonist in rat white adipocytes with little activity on β_1- and β_2-adrenoceptors (Grant *et al.,* 1994), appeared to be the least potent of the agonists used in the current study, producing about 50% relaxation at 10^{-4}M. All three β_3-adrenoceptor agonists were resistant to blockade with propranolol (10^{-6}M) suggesting they are acting at a receptor distinct from β_1- and β_2-adrenoceptors. In addition, ZD 7114 responses were unaffected by ICI 118551 (10^{-6}M), further supporting the idea that this agonist is acting at a third β-adrenoceptor subtype.

Another agonist used to demonstrate the presence of atypical β-adrenoceptors was BRL 37344. This compound has been demonstrated to stimulate lipolysis in both brown and white adipose tissues of the rat (Arch *et al.,* 1984; Wilson *et al.,* 1984) and is one of the most potent β_3-adrenoceptor agonists for relaxing several gut smooth muscle preparations from different animal species (see review Manara *et al.,* 1995b). However, in the present study, BRL 37344 was found to be less potent than both isoprenaline or ZD 7114, with BRL 37344 producing approximately 50% relaxation at 10^{-5}M. Also propranolol produced a small rightward shift of the BRL 37344 concentration-response curve, although this shift was comparable to that obtained with a second repeat curve to BRL 37344.

CGP 12177, a β_1-/β_2-adrenoceptor antagonist, is also an agonist at atypical β-adrenoceptors, stimulating thermogenesis in brown adipose tissues (Mohell & Dicker,

1989) and lipolysis in white adipose tissues (Langin *et al.,* 1991) of the rat. In terms of antagonist activity, CGP 12177 (10^{-7}M and 10^{-6}M) antagonized isoprenaline responses non-competitively (pK_B value of 7.7 obtained from the lowest concentration used) in the present study. CGP 12177 was also a full agonist in the mesenteric artery and was similar in efficacy and potency to ZD 7114 (pEC_{50} values being 5.0 and 5.3 respectively). The relaxant response to CGP 12177 was not antagonized by propranolol (10^{-6}M) thus confirming interaction of CGP 12177 with atypical β-adrenoceptors. Alprenolol is also an agonist at atypical β-adrenoceptors (guinea pig ileum, Blue *et al.,* 1990) and in the current study produced relaxations (pEC_{50} 5.6) which were not antagonized by propranolol (10^{-7}M and 10^{-6}M).

In addition to subtype-selective agonists, selective and competitive antagonists should be employed to classify receptor subtypes whenever possible. In an attempt to further characterize the atypical β-adrenoceptor, Blue *et al.* (1990) demonstrated that the β_1-/β_2-adrenoceptor antagonist, alprenolol, had antagonist activity at the atypical β-adrenoceptor in the guinea pig ileum. In the present study, alprenolol antagonized isoprenaline responses giving a pK_B value of 6.8 (at 10^{-6}M), which is lower than would be expected for effects at β_1- and β_2-adrenoceptors (i.e., pA_2 values 8.6-9.0). However, alprenolol had no effect on isoprenaline responses in the presence of either propranolol (10^{-6}M) alone or both atenolol (10^{-6}M) and ICI 118551 (10^{-6}M), used in order to eliminate any possible contribution from classical β-adrenoceptors. Blue *et al.* (1990) has suggested that the racemic form of alprenolol may be less able to discriminate multiple sites than the (-)-isomer. However, again isoprenaline responses were unaffected by (-)-alprenolol (10^{-6}M and $3x10^{-6}$M) in the presence of propranolol (10^{-6}M) in the present study. Similarly, the racemic form of alprenolol (10^{-6}M) did not antagonize responses to the β_3-adrenoceptor agonist ZD 7114 (in the presence of either propranolol or the combination of atenolol and ICI 118551), with (-)-alprenolol (10^{-6}M; in the presence of propranolol) producing only a three-fold rightward shift of the ZD 7114 concentration-response curve. Therefore, the atypical β-adrenoceptor in the mesenteric artery may differ from that present in gastrointestinal tissues. For example, in rat and guinea pig ileum, alprenolol competitively antagonized responses to isoprenaline giving pA_2 values of 6.50 and 6.47 respectively (Growcott *et al.,*

1993b; Blue *et al.,* 1990) and in the guinea pig ileum responses to ZD 7114 were antagonized by alprenolol (10^{-6}M) giving a pK_B of 6.53 (Growcott *et al.,* 1993a; all these experiments being carried out in the presence of α- and/or β-adrenoceptor antagonists).

An interesting observation in the study by Blue *et al.* (1990) was that although alprenolol was an antagonist at atypical β-adrenoceptors, it also displayed agonist activity in the guinea pig ileum, the alprenolol concentration-response curve spanning from 10^{-9}M-10^{-4}M. These authors suggested that at very high concentrations the response to alprenolol may be independent of β-adrenoceptors, but the first phase of the curve occurred at concentrations that elicited antagonist activity and was antagonized by nadolol (previously demonstrated to have a low affinity for atypical β-adrenoceptors in guinea pig ileum; Bond & Clarke, 1988) but not propranolol. The pA_2 value obtained for nadolol against alprenolol (4.3; Blue *et al.*, 1990) was similar to its low pA_2 values against responses to isoprenaline and BRL 37344 (4.3 and 4.7 respectively; Bond & Clarke, 1988) suggesting that it was blocking atypical β-adrenoceptors.

Therefore, isoprenaline-induced relaxations of the rat mesenteric artery were found to comprise a propranolol-sensitive component and a component resistant to blockade with increasing concentrations of this antagonist. The former may be mediated by β_2-adrenoceptors because of the antagonism of isoprenaline responses by ICI 118551, but not by atenolol. Based on the relaxant responses to novel β_3-adrenoceptor agonists, i.e., ZD 7114, ICI 215001, ZD 2079, BRL 37344, and also alprenolol and CGP 12177, the additional receptor mediating vasorelaxation to isoprenaline appears likely to be an atypical β-adrenoceptor.

Chapter 4

Rat thoracic aorta and pulmonary artery - functional studies

4.1 Introduction

The existence of atypical β-adrenoceptors has now been demonstrated in various tissues. Studies show that these receptors are not confined to adipose tissue and the gastrointestinal tract, but are also found in a number of other tissues, e.g., rat skeletal muscle (Challis *et al.,* 1988) and canine bronchial smooth muscle (Tamaoki *et al.,* 1993). Studies in the rat mesenteric artery have indicated the presence of atypical β-adrenoceptors in this tissue (**Chapter 3**). Atypical β-adrenoceptors are also functionally expressed in another blood vessel, the rat carotid artery (Oriowo, 1994), and it is possible that these receptors are widespread within the vasculature.

β-Adrenoceptors in the rat aorta have been characterized as predominantly of the β_2-subtype (O'Donnell & Wanstall, 1984). Doggrell (1990) observed that relaxations to pindolol in the rat aorta were not altered by 10^{-6}M ICI 118551. In a more recent study, it was shown that propranolol antagonized the vasorelaxant effect of isoprenaline in the rat aorta with a pK_B of 7.5 (Gray & Marshall, 1992) which is less than pK_B values of 8.5-9.0 expected from an action on conventional β_1- and β_2-adrenoceptors. These observations suggest atypical β-adrenoceptors may also be present in the rat aorta.

In the rat pulmonary artery, it has been shown that β_2-adrenoceptors predominate with a small number of β_1-adrenoceptors also present (O'Donnell & Wanstall, 1981; 1984; Shaul *et al.,* 1990). Radioligand binding studies have demonstrated that β-adrenoceptors on cultured endothelial cells of bovine pulmonary arteries are heterogeneous, with 25% of them being of the β_1-subtype and the remaining 75% consisting of β_2- or atypical β-adrenoceptors (Ahmad *et al.,* 1990).

In the present chapter, the existence of atypical β-adrenoceptors were further investigated in rat vascular smooth muscles to establish whether these receptors are more widely distributed within the vasculature. The present study focuses mainly on the rat thoracic aorta, while a few experiments were also carried out in the rat pulmonary artery.

4.2 Results

In rat aortic and pulmonary arterial rings, set up as described earlier (**Chapter 2**), phenylephrine ($3x10^{-7}$M and $3x10^{-8}$M respectively) evoked an increase in tone of endothelium-intact rings to 0.9±0.2g (n=4) and 0.23±0.02g (n=4) respectively. Isoprenaline produced a relatively rapid reduction in tone in both tissues with the effect of a given concentration reaching a maximum within approximately 180s after administration. **Figures 4.1.a** and **4.2.a** represent typical traces showing the contractile response to phenylephrine in the aorta and pulmonary artery respectively.

4.2.1 Relaxations to isoprenaline

Isoprenaline produced reproducible concentration-dependent relaxations of phenylephrine-induced contractions in rat aortic (pEC_{50} values 7.3±0.1 and 7.4±0.2 for first and second curves, respectively; n=4) and pulmonary arterial rings (pEC_{50} values 8.1±0.1 and 8.0±0.1 for first and second curves, respectively; n=4). **Figures 4.1.b** and **4.2.b** show typical relaxant responses to isoprenaline in rat aortic and pulmonary arterial rings respectively.

(a) Effect of propranolol, atenolol and ICI 118551

(i) Rat thoracic aorta

Propranolol (10^{-7}M, 10^{-6}M and $3x10^{-6}$M) shifted isoprenaline concentration-response curves to the right producing concentration-ratios of 8.1±1.8 (n=5), 7.3±0.2 (n=5), and 6.4±3.6 (n=4), respectively with no depression in the maximum response (**Figure 4.3.a**). Antagonism of isoprenaline responses by propranolol over this range of concentrations was non-competitive. A pK_B value derived from the lowest concentration of propranolol used was 7.8±0.1.

Atenolol had no effect on the isoprenaline concentration-response curve at concentrations of 10^{-7}M and 10^{-6}M (**Figure 4.3.b**). ICI 118551 produced shifts of 2.8±0.9 (n=4) and 11.0±3.3 (n=4) at 10^{-7}M and 10^{-6}M, respectively of the isoprenaline concentration-response curve (**Figure 4.3.c**), giving a pK_B value of 7.3±0.1 from the lowest concentration of ICI 118551 used.

(ii) Rat pulmonary artery

Propranolol (10^{-9}M-10^{-6}M) shifted the isoprenaline concentration-response curve to the right without reducing the maximum response (**Figure 4.4.a**). A Schild plot (**Figure 4.4.b**) revealed that this antagonism was non-competitive in nature, as demonstrated by the slope of the plot, which was less than 1 (0.6±0.1). A pA_2 value for propranolol, calculated from the x-intercept, gave a value of 9.8 and a pK_B value of 9.4±0.3 was calculated using 10^{-9}M propranolol.

Responses to isoprenaline were weakly antagonized by atenolol giving concentration-ratios of 2.0±0.4 (n=4), 2.8±0.9 (n=4) and 4.4±1.5 (n=4) at 10^{-8}M, 10^{-7}M and 10^{-6}M respectively (**Figure 4.5.a**). A pK_B value derived from the lowest concentration of atenolol used was 7.1±0.1. The shift in the isoprenaline concentration-response curves by ICI 118551, i.e., concentration-ratios of 3.9±1.4 (n=4), 7.4±1.1 (n=4) and 38.7±11.1 (n=4) at 10^{-8}M, 10^{-7}M and 10^{-6}M respectively, was also non-competitive (**Figure 4.5.b**). From the lowest concentration of ICI 118551 used, a pK_B value of 8.3±0.2 was obtained

4.2.2 Relaxations to procaterol

Procaterol (10^{-10}M-10^{-5}M) elicited concentration-dependent relaxations of phenylephrine ($3x10^{-7}$M)-contracted rat aortic rings. As in the mesenteric artery, procaterol was a partial agonist in the aorta, producing a maximum relaxation of approximately 69% at 10^{-5}M. Also, the procaterol concentration-response curve appeared to be of a biphasic nature in the rat aorta. Propranolol (10^{-7}M) did not produce antagonism of the responses at low procaterol concentrations (10^{-10}M-10^{-8}M; in fact, a shift to the left was observed), whereas at higher procaterol concentrations (10^{-7}M-10^{-5}M) a small reduction in the response was observed (**Figure 4.6**).

4.2.3 Relaxations to selective β_3-adrenoceptor agonists

The traces illustrated in **Figures 4.1.c,d** and **4.2.c,d** depict the relaxant responses elicited by cumulative concentrations of ZD 7114 and BRL 37344 in the rat thoracic aorta and pulmonary artery respectively. The response profiles obtained with ZD 7114 and BRL 37344 in these tissues were similar to those obtained in the rat mesenteric artery (**Chapter 3**), both agonists producing a response which was slow in onset and duration relative to isoprenaline.

ZD 7114 (10^{-9}M-10^{-4}M) and BRL 37344 (10^{-8}M-$3x10^{-5}$M) produced concentration-dependent relaxations in rat aortic and pulmonary arterial rings. Repeat concentration-response curves to ZD 7114 were reproducible in both tissues. ZD 7114 induced a maximum response in rat aortic and pulmonary arterial rings, which was approximately 100% of the tone induced by phenylephrine (pEC_{50} values being 5.4±0.1, n=4 and 6.2±0.1, n= 4 respectively).

With BRL 37344, a maximal relaxation was not reached at the highest concentration used ($3x10^{-5}$M; relaxations of 81.1%, n=5 and 66.3%, n=5 in aortic and pulmonary arterial rings respectively; limited supplies of this compound preventing the use of higher concentrations). Concentration-response curves to BRL 37344 in the rat thoracic aorta were non-sigmoidal (**Figure 4.7.a**). BRL 37344 produced reproducible concentration-dependent relaxations of phenylephrine-induced contractions in aortic rings, although in the pulmonary artery, a rightward shift of approximately three-fold was observed with the repeat curve (consecutive concentration-response curves to BRL 37344 being separated by a 1 hour interval; **Figure 4.7.b**).

(a) Effect of propranolol

(i) Rat thoracic aorta

The concentration-response curve to ZD 7114 appeared to be biphasic, the first phase of the curve occurring at concentrations of 10^{-9}M-$3x10^{-7}$M and the latter phase at 10^{-6}M-10^{-4}M. Pretreatment with propranolol (10^{-6}M) did not antagonize ZD 7114 responses, but produced a small shift of the concentration-response curve to the

left (**Figure 4.8.a**). Relaxant responses to BRL 37344, however, were unaffected by propranolol (10^{-6}M; **Figure 4.8.a**).

(ii) Rat pulmonary artery

Unlike the aorta, the concentration-response curve to ZD 7114 in the pulmonary artery was sigmoidal in nature and responses to ZD 7114 were unaffected by propranolol (10^{-6}M) in this tissue (**Figure 4.9.a**). With BRL 37344, a shift of approximately ten-fold to the right was observed in the pulmonary artery (**Figure 4.9.b**), a component of this shift probably being due to desensitization (i.e., a three-fold shift observed with repeat curves; see **Figure 4.7.b**).

4.2.4 Relaxations to CGP 12177 and alprenolol

CGP 12177 (10^{-9}-10^{-3}M) and alprenolol (10^{-9}M-10^{-4}M) relaxed rat aortic rings precontracted with phenylephrine (pEC_{50} values 5.7±0.1, n=4 and 5.3±0.1, n=5 respectively). The relaxant effect of CGP 12177 (10^{-9}M-10^{-3}M) was also investigated in the rat pulmonary artery and produced concentration-dependent relaxations of arterial rings (pEC_{50} 6.1±0.1, n=4).

(a) Effect of propranolol

(i) Rat thoracic aorta

The concentration-response curve to CGP 12177 in rat aortic rings appeared to be biphasic, the first phase of the curve occurring at 10^{-9}M-10^{-6}M and the latter phase at $3x10^{-6}$M-10^{-3}M (**Figure 4.10.a**). However, it was not possible to carry out a repeat curve in the same tissue since a significant inhibition of the phenylephrine-induced contraction was observed even after a 2 hour interval. Therefore, in this case paired experiments were carried out such that only one concentration-response curve was constructed in any one tissue. Pretreatment with propranolol (10^{-6}M) produced a shift of both phases of the CGP 12177 concentration-response curve (pK_B 6.4±0.4; **Figure 4.10.a**).

In the case of alprenolol, relaxations were largely unaffected by propranolol (10^{-7}M) in rat aortic rings (**Figure 4.10.b**).

(ii) Rat pulmonary artery

In rat pulmonary arterial rings, consecutive concentration-response curves to CGP 12177 were reproducible (pEC_{50} values being 6.1±0.1 and 6.2±0.1 for first and second curves respectively; n=4). Relaxant responses were not affected by propranolol in this tissue (**Figure 4.11**).

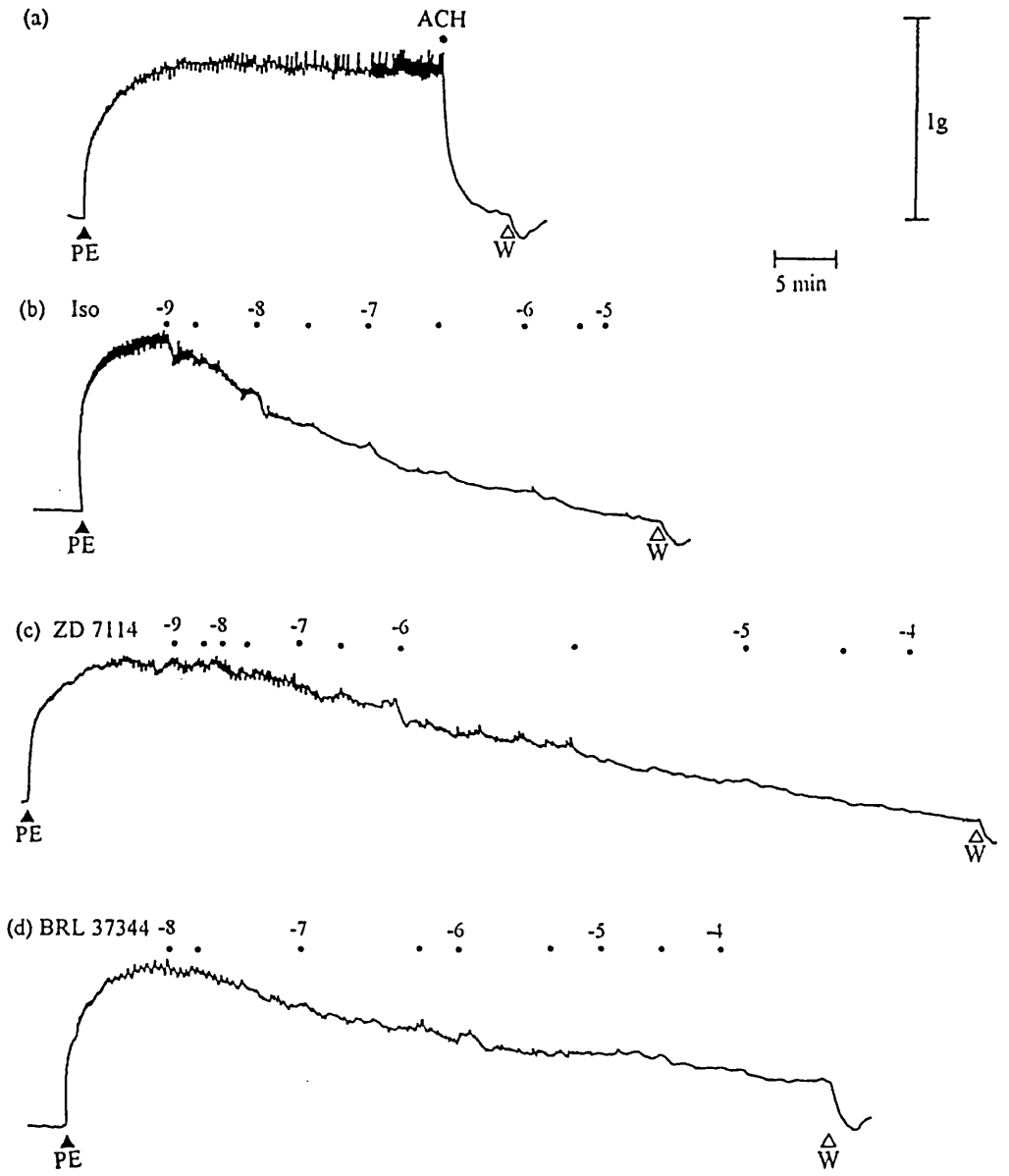


Figure 4.1 Traces showing (a) contractile activity of $3x10^{-7}$M phenylephrine (PE) and typical relaxant effects of cumulative addition of (b) isoprenaline (Iso), (c) ZD 7114 and (d) BRL 37344 in rings of rat thoracic aorta with intact endothelium. All rings were constricted with phenylephrine ($3x10^{-7}$M) at the point labelled PE The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

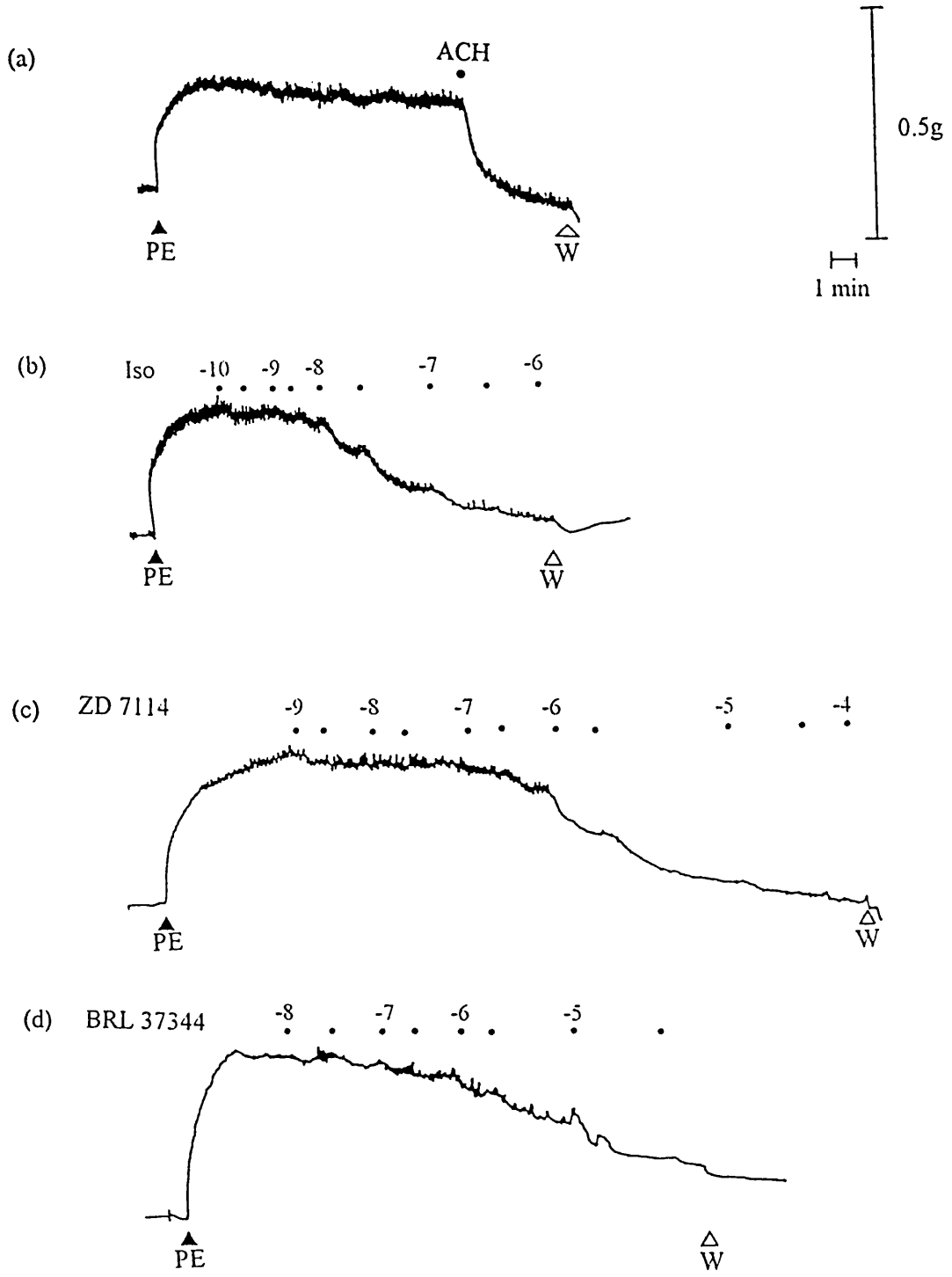


Figure 4.2 Traces showing (a) contractile activity of $3x10^{-8}$M phenylephrine (PE) and typical relaxant effects of cumulative addition of (b) isoprenaline (Iso), (c) ZD 7114 and (d) BRL 37344 in rings of rat pulmonary with intact endothelium. All rings were constricted with phenylephrine ($3x10^{-8}$M) at the point labelled PE The vasodilators were added at the points indicated in concentrations giving half-log molar increments.

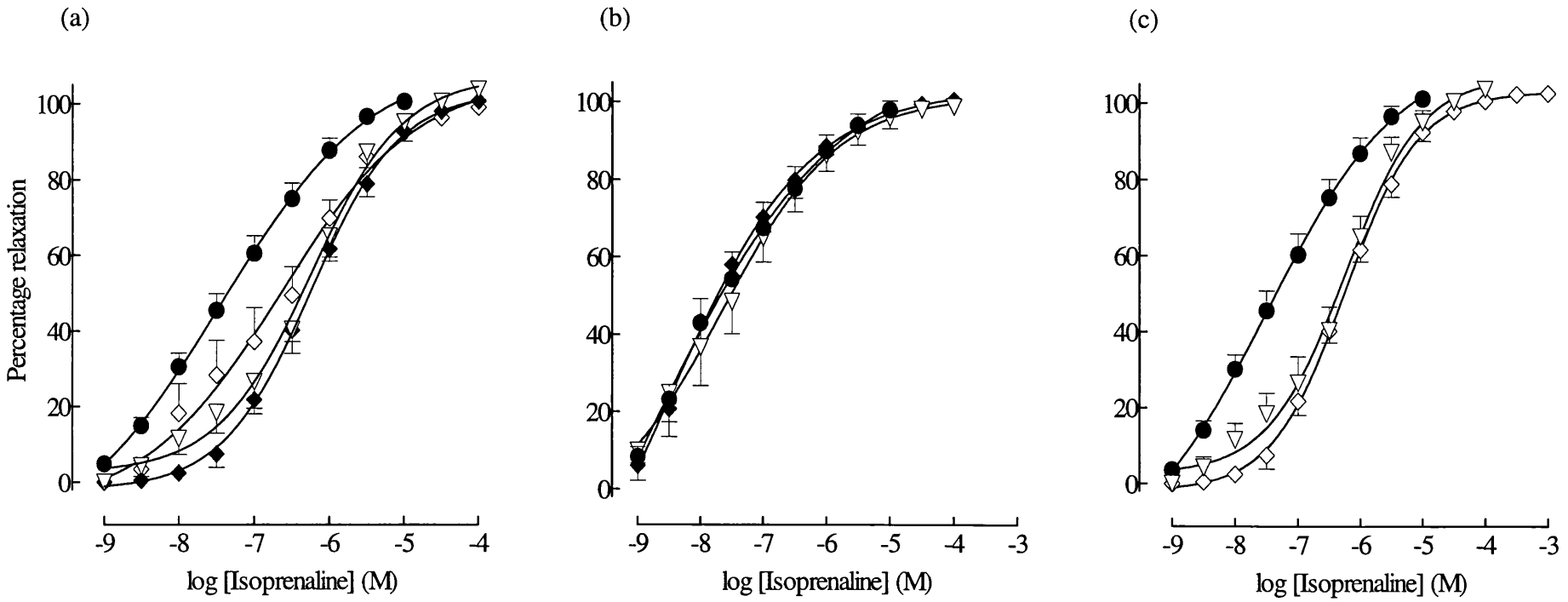


Figure 4.3 Relaxant effects of isoprenaline in rat aortic rings preconstricted with phenylephrine ($3x10^{-7}M$) in the absence and presence of (a) propranolol, (b) atenolol and (c) ICI 118551 (●, control; ▽, $10^{-7}M$; ◆, $10^{-6}M$; ◇, $3x10^{-6}M$). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-7}M$). Points represent the mean±s.e.mean of 4-6 separate experiments.

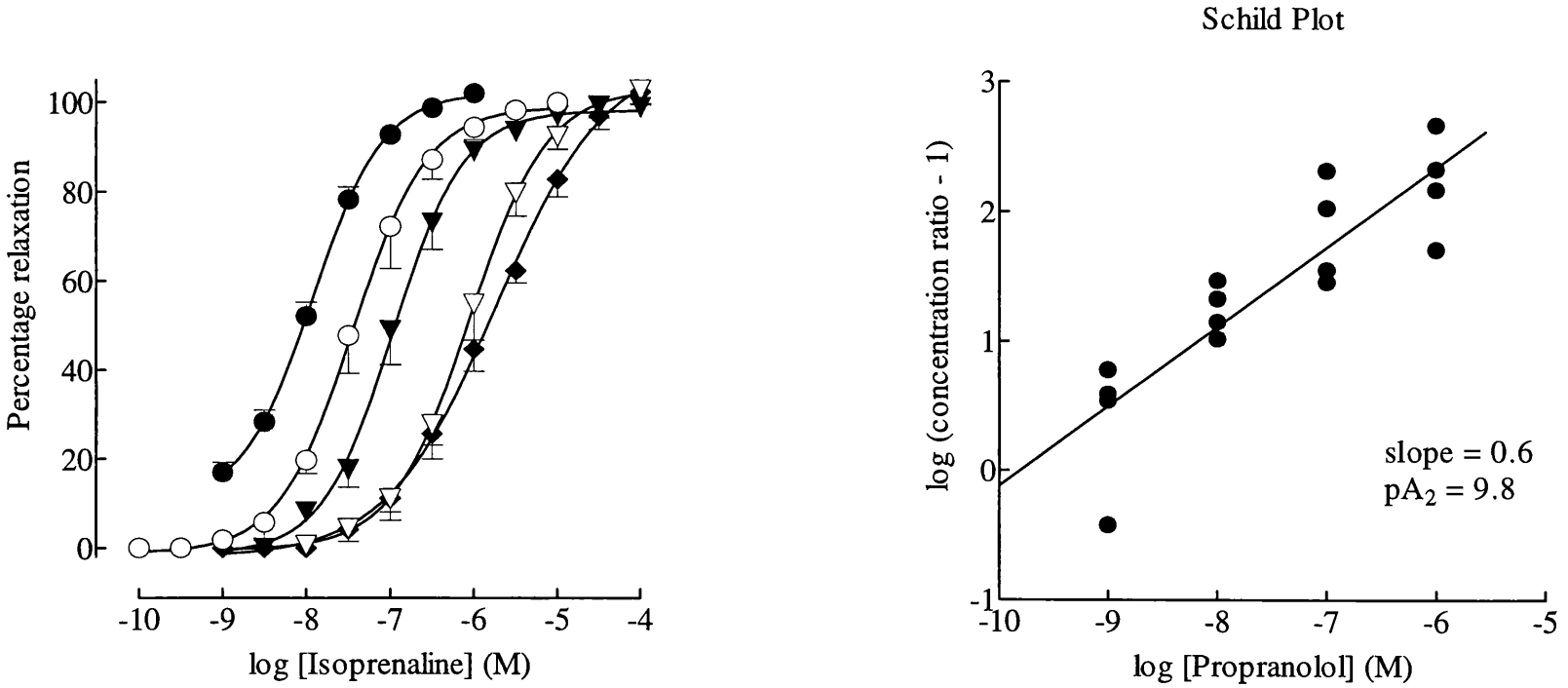


Figure 4.4 Relaxant effects of isoprenaline in rat pulmonary arterial rings preconstricted with phenylephrine ($3x10^{-8}$M) in the absence and presence of propranolol (●, control; ○, 10^{-9}M; ▼, 10^{-8}M; ▽, 10^{-7}M; ◆, 10^{-6}M). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-8}$M). Points represent the mean±s.e.mean of 4-5 separate experiments.

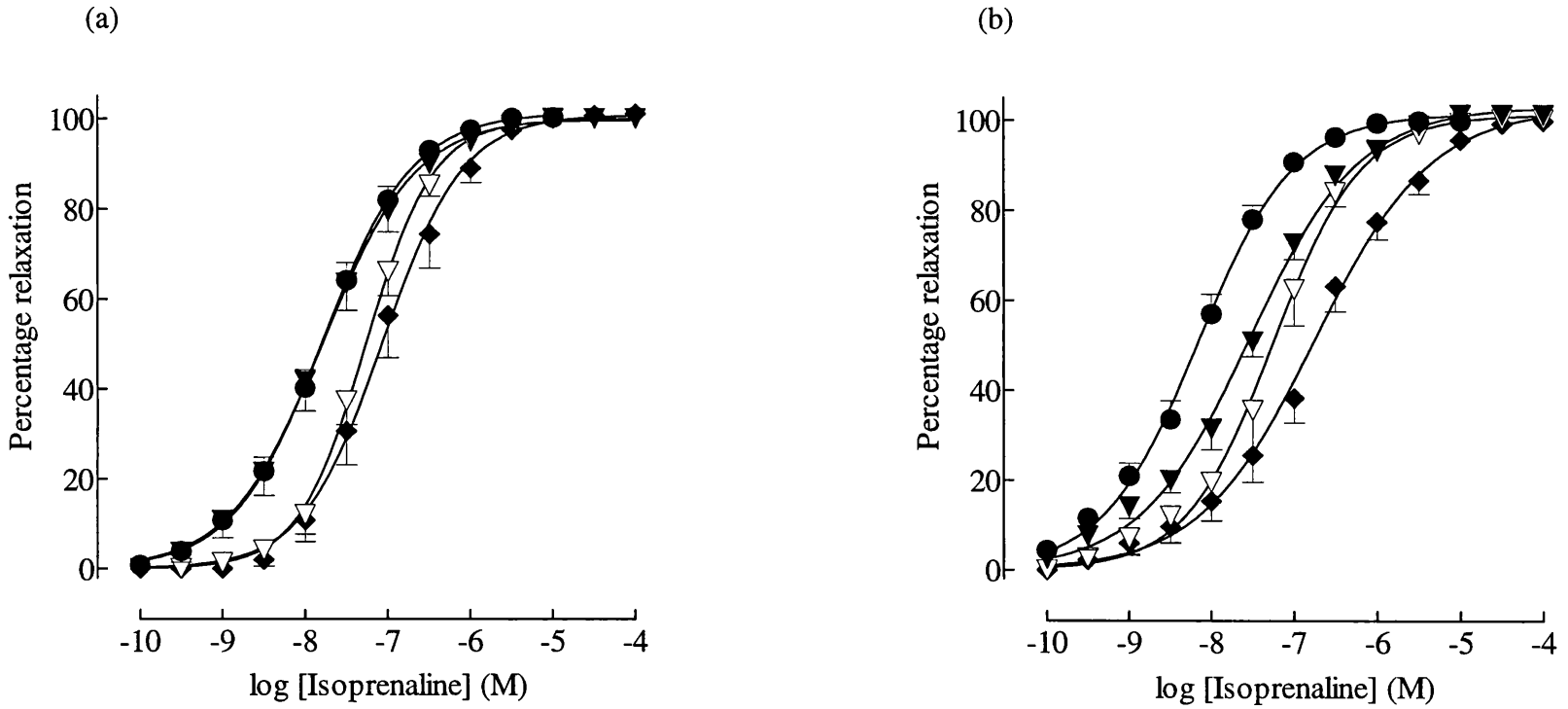


Figure 4.5 Relaxant effects of isoprenaline in rat pulmonary arterial rings preconstricted with phenylephrine ($3x10^{-8}$M) in the absence and presence of (a) atenolol and (b) ICI 118551 (●, control; ▼, 10^{-8}M; ▽, 10^{-7}M; ◆, 10^{-6}M). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-8}$M). Points represent the mean±s.e.mean of 4-5 separate experiments.

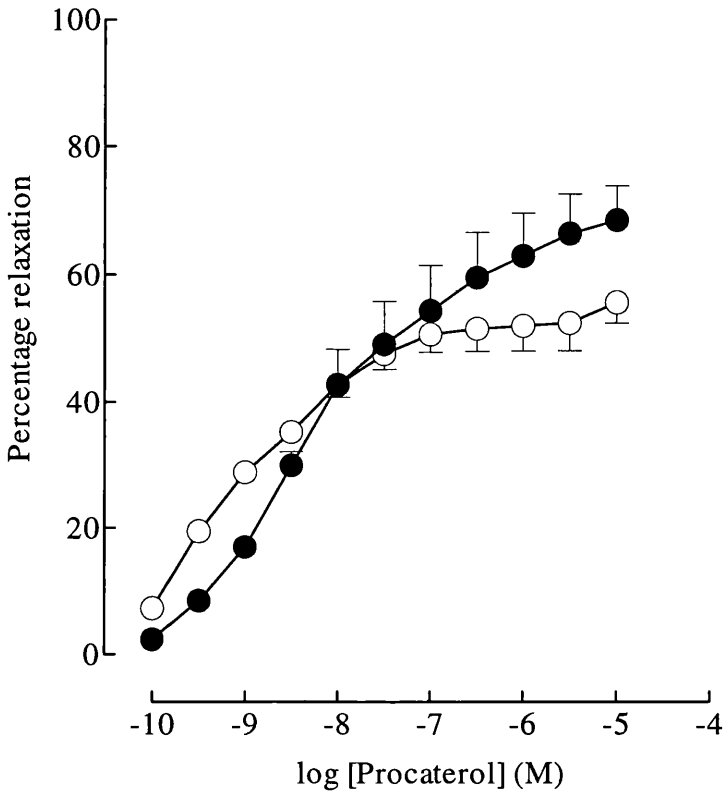


Figure 4.6 Relaxant effects of procaterol in rat aortic rings preconstricted with phenylephrine ($3x10^{-7}M$) in the absence (●) and presence of propranolol $10^{-6}M$ (O). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-7}M$). Points represent the mean±s.e.mean of 5 separate experiments.

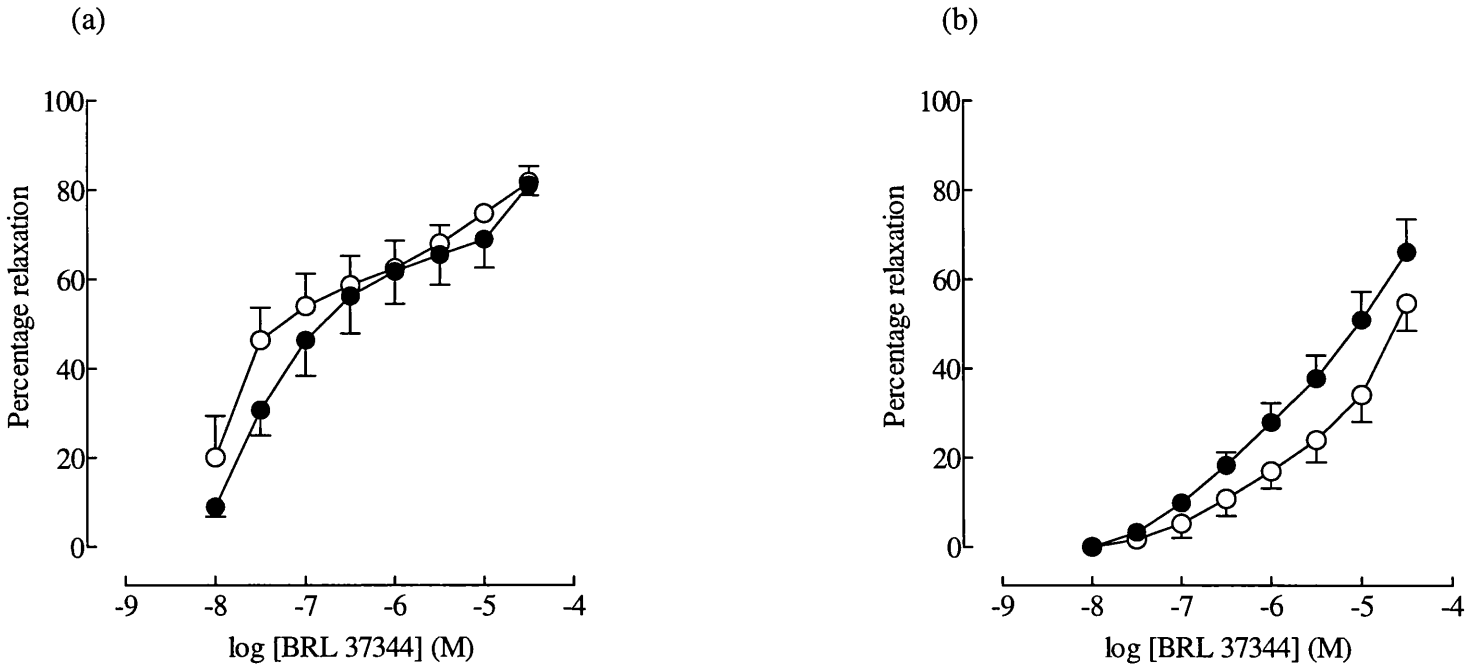


Figure 4.7 Repeat concentration-response curves to BRL 37344 in (a) rat aortic and (b) pulmonary arterial rings preconstricted with phenylephrine ($3x10^{-7}$M and $3x10^{-8}$M respectively; ●, control; ○, repeat curve following 60 min interval). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 4-6 separate experiments.

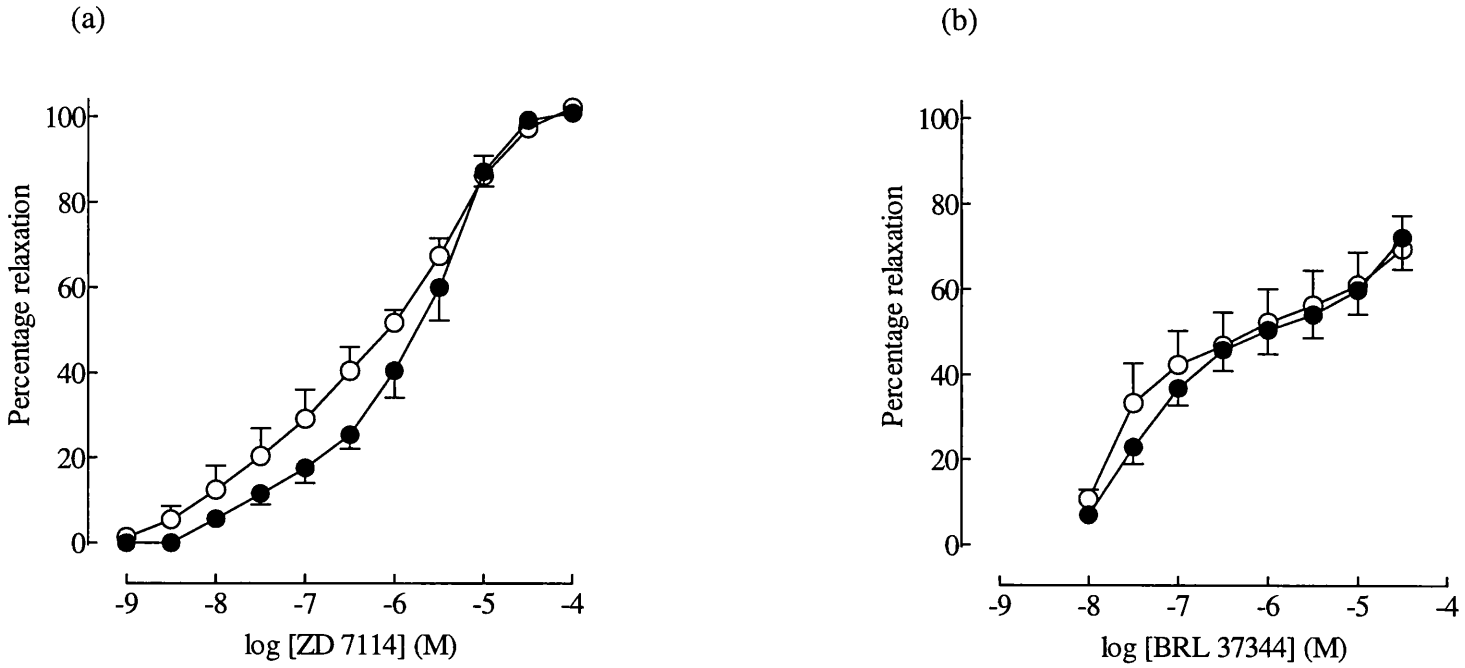


Figure 4.8 Relaxant effect of (a) ZD 7114 and (b) BRL 37344 in rat aortic rings preconstricted with phenylephrine ($3x10^{-7}$M) in the absence (●) and presence of propranolol 10^{-6}M (O). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-7}$M). Points represent the mean±s.e.mean of 6 separate experiments.

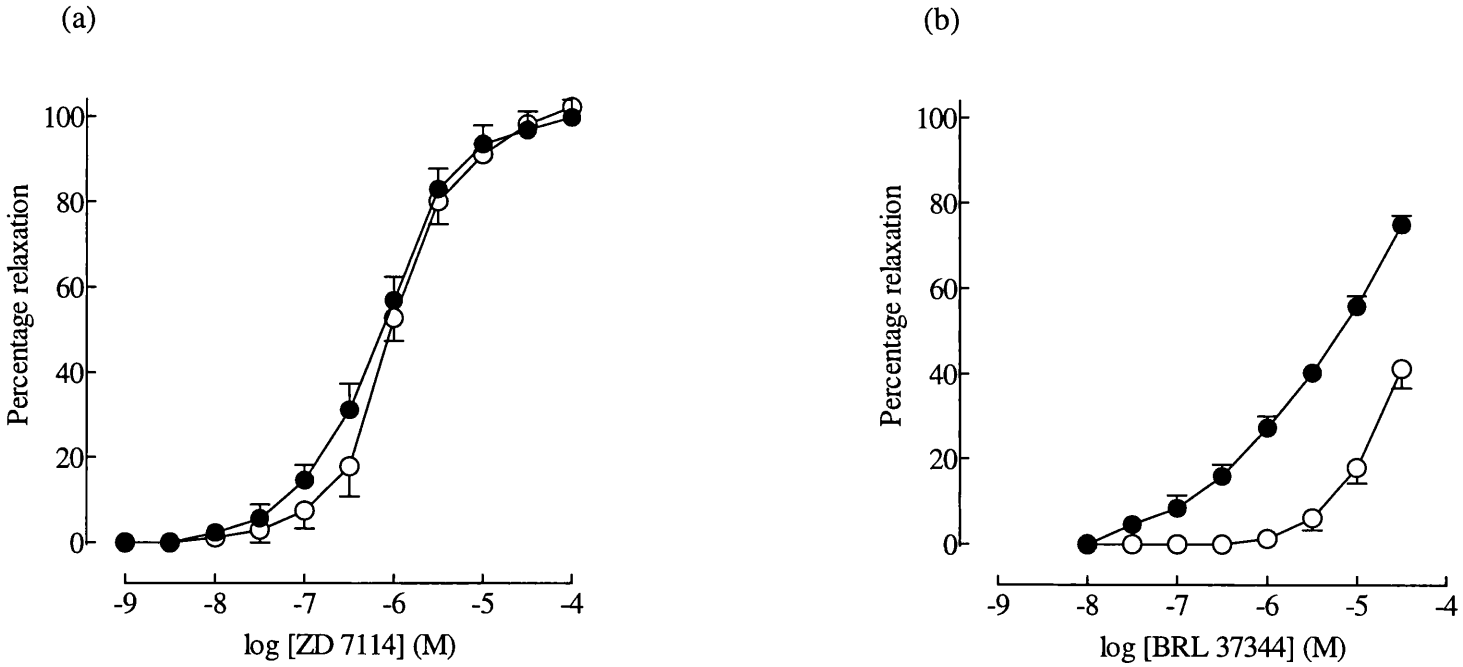


Figure 4.9 Relaxant effect of (a) ZD 7114 and (b) BRL 37344 in rat pulmonary arterial rings preconstricted with phenylephrine ($3x10^{-8}$M) in the absence (●) and presence of propranolol 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-8}$M). Points represent the mean±s.e.mean of 5 separate experiments.

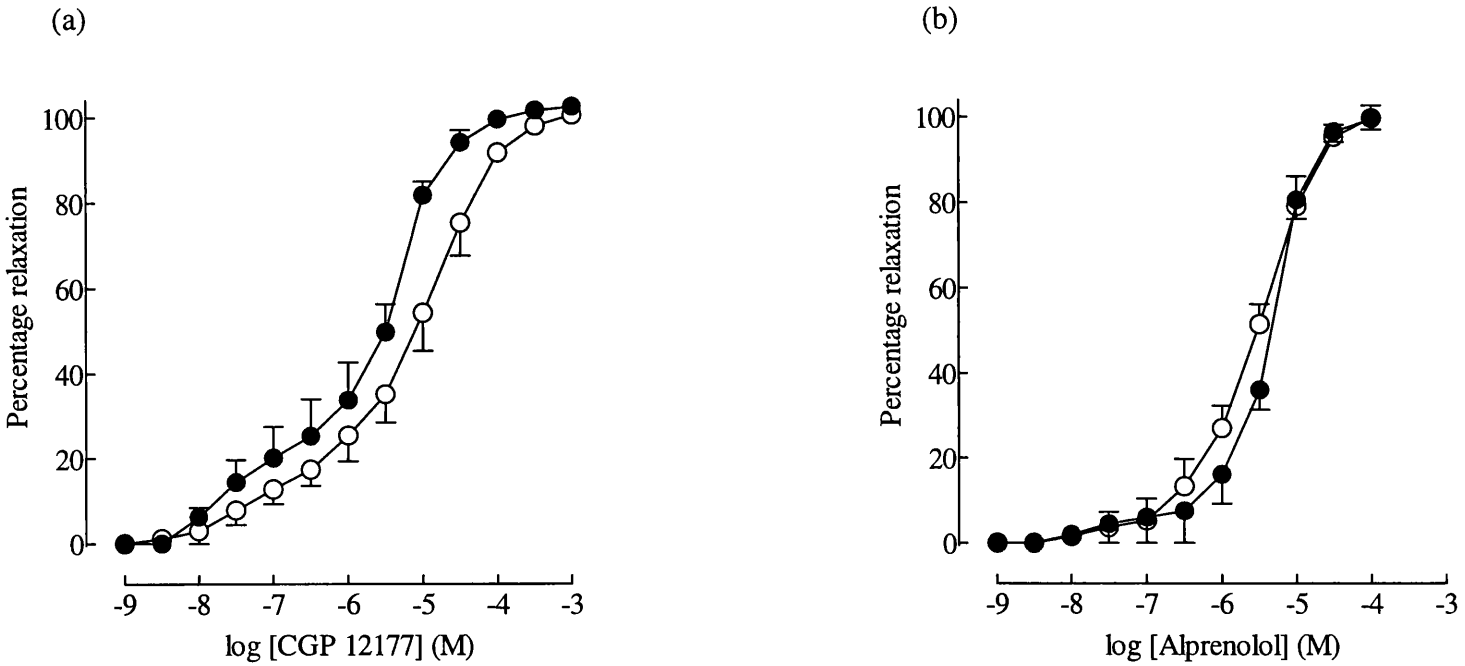


Figure 4.10 Relaxant effect of (a) CGP 12177 and (b) alprenolol in rat aortic rings preconstricted with phenylephrine ($3x10^{-7}$M) in the absence (●) and presence of propranolol 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-7}$M). Points represent the mean±s.e.mean of 4-5 separate experiments.

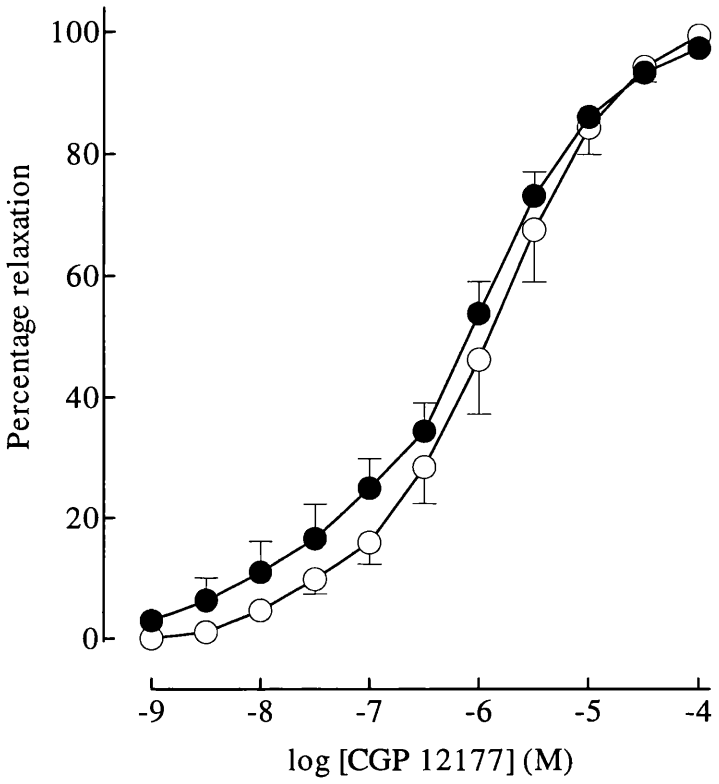


Figure 4.11 Relaxant effects of CGP 12177 in rat pulmonary arterial rings preconstricted with phenylephrine ($3x10^{-8}$M) in the absence (●) and presence of propranolol 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine ($3x10^{-8}$M). Points represent the mean±s.e.mean of 4 separate experiments.

4.3 Discussion

Increasing evidence suggests that in addition to β_1- and/or β_2-adrenoceptors, atypical β-adrenoceptors are also present in the vasculature. The present chapter looked at the possibility of the existence of atypical β-adrenoceptors in another two blood vessels, the rat thoracic aorta and pulmonary artery, the results indicating that atypical β-adrenoceptors are also present in these tissues.

Similar to the rat mesenteric artery (**Chapter 3**), isoprenaline responses were weakly antagonized by propranolol in the rat thoracic aorta, the nature of the antagonism being non-competitive (i.e. increasing shifts of the isoprenaline concentration-response curve were not observed with increasing propranolol concentrations).

The nature of the β-adrenoceptor subtype(s) involved in the 'propranolol-sensitive' component was determined using subtype-selective antagonists. The lack of effect of atenolol suggests that β_1-adrenoceptors do not contribute to the vasorelaxant effect of isoprenaline in the rat thoracic aorta. ICI 118551 antagonized responses to isoprenaline, but as with propranolol, the antagonism was non-competitive, these results being consistent with the idea that ICI 118551 is blocking a small proportion of β_2-adrenoceptors which contribute to the isoprenaline response. The β_2-adrenoceptor agonist, procaterol, was a partial agonist indicating that there may be a low β_2-adrenoceptor reserve in the aorta.

Although propranolol produced a surmountable antagonism of isoprenaline-induced relaxations in the rat pulmonary artery, the antagonism did not satisfy the criteria for competitive antagonism. The Schild plot gave a straight line with a slope of 0.6 which was significantly different from unity.

Atenolol weakly antagonized responses to isoprenaline (approximately 4-fold shift of the isoprenaline concentration-response curve at 10^{-6}M), and non-competitive antagonism was also observed with ICI 118551. These results would therefore confirm those obtained by other authors that the rat pulmonary artery contains a

mixed population of β-adrenoceptor subtypes, i.e., β_2-adrenoceptors appear to predominate together with a β_1-adrenoceptor population (O'Donnell & Wanstall, 1984; Shaul *et al.,* 1990).

Both ZD 7114 and BRL 37344 produced concentration-dependent relaxations of aortic and pulmonary arterial rings, ZD 7114 being the most potent, producing 100% relaxation at 10^{-4}M (pEC_{50} values 5.4 and 6.2 respectively). Limited supplies of BRL 37344 did not enable maximum relaxations to be achieved at the highest concentration used in either tissue, although BRL 37344 appeared to be the least potent of all the agonists used in the present study. BRL 37344 has been demonstrated as a potent stimulant of lipolysis in both brown and white adipose tissues of the rat (Arch *et al.,* 1984; Wilson *et al.,* 1984). In addition, BRL 37344 has been shown to potently relax a range of gastrointestinal smooth muscle preparations, e.g., rat ileum (Growcott *et al.,* 1993b), distal colon (McLaughlin & MacDonald, 1990; Kirkham & Kelly, 1992) and oesophagus (de Boer *et al.,* 1993) and guinea pig ileum (Bond & Clarke, 1988; Growcott *et al.,* 1993a). However, results obtained in the present study indicates that the potency of BRL 37344 appears to be lower compared with values previously reported for other tissues such as the rat distal colon (pEC_{50} 7.3, McLaughlin & MacDonald, 1990; pEC_{50} 7.1, Kirkham & Kelly, 1992) and oesophagus (pEC_{50} 8.3, de Boer *et al.,* 1993). The low potency of BRL 37344 has also been observed in the rat carotid artery (pEC_{50} 4.3, Oriowo, 1994) and mesenteric artery (**Chapter 3**). In addition, McLaughlin & MacDonald (1991) also reported a low potency (5.2) in the rat gastric fundus, but in a recent review a pEC_{50} value of 8.5 was reported by Manara *et al.* (1995b; authors' unpublished results) in the same tissue. The reason for this poor potency of BRL 37344 in rat vasculature compared to other tissues is not known, but may relate to differences in the nature of the atypical β-adrenoceptor. It is unlikely that the poor potency of BRL 37344 observed in rat vasculature relates to a low receptor density since ZD 7114 exhibited full agonist activity in the thoracic aorta, pulmonary (present chapter) and mesenteric artery (**Chapter 3**).

Responses to BRL 37344 were not inhibited by propranolol in the rat thoracic aorta, whereas in the pulmonary artery, BRL 37344 relaxations were antagonized by 10^{-6}M propranolol (approximately ten-fold shift, although a component of this shift was likely to be due to desensitization since a 3-fold shift was observed with repeat curves). This would suggest that a component of the response to BRL 37344 is mediated via β_1- and/or β_2-adrenoceptors. It is interesting to note that while some authors find that responses to BRL 37344 are completely insensitive to blockade with propranolol (guinea pig ileum, Bond & Clarke, 1988; rat carotid artery, Oriowo, 1994), others find that the actions of BRL 37344 may be weakly antagonized by propranolol (rat ileum, Growcott *et al.,* 1993b; rat distal colon, McLaughlin & MacDonald, 1990; rat jejunum, MacDonald *et al.,* 1994). However, the affinity of propranolol in these latter studies was lower than that obtained at β_1- and β_2-adrenoceptors.

The β_1-/β_2-adrenoceptor antagonist, CGP 12177 has been shown to have agonist activity at adipose β_3-adrenoceptors causing lipolysis and thermogenesis (see review, Arch & Kaumann, 1993). In addition, CGP 12177 has been shown to have vasodilator effects in the dog *in vivo* (Tavernier *et al.,* 1992; Berlan *et al.,* 1994) and exhibits cardiostimulant effects in the rat heart *in vitro* (Kaumann & Molenaar, 1996) and *in vivo* (Malinowska & Schlicker, 1996). Furthermore, CGP 12177 is a full agonist in the rat mesenteric artery (Chapter 3) and carotid artery (Oriowo, 1994). In the present chapter, CGP 12177 was also a full agonist in both the rat thoracic aorta (pEC_{50} 5.7) and pulmonary artery (pEC_{50} 6.1). While propranolol (10^{-6}M) antagonized the relaxant effect of CGP 12177 in the rat thoracic aorta (pK_B 6.4), CGP 12177 responses were largely unaffected by propranolol in the pulmonary artery. Alprenolol, another β_1-/β_2-adrenoceptor antagonist, which has been shown to be a full agonist in the guinea pig ileum (Blue *et al.,* 1990) and rat mesenteric artery (**Chapter 3**) also elicited relaxations in the rat thoracic aorta (pEC_{50} 5.3) which were resistant to blockade by propranolol (10^{-7}M).

It was interesting to note that, in the current studies, the concentration-response curves to ZD 7114, BRL 37344 and CGP 12177 were non-sigmoidal in the

rat thoracic aorta, suggesting that these agonists are interacting with more than one site. It seems unlikely that these compounds are interacting with β_1- and/ or β_2-adrenoceptors, as non-sigmoidal concentration-response curves were still observed following pretreatment with a high concentration (i.e., 10^{-6}M) of propranolol. It is not clear why this feature seems to be particularly apparent in the aorta, but not in the pulmonary artery.

Cross-desensitization to agonists has been observed in gastrointestinal tissues pretreated with BRL 37344. For example, in the rat distal colon and gastric fundus, pretreatment of the tissue with BRL 37344 resulted in a rightward shift of the concentration-response curve to isoprenaline and BRL 37344 (McLaughlin & MacDonald, 1990; 1991). However, Oriowo (1994) observed that pretreatment of tissues with BRL 37344 did not reduce either the potency or the maximum response to isoprenaline, CGP 12177 or salbutamol in the rat carotid artery. In the current study, desensitization to BRL 37344 was not observed in the rat thoracic aorta or mesenteric artery (**Chapter 3**), although in the pulmonary artery, a shift of approximately three-fold was observed with consecutive BRL 37344 concentration-response curves. This may suggest that the receptor coupling mechanism is irreversibly affected by BRL 37344 in tissues where desensitization is observed.

Whether or not the atypical β-adrenoceptors identified in rat vasculature belongs to the β_3-adrenoceptors or constitute a different class of atypical β-adrenoceptors remains to be answered. Studies in the rat isolated atria have demonstrated that the cardiostimulant effects of BRL 37344 are mostly mediated through β_1-adrenoceptors and marginally through β_2-adrenoceptors (Kaumann & Molenaar, 1996). Malinowska & Schlicker (1996) also observed that BRL 37344 caused tachycardia in the pithed rat that was blocked by a combination of CGP 20712A and ICI 118551 at concentrations that selectively blocked β_1- and β_2-adrenoceptors. Moreover, Kaumann & Molenaar (1996) demonstrated that the β_3-adrenoceptor antagonist, SR 59230A (Manara *et al.,* 1995a) caused blockade of agonist-induced colonic relaxation of the rat but hardly blocks the third cardiac β-adrenoceptor. Both these groups concluded that the atypical β-adrenoceptor identified

in the rat heart dose not belong to the group of β_3-adrenoceptors previously described. As mentioned, BRL 37344 has a very low potency in the rat thoracic aorta and pulmonary (present chapter), mesenteric (**Chapter 3**) and carotid artery (Oriowo, 1994) compared with values previously reported for other tissues.

To conclude, the low affinity of propranolol for agonist-mediated responses and the relaxations produced by ZD 7114, BRL 37344 and CGP 12177 is consistent with the existence of atypical β-adrenoceptors in the rat thoracic aorta and pulmonary artery. Whether these receptors are belong to the group of β_3-adrenoceptors previously described in adipose tissue and gastrointestinal tissues remains to be established. However, the recent introduction of antagonists selective for the β_3-adrenoceptor will assist in determining this.

Chapter 5

Effector mechanisms for atypical β-adrenoceptor mediated vasorelaxation

5.1 Introduction

The β-adrenoceptors belong to the superfamily of receptors that contain seven transmembrane domains and are coupled to G-proteins. Like β_1- and β_2-adrenoceptors, the β_3-adrenoceptor is known to be linked to adenylate cyclase activation via the stimulatory G_s protein, leading to the production of the second messenger cyclic AMP (Zaagsma & Hollenga, 1991).

Generally, isoprenaline and other β-adrenoceptor agonists have been regarded as endothelium-independent vasodilators mediating their effects by increasing cyclic AMP in the smooth muscle (Furchgott & Martin, 1985; Furchgott & Vanhoutte, 1989). This rise in cyclic AMP leads to activation of protein kinase A and subsequent myosin light chain kinase activation within the smooth muscle. Therefore, such a mechanism of vasorelaxation would not be dependent on the presence of an intact endothelium. However, increasing evidence suggests that at least part of the response to β-adrenoceptor agonists is endothelium-dependent (Kamata *et al.,* 1989; Gardiner *et al.,* 1991a, b; Gray & Marshall, 1992). These findings raised the possibility that the relaxant response to agonists such as isoprenaline is mediated partly by endothelial nitric oxide, which is released in response to β-adrenoceptors located on the endothelium.

Nitric oxide is synthesized from the terminal guanidino nitrogen of the amino acid, L-arginine. The enzyme, nitric oxide synthase, catalyzes the *de novo* synthesis of nitric oxide from L-arginine in vascular endothelial cells. N^G-nitro-L-arginine (L-NOARG) has been shown to be an effective inhibitor of the constitutive form of nitric oxide synthase (Ishii *et al.,* 1990; Gross *et al.,* 1990; Mulsch & Busse, 1990).

Compared with N^G-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase inhibitor used in many earlier experiments, L-NOARG is 10-100 times more potent as an inhibitor of the constitutive form of nitric oxide synthase (Ishii *et al.,* 1990; Gross *et al.,* 1990; Mulsch & Busse, 1990).

Nitric oxide stimulates the soluble form of guanylate cyclase leading to increases in the intracellular level of cyclic GMP (Gruetter *et al.,* 1981; Griffith *et al.,* 1985). It has been suggested that one mechanism by which nitric oxide mediates vascular relaxation is through this resulting increase in cyclic GMP levels. A cyclic GMP mechanism is supported by observations which show that 'arterial tissue' levels of cyclic GMP correlate well with the level of relaxation induced by a number of endothelium-dependent vasodilators (Rapoport & Murad, 1983; Ignarro *et al.,* 1984; Griffith *et al.,* 1985).

Besides being linked to cyclic AMP and nitric oxide/cyclic GMP mechanisms, increasing evidence suggests that β-adrenoceptors can induce vasorelaxation via a mechanism involving a coupling of the β-adrenoceptor and ATP-sensitive potassium channels (K_{ATP}-channels). Glibenclamide, an K_{ATP}-channel blocker (Sturgess *et al.,* 1985), has been found to antagonize relaxant responses to isoprenaline in the rat isolated perfused mesenteric arterial bed (Randall & McCulloch, 1995) and in rat aortic rings (Hüsken *et al.,* 1994), while electrophysiological evidence has demonstrated that isoprenaline causes hyperpolarization of the canine saphenous vein which is sensitive to glibenclamide (Nakishima & Vanhoutte, 1995). Also, a study on the rat basilar artery *in vivo* has demonstrated that noradrenaline acts via β_1-adrenoceptors to cause glibenclamide-sensitive vasodilatation (Kitazono *et al.,* 1993).

The objective of the present chapter was to investigate the role of the endothelium in the relaxant response to isoprenaline in the rat thoracic aorta, mesenteric and pulmonary artery. The effects of L-NOARG and glibenclamide on isoprenaline responses were also examined. In addition, the levels of cyclic AMP associated with isoprenaline- and ZD 7114-mediated vasorelaxation was studied in the rat thoracic aorta.

5.2 Materials and methods

5.2.1 Functional studies

Relaxations induced by isoprenaline and ZD 7114 were studied in endothelium-intact and -denuded rings of rat thoracic aorta, mesenteric and pulmonary artery. Cumulative concentration-response curves to these agonists were carried out using phenylephrine (or noradrenaline in some experiments) to preconstrict tissues. The effect of the NO synthase inhibitor, L-NOARG, on the contractions elicited by phenylephrine or noradrenaline and the relaxations induced by isoprenaline was also studied.

5.2.2 Cyclic AMP studies

In order to study the cyclic AMP accumulations associated with the relaxations induced by isoprenaline and ZD 7114, rat aortic rings were prepared as described in **Chapter 2** with either the endothelium remaining intact or being removed. To determine the optimum time-point for cyclic AMP accumulation, a single concentration of the agonist giving approximately 80% relaxation of the tone induced by phenylephrine ($3x10^{-7}M$) was added and the tissues removed into liquid nitrogen at various time-points. Cyclic AMP levels were determined by scintillation proximity assay (SPA; Amersham) using the acetylation protocol (**Chapter 2**). Some rings were incubated for 15 min with propranolol ($10^{-6}M$) before being contracted with phenylephrine and relaxed with a single concentration of either isoprenaline or ZD 7114. These tissues were removed into liquid nitrogen to determine the effect of propranolol on cyclic AMP levels.

5.3 Results

5.3.1 Functional studies

Aortic, mesenteric and pulmonary arterial rings denuded of endothelium showed no spontaneous activity (**Figure 5.1**), as was observed in intact rings. Endothelium-denuded rings were preconstricted with a ten-fold lower concentration of phenylephrine ($3x10^{-8}M$, $10^{-7}M$ and $3x10^{-9}M$ for aortic, mesenteric and pulmonary arterial rings respectively) so that the size of the contraction matched that obtained in intact rings ($3x10^{-7}M$, $10^{-6}M$ and $3x10^{-8}M$ phenylephrine respectively), unless

otherwise stated. Intact rings were relaxed by acetylcholine (10^{-6}M) by greater than 80%, while endothelium-denuded rings showed no response to acetylcholine.

5.3.2 Relaxations to isoprenaline in endothelium-denuded rings

(a) Rat thoracic aorta

(i) Maintaining size of phenylephrine-induced contractions

In this set of experiments, the size of the contractile response to phenylephrine in endothelium-denuded rings matched that obtained in intact rings. Isoprenaline (10^{-9}M-10^{-5}M) relaxed endothelium-denuded aortic rings, producing a maximum relaxation of approximately 51% of the phenylephrine ($3x10^{-8}$M)-induced contraction (**Figure 5.2.a**).

(ii) Tissues precontracted with phenylephrine or noradrenaline

In contrast to the results above, Gray & Marshall (1992) showed a complete lack of vascular relaxation produced by isoprenaline in rat aortic rings after removal of the endothelium. In this study, noradrenaline (10^{-7}M) was used as a preconstrictor (Gray & Marshall, 1992). In addition, O'Donnell & Wanstall (1987) have demonstrated that the choice and concentration of the preconstrictor can affect the responses to vasodilators in the rat thoracic aorta. In order to investigate this, concentration-response curves to isoprenaline were constructed using either $3x10^{-7}$M phenylephrine or 10^{-7}M noradrenaline in both endothelium-intact and -denuded rings. The contractile response to both phenylephrine ($3x10^{-7}$M) and noradrenaline (10^{-7}M) was significantly increased by removal of the endothelium (from 0.9±0.2g to 1.5±0.1g, n=4 and 1.1±0.1g to 1.6±0.1g, n=4 respectively).

pEC_{50} values for isoprenaline in endothelium-intact rings were similar whether $3x10^{-7}$M phenylephrine or 10^{-7}M noradrenaline was used as the contractile agent (7.4±0.1, n=4 and 7.1±0.2, n=4 respectively). Isoprenaline (10^{-9}M-10^{-4}M) produced relaxations of endothelium-denuded rings, the concentration-response curve to isoprenaline being significantly shifted to the right of the control curve and a decrease in the maximum response from 99% to 67% of the tone induced by phenylephrine ($3x10^{-7}$M) was observed (**Figure 5.2.b**). With 10^{-7}M noradrenaline as

the preconstrictor, isoprenaline-induced relaxations were significantly reduced in endothelium-denuded rings, a maximum response of approximately 25% was observed compared with a response of 98% in endothelium-intact rings (**Figure 5.2.c**).

(b) Rat mesenteric artery

(i) Maintaining size of phenylephrine-induced contractions

The size of the contraction in endothelium-denuded mesenteric arterial rings (10^{-7}M phenylephrine, 0.9±0.2g) matched that obtained in rings with intact endothelium (10^{-6}M phenylephrine, 1.0±0.2g). Isoprenaline (10^{-9}M-10^{-4}M) relaxed endothelium-denuded rings, producing a maximum relaxation of approximately 41% of the phenylephrine (10^{-7}M)-induced contraction (**Figure 5.3.a**). Contractions to isoprenaline (at concentrations greater than 10^{-6}M) were also observed in endothelium-denuded rings (**Figure 5.3.a**).

(ii) Tissues precontracted with noradrenaline

Noradrenaline ($3x10^{-7}$M) evoked an increase in tone of rat mesenteric arterial rings with intact endothelium to 0.6±0.1g (n=5). In endothelium-denuded rings, the contractor response was significantly increased to 1.1±0.1g (n=5) using the same concentration of noradrenaline ($3x10^{-7}$M). The concentration-response curve to isoprenaline (10^{-8}M-10^{-3}M) was shifted to the right (greater than 100-fold) and the maximum response to isoprenaline was reduced to approximately 62% in rings denuded of endothelium (**Figure 5.3.b**).

(c) Rat pulmonary artery

The size of the contractile response in endothelium-denuded pulmonary arterial rings ($3x10^{-9}$M phenylephrine, 0.24±0.03g) was similar to that obtained in rings with intact endothelium ($3x10^{-8}$M phenylephrine, 0.23±0.02g). Isoprenaline (10^{-10}M-10^{-5}M) relaxed pulmonary arterial rings independently of the endothelium, although a small rightward shift of approximately five-fold was observed with endothelium-denuded rings (pEC_{50} values being 7.7±0.1 and 7.1±0.2 in the presence and absence of the endothelium respectively; **Figure 5.4**).

5.3.3 Effect of propranolol on isoprenaline responses in endothelium-denuded rat aortic and mesenteric arterial rings

Propranolol (10^{-6}M) was found to have no significant effect on the isoprenaline concentration-response curve in endothelium-denuded aortic rings (**Figure 5.5.a**), whereas propranolol (10^{-6}M) shifted the relaxant response to isoprenaline to the right in mesenteric arterial rings while decreasing the maximum response from 41% to 12% of the phenylephrine-induced tone (**Figure 5.5.b**).

5.3.4 Effect of L-NOARG on isoprenaline responses in endothelium-intact rings

(a) Rat thoracic aorta

(i) Maintaining size of phenylephrine-induced contractions

Arginine analogues such as N^G-nitro-L-arginine (L-NOARG) have been shown to inhibit the synthesis of nitric oxide by competing with the substrate L-arginine for the enzyme nitric oxide synthase (Moore *et al.*, 1990; Ishii *et al.*, 1990). Responses to isoprenaline were unaffected by L-NOARG (10^{-5}M), the NO synthase inhibitor, in aortic rings with intact endothelium (pEC_{50} values being 7.4±0.1 and 7.4±0.1, n=4 in the absence and presence of L-NOARG respectively; **Figure 5.6.a**).

(ii) Tissues precontracted with phenylephrine or noradrenaline

In experiments where $3x10^{-7}$M phenylephrine was used to contract aortic rings in the presence of L-NOARG (10^{-5}M), an increase in the contractile response was observed (from 0.8±0.1g to 1.6±0.2g in the absence and presence of L-NOARG respectively). In addition, L-NOARG (10^{-5}M) shifted the relaxant response to isoprenaline to the right (approximately 27-fold) and produced a decrease in the maximum response of about 10% (**Figure 5.6.b**). In experiments where 10^{-7}M noradrenaline was used to precontract aortic rings, L-NOARG (10^{-5}M) shifted the isoprenaline response to the right while decreasing the maximum response from 98% to 58% (**Figure 5.6.c**). These last set of results are similar to those obtained by Gray & Marshall (1992). Therefore, it appears that the choice and concentration of the contractile agent also influences the effect of L-NOARG on isoprenaline responses.

(b) Rat mesenteric artery

The size of the contractile response to phenylephrine in the absence of 10^{-5}M L-NOARG (10^{-6}M phenylephrine, 0.8±0.1g, n=4) was comparable to that obtained in the presence of L-NOARG (10^{-7}M phenylephrine, 0.9±0.2g, n=4). L-NOARG (10^{-5}M) shifted the relaxant response to isoprenaline to the right while decreasing the maximum response from 99% to 51% of the tone induced by phenylephrine (**Figure 5.7**).

(c) Pulmonary artery

Contractions to phenylephrine ($3x10^{-8}$M and $3x10^{-9}$M) in pulmonary arterial rings were similar in the absence (0.24±0.03g) and presence of 10^{-5}M L-NOARG (0.21±0.03g). L-NOARG produced a two-fold shift in the isoprenaline concentration-response curve and reduced the maximum response to 78% (**Figure 5.8**).

5.3.3 Relaxations to ZD 7114 in endothelium-denuded rings

In all three tissues, ZD 7114 relaxed rings independently of the presence of the endothelium, the maximal relaxation being 100% of the phenylephrine-induced contraction (**Figure 5.9**). In the mesenteric artery, ZD 7114 concentration-response curves were similar in the presence and absence of the endothelium (pEC_{50} values being 5.3±0.1 and 5.4±0.6 respectively; **Figure 5.9.b**). However, in the aorta, a small rightward shift of the bottom portion of the ZD 7114 concentration-response curve was observed on removal of the endothelium (**Figure 5.9.a**), whereas a leftward shift of approximately two-fold was seen in endothelium-denuded pulmonary arterial rings (pEC_{50} values being 6.2±0.1 and 6.5±0.1 in the presence and absence of the endothelium respectively; **Figure 5.9.c**).

5.3.4 Effect of glibenclamide on isoprenaline responses in endothelium-intact aortic and mesenteric arterial rings

Glibenclamide, an K_{ATP}-channel blocker (Sturgess *et al.*, 1985) has been shown to antagonize isoprenaline responses in rat aortic rings (Hüsken *et al.*, 1994) and to inhibit relaxations to isoprenaline, dobutamine (β_1-selective) and terbutaline (β_2-selective) in the isolated perfused superior mesenteric arterial bed of the rat

(Randall & McCulloch, 1995). Both these studies indicate that a component of β-adrenoceptor mediated vasodilatation is linked to activation of K_{ATP}-channels. However, in the rat thoracic aorta, relaxant responses were largely unaffected by glibenclamide (10^{-6}M) and a small increase in the maximal relaxation was observed in the presence of glibenclamide (pEC_{50} values 7.2±0.2 and 7.2±0.2 in the absence and presence of glibenclamide respectively; **Figure 5.10.a**). In mesenteric arterial rings, glibenclamide (10^{-6}M) caused a small two-fold shift of the isoprenaline concentration-response curve to the right (pEC_{50} values being 6.2±0.2 and 6.1±0.3 in the absence and presence of glibenclamide respectively; **Figure 5.10.b**).

5.3.5 Cyclic AMP studies in the rat thoracic aorta

(a) Time course for cyclic AMP accumulation

Cyclic AMP levels in rat thoracic aortic rings with intact endothelium constricted with phenylephrine ($3x10^{-7}$M) were 534±66 fmol mg^{-1} protein. The levels of cyclic AMP in endothelium-denuded rings (preconstricted with a ten-fold lower concentration of phenylephrine, $3x10^{-8}$M) were not significantly different (522±85 fmol mg^{-1} protein) to endothelium-intact rings.

Isoprenaline (10^{-6}M) caused 78±8% relaxation at 180s in rings with intact endothelium. As this relaxant response developed, an increase in cyclic AMP levels was observed over 60s (838±362 fmol mg^{-1} protein; **Figure 5.11**).

The β_3-adrenoceptor agonist, ZD 7114 (10^{-5}M), caused a maximum relaxation of 64±4% which developed over 180s. As this relaxant response developed, cyclic AMP levels were increased 2-fold (1101±302 fmol mg^{-1} protein) at 60s above basal levels (**Figure 5.12**).

(b) Effect of endothelium removal on cyclic AMP levels induced by isoprenaline and ZD 7114

In rings denuded of endothelium, isoprenaline (10^{-6}M) produced a relaxant response of 24±2% in phenylephrine-($3x10^{-8}$M) constricted rings at 60s. However,

cyclic AMP levels measured at this time point did not differ significantly from basal levels in denuded rings (522±85 fmol mg^{-1} protein; **Figure 5.13**).

In the case of ZD 7114 (10^{-5}M), a relaxant response of 12±3% was produced at 60s and cyclic AMP levels (417±70 fmol mg^{-1} protein) were similar to those of control levels in endothelium-denuded rings (**Figure 5.13**).

(c) Effect of propranolol on relaxations and cyclic AMP levels induced by isoprenaline

In these experiments, the concentration of isoprenaline was increased by 30-fold, i.e., to $3x10^{-5}$M, so that the degree of relaxation in the absence of propranolol was comparable to that produced in the presence of propranolol (10^{-6}M). Control cyclic AMP levels in aortic rings in the presence of propranolol (10^{-6}M) were 692±147 fmol mg^{-1} protein. At 180s, isoprenaline ($3x10^{-5}$M) caused a relaxant response of 65±6%. Levels of cyclic AMP were elevated 2-fold (1389±481 fmol mg^{-1} protein) at 15s (**Figure 5.14**).

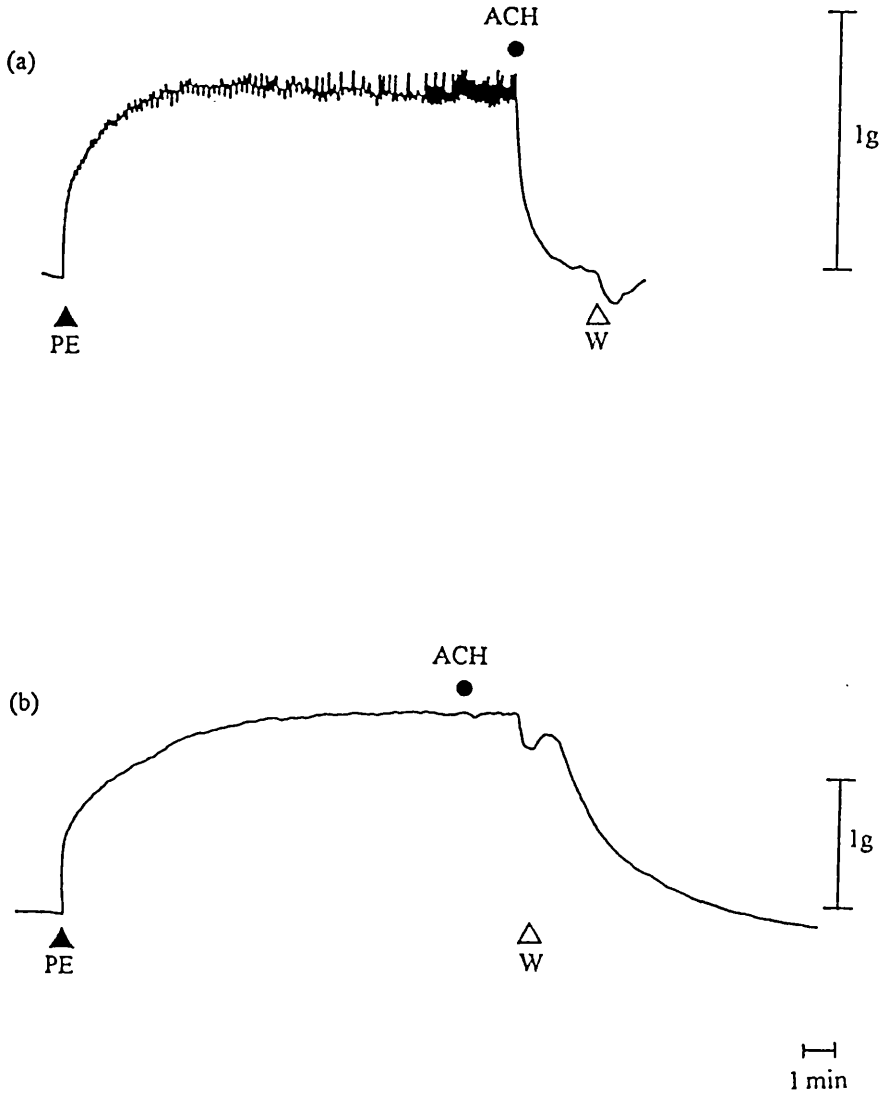


Figure 5.1 Traces showing contractile activity of $3x10^{-7}$M phenylephrine (PE) in rings of rat thoracic aorta with (a) intact endothelium and (b) denuded of endothelium. Note the absence of spontaneous activity and lack of relaxation to 10^{-6}M acetylcholine (ACH) in the ring denuded of endothelium when compared to the intact ring.

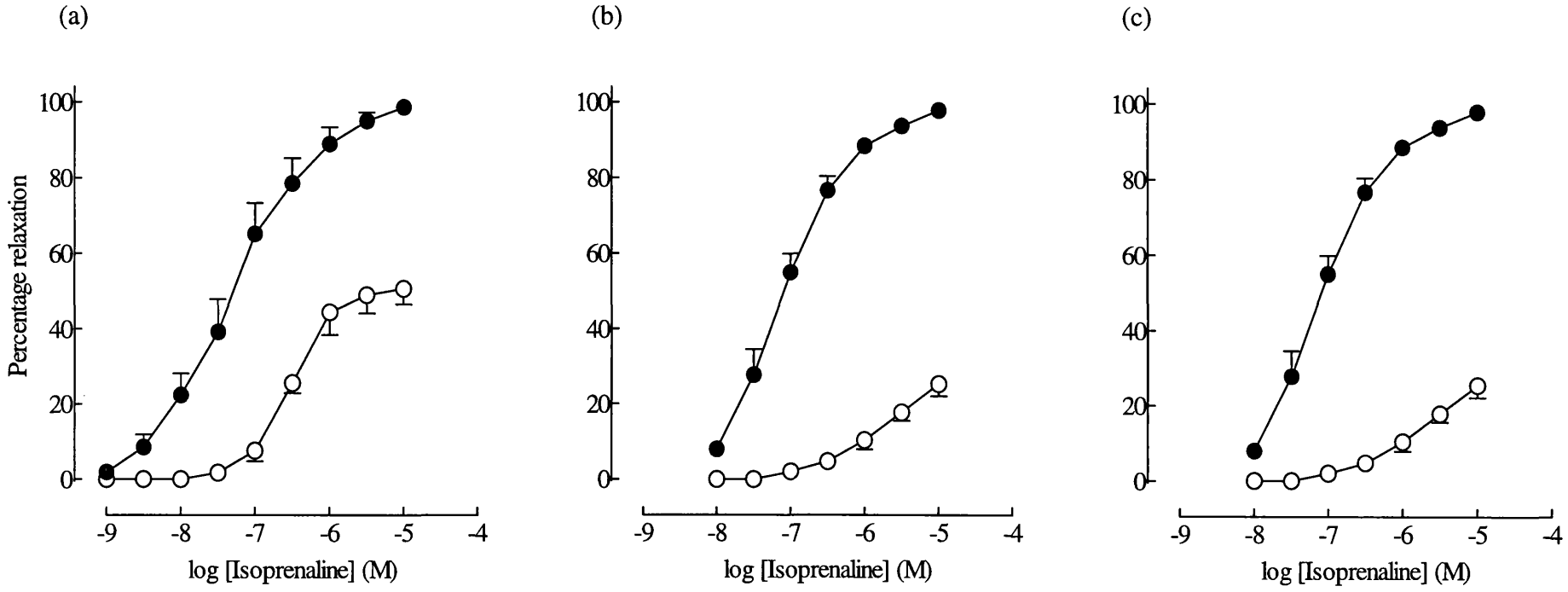


Figure 5.2 The effect of removal of endothelium on vasorelaxations induced by isoprenaline in rat aortic rings (●, endothelium-intact; O, endothelium-denuded) preconstricted with (a) phenylephrine ($3x10^{-7}$M, control and $3x10^{-8}$M, endothelium-denuded), (b) phenylephrine ($3x10^{-7}$M) or (c) noradrenaline (10^{-7}M). Results are expressed as percentage relaxation of the tone induced by phenylephrine or noradrenaline. Points represent the mean±s.e.mean of 4-6 separate experiments.

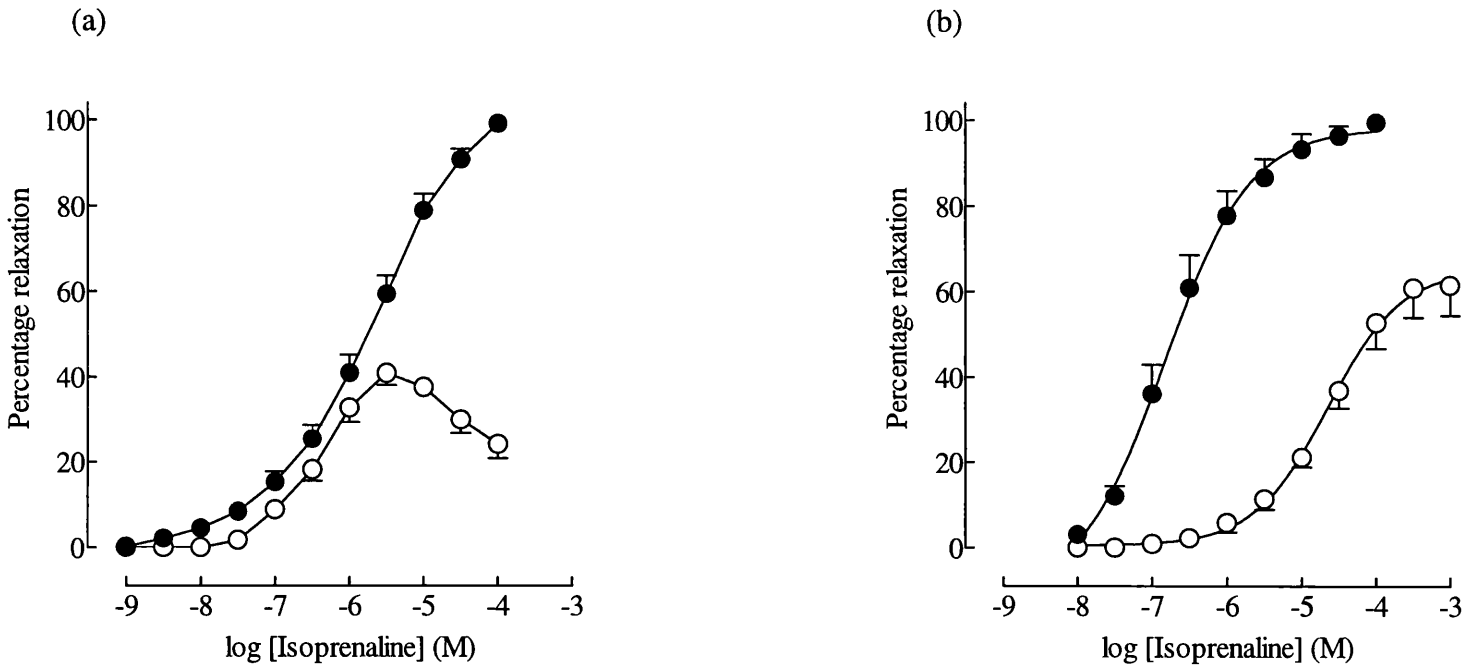


Figure 5.3 The effect of removal of endothelium on vasorelaxation induced by isoprenaline in rat mesenteric arterial rings (●, endothelium-intact; ○, endothelium-denuded) preconstricted with (a) phenylephrine (10^{-6}M, control and 10^{-7}M, endothelium-denuded) or (b) noradrenaline ($3x10^{-7}$M). Results are expressed as percentage relaxation of the tone induced by phenylephrine or noradrenaline. Points represent the mean±s.e.mean of 4-6 separate experiments.

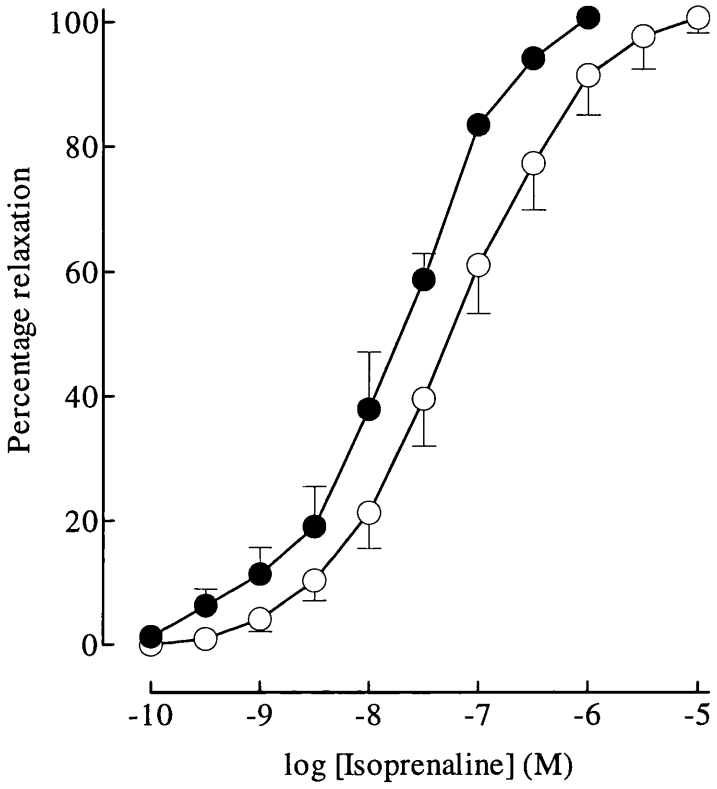


Figure 5.4 The effect of removal of endothelium on vasorelaxations induced by isoprenaline in rat pulmonary arterial rings (●, endothelium-intact; ○, endothelium-denuded) preconstricted with phenylephrine ($3x10^{-8}$M, control and $3x10^{-9}$M, endothelium-denuded). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 5 separate experiments.

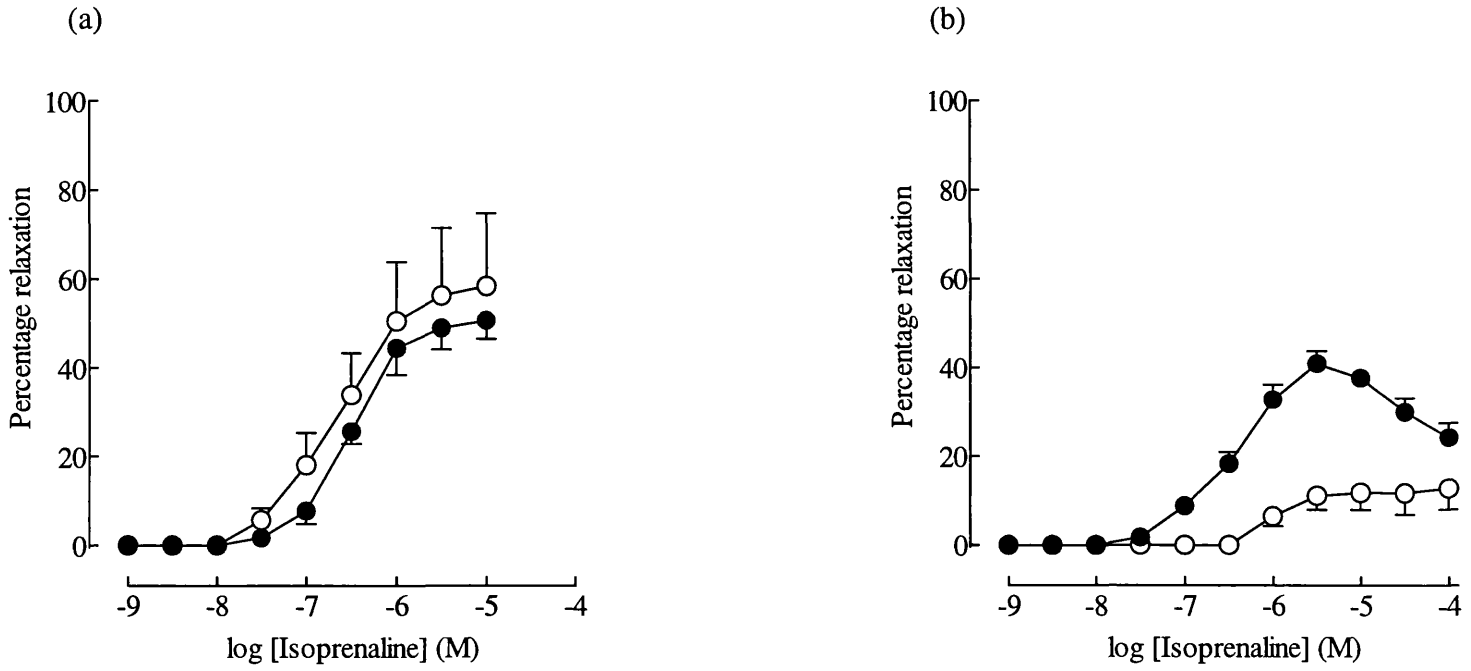


Figure 5.5 Relaxant effects of isoprenaline in endothelium-denuded (a) rat aortic and (b) mesenteric arterial rings, both preconstricted with phenylephrine (either $3x10^{-8}$M or 10^{-7}M respectively) in the absence (●) and presence of propranolol 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 4-5 separate experiments.

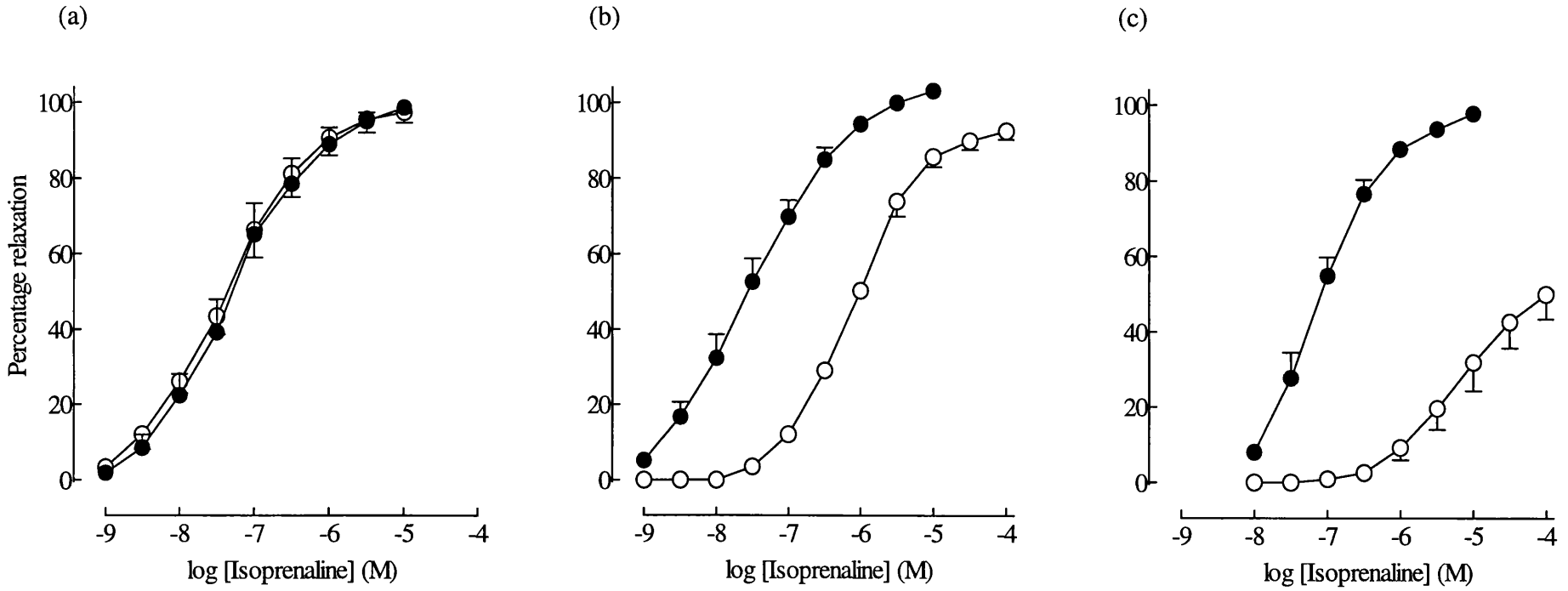


Figure 5.6 Relaxant effect of isoprenaline in rat aortic rings in the absence (●) and presence of L-NOARG 10^{-5}M (○) preconstricted with (a) phenylephrine ($3x10^{-7}$M, control and $3x10^{-8}$M, in the presence of L-NOARG), (b) phenylephrine ($3x10^{-7}$M) or (c) noradrenaline (10^{-7}M). Results are expressed as percentage relaxation of the tone induced by phenylephrine or noradrenaline. Points represent the mean±s.e.mean of 4-6 separate experiments.

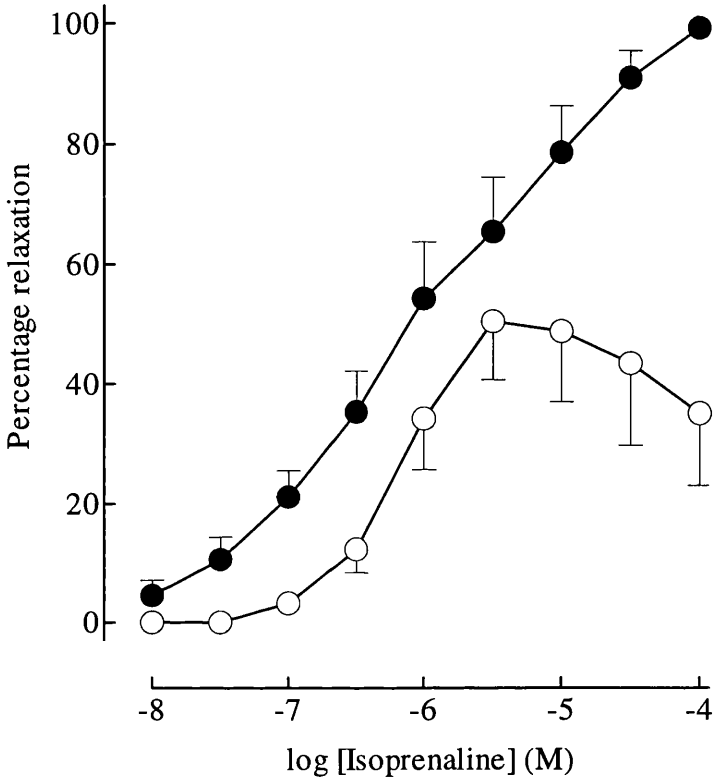


Figure 5.7 Relaxant effects of isoprenaline in rat mesenteric arterial rings in the absence (●) and presence of L-NOARG 10^{-5}M (O) preconstricted with (a) phenylephrine (10^{-6}M, control and 10^{-7}M, in the presence of L-NOARG). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 5 separate experiments.

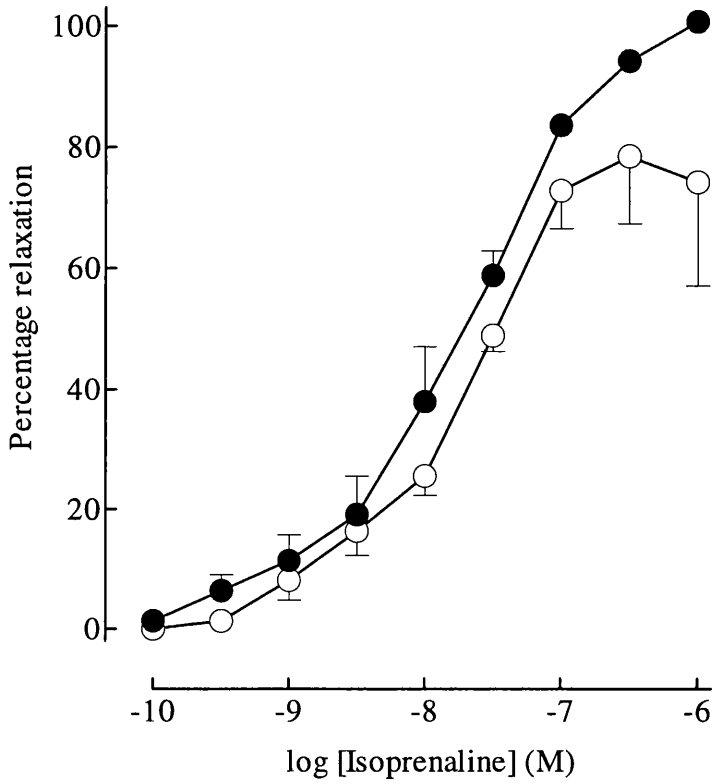


Figure 5.8 Relaxant effects of isoprenaline in rat pulmonary arterial rings in the absence (●) and presence of L-NOARG 10^{-5}M (O) preconstricted with (a) phenylephrine ($3x10^{-8}$M, control and $3x10^{-9}$M, in the presence of L-NOARG). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 6 separate experiments.

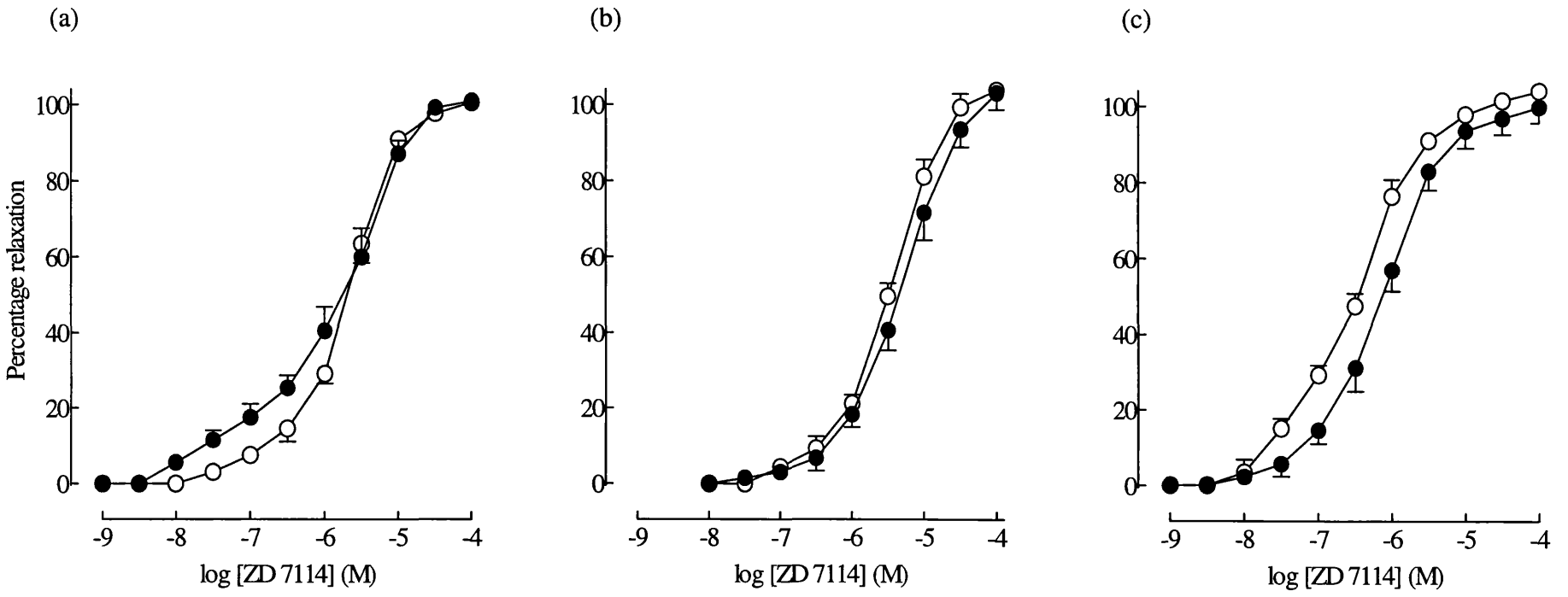


Figure 5.9 The effect of removal of endothelium on vasorelaxations induced by ZD 7114 in (a) rat aortic and (b) mesenteric and (c) pulmonary arterial rings (●, endothelium-intact; ○, endothelium-denuded) preconstricted with phenylephrine ($3x10^{-7}$M, control and $3x10^{-8}$M, endothelium-denuded for aortic rings; 10^{-6}M, control and 10^{-7}M, endothelium-denuded for mesenteric arterial rings; and $3x10^{-8}$M, control and $3x10^{-9}$M, endothelium-denuded for pulmonary arterial rings Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 4-6 separate experiments.

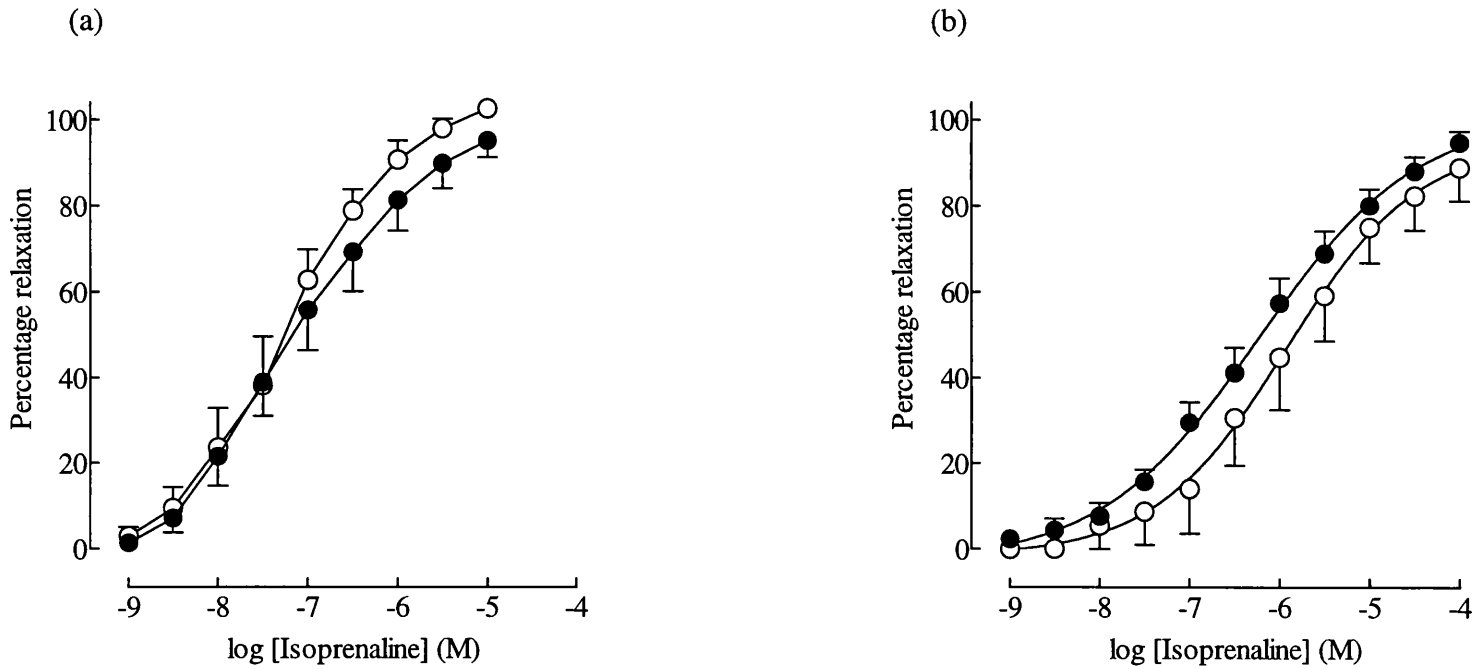


Figure 5.10 Relaxant effects of isoprenaline in (a) rat aortic and (b) mesenteric arterial rings preconstricted with phenylephrine ($3x10^{-7}$M and 10^{-6}M respectively) in the absence (●) and presence of glibenclamide 10^{-6}M (○). Results are expressed as percentage relaxation of the tone induced by phenylephrine. Points represent the mean±s.e.mean of 4-5 separate experiments.

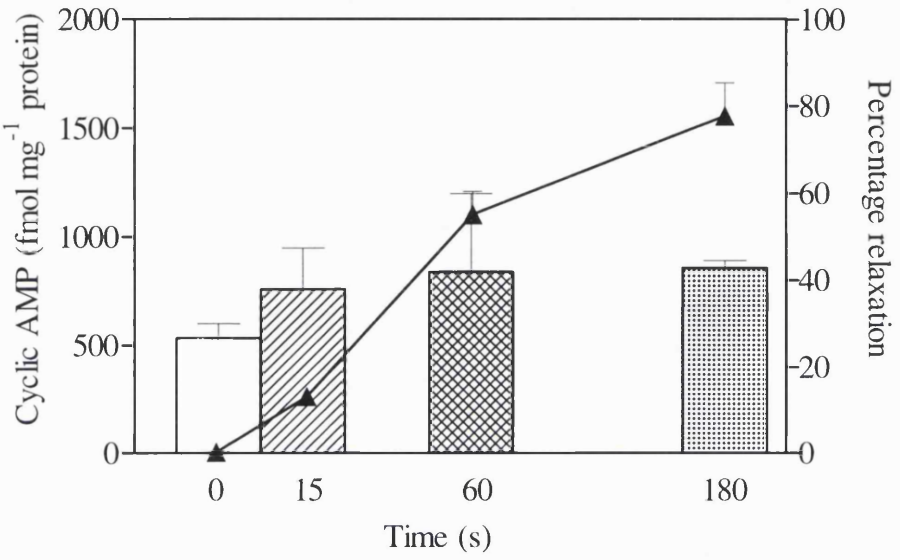


Figure 5.11 The effect of isoprenaline (10^{-6}M) on cyclic AMP levels and on vascular tone induced by phenylephrine ($3x10^{-7}$M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic AMP levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (▲) are expressed as percentage relaxation of tone induced by phenylephrine ($3x10^{-7}$M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

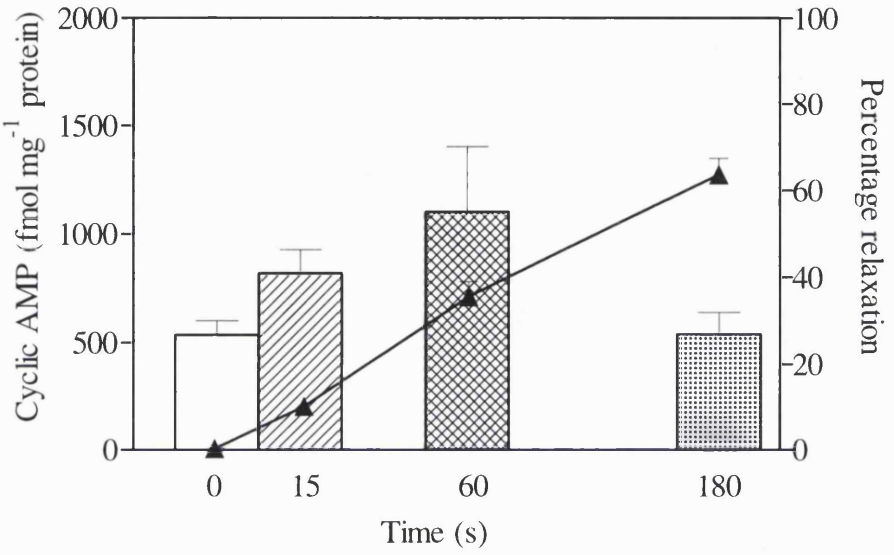


Figure 5.12 The effect of ZD 7114 (10^{-5}M) on cyclic AMP levels and on vascular tone induced by phenylephrine ($3x10^{-7}$M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic AMP levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (▲) are expressed as percentage relaxation of tone induced by phenylephrine ($3x10^{-7}$M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

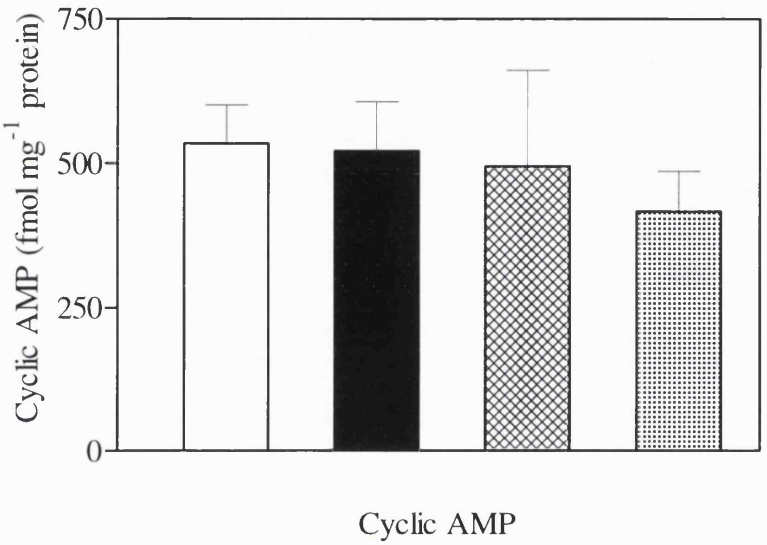


Figure 5.13 The effect of isoprenaline (10^{-6}M) and ZD 7114 (10^{-5}M) on cyclic AMP levels at 60s exposure in rat thoracic aortic rings denuded of endothelium and preconstricted with phenylephrine (10^{-6}M): ☐ represents basal levels of cyclic AMP in endothelium intact rings for comparison; ■ represents basal levels of cyclic AMP in rings denuded of endothelium; ⊠ represents levels of cyclic AMP after 60s exposure to isoprenaline (10^{-6}M) in rings denuded of endothelium; and ▦ represents levels of cyclic AMP after 60s exposure to ZD 7114 (10^{-5}M) in rings denuded of endothelium. Levels of cyclic AMP are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 3 and 6 separate experiments.

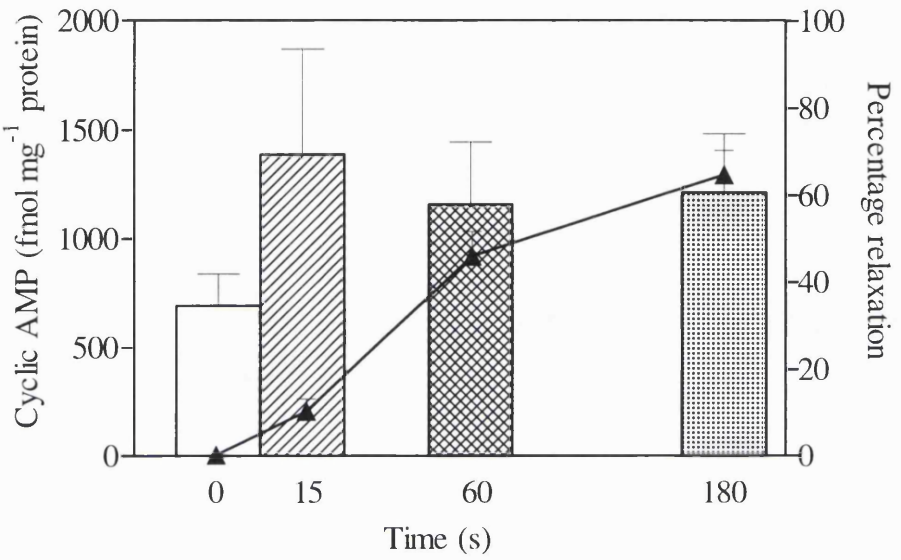


Figure 5.14 The effect of isoprenaline ($3x10^{-5}$M; in the presence of 10^{-6}M propranolol) on cyclic AMP levels and on vascular tone induced by phenylephrine ($3x10^{-7}$M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic AMP levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (▲) are expressed as percentage relaxation of tone induced by phenylephrine ($3x10^{-7}$M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

5.4 Discussion

Since Furchgott & Zawadzki (1980) described the obligatory role of the endothelium in the acetylcholine-induced relaxation of precontracted rabbit aortic strips, a number of vasodilator agents have been found to be endothelium-dependent (see reviews by Furchgott & Vanhoutte, 1989; Furchgott, 1990; Moncada *et al.,* 1991). Agents such as isoprenaline and other β-adrenoceptor agonists have generally been considered to act independently of the endothelium and mediate their actions via receptors on the smooth muscle cells, leading to an increase in cyclic AMP (Kukovetz *et al.,* 1981). But during recent years, increasing evidence suggests that at least a component of the response to β-adrenoceptor agonists is endothelium-dependent. These include: (1) the inhibition of salbutamol- and adrenaline-induced vasorelaxations *in vivo* in the rat by the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME; Gardiner *et al.,* 1991a, b); (2) inhibition of isoprenaline responses and complete prevention of vasorelaxation induced by dobutamine (β_1-adrenoceptor agonist) by L-NAME in rat mesenteric resistance arteries (Graves & Poston, 1993); and (3) complete lack of vasorelaxation induced by isoprenaline and salbutamol in rat aortic rings after removal of the endothelium (Gray & Marshall, 1992).

In this chapter it has been shown that isoprenaline mediates vasorelaxations in the rat thoracic aorta, mesenteric and pulmonary artery via an endothelium-independent and -dependent mechanism, whereas ZD 7114 causes relaxations which are independent of the presence of an intact endothelium.

In all of the tissues studied, an inhibition, but not an abolition, of isoprenaline responses was observed in endothelium-denuded rings suggesting that the response to isoprenaline in the rat aorta, mesenteric and pulmonary artery is mediated via both the smooth muscle and endothelium. In addition, isoprenaline-induced vasorelaxations were inhibited by L-NOARG in endothelium-intact mesenteric and pulmonary, but not aortic rings. Inhibition of isoprenaline responses by L-NOARG in the mesenteric and pulmonary artery implies that the L-arginine/nitric oxide pathway plays a role in the transduction mechanism. Other studies have also shown that nitric oxide may play

a role in the relaxation to β-adrenoceptor agonists. Graves & Poston (1993) demonstrated that β_1-adrenoceptor activation causes relaxation via nitric oxide release from the endothelium of rat mesenteric resistance arteries and studies suggest that the isoprenaline-induced relaxation in rat isolated cerebral arteries depends on an intact endothelial L-arginine/nitric oxide mechanism (Hempelmann & Ziegler, 1993). However, lack of inhibition of isoprenaline responses by L-NOARG in the aorta suggest that factors distinct from nitric oxide may contribute to the endothelium-dependent relaxation in this tissue. The present results contrast with those of Gray & Marshall (1992) who found that removal of the endothelium from rat aortic rings completely prevented the vascular relaxation induced by isoprenaline and also observed inhibition of isoprenaline responses by L-NOARG, while Moncada *et al.* (1991) observed that isoprenaline-induced relaxations in the rat thoracic aorta were neither endothelium-dependent nor affected by inhibitors of nitric oxide synthase.

5.4.1 Effect of endothelium removal

One explanation for the difference in results between the present study and that of Gray & Marshall (1992) in endothelium-denuded aortic rings is that the endothelium may not have been removed sufficiently to abolish the relaxant effects of isoprenaline in the present study. In a number of studies, where 'complete' removal of endothelium has been assumed (based on lack of relaxation to muscarinic agonists), there is a large variation in the relaxant response to isoprenaline, ranging from approximately 30% (Grace *et al.,* 1988) to 80% of the spasmogen-induced tone (Weir *et al.,* 1991) with nearly every intermediate value being reported (Martin *et al.,* 1986; Kamata *et al.,* 1989; Dainty *et al.,* 1990).

In most studies, complete removal of the endothelium is assessed by lack of response to a muscarinic agonist, usually acetylcholine. However, the use of muscarinic agonists to confirm endothelium loss is not entirely accurate since not all vasodilators have an equal sensitivity to endothelium loss, otherwise removal of the same proportion of the endothelium would abolish the responses to all endothelium-dependent vasodilators. One of the prerequisites in the current study has been that the observed lack of relaxation to acetylcholine in rings where the endothelium has been

removed, is taken as an indication that 'complete' removal of the endothelium has been achieved, after which experiments with isoprenaline are carried out. However, it is possible that a very small percentage of the endothelium has remained intact and whilst acetylcholine has failed to produce a relaxant response, isoprenaline may only require this very small proportion of intact endothelium to produce relaxations. Therefore, while muscarinic agonists such as acetylcholine are useful in determining the presence of a viable endothelium (i.e., by producing a maximum relaxation at a specific concentration), their use may not be ideal in confirming complete removal of endothelium and in some cases endothelium loss may be better assessed histologically.

Another possible explanation for the variation between the present results obtained in the rat thoracic aorta and those of Gray & Marshall (1992) is the choice of preconstrictor, phenylephrine being used in the current study, whereas noradrenaline was used in the latter investigation. Reports suggest that the choice and also the concentration of the spasmogen can influence the relaxant response to vasodilators in vasculature (Jones *et al.,* 1984; Furchgott, 1983; O'Donnell & Wanstall, 1987). Another difference between these two studies is that Gray & Marshall (1992) used the same concentration of noradrenaline to preconstrict endothelium-denuded rings, therefore producing a significant increase in the size of contraction compared with intact rings. However, in the present study, a ten-fold lower concentration of phenylephrine was employed to preconstrict denuded rings in order to match the size of contraction obtained in endothelium-intact rings.

In order to investigate these variations, experiments were carried out in the rat thoracic aorta, using the same concentration of either phenylephrine ($3x10^{-7}M$) or noradrenaline ($10^{-7}M$) in both endothelium-intact and -denuded rings. pEC_{50} values and the maximum response to isoprenaline in intact aortic rings, were similar in both cases. Although, responses to isoprenaline were not completely abolished in endothelium-denuded aortic rings preconstricted with noradrenaline in the current study, as has been reported (Gray & Marshall, 1992), there was a much greater

inhibition of isoprenaline responses when using noradrenaline as a preconstrictor compared with phenylephrine.

5.4.2 Effect of L-NOARG

Another difference between the present data and the report of Gray & Marshall (1992) is the lack of inhibition by L-NOARG of isoprenaline-induced relaxations in the present study suggesting that the endothelium-dependent component of isoprenaline relaxations does not involve nitric oxide. It is possible that factors such as prostacyclin and arachidonic acid may contribute to the endothelium-dependent relaxation in the rat aorta, although further experiments will be required to verify this. However, a recent study demonstrated that isoprenaline responses were not affected by indomethacin (10^{-5}M; a cyclo-oxygenase inhibitor) in the rat thoracic aorta, suggesting that none of the prostaglandins, in particular prostacyclin, are involved in mediating the relaxant effects of isoprenaline (Delpy *et al.,* 1996). Gray & Marshall (1992), on the other hand, suggested that isoprenaline-induced relaxations in the rat thoracic aorta involves the release of nitric oxide, in response to activation of endothelial β-adrenoceptors, mediated by cyclic AMP (Gray & Marshall, 1992). Experiments using the same concentration of phenylephrine or noradrenaline (which was used by Gray & Marshall, 1992), in the absence and presence of L-NOARG, resulted in a significant increase in spasmogen-induced tone, following pretreatment with L-NOARG. With phenylephrine, isoprenaline responses were significantly inhibited by L-NOARG and with noradrenaline, the inhibition was even greater (these latter results being similar to those obtained by Gray & Marshall, 1992). Delpy *et al.* (1996) also reported that isoprenaline-induced relaxations of rat aortic rings precontracted with phenylephrine (10^{-6}M) were inhibited by L-NAME, although these authors also used the same concentration of phenylephrine to preconstrict control tissues (10^{-6}M) and observed that contractions to phenylephrine had increased following pretreatment with L-NAME. Therefore, the effect of NO synthase inhibitors on responses to isoprenaline appears to be influenced by the choice of preconstrictor and also the size of the contractile response.

Responses to vasodilators other than isoprenaline have also been shown to be influenced by the choice of contractile agonist. Endothelium-dependent relaxation to acetylcholine in rat isolated mesenteric resistance arteries has been shown to be mediated by both NO and NO-independent repolarization (Garland & McPherson, 1992). However, recent studies demonstrated that the contribution of NO and NO synthase-independent repolarization to acetylcholine-evoked relaxation was determined by the choice of preconstrictor (Plane & Garland, 1996). In tissues precontracted with noradrenaline, acetylcholine-evoked relaxation and repolarization persist in the presence of L-NAME and L-NOARG (both $3x10^{-4}$M), whereas when U46619 was used to preconstrict tissues, relaxations to acetylcholine were entirely mediated by NO, independently of a change in membrane potential (Plane & Garland, 1996).

5.4.3 Cyclic AMP accumulations in the rat thoracic aorta

Like the β_1- and β_2-adrenoceptor, the β_3-adrenoceptor is coupled to the activation of adenylate cyclase via G_s, resulting in production of intracellular cyclic AMP (Zaagsma & Hollenga, 1991). A greater increase in cyclic AMP was observed with ZD 7114, although higher cyclic AMP levels may have been expected with isoprenaline which is most likely acting via β_2- and atypical β-adrenoceptors in this tissue.

Since the present studies suggest that isoprenaline (in part) and ZD 7114 mediate vasorelaxation via the smooth muscle, an increase in cyclic AMP levels would be expected in the absence of the endothelium. However, no rise in cyclic AMP was observed in tissues denuded of endothelium when exposed to isoprenaline or ZD 7114. These results would therefore suggest that the isoprenaline- and ZD 7114-mediated vasorelaxation is not linked to adenylate cyclase activation in the smooth muscle, although this is unlikely since all three β-adrenoceptor subtypes appear to be linked to adenylate cyclase activation via G_s.

The effect of isoprenaline ($3x10^{-5}$M) on cyclic AMP levels was also examined in the presence of propranolol (10^{-6}M) in order to block any contribution from β_2-

adrenoceptors. Isoprenaline produced a two-fold increase in cyclic AMP levels, which may represent, at least in part, an atypical β-adrenoceptor mediated increase in cyclic AMP. This increase is similar to that seen in the absence of propranolol but using a 30-fold lower concentration of isoprenaline (i.e. 10^{-6}M). However, it is difficult to interpret these results as controls using $3x10^{-5}$M isoprenaline alone (i.e., in the absence of propranolol) were not carried out.

5.4.4 ATP-sensitive potassium channels (K_{ATP}-channels)

Recent studies suggest that β-adrenoceptor agonists can also induce vasorelaxation via a mechanism involving an interaction between the β-adrenoceptor and K_{ATP}-channels. Jackson (1993) reported that these channels are important regulators of basal microvascular tone in both the hamster cheek pouch and cremaster muscle and found that, in the cheek pouch, glibenclamide resulted in reduced vasodilator responses to isoprenaline. Also, glibenclamide has been found to antagonize relaxant responses to isoprenaline in the rat isolated perfused mesenteric arterial bed (Randall & McCulloch, 1995) and in rat aortic rings (Hüsken *et al.*, 1994), and a study in the rat basilar artery in vivo demonstrated that noradrenaline acts via β_1-adrenoceptors to cause glibenclamide-sensitive vasodilatation (Kitazono *et al.*, 1993). Such findings, therefore, challenge the conventional view that β-adrenoceptors are solely coupled to adenylate cyclase and cyclic AMP formation. However, responses to isoprenaline in the current study were largely unaffected by glibenclamide (10^{-6}M) which would argue against the involvement of K_{ATP}-channels in relaxation mediated via β-adrenoceptors in the rat thoracic aorta and mesenteric artery. The reason for this difference in results between the present investigation and the reports mentioned are not clear. Although, Wistar rats were used in the reports of Randall & McCulloch (1995) and Hüsken *et al.* (1994) and Sprague-Dawley rats in the present study, further experiments would be necessary to determine whether these variations could account for the differences. It is unlikely that the concentration of glibenclamide used in the present study (10^{-6}M) was not high enough, since Hüsken *et al.* (1994) observed a decrease in maximal relaxations to isoprenaline of approximately 30% in rat aortic rings using the same concentration of glibenclamide.

5.4.5 Functional location of β-adrenoceptors

ZD 7114 produced relaxations independent of the presence of the endothelium in the aorta, mesenteric and pulmonary artery, whereas inhibition of isoprenaline responses was observed in endothelium-denuded rings suggesting that vasorelaxations to isoprenaline are mediated via both the endothelium and smooth muscle. Lack of inhibition of isoprenaline responses by propranolol (10^{-6}M) in endothelium-denuded rat aortic rings suggests that β_2-adrenoceptors are located on the endothelium and atypical β-adrenoceptors are present on the smooth muscle. The presence of β-adrenoceptors on endothelial cells has been demonstrated by autoradiography (Steinberg *et al.,* 1984; Stephenson & Summers, 1987; Molenaar *et al.,* 1988) and radioligand binding studies (Howell *et al.,* 1988; Freissmuth *et al.,* 1986; Emorine *et al.,* 1989). Therefore, these results are consistent with part of the response to isoprenaline being mediated by endothelial β-adrenoceptors.

In the case of the rat mesenteric artery, isoprenaline responses were antagonized by propranolol in endothelium-denuded rings, leaving a small residual response to isoprenaline. Assuming that this concentration of propranolol (i.e., 10^{-6}M) had a maximal effect, this would suggest that β_2-adrenoceptors are present on both the endothelium and smooth muscle. However, isoprenaline responses were found to comprise a propranolol-resistant component in the rat mesenteric artery in the presence of an intact endothelium (**Chapter 3**), which are presumably mediated via atypical β-adrenoceptors. Since ZD 7114 produced maximal relaxations in rings denuded of endothelium, atypical β-adrenoceptors are therefore likely to be located on the smooth muscle. However, the inhibition of isoprenaline responses by propranolol would not be expected if isoprenaline was mediating relaxations solely via atypical β-adrenoceptors in endothelium-denuded rings.

In general, the tendency has been to define vasodilators as either endothelium-dependent or endothelium-independent. For example, in the present study, ZD 7114 may be considered an endothelium-independent vasodilator, on the basis that it produced maximal responses after removal of the endothelium. However, this does not eliminate the possibility of a mixed response comprising both endothelium-

dependent and -independent components, as the endothelium-dependent component of vasorelaxation to ZD 7114 may be concealed by its endothelium-independent vasorelaxant effect. Furthermore, it has generally been accepted that endothelial cells contain only β_2-adrenoceptors (Howell *et al.*, 1988; Freissmuth *et al.*, 1986). However, radioligand binding studies in cultured bovine pulmonary arterial endothelial cells have shown that β_3-adrenoceptors may also be present on the endothelium (Ahmad *et al.*, 1990).

Chapter 6

Expression of β_3-adrenoceptor mRNA in rat and human tissues

6.1 Introduction

Emorine *et al.* (1989) provided the first molecular evidence for the existence of a third β-adrenoceptor subtype, by cloning a human gene which encoded a receptor resistant to blockade by conventional β-adrenoceptor antagonists and sensitive to novel β_3-adrenoceptor agonists. The mouse gene (Nahmias *et al.,* 1991) and the rat cDNA (Granneman *et al.,* 1991; Muzzin *et al.,* 1991) coding for the β_3-adrenoceptor were also cloned, several features supporting the concept that these cloned receptors are species homologues of the β_3-adrenoceptor rather than distinct β-adrenoceptor subtypes. The human and rodent β_3-adrenoceptor genes encode proteins of 408 (van Spronsen *et al.,* 1993) and 400 (Muzzin *et al.,* 1991; Granneman *et al.,* 1991; van Spronsen *et al.,* 1993) amino acids respectively. The mouse and rat β_3-adrenoceptor are 82% and 80% identical to the human β_3-adrenoceptor and the transmembrane sequence homology with human β_3-adrenoceptor being even greater, 93% for mouse β_3-adrenoceptor and 94% for rat β_3-adrenoceptor.

Isolation of the β_3-adrenoceptor gene has provided the means to detect the presence of the corresponding mRNA in tissues known to possess atypical β-adrenoceptors. β_3-Adrenoceptor mRNA has been detected in both brown and white adipose tissue of the rat (Granneman *et al.,* 1991), mouse (Nahmias *et al.,* 1991) and human (Granneman *et al.,* 1993; Krief *et al.,* 1993; Revelli *et al.,* 1993). In human, using reverse transcription-polymerase chain reaction (RT-PCR) analysis, expression has also been found in gallbladder, colon, ileum and heart (Krief *et al.,* 1993; Granneman *et al.,* 1993). However, in human heart, β_3-adrenoceptor mRNA expression was always associated with that of uncoupling protein (UCP), suggesting that the observed cardiac expression of β_3-adrenoceptor may be due to intrinsic fat

deposits (Krief *et al.,* 1993). A subsequent study demonstrated lack of β_3-adrenoceptor mRNA in all human tissues, including fat (Thomas & Liggett, 1993), although this was attributed to technical differences by other authors. Expression of the β_3-adrenoceptor gene has also been demonstrated in SK-N-MC human neuroblastoma cells by Northern blot analysis (Esbenshade *et al.,* 1992). More recent reports have demonstrated β_3-adrenoceptor mRNA expression in human stomach and prostate using ribonuclease protection assay (Berkowitz *et al.,* 1995) and brain by means of PCR analysis (Rodriguez *et al.,* 1995).

In the mouse, Northern blot analysis failed to detect any expression of β_3-adrenoceptor mRNA in non-adipose tissues, including ileum and colon (Nahmias et al., 1991). Northern blot analysis and ribonuclease protection assay of RNA from rat tissues, identified a low level of expression in ileum, but was not detected in skeletal muscle, heart, lung, kidney or cerebral cortex (Granneman *et al.,* 1991). Later studies, however, suggest that high levels of β_3-adrenoceptor mRNA are present in rat fundus (Granneman & Lahners, 1994; Cohen *et al.,* 1995).

Functional studies have indicated that atypical β-adrenoceptors are present in the vasculature. Therefore, the aim of this chapter was to investigate whether the mRNA for the β_3-adrenoceptor is present in rat and human vasculature, using RT-PCR analysis. In addition, a range of other rat and human tissues were examined for β_3-adrenoceptor mRNA expression. Since β_3-adrenoceptor mRNA is present in adipose tissue, the serine protease adipsin (Cook *et al.,* 1987), was used as a marker for white adipose tissue to check for fat contamination of other tissues. The use of adipsin as a marker for white adipose tissue has been described previously in rat tissues (Evans *et al.,* 1996).

6.2 Materials and methods

RNA preparation and the RT-PCR assay are detailed in **Chapter 2.**

6.3 Results

As shown in **Figure 6.1**, bands (406 bp) corresponding to hypoxanthine phosphoribosyltransferase (hprt) were observed in all of the tissues studied.

6.3.1 Rat tissues

Bands of an appropriate size were produced for mRNA coding for the β_3-adrenoceptor (580 bp) and adipsin (589 bp) in a range of rat tissues (**Figure 6.2**). However, as mRNA levels have not been quantitated in this study, it is not possible to make a direct comparison between β_3-adrenoceptor and adipsin mRNA levels. In addition to white adipose tissue, expression of β_3-adrenoceptor mRNA was found in all the gastrointestinal tissues examined, except for stomach. Rat tissues in which β_3-adrenoceptor mRNA was not detected include kidney, spleen, lung, liver, and bladder. Also, β_3-adrenoceptor mRNA was not detected in the heart or thoracic aorta. It should be noted that although PCR analysis using the β_3-adrenoceptor and adipsin primers gave single products consistent with expected size, the identity of the PCR products needs to be further confirmed by hybridization to independent probes (i.e., Southern blot analysis) when possible.

6.3.2 Human tissues

β_3-Adrenoceptor mRNA was detected in white adipose tissue (obtained from operations), but was not found in bladder, lung or any of the arteries, i.e., thoracic aorta, carotid, mesenteric and pulmonary artery (**Figure 6.3**).

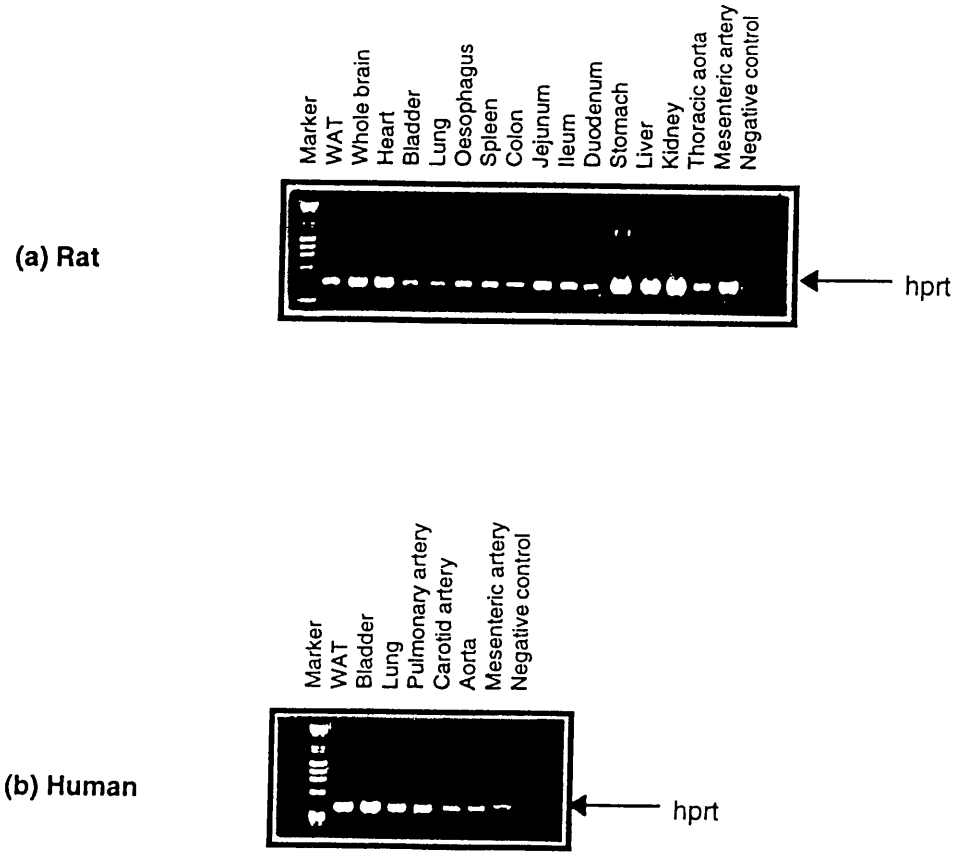


Figure 6.1 Expression of hprt in (a) rat and (b) human tissues. RT-PCR was carried out using the primers shown in **Table 2.3** (**Chapter 2**). Sizes of the products were determined from ethidium bromide stained gels by comparison with 1kB DNA ladder (Life Technologies).

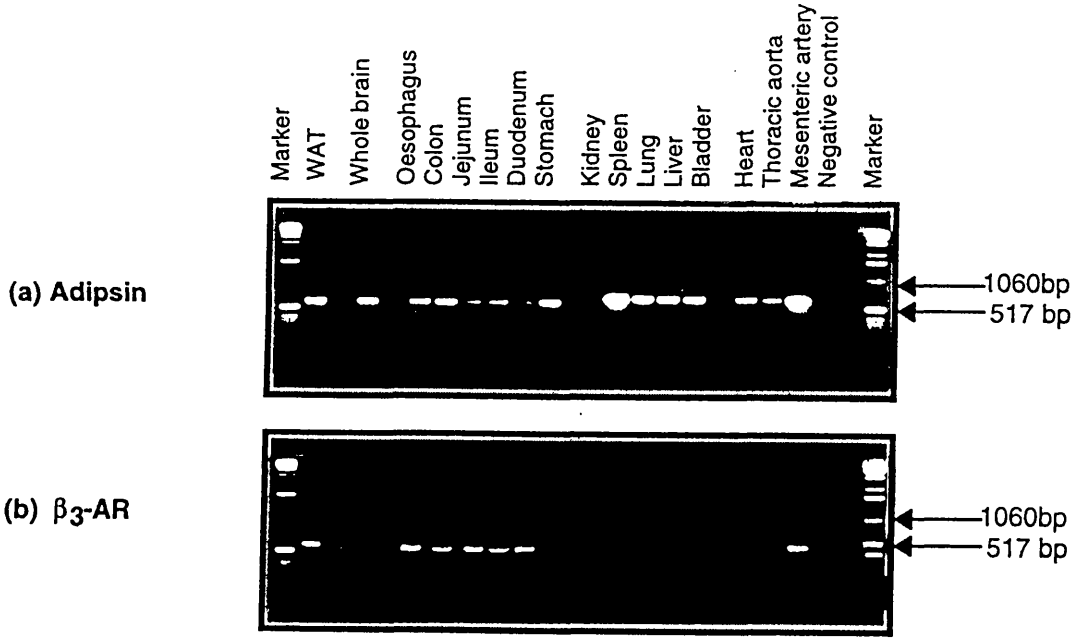


Figure 6.2 Expression of (a) adipsin and (b) β_3-adrenoceptor in rat tissues. RT-PCR was carried out using the primers shown in **Table 2.3** (**Chapter 2**). Sizes of the products were determined from ethidium bromide stained gels by comparison with 1kB DNA ladder (Life Technologies). Numbers on the right indicate marker sizes in bp.

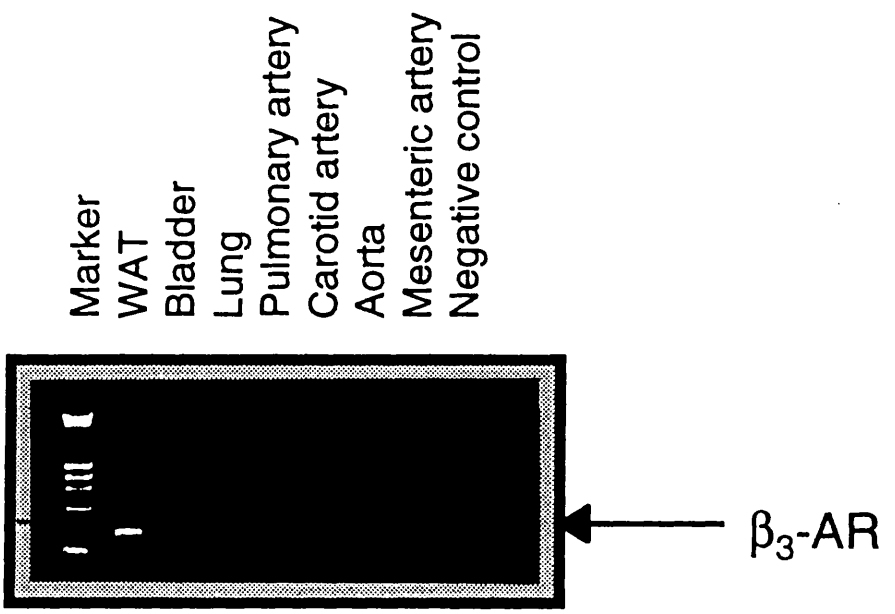


Figure 6.3 Expression of β_3-adrenoceptor in human tissues. RT-PCR was carried out using the primers shown in **Table 2.3** (**Chapter 2**). Sizes of the products were determined from ethidium bromide stained gels by comparison with 1kB DNA ladder (Life Technologies).

6.4 Discussion

In the present chapter, β_3-adrenoceptor mRNA expression was investigated in several rat and human tissues, in particular looking at expression in vasculature using PCR analysis.

6.4.1 Rat tissues

β_3-Adrenoceptor mRNA was detected in regions of the rat gastrointestinal tract, particularly the oesophagus, colon, jejunum and ileum. A recent study has also demonstrated β_3-adrenoceptor mRNA expression in rat ileum and colon, with high levels being present in the longitudinal/circular smooth muscle of both colon and ileum, and colon submucosa (Evans *et al.,* 1996). Functional evidence for β_3- or atypical β-adrenoceptors in the gastrointestinal tract has been demonstrated by various groups (see review, Arch & Kaumann, 1993). Studies in the rat oesophagus have demonstrated that these receptors receive a noradrenergic innervation therefore supporting a physiological role of the β_3-adrenoceptor (de Boer *et al.,* 1995). Also, *in vivo* studies have shown that activation of atypical β-adrenoceptors by SR 58611A inhibits motility of the rat proximal colon (Croci *et al.,* 1991). These observations are therefore consistent with the idea that these receptors may play a role in the regulation of gut motility via their relaxant effects (Bianchetti & Manara, 1990; van der Vliet *et al.,* 1990; McLaughlin & MacDonald, 1990; de Boer *et al.,* 1993; 1995; Growcott *et al.,* 1993b). Evans *et al.* (1996) also demonstrated high levels of β_3-adrenoceptor mRNA expression in the gastric fundus (some of which was attributed to fat), whereas in the present study β_3-adrenoceptor mRNA was not detected in the rat stomach. The reason for this is not clear, since functional studies have also shown that atypical β-adrenoceptors play a role in mediating both acid secretion (Canfield & Paraskeva, 1992) and relaxation of the rat fundus (McLauglin & MacDonald, 1991; Cohen *et al.,* 1995).

In the present study, expression of β_3-adrenoceptor mRNA was not detected in rat kidney, spleen, lung, liver or bladder. Evans *et al.* (1996) also reported that β_3-adrenoceptor mRNA is virtually undetectable in lung and liver. Similarly, in humans,

β_3-adrenoceptor mRNA was not found in lung, liver and also kidney (Berkowitz *et al.,* 1995).

β_3-Adrenoceptor mRNA was also detected in rat brain in the present study. A more detailed study by Summers *et al.* (1995) revealed that β_3-adrenoceptor mRNA levels were highest in hippocampus, cerebral cortex and striatum, with lower levels present in hypothalamus, brainstem and cerebellum. Studies carried out in humans have produced conflicting reports. While some authors have failed to detect human β_3-adrenoceptor mRNA in the human brain (Berkowitz *et al.,* 1995), others have demonstrated low levels to be present in several brain regions with infant brains containing about 100-fold more β_3-adrenoceptor mRNA than the adult brain (Rodriguez *et al.,* 1995). Functional evidence for atypical β-adrenoceptors in the CNS has been demonstrated by SR 58611A which was found to be active in animal models of depression including antagonism of the hypothermia induced by apomorphine or reserpine, potentiation of toxicity to yohimbine and reversal of learned helplessness (Simiand *et al.,* 1992). The effects were not antagonized by CGP 20712A or ICI 118551 and only by high doses of propranolol or alprenolol, these features being characteristic of atypical β-adrenoceptors.

In the present study, β_3-adrenoceptor mRNA was not found in rat heart and Evans *et al.* (1996) also observed that β_3-adrenoceptor mRNA was virtually undetectable in heart. Evidence for atypical β-adrenoceptors in the rat heart comes from the observation that a series of partial agonists related to pindolol cause positive chronotropic effects at concentrations much higher than those necessary to block β_1- and/or β_2-adrenoceptors (Kaumann *et al.,* 1979; Kaumann, 1989). More recent studies in the pithed rat demonstrated that CGP 12177 and cyanopindolol mediated positive chronotropic effects via atypical β-adrenoceptors (Malinowska & Schlicker, 1996). These authors suggested that this receptor was similar to the atypical β-adrenoceptor previously described by Kaumann and co-workers in isolated heart preparations (Kaumann, 1989). Also, recent functional studies have shown that while β_3-adrenoceptor agonists relaxed the rat colon at nanomolar concentrations, they were

ineffective at micromolar concentrations at the third cardiac β-adrenoceptor in rat atria (Kaumann & Molenaar, 1996). Furthermore, the β_3-adrenoceptor antagonist, SR 59230A (Manara et al., 1995a) was found to cause blockade of agonist-induced (i.e., BRL 37344, ZD 2079, CL 316243 and SR 58611A) colonic relaxation but hardly blocks the third cardiac β-adrenoceptor (Kaumann & Molenaar, 1996). Taken together, these results suggest that the atypical β-adrenoceptor demonstrated by functional studies in heart may not be the β_3-adrenoceptor.

Recent functional studies in humans have shown that the positive inotropic effects of (-)-CGP 12177 are resistant to blockade by (-)-propranolol but antagonized by (-)-bupranolol, which is consistent with the existence of atypical β-adrenoceptors in human atrial myocardium (Kaumann, 1996). As to whether these receptors resemble the cloned β_3-adrenoceptor remains to be established. Krief *et al.* (1993) demonstrated β_3-adrenoceptor mRNA expression in human heart, especially in left atrium, but this was also accompanied by mRNA for UCP, a marker of brown fat, and they attributed the β_3-adrenoceptor mRNA as located in fat tissue surrounding the atria. However, Krief *et al.* (1993) did not rule out the possibility that the β_3-adrenoceptor is present in a small number of specialized pacemaker cells.

Functional evidence for atypical β-adrenoceptors in isolated blood vessels, namely the rat thoracic aorta, mesenteric and pulmonary artery, has been demonstrated in this thesis (**Chapters 3** and **4**), and also by other workers (rat carotid artery, Oriowo, 1994; rat aorta, Doggrell, 1990; canine mesenteric vessels, Clark & Bertholet, 1983). Expression of β_3-adrenoceptor mRNA was not detected in the thoracic aorta in the present study, and although β_3-adrenoceptor mRNA appeared to be present in the mesenteric artery, there was also expression of adipsin mRNA, suggesting that the β_3-adrenoceptor signal may be contributed by fat associated with this blood vessel. Further experiments would be required to confirm these results and the possibility that, as in rat heart, the atypical β-adrenoceptor in vasculature characterized by functional studies may not correspond to the β_3-adrenoceptor previously described.

6.4.2 Human tissues

With the exception of adipose tissue (which was obtained from operations), β_3-adrenoceptor mRNA was not detected in any of the human tissues studied. This may be due to the time period between death and collection of the other tissue samples at post-mortem, i.e., 24-48 hours, during which time some breakdown of RNA is likely to occur. Although a signal for hprt was observed in all of the tissues studied, it is possible that there may be a low level of expression of β_3-adrenoceptor mRNA in humans. For example, it has been shown that humans have lower levels of β_3-adrenoceptor expression in fat compared to rats (Granneman *et al.,* 1993) and it has been suggested that under normal conditions, this β-adrenoceptor subtype may not be as important in humans as it is in rodents in the control of fatty acid mobilization (Emorine *et al.,* 1994). In a recent study using ribonuclease protection assay the presence of β_3-adrenoceptor mRNA in human white fat, gall bladder and small intestine was confirmed, and β_3-adrenoceptor expression was also found in stomach and prostate (Berkowitz *et al.,* 1995).

In conclusion, β_3-adrenoceptor mRNA was expressed in a number of rat tissues including adipose tissue, oesophagus, colon and small intestine, therefore supporting functional evidence that this receptor plays a role in lipolysis, thermogenesis and regulation of gut motility. The presence of β_3-adrenoceptor mRNA in rat brain also supports functional evidence that these receptors play a part in animal models of depression. In rat mesenteric artery, β_3-adrenoceptor mRNA expression was associated with expression of adipsin mRNA and may therefore be due to fat normally associated with the blood vessel. β_3-Adrenoceptor mRNA was not detected in rat heart or thoracic aorta therefore raising the possibility that the atypical β-adrenoceptor demonstrated by functional studies in vasculature and heart does not correspond to the β_3-adrenoceptor previously described. With the exception of human adipose tissue, β_3-adrenoceptor mRNA was not detected in any of the other tissues examined. This may be due to degradation of RNA prior to collection of tissue samples (since ideally tissue for such studies needs to be obtained as soon as possible following death) and/or low levels of β_3-adrenoceptor mRNA expression in humans.

Chapter 7

General discussion

The existence of atypical or β_3-adrenoceptors has been shown in a number of tissues, especially adipose and gastrointestinal tissues. In terms of their functional properties, the three main features have been: (i) resistance to blockade by β-adrenoceptor antagonists possessing high affinity for β_1- and/or β_2-adrenoceptors; (ii) selective stimulation by β_3-adrenoceptor agonists; and (iii) stimulation of these receptors by certain β_1- and β_2-adrenoceptor antagonists (see review, Arch & Kaumann, 1993). In addition, the recent introduction of antagonists selective for the β_3-adrenoceptor (Manara *et al.,* 1995a) should enable a more conclusive functional identification of these receptors.

In this thesis, the features described above have been demonstrated in rat vasculature, namely the thoracic aorta, mesenteric and pulmonary artery, therefore indicating atypical β-adrenoceptors are present in these tissues. In terms of the first criterion, non-competitive antagonism of isoprenaline responses by propranolol was observed in all of the tissues studied. In the case of the mesenteric artery and thoracic aorta, responses were found to comprise a propranolol-sensitive (most likely to be β_2-adrenoceptor mediated) and -insensitive response. Although propranolol produced a surmountable antagonism of isoprenaline-induced relaxation in the pulmonary artery, the antagonism did not satisfy the criteria for competitive antagonism (the slope of the Schild plot was significantly less than unity). Both the second and third criteria were also fulfilled, all three blood vessels were relaxed by β_3-adrenoceptor agonists, and both CGP 12177 and alprenolol were agonists in these tissues.

Therefore, the presence of a third β-adrenoceptor, referred to as the atypical β-adrenoceptor, has been demonstrated in rat vasculature. Whether the receptor described in the present study is the same as the β_3-adrenoceptor present in adipose

tissue and gut tissues or represents a distinct receptor population, remains to be established.

7.1 Evidence for a role of atypical β-adrenoceptors in the cardiovascular system

While this thesis has provided evidence for the existence of atypical β-adrenoceptors in isolated blood vessels, several *in vivo* studies also support the existence of these receptors in the vasculature. Furthermore, evidence suggests that a third β-adrenoceptor population, in addition to β_1- and β_2-adrenoceptors, also coexists in mammalian heart.

7.1.1 Vascular β-adrenoceptors

A number of studies have demonstrated that β_3-adrenoceptor agonists have vasodilator effects in the dog *in vivo*. Intravenous infusion of isoprenaline, BRL 37344 and CGP 12177 in dogs induced a positive chronotropic effect which was accompanied by an decrease in blood pressure with a relative order of potency of BRL 37344 > isoprenaline >> CGP 12177, suggesting involvement of atypical β-adrenoceptors (Tavernier *et al.,* 1992). However, the β_3-adrenoceptor mediated increase in heart rate in the anaesthetized dog is not a direct effect but occurs in response to the β_3-adrenoceptor mediated decrease in blood pressure (Tavernier *et al.,* 1992; Berlan *et al.,* 1994).

In anaesthetized, catecholamine-depleted dogs, BRL 37344 and ICI 215001 decreased hindlimb perfusion pressure and increased heart rate (Briscoe *et al.,* 1993). The positive chronotropic effects of these agonists were antagonized by a combination of the β-adrenoceptor antagonists, betaxolol and ICI 188551, suggesting that this effect is mediated via β_1- and/or β_2-adrenoceptors. However, the decrease in hindlimb perfusion pressure was unaffected by β-adrenoceptor antagonists, indicating β_3-adrenoceptors may mediate this effect.

Studies in anaesthetized and conscious dogs demonstrated that the peripheral vasodilatation induced by β_3-adrenoceptor stimulation, occurred mainly in the vessels of the skin and fat, even after propranolol pretreatment (Shen *et al.*, 1994).

7.1.2 Cardiac β-adrenoceptors

It is now well established that besides β_1-adrenoceptors, several mammalian hearts also possess functional β_2-adrenoceptors (Carlsson *et al.*, 1972; Kaumann, 1986). The involvement of β_1- and β_2-adrenoceptors in the modulation of heart function varies with heart region (Lemoine & Kaumann, 1986; Kaumann *et al.*, 1983; Molenaar *et al.*, 1990) and agonist (Lemoine *et al.*, 1985; 1989). Evidence suggests that human heart function is also stimulated through both β_1- and β_2-adrenoceptors (Gille *et al.*, 1985; Kaumann & Lemoine, 1987).

However, more recent studies suggest that a third β-adrenoceptor population, in addition to β_1- and β_2-adrenoceptors, also coexists in mammalian heart. The proposal was based on the *in vitro* cardiostimulant effects of non-conventional partial agonists (Kaumann, 1989). Non-conventional partial agonists were defined as β-adrenoceptor antagonists that exhibited agonist effects at concentrations much higher than those necessary to block β_1- and/or β_2-adrenoceptors (Kaumann, 1973; 1989; Kaumann & Blinks, 1980). The dissociation between blockade and stimulation of these cardiostimulant effects was observed in particular with pindolol and related compounds in isolated tissues of several species, e.g., rat, guinea pig, cat (Kaumann, 1989). Also, recent studies have demonstrated that the non-conventional partial agonists, (-)-CGP 12177 and cyanopindolol, produce positive chronotropic effects in the pithed rat, (Malinowska & Schlicker, 1996).

Evidence for a third cardiac β-adrenoceptor in humans has been less clear. Racemic pindolol causes sinoatrial tachycardia in humans (Man In't Veld & Schalekamp, 1981) which may be due, in part, to activation of a third sinoatrial β-adrenoceptor population (Kaumann, 1989). However, unlike its effects in feline atria, (-)-pindolol did not cause positive inotropic effects in human isolated atrium

(Kaumann & Lobnig, 1986), although this lack of effect observed in humans may be because the efficacy of (-)-pindolol for cardiac atypical β-adrenoceptors is low compared to other nonconventional partial agonists (Kaumann, 1989; Arch & Kaumann, 1993). More recent studies, however, demonstrated that (-)-CGP 12177 induced positive chronotropic effects in human isolated atrium (Kaumann, 1996). In addition, the resistance of these effects to blockade with (-)-propranolol and the antagonism of these effects by (-)-bupranolol is consistent with the existence of a third β-adrenoceptor population in human atrium (Kaumann, 1996).

Studies in human volunteers have shown that the ester BRL 35135 (which is de-esterified to BRL 37344 *in vivo*) causes tachycardia that is resistant to blockade with the β_1-adrenoceptor antagonist, bisoprolol but greatly inhibited by nadolol (presumably through sinoatrial β_2-adrenoceptors: Wheeldon *et al.,* 1994). However, a very small, but significant component of the chronotropic effect to BRL 35135 was resistant to blockade by nadolol (Wheeldon *et al.,* 1994) and could be mediated via β_3-adrenoceptors since the affinity of nadolol for β_3-adrenoceptors is particularly low.

7.2 Differences between the atypical β-adrenoceptor in rat vasculature and the 'β_3-adrenoceptor'

The question still remains of whether there is a single atypical β-adrenoceptor (the β_3-adrenoceptor) or a range of receptor subtypes. While atypical β-adrenoceptors characterized in adipose and gastrointestinal tissues have generally been defined as 'β_3-adrenoceptors', the term 'atypical β-adrenoceptor' has been used to describe the third β-adrenoceptor subtype present in rat vasculature in this thesis. Although the atypical β-adrenoceptors described in the present study have certain features in common with those expressed in other tissues, a number of differences also exist. For example, the potency of BRL 37344 appears to be particularly low in rat vasculature (present study; Oriowo, 1994) whereas it is one of the most potent β_3-adrenoceptor agonists for relaxing several intestinal preparations *in vitro* from different animal species (Manara *et al.,* 1995b). However, in addition to these tissue-specific differences in the potency of BRL 37344, there also appears to be species-specific

differences, e.g., BRL 37344 is a potent lipolytic agent when tested on rat, but not on guinea pig or human adipocytes (Carpené *et al.,* 1994). Also, BRL 37344 was found to have no effect on the human isolated colon (McLaughlin *et al.,* 1991; De Ponti *et al.,* 1996).

The β_1-/β_2-adrenoceptor antagonist, alprenolol, has been shown to be an antagonist at the atypical β-adrenoceptor in a number of tissues, e.g., guinea pig ileum (Blue *et al.,* 1990; Growcott *et al.,* 1993a; Tesfamariam & Allen, 1994), rat ileum (Growcott *et al.,* 1993b), rat proximal colon (Giudice *et al.,* 1989; Bianchetti & Manara, 1990) and rat caecum (Canfield & Abdul-Ghaffar, 1992). However, when investigated in the rat mesenteric artery, alprenolol did not antagonize responses to either isoprenaline (carried out in the presence of 10^{-6}M propranolol) or ZD 7114. This observation may therefore point to a different atypical β-adrenoceptor in rat vasculature. This could be examined further by looking at different blood vessels and investigating other β_1-/β_2-adrenoceptor antagonists, e.g., bupranolol, possessing affinity at the atypical β-adrenoceptor.

The failure to detect mRNA in rat vasculature using RT-PCR analysis, while it is consistently found in adipose tissue and gut muscle including colon (present study; Granneman *et al.,* 1993; Krief *et al.,* 1993; Evans *et al.,* 1996), also suggests that the atypical β-adrenoceptor described in rat vasculature may not correspond to the β_3-adrenoceptor.

7.3 Differences between the third cardiac β-adrenoceptor and the 'β_3-adrenoceptor'

Functional studies also suggest that the third cardiac β-adrenoceptor differs from the β_3-adrenoceptor. Recent studies have demonstrated that β_3-adrenoceptor agonists fail to activate the third cardiac β-adrenoceptor population indicating that these receptors are distinct from the β_3-adrenoceptor (Malinowska & Schlicker, 1996; Kaumann & Molenaar, 1996). In addition to this, Kaumann & Molenaar (1996) observed that the selective β_3-adrenoceptor antagonist SR 59230A failed to produce

potent antagonism of the stimulant effects of (-)-CGP 12177 in both right and left atria. In terms of molecular evidence, the absence of β_3-adrenoceptor mRNA in rat heart (Evans *et al.,* 1996) adds support to the hypothesis that the third cardiac β-adrenoceptor is distinct from the β_3-adrenoceptor.

7.4 Physiological function of the β_3-adrenoceptor

Evidence for the involvement of β_3-adrenoceptors in the control of gut motility has now been shown by a number of groups (see review by Manara *et al.,* 1995b). Also, molecular biology studies have demonstrated β_3-adrenoceptor mRNA expression in brown adipose tissue, where it is associated with UCP, in white adipose tissue and also in other human tissues, including colon and gallbladder (Krief *et al.,* 1993; Granneman *et al.,* 1993). Furthermore, the β_3-adrenoceptor protein has been detected in gallbladder using specific human anti-β_3-adrenoceptor antibodies (Guillaume *et al.,* 1994). These findings suggest that β_3-adrenoceptors may have a role in the control of lipid metabolism, possibly from fat assimilation in the digestive tract, to triglyceride storage and mobilization in adipose tissues (Krief *et al.,* 1993).

The involvement of the β_3-adrenoceptor in the regulation of energy balance is now beginning to be understood in rodents, but investigation of its functions in humans has just begun. It appears that under normal conditions, this β-adrenoceptor subtype may not be as essential in humans as it is in rodents in the control of fatty acid mobilization (Emorine *et al.,* 1994). However, further analysis of regulated expression of β_3-adrenoceptors should disclose conditions for their up-regulation in adipose tissue and they could become a target for anti-obesity and anti-diabetes drugs. Other therapeutic areas in which β_3-adrenoceptor agonists may provide potential drugs include disorders of gastrointestinal disorders such as irritable bowel syndrome (Manara & Bianchetti, 1990), inflammatory airways disease (Webber & Stock, 1992) and depression (Simiand *et al.,* 1992).

7.5 Physiological relevance of atypical β-adrenoceptors in vascular tissue

The physiological significance of atypical β-adrenoceptors in the vasculature remains to be established. It is possible that receptor may be involved in the regulation of blood flow to some organs. For example, Shen *et al.* (1994) observed that the peripheral vasodilatation induced by BRL 37344, occurred mainly in the vessels of the skin and fat in conscious dogs. Also, the work presented in this thesis has provided evidence for the existence of atypical β-adrenoceptors in conduit arteries and it is possible that further studies looking at resistance arteries may implicate a role for these receptors in blood pressure regulation. However, what has emerged from the present study is that any potential drugs being targeted for therapeutic applications will need to take potential vasodilator effects into account.

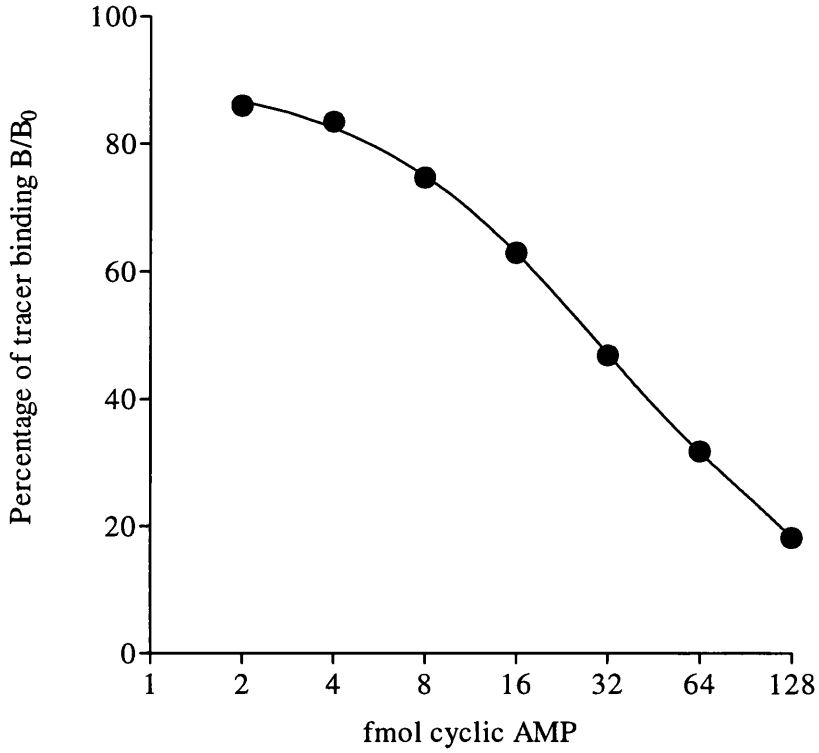
The present study has demonstrated that a third β-adrenoceptor population, referred to as the atypical β-adrenoceptor, which is distinct from β_1- and β_2-adrenoceptors, exists in rat vasculature. This atypical β-adrenoceptor may differ from the β_3-adrenoceptor, and the recent availability of selective β_3-adrenoceptor antagonists will help, in part, to establish this.

APPENDIX 1

SCINTILLATION PROXIMITY ASSAY

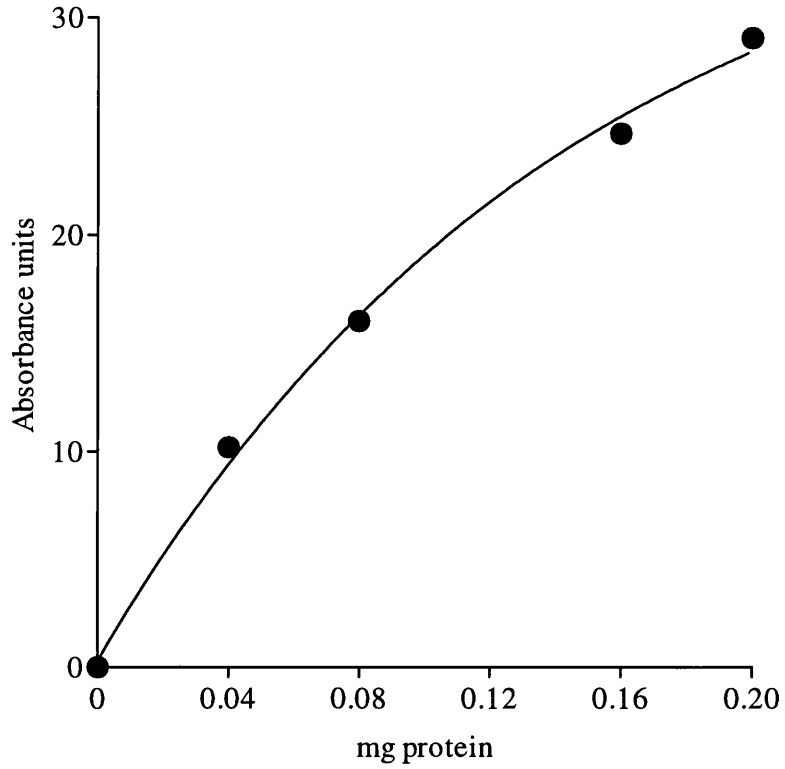
The table below shows data and calculations for the standard curve derived from an experiment using the cyclic AMP scintillation proximity assay kit (Amersham; acetylation protocol). **Graph 1** shows a standard curve for the cyclic AMP acetylation protocol.

	Mean counts per minute (CPM)	**CPM - NSB (B*)**	**B^*/B_0 x 100**
Non specific binding (NSB)	234.0	-	-
B_0	3950.5	3716.5	-
2 fmol cyclic AMP	3431.0	3197.0	86.0
4 fmol cyclic AMP	3334.0	3100.0	83.4
8 fmol cyclic AMP	3010.0	2776.0	74.7
16 fmol cyclic AMP	2573.8	2339.8	63.0
32 fmol cyclic AMP	1974.5	1740.5	46.8
64 fmol cyclic AMP	1415.5	1181.5	31.8
128 fmol cyclic AMP	910.8	676.8	18.2



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

PROTEIN DETERMINATION



Graph 2 Graph showing a standard curve for protein determination using the protocol of Lowry *et al.*, 1951 with bovine serum as control.

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