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Analysis of the effects of α_1-adrenoceptor antagonists on noradrenaline-mediated contraction of rat small mesenteric artery

[1]Pieter H. Van der Graaf, Nigel P. Shankley & James W. Black

Department of Analytical Pharmacology, King's College School of Medicine and Dentistry, Rayne Institute, 123 Coldharbour Lane, London SE5 9NU

1 In this study, we examined the interaction between noradrenaline (NA) and phenylephrine (PE) with seven antagonists (prazosin, tamsulosin, phentolamine, WB-4101, 5-methylurapidil, spiperone and HV 723) in an attempt to characterize the α_1-adrenoceptor population of the rat isolated small mesenteric artery (SMA) preparation.

2 Six of the seven antagonists investigated produced concentration-dependent, parallel, rightward shift of the NA concentration-effect (E/[A]) curves. The exception was tamsulosin, which produced significant decrease of the upper asymptote. In the case of 5-methylurapidil and HV723, the Schild plot slope parameters were not significantly different from unity over the range of concentrations used. However, the Schild plot slopes obtained for the other antagonists were all significantly greater than unity, inconsistent with expectations for simple competitive antagonism.

3 HV723, prazosin and tamsulosin were also tested using PE as an agonist. All three antagonists produced concentration-dependent, parallel, rightward shifts of the PE curves and Schild analysis yielded slope parameters not significantly different from unity. The pK_B estimates obtained for tamsulosin and prazosin were not significantly different from the pA_2 values obtained when NA was used as agonist. In the case of HV723, the 95% confidence intervals for the pK_B values yielded with NA and PE did not overlap ($pK_B = 8.80-9.13$ and $8.15-8.77$ for NA and PE, respectively).

4 In the absence of evidence to indicate that the steep Schild plots were due to failure to satisfy the basic criteria for quantitative analysis in a one-receptor system, we considered the possibility that the complexity was caused by an action of NA at inhibitory D_1 receptors. The selective D_1 receptor antagonists, SCH-23390 (10 nM), had no significant effect on the NA E/[A] control curve, but the apparent potency of 100 nM prazosin was reduced by $\sim$3.5 fold.

5 This study indicates that the steep Schild plots obtained from the interaction between NA and α_1-adrenoceptor antagonists were due to the simultaneous activation of inhibitory D_1 receptors by NA. Notwithstanding this complexity, our explanatory model of the system (see Appendix) suggests that the antagonist affinity values estimated in the absence of D_1 receptor block were not significantly affected by this other action of NA. The low affinity estimate obtained for prazosin suggests that the pharmacologically-defined α_{1L}-subtype operates in the SMA.

Keywords: α_1-Adrenoceptors; α_1-adrenoceptor antagonists; dopamine D_1 receptors; noradrenaline; phenylephrine; rat small mesenteric artery; Schild analysis

Introduction

It has become clear from radioligand binding and molecular biology experiments (see Hieble *et al.*, 1995) that there are at least three subtypes of α_1-adrenoceptors, now referred to as α_{1A} (previously known as α_{1C}), α_{1B} and α_{1D} (previously also known as α_{1A} or $\alpha_{1A/D}$). Various groups have shown that the α_1-adrenoceptor antagonist, prazosin, does not discriminate between these subtypes (see Ford *et al.*, 1994; Hieble *et al.*, 1995). In contrast, functional pharmacological studies on isolated tissue bioassays have resulted in a classification of α_1-adrenoceptors that is mainly based on the selectivity of prazosin. Digges & Summers (1983) showed that prazosin was 10 fold more potent in rat aorta ($pA_2 = 9.4$) than in portal vein ($pA_2 = 8.4$) when noradrenaline (NA) was used as agonist. Similar differences were also found with BE-2254 and phentolamine but not with yohimbine. At about the same time, Holck *et al.* (1983) reported that in rabbit main pulmonary artery, prazosin expressed a 10 fold higher affinity against clonidine ($pA_2 = 9.4$) than against methoxamine ($pA_2 = 8.4$). In this study, a similar difference was found with yohimbine. Subsequently, several authors presented a review of data from their own and other laboratories (Medget & Langer, 1984; Agrawal *et al.*, 1985; Drew, 1985; Flavahan & Vanhoutte, 1986) and noted the wide between-tissue variation in affinity reported for prazosin and yohimbine in functional studies. On the basis of this variation, Flavahan & Vanhoutte (1986) proposed the existence of two α_1-adrenoceptors, one with high affinity for prazosin and yohimbine and one with low affinity for these two ligands. These two subtypes became known as α_{1H} and α_{1L}-adrenoceptor, respectively (see McGrath & Wilson, 1988). More recently, Muramatsu and co-workers (1990) studied the effect of five ligands, classified as α_1-adrenoceptor antagonists, on the contractile responses to NA and phenylephrine (PE) in nine blood vessels from different species. On the basis of pA_2 values estimated for prazosin and yohimbine, the α_1-adrenoceptor population of six tissues could be classified as either α_{1H} or α_{1L}. In three dog vascular preparations, however, yohimbine displayed high affinity and prazosin relatively low affinity, inconsistent with the α_{1H}/α_{1L} scheme. Therefore, they concluded that a third receptor would be needed to account for their data. This subtype was designated α_{1N}-adrenoceptor and HV723 was proposed to be a selective antagonist (Mur-

[1]Author for correspondence at present address: Leiden/Amsterdam Center for Drug Research, Division of Pharmocology, Sylvius Laboratories, P.O. Box 9503, 2300 RA Leiden, The Netherlands.

amatsu *et al.*, 1990). Subsequently, in an attempt to reconcile the classification schemes from binding and functional studies the same group proposed to divide further the α_{1H}-subtype into α_{1A} and α_{1B} (Oshita *et al.*, 1991) or into α_{1A}, α_{1B} and α_{1C} (Muramatsu *et al.*, 1991; Muramatsu, 1995). Ford and coworkers (1994) have proposed a similar classification with four subtypes: three with high affinity for prazosin (α_{1A}, α_{1B} and α_{1D}) and one subtype which displays low affinity for prazosin, the α_{1L}-adrenoceptor.

It is believed that α_1-adrenoceptors play an important role in the physiological control of small artery diameter and therefore of blood pressure (see, for example, Cubeddu, 1988; Mulvany & Aalkjaer, 1990). However, the majority of pharmacological studies of α_1-adrenoceptors in vascular smooth muscle preparations has been confined to larger conduit vessels while α_1-adrenoceptors operating in resistance arteries have not yet been studied in detail. In this study, we describe our attempt to characterize the α_1-adrenoceptor population in a resistance vessel bioassay, the rat isolated small mesenteric artery (SMA) preparation. We used the same seven ligands (prazosin, tamsulosin, phentolamine, WB-4101, 5-methylurapidil, spiperone and HV 723) which, when employed in a previous study, provided evidence for α_1-adrenoceptor heterogeneity in the rat aorta (Van der Graaf *et al.*, 1993).

Preliminary accounts of these data were presented to the British Pharmacological Society (Van der Graaf, 1995b, 1996a,b).

Methods

Rat isolated small mesenteric artery preparation

Male Wistar rats (225–300 g) were killed by cervical dislocation and the mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 and ethylenediaminetetra-acetic acid (EDTA) 0.026. Six arterial trees were dissected from each mesenteric vascular bed and cleared from surrounding adipose tissue. From each arterial tree, a ~2 mm ring segment was mounted in a small vessel myograph (J.P. Trading, Aarhus, Denmark) with separated 6 ml organ baths (thermostatically controlled at 37 ± 0.5°C, containing the KHS and continuously gassed with 95% O_2 and 5% CO_2) as described by Mulvany & Halpern (1977). The endothelium was removed by gentle rubbing of the intimal surface with a thin, scoured, metal wire. Tissue responses were continuously measured as changes in isometric tension and displayed on potentiometric chart recorders.

Experimental protocol

Following a 30 min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure ($L_{100} = 239.3 \pm 0.8$ μm, $n = 367$) according to the standard procedure of Mulvany & Halpern (1977). After a further 15 min stabilization period, a calibration contraction (6.6 ± 0.3 mN) was obtained to 10 μM NA in each tissue and the absence of the endothelium was then confirmed by the lack of response to 10 μM 5-methylfurmethide, the acetylcholine M-receptor agonist. After a 15 min washout period, tissues were incubated for 90 min with either antagonist or vehicle. A block design was used to allocate treatments. Single agonist concentration-effect (E/[A]) curves were obtained by cumulative dosing at half or third-log unit concentration increments. Effects were expressed as percentage of the calibration response to 10 μM NA. Cocaine (30 μM) and timolol (6 μM) were present in all experiments with NA and PE to block neuronal uptake and β_1/β_2 adrenoceptors, respectively. Extraneuronal uptake did not appear to play a role in the SMA assay because it was shown in preliminary experiments that 10 μM corticosterone did not have a significant effect on the NA E/[A] curve (data not shown).

Analysis

Competitive analysis Individual agonist curve data were fitted to the Hill equation using an iterative, least-squares method:

$$E = \frac{\alpha \times [A]^{n_H}}{[A]_{50}{}^{n_H} + [A]^{n_H}}, \quad (1)$$

to provide estimates of midpoint slope (n_H), midpoint location ($[A]_{50}$, estimated as a logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's t test, as appropriate. Values of $P < 0.05$ were considered to be significant.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel, rightward shift of the agonist E/[A] curves with no change in upper asymptote, antagonist affinity estimates were obtained by fitting the individual midpoint location values obtained in the absence ($\log[A]_{50}$) and presence ($\log[A]_{50\,B}$) of antagonist (B) to the following derivation of the Schild equation as described previously (Black *et al.*, 1985).

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^b/10^{\log K_B}) \quad (2)$$

When the Schild plot slope parameter (b) was not significantly different from unity, then the data were re-fitted with b constrained to unity so that the antagonist dissociation equilibrium constant, K_B could be estimated as $\log K_B \pm$ s.e. For purposes of display, Schild plots were then constructed with slopes of unity and intersection of the abscissa scale at the $\log K_B$ calculated by the method above. When b was found to be significantly different from unity, then an empirical pA_2 value was estimated from the intercept with the log [B]-axis of the Schild plot using the line generated with the unconstrained slope.

Compounds

Compounds were obtained from the following sources: cocaine hydrochloride, 5-hydroxytryptramine hydrochloride (5-HT), (−)-noradrenaline hydrochloride (NA), phentolamine hydrochloride, (−)-phenylephrine hydrochloride (PE), prazosin hydrochloride and spiperone (Sigma Chemical Company Ltd., U.K.); 5-methylurapidil, SCH-23390 (**R**-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) and WB-4101 (N-[2-(2,6 dimethoxyphenoxy)-ethyl] -2, 3-dihydro-1, 4-benzodioxin-2-methanamine hydrochloride) (Research Biochemicals Incorporated, U.S.A.); tamsulosin (a gift from Yamanouchi Pharmaceutical Co. Ltd., Japan); HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)amino)propyl)benzene-acetonitrile fumarate) (a gift from Professor I. Muramatsu, Fukui Medical School, Japan); timolol maleate (Merck, Sharp & Dohme, U.K.); 5-methylfurmethide iodide (Wellcome Research Laboratories Ltd., U.K.).

NA, PE and 5-HT were dissolved and diluted in stoichiometric, aqueous ascorbic acid solution. Spiperone was dissolved initially in absolute ethanol to give a 2 mM stock solution and subsequently diluted in distilled water. Prazosin and 5-methylurapidil were dissolved initially in 50% ethanol to give 2 mM stock solutions and subsequently diluted in distilled water. All other drugs were dissolved in distilled water. NA, PE, 5-HT and phentolamine solutions were made up each day. All other drug stock solutions were stored below −20°C and diluted on the day of the experiment. The maximum volume of drug solution administered to the 6 ml organ baths did not exceed 150 μl, corre-

sponding to ~2.5% of the bath volume. Neither the vehicles nor the antagonists were found to produce significant effects on basal tone.

Results

Noradrenaline concentration-effect relation

Noradrenaline (NA) produced concentration-dependent contraction of SMAs (Figure 1) and the midpoint location ($p[A]_{50} = 6.64 \pm 0.04$), midpoint slope ($n_H = 1.41 \pm 0.07$) and upper asymptote ($\alpha = 119 \pm 7\%$) were estimated from curve data obtained from a typical, preliminary experiment ($n = 4$).

Effects of competitive α_1-adrenoceptor antagonists

Six of the seven antagonists investigated produced concentration-dependent, parallel, rightward shift of the NA E/[A] curves. The exception was tamsulosin, which produced significant decrease of the upper asymptote (Figure 2g). As judged by inspection of individual experimental traces, the antagonists did not appear to have an effect on the rate of contraction to NA (data not shown).

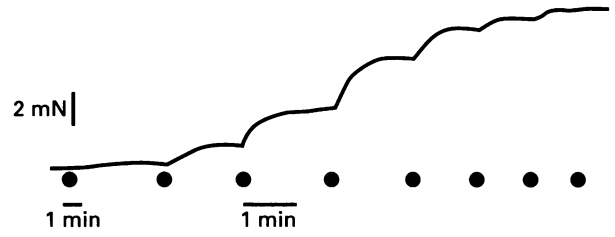


Figure 1 Representative experimental tracing showing the response of a rat isolated small mesenteric artery ($l_{100} = 239\,\mu m$) to noradrenaline (0.1 – 30 μM) administered by a cumulative dosing regimen at one third-log unit concentration increments (●). Note that the speed of the chart recorder was increased immediately following the third addition of noradrenaline, as indicated by the time bars.

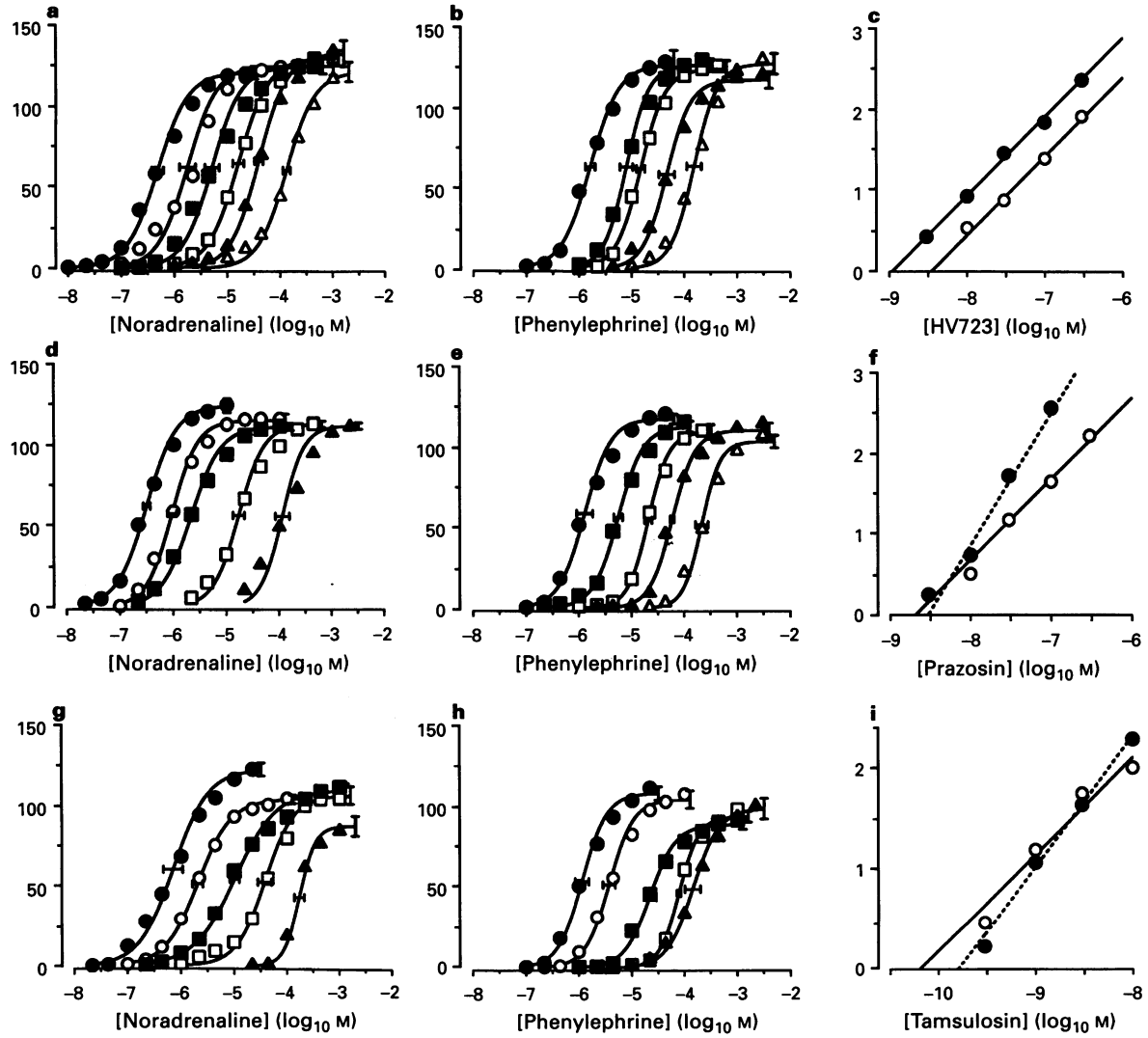


Figure 2 Concentration-effect curves obtained on rat small mesenteric arteries to noradrenaline (a, d, g) and phenylephrine (b, e, h) in the absence (●) and presence of (a, b) 3 (○), 10 (■), 30 (□), 100 (▲) and 300 nM (△) HV723; (d, e) 3 (○), 10 (■), 30 (□), 100 (▲) and 300 nM (△) prazosin; (g, h) 0.3 (○), 1 (■), 3 (□) and 10 nM (▲) tamsulosin. The lines shown superimposed on the mean data points ($n = 4-10$) were simulated using the Hill equation. Horizontal and vertical error (s.e.means) are shown on the mean midpoint and upper asymptote locations, respectively. Ordinates: Effect, expressed as percentage of a 10 μM noradrenaline calibration response. (c, f, i) Schild plots for the interaction between (c) HV723; (f) prazosin and (i) tamsulosin with noradrenaline (●) and phenylephrine (○). The solid and dashed lines shown superimposed on the mean data points were simulated using the parameters obtained from constrained and unconstrained model fits, respectively (Table 1). Ordinates: $\log_{10}$ (r-1), where r is the concentration ratio.

Table 1 Competitive analysis of the antagonism of noradrenaline and phenylephrine in the rat isolated small mesenteric artery

	Noradrenaline		
Antagonist	pK_B (pA_2) ± s.e.	b ± s.e.	d.f.
HV723	8.96 ± 0.08	0.98 ± 0.06	52
5-Methylurapidil	8.38 ± 0.11	1.04 ± 0.07	29
Prazosin	(8.5 ± 0.1)	1.63 ± 0.13*	43
Tamsulosin	(9.8 ± 0.2)	1.35 ± 0.09*	23
Phentolamine	(7.7 ± 0.1)	1.40 ± 0.09*	27
WB-4101	(8.4 ± 0.1)	1.52 ± 0.19*	22
Spiperone	(7.5 ± 0.2)	1.37 ± 0.12*	25
	Phenylephrine		
Antagonist	pK_B ± s.e.	b ± s.e.	d.f.
HV723	8.46 ± 0.15	0.96 ± 0.09	23
Prazosin	8.68 ± 0.08	1.12 ± 0.07	19
Tamsulosin	10.20 ± 0.15	1.10 ± 0.09	19

*b significantly greater than unity ($P<0.05$).

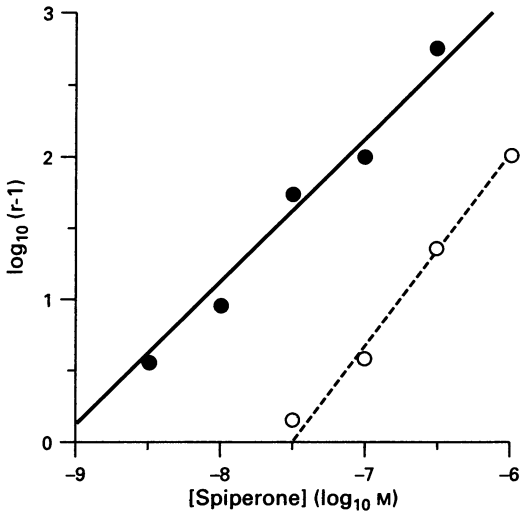


Figure 3 Schild plots for the interactions between spiperone with 5-HT (●) and noradrenaline (○). The solid and dashed lines shown superimposed on the mean ($n=5-6$) data points were simulated using the parameters (see text) obtained from constrained and unconstrained model fits, respectively.

The individual log $[A]_{50}$ values estimated in the absence and presence of antagonist within each experiment were fitted to the competitive model (Equation 2). In the case of 5-methylurapidil and HV723, the Schild plot slope parameters were not significantly different from unity over the range of concentrations used and pK_B values were estimated (Table 1). However, the Schild plot slopes obtained for the other antagonists were all significantly greater than unity, inconsistent with expectations for simple competitive antagonism (Table 1). The data obtained with three of the antagonists (HV723, prazosin and tamsulosin), chosen on the basis that they illustrate different patterns of α_1-adrenoceptor antagonism observed, are presented graphically in Figure 2.

HV723, prazosin and tamsulosin were also tested using the selective α_1-adrenoceptor agonist, phenylephrine (PE). All three antagonists produced concentration-dependent, parallel, rightward shifts of the PE curves (Figure 2) and Schild analysis yielded slope parameters not significantly different from unity (Table 1). The pK_B estimates obtained for tamsulosin and prazosin were not significantly different from the pA_2 values obtained when NA was used as agonists (Table 1). In the case of HV723, the 95% confidence intervals for the pK_B values yielded with NA and PE did not overlap ($pK_B = 8.80-9.13$ and $8.15-8.77$ for NA and PE, respectively), and therefore the estimates could be regarded as significantly different.

Investigation of explanations for the steep Schild plots: one-receptor system

Kenakin (1993) has reviewed three interpretative models in which a steep Schild plot can be generated when the agonist interacts with a single receptor population. Schild plots with slopes greater than unity can occur if the low concentration-ratios are underestimated, as is the case with insufficient antagonist incubation time or with the presence of a saturable antagonist uptake process. Steep Schild plots will also be found if the high concentration ratios are overestimated, as is the case if at high antagonist concentrations an additional inhibitory property is expressed (Kenakin, 1993). Three experiments were designed to test whether one of these conditions could explain the data obtained with NA in the SMA.

In the first experiment, the possibility that inadequate antagonist equilibration time was responsible was tested with a low (6 nM) concentration of prazosin, since the greatest effect of non-equilibrium would theoretically be seen at small concentration ratios (Kenakin, 1980). However, the rightward shift of the NA E/[A] curve produced by 6 nM prazosin after 90 min incubation (concentration-ratio = 5.0 ± 1.3, $n=6$) was not significantly increased when the incubation time was prolonged to 150 min (concentration ratio = 4.1 ± 1.3, $n=6$; $P>0.5$). Therefore, at least in the case of prazosin, inadequate antagonist incubation time was not the cause for the steep Schild plots.

The possibility of antagonist uptake was investigated using spiperone, a ligand which has high affinity not only for α_1-adrenoceptors but also for vascular 5-HT_2 receptors (Leff & Martin, 1986). It was reasoned that if antagonist uptake was the cause for the steep Schild plot obtained for the interaction between spiperone and NA, then over the same concentration-range spiperone should also yield Schild plot slopes greater than unity when tested as an antagonist of 5-HT. 5-HT produced concentration-dependent contraction of the SMA ($p[A]_{50} = 6.31 \pm 0.17$; $n_H = 1.97 \pm 0.09$; $\alpha = 100 \pm 7\%$, $n=5$). Spiperone (3–300 nM) produced concentration-dependent, parallel, rightward displacement of the 5-HT E/[A] curves and Schild analysis yielded a slope parameter not significantly different from unity (b = 1.08 ± 0.11, d.f. = 25; Figure 3). From the constrained fit, a pK_B of 9.14 ± 0.16 was estimated, similar to the value (9.28 ± 0.10) found by Leff & Martin (1986) for the antagonism of 5-HT by spiperone in rabbit aorta. The fact that spiperone behaved as a competitive antagonist of 5-HT but not of NA suggests that the presence of a saturable antagonist uptake process was not the cause of the complexity.

A third experiment was designed to expose inhibitory properties of the antagonists not related to competitive blockade of α_1-adrenoceptors. For this purpose, we investigated the effects of a high concentration of prazosin on the response of the SMA to 5-HT. Again, the outcomes did not provide an explanation for the steep Schild plots, since 100 nM prazosin had no effect on the Hill equation parameters of the 5-HT E/[A] curve ($p[A]_{50} = 6.69 \pm 0.16$ and 6.68 ± 0.11; $n_H = 1.80 \pm 0.19$ and 1.96 ± 0.11; $\alpha = 107 \pm 3\%$ and $110 \pm 2\%$, in the absence and presence of prazosin, respectively, $n=5$).

Investigation of explanations for the steep Schild plots: two-receptor system

Recently, we characterised an endothelium-independent, relaxant effect of NA in the precontracted SMA which appeared to be mediated by dopamine D_1 receptors (Van der Graaf *et al.*, 1995a). We have investigated whether this action might produce functional antagonism of α_1-adrenoceptor-mediated contractile responses and thus be the cause for the steep Schild plots obtained in the present study. We chose to use a single, high concentration of prazosin for this experiment because a

clear deviation from the simple competitive model was expected to be achieved only at high concentration ratios (see Figure 2f). The selective D_1 receptor antagonist, SCH-23390 (10 nM, ~20 fold its K_i value for dopamine-stimulated cyclic AMP formation in cultured smooth muscle cells obtained from rat mesenteric artery; Hall *et al.*, 1993), had no significant effect on the NA E/[A] curve (Figure 4). Prazosin (100 nM) produced parallel rightward shift of the NA E/[A] curve with an associated concentration-ratio of 79 (Figure 4). In the presence of 10 nM SCH-23390, the apparent potency of prazosin at this concentration was significantly reduced by ~3.5 fold (new concentration-ratio = 22, Figure 4). We demonstrated that this difference in concentration ratios could quantitatively account for the steep Schild plot obtained with prazosin as follows. It was assumed that the pA_2 value obtained in the presence of SCH-23390 provided an uncontaminated estimate of the pK_B for prazosin at the α_1-adrenoceptors in the SMA. Thus, the $[A]_{50}$ values of the curves obtained in the absence and presence of 100 nM prazosin alone were fitted to Equation (2) with the pK_B parameter constrained to the pA_2 value (8.33 ± 0.14) obtained from the rightward shift produced by 100 nM prazosin in the presence of 10 nM SCH-23390. Under these conditions, a value of 1.42 ± 0.10 was obtained for the slope parameter (b), which was not significantly different from the value obtained from the primary Schild analysis obtained with multiple concentrations of prazosin (b = 1.63 ± 0.13, Table 1).

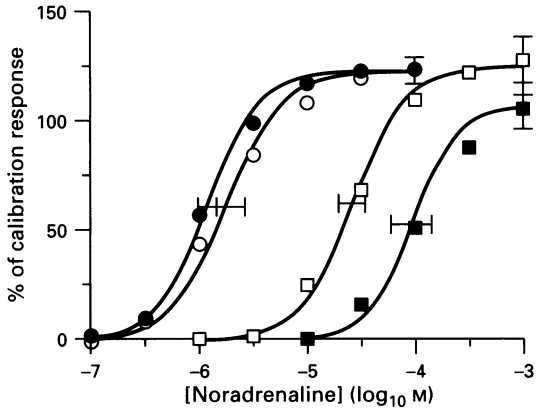


Figure 4 Concentration-effect curves obtained on rat small mesenteric arteries to noradrenaline in the absence (●) and presence of 10 nM SCH-23390 (○), 100 nM prazosin (■) and a combination of 10 nM SCH-23390 and 100nM prazosin (□). The lines shown superimposed on the mean data points ($n = 4$) were simulated using the Hill equation. Horizontal and vertical error (s.e.mean) bars are shown on the mean midpoint and upper asymptote locations, respectively. Effect is expressed as percentage of a 10 μM noradrenaline calibration response.

Discussion

Although it is recognised that mesenteric resistance arteries play an important role in the regulation of blood pressure (Furness & Marshall, 1974; Christensen & Mulvany, 1993), relatively few studies have provided a detailed quantitative characterization of α_1-adrenoceptors in the mesenteric vasculature. Recently, however, Williams & Clarke (1995) showed that that α_{1A}-adrenoceptor (previously known as α_{1C}-adrenoceptor, Hieble *et al.*, 1995) mediates vasoconstriction to NA and nerve stimulation in the isolated perfused mesentery of rat. This result was not entirely consistent with the conclusion from a previous study in the same preparation by Kong *et al.* (1994), who proposed that both the α_{1A} and α_{1B}-adrenoceptor are involved in the contractile responses. Most recently, using the assay employed in this study, Ipsen *et al.* (1995) found evidence for the presence of α_{1A}, α_{1B} and 'other α_1-subtypes' in the SMA. Furthermore, Piascik and co-workers (1994) have reported that they could detect mRNA for the α_{1a}, α_{1b} and α_{1d}-adrenoceptor subtypes in rat mesenteric resistance arteries. When we compared the pA_2 values obtained with NA as agonist (Table 1) with previously published affinities measured at the three cloned α_1-adrenoceptors, we found a significant correlation ($r^2 = 0.78$, $P < 0.01$) with the α_{1a} but not with the α_{1b} ($r^2 = 0.22$, $P > 0.2$) and α_{1d}-adrenoceptor subtype ($r^2 = 0.53$; $P > 0.05$; Figure 5). However, the pA_2 value (8.5) for prazosin in the SMA was about one log unit lower than the pK_i values at all three cloned α_1-adrenoceptor subtypes (α_{1a}: 9.5; α_{1b}: 9.7; α_{1d}: 9.5; Figure 5). This suggests that the characteristics of the α_1 adrenoceptor population mediating contraction of the isolated SMA do not fit completely into the currently accepted classification scheme (Hieble *et al.*, 1995) which recognises three (α_{1A}, α_{1B} and α_{1D}) α_1-adrenoceptors. Indeed, the data are more consistent with the profile of the pharmacologically-defined α_{1L}-subtype (Flavahan & Vanhoutte, 1986; McGrath & Wilson, 1988; Ford *et al.*, 1994). The significant, though small, difference between the pK_B estimates for HV723 obtained with NA and PE (Table 1, Figure 2c) could be indicative of het-

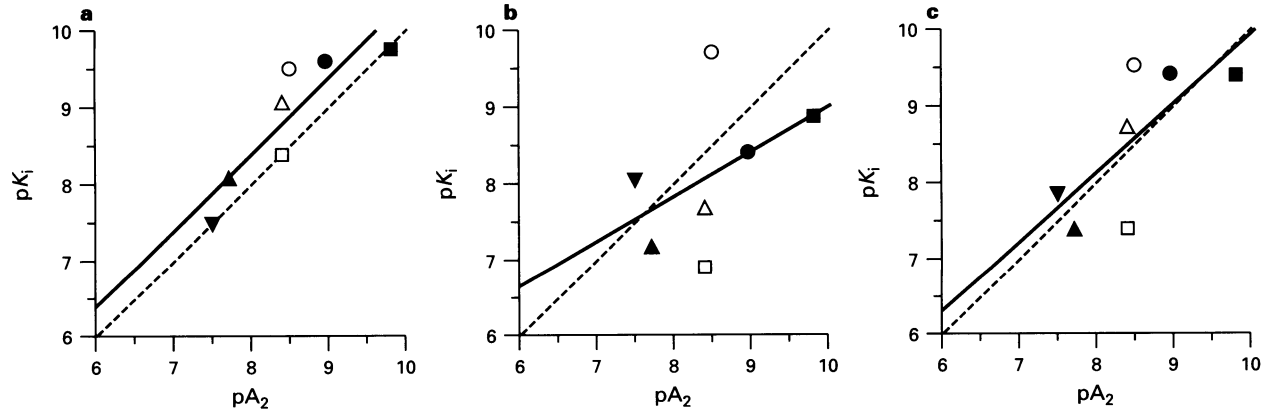


Figure 5 Relation between pA_2 estimates obtained in rat small mesenteric arteries using noradrenaline as agonist (Table 1) and previously published pK_i values for the displacement of [^{3}H]-prazosin at cloned (a) α_{1a}, (b) α_{1b} and (c) α_{1d}-adrenoceptor subtypes for HV723 (●), prazosin (○), tamsulosin (■), 5-methylurapidil (□), phentolamine (▲), WB-4101 (△) and spiperone (▼). With the exceptions of tamsulosin and HV723, pK_i values were obtained from Laz *et al.* (1994), who used rat cloned α_1-adrenoceptors expressed in COS-7 cells. The pK_i values for tamsulosin were taken from Taguchi & Michel (1995), who used rat cloned α_1-adrenoceptors expressed in COS-1 cells. To our knowledge, binding data of HV723 at all three rat cloned α_1-subtypes have not been published. Therefore, we used data provided by Blue *et al.* (1995), who published pK_i values for HV723 at bovine α_{1a}, hamster α_{1b} and rat α_{1d} cloned adrenoceptors expressed in rat-1 fibroblasts. The dashed lines represent the line of identity, the solid lines were obtained by linear regression.

erogeneity of this α_1-adrenoceptor class, which might be related to the subclassification postulated by Muramatsu and coworkers (1990; 1991).

The low affinity for prazosin estimated in the present study is not consistent with values found previously by Högestätt & Andersson (1984) and Nielsen & Mulvany (1990) in the same myographic assay of isolated SMA ($pA_2 = 9.58-9.84$ and 9.23 ± 0.16, respectively) or with the pA_2 value of 9.3 ± 0.1 obtained by Williams & Clarke (1995) in the rat perfused mesentery preparation. On the other hand, however, McPherson *et al.* (1984) have reported that prazosin antagonizes the pressor response of the rat perfused mesenteric bed to NA with low affinity ($pA_2 = 8.52 \pm 0.15$).

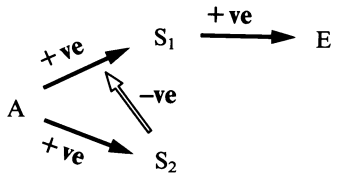
In contrast to the current work, steep Schild plots were not reported in previous studies in which the effects of α_1-adrenoceptor antagonists in the rat isolated SMA were analysed in a quantitative manner (Högestätt & Anderson, 1984; Pegram & Kardon, 1985; Nielsen & Mulvany, 1990; Ipsen *et al.*, 1995). However, inspection of the data presented by Nielsen & Mulvany (1990) suggests that the interaction between prazosin and NA may have been associated with a steep Schild plot. In the absence of evidence to indicate that the steep Schild plots were due to failue to satisfy the basic criteria for quantitative analysis in a one receptor system, we considered the possibility that the complexity was caused by an action of NA at a second receptor mediating a stimulus opposite to that generated by the α_1-adrenoceptors. Recently, we showed that NA, but not PE, activates dopamine D_1 receptors mediating relaxation in the SMA at concentrations which were applied in the present study (Van der Graaf *et al.*, 1995a). Furthermore, we showed that HV723, but not prazosin or tamsulosin, blocked this action with an apparent pA_2 value (~ 8) which was only one-log unit lower than the pK_B for α_1-adrenoceptors estimated in this study (Table 1). On the basis of these findings we speculated that the steep Schild plots were due to an action of NA at D_1 receptors, because they were not obtained when PE was used as the agonist nor when HV723 was used as the antagonist. This hypothesis was supported by the finding that the concentration-ratio produced by 100 nM prazosin was reduced by ~ 3.5 fold in the presence of a selective concentration of the D_1 receptor antagonist, SCH-23390 (Figure 4). Although it has been recognised before that a dual action of an agonist within one assay may produce steep Schild plots (Kenakin, 1981), to our knowledge the present data provide the first example of such a case. We have developed a model of functional antagonism which can account for the data by assuming that the efficacy of NA at α_1-adrenoceptors is reduced by a simultaneous action at D_1 receptors (see Appendix). In terms of speculating on the mechanism of this interaction, D_1 receptors in rat mesenteric artery vascular smooth muscle cells are known to be coupled to activation of adenylyl cyclase (Hall *et al.*, 1993). However, it has also been shown that D_1 receptors mediate various other cellular responses (see Kimura *et al.*, 1995; Sokoloff & Schwartz, 1995), for example stimulation of inositol phosphate production and mobilisation of Ca^{2+}. Furthermore, it has been reported that D_1 receptors can inhibit phosphatidyl inositol metabolism either directly (Wallace & Claro, 1990) or by elevating cyclic AMP levels (Undie & Friedman, 1994), which may be particularly relevant for the present study since stimulation of phosphoinositide hydrolysis is believed to be the predominant effector pathway of α_1-adrenoceptors. In addition, it has recently been shown that D_1 receptors can couple to G_o proteins inhibiting ion channel function (Kimura *et al.*, 1995).

In conclusion, the current analysis indicates that the steep Schild plots obtained from the interaction between NA and α_1-adrenoceptor antagonists were due to the simultaneous activation of inhibitory D_1 receptors by NA. Notwithstanding this complexity, our explanatory model of the system (see Appendix) suggests that the antagonist affinity values estimated in the absence of D_1 receptor block were not significantly affected by this other action of NA in the assay. The affinity estimate made for prazosin suggests that the α_1-adrenoceptor in the SMA expresses a pharmacological profile different from that of α_{1A}, α_{1B} and α_{1D}-adrenoceptors.

Appendix

Development of a model of functional antagonism to account for steep Schild plots

Furchgott's (1981) two-receptor model, which assumes that the individual stimuli (S_1 and S_2) produced by the action of an agonist at two receptors converge into an overall stimulus (S_T) by simple addition, can only describe Schild plots with slopes of unity or less. Therefore, in seeking to find an explanatory model for the current data, we developed a model in which one stimulus inhibits the action of the second. The simplest version, in which $S_T = S_1 - S_2$ (see Van den Brink, 1973; Szabadi, 1975; 1977), was rejected because it can produce steep Schild plots only under conditions where the agonist E/[A] curve shape was significantly changed. Therefore, we considered a model in which the second stimulus (S_2) inhibits the production of the first (S_1). This model can be represented schematically as follows:



The first step in the model building process was the assumption that activation of a receptor R_1 by the agonist (A) produces a stimulus (S_1) and that the pharmacological effect (E) is given as a saturable function of the amount of S_1:

$$S_1 = \frac{\alpha_1 \times [A]^m}{K_1^m + [A]^m} \quad (3)$$

$$E = \frac{E_m \times S_1^n}{1 + S_1^n} \quad (4)$$

where α_1, K_1 and m are the upper asymptote, midpoint location and slope parameter of the S_1/[A] function, respectively, E_m is the maximum effect and n is the slope parameter of the E/S_1 function. A competitive antagonist (B) was assumed to decrease the apparent potency, K_1, by the factor $(1 + [B]/K_{B1})$ in the usual manner. It was then assumed that the agonist also activates a second receptor (R_2) to produce stimulus S_2, which exerts an inhibitory effect on the formation of S_1. In terms of the model, this can be achieved by modifying one or more of the parameters of Equation (3). In order to minimize the complexity of the model, only one of these parameters was allowed to change. Simulations showed that changes in the slope parameter, m, produced too great an effect on the slope of the E/[A] curve compared to the midpoint location. The parameters K_{B1} and K_1 were not considered because we could not envisage how antagonist affinity (K_{B1}) could be changed by S_2 independently of agonist affinity, which is a component of K_1. Therefore, K_{B1} and K_1 would necessarily be changed simultaneously which would violate our constraint to change only one of the parameters. This left α_1 (maximum amount of S_1) as the parameter to be changed by S_2. Modification of α_1 allows for the most flexible changes in E/[A] curve shape to occur, since α_1 is a determinant of E/[A] curve location, slope and upper asymptote in the model. Without prejudice to mechanism, α_1 was multiplied by the factor $(1 - S_2)$ and another hyperbolic function was assumed to relate S_2 with [A] as follows:

$$S_2 = \frac{\alpha_2 \times [A]^q}{K_2^q + [A]^q} \quad (5)$$

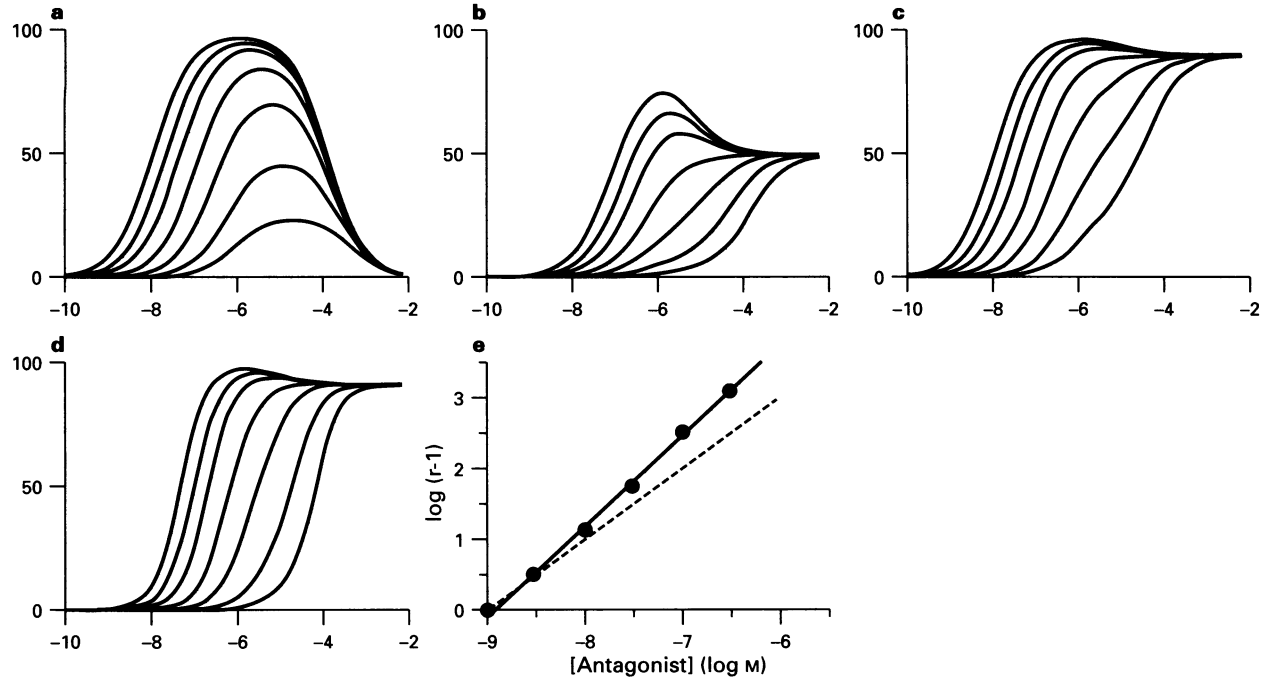


Figure 6 Model of functional antagonism simulations showing the effect of varying m, α_1, and α_2 in Equations (3)–(5) on the behaviour of a competitive antagonist (0, 1, 3, 10, 30, 100 and 300 nM). In the simulations, n (1), E_m (100), q (1), pK_1 (6), pK_2 (6) and pK_{B1} (9) were fixed and m, α_1 and α_2 were varied as follows: (a) m = 1, α_1 = 100, α_2 = 1; (b) m = 1, α_1 = 10, α_2 = 0.9; (c) m = 1, α_1 = 100, α_2 = 0.9; (d) m = 1.5, α_1 = 100, α_2 = 0.9. Abscissae: $\log_{10}$ [agonist] (M). Ordinates: Effect, expressed as percentage of E_m. (e) Schild plot corresponding to the simulation shown in (d). The solid line was obtained by fitting the estimated log $[A]_{50}$ values to the unconstrained competitive model (Equation (2), b = 1.3), the dashed line represents the expected Schild plot associated with simple competitive antagonism in a one-receptor system (b = 1).

where α_2 is a proportionality factor defining the maximal reduction in α_1 with values ranging between zero (no reduction) and unity (complete reduction possible) and K_2 and q are the midpoint location and slope parameter of the $S_2/[A]$ function.

The model simulations in Figure 6 show that the effect of a reduction of α_1 by S_2 is highly dependent on the initial values of α_1 and α_2. From Equations (3)–(5) it can be deduced that when $[A] \rightarrow \infty$, then the upper asymptote of the E/[A] curve is given by

$$E_{[A]\rightarrow\infty} = \frac{E_m \times (\alpha_1 \times (1 - \alpha_2))^n}{1 + (\alpha_1 \times (1 - \alpha_2))^n} \qquad (6)$$

In the case when $\alpha_2 = 1$, the effect at high agonist concentrations will always approach zero, independent of the value of α_1 (Figure 6a). However, in cases when $\alpha_2 < 1$, the effect of S_2 on the upper asymptote becomes dependent on the efficacy (α_1) of the agonist at the first receptor. In the case of a low efficacy agonist, the upper asymptotes of the E/[A] curves will approximate to a value that is significantly less than the upper asymptote in the absence of S_2 (Figure 6b). However, in the case of a high efficacy agonist (i.e. high values of α_1), S_2 will produce only a small reduction of the maximum response which may not be detected experimentally (Figure 6c). Nevertheless, under the conditions used for the simulations in Figure 6c, the changes in the curve shape in the presence of antagonist should be detectable in terms of rejecting the one-receptor competitive antagonism model. However, when a steeper $S_1/[A]$ or E/S_1 function is assumed for the same system (i.e. m and/or n > 1), the effect on the curve shape is more subtle and the antagonist could be concluded to be producing parallel, rightward shift (Figure 6d). In such cases, however, Schild analysis would reveal that concentration-ratios are greater than expected for simple competitive antagonism (Figure 6e). When the antagonist also binds to receptor R_2 with affinity K_{B2}, and thus modifies the apparent agonist potency (K_2) by a factor $(1 + [B]/K_{B2})$, the magnitude of the deviation of the Schild plot slope parameter from unity becomes dependent on the selectivity of the antagonist for R_1 and R_2. When the antagonist is nonselective, the amount of reduction of K_1 and K_2 is always the same and Schild plots with unit slope will be obtained.

In order to determine whether the model could account for the experimental data obtained in the present study, the mean E/[A] data for the interaction between NA and the five antagonists which yielded steep Schild plots (prazosin, tamsulosin, phentolamine, WB-4101 and spiperone) were fitted simultaneously to Equations (3)–(5) using the AR module (derivative-free, nonlinear regression) of the BMDP statistical software package (Dixon *et al.*, 1990). This provided single estimates of E_m, n, m, q, pK_1, pK_2 and α_2 and individual estimates of pK_B for each antagonist. In order to allow for differences in the location of the control curve between experiments, individual α_1 estimates were obtained for each antagonist data set. Initially, it was found that the model fits did not converge when n was allowed to

Table 2 Model of functional antagonism parameter estimates (± s.e.[1]) for the interaction between noradrenaline and five antagonists which yielded steep Schild plots in the rat small mesenteric artery

n	2 (Constrained)	
E_m	126 ± 6%	
m	1.0 ± 0.7	
q	0.6 ± 0.3	
α_1	104 ± 14[2]	
α_2	0.96 ± 0.06	
pK_1	4.7 ± 0.3	
pK_2	6.5 ± 0.8	
		pA_2 obtained from Schild analysis[3]
pK_B prazosin	8.5 ± 0.2	8.5 ± 0.1
pK_B tamsulosin	9.5 ± 0.2	9.8 ± 0.2
pK_B phentolamine	7.4 ± 0.2	7.7 ± 0.1
pK_B WB-4101	8.3 ± 0.2	8.4 ± 0.1
pK_B spiperone	7.2 ± 0.2	7.5 ± 0.2

[1] Standard errors on the parameter estimates were calculated using the scaling-up method described by Leff *et al.*, (1990). [2] Mean ± s.e.mean, calculated from the estimates obtained for each antagonist data set. [3] See Table 1.

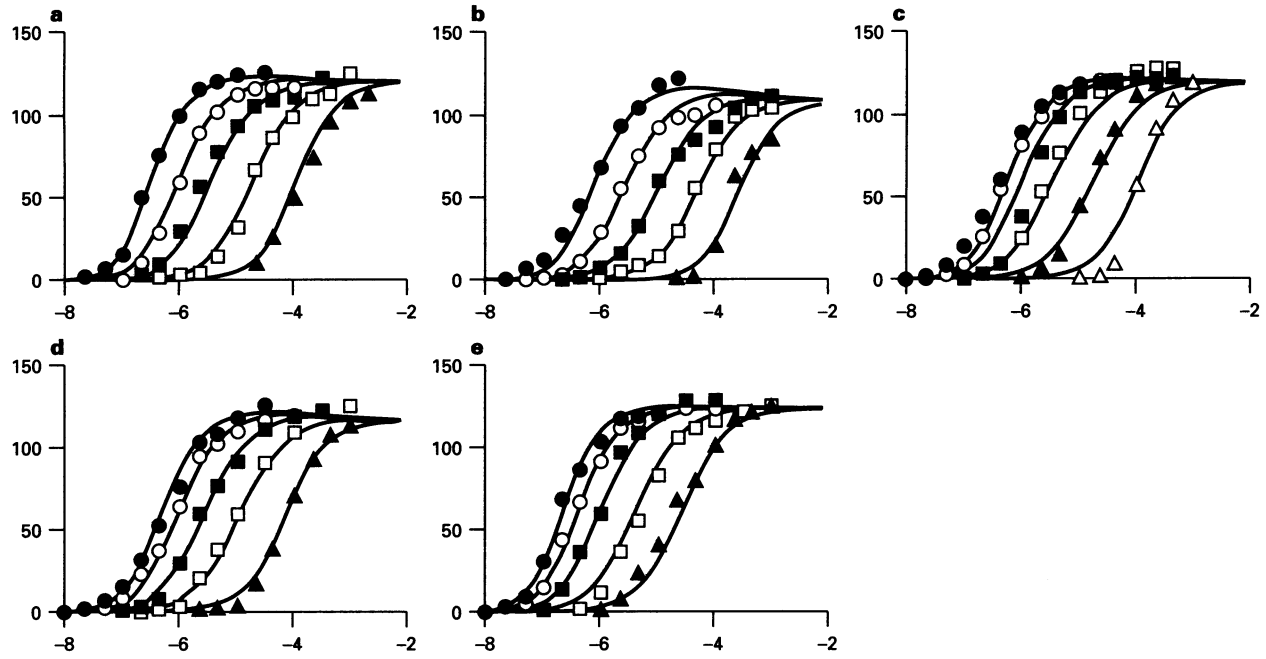


Figure 7 Simulations of the model of functional antagonism of the concentration-effect curves obtained on rat small mesenteric arteries to noradrenaline in the absence (●) and presence of (a) 3 (○), 10 (■), 30 (□) and 100 nM (▲) prazosin; (b) 0.3 (○), 1 (■), 3 (□) and 10 nM (▲) tamsulosin; (c) 0.01 (○), 0.03 (■), 0.1 (□), 0.3 (▲) and 1 μM (△) phentolamine; (d) 3 (○), 10 (■), 30 (□) and 100 nM (▲) WB-4101; (e) 0.03 (○), 0.1 (■), 0.3 (□) and 1 μM (▲) spiperone. The curves shown superimposed on the mean experimental data points were obtained using the parameter estimates summarised in Table 2. Abscissae: $\log_{10}$ [noradrenaline] (M). Ordinates: Effect, expressed as percentage of a 10 μM noradrenaline calibration response.

increase indefinitely. However, the goodness-of-fit, as judged by the residual sum of squares, improved only marginally with increasingly higher values of n and therefore the value of n was constrained to 2. The outcomes of the model fitting are summarised in Table 2 and were used for the simulations shown superimposed on the mean experimental data shown in Figure 7. As judged by eye, the model provided a good description of the data. The exception was the tamsulosin data set for which the model did not predict the marked depression of the maximum response obtained in the presence of high antagonist concentrations (Figure 7b). However, it has been reported previously that tamsulosin acts as a non-competitive antagonist of α_1-adrenoceptor-mediated responses in other vascular smooth muscle preparations (Takayanagi *et al.*, 1986; Furukawa *et al.*, 1995) and it is therefore possible that in the SMA, tamsulosin also behaves in a manner different from other α_1-adrenoceptor antagonists. The pK_B values estimated by the model were not significantly different from the pA_2 values obtained from Schild analysis (Table 2). Therefore, the model predicts that, at least for the compounds used in this study, the complexity was due to an overestimation of the high concentration-ratios and that the pA_2 values obtained from the steep Schild plots provide reliable estimates of antagonist affinity.

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