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Analysis of the activity of α_1-adrenoceptor antagonists in rat aorta

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1 In this study, the effects of seven α_1-adrenoceptor antagonists (tamsulosin, phentolamine, prazosin, WB-4101, 5-methylurapidil, spiperone and HV723) have been examined on the contractile response to noradrenaline (NA) and phenylephrine (PE) in rat isolated aorta.

2 NA and PE, when administered using a cumulative dosing schedule, both produced concentration-dependent contraction of aortic rings. It was possible to fit the individual concentration-effect (E/[A]) curve data to the Hill equation to provide estimates of the curve midpoint location ($p[A]_{50} = 7.74 \pm 0.10$ and 7.14 ± 0.18), midpoint slope ($n_H = 0.82 \pm 0.03$ and 0.99 ± 0.10) and upper asymptote ($\alpha = 3.2 \pm 0.3$ and 3.1 ± 0.2 g) parameters for NA and PE, respectively. However, the Hill equation provided a better fit to the E/[A] curve data obtained with another contractile agent, 5-hydroxytryptamine (5-HT) ($p[A_{50}] = 6.09 \pm 0.08$, $n_H = 1.49 \pm 0.09$, $\alpha = 2.6 \pm 0.3$ g), as judged by calculation of the mean sum of squares of the differences between the observed and predicted values.

3 All of the antagonists investigated produced concentration-dependent inhibition of the contractile responses of the aorta to NA and PE. Although no significant effects on the upper asymptotes of the E/[A] curves of any of the antagonists tested were detected, only tamsulosin and 5-methylurapidil did not have a significant effect on the slope (n_H) of the NA and PE E/[A] curves. The other antagonists produced significant steepening of the curves obtained with NA and/or PE.

4 Notwithstanding the fact that one of the basic criteria for simple competitive antagonism at a single receptor class was not always satisfied, the individual log $[A]_{50}$ values estimated in the absence and presence of antagonist within each experiment were fitted to the competitive model. The Schild plot slope parameters for the antagonism of NA and PE by phentolamine and HV723 were found to be significantly less than unity. The Schild plot slope parameters for the other antagonists were not significantly different from unity.

5 In the absence of evidence to suggest that the deviations from simple competitive antagonism were due to failure to satisfy basic experimental conditions for quantitative analysis, an attempt was made to see whether the data could be accounted for by an existing two-receptor model (Furchgott, 1981). The goodness-of-fit obtained with the two-receptor model was significantly better than that obtained with the one-receptor model. Furthermore, with the exception of the data obtained with phentolamine, the pK_B estimates for the two receptors were independent of whether NA or PE was used as agonist.

6 To determine which α_1-adrenoceptor subtypes may be associated with those defined by the two receptor model, the mean pK_B estimates obtained from the two-receptor model fit were compared with affinities measured by Laz *et al.* (1994) for rat cloned α_1-adrenoceptor subtypes expressed in COS-7 cells. The sum of squared differences of the data points from the line of identity was smallest for both pK_{B1} and pK_{B2} in the case of the $\alpha_{1a/d}$-adrenoceptor (now referred to as α_{1d}-adrenoceptor; Hieble *et al.*, 1995). Therefore, the complexity exposed in this study may be due to the expression of closely-related forms of the α_{1d}-adrenoceptor. However, relatively good matches were also found between pK_{B1} and α_{1c} and between pK_{B2} and α_{1b}. Therefore, on the basis of these data, it is not possible to rule out the involvement of all three α_1-adrenoceptors. The conflicting reports concerning the characteristics of the α_1-adrenoceptor population mediating contraction of the rat aorta may, at least in part, be due to the lack of highly selective ligands and to between-assay variation in the expression of multiple α_1-adrenoceptors.

Keywords: α_1-Adrenoceptors; Hill equation; noradrenaline; phenylephrine; rat aorta; Schild analysis; two-receptor model

Introduction

In the last decade it has become clear from radioligand binding, molecular biology and isolated tissue experiments that α_1-adrenoceptors are not a homogeneous class. Battaglia and co-workers (1983) reported that both phentolamine and WB-4101 inhibited [^{3}H]-prazosin binding in rat cerebral cortex in a manner that was inconsistent with these two ligands binding to a single receptor and suggested the existence of at least two α_1-adrenoceptors. Further studies by Morrow & Creese (1986) confirmed and extended these findings and binding sites with high and low affinity for WB-4101 and phentolamine were designated as α_{1A}- and α_{1B}-adrenoceptors, respectively. Subsequently, various ligands have been reported to discriminate between these two receptors with higher affinity for the α_{1A}-adrenoceptor, notably, 5-methylurapidil (Gross *et al.*, 1988) and (+)-niguldipine (Boer *et al.*, 1989). So far, three ligands have been suggested to display higher affinity for the α_{1B}-adrenoceptor, namely spiperone (Michel *et al.*, 1989), risperidone (Sleight *et al.*, 1993) and abanoquil (Perez *et al.*, 1994). In addition, the alkylating agent, chloroethylclonidine, has been proposed to inactivate irreversibly α_{1B}- but not α_{1A}-adrenoceptors (Han *et al.*, 1987).

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Molecular cloning studies have identified at least three different gene products which have been classified as α_1-adrenoceptors; α_{1a} from rat cerebral cortex (Lomasney *et al.*, 1991), α_{1b} from hamster DDT_1MF-2 smooth muscle cells (Cotecchia *et al.*, 1988) and α_{1c} from bovine brain (Schwinn *et al.*, 1990; 1991). Schwinn & Lomasney (1992) reported that the cloned α_{1a}-adrenoceptor has an identical sequence to a clone from rat brain which was originally termed α_{1d}-adrenoceptor by Perez *et al.* (1991) and was different from the pharmacologically-defined α_{1A}-adrenoceptor, suggesting that there are four α_1-adrenoceptor subtypes. However, various groups (e.g. Faure *et al.*, 1994; Forray *et al.*, 1994; Laz *et al.*, 1994; Michel & Insel, 1994; Perez *et al.*, 1994; Price *et al.*, 1994; Blue *et al.*, 1995; Esbenshade *et al.*, 1995; Pimoule *et al.*, 1995; Schwinn *et al.*, 1995) recently showed that the binding profile of the cloned α_{1c}-adrenoceptor corresponds to that of the pharmacologically-defined α_{1A}-subtype. This suggests that α_1-adrenoceptors can be satisfactorily classified into three subtypes, now referred to as α_{1A} (equivalent to the cloned α_{1c}), α_{1B} and α_{1D} (Hieble *et al.*, 1995). However, there is still no general agreement. For example, Kenny *et al.* (1994) have claimed that the rat cloned $\alpha_{1a/d}$-subtype is the pharmacologically-defined α_{1A}-adrenoceptor in rat vas deferens and cerebral cortex.

Pharmacological studies on isolated tissue bioassays have not resulted in a classification of α_1-adrenoceptors that is, as yet, congruent with the binding and cloning studies. Muramatsu and co-workers (1990) studied the effect of five ligands, classified as α_1-adrenoceptor antagonists, on the contractile responses to noradrenaline (NA) and phenylephrine (PE) in nine blood vessels from different species. They concluded that three receptor subtypes would be needed to account for their data: α_{1H} with high affinity for prazosin, α_{1N} with high affinity for HV723 and α_{1L} with low affinity for both ligands. 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). Similarly, Ford and co-workers (1994) proposed a 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.

There is no agreement on the characteristics of the α_1-adrenoceptor population mediating contraction of the rat aorta. In the past few years different groups have variously characterized the α_1-adrenoceptor(s) in this tissue as α_{1A} (Beckeringh & Brodde, 1989), α_{1B} (Han *et al.*, 1990; Eltze & Boer, 1992; Vargas *et al.*, 1993; Yazawa & Honda, 1993; Kong *et al.*, 1994; Piascik *et al.*, 1994; Testa *et al.*, 1995b), both α_{1A} and α_{1B} (Piascik *et al.*, 1991; Orsetti & DiStilo, 1994; Wenham & Marshall, 1994a), non-α_{1A}/non-α_{1B} (Mir & Fozard, 1990; Martinotti *et al.*, 1991; Li *et al.*, 1992; Oriowo & Ruffolo, 1992a; Aboud *et al.*, 1993; Fujimoto, 1993; Kearns *et al.*, 1994), α_{1D} (Ko *et al.*, 1994; Buckner *et al.*, 1995; Goetz *et al.*, 1995; Kenny *et al.*, 1995a; Testa *et al.*, 1995a), 'predominantly' α_{1D} (Kenny *et al.*, 1995b) or α_{1H} (Muramatsu *et al.*, 1990; 1991). Moreover, Rokosh *et al.* (1994) and Piascik *et al.* (1994) recently reported that they could detect mRNA for the α_{1b}, α_{1c} and α_{1d} adrenoceptors in the rat aorta.

In this paper we describe our attempt to make a contribution to this classification problem. Previously, our first indication of α_1-adrenoceptor heterogeneity in the rabbit aorta was given by small, but systematic, antagonist concentration-dependent changes in both the slopes of agonist concentration-effect curves and Schild plots (Martin, 1989). Therefore, we have performed a similar quantitative analysis of the interaction between a range of α_1-adrenoceptor antagonists and NA and PE in the rat isolated aorta assay. Four α_1-adrenoceptor antagonists were selected as representatives from different chemical classes: tamsulosin (a phenethylamine), phentolamine (an imidazoline), prazosin (a quinazoline) and WB-4101 (a benzodioxan). In addition, three other ligands were selected on the basis of their assigned selectivity for one or other of the proposed α_1-adrenoceptor subtypes (see above): 5-methylurapidil (α_{1A}-selective), spiperone (α_{1B}-selective) and HV723 (α_{1N}-selective).

A preliminary account of these data was presented to the British Pharmacological Society (Van der Graaf *et al.*, 1993).

Methods

Rat isolated aortic ring preparation

Male Wistar rats (225–300 g) were killed by cervical dislocation and the thoracic aorta was dissected. The aorta was mounted on a length of scoured, polythene tubing and placed in a Petri dish containing modified Krebs-Henseleit solution (KHS) of the following (mM) composition: NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.0, $CaCl_2$ 0.25 and ascorbic acid 0.1 to prevent oxidation of ligands added to the solution. The Ca^{2+} concentration for this assay is one tenth of that of standard KHS in order to eliminate the spontaneous phasic contractions of the aorta seen in standard KHS. The aorta was cleared from surrounding adipose tissue and the endothelium was removed by gentle rubbing of the intimal surface with the polythene tube. The effectiveness of this procedure was confirmed after completion of each concentration-effect curve by the lack of relaxant response to 10 μM 5-methylfurmethide, the acetylcholine M-receptor agonist. Six ring segments ($\sim$4 mm length) were prepared from each aorta and mounted between two stainless-steel wires in 20 ml organ baths, thermostatically controlled at 37 ± 0.5°C, containing modified KHS and continuously gassed with 95% O_2 and 5% CO_2. Tissue responses were continuously measured as changes in isometric tension (g) by use of Grass FT03C strain gauges and displayed on potentiometric chart recorders.

Experimental protocol

Following application of 2 g resting tension, tissues were allowed to stabilize for 60 min during which time the organ bath fluid was replaced four times with pre-warmed KHS at regular intervals. The resting tension was re-established once after 30 min. A block design was used to allocate treatments. Tissues were incubated for 90 min with antagonist or the appropriate vehicle. Single agonist concentration-effect (E/[A]) curves were obtained by cumulative dosing at half-log unit concentration increments. 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 aorta assay because it was shown in preliminary experiments that 30 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:

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

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 right-

ward shift of the agonist E/[A] curves with no change in upper asymptote, pK_B values were obtained by fitting the individual midpoint location values obtained in the absence ($\log[A]_{50}$) and presence ($\log[A]_{50B}$) of antagonist (B) to the following derivation of the Schild equation as described previously (Black *et al.*, 1985a):

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

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 [antagonist]-axis of the Schild plot using the line generated with the unconstrained slope.

Application of a two-receptor model Experimental data were fitted to a two-receptor model which is identical in algebraic formulation to the model derived by Furchgott (1981). Stephenson's (1956) concept of stimulus, S, is used to symbolize the product of an action of an agonist, A, at two receptors, R_1 and R_2, in the presence of a competitive antagonist, B, as follows:

$$S_1 = \frac{\alpha_1 \cdot [A]}{K_1 \cdot (1 + \frac{[B]}{K_{B1}}) + [A]} \quad (3)$$

$$S_2 = \frac{\alpha_2 \cdot [A]}{K_2 \cdot (1 + \frac{[B]}{K_{B2}}) + [A]} \quad (4)$$

where S_1 and S_2 are the stimuli produced by activation of receptors R_1 and R_2, respectively, α_1 and α_2 are the maximum concentrations of S_1 and S_2 and K_1 and K_2 are the midpoint location parameters of the functions. K_{B1} and K_{B2} are the antagonist dissociation equilibrium constants for the two receptors. Pharmacological effect (E) is assumed to be a saturable function of the sum of the stimuli, referred to as the total stimulus (S_T):

$$\frac{E}{E_M} = \frac{S_T{}^n}{1 + S_T{}^n} = \frac{(S_1 + S_2)^n}{1 + (S_1 + S_2)^n} \quad (5)$$

where E_M is the maximum effect and n is the midpoint slope parameter of the E/S_T function. Note that, in contrast to Furchgott's (1981) original formulation of the model, this notation does not include parameters which explicitly relate to affinity and efficacy of the agonist at the two receptors. For comparison, the data were also fitted to a one receptor-model given by Equation (1) with the midpoint location parameter ($[A]_{50}$) multiplied by the factor $(1 + [B]/K_B)$ in the usual way. An *F* test was then used to compare the goodness-of-fit obtained with the one and two-receptor models (see Motulsky & Ransnas, 1987).

The AR module (derivative-free, nonlinear regression) of the BMDP statistical software package (Dixon *et al.*, 1990) was used for the fitting procedures. Standard errors on the parameter estimates were calculated using the 'scaling-up' method described by Leff *et al.* (1990).

Compounds

Compounds were obtained from the following sources: cocaine hydrochloride, 5-hydroxytryptamine hydrochloride (5-HT), (−)-noradrenaline hydrochloride (NA), phentolamine hydrochloride, (−)-phenylephrine hydrochloride (PE), prazosin hydrochloride and spiperone: Sigma Chemical Company Ltd., U.K.; 5-methylurapidil 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 (previously known as YM617, (−)-5-[2(2-ethoxyphenoxy)ethyl]amino]propyl] - 2 - methoxybenzenesulphonamide hydrochloride): 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 & Sohme, 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 20 ml organ baths did not exceed 800 μl, corresponding to 4% of the bath volume. Neither the vehicles nor the antagonists were found to produce significant effects on basal tone.

Results

Noradrenaline and phenylephrine concentration-effect relations

Noradrenaline (NA) and phenylephrine (PE), when administered by a cumulative dosing schedule, both produced concentration-dependent contractions of the aortic rings (Figure 1). It was possible to fit the individual E/[A] curve data to the Hill equation to provide estimates of the curve midpoint location ($p[A]_{50} = 7.74 \pm 0.10$ and 7.14 ± 0.18, $P < 0.05$), midpoint slope ($n_H = 0.82 \pm 0.03$ and 0.99 ± 0.10, $P > 0.1$) and upper asymptote ($\alpha = 3.2 \pm 0.3$ g and 3.1 ± 0.2 g, $P > 0.5$) parameter values for NA ($n = 7$) and PE ($n = 7$), respectively (Figure 1a). However, the data obtained at high concentrations of the NA and PE appeared to deviate systematically from the curve fits (Figure 1a) as though the curve was not monotonic. It has been our experience that single receptor systems usually produce monotonic E/[A] curves (e.g. Black *et al.*, 1985b) and we investigated whether this curve-shape complexity was an inherent feature of the bioassay, that is independent of the receptor class coupled to the contraction. 5-HT was selected for the comparison because it is reported to produce contraction of the rat aorta by activating a homogeneous population of 5-HT_2-receptors (Cohen *et al.*, 1981; Killam *et al.*, 1990). The Hill equation provided a better fit to the 5-HT E/[A] curve data ($p[A_{50}] = 6.09 \pm 0.08$, $n_H = 1.49 \pm 0.09$, $\alpha = 2.6 \pm 0.3$ g, $n = 6$), as judged by eye (Figure 1a) and by calculation of the mean sum of squares (SS) of the differences between the observed and predicted values (mean SS = 0.012, 0.010 and 0.001 for NA, PE and 5-HT, respectively).

Another indication of differences in the modality of 5-HT receptor and α-adrenoceptor-mediated contraction was given by examination of the individual experimental traces (Figure 1b). It was found that the relationship between the time to plateau of the individual responses within the cumulative curves and the concentration for NA and PE was different from 5-HT. This was investigated further by comparing single exposure, low and high level, responses to NA (1 nM and 10 μM) and 5-HT (0.1 μM and 30 μM). The responses obtained with NA were significantly slower at the higher concentration. The rate of contraction (response/time to plateau) was

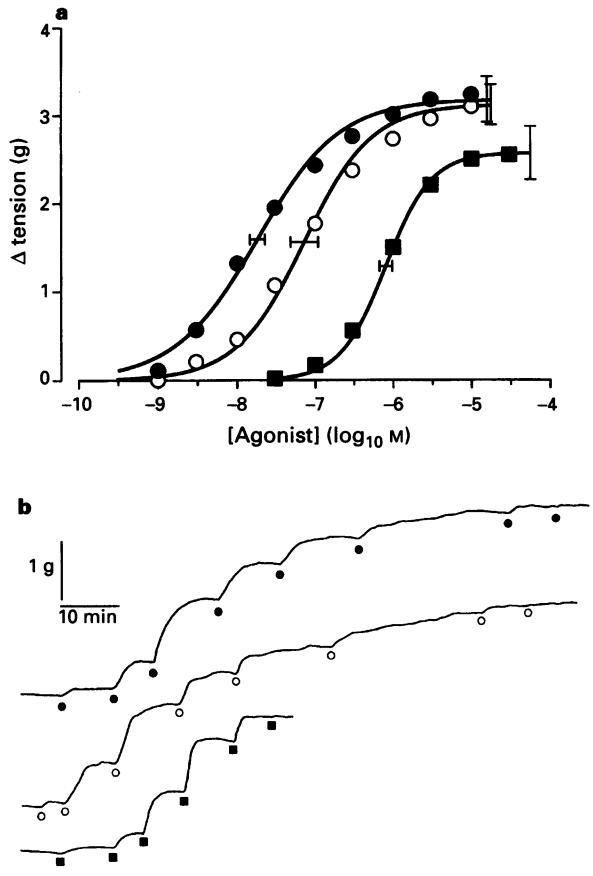

Figure 1 (a) Concentration-effect curves obtained on the rat aorta to noradrenaline (●, $n=7$), phenylephrine (○, $n=7$) and 5-HT (■, $n=6$). The lines shown superimposed on the mean experimental data points were simulated using the Hill equation (see text for parameter estimates). Horizontal and vertical error bars (s.e.mean) are shown on the mean midpoint and upper asymptote locations, respectively. (b) Representative experimental traces showing the response of the rat aorta to noradrenaline (●, 1 nM–3 μM), phenylephrine (○, 3 nM–10 μM) and 5-HT (■, 0.1–30 μM), administered using a cumulative dosing regimen at half-log unit concentration increments.

0.24 ± 0.03 and 0.084 ± 0.009 g min^{-1} for 1 nM ($n=4$) and 10 μM ($n=4$) NA, respectively ($P<0.005$, d.f. = 6). In contrast, an inverse relationship was found with 5-HT (rate of contraction $=0.021\pm0.003$ and 0.22 ± 0.04 g min^{-1} for 0.1 ($n=4$) and 30 μM ($n=3$), respectively ($P<0.005$, d.f. = 5).

Effects of competitive antagonists

All of the antagonists investigated produced concentration-dependent inhibition of the contractile responses of the aorta to NA and PE. The data from four of the interactions, chosen on the basis that they illustrate the different patterns of α_1-adrenoceptor antagonism observed, are presented graphically in Figure 2. Although no significant effects on the upper asymptotes of the E/[A] curves of any of the antagonists tested were detected, only tamsulosin and 5-methylurapidil (5-MU) did not have a significant effect on the slope (n_H) of the NA and PE E/[A] curves. The other antagonists produced significant steepening of the curves obtained with NA and/or PE as judged by the results of a one-way ANOVA on the Hill slopes (Figure 2 and Table 1). In fact, with one exception (PE in the presence of 30 nM prazosin), the NA and PE curves obtained in the presence of all the antagonist treatments were steeper than the corresponding control curves, although this effect was not always significant as tested. There did not appear to be a consistent relationship between antagonist-concentration and the change in Hill slope. The variation in the Hill slopes of the control curves between treatment groups was small and not significant for either NA ($n_H=0.81\pm0.02$–1.00 ± 0.12; $F_{6,34}=1.24$, $P>0.3$) or PE ($n_H=0.97\pm0.06$–1.08 ± 0.06; $F_{6,34}=0.36$, $P>0.8$).

The time to plateau of the individual responses to NA and PE did not appear to be altered in the presence of the antagonists at any concentration (data not shown).

One-receptor model fit

Notwithstanding the fact that one of the basic criteria for simple competitive antagonism at a single receptor class was not always satisfied, 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) and the Schild plot slope parameters for the antagonism of NA and PE by phentolamine and HV723 were found to be significantly less than unity. The Schild plot slope parameters for the other antagonists were not significantly different from unity over the range of concentrations used and apparent pK_B values were estimated (Figure 2 and Table 1).

Application of the two-receptor model

In the absence of evidence to indicate that the deviations from simple competitive antagonism were due to failure to satisfy basic experimental conditions for quantitative analysis, we attempted to account for the data using an existing two-receptor model (Furchgott, 1981, see Methods for details). In the first instance, the data obtained with NA as agonist and all seven of the antagonists was fitted simultaneously to the two-receptor model described in the Methods (Equations 3–5). To facilitate the model-fitting, the E/[A] data were self-normalised to the value of the curve upper asymptote, having established that there was no significant difference between the asymptotes in the absence and presence of the antagonists. Next, the data from each treatment group were expressed as mean values to reduce the data to a size which our computer could handle. In addition, because the control locations of NA curves were not significantly different between experiments (i.e. between each antagonist data set), the agonist concentrations were expressed normalised to the midpoint location of the control curve from each experiment. This process of data preparation had the effect of reducing the number of parameters to be estimated from 31 to 19 and the number of pairs (i.e. E, $[A]/[A]_{50\,control}$) of data points from approximately 2500 to 396. The model-fit satisfied its convergence criteria (SS = 2976; d.f. = 377) and estimates of K_{B1} and K_{B2} were obtained for each antagonist and a single, common, estimate of the agonist and system-dependent parameters, K_1, K_2 α_1, α_2 (estimated as logarithms) and n (Table 2). The goodness-of-fit obtained with the two-receptor model was significantly better ($F_{10,377}=35.45$; $P<0.001$) than that obtained when the same data set was fitted to the one-receptor model (SS = 5775; d.f. = 387). An example of the two-receptor model fit is shown in Figure 3 where the parameter estimates were used to simulate the curves shown superimposed on the mean experimental data obtained using phentolamine and prazosin.

The general applicability of the two-receptor model was tested by performing an identical fit of the data obtained using PE as agonist. Once again, the fit was significantly better than that obtained using the one-site model (SS = 2158 and 4123 for the two and one-receptor model, respectively, $F_{10,311}=28.3$, $P<0.001$). The parameter values obtained are presented in Table 2 and were used for the simulations shown in Figure 3. The slope parameter (n) of the function relating S_T to E in the two receptor model is independent of the agonist and, in agreement with this model assumption, the value estimated when PE was used as agonist was not significantly different from the value estimated using the NA data. A comparison was made of the pK_B values estimated in the two model fits (Figure 4). In agreement with expectations, the best-fit lines through plots of the pK_B values (Table 2)

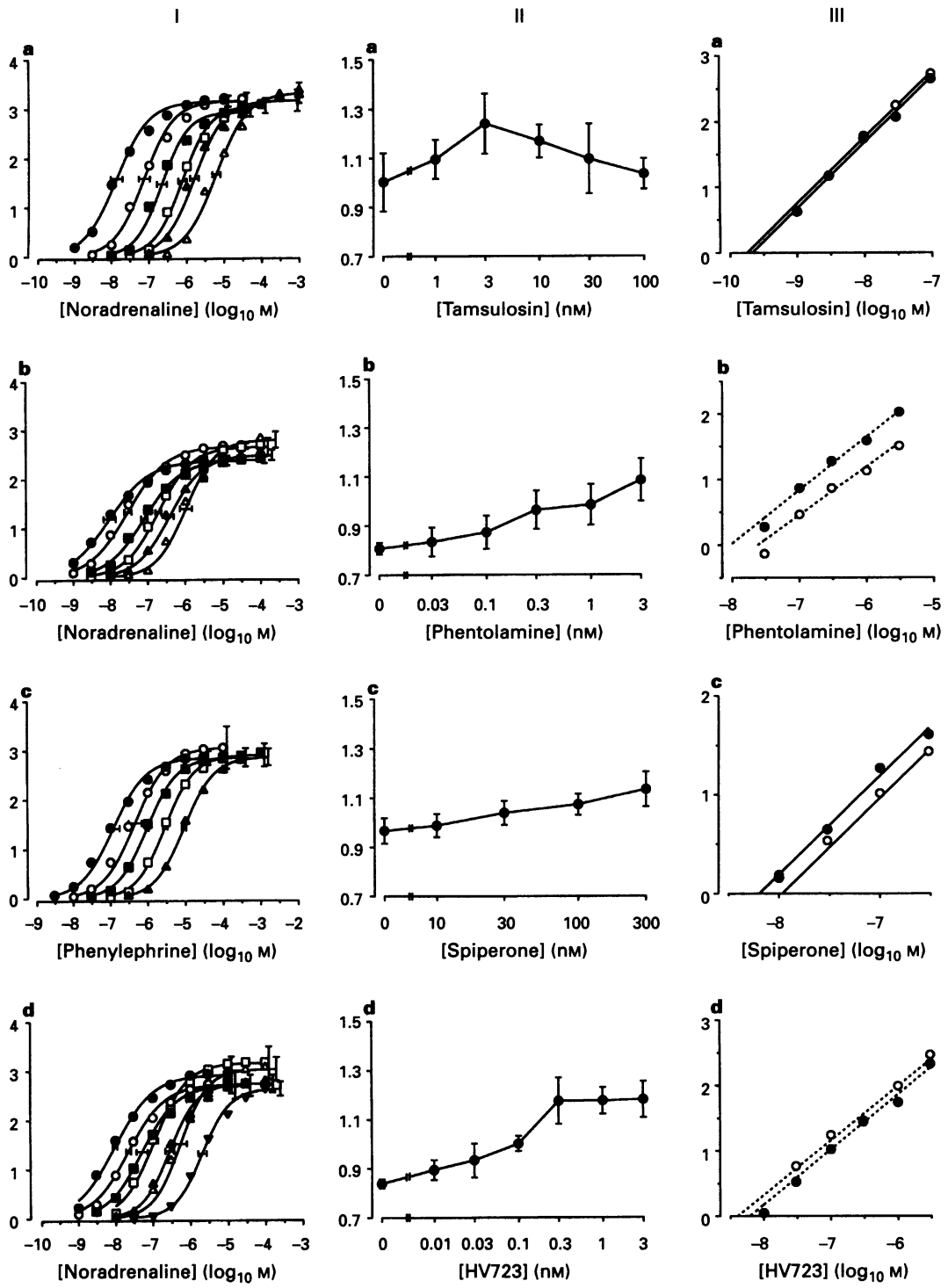

Figure 2 (I) Examples of different patterns of α_1-adrenoceptor antagonism in the rat aorta. (a) No significant steepening of E/[A] curves and corresponding Schild plot slope parameter not significantly different from unity: noradrenaline in the absence (●) and presence of 1 nM (○), 3 nM (■), 10 nM (□), 30 nM (▲) and 100 nM (△) tamsulosin ($n=5$). (b) No significant steepening of E/[A] curves and corresponding Schild plot slope parameter significantly less than unity: noradrenaline in the absence (●) and presence of 30 nM (○), 100 nM (■), 300 nM (□), 1 μM (▲) and 3 μM (△) phentolamine ($n=6$). (c) Significant steepening of E/[A] curves and corresponding Schild plot slope parameter not significantly different from unity: phenylephrine in the absence (●) and presence of 3 nM (○), 10 nM (■), 30 nM (□), 100 nM (▲) and 300 nM (△) spiperone ($n=5-6$). (d) Significant steepening of E/[A] curves and corresponding Schild plot slope parameter significantly less than unity: noradrenaline in the absence (●) and presence of 10 nM (○), 30 nM (■), 100 nM (□), 300 nM (▲), 1 μM (△) and 3 μM (▼) HV723 ($n=6-7$). The lines shown superimposed on the mean experimental data points were simulated using the Hill equation. Horizontal and vertical error bars (s.e.mean) are shown on the mean midpoint and upper asymptote locations, respectively. Ordinates: tension (g). (II) Hill slope parameter estimates (±s.e.mean), plotted as a function of antagonist concentration, from examples of different patterns of α_1-adrenoceptor antagonism in the rat aorta. The panels correspond to the data shown in Figure 2 (I). Ordinates: Hill slope estimates. (III) Schild plots for the interaction between (a) tamsulosin; (b) phentolamine, (c) spiperone and (d) HV723 with noradrenaline (●) and phenylephrine (○) in the rat aorta. The solid and dashed lines shown superimposed on the mean data points were simulated using the parameters obtained from the constrained and unconstrained fit, respectively (Table 1). Ordinates: $\log_{10}(r-1)$, where r is the concentration ratio.

Table 1 One-receptor model fitting parameters from the antagonism of noradrenaline (NA) and phenylephrine (PE) in the rat aorta (see text for details)

Antagonist	*Agonist*	*d.f.*	$pK_B^1 \pm s.e.$	$b \pm s.e.$	*Effect on the slope of the E/[A] curve*[2]
Tamsulosin	NA	28	9.67±0.10	0.98±0.06	–
	PE	33	9.75±0.09	1.04±0.05	–
Phentolamine	NA	33	(8.0±0.3)	0.81±0.06*	–
	PE	28	(7.6±0.3)	0.74±0.08*	steepening
Prazosin	NA	28	9.46±0.10	0.93±0.08	steepening
	PE	44	9.66±0.12	1.06±0.10	–
WB-4101	NA	41	8.72±0.07	0.98±0.05	steepening
	PE	25	8.88±0.10	0.96±0.09	steepening
5-MU	NA	28	7.27±0.07	0.98±0.04	–
	PE	22	7.59±0.06	0.97±0.04	–
Spiperone	NA	23	8.19±0.09	0.95±0.08	steepening
	PE	25	7.97±0.14	0.80±0.13	–
HV723	NA	43	(8.2±0.2)	0.84±0.05*	steepening
	PE	30	(8.4±0.3)	0.83±0.08*	–

[1] Estimated with b constrained to unity. When b was significantly different from unity an empirical pA_2 value (shown in parentheses) was estimated from the unconstrained fit.
[2] Hill slopes were compared by ANOVA.
*b significantly different from unity ($P<0.05$).

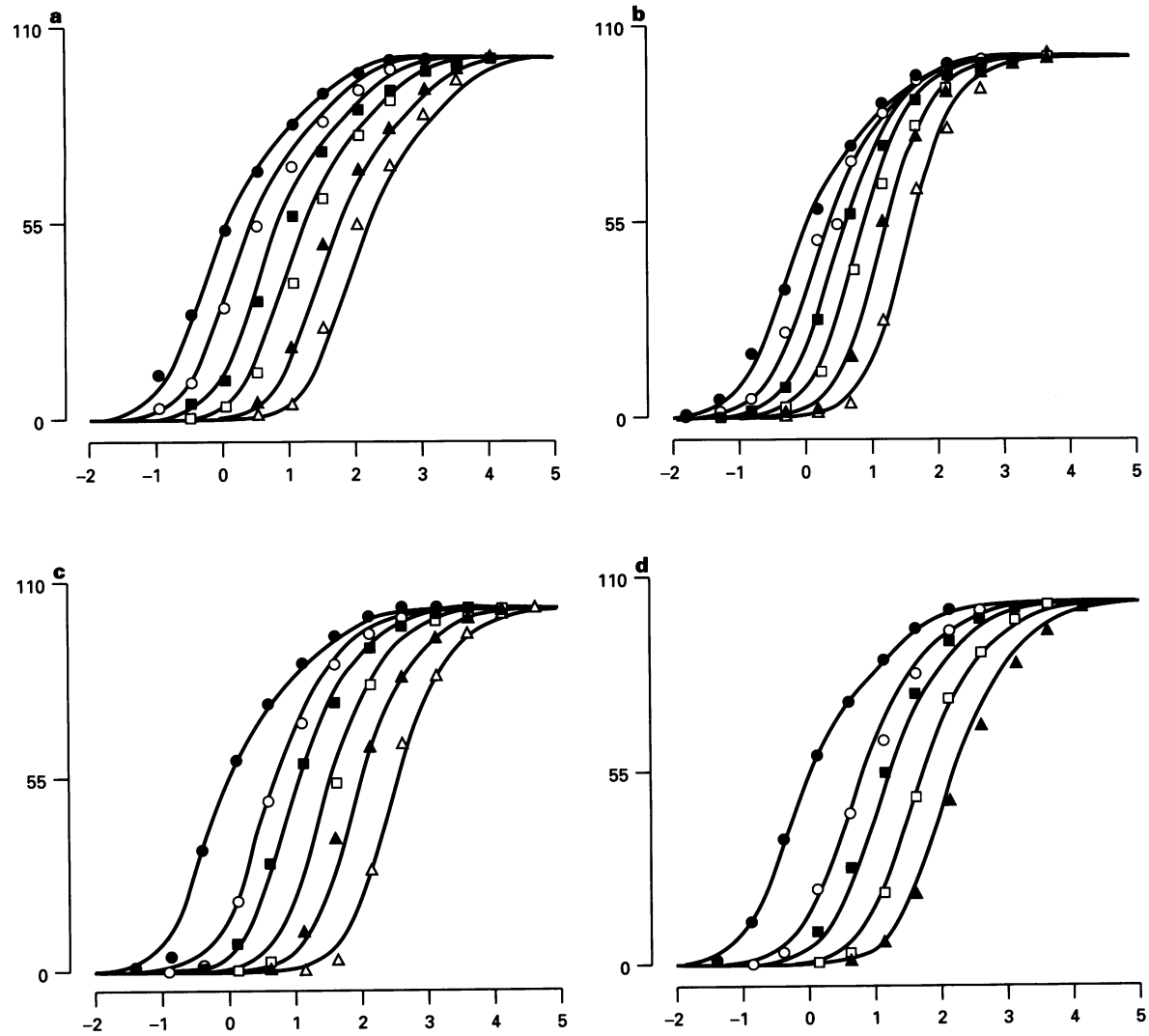

Figure 3 Two-receptor model simulations of the antagonism by 0 (●), 0.03 (○), 0.1 (■), 0.3 (□), 1 (▲) and 3 (△) μM phentolamine of (a) noradrenaline and (b) phenylephrine and by 0 (●), 1 (○), 3 (■), 10 (□), 30 (▲) and 100 (△) nM prazosin of (c) noradrenaline and (d) phenylephrine in the rat aorta. The lines shown superimposed on the mean, self normalised data points were simulated using the parameter estimates shown in Table 2. Ordinates: % of maximum response. Abscissae: $\log_{10}([A]/[A]_{50\ control})$.

Table 2 Two-receptor model fitting parameters from the antagonism of noradrenaline and phenylephrine in the rat aorta (see text for details)

		Noradrenaline	*Phenylephrine*
E_M (constrained)		105%	105%
n		1.5±0.2	1.3±0.1
log α_1		0.3±0.1	0.3±0.1
log α_2		0.9±0.2	1.1±0.2
logK_1		−0.1±0.2	0.1±0.2
logK_2		2.1±0.3	2.1±0.3
Tamsulosin	pK_{B1}	9.8±0.1	10.0±0.1
	pK_{B2}	9.3±0.2	9.3±0.2
Phentolamine	pK_{B1}	7.7±0.1	7.7±0.2
	pK_{B2}	7.5±0.2	6.2±0.2
Prazosin	pK_{B1}	9.7±0.1	9.8±0.1
	pK_{B2}	8.9±0.2	9.3±0.2
WB-4101	pK_{B1}	8.9±0.1	9.1±0.1
	pK_{B2}	8.3±0.2	8.4±0.2
5-MU	pK_{B1}	7.4±0.1	7.8±0.1
	pK_{B2}	6.8±0.2	7.1±0.2
Spiperone	pK_{B1}	8.3±0.1	8.3±0.1
	pK_{B2}	7.7±0.2	7.5±0.2
HV723	pK_{B1}	8.1±0.1	8.2±0.1
	pK_{B2}	7.4±0.2	7.6±0.2

estimated for both receptors using NA and PE were not significantly different from a line of identity, that is the slope parameters (1.0±0.1 and 1.1±0.3 for pK_{B1} and pK_{B2}, respectively) were not significantly different from unity and the y-intercepts (0.4±0.7 and −1.3±2.3 for pK_{B1} and pK_{B2}, respectively) were not significantly different from zero. Overall, therefore, the pK_B values estimated for the antagonists at the two receptors were independent of the agonist used. The only data point which appeared to deviate from the line of identity was the pK_{B2} value for phentolamine (Figure 4b).

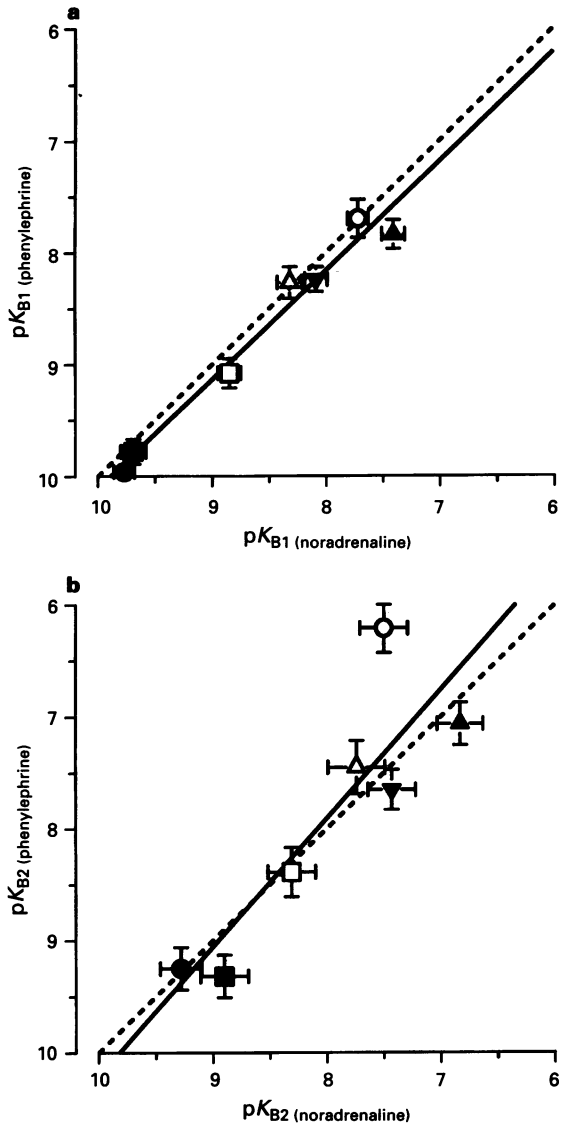

Figure 4 Relationship between the antagonist affinity estimates obtained from the two-receptor model fitting of the noradrenaline and phenylephrine data sets. The solid lines were obtained by linear regression (see text for parameter estimates) of the (a) pK_{B1} and (b) pK_{B2} value estimates (see Table 2) for tamsulosin (●), phentolamine (○), prazosin (■), WB-4101 (□), 5-methylurapidil (▲), spiperone (△) and HV723 (▼). The dashed lines represent the line of identity.

Discussion

In this study it was shown that a number of structurally-diverse ligands, previously classified as α_1-adrenoceptor blockers, did not behave as antagonists competing at a homogeneous receptor population in the rat isolated aorta assay. This conclusion was reached from the analysis of E/[A] curve shapes and the corresponding Schild plots. The Hill slope parameters of the E/[A] curves appeared to be more sensitive indicators of complexity than the corresponding Schild plots although there did not appear to be a consistent antagonist concentration-dependent effect on slope (Table 1, Figure 2). With one exception, the slopes of the curves obtained in the presence of antagonist were always greater than control curves, although not necessarily significant as tested by ANOVA. In contrast, deviations from the simple competitive model were only detected by Schild analysis when phentolamine and HV723 were used as antagonist.

Previously, several groups have examined the interaction between α_1-adrenoceptor agonists and antagonists on bioassays of rat thoracic aorta (see Introduction for references). Complex behaviour has been recognised in some of these reports. For example, multiphasic PE and methoxamine curves, obtained in the absence and presence of prazosin and WB-4101, were described by Piascik *et al.* (1991) who suggested that the tissue expressed at least two receptors. In fact, the same group (Piascik *et al.*, 1994), and Rokosh *et al.* (1994), subsequently reported that they could detect mRNA in the rat aorta which coded for all three, currently-recognised, α_1-adrenoceptor subtypes. Other signs of non-homogeneity were reported by Ölmez & Ilhan (1992) who estimated significantly different pA_2 values for the natural product, berberine, when PE and NA were used as agonists. Furthermore, Orsetti & DiStilo (1994) obtained a biphasic Schild plot slope with an overall slope of 0.48 from the interaction between NA and a phenylfuroxan analogue of prazosin, PFX3. Most recently, Kenny *et al.* (1995b) obtained a flat Schild plot (b=0.62) for the interaction between NA and the selective α_{1D}-adrenoceptor antagonist, BMY 7378. In addition, the authors reported that the NA E/[A] curves obtained 'in the presence of BMY 7378 were steeper than corresponding controls'. However, other groups have concluded that their rat aorta data were consistent with expectations for receptor homogeneity. For example, Ko *et al.* (1994) considered that data obtained from the interaction between NA and several antagonists 'clearly support the idea that the non-α_{1A}-, non-α_{1B}-adrenoceptor (the putative α_{1D}-adrenoceptor) exists in rat aorta'. It is not clear whether these authors performed a statistical analysis on the effect of the antagonist treatments on agonist curve shape. However, the data presented from that study appear to contain examples of antagonist-induced, agonist curve slope changes and, in the case of the interaction between NA and prazosin, evidence that the NA curves are biphasic. Recently, Testa *et al.* (1995b) investigated the interaction between NA and several antagonists which were used in this study (prazosin, tamsulosin, phentolamine, 5-MU and spiperone). These authors reported that 'the antagonists caused parallel, concentration-related, shifts to the

right of the NA concentration-response curves' and that 'the slopes of the Schild plots obtained with these compounds were found to be close to, and not significantly different from, unity, suggesting that simple competitive antagonism occurred'. It was concluded that 'most of the contraction' was mediated by α_{1B}-adrenoceptors. However, inspection of the competitive antagonist data set presented graphically in that paper, which shows a NA curve obtained in the absence and presence of a single concentration (10 nM) of prazosin, suggests that their data were not too dissimilar from those obtained in the current study, although the changes in the shape of the NA curve were not significant as tested in their analysis. The assay conditions employed in this study differ from previous literature methods only in terms of the Ca^{2+} concentration in the bathing solution. We reduce the Ca^{2+} concentration by tenfold to 0.25 mM to remove the tendency for the tissue to contract spontaneously and, upon addition of agonist, to exhibit rhythmic contractions.

Previously, Schild slopes of less than unity and steepening of E/[A] curves have been attributed to the presence of an agonist uptake process (Langer & Trendelenburg, 1969; Furchgott, 1972). However, in this study it is not very likely that this was the cause for the deviations from the simple competitive model, because $Uptake_1$ was inhibited by 30 μM cocaine and preliminary studies had shown that $Uptake_2$ does not affect the location or shape of the NA control curve in our rat aorta assay. Furthermore, the fact that steepening of both NA and PE E/[A] curves was not only apparent at high concentration ratios but, in every case, was also observed at concentration ratios even less than 5 indicates that the complexity was not due to the presence of a low-affinity uptake system. Timolol (6 μM) was present in all experiments to block β-adrenoceptors and the endothelium was always removed. In addition, the observation that a number of structurally-different antagonists produced steepening of both NA and PE

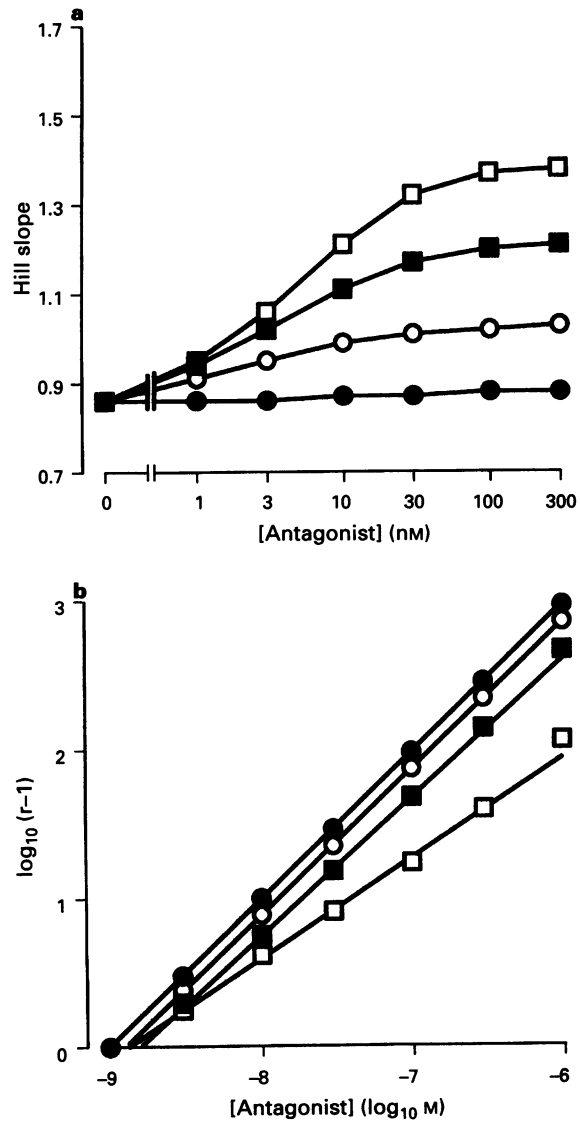

Figure 5 Two receptor model simulations showing the effect of varying antagonist selectivity on (a) Hill slopes and (b) Schild plots. The simulations were obtained by generating families of E/[A] curves at half-log unit [A] increments in the absence and presence of increasing concentrations of antagonist using the two receptor model parameters obtained for noradrenaline (Table 2). The antagonist selectivity was varied as follows: $pK_{B1}=9$, $pK_{B2}=9$ (●); $pK_{B1}=9$, $pK_{B2}=8.5$ (○); $pK_{B1}=9$, $pK_{B2}=8$ (■); $pK_{B1}=9$, $pK_{B2}=7$ (□). The Hill slopes were obtained by fitting the E/[A] curve data sets to Equation (1) and the Schild plots by fitting the corresponding log $[A]_{50}$ values to Equation (2). By this method, the Schild plot slope parameters were estimated as follows: 0.99 (●); 0.99 (○); 0.93 (■); 0.68 (□).

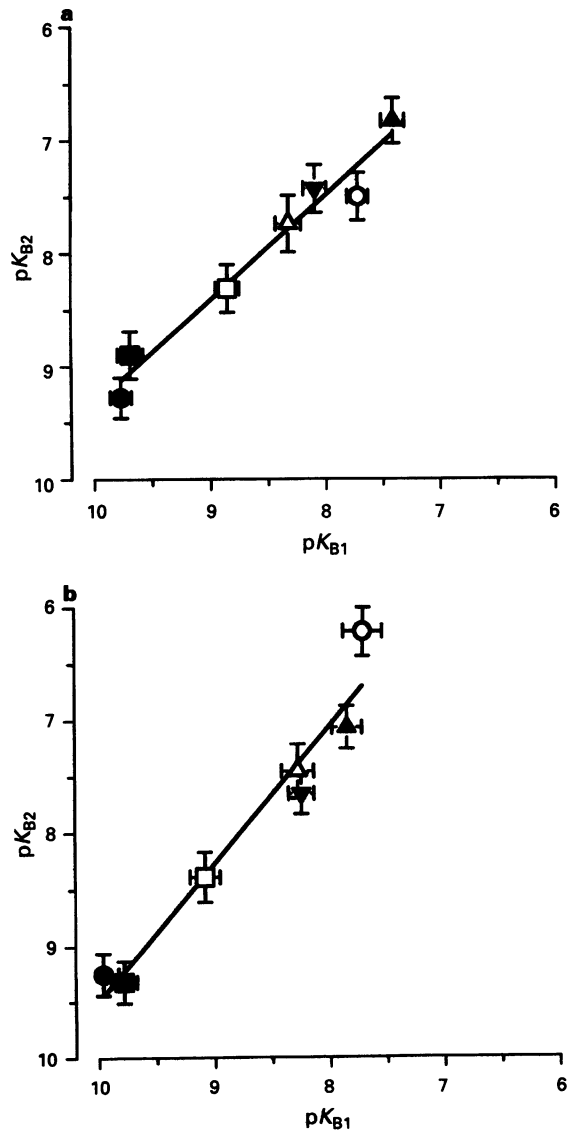

Figure 6 Relation between the pK_{B1} and pK_{B2} estimates for tamsulosin (●), phentolamine (○), prazosin (■), WB-4101 (□), 5-methylurapidil (▲), spiperone (△) and HV723 (▼) obtained from the two-receptor model fitting (Table 2). The lines were obtained by linear regression of the data obtained with (a) noradrenaline ($pK_{B2}=0.93\ pK_{B1}+0.08$; $r^2=0.97$ and (b) phenylephrine ($pK_{B2}=1.22\ pK_{B1}-2.70$; $r^2=0.95$).

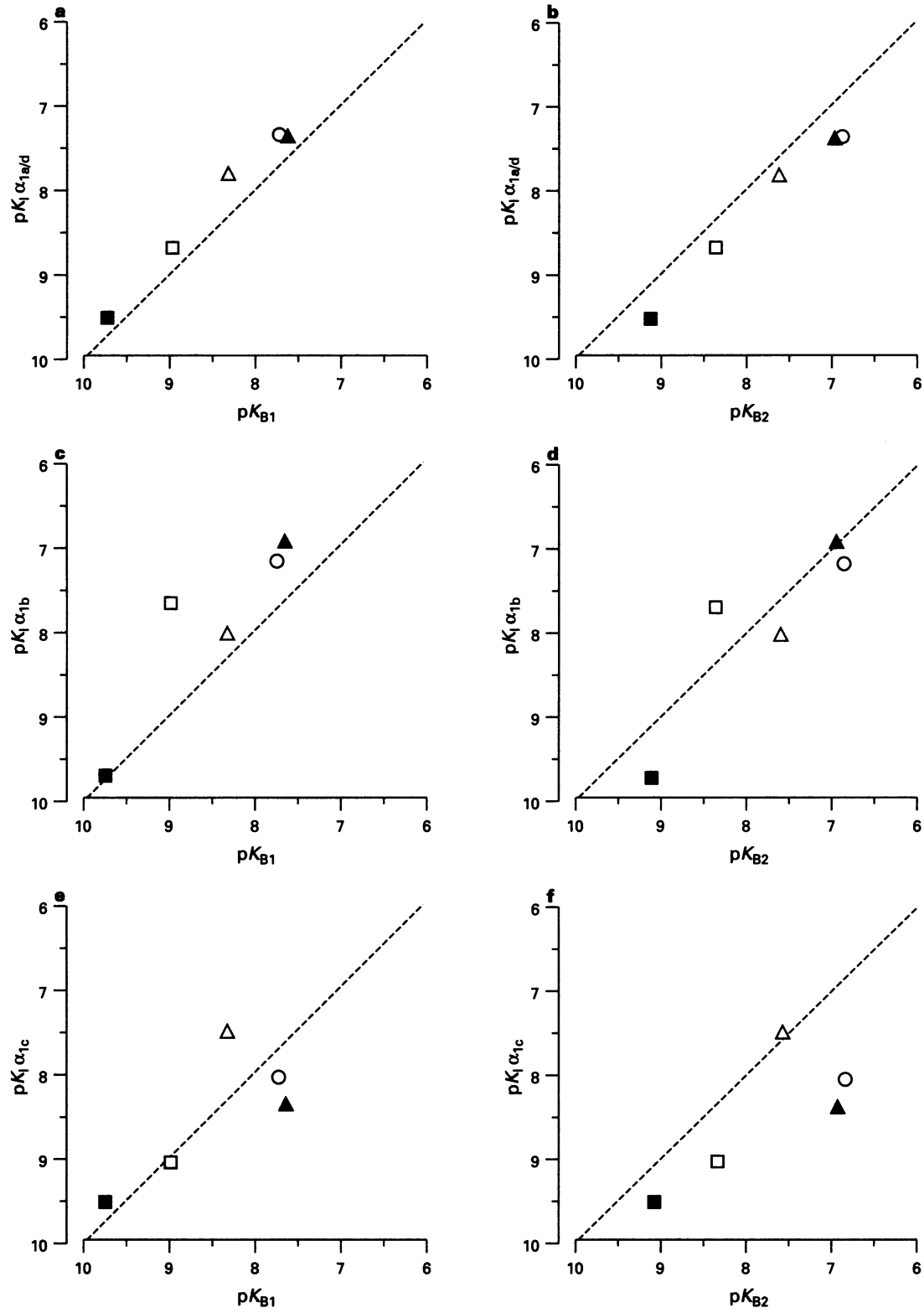

Figure 7 Relation of the mean pK_{B1} (a, c and e) and pK_{B2} (b, d and f) estimates for phentolamine (○), prazosin (■), WB-4101 (□), 5-methylurapidil (▲) and spiperone (△) obtained from the two-receptor model fitting of the noradrenaline and phenylephrine data sets (Table 2) and pK_i values for the displacement of [^{3}H]-prazosin at rat cloned $\alpha_{1a/d}$ (a and b), α_{1b} (c and d) and α_{1c}-adrenoceptor (e and f) subtypes expressed in COS-7 cells (Laz et al., 1994). The dashed lines represent the line of identity. The sum of squares of the differences between the mean pK_{B1} and pK_{B2} estimates and pK_i values were as follows,

	$\alpha_{1a/d}$	α_{1b}	α_{1c}
pK_{B1}	0.48	2.54	1.36
pK_{B2}	0.83	1.13	4.19

E/[A] curves suggests that the complexity was not due to a ligand-specific property. Thus, the current analysis provided objective grounds to reject the one-receptor model and an attempt was made to see to what extent the data could be accounted for by an existing two-receptor model (Furchgott, 1981). As judged by eye, the two-receptor model could account for all of the complexity in the data (Table 2, Figure 3). Furthermore, the goodness-of-fit obtained with the two-receptor model was significantly better than that obtained with the one-receptor models, as judged by applying an *F*-test which takes into account the reduced number of degrees of freedom due to the increased number of parameters associated with the more complex model (see Motulsky & Ransnas, 1987). As expected when the model is applicable, with the exception of the data obtained with phentolamine the model fit provided antagonist pK_B estimates for the two receptors which were independent of whether NA or PE was used as agonist (Figure 4 and Table 2). At present, we do not have an explanation for the finding that the phentolamine pK_{B2} estimate was agonist-dependent, although it may be an indication of further receptor heterogeneity in the system. If this were true, then the analysis would suggest that there are at least three functional α_1-adrenoceptors in the rat aorta. This would be consistent with the data from Rokosh *et al.* (1994) and Piascik *et al.* (1994) who detected mRNA for three α_1-adrenoceptor subtypes in this tissue.

According to the two-receptor model the effect of competitive antagonists on agonist curve shape is dependent on the antagonist and agonist receptor selectivity and the relative contribution of each of the two pathways to the production of pharmacological effect. An example of the relationship between Hill slope and antagonist concentration, simulated using the two-receptor model, is shown in Figure 5a. In the simulation, the agonist- and system-dependent model parameters were fixed at those estimated for NA in the model fit (Table 2). The antagonist selectivity for the two receptors was then varied. The simulation shows that a significant change in Hill slope would be experimentally-detectable only when the antagonist expresses greater then 3 fold selectivity for one of the receptor subtypes. The slope changes when the antagonist selectivity is less than 3 fold lie within the current experimental variance. Thus, the failure to observe a systematic relationship between antagonist concentration and Hill slope in our study was not surprising because the model fit indicated that none of the antagonists expressed more than a 10 fold selectivity. In fact, the changes in Hill slope would have been difficult to detect without curve fitting. Notwithstanding this, the model simulation of the corresponding Schild plots (Figure 5b) confirms that the Hill slope is a more sensitive indicator of receptor heterogeneity than the Schild plot slope parameter in this particular system. The simulation indicates that at least 10 fold antagonist selectivity is required to obtain a Schild plot slope parameter which would be significantly less than unity.

In an attempt to increase the chances of exposing any receptor heterogeneity, we included a range of ligands from different chemical classes. Thus, if the aorta did express multiple α_1-adrenoceptor subtypes, it was hoped that the different ligands would display a significant degree of variation in their selectivity for the subtypes. However, as shown in Figure 6, a significant correlation was found between the pK_B values estimated for the two receptors in the model fit. Thus, according to the analysis, all the antagonists expressed the same receptor selectivity ($K_{B1} < K_{B2}$) to a similar extent ($\sim$6 fold). In the absence of any other information, these data could be indicative of an antagonist-independent explanation for the data. Ruffolo and co-workers (Nichols & Ruffolo, 1988; Ruffolo & Oriowo, 1990; Oriowo & Ruffolo, 1992b; Oriowo *et al.*, 1992; Li *et al.*, 1992) have suggested that in the rat aorta a single α_1-adrenoceptor is coupled to two signal transduction pathways, one coupled to the transmembrane influx of extracellular Ca^{2+} and the other to the release of Ca^{2+} from intracellular stores. However, although mathematical models of two signal transduction processes activated by one receptor (see Nichols & Ruffolo, 1988) predict biphasic agonist curves, intuitively they cannot account for the most significant features of our data, namely antagonist-induced steepening of E/[A] curves and flat Schild plots.

It has also been suggested that the two different transduction pathways are responsible for the tonic (associated with translocation of extracellular Ca^{2+}) and phasic (associated with mobilisation of intracellular Ca^{2+}) components of the α_1-adrenoceptor-mediated muscle contraction described in this assay (Godfraind & Kaba, 1972; Godfraind *et al.*, 1982). However, Wenham & Marshall (1994b) recently showed that the sources of Ca^{2+} mobilised for the phasic and tonic contractions of the rat aorta to NA cannot be clearly subdivided, suggesting that both components of the contraction share similar mechanisms for Ca^{2+} utilization. We do not know whether the slowing of individual agonist responses with increasing concentrations of both NA and PE recorded in this study was related to the two transduction pathways or if this was due to another mechanism, for example the involvement of different receptors at low and high agonist concentrations, because there was no obvious effect of the antagonists on the time to plateau of the agonist responses.

Intuitively, we did not expect the almost constant difference between the model-derived pK_{B1} and pK_{B2} values for such a chemically-diverse range of antagonists (Figure 6). However, significant correlations have also been reported between affinity values measured in cell lines transfected individually with the three recognised α_1-adrenoceptor subtype mRNAs for a similar diverse group of antagonists (Lutz *et al.*, 1993). Thus, in the absence of evidence to support an alternative hypothesis, the presence of a heterogeneous α_1-adrenoceptor population appears to provide the best possible explanation for our data. In order to determine which α_1-adrenoceptor subtypes may be associated with the model receptors, R_1 and R_2, the mean pK_{B1} and pK_{B2} estimates obtained from the two-receptor model fit were compared with affinities measured at rat cloned α_1-adrenoceptor subtypes expressed in COS-7 cells for the five antagonists (phentolamine, prazosin, WB-4101, 5-methylurapidil and spiperone) which were used by Laz *et al.* (1994) and in the present study (Figure 7). We chose to use analysis of the sum of squared differences because a direct comparison of the data was required rather than estimation of correlation coefficients (see Michel & Insel, 1994). The sum of squared differences of the data points from the line of identity was smallest for both pK_{B1} and pK_{B2} in the case of the $\alpha_{1a/d}$-adrenoceptor (now referred to as α_{1d}-adrenoceptor, Hieble *et al.*, 1995; Figure 7). Therefore, the complexity exposed in this study may be due to the expression of closely-related forms of the α_{1d}-adrenoceptor, at least in terms of pharmacological profile. However, relatively good matches were also found between pK_{B1} and α_{1c} and between pK_{B2} and α_{1b} (Figure 7). Therefore, on the basis of these data it is not possible to rule out the involvement of all three α_1-adrenoceptors. The dissonance in the literature about the characteristics of the α_1-adrenoceptor population mediating contraction of the rat aorta may, at least in part, be due to the lack of highly selective ligands and to between-assay variation in the expression of multiple α_1-adrenoceptors. Although tissue and species differences in the expression of receptors are well-recognised, the general possibility of rhythmical and differential expression of receptor subtypes within one tissue is not. The physiological significance of this may be obscure but the implications for the interpretation of studies of receptor heterogeneity could be substantial. Ambiguity in results between laboratories, looked at in this way, might be important pointers to the dynamics of receptor processing and expression.

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