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Characterization of α_1-adrenoceptor subtypes mediating contractions to phenylephrine in rat thoracic aorta, mesenteric artery and pulmonary artery

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1 The subtype of α_1-adrenoceptor mediating contractions to phenylephrine of the rat thoracic aorta, mesenteric artery and pulmonary artery were investigated by use of antagonists which show selectivity between the cloned α_1-adrenoceptor subtypes in binding studies.

2 Cumulative concentration-contraction curves for phenylephrine were competitively antagonized in the rat thoracic aorta by prazosin (pA_2 9.9), WB4101 (pA_2 9.6), 5-methylurapidil (pA_2 8.1), benoxathian (pA_2 9.2) and indoramin (pA_2 7.4). These compounds were also competitive antagonists in the mesenteric and pulmonary arteries (except for 5-methylurapidil in the pulmonary artery), (prazosin pA_2 9.9 and 9.7; WB4101 pA_2 9.8 and 9.6; 5-methylurapidil pA_2 7.9 and pK_B estimate 8.0; benoxathian pA_2 8.8 and 9.3; indoramin pA_2 7.2 and 7.5, respectively).

3 RS 17053 was not a competitive antagonist in any blood vessel as Schild plot slopes were greater than unity. The pK_B estimates for RS 17053 were 7.1 in aorta, 7.0 in the mesenteric artery and 7.7 in the pulmonary artery.

4 The α_{1D}-subtype selective antagonist BMY 7378 appeared to be non-competitive with shallow Schild plot slopes. The data were better fitted with two lines in all tissues, with Schild plot slopes that were no longer different from unity, except in the pulmonary artery. The higher affinity site for BMY 7378 in the aorta had a pA_2 of 9.0, while it was 8.8 and 8.9 in the mesenteric and pulmonary arteries, respectively.

5 MDL73005EF acted in a non-competitive manner in all three blood vessels, with shallow Schild plot slopes. The pK_B estimates for MDL73005EF were 8.4 in aorta, 7.5 in the mesenteric artery and 8.0 in the pulmonary artery.

6 In all three blood vessels the functionally determined antagonist affinity estimates correlated best with published pK_i values for their displacement of [^{3}H]-prazosin binding on membranes expressing cloned α_{1d}-adrenoceptors compared with α_{1a}- or α_{1b}-adrenoceptors. The antagonist affinity estimates in the aorta, mesenteric and pulmonary arteries correlated highly with their previously published pA_2 values in rat aorta (α_{1D}) and less well with those for α_{1A}- and α_{1B}-adrenoceptors mediating contraction of the rat epididymal vas deferens and rat spleen, respectively.

7 The results of this study suggest that the contraction to phenylephrine of the rat thoracic aorta, mesenteric artery and pulmonary artery are mediated in part via the α_{1D}-subtype of adrenoceptor. The data for both BMY 7378 and MDL73005EF in all three blood vessels are consistent with receptor heterogeneity. However, the identity of the second site is unclear.

Keywords: α_1-Adrenoceptor subtype classification; rat thoracic aorta; rat mesenteric artery; rat pulmonary artery; BMY 7378; MDL73005EF; prazosin; WB 4101; 5-methylurapidil; RS 17053

Introduction

To date three α_1-adrenoceptor subtypes have been identified by pharmacological techniques with competitive antagonists and the alkylating agent chloroethylclonidine (CEC). The corresponding cDNA coding for these receptors had been cloned by means of molecular biological methods (Michel *et al*., 1995; Hieble *et al*., 1995). The IUPHAR recommendation for receptor nomenclature (Bylund *et al*., 1994; Hieble *et al*., 1995) refers to those receptors identified in native tissues by capital subscripts (α_{1A}, α_{1B} and α_{1D}) and the corresponding cloned receptors by lower case subscripts (α_{1a}, α_{1b} and α_{1d}).

The identity of the receptor subtype(s) mediating contraction to α_1-adrenoceptor agonists in the vasculature, especially in the rat aorta, has been controversial. When only two subtypes of α_1-adrenoceptors were recognized (α_{1A} and α_{1B}), pharmacological evidence suggested that the α_1-adrenoceptor(s) of the rat aorta were of either an α_{1B}-subtype (Han *et al*., 1990; Eltze & Boer, 1992; Kong *et al*., 1994; Testa *et al*., 1995) or both α_{1A}- and α_{1B}-subtypes (Piascik *et al*., 1991; Orsetti & Distillo, 1994; Wenham & Marshall, 1994). Other groups have suggested that the receptor was atypical and could not be classified as either α_{1A} or α_{1B} (Muramatsu *et al*., 1991; Aboud *et al*., 1993). The atypical nature of this receptor was based on the high affinity of α_{1A}-selective compounds such as 5-methylurapidil and WB4101 together with noradrenaline contractions being sensitive to CEC.

More recently a third α_1-adrenoceptor subtype (α_{1d}) and a selective antagonist, BMY 7378, have been identified. Subsequently the α_1-adrenoceptors of the rat aorta have been pharmacologically classified as of the α_{1D}-subtype (Saussy *et al*., 1994; Kenny *et al*., 1995) by use of BMY 7378, which has over 100 fold selectivity for the α_{1d}-clone compared with the α_{1a}- and α_{1b}-subtypes. However, the action of BMY 7378 was not consistent with competitive antagonism at a single site in the rat aorta, although it had a high affinity similar to that at the cloned α_{1d}-adrenoceptor (Kenny *et al*., 1995). Therefore the effect of BMY 7378 was suggested to be consistent with receptor population heterogeneity. In contrast to the aorta, BMY 7378 was found to be competitive in the rat epididymal vas deferens, spleen and human prostate but yielded lower affinities that were consistent with these tissue possessing α_{1A}- or α_{1B}-subtypes (Burt *et al*., 1995; Marshall *et al*., 1995).

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In the rat mesenteric (Han *et al*., 1990; Kong *et al*., 1994) and pulmonary arteries (Chen & Han, 1992), the α_1-adrenoceptor subtype mediating contractions to noradrenaline or phenylephrine has been described as being via a mixed population of α_{1A}- and α_{1B}-subtypes based partly on data with CEC and nifedipine. However, CEC may be less reliable as a tool for subtyping α_1-adrenoceptors than originally thought as the extent of alkylation depends on temperature, concentration and time of incubation (Michel *et al*., 1995). In addition, evaluation of the source of calcium required for the contraction (by use of the calcium channel blocker nifedipine) may be of limited value in characterizing α_1-adrenoceptor subtypes (Bylund *et al*., 1994). A very recent investigation suggested an α_{1D}-subtype mediating contraction in the rat mesenteric artery (Villalobos-Molina & Ibarra, 1996).

An alternative classification has been used to characterize α_1-adrenoceptors in the vasculature (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1990). Here the receptors are classed into α_{1H} (high affinity for prazosin), α_{1N} (high affinity for HV-723) and α_{1L} (low affinity for both antagonists). With this system the α_1-adrenoceptor in the rat aorta and mesenteric artery has been described as having a high affinity for prazosin (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1991).

BMY 7378 is proving to be an important compound in classifying α_1-adrenoceptors in the vasculature. Therefore, it is important to see if the apparent non-competitive action of BMY 7378 is specific to the rat aorta or a more general phenomenom which is also found in other rat blood vessels. Although there is no other compound as selective as BMY 7378 for the α_{1D}-adrenoceptor, for comparison we have also employed MDL73005EF which is about 30 fold selective for the α_{1D}-subtype (Saussy *et al*., 1996). Thus the aim of the present study was to characterize further and compare the α_1-adrenoceptor subtypes mediating phenylephrine contraction of the rat aorta, mesenteric and pulmonary arteries by use of a range of eight antagonists, including α_{1A}-selective 5-methylurapidil, indoramin and RS 17053 as well as the α_{1D}-selective BMY 7378.

Methods

Male Sprague Dawley rats (350–450 g) were stunned and killed by cervical dislocation. The blood vessels were dissected out, connective tissue removed and then cut into rings (3–5 mm long) which were denuded of endothelium by gentle abrasion with a serrated file. Rings were suspended in 10 ml organ baths containing Krebs of the following composition (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5 Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2, and D-glucose 11, maintained at 37°C and gassed with 95% O_2/5% CO_2. The Krebs solution also contained 10^{-5} M cocaine, 10^{-7} M rauwolscine and 10^{-7} M propranolol to block uptake$_1$ and inhibit α_2- and β-adrenoceptors, respectively. In addition, in experiments with the rat pulmonary artery, β-oestradiol 10^{-5} M was also added to inhibit extraneuronal uptake (uptake$_2$). Preliminary experiments showed that β-oestradiol on its own shifted the phenylephrine concentration-contractile response curve to the right in the aorta and mesenteric artery (results not shown) and therefore it was excluded from the Krebs solution when these tissues were used.

Arterial rings were placed across two tungsten wires (diameter of each 0.125 mm) under a tension of 0.5 g and the change in isometric tension was measured by use of Grass FT.03 force displacement transducers and recorded on a Grass 7D polygraph. The tissues were allowed to equilibrate for 75 min except for the pulmonary artery (100 min). During this time the tissue was washed two to three times and the tension re-adjusted to baseline.

Initially a concentration of phenylephrine producing a submaximal contraction (70–85% of maximum) was given to the rat thoracic aorta (10^{-7} M), mesenteric artery (3×10^{-7} M) and pulmonary artery (3×10^{-8} M). Once the response had stabilized (10–15 min) acetylcholine (10^{-6} M) was added to assess the integrity of the endothelium. If the contractions to phenylephrine were not maintained or relaxation (>5% of the phenylephrine induced tone) to acetylcholine was observed, the tissues were discarded.

Tissues were washed and left to recover for 30 min except for the pulmonary artery, which needed a longer time interval (40 min). This was followed by a cumulative concentration-response curve to phenylephrine in all tissues. The tissues were then washed to baseline (for either 30 min or 40 min, as above) and left to equilibrate for a further 30 min with the antagonist before the concentration-response curve to phenylephrine was repeated. An exception to this was RS 17053 where all tissues were incubated for 2 h as this time interval has been found to be necessary (Marshall *et al*., 1996). Some tissues had two successive phenylephrine curves (without antagonist) to check reproducibility of agonist effects. Two cumulative concentration-effect curves to K^+ (1.0–80.0 mM) were carried out in the mesenteric artery to assess the reproducibility of K^+ contractions. In other tissues BMY 7378 (3×10^{-6} M) was added for 30 min before the repeat K^+ concentration contractile response curve was performed. Also in the mesenteric artery, an inhibition curve to BMY 7378 was constructed by adding the antagonist in half log molar increments after the first dose-response curve to phenylephrine had reached a maximum. Each concentration of BMY 7378 was left in the bath till relaxation to that concentration of antagonist had reached a plateau. The effect of dimethylsulphoxide (DMSO) on phenylephrine concentration-response curves was also investigated.

Data analysis

The results were calculated as a percentage of the maximum response of the first concentration-effect curve to phenylephrine. Responses are plotted graphically as means from at least 4 separate experiments, with vertical lines representing s.e.mean. When error bars do not appear on the figures, this is because they are small and fall within the dimension of the symbols. Curves were fitted to all the data by non-linear regression by use of either Inplot or Prism (GraphPAD software San Diego, California, U.S.A.) to determine Hill slopes for the agonist concentration-response curves and to calculate pEC_{50} values ($-\log$ of the EC_{50} values). In all cases, including the data for BMY 7378 and MDL73005EF (in the presence of these antagonists there was, with some concentrations, an increase in the maximum responses, see results section), the 50% of the maximum for each concentration-response curve was used to calculate the EC_{50} value. The EC_{50} value in the presence and absence of antagonist in a single tissue was used to determine the concentration-ratio (CR).

pA_2 values were calculated by linear regression by use of GraphPAD Prism and were obtained from the x-intercept of the plot of log (CR−1) against log molar antagonist concentration (Arunlakshana & Schild, 1959), where slope was not different from unity. Where a slope appeared to be significantly different from one, an affinity estimate was obtained with the equation, $pK_B = \log(CR-1) - \log$ (molar antagonist concentration). The lowest concentration of the antagonist was used for this calculation. For the BMY 7378 results, in all three tissues two linear regressions were fitted to the data as this was found to be a better fit than a single linear regression. The sets of points at the point of inflexion are joined up (Figures 7a–c) to illustrate the 'plateau'. The pA_2 values or pK_B estimates obtained for the antagonists in the three rat blood vessels were plotted against their average pK_i values calculated from measurements with the expressed cloned subtypes (data from the literature) and against functionally determined antagonist affinities from a number of other tissues (data from the literature). Linear regression was used to correlate the pK_i and pA_2 values. A modified form of the Chen Prusoff equation given below was used to calculate a pK_B for BMY 7378 (in the mesenteric artery) by

use of the IC_{50} value determined from the antagonist inhibition curve (Leff & Dougall, 1993).

$$K_B = \frac{[IC_{50}]}{(2 + ([A]/[A_{50}])^n)^{1/n} - 1)}$$

In the above equation, K_B is the dissociation constant of the antagonist, IC_{50} refers to the concentration of the antagonist required to produce half maximal reduction of the response (phenylephrine contraction), [A] is the concentration of the agonist, $[A_{50}]$ is the concentration of the agonist producing half maximal response and n is the slope factor.

A paired *t* test was used to assess the significance of differences between (a) pEC_{50} values for the control and repeat curves, (b) change in the Hill slopes and (c) increases in the maximum responses, between control curves and that in the presence of an antagonist. A *P* value <0.05 was taken to indicate a statistically significant difference. Statistical analysis was performed by use of either Instat or Prism (GraphPAD software, San Diego, CA, U.S.A.).

Drugs and solutions

Prazosin hydrochloride and WB4101 (2(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride) were donated by Pfizer Central Research (Kent). Phenylephrine hydrochloride, cocaine hydrochloride, propranolol hydrochloride and β-oestrodiol, were obtained from Sigma. 5-Methylurapidil, benoxathian hydrochloride, rauwolscine hydrochloride and BMY 7378 dihydrochloride (8-(-2(-4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspiro(4,5)decane-7,-9,dionedihydrochloride) and MDL73005EF (8-[2-(1,4-benzodioxan-2-ylmethylamino)ethyl]-8-azaspiro[4,5]decane-7,9-dione hydrochloride) were obtained from RBI while RS 17053 (N-[2-(2cyclopropylmethoxy)ethyl]5-chloro-α, α-dimethyl-1 H-indole3-ethylamine hydrochloride) was donated by Roche Bioscience (U.S.A.).

Preparation of all stock solutions and their subsequent dilution were made in distilled water. Exceptions to this were RS 17053 and prazosin which were initially dissolved in dimethylsulphoxide (DMSO, 0.1%) then diluted in distilled water. Phenylephrine was prepared fresh each day, whereas a stock solution (10^{-2} M) of the antagonists was stored frozen in aliquots and thawed and diluted fresh daily.

Results

Phenylephrine produced concentration-dependent isometric contractions of the rat aorta (pEC_{50} 7.63 ± 0.09, mean $\pm$ s.e.mean; maximum tension 2.50 ± 0.22 g, $n=5$), mesenteric artery (pEC_{50} 7.51 ± 0.04; maximum tension 1.32 ± 0.20 g, $n=4$) and pulmonary artery (pEC_{50} 7.90 ± 0.14; maximum tension 0.40 ± 0.04 g, $n=4$). The pEC_{50} value for the second concentration response curve was not significantly different from the first in all three tissues ($P>0.05$). The concentration of DMSO equivalent to that added to organ baths with dilutions of either prazosin or RS 17053 did not alter the concentration-response curve to phenylephrine (data not shown).

Prazosin produced rightward shifts of the concentration-response curves to phenylephrine, with no depression of the maximum response, in all three blood vessels consistent with competitive antagonism (Figure 1 and Table 1). WB4101, 5-methylurapidil, benoxathian and indoramin were all competitive antagonists of the phenylephrine contractions in the aorta (Figures 2–5, Table 1). These antagonists were also competitive in both the mesenteric and the pulmonary arteries (Figures 2–5), with the exception of 5-methylurapidil in the latter vessel (see Table 1). In the pulmonary artery, 5-methylurapidil gave a Schild slope that was greater than unity (slope 1.14 ± 0.05), inconsistent with competitive antagonism. A pK_B estimate of 8.0 was obtained for 5-methylurapidil with the lowest concentration of the antagonist tested (10^{-7} M).

RS 17053 acted in a manner inconsistent with competitive antagonism in all three blood vessels. It had pA_2 values of 6.8, 6.8 and 7.3 but with slopes greater than unity in the aorta (slope 1.79 ± 0.16), mesenteric artery (slope 1.54 ± 0.27) and pulmonary artery (slope 1.54 ± 0.22), respectively (Figure 6). With the lowest concentration of RS 17053 tested (3×10^{-7} M, in all arteries), pK_B estimates were obtained for the three arteries (see Table 1).

BMY 7378 did not appear to be a competitive antagonist of phenylephrine contractions in any of the three blood vessels. In the presence of BMY 7378 there was an increase in the maximum response (aorta 3×10^{-9} M: mesenteric artery 3×10^{-6} M: pulmonary artery 10^{-8} M and 10^{-7} M; $P<0.05$) relative to the control concentration-effect curve to phenylephrine, although the magnitude of the change was not dependent on the antagonist concentration. In spite of the increase in the maximum contraction produced by a few of the antagonist concentrations, Schild plots were constructed. By use of all the concentrations of antagonist for the Schild plot, pA_2 values of 9.3 (aorta, slope 0.69 ± 0.07), 9.2 (mesenteric artery, slope 0.61 ± 0.05) and 9.3 (pulmonary artery, slope 0.63 ± 0.06) were obtained but with shallow slopes. (If results

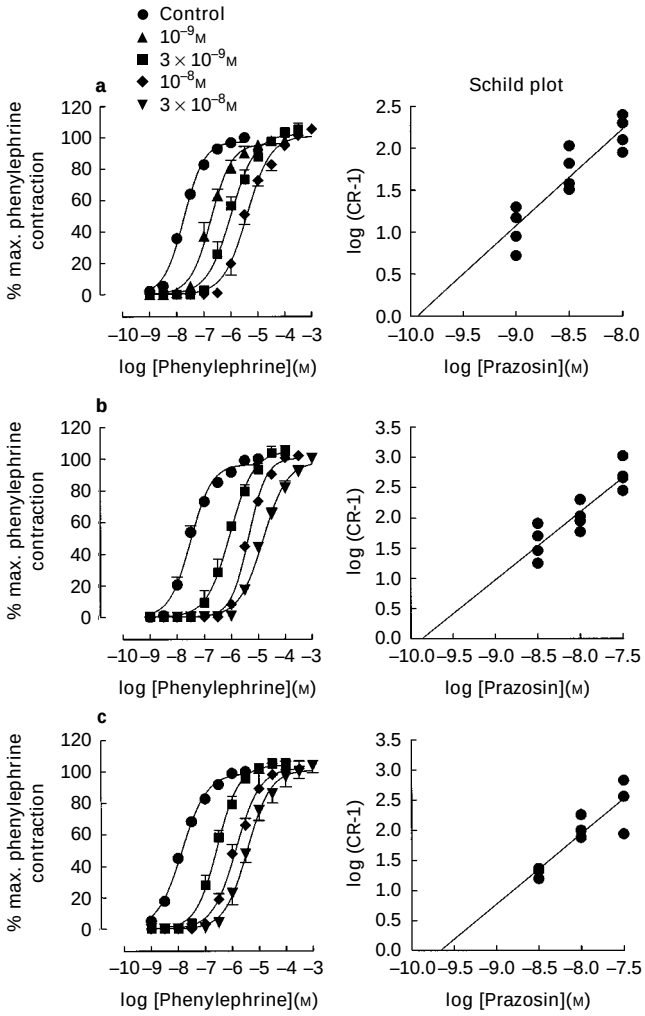

Figure 1 Concentration-response curves and Schild plots for antagonism of phenylephrine induced contraction by prazosin in (a) rat aorta, control (in the absence of prazosin), plus prazosin 10^{-9} M, 3×10^{-9} M and 10^{-8} M and in (b) rat mesenteric artery, control, plus prazosin 3×10^{-9} M, 10^{-8} M and 3×10^{-8} M and in (c) rat pulmonary artery, control, plus prazosin 3×10^{-9} M, 10^{-8} M and 3×10^{-8} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

Table 1 Comparison of antagonist pA_2 or pK_B* values on rat blood vessels with their published pK_i values calculated by use of measurements with cloned receptor subtypes

	pA_2/pK_B			pK_i**		
Antagonist	*Thoracic aorta*	*Mesenteric artery*	*Pulmonary artery*	α_{1a}	α_{1b}	α_{1d}
Prazosin	9.9 (1.15±0.16)	9.9 (1.12±0.17)	9.7 (1.17±0.17)	9.2±0.2	9.6±0.2	9.4±0.2
WB4101	9.6 (0.97±0.03)	9.8 (0.88±0.12)	9.6 (1.10±0.11)	9.5±0.3	8.2±0.1	9.2±0.1
5-Methylurapidil	8.1 (0.98±0.13)	7.9 (1.10±0.12)	8.0*	8.8±0.1	6.8±0.3	7.3±0.3
Benoxathian	9.2 (0.92±0.13)	8.8 (1.04±0.20)	9.3 (1.08±0.17)	9.0	7.8	8.7
Indoramin	7.4 (1.03±0.10)	7.2 (1.14±0.14)	7.5 (0.96±0.07)	8.2±0.3	7.3±0.1	6.8±0.2
RS 17053	7.1*	7.0*	7.7*	9.1	7.8	7.8
BMY 7378	9.0 (1.07±0.17)	8.8 (1.03±0.12)	8.9 (1.11±0.17)	6.6	7.2	9.4
MDL73005EF	8.4*	7.5*	8.0*	5.8	6.2	7.3

Values shown for pA_2/pK_B are mean (Schild plot slope±s.e.mean). **Data are mean values from, Faure *et al*., 1994; Forray *et al*., 1994b; Kenny *et al*, 1994a,b; Testa *et al*., 1994; Goetz *et al*., 1995 and Saussy *et al*., 1996. No s.e.mean are listed for compounds with only one or two values.

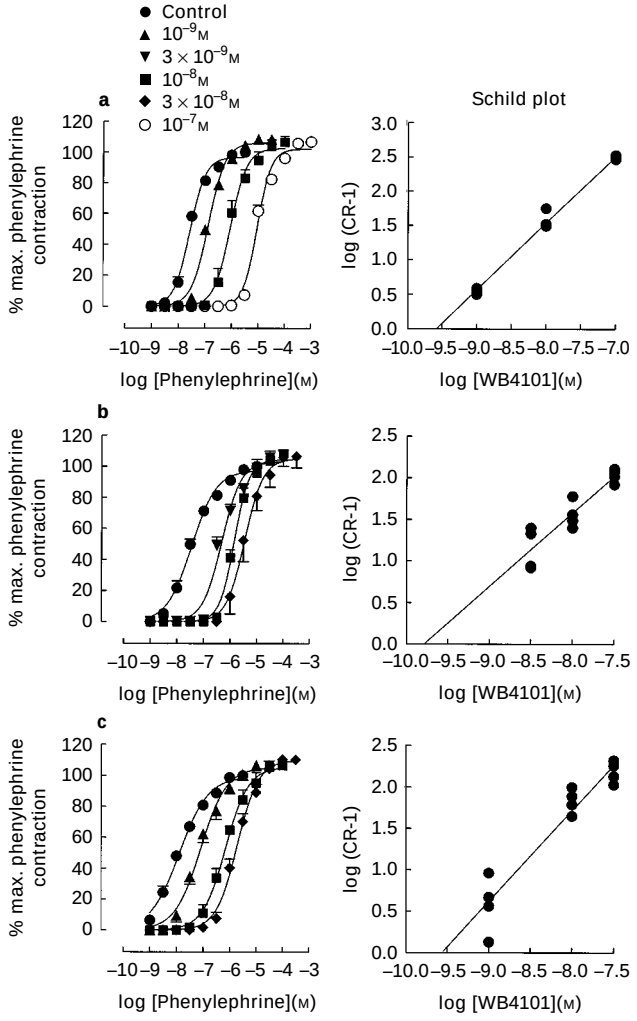

Figure 2 Concentration-response curves and Schild plots for antagonism of phenylephrine induced contraction by WB4101 in (a) rat aorta, control (in the absence of WB4101), plus WB4101 10^{-9} M, 10^{-8} M and 10^{-7} M and in (b) rat mesenteric artery, control, plus WB4101 3×10^{-9} M, 10^{-8} M and 3×10^{-8} M and in (c) rat pulmonary artery, control, plus WB4101 10^{-9} M, 10^{-8} M and 3×10^{-8} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

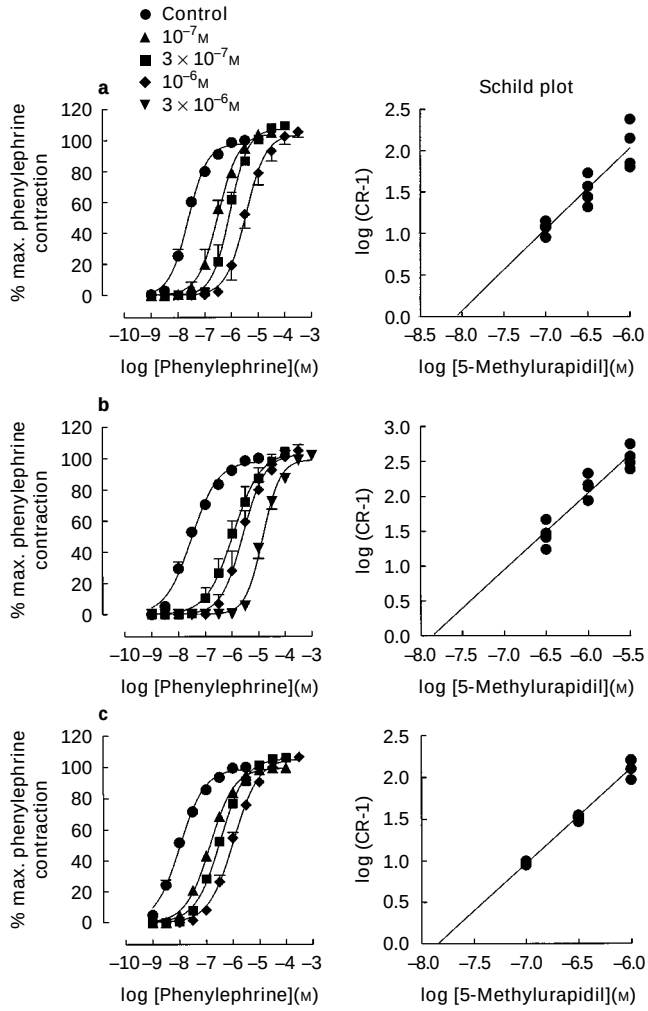

Figure 3 Concentration-response curves and Schild plots for antagonism of phenylephrine induced contraction by 5-methylurapidil in (a) rat aorta, control (in the absence of 5-methylurapidil), plus 5-methylurapidil 10^{-7} M, 3×10^{-7} M and 10^{-6} M and in (b) rat mesenteric artery, control, plus 5-methylurapidil 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M and in (c) rat pulmonary artery, control, plus 5-methylurapidil 10^{-7} M, 3×10^{-7} M and 10^{-6} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

where there was an increase in the agonist maximum response were omitted from the analysis e.g. 3×10^{-9} M of BMY 7378 in the aorta, the results were almost unaltered; BMY 7378 aorta pA_2 9.4, slope 0.67±0.10, mesenteric artery 9.4, slope 0.53±0.05, pulmonary artery pA_2 9.1 slope 0.63±0.05). However, inspection of the shallow Schild plots in the three arteries (Figure 7a–c) revealed discontinuity and suggests the data may be better fitted for two sites rather than one. Therefore, Schild regression was applied separately to the two (three in the case of the mesenteric artery) lower, and to the

three higher concentrations of BMY 7378 (shown on the same Schild plot). This yielded two slopes that were not different from unity in the aorta (slope 1.07 ± 0.17 and 1.01 ± 0.14) and mesenteric artery (slope 1.03 ± 0.12 and 1.02 ± 0.15). However, in the pulmonary artery the second component had a slope that was less than unity (slope 0.63 ± 0.13). A high affinity estimate for BMY 7378 was obtained in the aorta (9.0, slope 1.07 ± 0.17; Figure 7a), mesenteric artery (8.8, slope 1.03 ± 0.12; Figure 7b) and pulmonary artery (8.9, slope 1.11 ± 0.17; Figure 7c).

The increase in the maximum response to phenylephrine by BMY 7378 was investigated by studying the effect of this antagonist on K^+ contractions. K^+ produced reproducible concentration-dependent increases in tension of the rat mesenteric artery (EC_{50} for 1st and 2nd curves, 18.2 ± 1.4 mM and 19.1 ± 3.6 mM, respectively). Neither the EC_{50} nor the maximum response was altered in the presence of 3×10^{-6} M BMY 7378 ($P > 0.05$), a concentration which had significantly increased the maximum response to phenylephrine in this tissue.

BMY 7378 gave a shallow Schild plot slope in all three arteries and therefore an inhibition curve to this antagonist in the mesenteric artery was tried to see if a biphasic curve was produced. However, in contrast to the Schild plot seen for BMY 7378, the inhibition curve was monophasic, not in keeping with the action of BMY 7378 on two different sites. In the rat mesenteric artery BMY 7378 gave an IC_{50} of 1.4×10^{-6} M (from the inhibition curve, Figure 8). By use of a modified form of the Cheng Prusoff equation (which takes in to account the slopes of the concentration-response curves, Leff & Dougall, 1993) a pK_B of 8.5 was calculated.

MDL73005EF apparently acted in a non-competitive manner, with pA_2 values of 8.6, 7.6 and 8.2 in the rat aorta (slope 0.72 ± 0.06), mesenteric artery (slope 0.86 ± 0.05) and pulmonary artery (slope 0.79 ± 0.05), respectively (Figure 9a–c). With the lowest concentration of the antagonist, pK_B estimates were obtained for all three arteries (Table 1). Like BMY 7378, increases in the maximum responses were also observed in the presence of MDL73005EF at some concentrations (aorta, 10^{-6} M and 10^{-5} M; mesenteric artery, 10^{-6} M, 3×10^{-6} M and 10^{-5} M; pulmonary artery 10^{-7} M and 10^{-6} M, $P < 0.05$). (If results where there was an increase in the agonist maximum response were omitted from the analysis pA_2 values of 8.4 (slope 0.87 ± 0.42), 7.6 (slope 0.85 ± 0.21) and 8.1 (slope

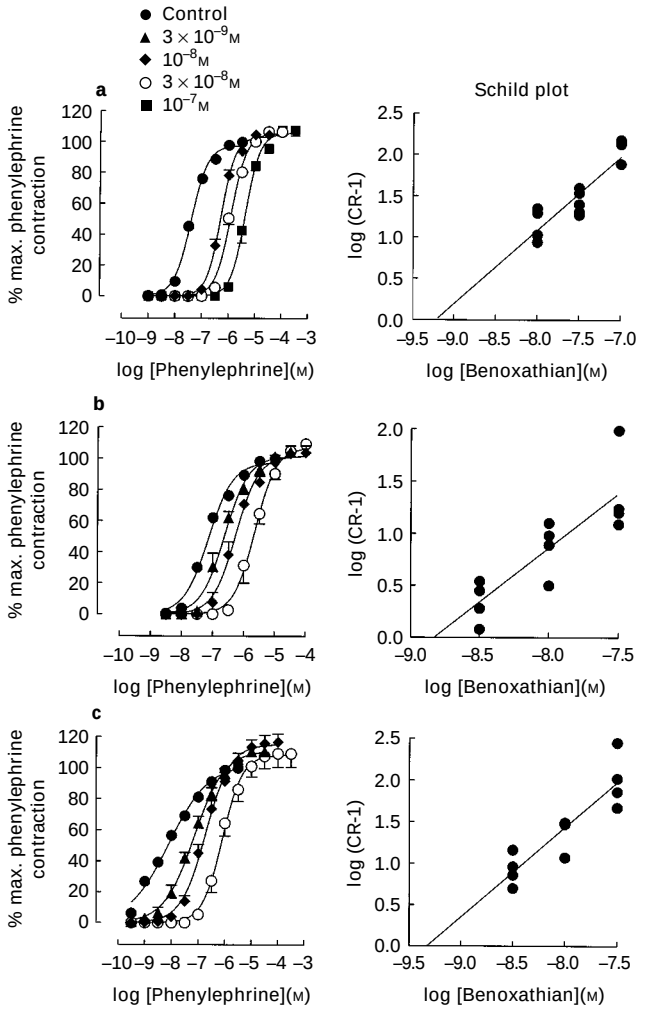

Figure 4 Concentration-response curves and Schild plots for antagonism of phenylephrine induced contraction by benoxathian in (a) rat aorta, control (in the absence of benoxathian), plus benoxathian 10^{-8} M, 3×10^{-8} M and 10^{-7} M and in (b) rat mesenteric artery, control, plus benoxathian 3×10^{-9} M, 10^{-8} M and 3×10^{-8} M and in (c) rat pulmonary artery, control, plus benoxathian 3×10^{-9} M, 10^{-8} M and 3×10^{-8} M. Each symbol represents the mean and vertical lines show s.e.meam of at least 4 separate experiments. Schild plots for each tissue were constructed with concentration-ratios from individual experiments.

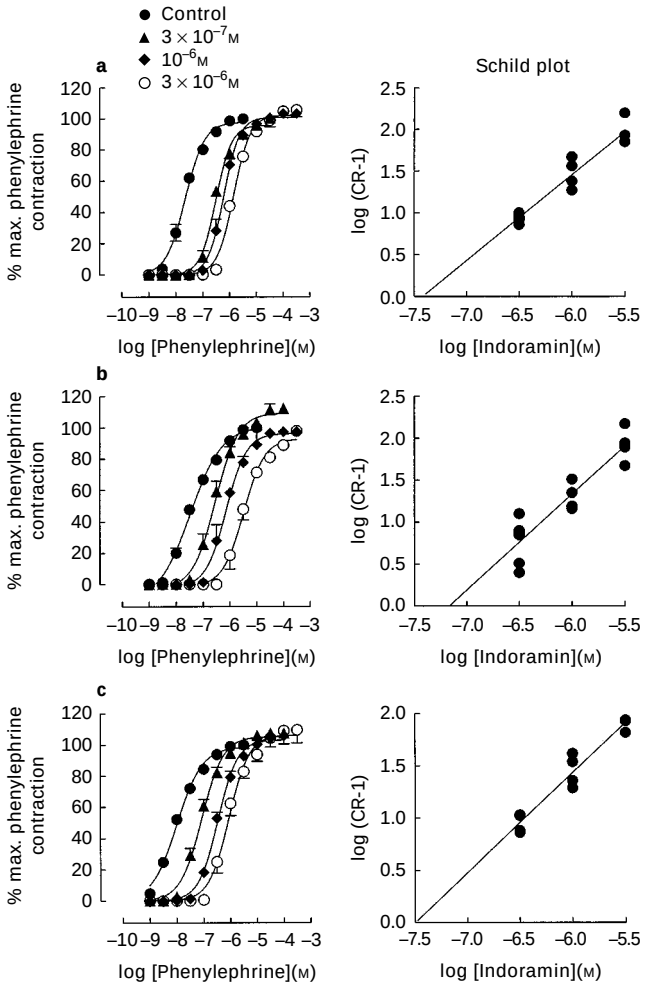

Figure 5 Concentration-response curves and Schild plots for antagonism of phenylephrine-induced contraction by indoramin in (a) rat aorta, control (in the absence of indoramin), plus indoramin 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M and in (b) rat mesenteric artery, control, plus indoramin 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M and in (c) rat pulmonary artery, control, plus indoramin 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

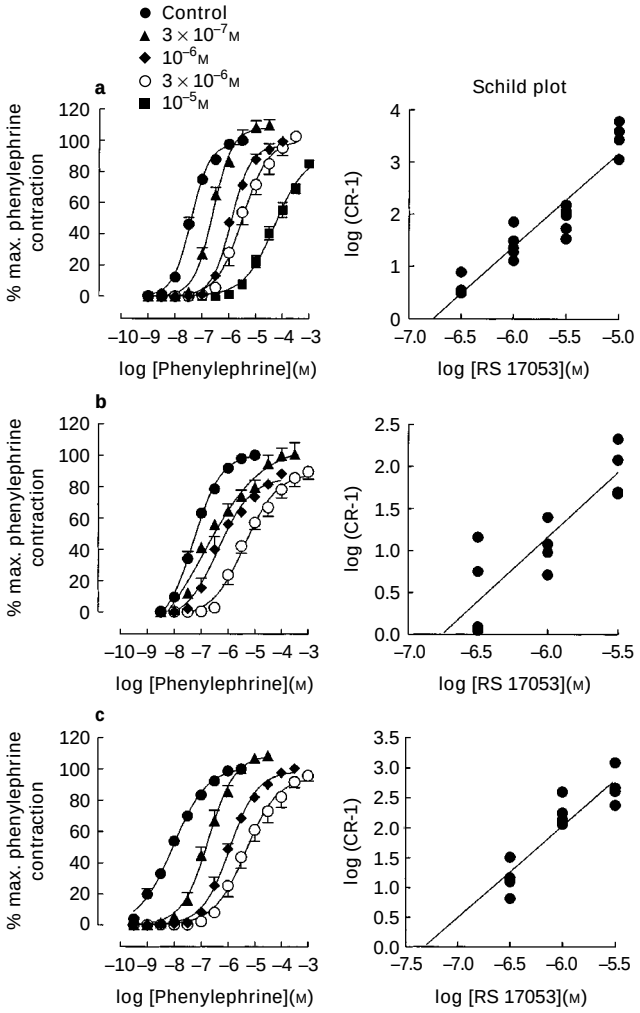

Figure 6 Concentration-response curves and Schild plots for antagonism of phenylephrine-induced contraction by RS 17053 in (a) rat aorta, control (in the absence of RS 17053), plus RS 17053 3×10^{-7} M, 10^{-6} M, 3×10^{-6} M and 10^{-5} M and in (b) rat mesenteric artery, control, plus RS 17053 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M and in (c) rat pulmonary artery, control, plus RS 17053 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

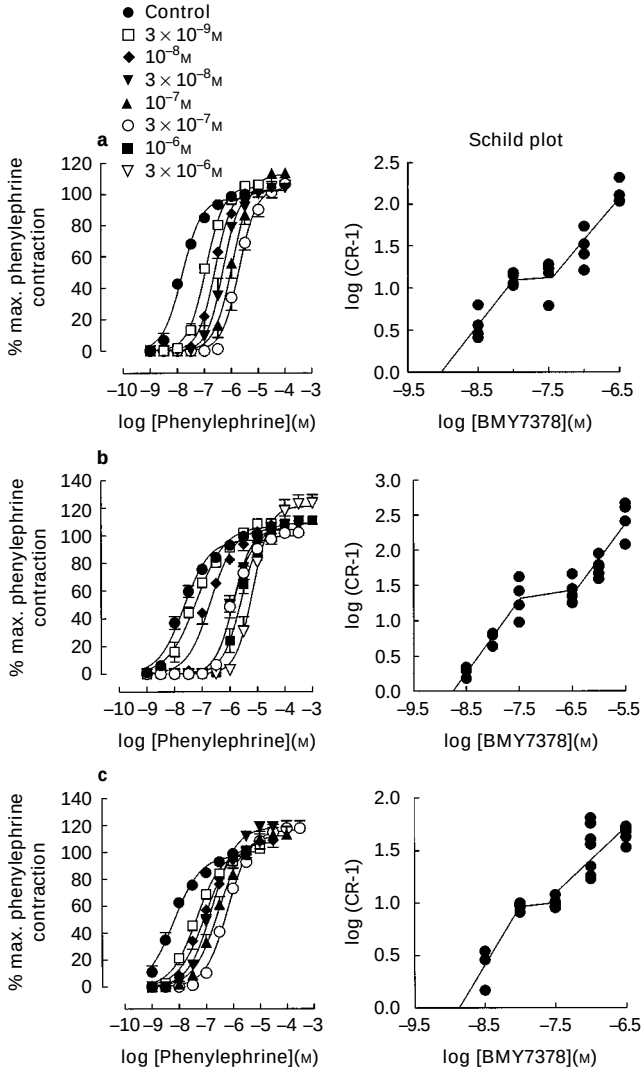

Figure 7 Concentration-response curves and Schild plots for antagonism of phenylephrine-induced contraction by BMY7378 in (a) rat aorta, control (in the absence of BMY7378), plus BMY7378 3×10^{-9} M, 10^{-8} M, 3×10^{-8} M, 10^{-7} M and 3×10^{-7} M and in (b) rat mesenteric artery, control, plus BMY7378 3×10^{-9} M, 10^{-8} M, 3×10^{-8} M, 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M and in (c) rat pulmonary artery, control, plus BMY7378 3×10^{-9} M, 10^{-8} M, 3×10^{-8} M, 10^{-7} M and 3×10^{-7} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments. Two linear regression lines are fitted to each set of data (see text). The sets of points at the point of inflection are joined up to illustrate the 'plateau'.

0.79 ± 0.07) were obtained in the rat aorta, mesenteric and pulmonary artery, respectively. These values (pA_2) are not very different from those observed with all concentrations of the antagonist).

In the aorta, the Hill slope of the phenylephrine concentration-response curves in the absence of antagonist was 1.39 ± 0.03 ($n=113$). In the presence of prazosin, WB4101, 5-methylurapidil, indoramin, benoxathian and BMY 7378 the Hill slope of the agonist concentration-response curves did not alter. However, RS 17053 (above 300 nM) decreased the Hill slope while MDL73005EF (30 nM and 1 μM) increased it ($P<0.05$). In the rat mesenteric and pulmonary artery, the Hill slopes of the control curves were 1.05 ± 0.02 ($n=122$) and 0.88 ± 0.02 ($n=120$), respectively. There was no change in the Hill slope with prazosin (except with 10^{-9} M in the mesenteric artery) or WB 4101. 5-Methylurapidil (3×10^{-7} M in mesenteric artery; 10^{-6} M in the pulmonary artery), indoramin (no change in the mesenteric artery; 10^{-6} M in the pulmonary artery), BMY 7378 (3×10^{-7} M and 10^{-6} M in mesenteric artery; 3×10^{-7} M in pulmonary artery) and MDL73005EF (3×10^{-7} M, 10^{-6} M and 3×10^{-6} M in mesenteric artery; 10^{-6} M and 10^{-5} M in pulmonery artery) caused an increase in the Hill slope only at some concentrations. RS 17053 produced a decrease in the Hill slope at concentrations greater than 3×10^{-7} M in the mesenteric artery and an increase was seen in the pulmonary artery at 3×10^{-7} M. Benoxathian produced no significant change in the Hill slope of phenylephrine concentration-response curves in the mesenteric artery, but increased it in a concentration-dependent manner in the pulmonary artery with all concentrations of benoxathian tested ($P<0.05$).

The affinity estimates for the antagonists in the three blood vessels (with the high affinity estimate for BMY 7378) were correlated with their affinity measured in ligand binding competition assays with membranes from cell lines expressing one or other of the cloned α_1-adrenoceptor subtypes (data from the literature shown in Table 1). The scattergrams for the aorta, mesenteric and pulmonary arteries show a clear relationship between the present functional data and the α_{1d}-subtype binding affinities (Figure 10). The values for the correlation coefficient r and the slope are given in Table 2. For

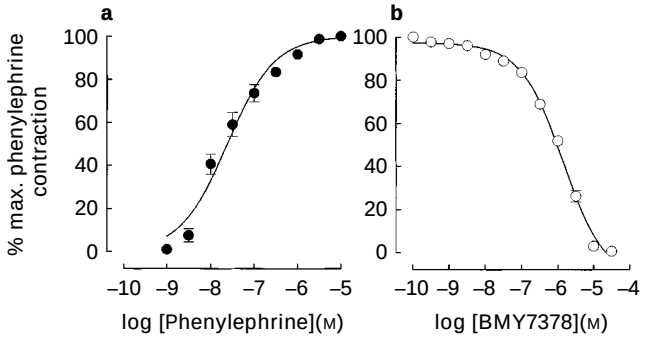

Figure 8 (a) Concentration-response curve to phenylephrine and (b) subsequent inhibition curve to BMY 7378 in the rat mesenteric artery. The inhibition curve to BMY 7378 was plotted as % maximum phenylephrine contraction of the initial control curve. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments.

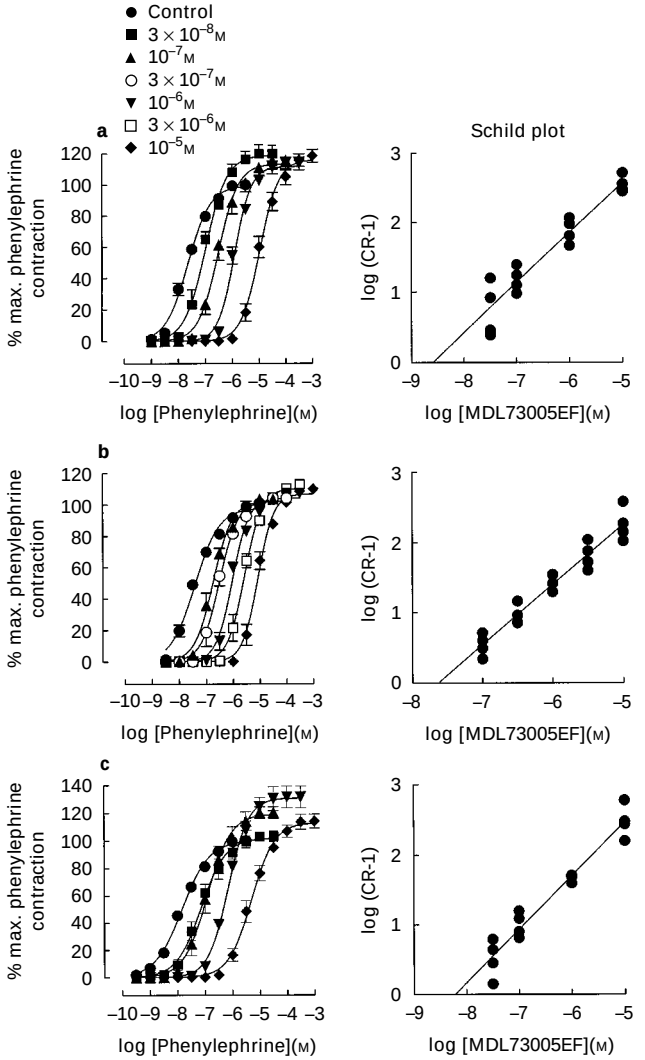

Figure 9 Concentration-response curves and Schild plots for antagonism of phenylephrine-induced contraction by MDL73005EF in (a) rat aorta, control (in the absence of MDL73005EF), plus MDL73005EF 3×10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M and in (b) rat mesenteric artery, control, plus MDL73005EF 10^{-7} M, 3×10^{-7} M, 10^{-6} M, 3×10^{-6} M and 10^{-5} M and in (c) rat pulmonary artery, control, plus MDL73005EF 3×10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M. Each symbol represents the mean and vertical lines show s.e.mean of at least 4 separate experiments. Schild plots for each tissue were constructed with the concentration-ratios from individual experiments.

each tissue the poorest correlation and lowest slopes occur with the α_{1a}-subtype while the best correlation is with the α_{1d}-clone.

The affinity estimates in the three rat blood vessels were also correlated with those from other functional studies from the literature (Table 2). There was a poor correlation between the vascular receptor and values from the epididymal vas deferens (Burt *et al*., 1995), an α_{1A}-subtype-mediated response, while the highest values were with previously published data in the rat aorta (α_{1D}; Kenny *et al*., 1995). (At present there is no other functional model of the α_{1D}-subtype which can be used for comparison). Intermediate values for correlation coefficients and slopes were found when comparing the blood vessels with an α_{1B}-mediated contraction (rat spleen; Burt *et al*., 1995).

Discussion

In this study a number of antagonists showing selectivity between α_1-subtypes was used to characterize the α_1-adrenoceptor mediating phenylephrine-induced contraction of three rat blood vessels. The selectivity of these antagonists has been confirmed in several studies with membranes from cell lines expressing one or other of the cloned receptor subtypes (Faure *et al*., 1994; Forray *et al*., 1994; Kenny *et al*., 1994a,b; Testa *et al*., 1994; Goetz *et al*., 1995; Saussy *et al*., 1996).

Prazosin, the non-subtype selective antagonist, was competitive in all three tissues with an affinity that is consistent with the contractile responses being mediated via α_1-adrenoceptors and in line with that found by others in rat aorta (Aboud *et al*., 1993; O'Rourke *et al*., 1995; Kenny *et al*., 1995).

In the aorta, affinities in this study for 5-methylurapidil, WB4101, benoxathian and indoramin were similar to values obtained previously (Aboud *et al*., 1993; Kenny *et al*., 1995; Villalobos-Molina & Ibarra, 1996; Buckner *et al*., 1996). RS 17053 was apparently non-competitive in action in the aorta in the present experiments as found previously (Marshall *et al*., 1996), although in contrast to the findings of Kenny *et al*. (1995). The relatively high affinities for WB4101 and benoxathian and the lower ones for 5-methylurapidil, indoramin and RS 17053 suggest a similarity of the α_1-adrenoceptor to the cloned α_{1d}-subtype. In agreement with this hypothesis, high affinity estimates were obtained with the α_{1d}-selective antagonists, BMY 7378 and MDL73005EF in the rat aorta similar to those found by others (Saussy *et al*., 1994; 1996; Goetz *et al*., 1995; Kenny *et al*., 1995; Villalobos-Molina & Ibarra, 1996) and therefore an α_{1D}-adrenoceptor appears to mediate the contractions to phenylephrine. It is of interest that the mRNA for what is now termed the α_{1d}-subtype has been demonstrated in this tissue (Perez *et al*., 1991).

In the other two vascular tissues, the mesenteric and pulmonary arteries, WB4101, benoxathian and indoramin were competitive and yielded affinity values that were similar to those in the aorta in this study. Therefore, these affinities aligned best with those at the cloned α_{1d}-subtype. 5-Methylurapidil was competitive in the mesenteric artery but in the pulmonary artery it yielded a Schild slope greater than unity. The reason for this deviation from competitiveness is unclear. However, it is unlikely to be due to lack of time to reach equilibrium as a 30 min period for this antagonist was sufficient in the other arteries. The affinities of WB4101 and 5-methylurapidil on the mesenteric artery were close to those in the literature (Han *et al*., 1990; Kong *et al*., 1994; Villalobos-Molina & Ibarra, 1996). RS 17053 was apparently non-competitive in the rat mesenteric and pulmonary artery, but the affinity estimates were consistent with the cloned α_{1d}-subtype. However, more recently α_{1A} adrenoceptors have been identified for which RS 17053 also displays a low affinity (rat portal vein, Marshall *et al*., 1996). Additionally, high affinity estimates were obtained for BMY 7378 and MDL73005EF in the two arteries, consistent with that at the cloned α_{1d}-subtype and the value for BMY 7378 on the mesenteric artery is similar to that

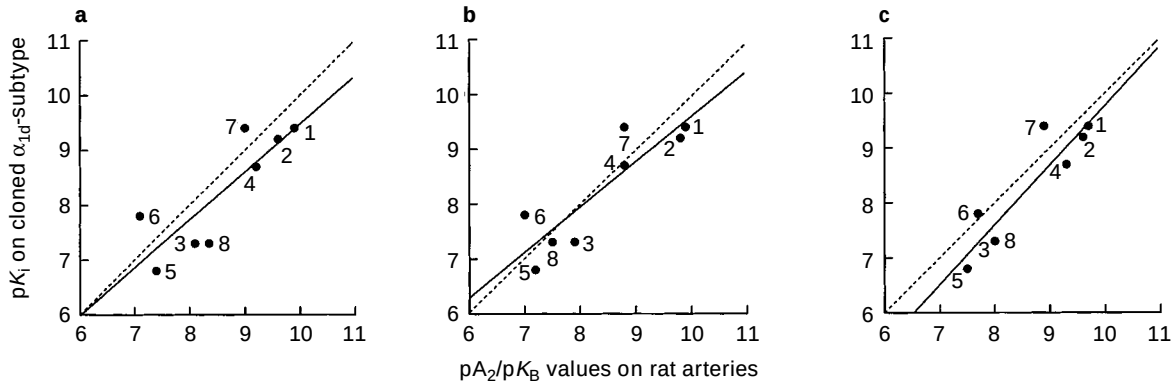

Figure 10 Correlation plots comparing average pK_i values calculated from measurements with cloned α_{1d}-adrenoceptor subtype with pA_2 or pK_B* values (from results section) in (a) rat thoracic aorta, (b) in rat mesenteric artery and (c) in rat pulmonary artery. The numbers on the plots denote the antagonists, prazosin (1), WB4101 (2), 5-methylurapidil (3), benoxathian (4), indoramin (5), RS17053* (6), BMY7378 (7) and MDL73005EF* (8). The solid line was fitted by use of linear regression and the dashed line has a slope of unity and passes through the origin.

Table 2 Correlation coefficients and slopes of the correlation plots for antagonist pA_2 or pK_B values on rat blood vessels with either their pK_i calculated from measurements with cloned subtypes or with pA_2 values from functional assays (α_{1A}, vas deferens; α_{1B}, spleen; α_{1D}, aorta)

	Rat aorta		*Rat mesenteric artery*		*Rat pulmonary artery*	
α_1-Subtype	*Correlation coefficient* (r)	*Slope*	*Correlation coefficient* (r)	*Slope*	*Correlation coefficient* (r)	*Slope*
α_{1a}	0.15	0.20±0.54	0.34	0.40±0.46	0.33	0.50±0.59
α_{1b}	0.55	0.55±0.34	0.68	0.61±0.27	0.68	0.78±0.35
α_{1d}	0.84	0.87±0.23	0.88	0.82±0.18	0.91	1.09±0.20
α_{1A}-Vas deferens	0.15	0.04±0.42	0.10	0.08±0.39	0.16	0.18±0.49
α_{1B}-Spleen	0.69	0.47±0.22	0.74	0.47±0.19	0.68	0.54±0.26
α_{1D}-Aorta	0.97	0.99±0.10	0.96	0.93±0.12	0.93	1.11±0.20

Data for the cloned subtypes are mean values from, Faure *et al*., 1994; Forray *et al*., 1994; Kenny *et al*., 1994a,b; Testa *et al*., 1994; Goetz *et al*., 1995 and Saussy *et al*., 1996. No s.e.mean are listed for compounds with only one or two values. Data for the rat vas deferens and spleen are from Burt *et al*., 1995 and that for the rat aorta are from Kenny *et al*., 1995.

previously obtained (Villalobos-Molina & Ibarra, 1996). Thus, the results with eight antagonists in all three blood vessels are consistent with the α_{1D}-subtype.

One feature of the action of BMY 7378 which has been demonstrated previously in the aorta (Kenny *et al*., 1995), is that it did not act in a simple competitive manner, having a low Schild plot slope. In the literature sometimes the action of the antagonist has been described as competitive but in these cases the slope of the Schild plot was not shown (Saussy *et al*., 1994; Villalobos-Molina & Ibarra, 1996).

High affinity estimates were obtained for MDL73005EF against phenylephrine contractions in all three arteries. The pK_B estimate for this antagonist varied by nearly 10 fold between the three tissues but, nonetheless, the results are in line with responses being mediated via the α_{1D}-adrenoceptor. However, MDL73005EF did not act in a simple competitive manner in any of the three blood vessels, displaying shallow Schild plot slopes in all cases which is consistent with receptor heterogeneity.

While the data from all antagonists contribute to the conclusions, it is the results with the selective α_{1D}-adrenoceptor antagonist BMY 7378 which are particularly interesting. Inspection of the Schild plot analysis suggested that while the overall Schild plot slope was less than unity, the data from each blood vessel might be resolved into two components consistent with the involvement of more than one receptor. There is the possibility that the discontinuity seen in the plots may be due to an additional effect of BMY 7378, for example the increase in the maximum responses, although this seems unlikely for a number of reasons. Firstly, the increases in the maximum responses are not concentration-dependent (see Results). Secondly, BMY 7378 had no effect on the K^+ contractions (mesenteric artery) suggesting that BMY 7378 does not enhance the general contractility of the tissue. Thirdly, an increase in the maximum response was also seen in the presence of MDL73005EF, although the discontinuity seen in the Schild plots for BMY 7378 was not observed with this antagonist (MDL73005EF) in any of the tissues. The difference in results between these two antagonists may, at least in part, reflect the lower α_{1D}-selectivity of MDL73005EF compared with BMY 7378 (30 versus 100 fold).

Changes in the Hill slopes of the agonist concentration-response curve can occur, in the presence of a subtype selective antagonist, if the response to the agonist is mediated via a heterogeneous receptor population. In this study both the α_{1D}-selective antagonists produced an increase in the Hill slope in all arteries at some concentrations, with the exception of the aorta where an increase was not observed with BMY 7378. However, there was no particular pattern as to which concentrations produced this effect. Data from Schild analysis suggest the presence of a heterogenous receptor system in all three tissues and these are distinguished by the α_{1D}-selective antagonists used in this study. Therefore, it would appear that measuring the deviation of the Schild slope from unity is a more sensitive indicator of receptor heterogeneity than the change in Hill slope of an agonist concentration-response curve. Conversely some antagonists gave Schild plots consistent with competitive antagonism but also altered the Hill slopes with a few concentrations in the mesenteric and pulmonary artery and the significance of these changes is unclear. However, with benoxathian in the pulmonary artery an increase in Hill slope was observed in all concentrations tested,

suggesting possible receptor heterogeneity although with Schild analysis the antagonist appeared to be competitive. The reason why a consistent change in Hill slope was obtained with this antagonist in only one out of the three blood vessels is not apparent.

An antagonist inhibition curve was used to see if the nature of the inhibition of phenylephrine contraction by BMY 7378 was consistent with responses being mediated via a single or a heterogeneous receptor population. As discussed above, in the mesenteric artery Schild analysis of the BMY 7378 data revealed a pronounced flattening of the Schild plot resulting in a shallow Schild slope (Figure 7b), consistent with a heterogeneous receptor population. In contrast a monophasic concentration-inhibition curve to BMY 7378 was obtained which does not support the idea that BMY 7378 was acting at more than one site. However, one possible reason may be that phenylephrine may also have different potencies for the two sites. Thus, if the control maximum of the phenylephrine curve used for the inhibition curve is only mediated via α_1-adrenoceptors for which phenylephrine and BMY 7378 have a high affinity, then the resulting inhibition curve is likely to be monophasic. On the other hand, the additional site should be involved in the contraction to phenylephrine in the presence of BMY 7378 which gave a concentration-ratio of just over 10. The calculated pK_B of 8.5 (from inhibition curve) was similar to the high affinity value for BMY 7378 from Schild analysis (8.8). Under these limited conditions the antagonist-inhibition curve was not as good as identifying receptor heterogeneity as Schild analysis. On the other hand, a different experimental design might be more sensitive at distinguishing the subtypes (Barlow *et al*., 1997).

Nevertheless, the results from Schild analysis, with both BMY 7378 and MDL73005EF are consistent with more than one receptor mediating contractions in these tissues. In the rat aorta for instance, the presence of receptor heterogeneity has been suggested by more than one group of researchers. Early workers suggested a mixed receptor population (α_{1A}- and α_{1B}-subtypes, Piascik *et al*., 1991; Orsetti & Distillo, 1994; Wenham & Marshall, 1994), although the α_{1D}-subtype had not been distinguished in tissues at this time and therefore data interpretation did not include this possibility. The initial suggestion of receptor heterogeneity in the rat aorta (i.e., into α_{1A} and α_{1B}) may, at least in part, reflect this limitation as the α_{1D}-adrenoceptor exhibits some characteristics of both α_{1A}- (e.g. high affinity for WB4101) and α_{1B}-subtypes (e.g. low affinity for 5-methylurapidil). More recently the α_{1D}-subtype was suggested to mediate predominantly the noradrenaline response in the rat aorta, although the presence of another receptor could not be excluded (Kenny *et al*., 1995).

The α_1-adrenoceptors in the rat aorta recently have been compared with those in the rabbit ear artery (Fagura *et al*., 1997). Consistent with the findings of the present study, it was shown that BMY 7378 gave shallow Schild plot slopes in the aorta and ear artery which are consistent with receptor heterogeneity. A two receptor model yielded values of 9.0 for BMY 7378 at the first site and 7.0 for the lower affinity site in the aorta (Fagura *et al*., 1997). In the ear artery the low Schild slope for BMY 7378 against phenylephrine gave a pA_2 of 6.1, inconsistent with a role for the α_{1D}-adrenoceptor subtype. Nevertheless, this is from a different species to the vessels used in the present work.

The evidence therefore supports the view that more than one subtype of α_1-adrenoceptor is responsible for mediating contractions to phenylephrine in these rat blood vessels. However, the identity of the second site is uncertain. Firstly, the second site identified by BMY 7378 appears to be an α_1-adrenoceptor, as prazosin had a high affinity and was competitive. Secondly, it seems unlikely that this site is similar to the α_{1A}-subtype because the α_{1A}-selective antagonist 5-methylurapidil has a lower affinity than would be expected if a component of the response were mediated through that subtype. In addition, the lower affinity of RS 17053 is not in keeping with the second site being a classical α_{1A}-adrenoceptor with high affinity for this antagonist (Marshall *et al*., 1996). Thirdly, the high affinity displayed by WB4101 against phenylephrine contractions in all three arteries makes the α_{1B}-adrenoceptor an unlikely candidate for the second subtype. Fourthly, there is the possibility that BMY 7378 is differentiating between populations of α_{1D}-adrenoceptors. A related phenomenon for the α_{1A}-adrenoceptor has been demonstrated where RS 17053 was shown to differentiate between subtypes of this receptor in the rat epididymal vas deferens compared with rat hepatic portal vein and human prostate (Marshall *et al*., 1996). Finally, the second site might not be similar to any of the presently identified three cloned subtypes. The present evidence does not allow any conclusion to be reached.

The heterogeneity of receptors described in these three rat blood vessels may partly depend on the use of phenylephrine as the agonist, as mentioned above. Functional differences in α_1-adrenoceptor populations have been described recently with different agonists (Fagura *et al*., 1997). It is known that imidazoline compounds show some preference for the α_{1A}-subtype (Minneman *et al*., 1994; Horie *et al*., 1995). The use of these and other agonists may help to clarify which α_1-subtype is present in addition to the α_{1D}-adrenoceptor.

In conclusion, the high affinity of prazosin for phenylephrine contractions of the rat thoracic aorta, mesenteric artery and pulmonary artery are in line with the responses being mediated via α_1-adrenoceptor(s). Results with BMY 7378 and a range of other antagonists show that it is mainly the α_{1D}-subtype that mediates the contractile responses to phenylephrine in all three blood vessels. In addition, a part of the α_1-adrenoceptor-mediated response appears to be through a receptor other than the α_{1D}-subtype with high affinity for BMY 7378.

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