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Pharmacological classification of α_1-adrenoceptors mediating contractions of rabbit isolated ear artery: comparison with rat isolated thoracic aorta

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1 The present study attempted to classify pharmacologically the α_1-adrenoceptor subtype(s) present in two isolated, vascular ring preparations, the rabbit ear artery and rat thoracic aorta.

2 In the ear artery, the agonist effects of phenylephrine were antagonized by 5-methyl urapidil ($pA_2 = 7.90$; Schild slope = 0.85) and BMY 7378 ($pA_2 = 6.11$; Schild slope = 0.80) but not in a simple competitive manner. The shallow Schild slopes are consistent with the activation of a heterogeneous receptor population. Indeed the 5-methyl urapidil data set could be fitted to a two-receptor model yielding a high antagonist affinity (pK_{BH}) estimate of 7.85 and a low affinity (pK_{BL}) estimate of 6.03.

3 The effects of clonidine in the ear artery were competitively antagonised by 5-methyl urapidil ($pK_B = 7.91$) and BMY 7378 ($pK_B = 5.53$). These data are consistent with contractions to clonidine being mediated by a single receptor subtype.

4 In the aorta, the effects of phenylephrine were antagonized by 5-methyl urapidil ($pA_2 = 7.95$; Schild slope = 1.11) and BMY 7378 ($pA_2 = 9.08$; Schild slope = 0.73). Neither data set was consistent with a simple competitive interaction. The BMY 7378 data suggested again, that phenylephrine was acting at a heterogeneous receptor population. Subsequent analysis by the two-receptor model yielded a high affinity (pK_{BH}) estimate of 8.95 and a low affinity (pK_{BL}) estimate of 7.00.

5 The alkylating agent, chloroethylclonidine (CEC) elicited concentration-dependent contractions in the ear artery with a potency ($p[A]_{50}$) of 5.57. Pretreatment of this tissue with CEC (5 μM, 30 min incubation) had no effect on subsequent responses to phenylephrine. In contrast, in the aorta, CEC demonstrated no agonism but pretreatment with this agent (5 μM, 15 min incubation) caused a rightward shift and depression of subsequent phenylephrine concentration-effect curves.

6 The affinity of clonidine in the rabbit ear artery ($pK_A = 6.17$) was found to be significantly different to its affinity in the rat thoracic aorta ($pK_A = 7.12$) suggesting that this agonist activates different α_1-adrenoceptor subtypes in the two tissues.

7 These results suggest that heterogeneous populations of α_1-adrenoceptors are present in both tissues. In the ear artery, the profile of antagonist and agonist activity is most consistent with α_{1A}-adrenoceptors being the predominant receptor subtype. The second receptor population does not appear to correspond to any of the recognized α_1-adrenoceptor subtypes. In the aorta α_{1D}-adrenoceptors appear to predominate, with α_{1A}-adrenoceptors being the most likely candidate for the second receptor population.

Keywords: Rabbit ear artery; rat thoracic aorta; α_1-adrenoceptors; receptor classification; BMY 7378; 5-methyl urapidil; chloroethylclonidine; phenylephrine; clonidine

Introduction

The subdivision of α_1-adrenoceptors into α_{1A} and α_{1B}-subtypes (Morrow & Creese, 1986; Han *et al*., 1987) was based initially on both functional and binding studies, in which a number of competitive α_1-adrenoceptor antagonists, such as 5-methyl urapidil, were shown to have higher affinity for the α_{1A}-subtype than the α_{1B}-subtype (Gross *et al*., 1988; Minneman *et al*., 1988). Further evidence to support this subdivision was provided by studies which demonstrated that chloroethylclonidine (CEC) selectively alkylated the α_{1B}-subtype (Han *et al*., 1987; Minneman *et al*., 1988).

More recently, with the advent of molecular biological techniques, the above classification scheme has been extended to include α_{1C} and α_{1D}-subtypes (Cotecchia *et al*., 1988; Schwinn *et al*., 1990; Lomasney *et al*., 1991; Perez *et al*., 1991). However, detailed pharmacological studies, with a number of selective ligands, have indicated that the classical α_{1A}-adrenoceptor and cloned α_{1C}-adrenoceptor are identical (Ford *et al*., 1994; Forray *et al*., 1994a; Blue *et al*., 1995). These proposals have recently been accepted by the IUPHAR committee on nomenclature for adrenoceptors (Hieble *et al*., 1995) and currently α_1-adrenoceptors are subdivided into α_{1A}, α_{1B} and α_{1D}-subtypes.

The subtype(s) of α_1-adrenoceptor present on the rat thoracic aorta has undergone extensive research. It has been variously classified as containing: a homogeneous population of α_{1B}-receptors (Han *et al*., 1990; Eltze & Boer, 1992; Testa *et al*., 1995); both α_{1B} and α_{1A}-subtypes (Piascik *et al*., 1991a); a population of α_1-adrenoceptors distinct from both these subtypes (Mir & Fozard, 1989; Aboud *et al*., 1993). Recently a selective α_{1D}-antagonist, BMY 7378, has been described and was shown to have a high affinity in the aorta (Saussy *et al*., 1994). Further work by Kenny *et al*. (1995) has shown that in the aorta the functional affinity of several antagonists, including BMY 7378, correlates well with binding affinities at cloned human α_{1D}-adrenoceptors but not with α_{1A} or α_{1B}-subtypes.

In contrast, the classification of α_1-adrenoceptor subtype(s) present in the rabbit ear artery has received much less attention. Some early work compared the α_1-adrenoceptor population present in this tissue with that of the rabbit thoracic aorta and suggested that they were different (Purdy *et al*., 1982; Purdy & Stupecky, 1984). However, at that time, selective α_{1A} and α_{1D}-adrenoceptor ligands such as those mentioned above were not available, precluding definitive classification of the α_1-adrenoceptors in the rabbit ear artery.

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The objective of this study was to re-examine the α_1-adrenoceptor subtype(s) present in the rabbit ear artery in light of the current knowledge of α_1-adrenoceptor subtypes and the selective ligands available. The study aimed to classify the α_1-subtype(s) present with the use of two selective α_1-adrenoceptor competitive antagonists, 5-methyl urapidil (α_{1A}-selective) and BMY 7378 (α_{1D}-selective), an irreversible antagonist, chloroethylclonidine (CEC) and two different chemical classes of agonist, the phenylethylamine, phenylephrine and the imidazoline, clonidine. In order to aid this process a comparison was made with the more extensively studied rat thoracic aorta.

Methods

Tissue preparation

Rabbit ear artery Male New Zealand White rabbits (2.0–3.5 kg) were killed by injection of sodium pentobarbitone (Euthatal, 100 mg kg^{-1}) into a marginal ear vein. The ears were removed and the central ear artery located at the base of the ears. A small section of the artery was cleared of connective tissue and an incision was made to allow the insertion of a nylon cannula (external diameter 0.75 mm). The cannula had been lightly scored with a scalpel blade to remove the vessel endothelium. The artery, mounted on the cannula, was dissected from the ear, cleared of connective tissue and cut into 5 mm rings.

Rat thoracic aorta Male Charles River rats (300–400 g) were killed by injection of sodium pentobarbitone (Euthatal, 200 mg kg^{-1}, containing 500 units of heparin) into the peritoneal cavity. The thoracic aorta was removed and after being cleared of adherent connective tissue, was cut into rings 3 mm in length. These were gently rubbed between the index finger and thumb ten times, to remove the endothelium.

Experimental protocols

General Tissues were mounted horizontally between two tungsten wire hooks in 10 ml organ baths. The lower hook was attached to a fixed support in the organ bath and the upper hook was connected to an Ormed Beam transducer, to record changes in isometric force. Tissues were bathed in Krebs solution of the following composition (mM): NaCl 117.56, KCl 5.36, NaH_2PO_4 1.15, $MgSO_4$ 1.18, glucose 11.10, $NaHCO_3$ 25.00, $CaCl_2$ 2.55. Cocaine (30 μM) and propranolol (1 μM) were also included in the Krebs solution, the former to inhibit the uptake$_1$ process and the latter to avoid any complications resulting from β-adrenoceptor activation. The Krebs solution used for the rat aorta experiments also contained indomethacin (2.8 μM) to prevent the formation of cyclo-oxygenase products. We have found that this treatment helps to reduce the spontaneous phasic contractions that are characteristic of this tissue (Van der Graaf *et al*., 1996). The tissues were maintained at 37°C and continually gassed with 5% CO_2 in oxygen.

An initial force of 1.0 g was applied to all tissues and this was reinstated over a 60 min equilibration period until it remained steady. During this time, the tissues were washed three times with fresh Krebs solution. All agonist concentration-effect (E/[A]) curves were constructed by cumulative additions of agonist at 0.5 $\log_{10}$ unit increments. A paired curve design was used with a period of 60 min between the first and second E/[A] curves. Changes in isometric force were recorded on Advance Bryans 3-channel chart recorders. Responses were expressed as percentage of the first curve maximum.

Rat aortic rings were contracted with a maximal concentration of phenylephrine (1 μM) followed by exposure to acetylcholine (10 μM) to test for the funtional state of the endothelium. Tissues were only included in this study if the vasorelaxation to acetylcholine was less than 10% of the phenylephrine contraction. Rabbit ear artery rings were not routinely tested for the absence of endothelium as previous experiments have shown the cannulation process to remove effectively the funtional endothelium from this tissue (O'Connor *et al*., 1990).

Competitive antagonist studies E/[A] curves to either phenylephrine or clonidine were constructed. After being washed, tissues were incubated with either 5-methyl urapidil (0.03–10 μM); BMY 7378 (3 nM–100 μM) or vehicle for a period of 60 min. Subsequently, a second E/[A] curve to either phenylephrine or clonidine was constructed. In the rat aorta, the antagonist effects were only studied against phenylephrine.

Effect of chloroethylclonidine on phenylephrine E/[A] curves An E/[A] curve to phenylephrine was constructed in each tissue. After being washed, ear artery and aortic rings were exposed to 5 μM chloroethylclonidine (CEC) or vehicle for a period of 30 or 15 min, respectively. Following removal of the irreversible antagonist by several exchanges of the Krebs solution, a second E/[A] curve to phenylephrine was constructed.

Estimation of the affinity and efficacy of clonidine The affinity and efficacy of clonidine were estimated in the rabbit ear artery by the receptor inactivation method (Furchgott, 1996). E/[A] curves to clonidine were constructed and after being washed, tissues were exposed to either the irreversible antagonist phenoxybenzamine (Pbz, 1–10 nM) or vehicle (ethanol) for a period of 15 min. Tissues were then washed several times with fresh Krebs solution over a period of 45 min to remove excess Pbz. A second E/[A] curve to clonidine was then constructed.

The affinity and efficacy of clonidine in the rat aorta were estimated by the comparative method (Barlow *et al*., 1967). E/[A] curves to phenylephrine were constructed. Tissues were then washed several times with fresh Krebs solution, over a period of 1 h, before E/[A] curves to clonidine were constructed.

Drugs and materials

(−)-Phenylephrine hydrochloride, (±)-propranolol hydrochloride and indomethacin were purchased from the Sigma Chemical Company. Cocaine hydrochloride was purchased from May & Baker. Clonidine hydrochloride, BMY 7378 dihydrochloride 8-[2-[4-(2-Methoxyphenyl)-1-piperozynl] ethyl]-8-azaspiro[4.5]decone-7,9-dione, 5-methyl urapidil hydrochloride, phenoxybenzamine hydrochloride and chloroethylclonidine hydrochloride were purchased from Research Biochemicals incorporated.

Indomethacin was dissolved in 10% w/v Na_2CO_3. Phenoxybenzamine was dissolved in ethanol and made up immediately before use. All other drugs were dissolved in distilled water.

Data analysis

Logistic curve fitting In control experiments designed to determine any time-dependent changes in tissue sensitivity and in competitive antagonist experiments, individual E/[A] curves were fitted to the following form of the Hill equation:

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

where β is the basal effect level and α, $[A]_{50}$ and n_H are the asymptote, location and slope parameters respectively (location parameters were estimated as negative logarithms, that is $p[A]_{50}$). In the present study, [A] refers to the agonist, phenylephrine or clonidine and β is the effect level produced by 5-methyl urapidil or BMY 7378.

Antagonist affinity estimation Concentration-ratios (r) were calculated from the $[A]_{50}$ estimates for each pair of curves (control and in the presence of antagonist, B) and fitted to equation 2 (Arunlakshana & Schild, 1959).

$$\log_{10}(r-1) = n\log_{10}[B] + pK_B \quad (2)$$

Each experiment was analysed in this manner and if the mean Schild slope parameter (n) was not significantly different from unity it was constrained to this value in each experiment, in order to estimate pK_B ($-\log_{10} K_B$). If n was significantly less than unity and a clear inflection was evident in the data, concentration-ratios were fitted to equation 3:

$$\log_{10}(r-1) = \log_{10}[B] - \log_{10}\left\{\frac{(\sigma_H K_{BH} + \sigma_L K_{BL})[B] + K_{BL}K_{BH}}{[B] + \sigma_H K_{BL} + \sigma_L K_{BH}}\right\} \quad (3)$$

This modified Schild equation (Lemoine & Kaumann, 1983) allows estimation of the affinities of an antagonist for two distinct receptor subtypes activated by an agonist, where r is as defined above, [B] is the concentration of antagonist, K_{BL} is the equilibrium dissociation constant of the antagonist for the low affinity receptor subtype; K_{BH} is the equilibrium dissociation constant of the antagonist for the high affinity receptor subtype; σ_L and σ_H are the fractional stimuli elicited by the agonist through the two receptor subtypes. The sum of the fractional stimuli was constrained to unity (i.e. $\sigma_L = 1-\sigma_H$) for fitting purposes.

In all other cases where n was significantly different from unity, data were fitted to equation 2 and a pA_2 value estimated.

Operational model-fitting In order to estimate agonist efficacies and affinities experimental E/[A] curve data were fitted with the operational model of agonism (Black & Leff, 1983; Black *et al*., 1985):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (4)$$

in which E and [A] are the pharmacological effect and the concentration of the agonist respectively; E_m is the maximum possible effect; K_A is the agonist dissociation constant (this was estimated as the negative logarithm, that is, pK_A); τ is the efficacy of the agonist (estimated as a logarithm) and n determines the steepness of the occupancy-effect relation.

The fitting procedures employed are described in detail elsewhere (Leff *et al*., 1990); only brief details are given here. In the case of the inactivation method, the E/[A] curve data for the agonist before and after receptor inactivation were fitted to equation 4 providing a common estimate of E_m, n and K_A, and a value of τ for each curve in the pair.

For the comparative method, the partial agonist E/[A] curve data were fitted to the operational model (equation 4) simultaneously to fitting the full agonist data to a Hill equation of the form:

$$E = \frac{E_m [A]^n}{[A]_{50}{}^n + [A]^n} \quad (5)$$

where E_m and n are as defined above and $[A]_{50}$ is the location parameter for the full agonist curve. (Location parameters were estimated as negative logarithms, that is $p[A]_{50}$). This allows K_A and τ for the partial agonist to be estimated as well as E_m and n from each pair of curves.

Data were fitted to the two-receptor model of Lemoine & Kaumann (1983) (Equation 3) by use of the data analysis package 'KaleidaGraph' on a Macintosh Centris 650 computer. All other data fitting procedures were carried out with the statistical package 'BMDP' on a Vax 11/780 mainframe computer. Results are expressed and plotted as mean values ± s.e.mean (vertical lines). In all figures the lines drawn through the data points are the results of either logistic curve fitting or operational model-fitting. Statistical differences were assessed by the use of Student's *t* test and considered significant at the level of $P<0.05$.

Results

Paired agonist control E/[A] curve data

For each different series of experiments the relevant paired agonist (phenylephrine or clonidine) control data were generated. Examples of these data are shown in Figures 1a, 8a and c. In general, these paired E/[A] curves were superimposable. However, in some instances small but significant changes in one or more of the E/[A] curve parameters were observed. The most notable change was an increase in the asymptote of the second clonidine E/[A] curve in the experiments designed to study the antagonist effects of 5-methyl urapidil in the rabbit ear artery (see below). Since all of these apparent time-dependent changes were variable and small, we chose not to correct for them in the subsequent analyses.

Estimation of antagonist affinities

Rabbit ear artery Exposure of this tissue to either 5-methyl urapidil or BMY 7378 resulted in small initial contractile responses. These responses usually faded to baseline during the 60 min incubation period but in some instances residual levels of induced tone were still present when agonist E/[A] curves were constructed (see Figures 1b, 3a and 4a). The cause of these contractions remains unclear, but they are unlikely to be related to the known activity of these agents on 5-hydroxytryptamine $(5\text{-HT})_{1A}$ receptors, since they occurred in a concentration range which does not correspond to their 5-HT_{1A} affinities (Grob *et al*., 1987; Goetz *et al*., 1995). 5-Methyl urapidil (0.03–3 μM) and BMY 7378 (3–100 μM) caused rightward displacements of phenylephrine E/[A] curves (Figures 1b and 2a). In the case of BMY 7378 the shifts observed were parallel in nature. In contrast, statistical analysis of the 5-methyl urapidil data indicated a significant decrease in the slope parameter of phenylephrine E/[A] curves in the presence of some concentrations (0.1, 0.3 and 1 μM) of this antagonist. Schild analysis of these data yielded pA_2 estimates of 7.90 ± 0.09 ($n=6$) and 6.11 ± 0.14 ($n=5$), respectively (Figures 1c and 2b). The Schild slope parameters were found to be significantly less than unity for both 5-methyl urapidil (0.85 ± 0.03, $n=6$) and BMY 7378 (0.80 ± 0.07, $n=5$). Data from the 5-methyl urapidil experiments were fitted to the two-receptor model (equation 3) of Lemoine & Kaumann (1983) and yielded antagonist affinity estimates, $pK_{BH} = 7.85 \pm 0.11$ and $pK_{BL} = 6.03 \pm 0.19$ and a fractional stimulus estimate (σ_H) of 0.98 ± 0.004 ($n=3$). An example of one such fit is given in Figure 1d. The BMY 7378 data could not be fitted to the two receptor model as no clear inflection was visible in the Schild plot data.

As stated above the paired clonidine control E/[A] data in the 5-methyl urapidil series of experiments indicated that there was a significant increase in the asymptote of the second curve (1st curve: $\alpha = 101.17 \pm 0.79$; 2nd curve: $\alpha = 121.81 \pm 6.67$; $n=6$). This apparent time-dependent change was also observed in the presence of 5-methyl urapidil. Taking this into account, both 5-methyl urapidil (0.03–3 μM) and BMY 7378 (10-100 μM) caused concentration related, parallel, rightward displacements of clonidine E/[A] curves (Figures 3a and 4a). Schild analysis of both data sets yielded slope parameters which were not significantly different from unity: 1.05 ± 0.10 ($n=6$) and 1.16 ± 0.07 ($n=5$) for 5-methyl urapidil and BMY 7378 respectively (Figures 3b and 4b). When Schild slopes were constrained to unity the estimated pK_B values were 7.91 ± 0.04 ($n=6$) and 5.53 ± 0.06 ($n=5$) for 5-methyl urapidil and BMY 7378, respectively.

Rat aorta Neither 5-methyl urapidil (0.1–10 μM) nor BMY 7378 (3–300 nM) produced a contractile response in this

tissue. Both antagonists caused concentration-related, parallel, rightward displacements of phenylephrine E/[A] curves (Figures 5a and 6a). Schild analysis of these data gave pA_2 estimates of 7.95 ± 0.05 and 9.08 ± 0.12 for 5-methyl urapidil and BMY 7378, respectively ($n = 5$, Figures 5b and 6b). The Schild slope parameters in both cases were found to be significantly different from unity, being 1.11 ± 0.03 for 5-methyl urapidil and 0.73 ± 0.08 for BMY 7378. Experimental data for BMY 7378 experiments were fitted to the two receptor model (equation 3) of Lemoine & Kaumann (1983) and gave antagonist affinity estimates of: $pK_{BH} = 8.95 \pm 0.11$ and $pK_{BL} = 7.00 \pm 0.18$ and a fractional stimulus estimate (σ_H) of 0.94 ± 0.02 ($n = 4$). An example of one such fit is given in Figure 6c.

Effects of CEC

The effect of CEC on phenylephrine E/[A] curves was assessed in both tissues. Incubation with and subsequent washout of CEC (5 μM for 30 min) caused no rightward shift or depression of phenylephrine E/[A] curves in the ear artery ($n = 5$,

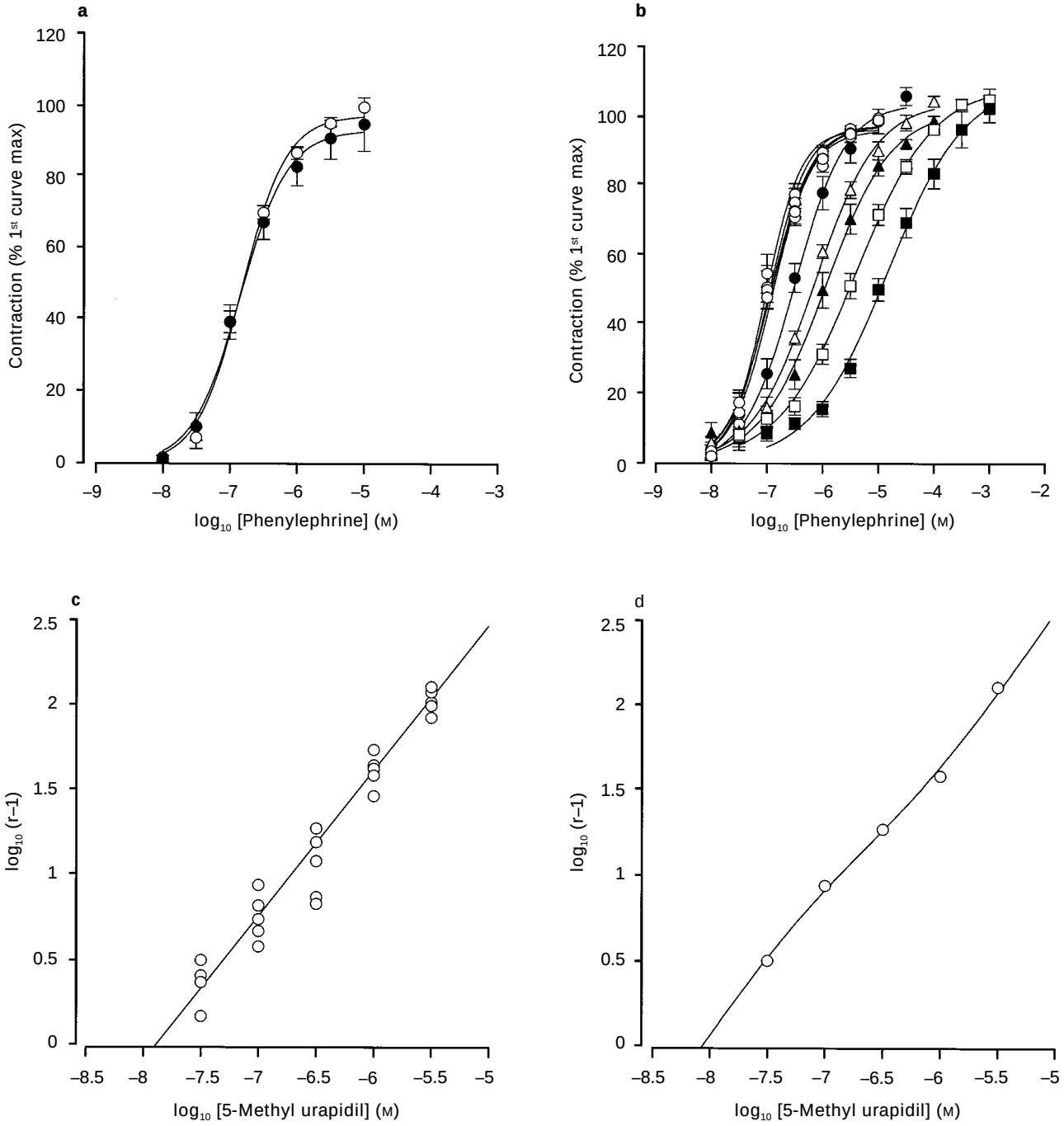

Figure 1 Antagonism of phenylephrine by 5-methyl urapidil in the rabbit ear artery. (a) Paired phenylephrine control E/[A] curves: 1st curve (○); 2nd curve (●); (b) E/[A] curves to phenylephrine in the presence of 5-methyl urapidil (μM): 0.03 (●), 0.1 (△), 0.3 (▲), 1 (□) and 3 (■). For ease of display the graph shows the first-curves performed to phenylephrine for each antagonist treatment using the same symbol (○). Points shown are the mean and vertical lines s.e.mean of 6 experiments. (c) Schild plot for 5-methyl urapidil versus phenylephrine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of pA_2 and slope and these were averaged to obtain values of 7.90 ± 0.09 and 0.85 ± 0.03, respectively ($n = 6$). (d) Schild plot for a single 5-methyl urapidil versus phenylephrine experiment. The line drawn through the points is the result of a two receptor model fitting procedure (see Methods), yielding antagonist affinity estimations of $pK_H = 8.03$ and $pK_L = 6.07$; and fractional stimuli of $\sigma_H = 0.97$ and $\sigma_L = 0.03$.

Figure 7a). However, when tissues were exposed to CEC (5 μM) a substantial contractile response was elicited ($47.86 \pm 7.67\%$ of the phenylephrine maximum, $n = 5$). In a separate series of experiments E/[A] curves to CEC were constructed following the generation of phenylephrine E/[A] curve data. Analysis of these data gave the following curve parameters for CEC: $p[A]_{50} = 5.57 \pm 0.11$, $n_H = 1.59 \pm 0.13$ and $\alpha = 77.91 \pm 2.18\%$ ($n = 4$, Figure 7b). The addition of prazosin (30 nM) at the 'top' of the CEC E/[A] curves reversed the contractile responses (data not shown).

CEC (5 μM for 15 min) produced a 4 fold rightward shift and a 20% depression of the phenylephrine E/[A] curves in the aorta ($n = 4$, Figure 7c). These effects could be protected against by pretreatment with 30 nM prazosin (data not shown). In an initial 'dose-ranging' study with CEC, higher con-

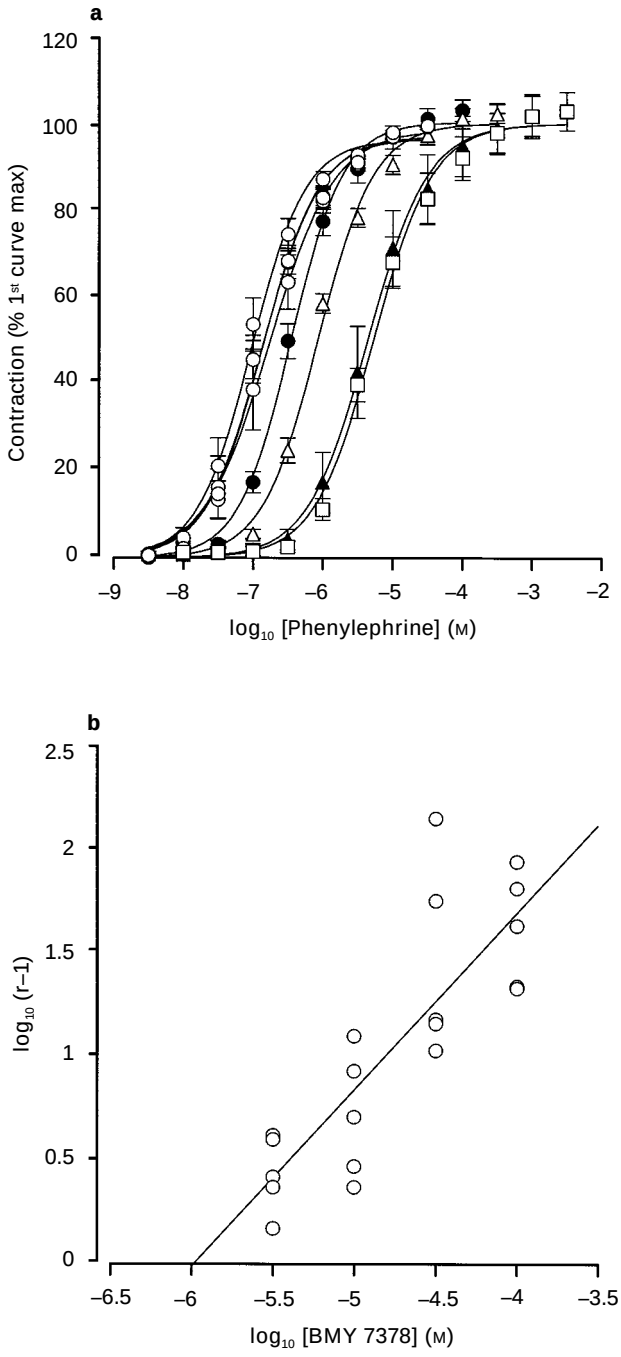

Figure 2 Antagonism of phenylephrine by BMY 7378 in the rabbit ear artery: (a) E/[A] curves to phenylephrine in presence of BMY 7378 (μM): 3 (●), 10 (△), 30 (▲) and 100 (□). For ease of display the graph shows the first-curves performed to phenylephrine for each antagonist treatment using the same symbol (○). The graph represents the mean and vertical lines s.e.mean of 5 experiments. (b) Schild plot for BMY 7378 versus phenylephrine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of pA_2 and slope and these were averaged to obtain values of 6.11 ± 0.14 and 0.80 ± 0.07, respectively ($n = 5$).

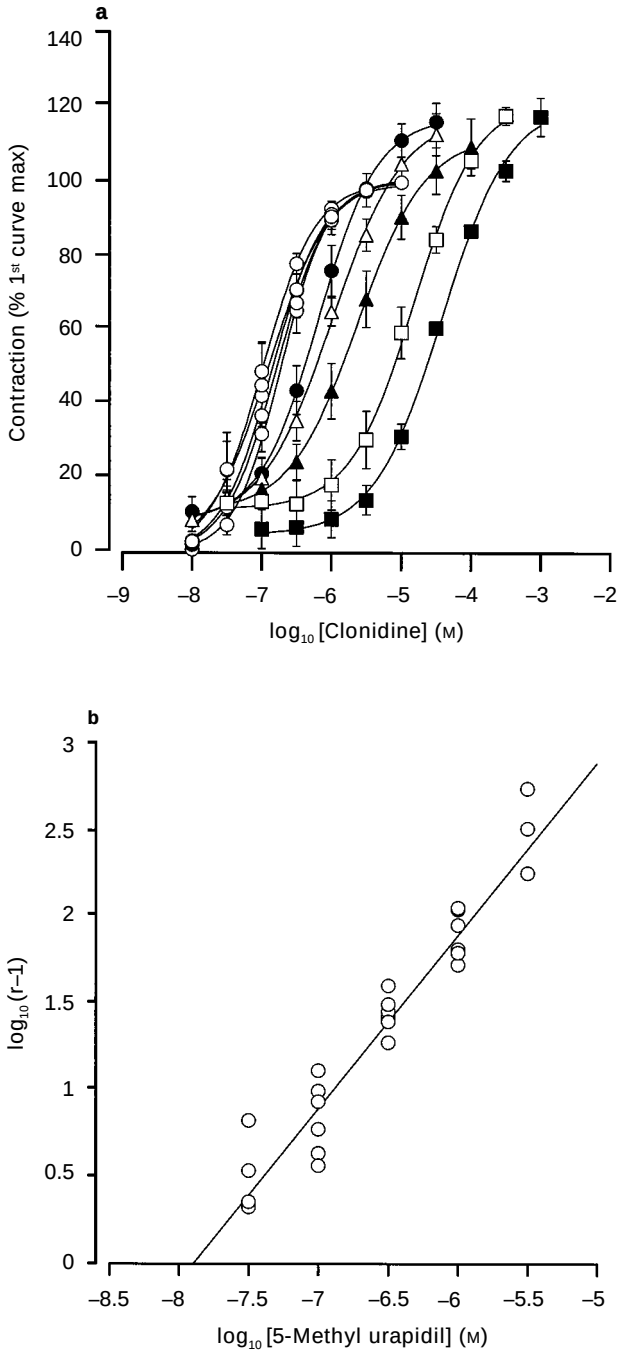

Figure 3 Antagonism of clonidine by 5-methyl urapidil in the rabbit ear artery: (a) E/[A] curves to clonidine in the presence of 5-methyl urapidil (μM): 0.03 (●), 0.1 (△), 0.3 (▲), 1 (□) and 3 (■). For ease of display the graph shows the first-curves performed to clonidine for each antagonist treatment using the same symbol (○). Points shown are the mean and vertical lines s.e.mean of 3–6 experiments. (b) Schild plot for 5-methyl urapidil versus clonidine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of affinity (pK_B) and slope which were subsequently averaged. The slope parameter was found not to be significantly different from unity (1.05 ± 0.10), and this was subsequently constrained to unity to estimate a pK_B of 7.91 ± 0.04 ($n = 6$).

centrations of this agent (30 μM and 100 μM) caused almost complete depression of the phenylephrine E/[A] curve but appeared to unmask a second CEC-insensitive phenylephrine curve. The potency ($p[A]_{50}$) of this second phase was approximately 5.0 with a maximum response in the region of 60% of the control response. Addition of prazosin (30 nM) at the 'top' of these curves reversed the contractile responses (data not shown).

Estimation of the affinity and efficacy of clonidine

Rabbit ear artery Phenoxybenzamine (Pbz, 1 – 10 nM) caused a 7 fold rightward shift and 60% depression of clonidine E/[A] curves ($n=5$, Figure 8b). Analysis of the data as described in the methods section gave average affinity (pK_A) and efficacy (τ) estimates of 6.17 ± 0.12 and 5.50 (log $\tau = 0.74 \pm 0.14$) ($n=5$), respectively.

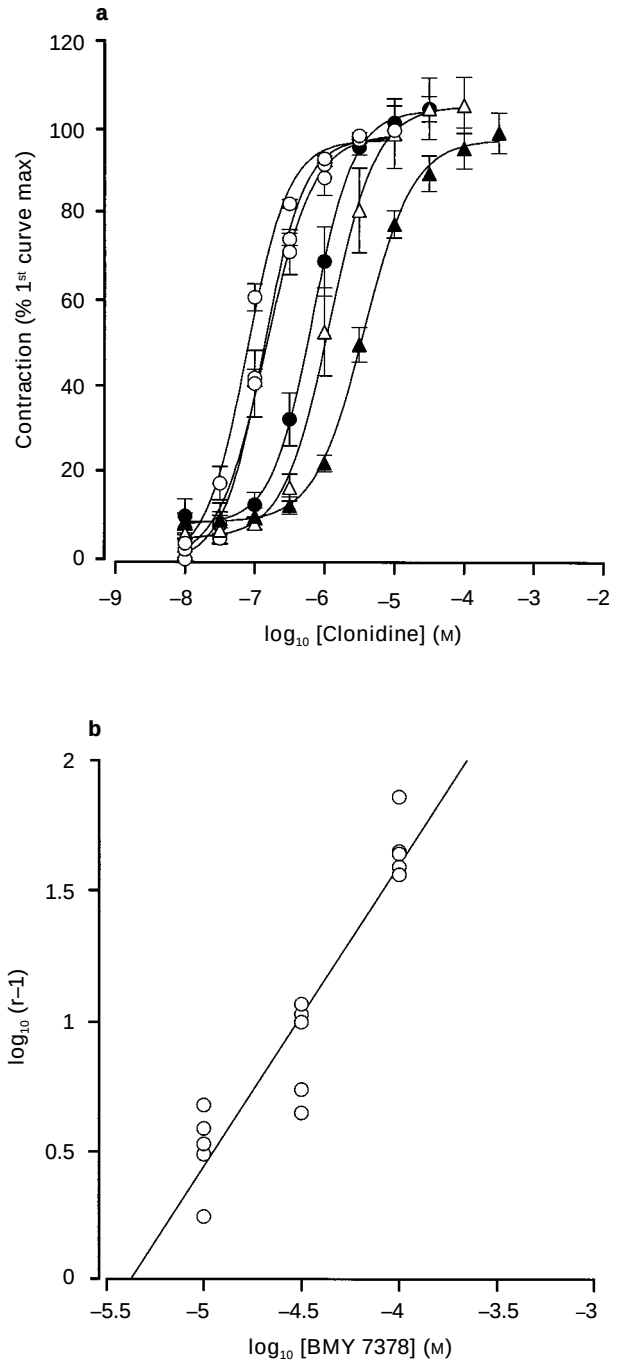

Figure 4 Antagonism of clonidine by BMY 7378 in the rabbit ear artery: (a) E/[A] curves to clonidine in the presence of BMY 7378 (μM): 10 (●), 30 (△) and 100 (▲). For ease of display the graph shows the first-curves performed to clonidine for each antagonist treatment using the same symbol (○). Points shown are the mean and vertical lines s.e.mean of 5 experiments. (b) Schild plot for BMY 7378 versus clonidine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of affinity (pK_B) and slope which were subsequently averaged. The slope parameter was found not to be significantly different from unity (1.16 ± 0.07), and this was subsequently constrained to unity to estimate a pK_B of 5.53 ± 0.06 ($n=5$).

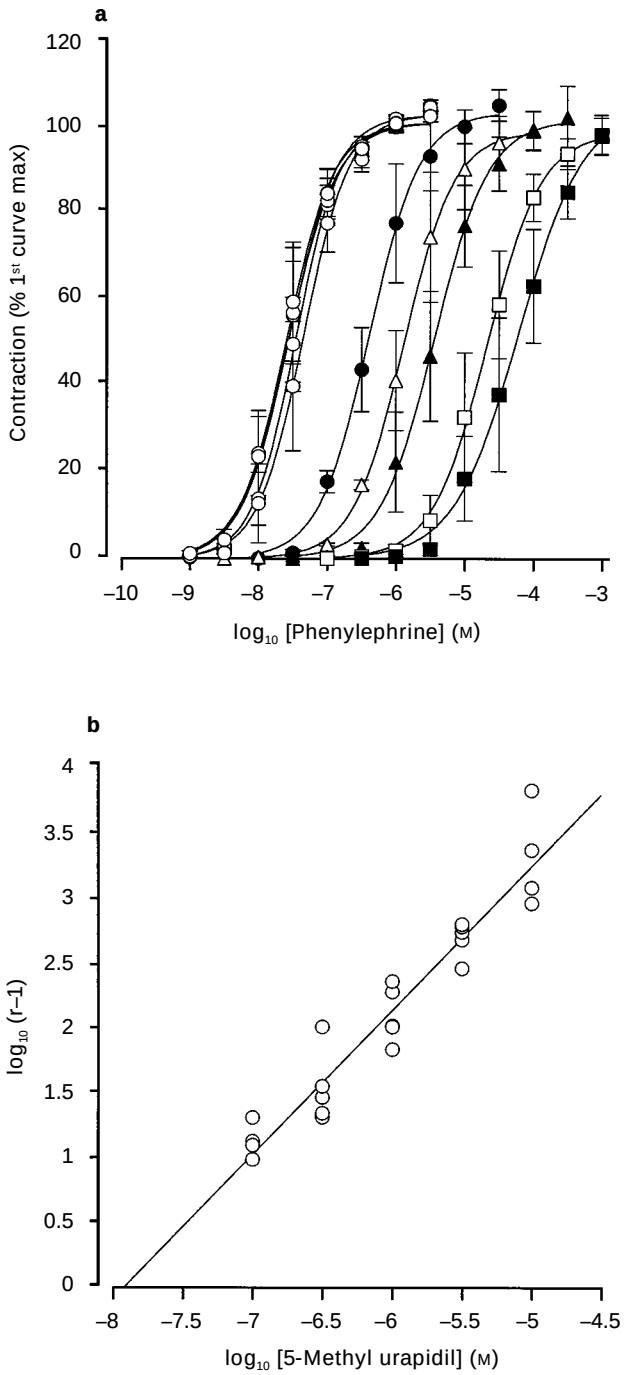

Figure 5 Antagonism of phenylephrine by 5-methyl urapidil in the rat thoracic aorta: (a) E/[A] curves to phenylephrine in the presence of 5-methyl urapidil (μM): 0.1 (●), 0.3 (△), 1 (▲), 3 (□) and 10 (■). For ease of display the graph shows the first-curves performed to phenylephrine for each antagonist treatment using the same symbol (○). Points shown are the means and vertical lines s.e.mean of 5 experiments. (b) Schild plot for 5-methyl urapidil versus phenylephrine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of pA_2 and slope and these were averaged to obtain values of 7.95 ± 0.05 and 1.11 ± 0.03, respectively ($n=5$).

Rat aorta Clonidine produced a partial agonist response ($\alpha = 42.73 \pm 7.97\%$) compared to phenylephrine with $p[A]_{50} = 6.97 \pm 0.08$ and $n_H = 1.41 \pm 0.27$ ($n = 5$, Figure 8d). Analysis of the data as described in the methods section gave the following affinity and efficacy estimates ($n = 5$): $pK_A = 7.12 \pm 0.13$; $\tau = 0.91$ ($\log \tau = -0.04 \pm 0.08$), respectively.

The affinity estimates for clonidine in the two tissues were significantly different.

Discussion

The aim of this study was to classify the α_1-adrenoceptor population eliciting contractile responses in the rabbit isolated ear artery and to compare it with that mediating the same response in the rat isolated aorta. To this end we chose to use a variety of pharmacological tools and procedures. Thus, in both tissues the affinities of the competitive antagonists 5-methyl urapidil and BMY 7378 were estimated, the effects of the putative irreversible antagonist CEC on responses to phenylephrine were studied and the affinity (and efficacy) of the agonist clonidine was estimated. The results of each of these studies are discussed below (see Table 1 for summary).

Competitive antagonist studies

5-Methyl urapidil has been widely used as a pharmacological tool for the classification of α_1-adrenoceptors. Its affinity for the α_{1A}-subtype (pK_B approximately 8.5; see Table 2) is ap-

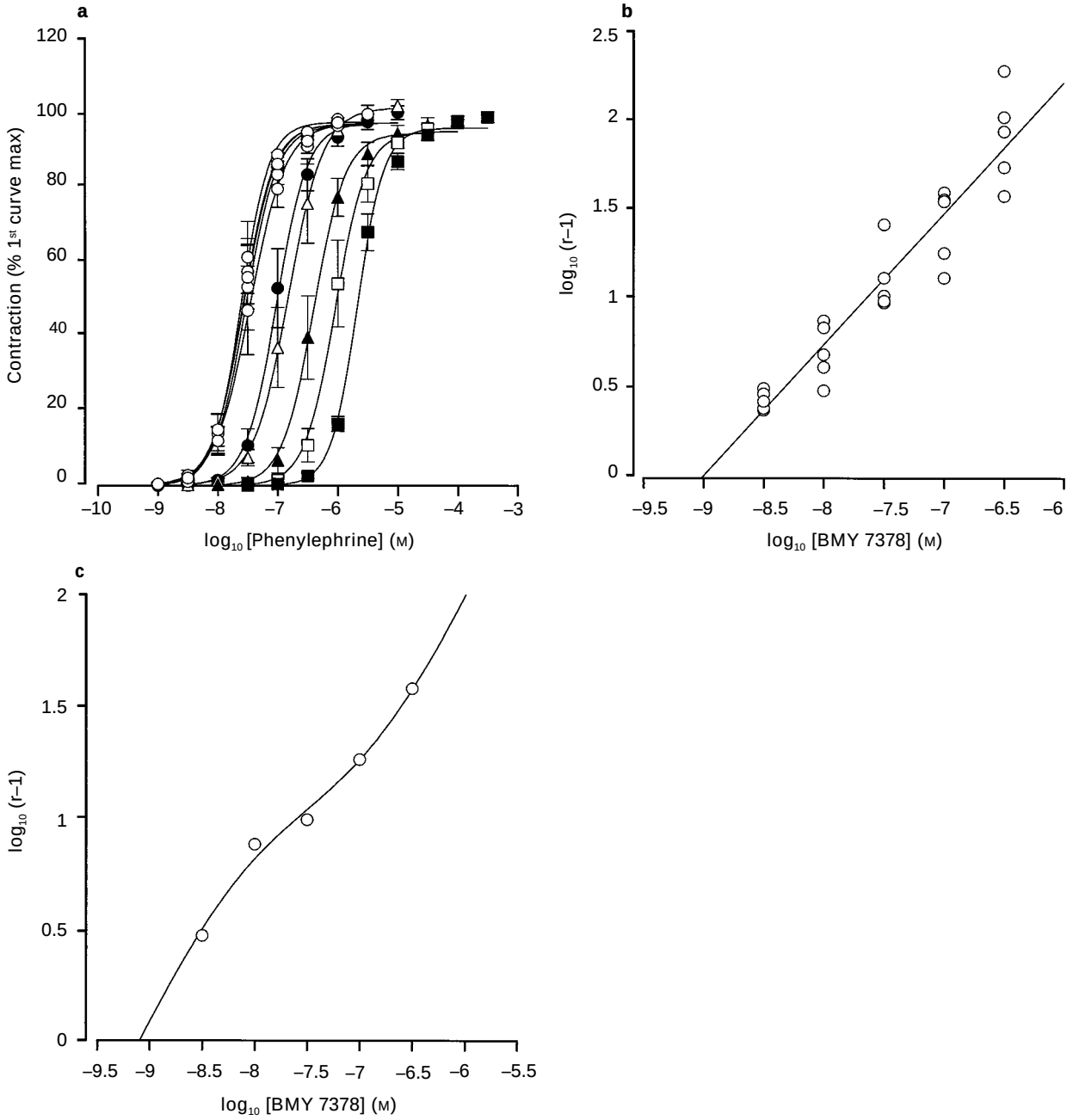

Figure 6 Antagonism of phenylephrine by BMY 7378 in the rat thoracic aorta. (a) E/[A] curves to phenylephrine in the presence of BMY 7378 (nM): 3 (●), 10 (△), 30 (▲), 100 (□) and 300 (■). For ease of display the graph shows the first-curves performed to phenylephrine for each antagonist treatment using the same symbol (○). Points shown are the mean and vertical lines s.e.mean of 5 experiments. (b) Schild plot for BMY 7378 versus phenylephrine. For display purposes all the data are presented on a single plot. However, in practice each experiment yielded a single estimate of pA_2 and slope and these were averaged to obtain values of 9.08 ± 0.12 and 0.73 ± 0.08, respectively ($n = 5$). (c) Schild plot for a single BMY 7378 versus phenylephrine experiment. The line drawn through the points is the result of a two receptor model fitting procedure (see Methods), yielding antagonist affinity estimations of $pK_H = 9.17$ and $pK_L = 6.87$; and fractional stimuli of $\sigma_H = 0.92$ and $\sigma_L = 0.08$.

proximately 10 fold greater than for the α_{1D}-subtype (pK_B approximately 7.6; see Table 2) and some 100 fold greater than for the α_{1B}-subtype (pK_B approximately 6.7; see Table 2). In this study, the estimated affinities of 5-methyl urapidil in both the ear artery ($pA_2 = 7.90$ (phenylephrine); $pK_B = 7.91$ (clonidine)) and the aorta ($pA_2 = 7.95$ (phenylephrine)) were similar and close to the value expected for an interaction at α_{1D}-adrenoceptors. However, in only one case, namely the interaction of clonidine and 5-methyl urapidil in the rabbit ear artery, did the data conform to simple competition. When phenylephrine was employed as the agonist the resultant Schild slope parameter was significantly less than unity in the ear artery and significantly greater than unity in the aorta. Shallow Schild slopes are often the result of an agonist eliciting its effects by interacting with two (or more) receptor types for which the antagonist under study has differential affinities (Kenakin, 1982). We therefore attempted to fit the former data to an appropriate two-receptor model (Lemoine & Kaumann, 1983) and found that in most instances an acceptable fit was obtained (see Figure 1d). These analyses yielded two affinity estimates for 5-methyl urapidil, a high affinity value (pK_{BH}) of 7.85 and a low affinity value (pK_{BL}) of 6.03. They also indicated that the effects of phenylephrine were predominately ($\sigma_H = 0.98$) mediated through the receptor type for which 5-methyl urapidil had high affinity. The above pK_H estimate from the phenylephrine analysis and the pK_B estimate of 7.91 obtained when clonidine was used are similar. This suggests that the latter agonist elicits contractions of the ear artery by interacting with a single receptor type, the same type that predominately mediates the response to phenylephrine.

As stated above, the interaction of phenylephrine and 5-methyl urapidil in the aorta also did not conform to simple competition. In this case, the Schild slope (1.11) was significantly steeper than unity. The reasons for this deviation

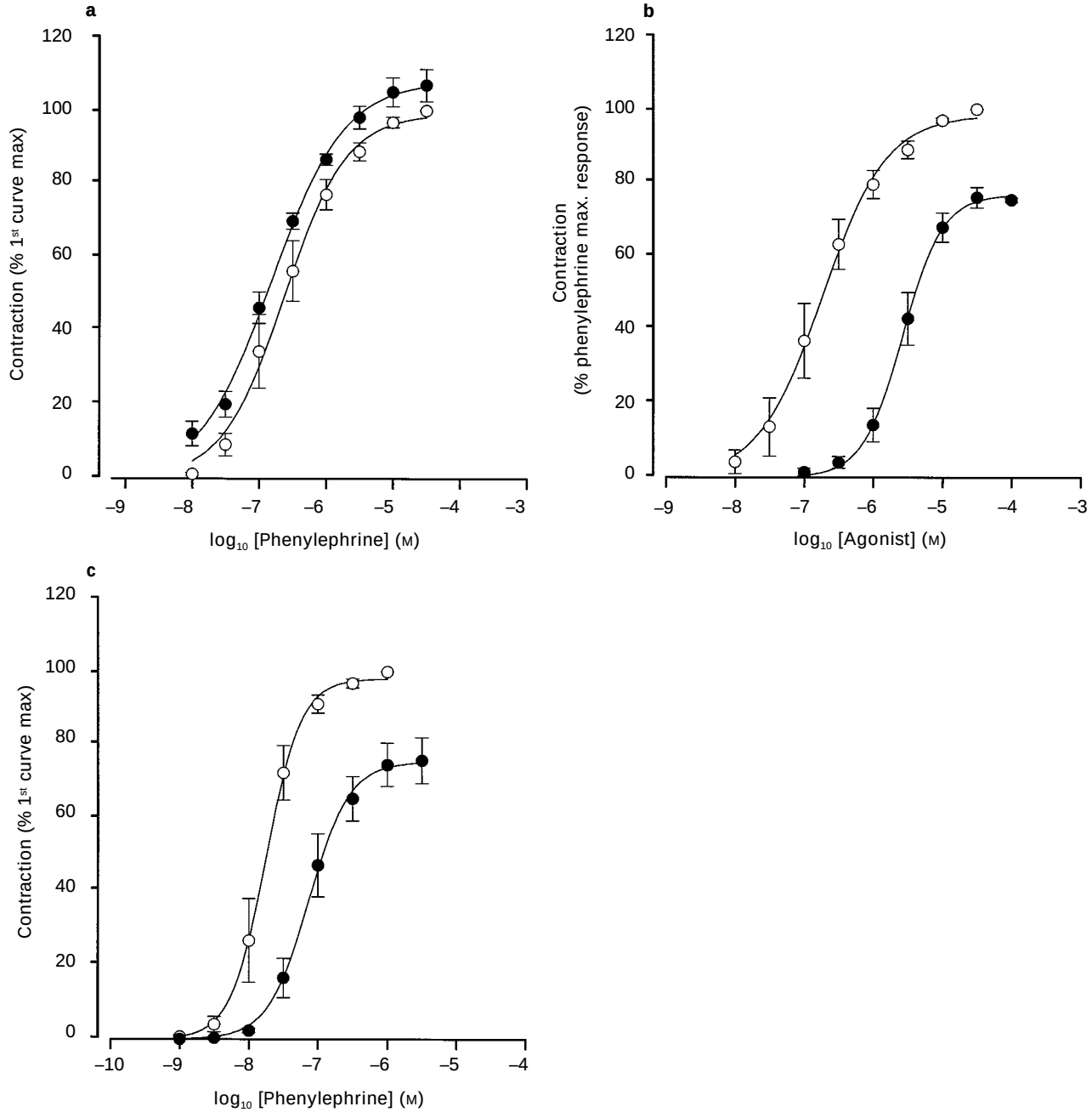

Figure 7 Effects of chloroethylclonidine (CEC): (a) phenylephrine E/[A] curves were obtained before (○) and after 30 min exposure to 5 μM (●) CEC in the rabbit ear artery. (b) E/[A] curves to phenylephrine (○) and CEC (●) in the rabbit ear artery. (c) Phenylephrine E/[A] curves were obtained before (○) and after 15 min exposure to 5 μM (●) CEC in the rat thoracic aorta. Points shown are the mean and vertical lines s.e.mean of 4–5 experiments.

from competition are unclear. Nevertheless, the estimated pA_2 value of 7.95 is consistent with other values obtained in this tissue (Aboud *et al*., 1993; Saussy *et al*., 1994; Kenny *et al*., 1995).

In summary, taken in isolation, the data obtained with 5-methyl urapidil suggest that in the rabbit ear artery and the rat aorta, agonist-induced contractions are predominately mediated by activation of α_{1D}-adrenoceptors. However, in the ear artery the presence of a second receptor population was revealed when phenylephrine was employed as the agonist. The estimated affinity of 5-methyl urapidil for this receptor type ($pK_{BL} = 6.03$) is not consistent with its affinity for any of the recognised α_1-adrenoceptor subtypes (see Table 2). However, it may be similar to the '5-methyl urapidil low affinity receptor' recently obtained in the isolated perfused kidney of the rat (Blue *et al*., 1995).

The classification proposed above becomes more difficult to sustain when the data obtained with BMY 7378 are taken into account. BMY 7378 is a selective α_{1D}-adrenoceptor antagonist exhibiting 100 fold selectivity over α_{1A} and α_{1B}-subtypes (Saussy *et al*., 1994; Goetz *et al*., 1995; Kenny *et al*., 1995) (see Table 2). In contrast to 5-methyl urapidil, Schild analyses with this antagonist yielded quite different affinity values in the two vascular preparations. In the ear artery, affinity estimates of 6.11 (pA_2) and 5.53 (pK_B) were obtained, with phenylephrine and clonidine as the respective agonists. In the aorta the in-

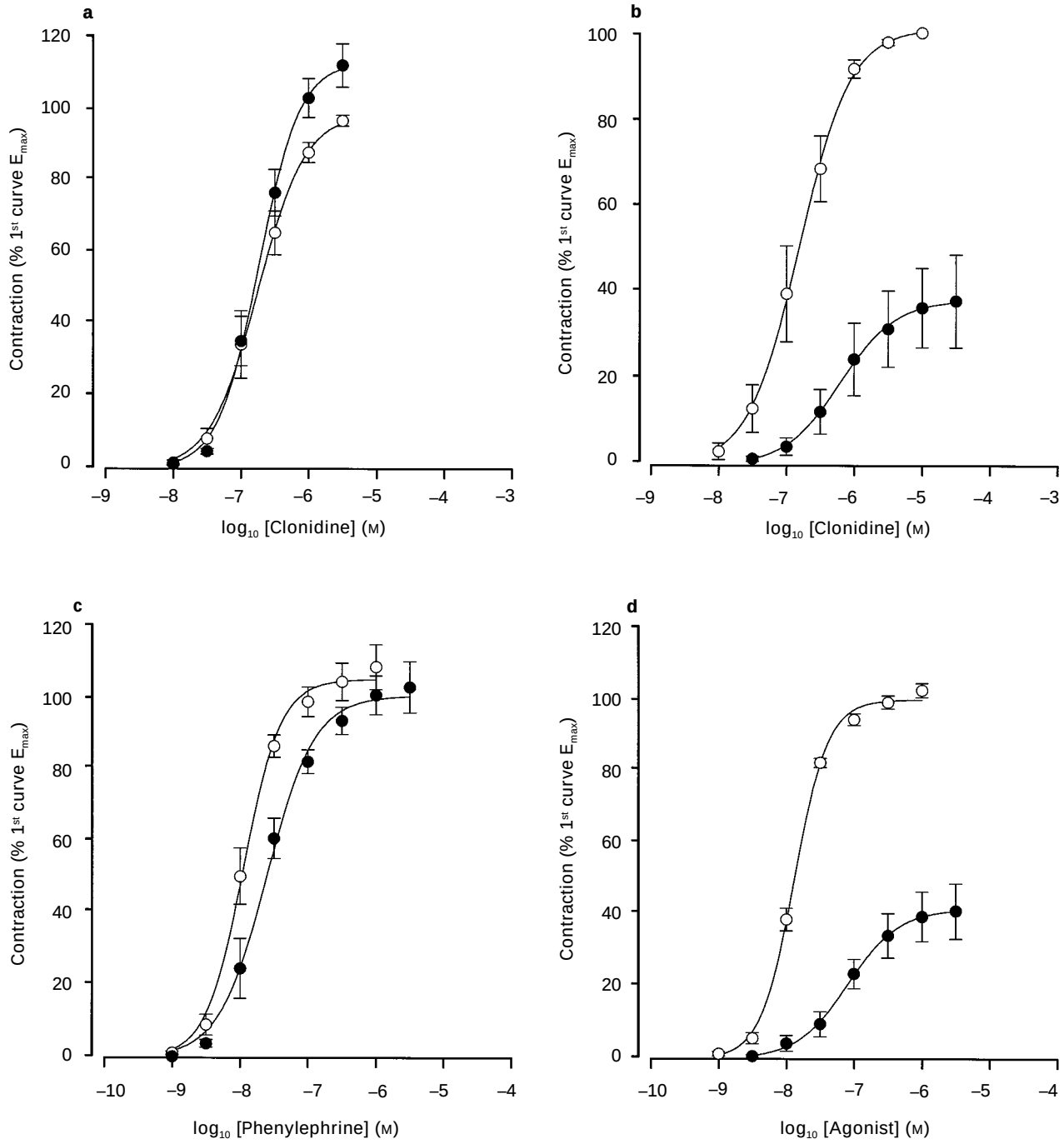

Figure 8 Estimation of the affinity and efficacy of clonidine by Operational model-fittting. Receptor inactivation analysis in the rabbit ear artery: (a) paired clonidine control E/[A] curves: 1st curve (○); 2nd curve (●); (b) clonidine E/[A] curves were obtained before (○) and after 15 min exposure to phenoxybenzamine (1–10 nM) (●). Comparative analysis in the rat aorta: (c) paired phenylephrine control E/[A] curves: 1st curve (○); 2nd curve (●); (d) E/[A] curves to phenylephrine (○) and clonidine (●). Points shown are the mean and vertical lines s.e.mean of 5 experiments. The affinity (pK_A) estimates were 6.17 ± 0.12 and 7.12 ± 0.13, in the ear artery and aorta, respectively ($n = 5$).

Table 1 Summary of analysis of α_1-adrenoceptor antagonists and agonists in the rabbit ear artery and rat thoracic aorta

Antagonist	*Agonist*	*Rabbit ear artery*	*Rat aorta*
5-Methyl urapidil	Phenylephrine	$pA_2 = 7.90 \pm 0.09$, slope $= 0.85 \pm 0.03$ ($pK_{BH} = 7.85 \pm 0.11$, $pK_{BL} = 6.03 \pm 0.19$)*	$pA_2 = 7.95 \pm 0.05$, slope $= 1.11 \pm 0.03$
	Clonidine	$pK_B = 7.91 \pm 0.04$	NT
BMY 7378	Phenylephrine	$pA_2 = 6.11 \pm 0.14$, slope $= 0.80 \pm 0.07$	$pA_2 = 9.08 \pm 0.12$, slope $= 0.73 \pm 0.08$ ($pK_{BH} = 8.95 \pm 0.11$, $pK_{BL} = 7.00 \pm 0.18$)*
	Clonidine	$pK_B = 5.53 \pm 0.06$	NT
CEC	Phenylephrine	Insensitive#	Sensitive
Agonist			
Clonidine		$pK_A = 6.17 \pm 0.12$	$pK_A = 7.12 \pm 0.13$

*Antagonist affinity estimations resulting from a two-receptor, model fitting procedure (see Methods). #CEC is an agonist ($p[A]_{50} = 5.57$). Results are presented as mean ± s.e.mean of 3–6 experiments. NT = not tested.

Table 2 Summary of published affinity data for α_1-adrenoceptor antagonists and agonists used in this study

	α_{1A}	α_{1B}	α_{1D}
5-Methyl urapidil[a]	8.5	6.7	7.6
BMY 7378[b]	6.6	7.2	9.4
CEC sensitivity[c]	±*	++++	+++
Clonidine[d]	5.9	6.7	ND

[a] pK_i values determined in human α_1-subtype clones with both [^{3}H]-prazosin and [^{125}I]-HEAT as ligands. Values represent mean of data taken from Schwinn *et al.* (1995); Forray *et al.* (1994a); Kenny *et al.* (1995). [b] pK_i values determined in human α_1-subtype clones with [^{125}I]-HEAT as the ligand (Goetz *et al.*, 1995). [c] Data taken from Hieble *et al.* (1995). *Sensitivity to CEC may be species-dependent (Schwinn *et al.*, 1990; Garcia-Sainz *et al.*, 1992; Forray *et al.*, 1994b). [d] Functional affinity (pA_2) estimates were determined in rat vas deferens and rat spleen (Ruffolo *et al.*, 1980) containing α_{1A} and α_{1B}-adrenoceptors, respectively. ND = not determined.

teraction of phenylephrine and BMY 7378 yielded a pA_2 of 9.08. As was the case with 5-methyl urapidil, the only interaction that conformed to simple competition was that with clonidine in the ear artery. The interactions with phenylephrine in both tissues were consistent with this agonist activating two distinct receptor populations (Schild slope parameters of 0.80 and 0.73 in the ear artery and aorta, respectively). In the aorta the data could be fitted to the model of Lemoine & Kaumann (1983) (see Figure 6c) yielding a pK_{BH} value of 8.95 and a pK_{BL} value of 7.00. The estimated σ_H value of 0.94 indicated that the effect of phenylephrine was predominantly mediated by the receptor type for which BMY 7378 has higher affinity. The corresponding data in the ear artery were not amenable to such analysis. However, the failure of 100 μM BMY 7378 to produce a significantly greater shift than 30 μM BMY 7378 (see Figure 2a) is consistent with phenylephrine interacting with two distinct receptor populations, at one of which BMY 7378 has very low affinity.

The relative similarity of the affinity estimates obtained in the ear artery when phenylephrine ($pA_2 = 6.11$) and clonidine ($pK_B = 5.53$) are used, supports the data obtained previously with 5-methyl urapidil, indicating that clonidine activates a single receptor population in this tissue whereas phenylephrine activates two distinct receptor populations.

In summary, the data obtained with BMY 7378 provide further evidence to support the presence of α_{1D}-adrenoceptors in the aorta, the pK_{BH} value of 8.95 being consistent with data obtained for this receptor subtype previously (Saussy *et al.*, 1994; Kenny *et al.*, 1995). The pK_{BL} value of 7.0 obtained in this analysis could result from an interaction with either α_{1A} or α_{1B}-adrenoceptors (see Table 2). These data which indicate a heterogeneous receptor population in this tissue are in accord with previous studies (Van der Graaf *et al.*, 1994; 1996).

In the ear artery the affinity estimates of 5.53 and 6.11 obtained against clonidine and phenylephrine do not correspond closely with the affinity of BMY 7378 at any of the recognized α_1-adrenoceptor subtypes (see Table 2). However, they are most similar to the values found at α_{1A}-adrenoceptors. In addition, the data obtained with phenylephrine suggest that BMY 7378 interacts with a second receptor population in the ear artery with very low affinity. This receptor type does not appear to correspond to any of the recognized α_1-adrenoceptor subtypes (see Table 2).

The effects of CEC

The various α_1-adrenoceptor subtypes exhibit different sensitivities to the alkylating effects of CEC (Perez *et al.*, 1991; Forray *et al.*, 1994a; Schwinn *et al.*, 1995). α_{1B}-Adrenoceptor and α_{1D}-adrenoceptor subtypes are both effectively inactivated by CEC-treatment, the former subtype being relatively more sensitive (Perez *et al.*, 1991). In contrast, α_{1A}-adrenoceptors are considerably more resistant to this alkylating agent, although species-dependent variations in sensitivity have been obtained (Schwinn *et al.*, 1990; Garcia-Sainz *et al.*, 1992; Forray *et al.*, 1994b) (see Table 2). In the present study, CEC showed strikingly different actions in the two vascular preparations. In the ear artery, it was an agonist with a potency ($p[A]_{50}$) value of 5.57. These contractions, which have been described previously (Tian *et al.*, 1990; Oriowo *et al.*, 1992), were prazosin-sensitive indicating that they were mediated by α_1-adrenoceptors. However, it was still conceivable that underlying these agonist responses, CEC was inactivating one of the two α_1-adrenoceptor populations present in this tissue. We, therefore, studied the effects of a 30 min exposure to 5 μM CEC on subsequent responses to phenylephrine. Such treatment had no effect on phenylephrine E/[A] curve parameters (see Figure 7a), suggesting that the α_1-adrenoceptor subtypes present in the ear artery are insensitive to CEC-inactivation. In contrast, in the aorta CEC exhibited no agonism. Furthermore, in this tissue a 15 min exposure to 5 μM CEC caused a significant rightward shift and depression of the phenylephrine E/[A] curves (see Figure 7c), the classic profile of irreversible antagonism. Interestingly, some of the tissues exposed to CEC produced a marked biphasic phenylephrine E/[A] curve, a phenomenon that has been observed previously (Tian *et al.*, 1990; Piascik *et al.*, 1991b). The high potency phase of these curves presumably corresponds to that mediated by α_{1D}-adrenoceptors, the low potency phase may correspond to that mediated by the second receptor population, revealed previously by BMY 7378. Increasing the concentration of CEC further (30 μM and 100 μM) resulted in complete abolition of the high potency phase of phenylephrine's agonism, leaving the lower potency relatively unaffected (data not shown).

In summary, CEC behaved as an agonist in the ear artery and as an irreversible antagonist in the aorta. In the former tissue its failure to effect responses to phenylephrine suggests that α_{1A}-adrenoceptors predominate. In contrast, in the aorta, phenylephrine-induced contractions were sensitive to CEC-inactivation lending further support to the contention that α_{1D}-

adrenoceptors represent the major subtype present in this tissue. The second phase of phenylephrine's agonism, revealed by CEC-treatment, was apparently insensitive to inactivation by this agent consistent with it being mediated by α_{1A}-adrenoceptors.

Estimation of the affinity of clonidine

The competitive antagonist studies discussed above have indicated that clonidine, unlike phenylephrine, mediates its contractile responses in the ear artery through a single receptor subtype, the same type that predominantly mediates the response to phenylephrine. The affinity (pK_A) of clonidine at this receptor subtype was estimated to be 6.17. This affinity value is consistent with other values obtained in the ear artery (Purdy & Stupecky, 1984; Oriowo *et al*., 1991) and is similar to that found in the rat vas deferens (Ruffolo *et al*., 1980b; see Table 2), a tissue shown to contain α_{1A}-adrenoceptors (Eltze & Boer, 1992; Aboud *et al*., 1993; Burt *et al*., 1995). In comparison affinity of clonidine at α_{1B}-adrenoceptors in the rat spleen is significantly higher ($pK_A = 6.73$; Ruffolo *et al*., 1980b; see Table 2). To our knowledge an estimate of the affinity of clonidine in a tissue or cell system containing a homogeneous population of α_{1D}-adrenoceptors has not been made. However, the similarity of our estimate in the ear artery to that in the vas deferens suggests that the effects of clonidine are mediated by α_{1A}-adrenoceptors.

The affinity (pK_A) estimate obtained in the aorta (7.12) was significantly higher than that in the ear artery, suggesting that different receptors mediate the effects of clonidine in the two tissues. However, it should be noted that in performing the comparative analysis in the aorta, we made the assumption that clonidine and phenylephrine activate the same, single receptor population. As it appears that under control conditions phenylephrine predominantly activates α_{1D}-adrenoceptors ($\sigma_H = 0.94$) the implicit assumption is that clonidine also acts at α_{1D}-adrenoceptors. The affinity (pK_A) estimate obtained (7.12) lends support to this suggestion as this value is considerably higher than that obtained in the rat vas deferens (6.17; Ruffolo *et al*., 1980b; see Table 2) and slightly higher than that obtained in the rat spleen (6.73; Ruffolo *et al*., 1980b; see Table 2). However, it is somewhat lower than that obtained by other workers in this tissue using similar methodology (mean $pK_A = 7.30$; Ruffolo *et al*., 1979; 1980a).

In summary, the data obtained with clonidine provide further evidence to suggest that the predominant α_1-adrenoceptor in the ear artery is of the α_{1A}-subtype and in the aorta is of the α_{1D}-subtype.

Conclusions

The above data suggest that heterogeneous populations of α_1-adrenoceptors exist in both the rabbit ear artery and the rat thoracic aorta. The moderate affinity of 5-methyl urapidil and high affinity of BMY 7378 suggest that CEC-sensitive phenylephrine contractions are predominantly mediated by α_{1D}-adrenoceptors in the aorta. The CEC-insensitive component of the phenylephrine response in this tissue was antagonised with low affinity by BMY 7378 and is probably mediated by α_{1A}-adrenoceptors. Further studies performed after CEC treatment are required to substantiate this conclusion.

The classification of the α_1-adrenoceptor population in the ear artery is less certain since the pharmacological profile obtained does not correspond very closely to any of the known α_1-adrenoceptors. Nevertheless, the low affinities of BMY 7378 and clonidine, moderate affinity of 5-methyl urapidil and the insensitivity to CEC alkylation are most consistent with the suggestion that agonist-induced contractions are predominantly mediated by α_{1A}-adrenoceptors. The other α_1-adrenoceptor present in this tissue cannot be easily reconciled with current α_1-adrenoceptor subtypes. Further studies are required to establish if the atypically low affinity estimates obtained for 5-methyl urapidil and BMY 7378 in this tissue are the consequence of species variations or whether they represent 'real' receptor differences.

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