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# $\alpha_1$-Adrenoceptor antagonist properties of CGP 12177A and other $\beta$-adrenoceptor ligands: evidence against $\beta_3$- or atypical $\beta$-adrenoceptors in rat aorta

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**1** The $\alpha_1$-adrenoceptor antagonist properties of the $\beta$-adrenoceptor nonconventional partial agonist, CGP 12177A, was investigated in functional assays in rat aorta and in radioligand binding assays in rat cerebral cortical membranes. In addition, binding affinities of other $\beta$-adrenoceptor ligands were measured to investigate any correlation between $\alpha_1$-adrenoceptor affinity and relaxant potency in phenylephrine-constricted rings.

**2** In functional studies, CGP 12177A produced parallel rightward shifts of the phenylephrine CRC with no reduction in the maximum responses. Schild regression analysis gave a straight line with a slope of 0.95 (95% CL: 0.87–1.04), suggesting reversible competitive antagonism, and gave a $pK_B$ value of 5.26. In contrast, CGP 12177A ($\leqslant 300\,\mu$M) had no effect on contraction induced by the thromboxane-mimetic, U46619.

**3** In binding studies, CGP 12177A competed monophasically with [$^3$H]prazosin binding (Hill slope, 0.95, 95% CL: 0.76–1.13), giving a $pK_i$ value of 5.48, in good agreement with the $pK_B$ from functional studies.

**4** Competition experiments with various other $\beta$-adrenoceptor ligands showed that they all displaced [$^3$H]prazosin in a manner consistent with one-site competition. $pK_i$ values were as follows: SR 59230A, 6.25; cyanopindolol, 6.33; bupranolol, 6.35; alprenolol, 5.90; propranolol, 5.80; BRL 37344, 5.50; ICI 118551, 5.55; CGP 20712A, 5.26. The $pK_i$ values correlated well with the $pEC_{50}$ values for relaxation of phenylephrine-constricted rat aorta obtained previously ($r^2 = 0.984$, $P < 0.0001$).

**5** In conclusion, relaxant effects of CGP 12177A and other $\beta$-adrenoceptor ligands in phenylephrine-constricted rat aorta can be attributed to $\alpha_1$-adrenoceptor blockade and are unrelated to effects at $\beta_3$-adrenoceptors or atypical $\beta$-adrenoceptors.
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**Keywords:** $\beta$-Adrenoceptors; $\beta_3$-adrenoceptors; low affinity state of $\beta_1$-adrenoceptors; atypical $\beta$-adrenoceptors; rat aorta; CGP 12177A; nonconventional partial agonists; $\alpha_1$-adrenoceptors

**Abbreviations:** $B_{max}$, total number of receptor binding sites; CL, confidence limits; CRC, concentration–response curve; DMSO, dimethyl sulphoxide; $E_{max}$, maximum response; $IC_{50}$, concentration (M) of competing ligand that inhibits binding of radioligand by 50%; $pEC_{50}$, negative logarithm of the concentration (M) of relaxant that produces 50% of its maximum response; $pK_B$, negative logarithm of the equilibrium dissociation constant, obtained from functional experiments; $pK_D$, negative logarithm of the equilibrium dissociation constant, obtained from saturation experiments; $pK_i$, negative logarithm of the equilibrium dissociation constant, obtained from competition experiments; s.e.m., standard error of the mean

## Introduction

The term 'atypical $\beta$-adrenoceptor' was originally used to describe $\beta$-adrenoceptors not corresponding to the $\beta_1/\beta_2$ classification (Arch *et al*., 1984). Later, a third $\beta$-adrenoceptor was cloned and the pharmacological properties of this $\beta_3$-adrenoceptor were shown to correspond to those previously attributed to 'atypical' $\beta$-adrenoceptors (Emorine *et al*., 1989). $\beta_3$-Adrenoceptors are pharmacologically characterised by (i) low affinity of classical $\beta$-adrenoceptor antagonists, (ii) activation by selective agonists, such as BRL 37344 (Arch *et al*., 1984), (iii) activation by 'nonconventional partial agonists' (potent $\beta_1$-/$\beta_2$-adrenoceptor antagonists with $\beta_3$-agonist activity at higher concentrations (Kaumann, 1989) such as cyanopindolol (Engel *et al*., 1981) and CGP 12177A (Mohell & Dicker, 1989)) and (iv) blockade by selective $\beta_3$-adrenoceptor antagonists such as SR 59230A (Manara *et al*., 1996). A further atypical $\beta$-adrenoceptor, designated the putative $\beta_4$-adrenoceptor, was also characterised in the heart (Kaumann, 1989; Kaumann & Molenaar, 1996; Malinowska & Schlicker, 1996) and in adipose tissue (Galitzky *et al*., 1997), which shared properties (i) and (iii) above but not (ii) and (iv).

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However, the pharmacology of the putative $\beta_4$-adrenoceptor is not seen in $\beta_1$-adrenoceptor knockout mice (Konkar *et al*., 2000; Kaumann *et al*., 2001) and it is now understood that this receptor corresponds to a low-affinity state of the $\beta_1$-adrenoceptor (Kompa & Summers, 1999; Konkar *et al*., 2000; Kaumann *et al*., 2001). Further atypical $\beta$-adrenoceptors, corresponding to different affinity states of the $\beta$-adrenoceptor, have recently been postulated (Kozlowska *et al*., 2003; Molenaar, 2003).

A number of studies have suggested the presence of $\beta_3$-adrenoceptors and/or atypical $\beta$-adrenoceptors in vascular smooth muscle. For example, in rat isolated aorta $\beta_3$-adrenoceptor agonists and/or nonconventional partial agonists have been shown to have vasorelaxant properties (Oriowo, 1995; Sooch & Marshall, 1997; Shafiei & Mahmoudian 1999; Trochu *et al*., 1999; Brawley *et al*., 2000; Rautureau *et al*., 2002). Shafiei & Mahmoudian (1999) found that relaxations to the nonconventional partial agonist, cyanopindolol, were not blocked by the selective $\beta_3$-adrenoceptor agonist, SR 59230A and suggested that the effects of cyanopindolol may be mediated by the 'putative $\beta_4$-adrenoceptor'. Our own previous studies (Brawley *et al*., 2000) also found that relaxations to BRL 37344 and CGP 12177A were not blocked by SR 59230A and we similarly suggested the presence of the 'putative $\beta_4$-adrenoceptor'. More recently, however, we showed that relaxations to BRL 37344 and nonconventional partial agonists occurred in phenylephrine-constricted but not $PGF_{2\alpha}$-constricted aortic rings and thus appeared to be specific for an $\alpha_1$-adrenoceptor-mediated contraction (Brahmadevara *et al*., 2003a). Furthermore, $\beta$-adrenoceptor antagonists also relaxed phenylephrine-constricted but not $PGF_{2\alpha}$-constricted rings. These results suggested that relaxations to the $\beta_3$-adrenoceptor agonist, BRL 37344, the nonconventional partial agonists and $\beta$-adrenoceptor antagonists in phenylephrine-constricted rat aorta were not related to effects at $\beta_3$- or atypical $\beta$-adrenoceptors but could be related to interference with the $\alpha_1$-adrenoceptor signalling pathway (Brahmadevara *et al*., 2003a). A common factor in the previous studies showing relaxant effects of $\beta_3$-adrenoceptor agonists or nonconventional partial agonists in rat aorta was the use of phenylephrine, or noradrenaline, as preconstricting agents (Oriowo, 1995; Sooch & Marshall, 1997; Shafiei & Mahmoudian 1999; Trochu *et al*., 1999; Brawley *et al*., 2000; Rautureau *et al*., 2002).

The aim of the present study was therefore to investigate the affinity of the nonconventional partial agonist, CGP 12177A, at $\alpha_1$-adrenoceptors both in functional assays in rat aorta and in radioligand binding assays in rat cerebral cortical membranes. Binding affinities of other $\beta$-adrenoceptor ligands used in our previous study were also measured to investigate any correlation between $\alpha_1$-adrenoceptor affinity and relaxant potency in phenylephrine-constricted rings. Rat cerebral cortical membranes, rather than aortic membranes, were used for the radioligand binding assays since the aorta provides a low amount of tissue, has a relatively low $\alpha_1$-adrenoceptor density and has high nonspecific binding. Although different $\alpha_1$-adrenoceptor subtypes are present in cerebral cortex and in aorta, our results show that the $\beta$-adrenoceptor ligands tested are non-subtype selective. Preliminary accounts of these results have been presented to the British Pharmacological Society (Brahmadevara *et al*., 2003b).

# Methods

Animal experiments were carried out in concordance with the Animals (Scientific Procedures) Act, 1986. Male Wistar rats (200–250 g) were stunned and killed by cervical dislocation followed by exsanguination. A total of 12 rats were used for functional studies: the thoracic aorta was isolated, removed carefully to prevent endothelium damage and cleared of fat and connective tissue. The whole cerebral cortex was removed from three rats for the binding experiments.

## *Functional studies*

Aortae were cut into 3 mm ring segments and mounted on stainless steel wires in 20 ml organ baths containing Krebs' medium with the following composition (mM): NaCl, 119; KC1, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; $KH_2PO_4$, 1.2; D-glucose, 11.1. The medium was maintained at 37°C and gassed continuously with 95% $O_2$ and 5% $CO_2$. Each tissue was placed under 1.5 g resting tension and equilibrated for 60 min prior to the execution of experimental protocols. During this period, tissues were washed with Krebs every 15 min and tension was readjusted to 1.5 g. Isometric muscle tension was recorded with Grass transducers and displayed on a Goerz Servogor 400 oscillograph.

After the equilibration period, artery rings were constricted with a submaximal concentration of phenylephrine (0.6 $\mu$M) or U46619 (15–20 nM) and the function of endothelium was defined by the presence of at least 80% relaxation in response to acetylcholine (1 $\mu$M). After washout, some tissues were incubated with CGP 12177A for 30 min with control tissues receiving vehicle treatment. Then, cumulative concentration–response curves (CRCs) to phenylephrine or U46619 were obtained.

## *Membrane preparation*

Cerebral cortical tissues were homogenised in 20 volumes of ice-cold homogenisation buffer (50 mM Tris, 5 mM $Na_2EDTA$, pH 7.4) using an Ultraturrax homogeniser (13,500 rpm, 15 s × 2). The homogenate was centrifuged for three times at 20,000 rpm for 15 min at 4°C and the pellet was resuspended each time in 30 volumes of assay buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4). After the final centrifugation, the supernatant was aspirated and the pellet was finally resuspended in assay buffer. Membrane preparations were pooled and protein concentration was determined according to Bradford (1976) before aliquoting and storing at −70°C.

## *Radioligand binding studies*

Binding experiments were performed using [$^3$H]prazosin (specific activity 77.0 Ci mmol$^{-1}$, Amersham Biosiences, U.K.). Aliquots of rat cortical membranes were thawed and diluted to a protein concentration of 0.2 mg ml$^{-1}$. Each assay was performed in a total volume of 250 $\mu$l for 45 min at room temperature. The reaction was terminated by filtration through Whatman filters in a 24-well Brandel cell harvester. The filters were previously soaked in 0.1% polyethyleneimine solution to reduce the binding of the radioligand to filters. After terminating the reaction, wet filters were placed in 5 ml of scintillation cocktail (OptiScint HiSafe, Fisher Scientific, U.K.) and

radioactivity was determined in a liquid scintillation counter (LS 6500 Scintillation System, Beckman). Nonspecific binding was determined in the presence of phentolamine (25 $\mu$M). Saturation experiments were carried out to determine the p$K_D$ (negative logarithm of the equilibrium dissociation constant) of [$^3$H]prazosin and the $B_{max}$ (total number of receptor binding sites). Competition experiments were carried out with prazosin and various $\beta$-adrenoceptor ligands to determine $IC_{50}$ (concentration (M) of competing ligand that inhibits binding of radioligand by 50%) and hence p$K_i$ (negative logarithm of the equilibrium dissociation constant) values. A concentration of 0.16 nM [$^3$H]prazosin was used in the competition experiments.

## Drugs

The following drugs were dissolved in distilled water: (±)-propranolol hydrochloride, alprenolol hydrochloride, phentolamine hydrochloride, prazosin hydrochloride (all from Sigma, Poole, Dorset, U.K.), CGP 12177A hydrochloride ((±)-4-(3-tbutylamino-2-hydroxypropoxy)-benzimidazol-2-one hydrochloride) (gift from Novartis Pharma, Basle, Switzerland), BRL 37344 ((R*, R*)-(±)-4-[2-[(3-chlorophenyl)-2-hydroxyethyl) amino] propyl] phenoxyacetic acid) (Tocris Cookson, Bristol, U.K.), (±)-cyanopindolol hemifumarate (Tocris Cookson), CGP 20712A (2-hydroxy-5 (2-((2-hydroxy-3-4 ((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl)-phenoxy) propyl) amino) ethoxy) – benzamide monomethane sulphonate) (gift from Novartis Pharma), (−)-bupranolol hydrochloride (gift from Schwartz Pharma, Monheim, Germany), ICI 118551 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) (Tocris Cookson). [$^3$H]prazosin (specific activity 77.0 Ci mmol$^{-1}$, Amersham Biosiences, U.K.) was dissolved in assay buffer. Pindolol (Sigma) was dissolved in 0.1 M HCl. U46619 (U46619, 9,11-dideoxy-11$\alpha$, 9$\alpha$-epoxy-methano prostaglandin $F_{2\alpha}$) (Biomol, Pennsylvania, U.S.A.) was dissolved in 100% ethanol. SR 59230A (3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylamino]-2*S*-2-propanol oxalate) (Sigma) was dissolved in 40% dimethyl sulphoxide (DMSO).

### *Calculations and statistical analysis*

Contractile responses to agonists were calculated in milligram tension and expressed as mean ± s.e.m. (standard error of the mean). The mean concentration response curves to agonists were analysed by fitting to a four parameter logistic equation (given below) using nonlinear regression (Graph Pad Prism),

$$Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\log EC_{50} - X)P}}$$

where $X$ is the logarithm of molar concentration of the relaxant, $Y$ is the response and $P$ is the Hill slope. $E_{max}$ and $pEC_{50}$ values were obtained where $E_{max}$ is the maximum contraction obtained and $EC_{50}$ is the concentration (M) of agonist that produces 50% of its maximum response. Concentration ratios ($r$) were determined from $EC_{50}$ values. Antagonist affinity for CGP 12177A was obtained from the $x$-intercept of the plot of $\log(r-1)$ and log[CGP 1277A] (Arunlakshana & Schild, 1959) after linear regression and was expressed as a p$K_B$ (negative logarithm of the equilibrium dissociation constant) since the slope of the plot was not significantly different from unity.

For binding studies, results are expressed as mean ± s.e.m. of $n$ experiments. Each experiment was performed in duplicate. Data were analysed using nonlinear curve fitting (Graphpad Prism) using a one-site binding (hyperbola) to obtain $K_D$ and $B_{max}$ values. $IC_{50}$ values were determined by fitting the competition curve with a nonlinear regression analysis (one-site fit) and $K_i$ values were calculated from the equation of Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + ([\text{ligand}]/K_D)}$$

where [ligand] is the concentration of radioligand and $K_D$ is the equilibrium dissociation constant of the radioligand.

For statistical analyses, $E_{max}$ and $pEC_{50}$ were compared using one-way analysis of variance followed by the Tukey multiple comparison post test for comparison of three or more groups. $P<0.05$ was considered to be significant.

## Results

### *Functional studies*

In functional studies, preincubation of rat aortic rings with CGP 12177A (30, 100 and 300 $\mu$M) produced parallel

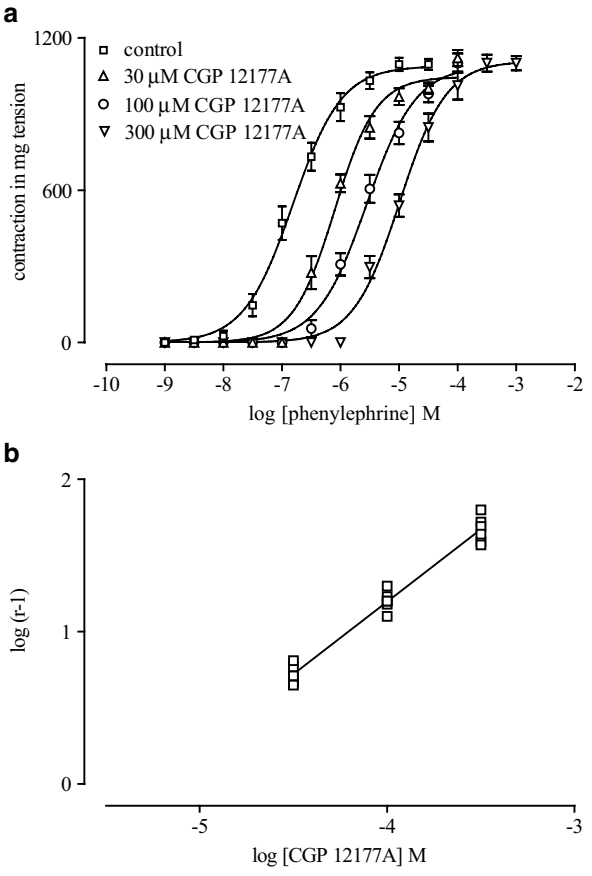


**Figure 1** (a) Effect of CGP 12177A on contractile responses to phenylephrine in rat thoracic aorta. Values are mean ± s.e.m. of six observations for each curve. (b) Schild plot for CGP 12177A against phenylephrine. Regression analysis gave a slope of 0.95 (95% CL: 0.87–1.04) and a p$K_B$ of 5.26 ($n=18$).

rightward shifts of the phenylephrine CRC with no reduction in the maximum responses (Figure 1a). Schild regression analysis gave a straight line with a slope of 0.95 (95% CL: 0.87–1.04) (Figure 1b), suggesting reversible competitive antagonism, and a $pK_B$ value of 5.26. In contrast, preincubation with the same concentrations of CGP 12177A (30, 100 and 300 $\mu$M) had no effect on U46619-mediated contraction (e.g. $pEC_{50}$s: U46619 control, $7.59 \pm 0.02$; U46619 + CGP 12177A 300 $\mu$M, $7.59 \pm 0.04$, $n = 6$, $P > 0.05$) (Figure 2).

### *Binding studies*

In binding studies, saturation experiments with [$^3$H]prazosin yielded a $pK_D$ of $9.79 \pm 0.04$ and a $B_{max}$ of $149.0 \pm 6.1$ fmol mg$^{-1}$ protein ($n = 3$) (Figure 3a). In competition experiments, unlabelled prazosin competed monophasically (Hill slope, 0.82, 95% CL: 0.63–1.01) with [$^3$H]prazosin binding, giving a $pK_i$ value of $9.83 \pm 0.12$ ($n = 3$) (Figure 3b). CGP 12177A also competed monophasically (Hill slope, 0.95, 95% CL: 0.76–1.13) with [$^3$H]prazosin binding, giving a $pK_i$ value of $5.48 \pm 0.17$ ($n = 3$) (Figure 3c).

Competition experiments with a number of other $\beta$-adrenoceptor ligands, which were previously shown to produce relaxation in phenylephrine-constricted rat aorta (Brahmadevara *et al*., 2003a), showed that they all displaced [$^3$H]prazosin in a manner consistent with one-site competition (Table 1). The $pK_i$ values correlated well with the $pEC_{50}$ values for relaxation of phenylephrine-constricted rat aorta obtained previously (Figure 4).

## Discussion

In the present study, functional experiments showed that preincubation of rat aortic rings with CGP 12177A shifted the CRC of phenylephrine to the right with no effect on the U46619 CRC. This is in agreement with our previous observation that CGP 12177A relaxed phenylephrine-constricted, but not $PGF_{2\alpha}$-constricted rat aorta (Brahmadevara *et al*., 2003a). Moreover, Schild analysis showed that the action of CGP 12177A was consistent with reversible competitive antagonism at $\alpha_1$-adrenoceptors.

Binding experiments in rat cerebral cortex membranes confirmed that CGP 12177A binds to $\alpha_1$-adrenoceptors.

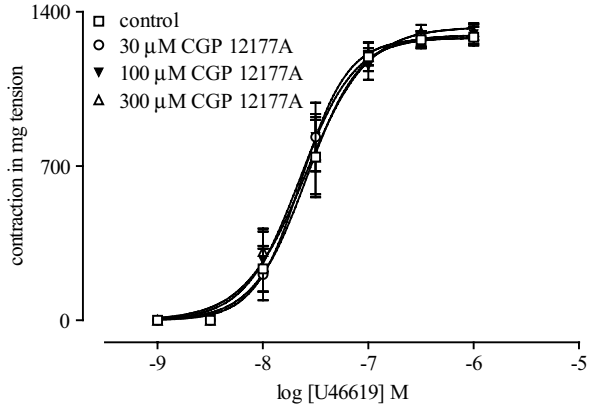


**Figure 2** Effect of preincubation with CGP 12177A on contractile responses to U46619 in rat thoracic aorta. Values are mean ± s.e.m. of six observations for each curve.

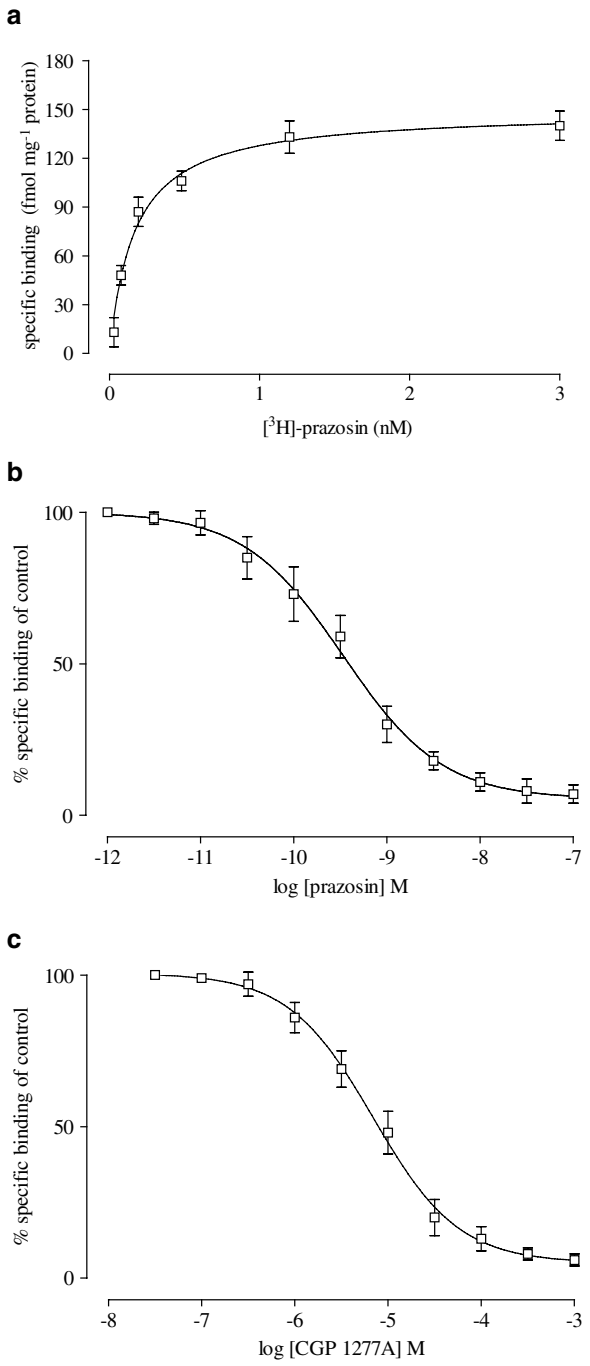


**Figure 3** (a) Saturation binding curve for [$^3$H]prazosin in rat cortex membranes. (b, c) Competition experiments. Displacement of [$^3$H]prazosin binding in rat cerebral cortex by (b) prazosin and (c) CGP 12177A. Values are mean ± s.e.m. ($n = 3$). Nonspecific binding was defined by phentolamine (25 $\mu$M).

[$^3$H]prazosin bound to rat cerebral cortical membranes in a specific, saturable manner, with a $K_D$ and $B_{max}$ in agreement with values in the literature (Salles & Badia, 1994). Unlabelled prazosin competed monophasically with [$^3$H]prazosin and the $pK_i$ obtained was in agreement with the $pK_D$ from the saturation study, validating the competition assay. CGP 12177A competed monophasically with [$^3$H]prazosin and the $pK_i$ value of 5.48 obtained is in good agreement with the $pK_B$ value of 5.26 obtained from the functional studies.

**Table 1** Displacement of [$H^3$]prazosin from $\alpha_1$-adrenoceptors in rat cortical membranes by $\beta$-adrenoceptor ligands (competition experiments)

| β-*Adrenoceptor ligand* | p$K_i$ (±*s.e.m.*) | *Hill slope (95% CL)* |
|---|---|---|
| SR 59230A | 6.25±0.20 | 0.88 (0.68–1.07) |
| Cyanopindolol | 6.33±0.17 | 0.94 (0.76–1.11) |
| Bupranolol | 6.35±0.22 | 0.90 (0.76–1.05) |
| Alprenolol | 5.90±0.23 | 0.94 (0.77–1.10) |
| Propranolol | 5.80±0.19 | 1.05 (0.82–1.28) |
| BRL 37344 | 5.50±0.21 | 0.96 (0.82–1.09) |
| ICI 118551 | 5.55±0.19 | 1.05 (0.80–1.29) |
| CGP 12177A | 5.48±0.17 | 0.95 (0.76–1.13) |
| CGP 20712A | 5.26±0.16 | 0.99 (0.69–1.28) |

Values are means, $n=3$.
Nonspecific binding was defined using phentolamine (25 $\mu$M).

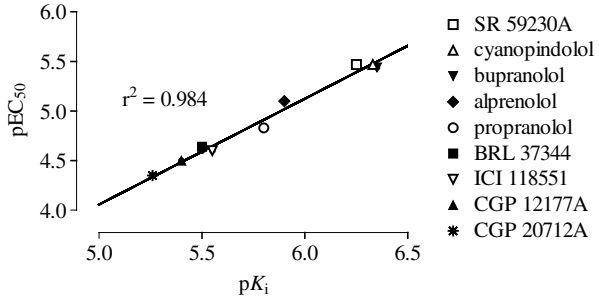


**Figure 4** Correlation between binding affinity at $\alpha_1$-adrenoceptors in rat cortical membranes (p$K_i$) and relaxant potency in phenylephrine-constricted rat aorta (p$EC_{50}$). p$K_i$ values were obtained from this study (Table 1). p$EC_{50}$ values were obtained from Brahmadevara *et al*. (2003a).

In our previous study (Brahmadevara *et al*., 2003a), other compounds that produced relaxation of phenylephrine-constricted aortic rings were: the nonconventional $\beta$-adrenoceptor partial agonists, cyanopindolol, pindolol and alprenolol; the $\beta_3$-adrenoceptor agonist, BRL 37344; the $\beta$-adrenoceptor antagonists, bupranolol, SR 59230A, propranolol, ICI 118551 and CGP 20712A. Competition studies against [$^3$H]prazosin showed that they all competed monophasically with prazosin and that their p$K_i$ values for binding to $\alpha_1$-adrenoceptors correlated strongly with their potency in producing relaxation of phenylephrine-constricted aortae. There are previous reports in the literature of $\alpha$-adrenoceptor blocking activity of $\beta$-adrenoceptor blocking agents (Gulati *et al*., 1968; 1973). The reported p$A_2$ of propranolol against methoxamine in rabbit aortic strip was 5.17 (Gulati *et al*., 1968), a little lower than, but not inconsistent with, the p$K_i$ obtained in the present study (5.8).

It would appear that all the $\beta$-adrenoceptor ligands tested are non-subtype selective for $\alpha_1$-adrenoceptors since the predominant subtype in rat aorta is $\alpha_{1D}$- (Kenny *et al*., 1995; Testa *et al*., 1995) and in rat cerebral cortex $\alpha_{1A}$- and $\alpha_{1B}$-adrenoceptors are predominant (Morrow & Creese, 1986; Salles & Badia, 1994). Further support for this is found with experiments in rat femoral resistance arteries, where the predominant subtype is $\alpha_{1A}$- (Jarajapu *et al*., 2001; Zacharia *et al*., 2004). In these arteries, CGP 12177A also produced relaxation of phenylephrine-constricted but not U46619-constricted rings with a p$EC_{50}$ value close to that found in rat aorta (manuscript in preparation/unpublished data).

These results show that relaxations of phenylephrine-constricted aortic smooth muscle to high concentrations of the $\beta_3$-adrenoceptor agonist, BRL 37344, or to nonconventional partial $\beta$-adrenoceptor agonists, such as CGP 12177A and cyanopindolol, are not due to activation of $\beta_3$- or atypical $\beta$-adrenoceptors and are adequately explained by $\alpha_1$-adrenoceptor blockade. Two recent papers appear to contradict the conclusion that $\beta_3$- or atypical $\beta$-adrenoceptors do not mediate relaxation in rat aorta. Firstly, Rautureau *et al*. (2002) showed that the $\beta_3$-adrenoceptor agonist, SR 58611A, produced relaxation of phenylephrine-constricted rat aorta which was inhibited by potassium channel blockers and thus was not simply due to $\alpha_1$-adrenoceptor blockade. We had previously shown that SR 58611A, in contrast to BRL 37344, produced relaxation of $PGF_{2\alpha}$-constricted rings, an effect which was not blocked by the $\beta_3$-adrenoceptor antagonist SR 59230A (Brahmadevara *et al*., 2003a) and thus we are in agreement that SR 58611A produces relaxation by a mechanism other than $\alpha_1$-adrenoceptor blockade. Secondly, de Groot *et al*. (2003) reported that nebivolol, a $\beta_1$-adrenoceptor antagonist with nitric oxide-dependent vasodilator properties (Ritter, 2001), relaxed U46619-induced constricted aortic rings by a mechanism attributed to $\beta_3$-adrenoceptor-stimulated release of nitric oxide. Our present results, taken together with our previous observation that the potent $\beta_3$-adrenoceptor agonist, CL 316243 (Bloom *et al*., 1992), produced no relaxation of phenylephrine- or U46619-constricted aortic rings (Brahmadevara *et al*., 2003a), would suggest that a mechanism other than $\beta_3$-adrenoceptor stimulation is involved but further studies on nebivolol are required to resolve this.

Also in contrast to the current findings, Kozlowska *et al*. (2003) concluded that relaxations to the nonconventional partial agonists, cyanopindolol and CGP 12177A and to the $\beta_3$-adrenoceptor agonist ZD 2079 in phenylephrine-constricted rat mesenteric artery were due to atypical $\beta$-adrenoceptors, different from $\beta_3$-adrenoceptors and from the low-affinity state of $\beta_1$-adrenoceptors (Kozlowska *et al*., 2003). These conclusions were based on the antagonism of cyanopindolol, CGP 12177A and ZD 2079 by high concentrations of bupranolol, CGP 20712A and SR 59230A. However, the potencies of cyanopindolol and CGP 12177A reported by Kozlowska (p$D_2$s of 5.45 and 4.19) are almost identical to the potencies reported previously by ourselves in rat aorta (Brahmadevara *et al*., 2003a: p$D_2$s of 5.47 and 4.38) and correspond to concentrations causing $\alpha_1$-adrenoceptor blockade as shown in the present study (p$K_i$s of 6.33 and 5.48 at $\alpha_1$-adrenoceptors). The high concentrations of bupranolol, CGP 20712A and SR 59230A used by Kozlowska *et al*. (2003) would also cause significant $\alpha_1$-adrenoceptor blockade according to our binding data and indeed they did report depression of the phenylephrine constriction. Thus, the evidence that bupranolol, CGP 20712A and SR 59230A are blocking the effects of cyanopindolol, pindolol and ZD 20729 at atypical $\beta$-adrenoceptors is subject to complicating interpretations. Kozlowska *et al*. (2003) also showed that cyanopindolol relaxed 5-hydroxytryptamine (5-HT)-constricted arteries, in order to rule out a specific effect of cyanopindolol on $\alpha_1$-adrenoceptor-induced constriction. However, the 5-HT receptor mediating contraction in the mesenteric artery is reported to be the 5-$HT_{2A}$ receptor (Watts *et al*., 1996) at which cyanopindolol has

affinity (p$K_i$, 4.5 (Hoyer, 1989)). Thus, the high concentration of cyanopindolol (0.1 mM) used to cause relaxation of the 5-HT-constricted mesenteric arteries would be sufficient to block the 5-$HT_{2A}$ receptor and this may account for the relaxation.

In conclusion, relaxant effects of CGP 12177A and other $\beta$-adrenoceptor ligands in phenylephrine-constricted rat aorta can be attributed to $\alpha_1$-adrenoceptor blockade and are unrelated to effects at $\beta_3$-adrenoceptors or atypical $\beta$-adrenoceptors. The present results suggest that $\alpha_1$-adrenoceptor agonists are not suitable preconstrictors for the investigation of $\beta_3$- or atypical $\beta$-adrenoceptors in blood vessels.

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