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# Investigation of the actions of chloroethylclonidine in rat aorta

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1 The interaction between chloroethylclonidine (CEC) and noradrenaline (NA) has been examined at $\alpha$-adrenoceptors mediating contractions of rat aorta.

2 In rat aorta, the competitive antagonist prazosin, over the concentration-range 0.01 - 10 $\mu$M, produced concentration-dependent shifts in the contractile potency of NA, so that there was no component of the NA contraction resistant to prazosin.

3 The irreversible $\alpha_1$-adrenoceptor antagonists, phenoxybenzamine (PBZ) (1 - 10 $\mu$M) and benextramine (10 $\mu$M) produced shifts in potency of NA and reduced the maximum response in a concentration-dependent manner.

4 The irreversible $\alpha_1$-adrenoceptor antagonist, CEC (100 $\mu$M), produced a non-parallel shift in the NA concentration-response curve so that low concentrations of NA produced relatively small contractions but relatively high concentrations produced further contractions, so that the maximum response was not significantly reduced.

5 The combination of CEC pretreatment and subsequent prazosin (0.1 $\mu$M) produced a parallel shift in the potency of NA. However, prazosin (10 $\mu$M) failed to produce any further effect on the response to high concentrations of NA following CEC pretreatment. Hence, a component of the contraction to NA in the presence of CEC was resistant to subsequent prazosin. Likewise, this component was resistant to a combination of prazosin (10 $\mu$M) and yohimbine (10 $\mu$M).

6 Receptor protection experiments were carried out in which tissues were exposed to NA (100 $\mu$M), yohimbine (10 $\mu$M) or prazosin (0.1 $\mu$M) prior to and during exposure to CEC. Receptor protection with NA, yohimbine or prazosin (0.1 $\mu$M), followed by washout prevented the shift in potency of NA produced by CEC.

7 Further experiments examined the effects of prazosin (10 $\mu$M) on responses to NA following receptor protection with NA (100 $\mu$M), yohimbine (10 $\mu$M), prazosin (10 $\mu$M), or xylazine (100 $\mu$M). In receptor protection studies with NA, subsequent prazosin (10 $\mu$M) produced a shift in response to NA following CEC which was not signficantly different from the shift produced by prazosin alone in the absence of receptor protection. In receptor protection studies with prazosin, yohimbine or xylazine, subsequent prazosin (10 $\mu$M) produced shifts in the response to NA following CEC which were significantly less than the shift produced by prazosin alone in the absence of receptor protection.

8 It is concluded that CEC has two actions in the rat aorta. Firstly, it behaves as an irreversible $\alpha_1$-adrenoceptor antagonist, reducing the response to low concentrations of NA (up to 10 $\mu$M). However, after exposure to CEC, concentrations of NA of 10 $\mu$M and above produced contractions resistant to prazosin. This resistant component was still present following receptor protection with $\alpha_1$- or $\alpha_2$-adrenoceptor antagonists, but absent following receptor protection with NA. Hence, the latter response may represent an irreversible agonist interaction between CEC, NA and $\alpha$-adrenoceptors which cannot be affected by subsequent competitive antagonism, but which can be prevented by receptor protection with the agonist NA prior to CEC.

**Keywords:** Rat aorta; $\alpha_1$-adrenoceptors; chloroethylclonidine; prazosin; WB 4101; benoxathian; 5-methyl-urapidil

## Introduction

$\alpha_1$-Adrenoceptors have been subdivided into $\alpha_{1A}$ and $\alpha_{1B}$-adrenoceptor subtypes based on affinities of a series of ligands for binding sites in rat cerebral cortex, hippocampus, vas deferens and spleen (Morrow & Creese, 1986; Han *et al.*, 1987; Gross *et al.*, 1988; see Docherty, 1989), and based on the ability of the alkylating agent chloroethylclonidine (CEC) to inactivate the $\alpha_{1B}$- but not the $\alpha_{1A}$-subtype. Based on this classification, it has been suggested that contractions of rat vas deferens to exogenous noradrenaline (NA) or adrenaline are mediated predominantly by $\alpha_{1A}$-adrenoceptors (Han *et al.*, 1987; Hanft & Gross, 1989; Aboud *et al.*, 1993), whereas contractions to NA in rat spleen are mediated predominantly by $\alpha_{1B}$-adrenoceptors (Han *et al.*, 1987; Aboud *et al.*, 1993).

Molecular cloning techniques have revealed the existence of possibly 4 genes coding for $\alpha_1$-adrenoceptors: $\alpha_{1a}$ (rat: Lomasney *et al.*, 1991), $\alpha_{1b}$ (hamster: Cotecchia *et al.*, 1988; rat: Voigt *et al.*, 1990), $\alpha_{1c}$ (bovine: Schwinn *et al.*, 1990), $\alpha_{1d}$ (rat: Perez *et al.*, 1992). However, it is likely that there are only 3 distinct receptors and that the $\alpha_{1a}$- and $\alpha_{1d}$-sites are similar and can be defined as the $\alpha_{1D}$-ligand binding site (see Perez *et al.*, 1992; Kenny *et al.*, 1994). The cloned $\alpha_{1c}$-receptor has now been shown to represent the $\alpha_{1A}$-adrenoceptor ligand binding site, which might be more appropriately termed $\alpha_{1C}$ (Rokosh *et al.*, 1994).

The subtype of $\alpha_1$-adrenoceptor present in the rat aorta has been variously classified as $\alpha_{1B}$ (Han *et al.*, 1990), both $\alpha_{1A}$ and $\alpha_{1B}$ (Tian *et al.*, 1990: Piascik *et al.*, 1991) or atypical (Muramatsu *et al.*, 1991; Aboud & Docherty, 1992; Oriowo & Ruffolo, 1992; Aboud *et al.*, 1993), based largely on the actions of CEC. In one study, CEC significantly reduced the maximum response to NA (Han *et al.*, 1990), but in most studies of rat aorta, CEC failed to reduce the maximum response to NA, but caused an approximately parallel shift (Oriowo & Bevan, 1990; Tian *et al.*, 1990; Muramatsu *et al.*, 1991; Oriowo & Ruffolo, 1992; Aboud *et al.*, 1993) or a clearly non-parallel shift in the concentration-response curve (Piascik *et al.*, 1991). The con-

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tractile response of rat aorta to NA following CEC pretreatment has been reported to be resistant to α-adrenoceptor antagonism (Oriowo & Bevan, 1990; Oriowo & Ruffolo, 1992). In one study, CEC also produced direct contractions of the smooth muscle (Muramatsu *et al.*, 1991). In other tissues, CEC has been reported to be an irreversible agonist at $\alpha_2$-adrenoceptors, both prejunctionally (Bultmann & Starke, 1993) and postjunctionally (Nunes & Guimaraes, 1993).

The object of this study was to re-examine the actions of CEC in rat aorta, comparing its effects to those of other irreversible antagonists such as phenoxybenzamine and benextramine.

## Methods

Male Wistar rats (200–300 g) were obtained from Trinity College Dublin, and the aorta was used as outlined below.

### *Rat aorta*

Aortic rings, 3–5 mm in length, were gently rubbed to remove the endothelium and attached to myograph transducers under 1 g tension in organ baths at 37°C in Krebs-Henseleit solution of the following composition: (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.0, EDTA 0.03 and ascorbic acid 0.28. Cocaine (3 μM), propranolol (3 μM) and indomethacin (10 μM) were also present.

Tissues were contracted with KCl (40 mM), exposed to ACh (10 μM) to test for absence of endothelium-dependent relaxations, and washed. Bathing fluid was then changed every 15 min for the next hour. Tissues were then contracted with NA administered cumulatively in 0.5 log unit increments beginning with 1 nM. Once a maximum response to NA had been obtained, tissues were washed and bathing fluid was changed every 15 min for 120 min, for the last 60 min of which tissues were incubated with various concentrations of competitive antagonists (prazosin, WB 4101, 5-methyl-urapidil, benoxathian, phentolamine or spiperone) or vehicle (one tissue from each animal received vehicle). Tissues were then contracted with NA in the continuing presence of antagonist or vehicle. In all experiments, responses in the second concentration-response curve were expressed as a percentage of responses obtained in the first concentration-response curve. Antagonist $pA_2$ values were obtained from the $\chi$-intercept of the plot of log (agonist dose ratio − 1) against log antagonist concentration, where the slope was not significantly different from negative unity (Arunlakshana & Schild, 1959). For yohimbine and xylazine, an apparent $pA_2$ was calculated from the effects of a single concentration from the equation $pA_2 = [B]/(DR-1)$, where [B] is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist.

In another series of experiments, tissues were incubated with CEC (100 μM) for 30 min, phenoxybenzamine (PBZ, 1 or 10 μM) for 10 min, or benextramine (10 μM) for 30 min, beginning 60 min after wash-out of the first NA concentration-response curve. After washing for another 60 min, a concentration-response curve to NA was repeated. In experiments examining the interaction between prazosin or yohimbine and CEC, some tissues were exposed to CEC for 30 min, washed, and a second concentration-response curve to NA was obtained following addition of prazosin or yohimbine (or both) for 60 min, or 45 min after exposure to PBZ (10 μM) for 10 min.

In receptor protection experiments, following the first concentration-response curve to NA, tissues were exposed to NA (100 μM), yohimbine (10 μM), prazosin (0.1 or 10 μM) or xylazine (100 μM) for 15 min before, and during the 30 min exposure to CEC. Following washout, experiments were carried out as outlined above.

### *Drugs*

Acetylcholine chloride (Sigma, Poole, Dorset); amidephrine hydrochloride (gift: Bristol-Myers Squibb, Wallingford, CT, U.S.A.); benextramine hydrochloride (Research Biochemicals, Natick, MA, U.S.A.); benoxathian hydrochloride (Research Biochemicals); chloroethylclonidine (Research Biochemicals); cocaine hydrochloride (Sigma); corticosterone (Sigma); 5-methyl-urapidil (gift: Byk Guilden, Konstanz); nifedipine (Sigma); (−)-noradrenaline bitartrate (Sigma); phenoxybenzamine hydrochloride (Research Biochemicals); phentolamine mesylate (Sigma); (−)-phenylephrine hydrochloride (Sigma); spiperone (Sigma); prazosin hydrochloride (gift; Pfizer, Sandwich, U.K.); (±)-propranolol hydrochloride (Sigma); WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride: Research Biochemicals); xylazine hydrochloride (gift; Bayer, Ireland).

Drugs were dissolved in distilled water, except for corticosterone and nifedipine (100% ethanol) and phenoxybenzamine (tartaric acid 1 mM).

**Table 1** Potencies of antagonists against contractions to noradrenaline (NA) in rat aorta

| *Antagonist* | $pA_2$ (95% confidence limits) | *No. of concentrations* | *Slope* |
|---|---|---|---|
| Prazosin | 9.45 (8.90–10.12) | 4 | −1.06 ± 0.21 |
| WB4101 | 9.21 (8.44–10.16) | 5 | −0.95 ± 0.15 |
| Benoxathian | 8.55 (7.86–9.58) | 4 | −1.10 ± 0.21 |
| 5-Methyl-urapidil | 8.12 (7.20–10.99) | 4 | −0.89 ± 0.30 |
| Spiperone | 7.40 (6.52–9.13) | 4 | −1.18 ± 0.26 |
| Phentolamine | 7.00 (6.28–8.15) | 3 | −1.17 ± 0.27 |
| Yohimbine | 6.37 ± 0.27 | | |
| Xylazine | 5.40 ± 0.42 | | |

Potencies are expressed as $pA_2$ values (and 95% confidence limits) from Schild plots based on the effects of 3–5 concentrations of antagonist in 9–20 experiments. Also shown are the apparent $pA_2$ values (and 95% confidence limits) of yohimbine and xylazine obtained from the effects of a single concentration (10 μM).

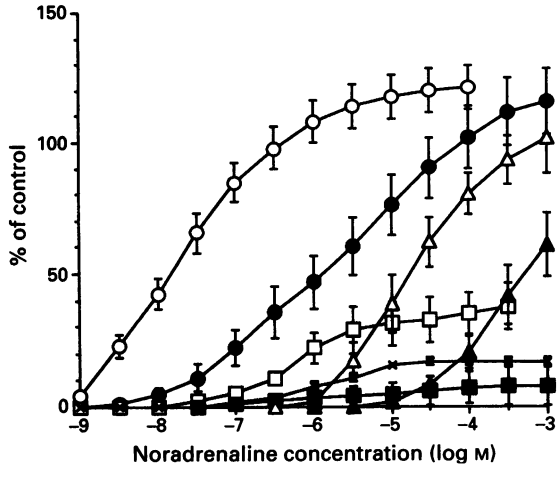


**Figure 1** Effects of chloroethylclonidine (CEC), phenoxybenzamine (PBZ), benextramine, prazosin or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 4 experiments are shown. Symbols: vehicle (○); CEC (100 μM) (●); PBZ (1 μM) (□), (10 μM) (■); benextramine (10 μM) (×); prazosin (0.1 μM) (△), (10 μM) (▲).

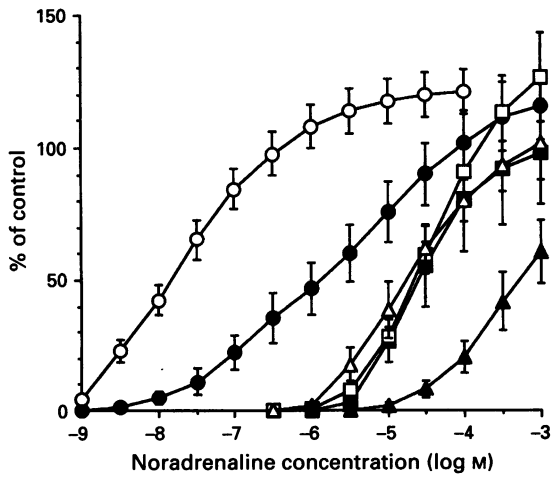


**Figure 2** Effects of chloroethylclonidine (CEC), prazosin, prazosin subsequent to CEC, or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 4 experiments are shown. Symbols: vehicle (○); CEC (100 μM) (●); prazosin (0.1 μM) (△), (10 μM) (▲); prazosin (0.1 μM) subsequent to CEC (□); prazosin (10 μM) subsequent to CEC (■).

### *Statistics*

Values are arithmetic mean ± s.e.mean, or geometric mean and 95% confidence limits. The 95% confidence limits were calculated from the standard deviation, except in the case of $pA_2$ values (95% limits of the intercept of Schild plot: MacFounds package). Agonist $pD_2$ values ($-\log EC_{50}$, $EC_{25}$ or $EC_{75}$) and contractions (% of control), or antagonist $pA_2$ values, were compared between tissues, and were compared with the effects of vehicle by a Student's *t* test for unpaired or paired data, where appropriate, and by ANOVAR analysis (Instat package).

## Results

### *Competitive antagonists*

NA produced isometric contractions with a $pD_2$ of 7.41 ± 0.12 (mean and 95% confidence limits, −log M, $n = 130$) and a maximum contraction of 0.90 ± 0.03 g ($n = 130$). Prazosin, WB 4101, benoxathian, 5-methyl-urapidil, phentolamine and spiperone produced concentration-dependent shifts in the potency of NA without reducing the maximum response. Schild plots were constructed from the effects of a range of concentrations of each antagonist, and since the slope of these regressions did not differ significantly from negative unity, $pA_2$ values were calculated (see Table 1). The effects of prazosin (0.1 and 10 μM) on contractions to NA are shown in Figure 1. Prazosin (0.1 μM) produced a parallel shift in the potency of NA without significantly altering the maximum response, and prazosin (10 μM) produced a shift of such magnitude that a maximum response to NA could not be obtained (see Figure 1).

### *Irreversible antagonists and CEC*

The effects of the irreversible antagonists PBZ and benextramine were also examined against contractions to NA. PBZ (1 μM) significantly shifted the potency of NA and significantly

**Table 2** Effects of receptor protection on the response to noradrenaline (NA 100 μM), expressed as a percentage of the maximum response to NA in the first (control) concentration-response curve

| *Drug combination* | *NA* (100 μM) | n |
|---|---|---|
| Vehicle (no receptor protection) | | |
| Prazosin (10 μM) | 20.4 ± 6.2%[d] | 8 |
| CEC (100 μM), prazosin (10 μM) | 82.8 ± 11.9%[b] | 8 |
| NA (100 μM) protection | | |
| Prazosin (10 μM) | 48.7 ± 9.1%[a,c] | 8 |
| CEC (100 μM), prazosin (10 μM) | 25.9 ± 8.9%[c] | 6 |
| Prazosin (10 μM) protection | | |
| Prazosin (10 μM) | 15.6 ± 6.0%[d] | 6 |
| CEC (100 μM), prazosin (10 μM) | 66.1 ± 17.2%[a] | 9 |
| Yohimbine (10 μM) protection | | |
| Prazosin (10 μM) | 54.2 ± 9.9%[a] | 6 |
| CEC (100 μM), prazosin (10 μM) | 55.7 ± 13.3%[a] | 6 |
| Xylazine (100 μM) protection | | |
| Prazosin (10 μM) | 66.8 ± 19.4%[a] | 5 |
| CEC (100 μM), prazosin (10 μM) | 89.8 ± 6.3%[b] | 4 |

Values are as shown in Figures 6–9, and are mean ± s.e.mean. Superscripts denote responses significantly different from response to NA obtained in the presence of prazosin alone (a,b) or in the presence of prazosin following chloroethylclonidine CEC (c,d) (Student's *t* test and Anovar analysis: $^{a,c}P < 0.05$, $^{b,d}P < 0.001$).

reduced the maximum response ($P<0.001$ from effects of vehicle), while PBZ (10 $\mu$M) and benextramine (10 $\mu$M) significantly reduced the maximum response ($P<0.001$) (NA potency not calculated due to the small contractions) (see Figure 1). CEC (100 $\mu$M) did not itself produce contractions and failed to reduce the maximum response to NA but significantly shifted the potency of NA ($P<0.001$) (Figure 1). However, CEC did not produce a clearly parallel shift in the concentration-response curve to NA, but shifted the response to high concentrations of NA more than the response to low concentrations. CEC produced a $1.63 \pm 0.24$ log unit shift ($n=13$) in NA potency measured at the $EC_{25}$ level, but a $2.04 \pm 0.14$ log unit shift in NA potency measured at the $EC_{75}$ level (Student's $t$ test for paired data, $P<0.01$) (see Figures 1 and 2). The difference between CEC and prazosin (0.1 $\mu$M) in shifting the NA concentration-response curve was accentuated (see Figure 2), because prazosin tended to shift the NA $EC_{25}$ ($3.04 \pm 0.18$ log unit shift, $n=5$) more than the $EC_{75}$ ($2.61 \pm 0.26$ log unit shift; Student's $t$ test for paired data, $P<0.01$).

Prazosin (0.1 $\mu$M), given after exposure to CEC, abolished the effects of low concentrations of NA so that the combination of CEC and prazosin produced an approximately parallel shift in the NA concentration-response curve (Figure 2). However, a higher concentration of prazosin (10 $\mu$M), given after exposure to CEC, produced no further effect (Figure 2). Hence, prazosin (10 $\mu$M) had a lesser effect on the response to NA following exposure to CEC: the response to NA (100 $\mu$M) was $20.4 \pm 6.2\%$ of control ($n=8$) following prazosin, but $82.8 \pm 11.8\%$ of control ($n=8$) following prazosin subsequent to CEC ($P<0.01$) (Figure 2 and Table 2). Exposure to PBZ (10 $\mu$M) for 10 min in vehicle experiments virtually abolished contractions to NA (Figure 3). However, PBZ (10 $\mu$M), given after CEC, failed to affect the component of the response to NA in CEC-treated tissues which was resistant to prazosin (Figures 2 and 3).

The $\alpha_2$-adrenoceptor antagonist, yohimbine (10 $\mu$M) significantly shifted the potency of NA ($P<0.001$ from effects of vehicle), although the potency of yohimbine was consistent with $\alpha_1$-adrenoceptor antagonism (Figure 4 and Table 1). Following exposure to CEC, yohimbine produced a significant shift in the potency of NA, especially the lower portion of the concentration-response curve (NA potency at the $EC_{25}$ level: $P<0.05$ from effects of CEC alone), but the combination of yohimbine and prazosin (both 10 $\mu$M) produced no further effect than that produced by prazosin alone (compare Figures 2 and 4). Hence, a component of the response to NA following CEC was resistant to the combination of prazosin and yohimbine.

The response to NA following CEC which was resistant to prazosin could be abolished by the calcium entry blocker, nifedipine (10 $\mu$M) (data not shown).

### *Receptor protection experiments*

Exposure to NA (100 $\mu$M) or yohimbine (10 $\mu$M) for 45 min followed by washout for 1–2 h had no significant effect on subsequent response to NA (Figure 5 shows results obtained with yohimbine). However, following exposure to prazosin (10 $\mu$M), responses to NA were markedly affected even after 1–2 h washout (data not shown). Following receptor protection with prazosin (0.1 $\mu$M), responses to NA recovered after washout. When CEC was administered during receptor protection with NA (100 $\mu$M), prazosin (0.1 $\mu$M) or yohimbine (10 $\mu$M), it had no significant effect on responses to NA following washout for 1 h (data for yohimbine protection shown in Figure 5).

When CEC (100 $\mu$M) was administered during receptor protection with NA (100 $\mu$M), prazosin (10 $\mu$M) given subsequent to CEC caused a large shift in potency of NA similar to that occurring in experiments without CEC pretreatment: responses to NA (100 $\mu$M) were not significantly different following prazosin whether or not CEC had been administered

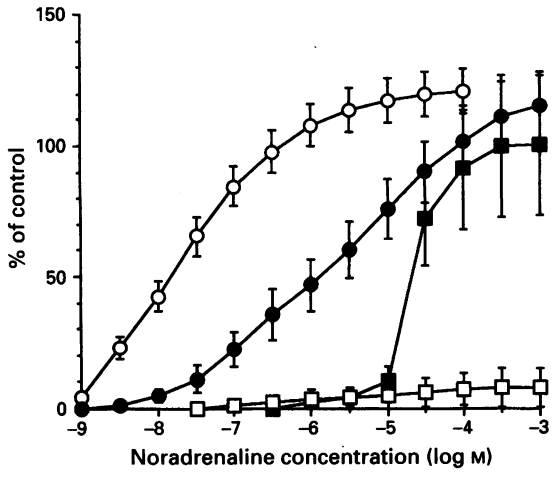


**Figure 3** Effects of chloroethylclonidine (CEC), phenoxybenzamine (PBZ), PBZ subsequent to CEC, or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 4 experiments are showm. Symbols: vehicle (○); CEC (100 $\mu$M) (●); PBZ (10 $\mu$M) (□); PBZ (10 $\mu$M) subsequent to CEC (■).

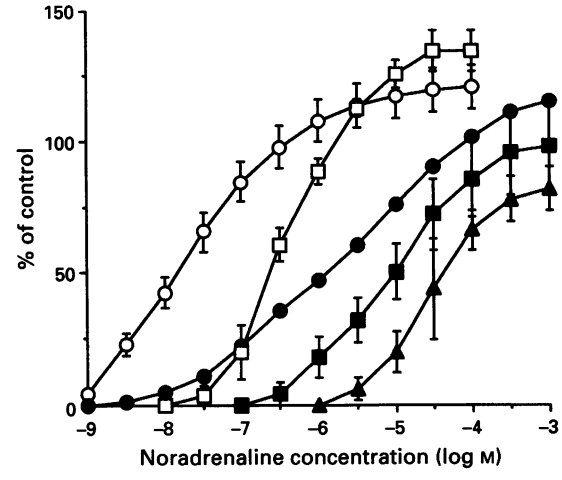


**Figure 4** Effects of chloroethylclonidine (CEC), yohimbine, yohimbine or yohimbine and prazosin subsequent to CEC, or vehicle on contractions to NA in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 4 experiments are shown. Symbols: vehicle (○); CEC (100 $\mu$M) (●); yohimbine (10 $\mu$M) (□); yohimbine (10 $\mu$M) subsequent to CEC (■); yohimbine (10 $\mu$M) and prazosin (10 $\mu$M) subsequent to CEC (▲).

(Figure 6 and Table 2). However, there was still a component of the response to NA following CEC which was resistant to prazosin when the receptor protection experiments were carried out with prazosin (10 $\mu$M), yohimbine (10 $\mu$M), or xylazine (100 $\mu$M): responses to NA (100 $\mu$M) were significantly smaller following prazosin in the absence of receptor protection than responses to NA following receptor protection with prazosin, yohimbine or xylazine, exposure to CEC and subsequent prazosin (see Figures 7–9 and Table 2).

Although prazosin protection did not significantly affect the response to prazosin following NA, as compared to the effects of prazosin alone (Figure 7 and Table 2), protection with NA, yohimbine or xylazine did affect the response to subsequent prazosin (Figures 6, 8 and 9, Table 2). Hence, although yo-

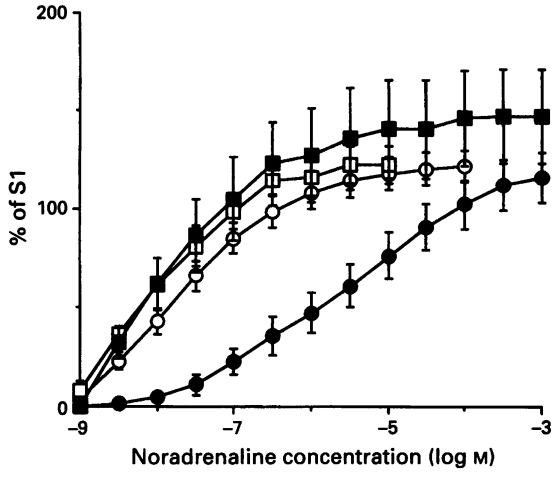


**Figure 5** Influence of receptor protection with yohimbine (10 $\mu$M) on effects of chloroethylclonidine (CEC) or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 4 experiments are shown. Receptor protection with yohimbine: symbols: vehicle (□); CEC (100 $\mu$M) (■). For comparison, responses to NA without receptor protection are also shown: vehicle (○), CEC (100 $\mu$M) (●).

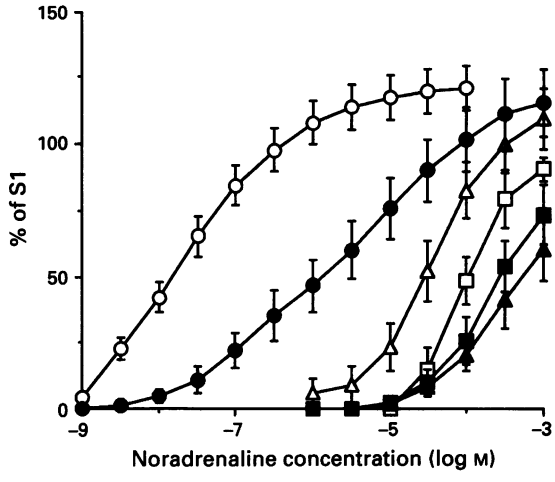


**Figure 6** Influence of receptor protection with noradrenaline (NA, 100 $\mu$M) on effects of chloroethylclonidine (CEC), prazosin or vehicle on contractions to NA in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Means with s.e. of mean from at least 6 experiments are shown. Symbols: receptor protection with NA: prazosin (10 $\mu$M) (□); prazosin (10 $\mu$M) following CEC (■). For comparison, responses to NA without receptor protection are also shown: vehicle (○); CEC (100 $\mu$M) (●); prazosin (10 $\mu$M) (▲); prazosin (10 $\mu$M) following CEC (△).

himbine or xylazine did not prevent the occurrence of a response to NA following CEC resistant to prazosin, the effect of prazosin on the response to NA after these agents was the same whether or not CEC had been administered (Figures 8 and 9). For prazosin, results were more clear: prazosin protection did not prevent the resistant component of the response to NA after CEC, and, not surprisingly, prior prazosin did not affect the response to subsequent prazosin (Figure 7).

### *Xylazine and clonidine as agonist or antagonist*

The $\alpha_2$-adrenoceptor selective agonist, xylazine, produced contractions in relatively high concentrations (10 $\mu$M and

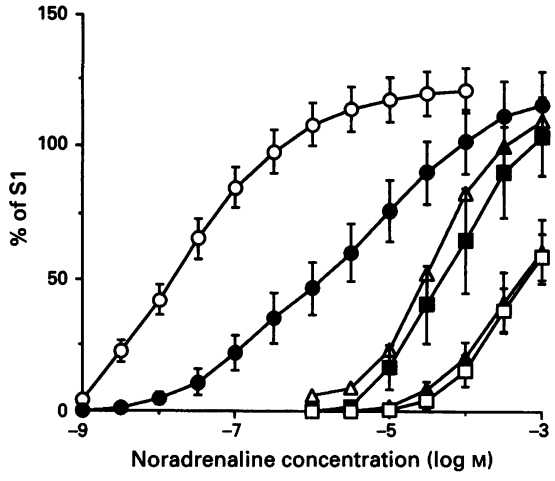


**Figure 7** Influence of receptor protection with prazosin (10 $\mu$M) on effects of chloroethylclonidine (CEC), prazosin, yohimbine or vehicle on contractions to NA in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Mean values with s.e. of mean from at least 6 experiments are shown. Symbols: receptor protection with prazosin: prazosin (10 $\mu$M) (□), prazosin (10 $\mu$M) following CEC (■). For comparison, responses to NA without receptor protection are also shown: vehicle (○), CEC (100 $\mu$M) (●), prazosin (10 $\mu$M) (▲), prazosin (10 $\mu$M) following CEC (△).

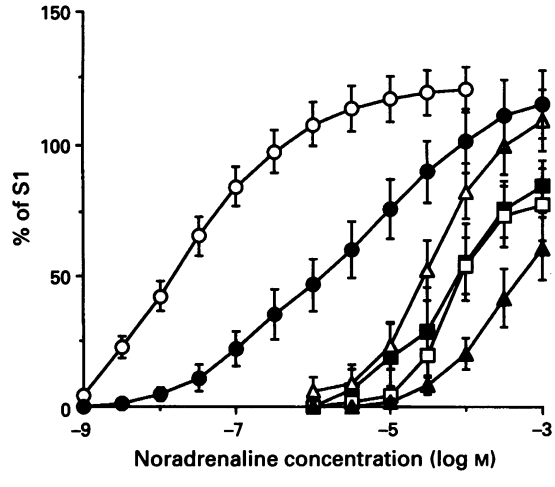


**Figure 8** Influence of receptor protection with yohimbine (10 $\mu$M) on effects of chloroethylclonidine (CEC), prazosin or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Vertical bars represent s.e. of mean from at least 6 experiments. Symbols: receptor protection with yohimbine: prazosin (10 $\mu$M) (□); prazosin (10 $\mu$M) following CEC (■). For comparison, responses to NA without receptor protection are also shown: vehicle (○); CEC (100 $\mu$M) (●); prazosin (10 $\mu$M) (▲), prazosin (10 $\mu$M) following CEC (△).

above) with maximum contraction in control experiments of 0.37 ± 0.09 g ($n$ = 6) (Figure 10), but these contractions were not antagonized by yohimbine (10 $\mu$M) and even prazosin (10 $\mu$M) produced only a small non-significant shift in the response to xylazine (data not shown). As an antagonist of contractions to NA, xylazine had relatively low potency with an apparent $pA_2$ (from the effects of a single concentration of xylazine) of 5.40 ± 0.42 ($n$ = 4) (Table 1).

The $\alpha_2$-adrenoceptor selective agonist, clonidine, produced concentration-dependent contractions of aorta with a maximum contraction in control experiments of 0.70 ± 0.07 g

($n=6$). The concentration-response curve to clonidine was significantly shifted by prazosin (10 $\mu$M) (Figure 10). However, subsequent to CEC, the response to clonidine was resistant to prazosin: the response to clonidine (300 $\mu$M) was significantly greater in the presence of prazosin after CEC (124.7 ± 7.1%, $n=4$) than in the presence of prazosin without prior exposure to CEC (38.7 ± 17.5%, $n=4$, $P<0.01$) (Figure 10). The shift in clonidine potency produced by prazosin alone was 2.32 ± 0.30 log units (prazosin apparent $pA_2$ of 7.32 ± 0.30), but subsequent to CEC the shift produced by prazosin was 1.36 ± 0.32 log units (prazosin apparent $pA_2$ of 6.36 ± 0.32) (Student's $t$ test: $P<0.01$).

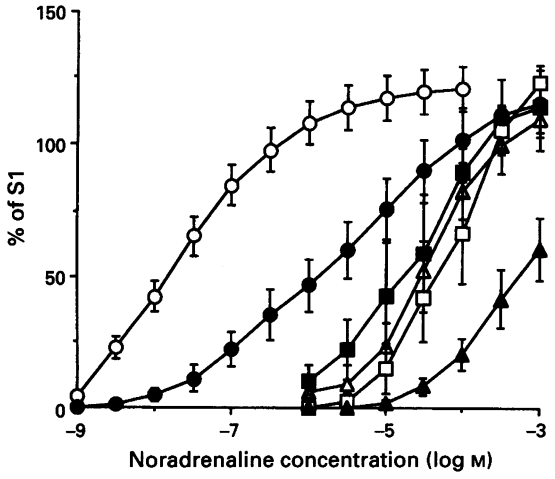


**Figure 9** Influence of receptor protection with xylazine (100 $\mu$M) on effects of chloroethylclonidine (CEC), prazosin or vehicle on contractions to noradrenaline (NA) in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Mean values with s.e. of mean from at least 4 experiments are also shown. Symbols: receptor protection with xylazine: prazosin (10 $\mu$M) (□); prazosin (10 $\mu$M) following CEC (■). For comparison, responses to NA without receptor protection are also shown: vehicle (○), CEC (100 $\mu$M) (●), prazosin (10 $\mu$M) (▲), prazosin (10 $\mu$M) following CEC (△).

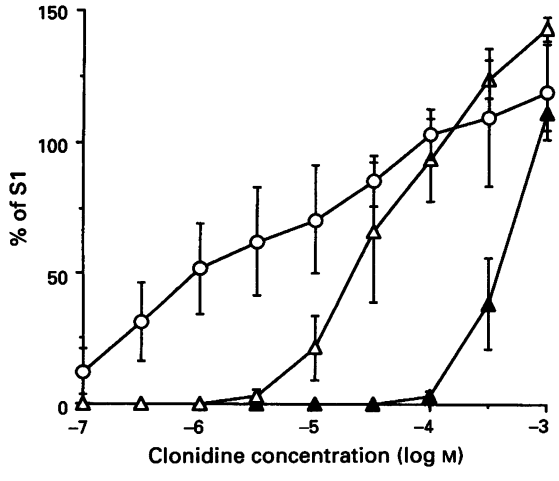


**Figure 10** Effects of prazosin and chloroethylclonidine (CEC) on contractions to clonidine in rat aorta. Responses in the presence of antagonist or vehicle are expressed as a percentage of the maximum response obtained in the first (control) concentration-response curve. Mean values with s.e. of mean from at least 3 experiments are shown. Symbols: vehicle (○); prazosin (10 $\mu$M) (▲); prazosin (10 $\mu$M) following CEC (△).

### *Correlation with ligand binding sites*

Antagonist $pA_2$ values shown in Table 1 were compared with antagonist affinities obtained from published ligand binding studies. For 5 antagonists (those in Table 1 except benoxathian, yohimbine and xylazine), correlations with the functional receptor of rat aorta were better for $\alpha_{1D}$- ($r=0.95$, $P<0.05$) and $\alpha_{1C}$- ($r=0.97$, $P<0.01$) than for the $\alpha_{1B}$-ligand binding site ($r=0.40$, non significant), using the ligand binding results of Testa *et al.* (1993). For 6 antagonists (those in Table 1 except yohimbine and xylazine), the correlations were better for the $\alpha_{1D}$- ($r=0.85$, $P<0.05$) and $\alpha_{1C}$-ligand binding site ($r=0.88$, $P<0.05$), than for the $\alpha_{1B}$- ($r=0.58$, non significant) using the ligand binding results of Kenny *et al.* (1994), with [$^3$H]-prazosin as radioligand. With [$^{125}$I]-HEAT as radioligand, the correlations with the functional receptor of rat aorta for the $\alpha_{1D}$- ($r=0.96$, $P<0.01$) and $\alpha_{1C}$-ligand binding site ($r=0.85$, $P<0.05$) were slightly different (Kenny *et al.*, 1994).

## Discussion

In this study, we have looked at the subtypes of α-adrenoceptor mediating contractions of the rat aorta to exogenous NA, and more particularly at the actions of CEC.

CEC has been used as a diagnostic tool in the identification of $\alpha_1$-adrenoceptor subtypes. CEC binds to all subtypes of $\alpha_1$-adrenoceptor (Michel *et al.*, 1993) but binds irreversibly, to varying degrees, to all except $\alpha_{1A}$-adrenoceptors. In the present study of rat aorta, CEC alone did not cause direct contractions but there was a complex interaction between CEC and NA. CEC neither caused a decrease in the maximum response to NA, as would be expected for an irreversible antagonist such as PBZ, nor produced a parallel shift in the potency of NA, as would be expected for a competitive antagonist such as prazosin. CEC produced a non-parallel shift in the NA concentration-response curve so that the response to low concentrations of NA was shifted less than the response to high concentrations, but the maximum response was not significantly reduced. Prazosin (0.1 $\mu$M) eliminated the first component of the response to NA following CEC, but even prazosin (10 $\mu$M) failed to produce any effect against high concentrations of NA following CEC. This $\alpha_1$-adrenoceptor antagonist resistant response to NA in rat aorta following CEC has been reported previously (Oriowo & Bevan, 1990; Oriowo & Ruffolo, 1992). Hence, the first component of the response to NA following exposure to CEC is clearly $\alpha_1$-adrenoceptor-mediated but the second component of the response to NA was resistant to prazosin following exposure to, but not in the absence of, CEC. Receptor protection with NA, yohimbine or prazosin prevented the inhibitory effects of CEC against low concentrations of NA (the first component), suggesting that this effect of CEC against low concentrations of NA is by irreversible $\alpha_1$-adrenoceptor antagonism. Receptor protection with NA left no component of the response following CEC resistant to prazosin.

From these results, three things are clear. Firstly, CEC interacts with low concentrations of NA as an irreversible $\alpha_1$-adrenoceptor antagonist. Secondly, all actions of NA following CEC involve α-adrenoceptors since the effects of CEC are prevented by receptor protection with NA. Thirdly, the response to NA resistant to prazosin, which occurred only after exposure to CEC, must also involve α-adrenoceptors, but since receptor protection with prazosin (10 $\mu$M) or yohimbine (10 $\mu$M) was ineffective, this may suggest that CEC may interact as an agonist (or partial agonist) in a way which is more easily prevented by using an agonist in receptor protection. It is well known that coupling of the receptor to the G-protein increases receptor affinity for agonists (see Dolphin, 1987), so that the interaction between CEC and NA may be at this conformation of the receptor.

Other authors have reported irreversible agonism by CEC at $\alpha_2$-adrenoceptors (Bultmann & Starke, 1993; Nunes &

Guimaraes, 1993). However, whereas Nunes & Guimaraes (1993) found that CEC contracted the dog saphenous vein with a maximum response approximately 75% of that to phenylephrine, the present study of rat aorta found no contractile response to CEC alone. Although most studies of rat aorta report no direct contraction to CEC (see Introduction), direct contractions were reported in one study (Muramatsu *et al.*, 1991) and were found to be nifedipine-sensitive. In our studies, clear contractile responses to CEC were observed in only 3 experiments (Authors' unpublished observations).

In our receptor protection studies, NA gave clear protection, and the $\alpha_1$-adrenoceptor antagonist, prazosin, gave no protection against the occurrence of a prazosin-resistant component to the response to NA subsequent to CEC. Results with the $\alpha_2$-adrenoceptor antagonist, yohimbine, were less clear cut: yohimbine caused the response to NA to be resistant to prazosin whether or not CEC had been administered. Hence, we also investigated the actions of the $\alpha_2$-adrenoceptor selective agonist, xylazine (Docherty & Hyland, 1985). Xylazine contracted the aorta only in relatively high concentrations, making it difficult to carry out interaction experiments, but these contractions were not blocked by yohimbine and resistant to prazosin. Contractions to xylazine were abolished by nifedipine. Xylazine was a weak antagonist at $\alpha_1$-adrenoceptors, and so was used in receptor protection studies. Receptor protection studies with xylazine were inconclusive, since, like yohimbine, xylazine caused the response to NA to be resistant to prazosin whether or not CEC had been administered. These results suggest that xylazine and, more surprisingly yohimbine, behave like CEC in causing a response to NA subsequent to CEC resistant to prazosin, but tell us nothing of the receptor involved: the concentrations of these agents used was sufficient to affect $\alpha_1$-adrenoceptors.

In terms of competitive antagonists, the receptor mediating NA-induced contractions of the rat aorta resembled both the $\alpha_{1D}$- and $\alpha_{1C}$-ligand binding sites (ligand binding data of Testa *et al.*, 1993 and Kenny *et al.*, 1994), ignoring the effects of CEC (since the original $\alpha_{1A}$-ligand binding site, coded for by the $\alpha_{1c}$-gene, is relatively resistant to CEC: Han *et al.*, 1987). However, susceptibility to CEC is relative and is time and dose-dependent (see Michel *et al.*, 1993; Kenny *et al.*, 1994). Studies of gene expression in rat aorta show the presence of $\alpha_{1B}$-, $\alpha_{1D}$- and $\alpha_{2A}$-adrenoceptors (Ping & Faber, 1993), or $\alpha_{1C}$ and $\alpha_{1D}$ (Rokosh *et al.*, 1994). Hence, the present results would tend to suggest that $\alpha_{1D}$-adrenoceptors are the primary receptors involved in contractions, although an involvement of $\alpha_{1C}$ could not be ruled out. The involvement of an $\alpha_{2A}$-adrenoceptor (also termed $\alpha_{2D}$ in the rat) at which prazosin has low potency (see Smith & Docherty, 1992), would be consistent with the inability of prazosin to protect against the effects of CEC, although the effects of the $\alpha_2$-adrenoceptor-selective agents neither prove nor disprove this view.

In conclusion, CEC appears to have two actions on the rat aorta, irreversible antagonism at $\alpha_1$-adrenoeptors, and irreversible partial agonism in combination with NA at $\alpha$ adrenoceptors. Only receptor protection with NA prevented this latter interaction, suggesting that interaction at the agonist binding conformation of the receptor was necessary.

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