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# Unusual α-adrenoceptor subtype in canine saphenous vein: comparison to mesenteric vein

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**1** We investigated the nature of the adrenoceptors in the dog saphenous vein (DSV) and dog mesenteric vein (DMV) to determine the nature of the unexpected interactions of phenylephrine and methoxamine with rauwolscine in the DSV, i.e. the ability of the putative $\alpha_2$-adrenoceptor antagonist to inhibit competitively contractions to these $\alpha_1$-agonists. Radioligand binding studies were performed in parallel with contractility studies.

**2** Functionally, in the DSV, phenylephrine and methoxamine-induced contractions were antagonized by rauwolscine with Schild slopes of $-0.52$ and $-0.46$, respectively and apparent $pA_2$ values of 8.5 and 9.2, respectively. Such antagonism was not observed in the DMV. In the DSV, prazosin competes for [$^3$H]-rauwolscine binding sites with a high and a low affinity binding site ($K_i$ of $1.49 \pm 0.65$ and $94.7 \pm 51$ $\mu$M, $n=6$, respectively).

**3** Pretreatment with 100 $\mu$M chloroethylclonidine (CEC) for 15 min abolished [$^3$H]-prazosin binding in microsomes from both veins and reduced binding ($B_{max}$) of [$^3$H]-rauwolscine in microsomes by $55.1 \pm 0.8\%$ ($n=3$) in the DSV but did not affect the $B_{max}$ in the DMV. CEC pretreatment in the venular rings denuded of endothelium caused persistent contraction in the DSV but not in the DMV. In the DSV, CEC appeared to interact with a single [$^3$H]-rauwolscine binding site. In both the DSV and the DMV, CEC (100 $\mu$M) caused a significant shift in the $EC_{50}$ values for phenylephrine and methoxamine. Maximum responses in the DMV were significantly attenuated while those in the DSV were unaffected when total tension was considered.

**4** Studies of the functional interactions of the DSV and the DMV with WB 4101 or 5-methylurapidil (5-MU) suggested the presence of $\alpha_{1D}$-adrenoceptors in the DSV and $\alpha_{1A}$-adrenoceptors in the DMV. The receptors inactivated by CEC in the DMV and DSV may represent some or all of the receptors with properties of $\alpha_{1D}$ and $\alpha_{1A}$-receptors present in the two veins. Studies of radioligand binding interactions of these two antagonists with [$^3$H]-prazosin, were consistent with the presence of some $\alpha_{1D}$-receptors in DSV and $\alpha_{1A}$-receptors in DMV. These findings raise questions about the selectivity of CEC in differentiating $\alpha_1$-adrenoceptor subtypes.

**5** B-HT 920 caused contractions in the DSV smaller than those to the $\alpha_1$-agonists but the maximum was not affected by CEC pretreatment. The $EC_{50}$ values were shifted to the left after CEC. In radioligand binding studies, B-HT 920 competition for [$^3$H]-rauwolscine binding was not significantly affected by CEC pretreatment.

**6** These results suggest the presence of unusual α-adrenoceptors in the DSV. In addition to $\alpha_2$-adrenoceptors, receptors recognizing rauwolscine as well as prazosin, WB 4101, phenylephrine and methoxamine and susceptible to inactivation by CEC are present. They appear to be, in part, unusual $\alpha_{1D}$-adrenoceptors.

**Keywords:** [$^3$H]-rauwolscine; prazosin; phenylephrine; methoxamine; chloroethylclonidine; α-adrenoceptor; canine saphenous vein; canine mesenteric vein

## Introduction

$\alpha_2$-Adrenoceptors have been recognised functionally by their selectivity toward certain agonists (e.g. UK-14304, B-HT 920) and certain antagonists (e.g. yohimbine or rauwolscine). $\alpha_1$-Adrenoceptors have been distinguished by their selective interactions with different agonists (e.g. phenylephrine or methoxamine) and antagonists (e.g. prazosin, 5-methylurapidil (5-MU), WB 4101; see Ruffolo *et al.*, 1991; Bylund, 1992; MacKinnon *et al.*, 1994). Although these selective functional interactions have retained their validity over many tissues, there has been less certainty that different affinities of agonists in binding to $\alpha_1$- and $\alpha_2$-adrenoceptors accounted for their functional selectivities (Agrawal & Daniel, 1985; Daniel *et al.*, 1991). Indeed, in some vascular tissues, there was little difference in $IC_{50}$ values for $\alpha_1$-adrenoceptor agonist competitive interactions with [$^3$H]-prazosin and [$^3$H]-rauwolscine binding sites. Moreover, in the canine saphenous vein (DSV), there has been an unexplained ability of yohimbine or rauwolscine to antagonize competitively phenylephrine-induced contractions (reviewed in Hicks *et al.*, 1991). In membranes from canine mesenteric vein (DMV), we found similar $IC_{50}$ values for phenylephrine to inhibit [$^3$H]-rauwolscine and [$^3$H]-prazosin binding (Daniel *et al.*, 1995), but similar studies have not been carried out in the DSV. Moreover, prazosin has been reported to compete for [$^3$H]-rauwolscine binding sites with high affinity at some classes of $\alpha_2$-adrenoceptors ($\alpha_{2B}$ and $\alpha_{2C}$); functional inhibition at these sites by prazosin is not established (reviewed in MacKinnon *et al.*, 1994).

Chloroethylclonidine (CEC) although related to an agonist somewhat selective for $\alpha_2$-adrenoceptors, clonidine, has become widely used because of its property to inactivate $\alpha_{1B}$-

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adrenoceptors irreversibly, leaving $\alpha_{1A}$-adrenoceptors intact (Han *et al.*, 1987). Until recently (Michel *et al.*, 1993), its interactions with $\alpha_2$-adrenoceptors in ligand binding were unreported. This recent study showed that certain $\alpha_2$-adrenoceptor subtypes are similarly inactivated in part, while others interact with CEC but are not inactivated by it.

The objectives of this study were to compare $\alpha_2$-adrenoceptors in the DMV and the DSV, to determine the interactions of phenylephrine and methoxamine with rauwolscine functionally and at rauwolscine binding sites in membranes from the two veins, and to determine if any of the $\alpha_2$-adrenoceptors were susceptible to CEC inactivation.

## Methods

### *Animals*

Healthy dogs (10–30 kg), irrespective of gender, were used. They were killed with an overdose of pentobarbitone (100 mg $kg^{-1}$, i.v.) after an overnight fast. These procedures were approved by the University Animal Care Committee. Tissues (DMV and DSV) were rapidly removed and placed either in oxygenated Krebs (95% $O_2$/5% $CO_2$) solution (composition in mM: NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, glucose 11) for functional studies or in sucrose MOPS (250 mM sucrose and 10 mM 3-[N-morpholino]propane-sulphonic acid (MOPS)) at pH 7.2 at 4°C for membrane preparation.

### *Dissection for membrane preparation*

The procedures have been described by Kwan *et al.* (1983) and by Shi *et al.* (1989a,b; 1990). The procedures were as follows. The mesenteric bed was removed and dissected on filter paper placed on an ice cold dissecting dish. The ends of branches, fat and connective tissue were trimmed and the vessels were separated. After locating an open end of a mesenteric vein, the branches were gently pulled out of the tissue one by one with two pairs of forceps. Separated blood vessels were placed immediately in ice-cold sucrose-MOPS, washed free from blood, blotted on filter paper, weighed and stored at −20°C for a maximum of two months. Mesenteric veins from a single animal provided about 0.2–0.4 g of tissue and yielded 600–1200 μg $g^{-1}$ tissue membrane protein. Saphenous vein from one dog on the other hand, provided about 0.5–1.0 g tissue with a protein yield in our microsomal fraction of only 268 ± 23 μg $g^{-1}$ tissue ($n = 15$) and therefore at least 8 animals were needed for membrane preparation.

### *Membrane preparation and characterization*

The isolated tissue (usually collected from 8–10 dogs) was minced in sucrose-MOPS solution in ratio 10:1 (ml:g wet tissue) and homogenized with a Polytron PT-20 (Brinkman Instruments Co., Switzerland) 15 s at 15,000 r.p.m. The homogenate was separated into various fractions by differential centrifugation, routinely used in this laboratory (Kwan *et al.*, 1983). The homogenate was centrifuged at 900 *g* for 10 min to obtain the post nuclear supernatant (PNS), which was filtered through gauze and centrifuged at 10,000 *g* for 10 min to remove mitochondria. The supernatant was separated by centrifugation at 105,000 *g* for 40 min. The crude microsomal pellet was resuspended in sucrose-MOPS buffer in glass homogenizer. A final centrifugation of 10,000 *g* for 10 min removed residual mitochondrial fragments. The microsomal supernatant (MIC II) was divided into aliquots and kept in the freezer (−20°C) for later studies.

### *CEC pretreatment*

The microsomal fraction was incubated for 15 min with 100 μM CEC at 37°C. The treatment was terminated by adding 20 ml of ice-cold sucrose-MOPS buffer. After high speed centrifugation (105,000 *g* for 40 min), the pellet was washed twice and resuspended in sucrose-MOPS with only two strokes in a Teflon glass homogenizer. Membranes used for control experiments were processed in parallel in CEC-free sucrose MOPS.

### *Binding procedures*

Prazosin and rauwolscine radioligand receptor binding studies were performed in triplicate. The concentrations of [$^3$H]-prazosin and [$^3$H]-rauwolscine used were around their $K_D$ values, 0.5 and 5 nM, respectively (Shi *et al.*, 1989a). The incubation medium (total volume, 250 μl) contained 100 μl Mg-MOPS buffer (500 mM MOPS and 10 mM $MgCl_2$, pH 7.2) or 10 μM phentolamine (for non-specific binding), 25 μl of sucrose-MOPS and 25 μl of radioligand diluted in Mg-MOPS from stock solution. In the competition experiments, 25 μl sucrose-MOPS volume was replaced with a tested drug of different concentration (usually $10^{-12}$ to $10^{-4}$ M) for each point. A 30 min incubation at 25°C was started by adding 100 μl of membrane fraction with a protein content of 35–70 μg. The reaction was terminated by addition of 4 ml of ice-cold Mg-MOPS buffer to the test tube. The reaction mixture was filtered rapidly through pre-soaked (Mg-MOPS buffer) Schleicher & Schuell GR No. 20 Glass Fibre filters (Mandel) on Millipore filtration manifolds and the test tube was washed three times with 4 ml of wash buffer which was then filtered. The radioactive filters in 4 ml liquid scintillation cocktail (Ready Safe, Beckman) were counted on a beta counter Beckman, model LS 6800 with efficiency of 40%.

All data were expressed as specific binding which was calculated as the radioactivity displaceable by 10 μM phentolamine. Non-specific binding was carried out in parallel incubation with total binding and always in triplicates.

### *Data handling for binding*

The saturation and drug displacement studies were analysed by EBDA computer programme (McPherson, 1983) and LIGAND programme (Munson & Rodbard, 1980). $K_D$ (concentration of the drug at which 50% of the receptors are occupied) or $K_i$ (equilibrium dissociation constant of the competing ligand, usually studied when the concentration of the labelled ligand is near its $K_D$ value) values derived from these programmes were accepted if the Hill slopes computed by the programmes were not significantly different from 1. If these values were less than one, the possibility of the occurrence of two binding sites was evaluated with CDATA 87 (ver. 1.1, EMF Software) and accepted if the residual error was significantly reduced by a two site fit.

### *Muscle bath procedures*

Veins were gently dissected from surrounding tissue and placed immediately in oxygenated Krebs solution at 25°C. The endothelium was gently removed from rings of vein about 4 mm in width with the teeth of a pair of forceps and rings were then mounted in a 15 ml organ bath and connected to a force transducer (Grass FT03C) with output to a chart recorder (Gould 2800 or Beckman). The presence or absence of functional endothelial cells was tested by use of 1 μM carbachol on rings precontractred with 60 mM $K^+$.

The organ baths and Krebs solution were bubbled continuously with 95% $O_2$/5% $CO_2$ and warmed to 37°C. The rings of the DSV and the DMV were equilibrated for 20 min before they were stretched to their optimal resting force of around 3 g and 1 g, respectively, for active contractile responses. Stimulation of the arteries with 100 mM $K^+$ added hypertonically was repeated every 15–20 min until reproducible contractions were obtained. Cumulative concentration-effect curves were then constructed to the agonists. Antagonists were incubated with the rings of tissue for 30 min

before agonist concentration-effect curves were reconstructed. Control tissue rings without antagonist were studied in each experiment as time controls. When significant shifts in these control concentration-response curves occurred, the data were discarded.

### *Data handling for contractions*

Contractions were related to the response (set at 100%) to 100 mM $K^+$ unless otherwise stated. $EC_{50}$ values were estimated by fitting each concentration-response curve with a sigmoidal curve (logistic function) using MicroCal Software Origin (Northampton, MA, U.S.A.). In cases where treatment (e.g. CEC) caused a basal constriction, $EC_{50}$ values were calculated from the new baseline. Schild plots were constructed in some cases (Figure 1) and evaluated by least square fit linear regression.

### *Chemicals*

[$^3$H]-prazosin (Sp.Act. 76.2) and [$^3$H]-rauwolscine (Sp.Act. 77.9–83.00) were purchased from NEN Research Products (Boston, MA, U.S.A.). Phenylephrine, phentolamine, methoxamine, prazosin were Sigma products (St. Louis, MO, U.S.A.). CEC (chloroethylclonidine), 5-MU (5-methylurapidil) and WB 4101 (N-[2(2,6-dimethoxyphenoxy)-ethyl]1,4-benzodioxane-2-methylamine) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Rauwolscine was obtained from Carl Roth KG (Karlsruhe, Germany). B-HT 920 ([2-amino-6-allyl-5,6,7,8-tetrahydro-4*H*-thiazolo-(4,5)azepine]) was obtained from Boehringer Ingleheim (Laval, Quebec, Canada).

### *Statistics*

Data are expressed as means ± standard error of the mean (s.e.mean). Significant differences were considered to be those in which $P<0.05$. The significant difference was calculated by Student's *t*-test (unpaired), the Mann Whitney's nonparametric unpaired test (two-tailed) or one-way analysis of variance where appropriate. When the *F* ratio was significant, significance of the differing pairs was evaluated by Bonferroni's method.

## Results

### *Functional studies*

*Effects of rauwolscine on phenylephrine and methoxamine-induced contractions* In the endothelium-denuded rings of DMV, 30 nM rauwolscine pretreatment did not have a statistically significant effect on the $EC_{50}$ values for phenylephrine (3.5 ± 0.6 μM, $n=6$ vs 4.1 ± 0.4 μM, $n=7$) or methoxamine (9.8 ± 4.2 μM, $n=7$ vs 10.5 ± 3.3 μM, $n=7$). Maximal responses (% of 100 mM $K^+$ contraction) to methoxamine (214.4 ± 33.7, $n=7$) and phenylephrine (226.7 ± 27.4, $n=6$) in the DMV were unaffected by 30 nM rauwolscine (192.7 ± 26.6, $n=7$ and 207 ± 31.5, $n=7$, respectively).

In endothelium-denuded rings of the DSV, rauwolscine antagonized phenylephrine-induced contractions in a concentration-dependent manner and competitively (Figure 1a). The $EC_{50}$ values were estimated using a Sigmoidal curve fitting function from which the dose-ratio values were obtained. The Schild plot fitted with a linear regression shows a slope of −0.52 with an x-axis intercept of 3.1 nM yielding an apparent $pA_2$ value of 8.5 (Figure 1b).

Concentration-response curves constructed for methoxamine in the presence of 3, 30 and 300 nM rauwolscine also caused a rightward shift in a concentration-dependent manner. The mean $EC_{50}$ value for controls was 1.59 ± 0.26 μM ($n=39$) while the $EC_{50}$ values in the presence of 3, 30 and 300 nM rauwolscine were 5.56 ± 0.96 μM ($n=12$), 8.26 ± 1.02 μM ($n=18$) and 34.76 ± 8.1 μM ($n=9$), respectively. The Schild plot for methoxamine against rauwolscine had a slope of −0.46 with an x-axis intercept of 0.54 nM and an apparent $pA_2$ value of 9.2 (Figure 1b). The methoxamine and rauwolscine interacted functionally like phenylephrine and rauwolscine; i.e. both phenylephrine and methoxamine recognized a similar receptor or receptors, not present in the DMV.

*Effects of CEC on phenylephrine and methoxamine-induced contractions* CEC caused persistent concentration-dependent contraction in the DSV but little or no contraction in the DMV. The contraction in the DSV did not wash out over several hours. CEC (100 μM), about 15 min after addition caused a contraction that was about 120% of the $K^+$ response

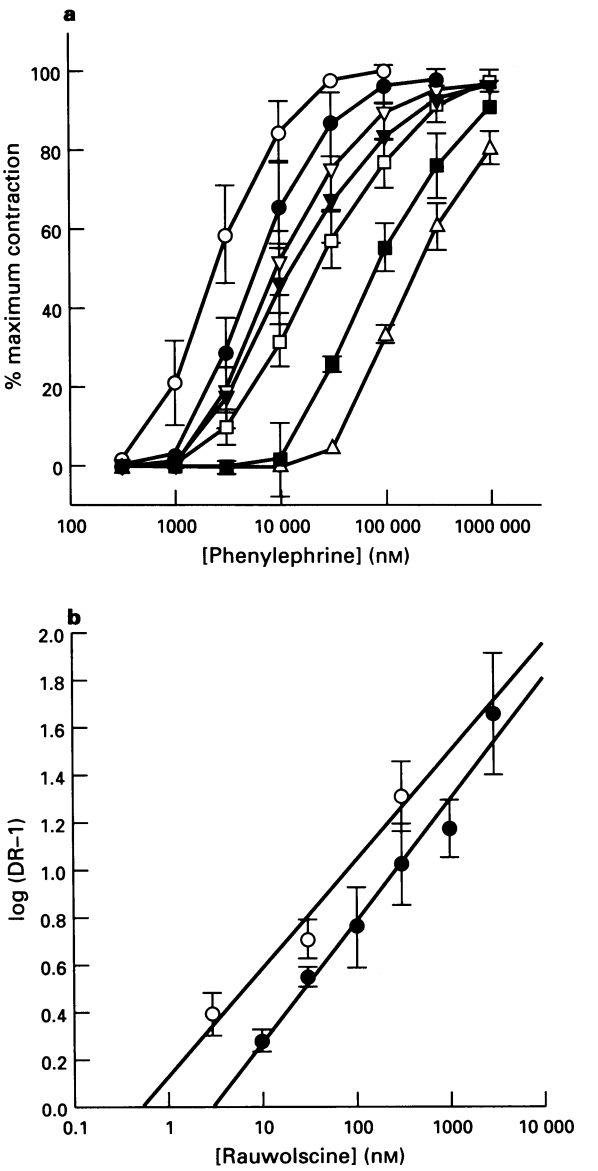


**Figure 1** (a) Competitive effects of rauwolscine on phenylephrine contractions in endothelium-denuded DSV ($n=5-7$). Rightward shift of the phenylephrine concentration-response curve was observed in a concentration-dependent manner with rauwolscine (● 10 nM; ▽ 30 nM; ▼ 100 nM; □ 300 nM; ■ 1 μM; △ 3 μM) compared with controls (○). Mean values with s.e.mean are shown. (b) Schild plots were generated from fitting a linear regression through points obtained from phenylephrine or methoxamine concentration-response curves in the absence and in the presence of rauwolscine. For phenylephrine (●), the Schild plot has a slope of 0.52 and a $pA_2$ value of 8.5. For methoxamine (○), the Schild plot has a slope of 0.46 and a $pA_2$ value of 9.2.

in the DSV (Figure 2a,b). This suggests that there is a functional receptor recognized and activated by CEC in the DSV which is absent in the DMV.

Table 1 shows that treatment of the DSV with CEC (100 μM) caused a small but statistically significant shift of the $EC_{50}$ values for phenylephrine (1.4 μM to 3.5 μM) and methoxamine (2.4 μM to 6.5 μM). In the DMV, the same manipulation caused a larger shift of the $EC_{50}$ values for phenylephrine from 2.8 μM to 178 μM ($P<0.05$) and for methoxamine from 7.4 μM to 21 μM ($P<0.05$). There was also a significant reduction in the maximum responses in the DMV to these agents to 51.3% of control for phenylephrine and to 44.2% of control for methoxamine. Reduction in the maximum responses was not observed in the DSV but the basal constriction which we previously reported to be prazosin- and rauwolscine-sensitive (Low *et al.*, 1994) complicated the interpretation of responses to these agonists.

*Effects of CEC pretreatment on B-HT 920 responses* In the presence of 30 nM prazosin, CEC caused a basal constriction

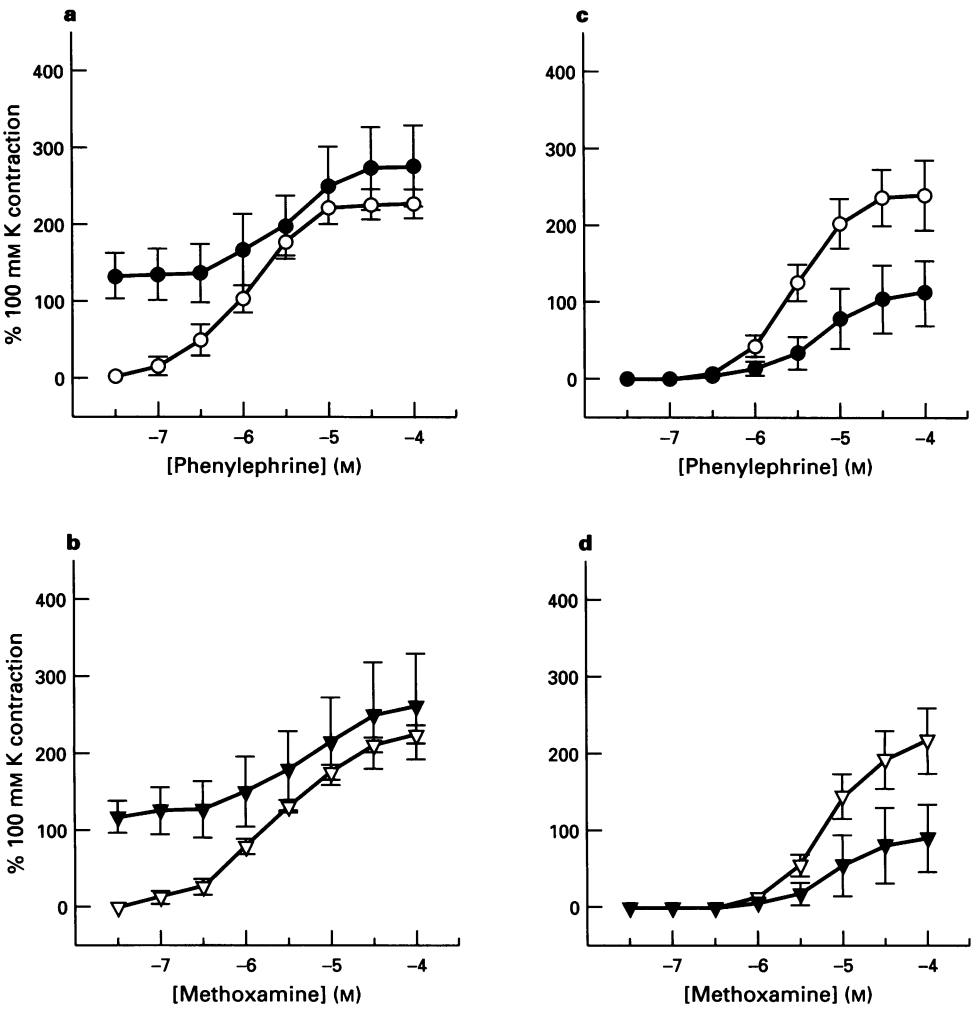


**Figure 2** Concentration-response curves to phenylephrine (a,c) and methoxamine (b,d) in the absence and presence of 100 μM CEC pretreatment for 15 min prior to the construction of the concentration-response curves in the DSV (a, $n=7-8$; b, $n=6-10$) and the DMV (c, $n=8$; d, $n=7-8$) where open symbols denote controls and closed symbols denote responses from CEC-pretreated rings. Circles denote phenylephrine while triangles denote methoxamine. CEC pretreatment caused a basal constriction in the DSV but not in the DMV. Responses are expressed as a percentage of 100 mM KCl contraction. Values are mean ± s.e. mean.

**Table 1** Effects of CEC (100 μM) on $EC_{50}$ and maximum responses for phenylephrine and methoxamine in the DSV and the DMV

| Tissue | Drug | $EC_{50}$ (μM) Control | CEC (100 μM) | Maximal response (% 100 mM $K^+$ contraction) Control | CEC (100 μM) |
|---|---|---|---|---|---|
| DSV | Methoxamine | 2.43 ± 0.48 (10) | 6.5 ± 1.62 (6)* | 225.9 ± 11.4 (10) | 261.8 ± 68.7 (6)$^{NS}$ |
| | Phenylephrine | 1.41 ± 0.22 (9) | 3.5 ± 0.77 (7)* | 240 ± 20.6 (8) | 274.2 ± 52.5 (7)$^{NS}$ |
| DMV | Methoxamine | 7.4 ± 1.43 (8) | 21.32 ± 7.08 (6)* | 217.1 ± 42.6 (8) | 96 ± 47.4 (7)* |
| | Phenylephrine | 2.8 ± 0.3 (8) | 178.19 ± 119.77 (7)* | 241 ± 39.1 (8) | 123.7 ± 38.6 (7)* |

Values are means ± s.e.mean; $n$ is given in parentheses.
*$P<0.05$ compared with controls.
NS not significantly different from controls.

in the DSV ($P<0.05$). Addition of B-HT 920 in concentrations ranging from 10 nM to 100 μM caused a concentration-dependent contraction in 5 veins before as well as after CEC. The $EC_{50}$ values estimated from these curves showed a statistically significant leftward shift after CEC pretreatment (control: 92.3±26.1, $n=4$; CEC-treated: 12.7±4.8, $n=5$).

Maximal responses (% of 100 mM $K^+$ contraction) to B-HT 920 in control (111.7±8.0%, $n=5$) and CEC-treated (123.2±14.2%, $n=5$) were not significantly different. However, these maximal responses obtained from B-HT 920 stimulations were significantly smaller compared to maximal responses to phenylephrine or methoxamine (cf. Figure 2).

*Effects of prazosin on contractions to phenylephrine and methoxamine* Phenylephrine and methoxamine concentration-response curves were constructed in the absence and presence of prazosin (30 nM) in the DSV and the DMV. Table 2 summarizes the results. Prazosin (30 nM) caused a significant rightward shift of the phenylephrine and methoxamine concentration-response curves in the DSV as evident from the approximately 4 fold increase in the $EC_{50}$ values. In the DMV, there was a 7–8 fold increase in the $EC_{50}$ value for both phenylephrine and methoxamine. The apparent $K_B$ values of prazosin against phenylephrine and methoxamine were not significantly different from each other in the DSV or the DMV (see Table 2). However, in the DSV, the apparent $K_B$ of prazosin against methoxamine in an additional study ($n=4$) increased significantly by 2.3 fold when 300 nM prazosin rather than 30 nM was used. The shift in the concentration-response curve by the 270 nM increment of prazosin was consistent with a $K_B$ of 200±90 nM ($n=4$) instead of the $K_B$ of 17.3±3.4 nM ($n=7$) when 30 nM prazosin was present, suggesting more than one interaction site between prazosin and methoxamine.

*Effects of other antagonists on contractions to phenylephrine* WB 4101 at 3 or 30 nM concentrations ($n=5$) inhibited phenylephrine-induced contractions of the DSV yielding $K_B$ of WB 4101 against phenylephrine of 0.58±0.37 nM and 0.84±0.42 nM, respectively. These values were not significantly different. In the DMV, $K_B$ values of WB 4101 against phenylephrine and methoxamine at 3 or 30 nM concentrations ($n=6$) were 0.24±0.1 nM and 1.0±0.34 nM, respectively. These values were significantly different ($P<0.05$). Moreover, at 300 nM ($n=4$), the $K_B$ value of WB 4101 against phenylephrine was 6.1±3.6 nM, again significantly different from this value at 3 or 30 nM.

In the DSV, the $K_B$ values of 5-MU at 0.3 or 3 μM concentrations ($n=4$) against phenylephrine-induced contractions were 0.62±0.43 μM and 0.9±0.13 μM, respectively. These values were not significantly different. In the DMV, the $K_B$ of 5-MU at 3, 30 or 300 nM concentrations against phenylephrine were 7.8±4.7 nM ($n=5$), 45±15 nM ($n=5$) and 220±70 nM ($n=4$), respectively. The values at the lowest and highest concentrations were significantly different ($P<0.05$).

## *Binding studies*

*Effects of CEC-pretreatment on [$^3$H]-prazosin and [$^3$H]-rauwolscine binding sites in the DSV and the DMV* Table 3 shows $K_d$, $B_{max}$ and Hill slope values for [$^3$H]-prazosin and [$^3$H]-rauwolscine binding in microsomes from both the DSV and the DMV. $K_d$ values from [$^3$H]-prazosin and [$^3$H]-rauwolscine saturation studies were not different between the two sets of membranes. Also, in both cases, Hill slopes were not significantly different from unity. However, $B_{max}$ values for both [$^3$H]-prazosin and [$^3$H]-rauwolscine were much higher in the DSV (385±107 and 832±168 fmol mg$^{-1}$ protein, respectively) than in the DMV (153±30 and 245±67 fmol mg$^{-1}$ protein, respectively). These values are consistent with those previously reported by Shi *et al.* (1989a,b).

Pretreatment with CEC abolished [$^3$H]-prazosin binding in microsomes from both veins (Table 3). It also reduced binding of [$^3$H]-rauwolscine in microsomes of the DSV by 55.1±0.8% but did not affect the density of binding of [$^3$H]-rauwolscine in

**Table 2** $EC_{50}$ values for phenylephrine and methoxamine in the absence and presence of 30 nM prazosin and calculated dose-ratio (DR) and $K_B$ of prazosin against phenylephrine and methoxamine

| | *$\alpha_1$-Agonists* | *Phenylephrine* | *Methoxamine* |
|---|---|---|---|
| DSV | $EC_{50}$ (μM) | 1.14±0.1 (7) | 2.4±0.89 (6) |
| | +Prazosin (30 nM) | 4.22±1.01 (7)* | 10.55±0.65 (6)* |
| | DR | 3.69±0.74 (7) | 4.04±3.65 (6) |
| | $K_B$ (nM) | 17.29±3.96 (7) | 11.03±5.44 (6)$^{NS}$ |
| DMV | $EC_{50}$ (μM) | 2.8±0.49 (7) | 12.27±3.46 (6) |
| | +Prazosin (30 nM) | 21.15±4.29 (7)* | 86.89±22.85 (6)* |
| | DR | 8.88±2.28 (7) | 7.69±2.16 (6) |
| | $K_B$ (nM) | 6.56±2.08 (7) | 5.98±1.01 (6)$^{NS}$ |

Values are means±s.e.mean; $n$ is given in parentheses.
*$P<0.05$ compared with controls.
NS not significantly different between phenylephrine and methoxamine.

**Table 3** Effects of CEC (100 μM) pretreatment on binding parameter for [$^3$H]-prazosin and [$^3$H]-rauwolscine in the DMV and the DSV

| | *[$^3$H]-prazosin* | | | | *[$^3$H]-rauwolscine* | | |
|---|---|---|---|---|---|---|---|
| *Tissue* | n | $K_d$ (nM) | $B_{max}$ (fmol mg$^{-1}$) | $n_H$ | n | $K_d$ (nM) | $B_{max}$ (fmol mg$^{-1}$) | $n_H$ |
| DSV control | 3 | 2.6±1.2 | 385.1±106.7 | 0.91±0.06 | 3 | 5.4±0.9 | 832.4±168.2 | 1.00±0.05 |
| CEC-treated | 4 | No binding | No binding | No binding | 3 | 5.4±1.4 | 374.1±81.35* | 0.96±0.01 |
| DMV control | 3 | 0.8±0.2 | 152.5±30.2 | 0.97±0.02 | 3 | 6.6±2.4 | 244.7±66.6 | 0.96±0.02 |
| CEC-treated | 5 | No binding | No binding | No binding | 3 | 5.7±1.9 | 234.0±118.6$^{NS}$ | 1.00±0.01 |

Values are means±s.e.mean.
*$t=5.274$ with 2 degrees of freedom, $P=0.034$ (two-tailed) compared with controls.
NS not significantly different from controls.

the DMV microsomes (Table 3). However, in competition studies, CEC appeared to interact with only one [$^3$H]-rauwolscine binding site: $K_i = 8.1 \pm 0.6$ μM; $n_H = 0.89 \pm 0.17$; $n = 3$, suggesting that the sites resistant to CEC were not easily distinguished from the sites sensitive to CEC.

*Effects of CEC-pretreatment on competition between phenylephrine or methoxamine and [$^3$H]-rauwolscine in the DSV and the DMV* Concentrations of [$^3$H]-rauwolscine near the $K_d$ values were used (Shi *et al.*, 1989b) and the effects of CEC pretreatment on the interactions of phenylephrine and methoxamine with [$^3$H]-rauwolscine binding sites studied. Table 4 shows that the $IC_{50}$ values from 3 experiments for phenylephrine as a competitor for [$^3$H]-rauwolscine binding sites in microsomes from the DSV increased from $8.2 \pm 1.9$ μM to $93.8 \pm 38$ μM after CEC pretreatment. In microsomes from the DMV, $IC_{50}$ values before and after CEC treatment were not significantly different ($34 \pm 8.7$ μM vs $25.5 \pm 6.7$ μM). In all cases, the Hill slopes were less than unity. In contrast, values derived from methoxamine interactions with [$^3$H]-rauwolscine binding in microsomes from the two veins were unaffected by CEC pretreatment (Table 4). Maximum displacements of [$^3$H]-rauwolscine in the DSV by phenylephrine and methoxamine were 92% and 70%, respectively.

*[$^3$H]-rauwolscine displacement by prazosin in the DSV* The displacement of [$^3$H]-rauwolscine by prazosin was carried out in 6 experiments. Analysis showed a slope of $0.55 \pm 0.04$ and 2 binding sites: a high and a low affinity binding site with a $K_i$ of $1.49 \pm 0.65$ and of $94.7 \pm 51$ μM, respectively. The $IC_{50}$ was $4.43 \pm 1.31$ μM.

*Competition between B-HT 920 and [$^3$H]-rauwolscine binding and the effects of CEC* Table 5 summarizes the effects of CEC pretreatment on the competition between B-HT 920 and [$^3$H]-rauwolscine binding. CEC-pretreatment at 100 μM for 15 min did not significantly affect the $IC_{50}$, $K_n$ or $n_H$ from 4 independent experiments. Maximum displacement of [$^3$H]-rauwolscine by B-HT 920 was estimated to be 92% from the displacement curve, similar to displacement of [$^3$H]-rauwolscine by phenylephrine.

*Other competition studies* In the DSV, WB 4101, competing against [$^3$H]-prazosin binding, yielded an $IC_{50}$ of $2.9 \pm 1.4$ nM with a Hill slope of $0.40 \pm 0.13$ ($n = 4$). In two of these studies sufficient data were obtained to fit two binding sites, one with an affinity less than 1 nM and the other with an affinity of about 10 nM. In the DMV, the values were: $IC_{50}$, $5.2 \pm 4.9$ μM and Hill slope, $0.32 \pm 0.06$, ($n = 4$). Two binding sites could be delineated with $K_i$ values of $0.5 \pm 0.21$ nM and $17 \pm 13$ μM. The proportions of high and low affinity sites were $54.1 \pm 9.3$% and $51.4 \pm 3.9$%, respectively.

In the DSV, 5-MU up to $10^{-4}$ M did not displace [$^3$H]-prazosin in 4 experiments. However, in the DMV, the $IC_{50}$ value from 7 experiments with this agent was $17.9 \pm 10.8$ nM with a Hill slope of $0.49 \pm 0.07$. In some experiments two binding sites were delineated; the values for the high and low affinity sites were $\approx 5$ and 100 nM respectively.

## Discussion

Both the DSV and the DMV contract to the selective $\alpha_2$-adrenoceptor agonist, B-HT 920 (Shi *et al.*, 1989a,b) and their [$^3$H]-rauwolscine binding sites have been presumed to represent $\alpha_2$-adrenoceptors. The receptors in membranes of these veins are not distinguished by their affinities ($K_D$) to [$^3$H]-rauwolscine (5–7 nM). Data from this study show for the first time that some of these [$^3$H]-rauwolscine binding sites in the DSV are unusual and are not present in the DMV.

About half of the [$^3$H]-rauwolscine binding sites in the DSV membranes were inactivated by pretreatment with CEC and the $IC_{50}$ of the residual site for phenylephrine was 10 fold higher than in control membranes. In the DMV membranes, CEC pretreatment affected neither the density of [$^3$H]-rauwolscine binding nor the ability of phenylephrine to compete for these [$^3$H]-rauwolscine binding sites. Methoxamine interaction with [$^3$H]-rauwolscine in both these veins was unaffected by CEC treatment. Thus, [$^3$H]-rauwolscine binding sites with unusual properties occurred in the DSV but not in the DMV. Functional studies suggested that these unusual binding sites in the DSV were involved in the contractile responses to phenylephrine and methoxamine as evident from the rightward shift of the $EC_{50}$ values with increasing concentrations of rauwolscine (Figure 1). In the DMV, functional responses to phenylephrine and methoxamine were not antagonized by rauwolscine as is the case in most vascular tissues.

How should these unusual sites in the DSV be classified? After intensive functional study of the α-adrenoceptors of the DSV, Hicks *et al.* (1991) concluded that there were several subtypes present: an $\alpha_{1A}$-adrenoceptor activated by phenylephrine and antagonized by WB 4101 with high affinity, an $\alpha_{2A}$-adrenoceptor activated by B-HT 920 and antagonized by yohimbine and idazoxan, and an atypical adrenoceptor with some characteristics of the $\alpha_{1B}$-adrenoceptor but at which yohimbine was a functional antagonist. Until recently, adrenoceptor binding sites inactivated by CEC were considered to be $\alpha_{1B}$-adrenoceptors. Since Hicks *et al.* (1991) showed that phenylephrine caused synthesis of 1,4,5-inositol-trisphosphate ($IP_3$) in this vein and that this effect was antagonized, like the contractions, by yohimbine (a close analogue of rauwolscine),

**Table 5** Competition between B-HT 920 and [$^3$H]-rauwolscine in the DSV and the effects of CEC (100 μM, 15 min) pretreatment

| | *B-HT 920* | *B-HT 920 + CEC* (100 μM) |
|---|---|---|
| $IC_{50}$ (μM) | $1.31 \pm 0.51$ | $2.64 \pm 1.41$ |
| $K_i$ (μM) | $0.64 \pm 0.25$ | $1.3 \pm 0.7$ |
| $n_H$ | $0.5 \pm 0.08$ | $0.33 \pm 0.03$ |
| $n$ | 4 | 4 |

Values are means ± s.e.mean.

**Table 4** Effects of CEC (100 μM) pretreatment on competition between phenylephrine or methoxamine and rauwolscine in the DMV and the DSV

| | *Phenylephrine* | | | | *Methoxamine* | | | |
|---|---|---|---|---|---|---|---|---|
| *Tissue* | $IC_{50}$ (μM) | $K_i$ (μM) | $n_H$ | n | $IC_{50}$ (μM) | $K_i$ (μM) | $n_H$ | n |
| DSV control | $8.2 \pm 1.9$ | $4.1 \pm 1.0$ | $0.57 \pm 0.03$ | 3 | $334.8 \pm 132.4$ | $167.6 \pm 66.5$ | $0.61 \pm 0.05$ | 4 |
| CEC-treated | $93.8 \pm 38$* | $47.0 \pm 19.1$* | $0.78 \pm 0.02$* | 3 | $497.8 \pm 214.8$ | $247.7 \pm 107.1$ | $0.77 \pm 0.18$ | 4 |
| DMV control | $34.0 \pm 8.7$ | $14.6 \pm 5.9$ | $0.91 \pm 0.08$ | 4 | $206.3 \pm 40.5$ | $103.0 \pm 20.3$ | $0.95 \pm 0.001$ | 3 |
| CEC-treated | $25.5 \pm 6.7$ | $12.7 \pm 3.3$ | $0.76 \pm 0.03$ | 4 | $228.7 \pm 73.7$ | $114.5 \pm 36.8$ | $0.82 \pm 0.03$ | 3 |

Values are means ± s.e.mean.
*$P < 0.05$ compared with controls.

one might conclude that these are atypical $\alpha_{1B}$-adrenoceptors which recognise rauwolscine and yohimbine as well as prazosin.

However, a recent study (Michel *et al.*, 1993) found that some classes of $\alpha_2$-adrenoceptors are also inactivated by CEC. For example, $\alpha_{2A}$-adrenoceptors in platelets and $\alpha_2$-$C_{10}$ (also $\alpha_{2A}$ in subtype) as well as $\alpha_2$-C4 ($\alpha_{2C}$ in subtype) adrenoceptors expressed in the host cell line, LM ($tk^-$) cells, were reduced in number by CEC pretreatment (32.6%, 63.8% and 47.3%, respectively). Michel *et al.* (1993) also found that additional $\alpha_2$-adrenoceptor subtypes interacted with CEC but were not inactivated by it. In a study of $\alpha_2$-adrenoceptors in canine mesenteric nerve membranes, we (Daniel *et al.*, 1995) showed that our procedure of treatment with CEC did not reduce $B_{max}$ values for classical $\alpha_{2A}$-adrenoceptors in platelets studied in comparative fashion. In this study of DSV, CEC competed with all [$^3$H]-rauwolscine binding sites as if it recognized a single site, not high and low affinity sites. Also, CEC pretreatment abolished all detectable [$^3$H]-prazosin binding in both the DSV and the DMV membranes, but did not affect the competition between B-HT 920 and [$^3$H]-rauwolscine. Thus, it is also possible that the DSV has an atypical $\alpha_1$-adrenoceptor which recognises phenylephrine, methoxamine, CEC, prazosin and rauwolscine, but not B-HT 920, with measurable affinity and efficacy and initiates release of $IP_3$. Prazosin competed with [$^3$H]-rauwolscine in the DSV and analysis revealed a higher (1.5 $\mu$M $K_i$) and a lower (95 $\mu$M $K_i$) affinity binding site. These data suggested that, although there are rauwolscine binding sites present in the DSV which recognize prazosin as well as rauwolscine, these sites do not appear to be those at which [$^3$H]-prazosin bound (2.6 nM $K_d$) or interacted with agonists (see below).

As noted above, Hicks *et al.* (1991) suggested that the DSV contained $\alpha_{1A}$-adrenoceptors, based on their susceptibility to WB 4101. Our studies, summarized in Table 6, confirmed the high potency of WB 4101 as an antagonist to phenylephrine and showed it to be a high affinity competitor to [$^3$H]-prazosin binding. However, this receptor subtype, as now reclassified to be the $\alpha_{1A/C}$ subtype (Perez *et al.*, 1994), is supposed to have high affinity for the antagonist, 5-MU, while the $\alpha_{1D}$ subtype is distinguished by a lower affinity for this antagonist. Our functional and corresponding binding studies both showed in the DSV (Table 6) that 5-MU is a weak antagonist to phenylephrine-induced contractions and a weak competitor at [$^3$H]-prazosin binding sites. This suggests that the DSV contains an $\alpha_1$-adrenoceptor with properties of the $\alpha_{1D}$-subtype; i.e., WB 4101 was a potent and 5-MU was a weak antagonist (Perez *et al.*, 1994). In the DMV, at least a fraction of the $\alpha_1$-adrenoceptors was $\alpha_{1A}$ in subtype, based on the same criteria. Functionally, CEC reduced maximum responses to phenylephrine and methoxamine and shifted the $EC_{50}$ values to the right in the DMV as expected if $\alpha_{1B}$-adrenoceptors were present and inactivated by CEC. In the DSV, CEC shifted the $EC_{50}$ values slightly but significantly to the right for both phenylephrine and methoxamine. These results appear to be consistent with the existence of some $\alpha_{1B}$-adrenoceptors in the DMV and the DSV. However, Minneman *et al.* (1994) recently reported that, when hamster $\alpha_{1B}$-receptors were expressed in human embryonic kidney cells, phenylephrine and methoxamine had as low or lower potency ($IC_{50}$) in competition with [$^{125}$I]-BE2254, an $\alpha_1$ antagonist, than when these agonists competed with this ligand for binding to bovine $\alpha_{1C}$ or rat $\alpha_{1A}$-receptors expressed in these cells. Therefore, it is unclear if inactivation of $\alpha_{1B}$-adrenoceptors, leaving $\alpha_{1A}$ or $\alpha_{1D}$-receptors, could explain this finding. The nature of the CEC-resistant responses to $\alpha_1$-adrenoceptor agonists in the DMV has not been studied further. Presumably, they are mediated by residual $\alpha_1$-adrenoceptors, of low affinity for prazosin not detected in binding sites. Whether the CEC-sensitive receptors in DMV are $\alpha_{1A}$-adrenoceptors susceptible to CEC under our experimental conditions or $\alpha_{1B}$-adrenoceptors susceptible to WB 4101 requires further study.

In the DSV, the interpretation of results is complicated by the persistent contraction induced by CEC. This contraction could not be washed out. Its nature has been studied and reported elsewhere (Low *et al.*, 1994); it seems to result from activation of both $\alpha_1$- and $\alpha_2$-adrenoceptors, perhaps better described as prazosin- and rauwolscine-sensitive sites. Both phenylephrine and methoxamine caused additional contraction above that caused by CEC and the maximum contractions reached in the presence of each agonist were unchanged from control values (Figure 2). It seems likely that CEC persistently activated and bound to the unusual $\alpha_1$-adrenoceptors in the DSV which are also rauwolscine binding sites. Additional contractions were produced by phenylephrine or methoxamine acting on residual receptors with lower rauwolscine sensitivity which remain functional after CEC treatment.

Since the $EC_{50}$ values for contractions to methoxamine and phenylephrine were shifted to the right and maximum responses decreased by CEC pretreatment or competitively antagonized by WB 4101 or 5-MU in the DMV (Table 1), but were unaffected by rauwolscine, it seem reasonable to suggest

**Table 6** Functional and radioligand binding data from DSV and DMV with phenylephrine (PE), WB 4101 (WB), 5-methylurapidil (5-MU), B-HT 920 (BHT), prazosin (Praz), CEC and rauwolscine (Rauw)

| *Tissue* | *PE/WB** | *[$^3$H]-Praz/WB* | *PE/5-MU** | *[$^3$H]-Praz/5-MU* | *BHT/CEC* | *[$^3$H]-Rauw/Praz* |
|---|---|---|---|---|---|---|
| DSV | *3 nM $K_B$ 0.58±0.37 nM ($n$=5) *30 nM $K_B$ 0.84±0.42 nM ($n$=5) $P$>0.05 | $IC_{50}$ 2.9±1.4 nM ($n$=4) Hill slope 0.4±0.13 $K_{iH}$ 0.6 nM | *0.3 μM $K_B$ 0.62±0.43 μM ($n$=4) *3 μM $K_B$ 0.9±0.13 μM ($n$=4) $P$>0.05 | No displacement ($n$=4) | $EC_{50}$ shifted from 92.3±26.1 μM to 12.7±4.8 μM $P$<0.05 | $IC_{50}$ 4.43±1.3 μM ($n$=6) Hill slope 0.55±0.04 $K_i$ high 1.49±0.65 μM $K_i$ low 94.7±51 μM |
| DMV | *3 nM $K_B$ 0.24±0.1 nM ($n$=6) *30 nM $K_B$ 1.0±0.3 nM ($n$=6) *300 nM $K_B$ 6.1±3.6 nM ($n$=3) $P$<0.05 | $IC_{50}$ 5.2±4.9 μM ($n$=4) Hill slope 0.32±0.06 $K_i$ high 0.5±0.21 nM $K_i$ low 17±13 μM | *3 nM $K_B$ 7.8±4.7 nM ($n$=5) *30 nM $K_B$ 45±15 nM ($n$=5) *300 nM $K_B$ 220±70 nM ($n$=4) $P$<0.05 | $IC_{50}$ 17.9±10.8 nM ($n$=6) Hill slope 0.51±0.2 $K_{iH}$ 5 nM | | |

Values are means±s.e.mean.
*Denotes concentration of antagonist used.

that the $\alpha_{1B}$- or $\alpha_{1A}$-adrenoceptors in that vein are not rauwolscine-sensitive. This blood vessel, like the DSV, also appears to have additional $\alpha_1$-adrenoceptors with low affinity for prazosin which account for the CEC-resistant responses.

In agreement with Hicks *et al.* (1991), $\alpha_2$ (probably $\alpha_{2A}$)-adrenoceptors exist in the DSV. This suggestion is based on the efficacy of B-HT 920 as an agonist and competitor for [$^3$H]-rauwolscine binding, with its apparent affinity unaffected by CEC. This suggestion needs further experimental evaluation since it implies that both CEC-sensitive and -insensitive sites of rauwolscine binding had similar affinities to B-HT 920.

Although a definitive interpretation of the nature of $\alpha_1$-adrenoceptors in DSV is not possible, these receptors were unusual in several respects. Firstly, they recognized rauwolscine in that this agent antagonized contractions to phenylephrine and methoxamine competitively with a potency at lower agonist concentrations similar to that expected from the $K_d$ value (5 nM) for rauwolscine binding (see Figure 1). We suggest that CEC acted on the same unusual α-adrenoceptors in the DSV through which rauwolscine-susceptible responses to phenylephrine and methoxamine occurred. At higher agonist concentrations, rauwolscine was less potent a functional antagonist, possibly acting at CEC-resistant receptors.

Secondly, those receptors utilized by lower concentrations of the two agonists also recognized prazosin and prazosin competitively antagonized the contractions induced by these agonists. However, the apparent $K_B$ values of prazosin against phenylephrine and methoxamine were somewhat higher (17 nM for phenylephrine and 11 nM for methoxamine) than expected from the $K_d$ value (2.6 nM) for prazosin binding. These $K_B$ values were however much lower than either of the $K_i$ values from prazosin competition for [$^3$H]-rauwolscine binding (which were in the range of μM and higher). Presumably, prazosin does not compete with rauwolscine at the same site on the receptor as that at which agonists interact. In any case, it is clear that the agonists, prazosin and rauwolscine do not interact at a single binding site.

Thirdly, the receptors at which phenylephrine and methoxamine induce rauwolscine-sensitive contraction are altered functionally, perhaps inactivated, by CEC. CEC pretreatment produced altered functional interactions (increased $EC_{50}$) and altered binding interactions (abolition of detectable prazosin binding and reduced rauwolscine binding as well as increased $IC_{50}$ values for the competition of phenylephrine for rauwolscine binding). The change in $IC_{50}$ value for methoxamine was not significant. The resistance of some rauwolscine binding sites to CEC inactivation does not establish definitively the existence of two classes of rauwolscine binding sites. CEC itself appeared to interact with only one such site and the apparent $K_i$ for this site was similar to those reported by Minneman *et al.* (1994) for clonidine at cloned and expressed $\alpha_{1B}$- and $\alpha_{1A/C}$-receptors. B-HT 920 interacted similarly with CEC-sensitive and CEC-resistant binding sites.

Fourthly, these phenylephrine- and methoxamine-activated receptors recognized WB 4101 but not 5-MU with high affinity, consistent with their classification as $\alpha_{1D}$ subtype (see Table 6). Since these receptors were affected and possibly inactivated by CEC and their activation induces $IP_3$ formation (Hicks *et al.*, 1991), they may not be classical $\alpha_{1D}$-adrenoceptors. However, Minneman *et al.* (1994) reported that, when expressed in human embryonic kidney cells, several $\alpha_1$-adrenoceptor subtypes could activate $IP_3$ formation. Perez *et al.* (1994) reported that in transfected Cos-1 cells 100 μM CEC inactivated 56% of $\alpha_{1D}$-adrenoceptors. If these results can be applied to the DSV adrenoceptors, they admit the possibility that the receptors activated by phenylephrine and by methoxamine in this tissue are $\alpha_{1D}$-adrenoceptors with a high affinity for rauwolscine; then residual rauwolscine binding sites after CEC pretreatment may be $\alpha_{2A}$ as proposed by Hicks *et al.* (1991).

Maximum responses in functional experiments to B-HT 920 in the DSV were unaffected by CEC pretreatment although the $EC_{50}$ values showed a leftward shift following CEC pretreatment. The inconsistent results between binding and functional studies may be due to the complication created by the CEC-induced basal contraction in the DSV; e.g., potentiation of $\alpha_2$-adrenoceptor agonist reponses by partial contraction or by partial receptor occupancy (Shimamoto *et al.*, 1993).

In conclusion, these studies show that there is an α-adrenoceptor in the DSV at which phenylephrine and methoxamine, can initiate contractile responses, antagonized by rauwolscine or prazosin acting at different sites on the receptor. It appears to be affected by CEC, being partially inactivated or irreversibly activated. It may represent an unusual $\alpha_{1D}$-adrenoceptor which can recognise and respond to $\alpha_2$ antagonists such as yohimbine or rauwolscine. Further studies of these unique adrenoceptors are clearly warranted.

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